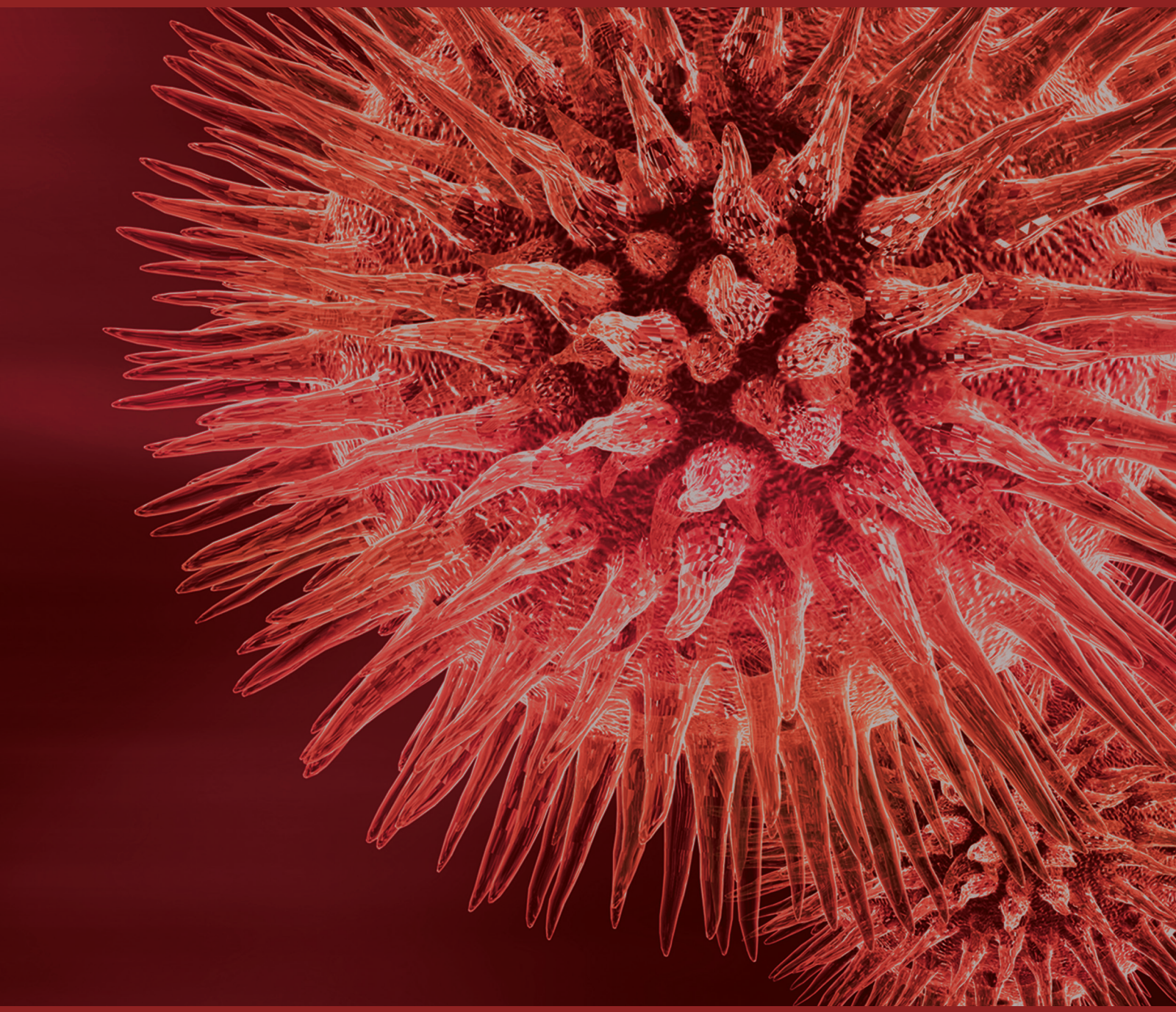
BioMed Research International

Cancer Immunology and Immunotherapy

Guest Editors: Mohammad Owais, Swaleha Zubair, Anshu Agrawal, and Yung-Fu Chang



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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 393454, 2 pages
http://dx.doi.org/10.1155/2015/393454

Editorial

Cancer Immunology and Immunotherapy

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Received 10 May 2015; Accepted 10 May 2015

The study of molecular and cellular interplays between immune system and cancerous cells is gaining tremendous momentum across the globe. Concomitantly, with the better insight into the intricacies of cancer immunology, immunotherapeutic approaches to deal with cancer have garnered tremendous boost in the recent past; reckoning with these, it is timely to analyse their potentialities either as standalone stratagem or in conjunction with traditional cancer therapeutic modalities. The comprehensive review by M. L. Santangelo et al. entitled "Immunosuppression and Multiple Primary Malignancies in Kidney-Transplanted Patients: A Single-Institute Study" provides an overview of immunotherapeutic interventions for metastatic renal cell carcinoma (RCC) as well as updating the readers on the recent developments in the field.

Further, the article entitled "Immunotherapy for Bone and Soft Tissue Sarcomas" by T. Uehara et al. enlightens the readers on immunotherapeutic strategies against bone and soft tissue sarcomas and metastatic prostate cancer under various stages of trials, besides highlighting their roles as an adjunct to traditional therapeutic modalities.

Natural killer (NK) cells have long been hypothesized to play a pivotal role in the development of new immunotherapeutic strategies to combat variety of cancers. In this regard, the article titled "'Adherent' versus Other Isolation Strategies for Expanding Purified, Potent, and Activated Human NK Cells for Cancer Immunotherapy" by S. R. Selvan and J. P. Dowling introduces a simple methodology for isolation and expansion of NK cells for adoptive cell therapies. Moreover, the researchers also equate potentialities of the newly introduced method with various published protocols to underline its effectiveness thereof. In the arena of NK cell based immunotherapies, the article "NKG2D and DNAM-1 Ligands: Molecular Targets for NK Cell-Mediated Immunotherapeutic Intervention in Multiple Myeloma" by C. Fionda et al. has come up with satisfactory results to further potentiate NK cell based immunotherapies. In their article of this special issue, the authors collate and discuss the molecular pathways whereby various chemotherapeutic agents could regulate the expression of NK cell activating ligands in multiple myeloma cells. Further, in a manner similar to NK cells, dendritic cells are also exploited in adoptive cell therapies; the manuscript "Dendritic Cell-Based Immunotherapy Treatment for Glioblastoma Multiforme" by L. Yang et al. discusses DCs based immunotherapeutic interventions for glioblastoma multiforme.

It is in general consensus that immunodeficiencies are associated with higher risk of cancer susceptibility; nevertheless, there remains paucity of reports on the association of immunodeficiencies with the development of multiple primary malignancies. In this regard, the study by R. Raman and D. Vaena illuminated the relationship between immunodeficiency status of the patient, related to kidney transplant in particular, and occurrence of multiple primary malignancies; nonetheless, further evidences are required to firmly establish the linkages between immune status of the recipient and its correlation with incidences of malignancy.

With continuous efforts laid down to better dissect the interplay between immune system and tumors, significant progresses have been made in the recent past, albeit much

have been unveiled; nevertheless, the drive continues to explore more and more. To this end, the manuscript by N. Vigneron provides a better insight into the ins and outs of tumor-immune system interrelationships highlighting the recent understandings gained in the field. Further, the article by S. Stigliani et al. suggests that expressions of FOXP3, CD14, and ARG1 in neuroblastoma tumor tissue from high-risk patients are significantly associated with event-free and overall survival. Besides, C. Li et al. investigate the association of CXCL13 (C-X-C motif chemokine 13) with hepatocellular carcinoma (HCC) and the authors further suggest that the correlation of CXCL13 with progression of HCC is related to the activation of Wnt/β-catenin pathway and the facilitation of IL-12, IL-17, and IgG4. Ascertaining their role in progression of HCC, the authors anticipate that CXCL13 could be a potential target for the diagnosis and treatment of HCC. Further, the report by A. Curioni-Fontecedro et al. highlights the intratumoral heterogeneity of MAGE-C1/CT7 and MAGE-C2/CT10 expression in mucosal melanoma. The article by Y. Nishimura et al. investigates the immunological effects of asbestos exposure and analyzes immune functions of patients with mesothelioma, thereby signifying that there occurs functional alteration in natural killer cells and cytotoxic T lymphocytes upon asbestos exposure as well as in malignant mesothelioma patients, while the manuscript by Z. Liu et al. investigates the regulative effects of microRNA-451a (miR-451a) on cell proliferation and sensitivity to tamoxifen in breast cancer cells. Further, P. Johnson et. al. from Cancer Research UK Clinical Centre have highlighted that it is equally important to delineate the metrics that are appropriate to annotate the significance of new cancer therapeutics modalities. The study also provided an insight into the intricacies that are the same and further ascertains that the mean overall survival, cure fraction, and overall survival rate at landmark time points represent the more appropriate endpoints.

The recent conceptual and technical footing of cancer immunology has paved ways to discover innovative cancer immunotherapies to treat and retard progression of the disease. It is widely accepted that the gamut of genetics and epigenetics changes occurring in tumors provides diverse set of antigenic repertoire that the immune system can exploit to distinguish tumor cells from their normal healthy counterparts. Moreover, studies continue to explore various genetic factors that increase the risk for cancer; the article by Y. Liu et al. studied the polymorphisms of nuclear factor-kappa B (NFκB) and its inhibitor (IκBα) and their synergistic outcome on nasopharyngeal carcinoma (NPC) predisposition. From their study, authors anticipate that genetic variants in NFκB1 (rs28362491del>ins ATTG) and IκBα (rs696G>A) and their synergistic outcome contribute to NPC susceptibility. Further, the manuscript by I. Silvestri et al. embarks on the importance of insight into the intricacies of antigenic peptide presentation in immunotherapy as well as in vaccine delivery.

Reckoning with the recent efforts devoted to developing superior strategies to fight against various diseases, over the years, there has been great wave of enthusiasm regarding employment of immunomodulators to combat various untamed diseases. In fact the strategy is high on pharma agenda and various immunomodulators especially naturally derived agents have been explored against various ailments including cancer. Considering the impact of immunomodulators in the field on cancer immunotherapies, W.-J. Wang et al. illuminated the role of mushroom β-Glucan to immunomodulate tumor associated macrophages in Lewis Lung Carcinoma. Moreover, the article by A. Ito et al. updates the readers on the clinical development of immunomodulators or immune checkpoint inhibitors. Further, the manuscript by Q. Guo et al. delineates the effect and molecular mechanisms of traditional Chinese medicine (TCM) on regulating tumor immunosuppressive microenvironment (TIM) revealing bidirectional and multitargeting features of TCM on TIM.

Finally, the pioneer work by O. Kurtenkov et al. for the first time revealed the fact that increased sialylation of anti-Thomsen-Friedenreich (TF) Antigen (CD176) antibodies is strongly linked to gastric cancer; reckoning with this, the authors anticipate that the important biomolecule can be employed as a novel biomarker for cancer detection and prognosis.

Besides active regimens to control and cure one of the most dreadful diseases across the world, the immunological intervention to dissipate various forms of the cancer seems to be the promising ordeal. The timely coverage of the various cancer cells versus host immune system interplay by some of the leading experts of the field will certainly take us to the state of affairs that will offer a great input to control this important ailment.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 183523, 8 pages
http://dx.doi.org/10.1155/2015/183523

Research Article

Immunosuppression and Multiple Primary Malignancies in Kidney-Transplanted Patients: A Single-Institute Study

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Received 31 May 2014; Revised 21 August 2014; Accepted 30 September 2014

Academic Editor: Mohammad Owais

Immunodeficiency is associated with higher cancer incidence. However, it is unknown whether there is a link between immunodeficiency and development of multiple primary malignancies. In the present study we analyse this link focusing on kidney-transplanted patients, as they are at higher risk of developing cancer due to the chronic assumption of immunosuppressants. We followed up 1200 patients who underwent kidney transplantation between 1980 and 2012. A total of 77/1200 kidney-transplanted patients developed cancer and 24 of them developed multiple cancers. Most multiple cancers were synchronous with a nonsignificant association between cancer and rejection episodes. In the general cancer population, one-ninth of patients are at higher risk of developing a second tumor over a lifetime; hence it would be reasonable to conclude that, from a merely theoretical and statistical viewpoint, long-term transplanted patients potentially have a higher risk of developing MPMs. However, data did not confirm this assumption, probably because these patients die before a second primary malignancy appears. Despite many observations on the increased incidence of different tumor types in immunodeficient patients and despite immunosuppression certainly being a predisposing factor for the multicancer syndrome, data so far are not robust enough to justify a correlation between immunodeficiency and multiple primary malignancies in transplanted patients.

1. Introduction

Renal transplantation is the gold standard procedure for patients with end-stage renal diseases. The increasing success of such an approach is partly due to the use of increasingly active immunosuppressive drugs, which have largely lowered the rate of rejection and improved outcome [1]. However, the chronic use of immunosuppressive drugs leads to an increased cancer incidence [2]. A link between cancer development and immunosuppression in transplanted patients is well recognized. Over the past decades, a growing body of evidence has emerged demonstrating the dual role of the immune system in cancer, being involved both in tumor development (via chronic inflammation through the innate immune system) and in tumor elimination and control (through the adaptive immune system) [3]. As an example, renal cell carcinoma has been traditionally considered immunogenic, as it does occur at a higher incidence in immunosuppressed patients [4]. Also, this tumor type is traditionally considered more responsive to immunotherapy. Therefore, it is now well recognized that immunosuppressive drugs used in transplanted patients may induce immune defects, thus compromising the immune response and facilitating the development of a secondary immunodeficiency

(ID) which can ultimately ease cancer onset [5]. Also, the incidence of multiple primary malignancies (MPMs) is increasing in the general population and it is expected to further increase in the coming years. The definition of MPMs requires that each tumor be a solid tumor, have a histopathological diagnosis of malignancy, be topographically distinct from another one, and not include tumor that are metastases of the primary. In terms of time, they are classified as simultaneous (i.e., both tumors appear at the same time), synchronous (i.e., the second tumor appears within six months from the first tumor), and metachronous (i.e., the second tumor appears more than six months after the first tumor) [6]. Having said that, it has to be considered that should a transplanted patient develop a tumor, recover from it, and continue to receive immunosuppressive treatment, the risk of developing MPMs is potentially higher as compared to the general population [7]. It is in this perspective that the problem of MPMs in transplanted patients should be considered. In this paper we focus on the link between secondary immunodeficiency and the onset of MPMs in transplanted patients, who are—by definition—patients at higher risk of developing cancer due to the chronic assumption of immunosuppressants.

To test the relationship between immunosuppression and MPMs, we specifically selected kidney-transplanted patients for several reasons. Firstly, among solid organ transplanted patients, kidney recipients represent the most numerous group, with the longest follow-up (kidney transplantation was the first solid organ transplant carried out; from a single cadaver donor it is generally possible to obtain two kidneys for two different kidney recipients; living kidney donation is a perfectly codified procedure which is carried out worldwide). Therefore, this is a representative population. Secondly, the median overall survival of kidney transplant recipients is long (more than 10 years) and, accordingly, so is the exposure to immunosuppressive drugs; hence, in these patients it is possible to evaluate whether there is a correlation between immunosuppression and cancer(s) development over an adequate timeframe. Thirdly, after kidney transplantation, immunosuppressants are generally used at full dosage, thus making it possible to evaluate their real effects on tumorigenesis. Last but not least, in this population, transplanted organ failure does not inevitably lead to death, as it is possible to return to dialysis. Therefore, in these patients, the natural history of the disease may be also evaluated after reduction/modification/interruption of immunosuppressive drugs. For all the above-mentioned reasons, we analyzed a consecutive series of patients undergoing kidney transplantation at our institute.

2. Material and Methods

Through the analysis of medical records collected in our department, we retrospectively examined 1200 kidney-transplanted patients (745 male and 455 female) followed up at Federico II University between 1980 and 2012. The median follow-up was 10 years and the average graft survival 8 years. Patients' age ranged between 18 and 65 years and they were homogenous for donor/recipient immunology (e.g., mismatch index), number of rejection events, and immunosuppressive therapy. In our population, different drugs and associations were used taking advantage of their different mechanisms of actions: corticosteroids (the oldest immunosuppressants), azathioprine (an old antiproliferative immunosuppressive drug), calcineurin inhibitors (cyclosporine and tacrolimus are the most used as maintenance therapy; they primarily suppress the activation of T lymphocytes by inhibiting the production of cytokines, specifically IL-2), basiliximab (an IL-2 receptor antagonist generally used as induction therapy), mycophenolate (a new antiproliferative agent that interferes with DNA replication, producing cytostatic effects on both T and B cells; it is generally used as a "third agent" in triple immunosuppressive regimens), mammalian target of rapamycin inhibitors (also called m-TOR inhibitors; they usually represent an alternative to the long-term calcineurin inhibitor-based regimen and its side effects). Drugs and associations have varied over time. In general we used corticosteroids and azathioprine from 1980 to 1984; corticosteroids, calcineurin inhibitors, and azathioprine from 1984 to 1998; corticosteroids, calcineurin inhibitors, basiliximab, mycophenolate, and mammalian target of rapamycin from 1998 to 2013. We did not at any time use thymoglobulin.

3. Results

Among 1200 kidney-transplanted patients, 77 patients (6.4%) [57 males (4.7%) and 20 females (1.7%)], developed a cancer. A total of 53 patients (4.4% of global series and 69% of cancer patients) developed a single cancer. A total of 20 patients were diagnosed with a skin cancer (including melanoma) and 33 patients with no skin cancer, representing 1.7% and 2.7% of global series and 26% and 43% of cancer patients, respectively. It is worth noting that 24 patients (2% of global series and 31% of cancer patients) presented with multiple cancers: 20 multiple skin cancers (including melanoma) and 4 solid MPMs, representing 1.6% and 0.3% of global series and 25.9% and 5.1% of cancer patients, respectively (Table 1). With regard to the latter group, we observed the following associations: prostate/kidney cancer (synchronous, surgically treated, 2-year disease-free interval); Kaposi sarcoma/gastric MALToma (metachronous, medical and surgical treatment, 1-year disease-free interval, exitus at 18 months); lung cancer/squamous skin carcinoma (metachronous, medical and surgical treatment, disease-free at 8 months, exitus at 14 months), and colon cancer/squamous skin carcinoma (metachronous, surgically treated, 18-month disease-free interval). Multiple cancer associations and their onset time are detailed in Figures 1 and 2. Data on the association between cancer and rejection episodes are not significant: rejection episodes were only found in four patients and only one of them developed a second cancer.

4. Discussion

Today the high standards in surgical, anaesthesiological, and intensive-care procedures as well as in the clinical

Table 1: Characteristics of patients with MPMs.

Patient	Sex	Year of kidney transplant	Patient's age at transplant	Immunosuppressive drugs used	Acute rejection events	Type of first tumor	Date of first tumor	Type of second tumor	Date of second tumor	Return to dialysis (year)	Status (last follow-up)
1	M	1995	39	CCS + CyA + Myc	No	BCC	2001	Melanoma	2006	No	Alive (2012)
2	M	1987	25	CCS + CyA	No	SCC	2008	SCC	2008	Yes (2010)	Alive (2012)
3	M	2001	64	CCS + CyA + Myc	No	BCC	2006	SCC	2006	Yes (2011)	Alive (2012)
4	M	2003	62	CCS + FK > > Rap + CCS	No	Prostate Ca	2010	Kidney Ca	2010	No	Alive (2012)
5	M	2001	55	CCS + CyA + Myc	No	BCC	2002	SCC	2002	No	Alive (2010)
6	M	1988	45	CyA > Rap + CCS	No	Kaposi	2004	Gastric MALToma	2005	Yes (2006)	Dead (2006)
7	M	2001	55	CCS + FK + Myc	No	BCC	2003	SCC	2003	No	Alive (2012)
8	M	1992	42	CCS + CyA	No	SCC	2005	BCC + SCC	2005	No	Alive (2012)
9	M	1997	39	CyA + Aza	No	SCC	2003	Melanoma	2012	No	Alive (2012)
10	M	1995	51	CCS + CyA + Myc	No	BCC	2005	BCC + SCC	2010/2011	No	Alive (2012)
11	F	2004	53	CCS + CyA > > Rap + CCS	No	Lung Ca	2005	SCC	2006	Yes (2007)	Dead (2007)
12	M	1998	56	CCS + CyA	No	SCC	1999	BCC	2007	No	Alive (2012)
13	M	1992	18	CCS + CyA + Myc	Yes	BCC	2000	SCC	2000	Yes (2008)	Alive (2012)
14	M	2001	61	CCS + Rap + Myc	No	SCC	2006	SCC	2007	No	Alive (2007)
15	M	1989	29	CyA + Aza > > Rap + CCS	No	BCC	2007	SCC	2007	No	Alive (2012)
16	M	2005	43	CCS + CyA	No	Melanoma	2008	BCC	2008	No	Alive (2012)
17	M	1999	59	CCS + CyA + Myc	No	BCC	2004	SCC	2004	No	Alive (2011)
18	M	1986	46	CCS + CyA	No	SCC	1997	Melanoma	2004	Yes (2004)	Dead (2007)
19	M	2000	35	CCS + CyA + Myc	No	SCC	2006	BCC	2012	No	Alive (2012)
20	M	1994	38	CCS + CYA	No	SCC	2000	SCC	2000	Yes (2010)	Alive (2012)
21	F	1999	56	CCS + CYA	No	SCC	2004	BCC	2004	No	Alive (2012)
22	F	1996	49	FK + Aza > > FK	No	BCC	2001	BCC	2001	No	Alive (2012)
23	F	1987	56	CCS + FK	No	BCC	1998	SCC	2006	Yes (2011)	Alive (2012)
24	M	2005	60	CCS + FK > > Rap > FK	No	SCC	2006	Colon Ca	2011	Yes (2012)	Alive (2012)

M: male; F: female.
CCS: corticosteroids; Aza: azathioprine; CyA: cyclosporine; FK: tacrolimus; Myc: mycophenolate and derivatives; Rap: rapamycin and derivatives.
>: switch to other drug(s).
Ca: carcinoma; SCC: squamous cell carcinoma; BCC: basal cell carcinoma; Kaposi: Kaposi sarcoma; MALToma: neoplasm of mucosa associated lymphoid tissue.

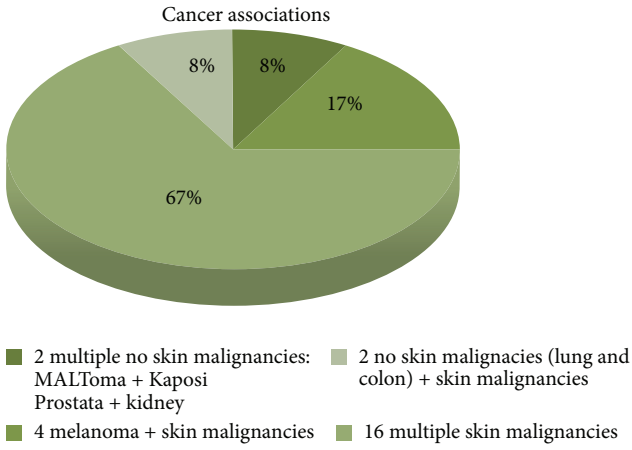

Figure 1: Cancers associations among 24 patients in 1200 kidney-transplant patients

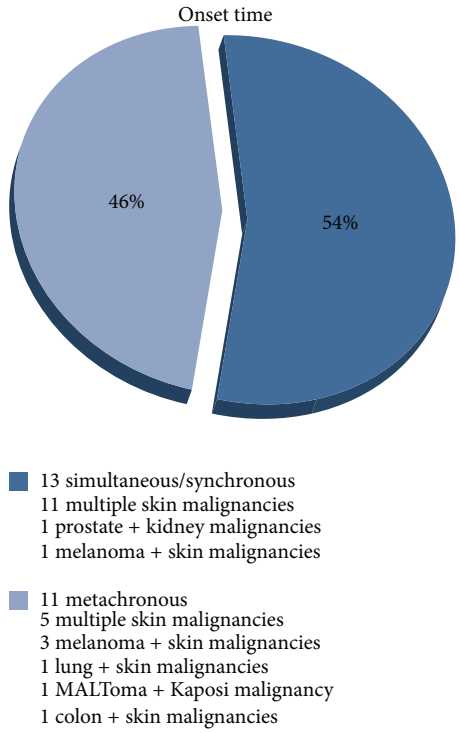

Figure 2: Onset time of MPMs among 24 patients in 1200 kidney-transplant patients

management of patients undergoing transplantation enable extremely positive results to be obtained in terms of short- and medium-term survival for both organs and patients [8–13]. However, these results have been partially nullified by the long-term complications reported in these patients, especially the development of cancer. Incidence, aggressiveness, and worse prognosis of tumors appear to be remarkably increased in this group of patients as compared to the general population of corresponding age [7, 14–16]. It has been calculated that tumor prevalence at 10 years after kidney transplant ranges from 20% to 30%, with peaks also over 45% at 20 years [16–21]. Among organ transplant recipients the main factor facilitating cancer onset is certainly the immunosuppressive treatment. Indeed, the neoplastic risk is also increased in people treated with immunosuppressants for reasons other than transplantation [20]. Besides the indirect oncogenic effect exerted by all immunosuppressive drugs, which alter the immune response, recent studies have shown that some immunosuppressants (i.e., calcineurin inhibitors, azathioprine, and thymoglobulin) exert a direct oncogenic effect. Calcineurin inhibitors promote oncogenesis, neoplastic growth, and metastasization by inhibiting DNA repair and apoptosis and by stimulating the production of transforming growth factor-beta (TGF-beta) and vascular endothelial growth factor (VEGF). Azathioprine and its derivatives are able to increase DNA damage caused by UV and to inhibit DNA repair. Thymoglobulin seems to foster genetic mutations induced by oncoviruses (Figure 3). In this context, it is worth noting that, in transplanted patients, some tumor types may show a regression if immunosuppressive therapy is withdrawn or changed/enriched with drugs such as mTOR inhibitors and mycophenolate [22, 23]. However, while the use of mycophenolate was associated with a reduced cancer incidence, probably because its administration is correlated to calcineurin inhibitor dose reduction, mTOR inhibitors have shown a direct antineoplastic effect. These properties—together with a reduced nephrotoxicity—have led to an extended use of these drugs [24–35].

However, in transplanted patients, immunosuppressive therapy is essential to avoid graft rejection, which ultimately results in reduced morbidity and mortality. There is a huge variability among different classes of immunosuppressive drugs, which work through different mechanisms on the immune system. When analyzing the association between immunosuppressive therapy and increased cancer risk, different aspects have to be considered, such as the duration of immune suppressive therapy, the intensity of treatment, and the drug(s) used. Yet, such a huge variability in terms of clinical studies' fragmentation, uniqueness of each single patient, different therapeutic approaches in different transplant centers, the switch from one immunosuppressive protocol to another, and—last but not least—the pressure exercised by pharmaceutical firms has led to contrasting results. Also, it is worth remembering that the lifespan of transplanted patients is longer; accordingly the time frame these people stay on immunosuppressive treatment is longer too, with augmented exposure to oncogenic factors and viral infections (Figure 4). Furthermore, transplanted patients nowadays have a longer life expectancy and may reach the age at which the neoplastic risk is naturally higher, when the transplant is not already performed in aged people [19]. Overall, the increased cancer risk after renal transplantation is now well recognized [36–40]. So, the association between pharmacological immune suppression and increased risk of cancer continues to be a much-discussed topic [41]. It has been calculated that if malignant tumors carried a lower mortality rate and were more uniformly distributed in the general population, we could still expect to find that 1 in 9 cancer patients would develop a second cancer over a lifetime and that within this group 1 in 27 patients will probably develop a third primary

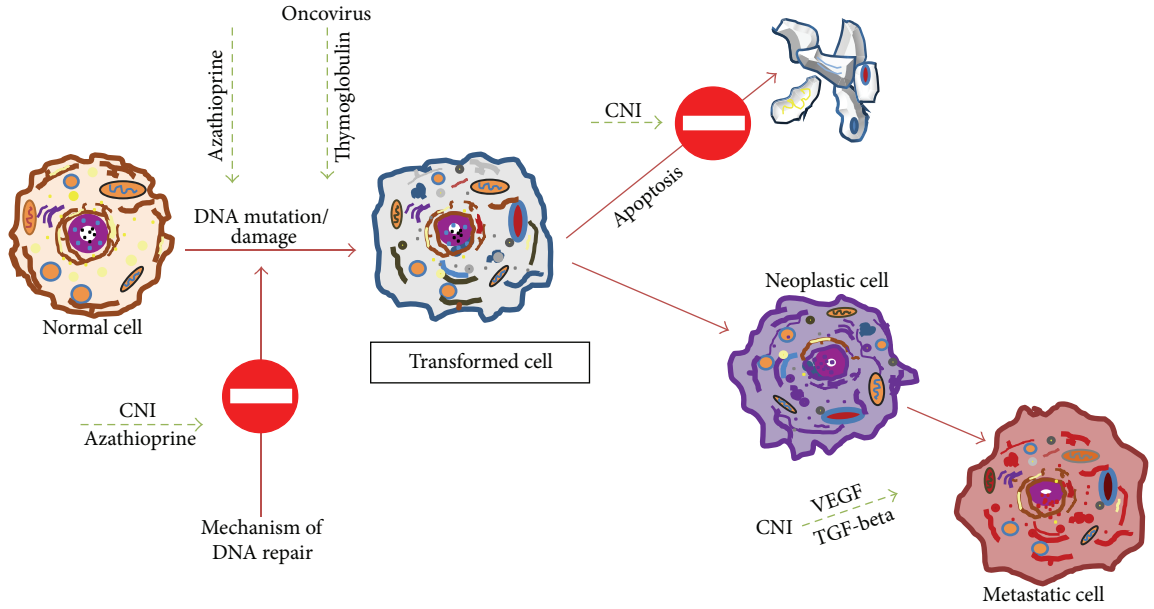

Figure 3: Schematic representation of some oncogenic mechanisms of calcineurin inhibitors (CNI), azathioprine, and thymoglobulin.

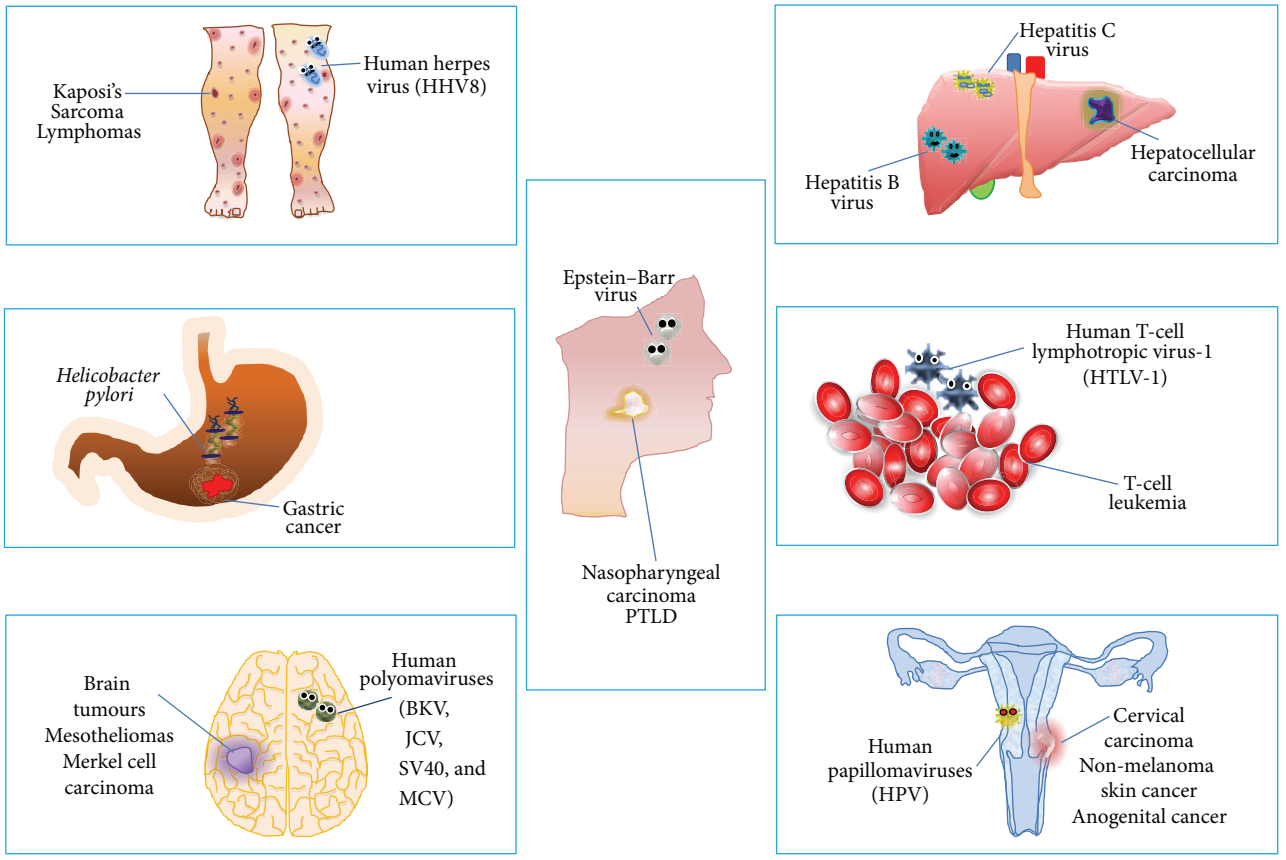

Figure 4: Oncoviruses and potentially related cancers.

cancer [6, 41–46]. This statistical projection obviously refers to the general population. Therefore, it would be logical to conclude that, from a merely theoretical and probabilistic point of view, immunocompromised patients potentially have a higher risk of developing MPMs [6, 24, 42, 46–48]. Transplanted patients treated with immunosuppressants may develop multiple cancers in three different conditions: (1) patients with a previous diagnosis of cancer who undergo transplantation and, afterwards, present with a new cancer during follow-up; (2) patients with a previous diagnosis of cancer who undergo transplantation and then present with a new cancer transmitted by the donor; (3) patients developing MPMs after transplantation (those reported in our study). But real life differs from theory, even when the theory has valid bases. To the best of our knowledge, only one study has specifically looked at the incidence of MPMs in transplanted patients [49]. In this study, transplanted patients did not show a statistically significant higher risk of developing MPMs as compared to the corresponding general population. Also, our experience together with a careful review of the literature does not support the hypothesis that immunocompromised patients are more likely to develop MPMs. The reasons for this might simply lie in the fact that kidney-transplanted patients probably die before a new "second primary malignancy" appears or, alternatively, returning to dialysis, they interrupt immunosuppressive therapy, thus limiting the exposure to oncogenic effects of such drugs over time. Furthermore, it is very hard to find transplanted patients surviving a first cancer who keep on taking immune suppressive treatment long enough to develop a second primary cancer as a consequence of iatrogenic immune deficiency. Indeed, until recently, kidney transplant recipients who developed a tumor were treated according to medical/surgical approaches, which included—among others—immunosuppressant withdrawal and, accordingly, return to dialysis. Nowadays, the chance to have recourse to immunosuppressants such as mycophenolate and mTOR inhibitors has allowed a large number of kidney-transplanted patients who develop a tumor to recover by maintaining the function of the transplanted organ. Hence, we are observing a group of kidney-transplanted patients at higher risk of developing a second tumor, as they recovered from the first one without interrupting immunosuppressive therapy. However, the follow-up of these patients is still limited; therefore, it is not yet possible to evaluate the actual incidence of second tumors.

5. Conclusions

Despite many observations regarding the increased incidence of different tumor types in immunosuppressed patients and despite the fact that immunosuppression is a predisposing factor for multicancer syndrome, at least theoretically, so far there are no significant statistical data indicating a clear correlation between immunosuppression and MPMs. We may therefore assume that it is hard to diagnose a second cancer in immunocompromised patients because of their shorter life expectancy. From the few reports found in the literature and from our experience, we can conclude that MPMs in immunosuppressed patients are more frequently simultaneous/synchronous, usually have a viral etiology, and regard the same organ or tissue, the skin is the most affected tissue with a predominance of spinocellular carcinomas over basocellular carcinomas (exactly the opposite of what is observed in the general population), and at least one cancer is readily detectable (e.g., skin cancer), thereby facilitating an early diagnosis and treatment. It is our opinion that the treatment of MPMs in immunosuppressed patients should be as intensive as possible, in order to obtain a complete recovery. Moreover, it might be useful to suspend the immunosuppressive treatment or switch to other drugs such as m-TOR inhibitors; this therapeutic approach has so far yielded good results. In conclusion, available clinical and epidemiological data allow immunosuppression to be considered as a cancer risk factor. However, so far there is no sufficient evidence to conclude that immunosuppression eases the onset of MPMs. Hence, even if MPMs do not seem to be a real problem today, they may become an important issue in the near future, when new treatments and stricter follow-up guarantee longer life expectancy in immunosuppressed patients diagnosed with cancer. Therefore, in potentially immunocompromised patients (e.g., kidney transplant candidates), great relevance must be given to preventive measures against oncoviral infections (e.g., a vaccination program, as has already been established for HBV and HPV); implementation of procedures aiming at reducing the exposure to environmental oncogenic factors (e.g., drugs, cigarette smoking, alcohol, sun exposure, etc., as is already recommended to our patients); strict follow-up programs with special attention to apparatus/organs (e.g., genitourinary, skin, thyroid, liver, blood, and bones) at higher cancer risk in such patients and—last but not least—it is important to try to reduce the dosage of immunosuppressive drugs as much as possible (especially for calcineurin inhibitors, azathioprine, and thymoglobulin, for which a direct oncogenic effect has been proven), without exposing the patient to the risk of graft rejection. This paradigm should aim at immunomodulation rather than immunosuppression, which might be the true gold standard of such a therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] I. Rama and J. M. Grinyó, "Malignancy after renal transplantation: the role of immunosuppression," *Nature Reviews Nephrology*, vol. 6, no. 9, pp. 511–519, 2010.

[2] E. A. Engels, R. M. Pfeiffer, J. F. Fraumeni Jr. et al., "Spectrum of cancer risk among US solid organ transplant recipients," *The Journal of the American Medical Association*, vol. 306, no. 17, pp. 1891–1901, 2011.

[3] L. Zitvogel, A. Tesniere, and G. Kroemer, "Cancer despite immunosurveillance: immunoselection and immunosubversion," *Nature Reviews Immunology*, vol. 6, no. 10, pp. 715–727, 2006.

[4] I. Penn, "Tumors of the immunocompromised patient," *Annual Review of Medicine*, vol. 39, pp. 63–73, 1988.

[5] D. Berg and C. C. Otley, "Skin cancer in organ transplant recipients: epidemiology, pathogenesis, and management," *Journal of the American Academy of Dermatology*, vol. 47, no. 1, pp. 1–17, 2002.

[6] N. Carlomagno, M. L. Santangelo, R. Mastromarino, A. Calogero, C. Dodaro, and A. Renda, "Rare multiple primary malignancies among surgical patients—a single surgical unit experience," *Ecancermedicalscience*, vol. 8, article 438, 2014.

[7] G. Tessari, L. Naldi, L. Boschiero et al., "Incidence of primary and second cancers in renal transplant recipients: a multicenter cohort study," *American Journal of Transplantation*, vol. 13, no. 1, pp. 214–221, 2013.

[8] J. Chinen, D. Anmuth, A. R. K. Franklin, and W. T. Shearer, "Long-term follow-up of patients with primary immunodeficiencies," *The Journal of Allergy and Clinical Immunology*, vol. 120, no. 4, pp. 795–797, 2007.

[9] P. de Rosa, M. Santangelo, A. Ferrara et al., "Suboptimal kidney: the experience of a single transplant unit," *Transplantation Proceedings*, vol. 36, no. 3, pp. 488–490, 2004.

[10] M. Santangelo, P. de Rosa, S. Spiezia et al., "Healing of surgical incision in kidney transplantation: a single transplant center's experience," *Transplantation Proceedings*, vol. 38, no. 4, pp. 1044–1046, 2006.

[11] P. De Rosa, M. Santangelo, A. Scala et al., "Difficult vascular conditions in kidney transplantation," *Transplantation Proceedings*, vol. 38, no. 4, pp. 1040–1043, 2006.

[12] M. Santangelo, M. Zuccaro, P. de Rosa et al., "Older kidneys donor transplantation: five years' experience without biopsy and using clinical laboratory and macroscopic anatomy evaluation," *Transplantation Proceedings*, vol. 39, no. 6, pp. 1835–1837, 2007.

[13] M. Santangelo, M. Clemente, S. Spiezia et al., "Wound complications after kidney transplantation in nondiabetic patients," *Transplantation Proceedings*, vol. 41, no. 4, pp. 1221–1223, 2009.

[14] J. Dantal, M. Hourmant, D. Cantarovich et al., "Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: randomised comparison of two cyclosporin regimens," *The Lancet*, vol. 351, no. 9103, pp. 623–628, 1998.

[15] P. N. Harden, "Annual incidence and predicted risk of nonmelanoma skin cancer in renal transplant recipients," *Transplantation Proceedings*, vol. 33, no. 1-2, pp. 1302–1304, 2001.

[16] R. P. Carroll, H. M. Ramsay, A. A. Fryer, C. M. Hawley, D. L. Nicol, and P. N. Harden, "Incidence and prediction of nonmelanoma skin cancer post-renal transplantation: a prospective study in Queensland, Australia," *American Journal of Kidney Diseases*, vol. 41, no. 3, pp. 676–683, 2003.

[17] *Proceedings of the IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Epstein-Barr Virus and Kaposi's Sarcoma Herpesvirus/Human Herpesvirus 8. Lyon, France, June 1997*, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, World Health Organization, International Agency for Research on Cancer.

[18] EBPG Expert Group on Renal Transplantation, "European best practice guidelines for renal transplantation. Section IV: Long-term management of the transplant recipient. IV.1. Organization of follow-up of transplant patients after the first year," *Nephrology Dialysis Transplantation*, vol. 17, supplement 4, pp. 3–4, 2002.

[19] A. G. R. Sheil, "Organ transplantation and malignancy: inevitable linkage," *Transplantation Proceedings*, vol. 34, no. 6, pp. 2436–2437, 2002.

[20] A. Gutierrez-Dalmau and J. M. Campistol, "Immunosuppressive therapy and malignancy in organ transplant recipients: a systematic review," *Drugs*, vol. 67, no. 8, pp. 1167–1198, 2007.

[21] J. N. Bouwes Bavinck, D. R. Hardie, A. Green et al., "The risk of skin cancer in renal transplant recipients in Queensland, Australia: a follow-up study," *Transplantation*, vol. 61, no. 5, pp. 715–721, 1996.

[22] A. Gaumann, H. J. Schlitt, and E. K. Geissler, "Immunosuppression and tumor development in organ transplant recipients: the emerging dualistic role of rapamycin," *Transplant International*, vol. 21, no. 3, pp. 207–217, 2008.

[23] R. Robson, J. M. Cecka, G. Opelz, M. Budde, and S. Sacks, "Prospective registry-based observational cohort study of the long-term risk of malignancies in renal transplant patients treated with mycophenolate mofetil," *American Journal of Transplantation*, vol. 5, no. 12, pp. 2954–2960, 2005.

[24] J. Dantal and E. Pohanka, "Malignancies in renal transplantation: an unmet medical need," *Nephrology Dialysis Transplantation*, vol. 22, supplement 1, pp. i4–i10, 2007.

[25] J. M. Campistol, V. Cuervas-Mons, N. Manito et al., "New concepts and best practices for management of pre- and post-transplantation cancer," *Transplantation Reviews*, vol. 26, no. 4, pp. 261–279, 2012.

[26] M. Rodríguez-Perálvarez, M. de La Mata, and A. K. Burroughs, "Liver transplantation: immunosuppression and oncology," *Current Opinion in Organ Transplantation*, vol. 19, no. 3, pp. 253–260, 2014.

[27] W. S. Asch and M. J. Bia, "Oncologic issues and kidney transplantation: a review of frequency, mortality, and screening," *Advances in Chronic Kidney Disease*, vol. 21, no. 1, pp. 106–113, 2014.

[28] J. R. Chapman, A. C. Webster, and G. Wong, "Cancer in the transplant recipient," *Cold Spring Harbor Perspectives in Medicine*, vol. 3, no. 7, 2013.

[29] M. J. Bottomley and P. N. Harden, "Update on the long-term complications of renal transplantation," *British Medical Bulletin*, vol. 106, no. 1, pp. 117–134, 2013.

[30] P. Piselli, G. Busnach, L. Fratino et al., "De novo malignancies after organ transplantation: focus on viral infections," *Current Molecular Medicine*, vol. 13, no. 7, pp. 1217–1227, 2013.

[31] P. A. Dugue, M. Rebolj, P. Garred, and E. Lynge, "Immunosuppression and risk of cervical cancer," *Expert Review of Anticancer Therapy*, vol. 13, no. 1, pp. 29–42, 2013.

[32] S. M. Hosseini-Moghaddam, A. Soleimanirahbar, T. Mazzulli, C. Rotstein, and S. Husain, "Post renal transplantation Kaposi's sarcoma: a review of its epidemiology, pathogenesis, diagnosis, clinical aspects, and therapy," *Transplant Infectious Disease*, vol. 14, no. 4, pp. 338–345, 2012.

[33] C. Ponticelli, "Present and future of immunosuppressive therapy in kidney transplantation," *Transplantation Proceedings*, vol. 43, no. 6, pp. 2439–2440, 2011.

[34] M. Suthanthiran, M. Hojo, M. Maluccio, D. J. Boffa, and F. L. Luan, "Post-transplantation malignancy: a cell autonomous mechanism with implications for therapy.," *Transactions of the American Clinical and Climatological Association*, vol. 120, pp. 369–388, 2009.

[35] P. Karran and N. Attard, "Thiopurines in current medical practice: molecular mechanisms and contributions to therapy-related cancer," *Nature Reviews Cancer*, vol. 8, no. 1, pp. 24–36, 2008.

[36] P. Piselli, D. Serraino, G. P. Segoloni et al., "Risk of de novo cancers after transplantation: results from a cohort of 7217 kidney transplant recipients, Italy 1997–2009," *European Journal of Cancer*, vol. 49, no. 2, pp. 336–344, 2013.

[37] P. Pedotti, M. Cardillo, G. Rossini et al., "Incidence of cancer after kidney transplant: results from the North Italy transplant program," *Transplantation*, vol. 76, no. 10, pp. 1448–1451, 2003.

[38] I. Penn, "Tumors after renal and cardiac transplantation," *Hematology/Oncology Clinics of North America*, vol. 7, no. 2, pp. 431–445, 1993.

[39] D. Serraino, P. Piselli, C. Angeletti et al., "Risk of Kaposi's sarcoma and of other cancers in Italian renal transplant patients," *British Journal of Cancer*, vol. 92, no. 3, pp. 572–575, 2005.

[40] E. Tatar, B. Sarsik, O. Gungor et al., "Multiple unrelated malignancies following renal transplantation: an evaluation of four cases," *Internal Medicine*, vol. 52, no. 6, pp. 673–677, 2013.

[41] P. O'Donovan, C. M. Perrett, X. Zhang et al., "Azathioprine and UVA light generate mutagenic oxidative DNA damage," *Science*, vol. 309, no. 5742, pp. 1871–1874, 2005.

[42] E. Strocchi, R. V. Iaffaioli, G. Facchini et al., "Stop-flow technique for loco-regional delivery of high dose chemotherapy in the treatment of advanced pelvic cancers," *European Journal of Surgical Oncology*, vol. 30, no. 6, pp. 663–670, 2004.

[43] P. Piscitelli, A. Santoriello, F. M. Buonaguro et al., "Incidence of breast cancer in Italy: mastectomies and quadrantectomies performed between 2000 and 2005," *Journal of Experimental and Clinical Cancer Research*, vol. 28, no. 1, article 86, 2009.

[44] L. Insabato, M. Siano, A. Somma, R. Gentile, M. Santangelo, and G. Pettinato, "Extrapleural solitary fibrous tumor: a clinicopathologic study of 19 cases," *International Journal of Surgical Pathology*, vol. 17, no. 3, pp. 250–254, 2009.

[45] A. Tortoriello, G. Facchini, F. Caponigro et al., "Phase I/II study of paclitaxel and vinorelbine in metastatic breast cancer," *Breast Cancer Research and Treatment*, vol. 47, no. 1, pp. 91–97, 1998.

[46] S. Maurea, M. Fusari, M. Imbriaco et al., "Pitfalls in diagnostic imaging of cystic pancreatic masses: a case of true cystic lesion mimicking a mucinous cystadenoma," *Journal of the Pancreas*, vol. 13, no. 1, pp. 83–86, 2012.

[47] H. M. Kauffman, W. S. Cherikh, M. A. McBride, Y. Cheng, and D. W. Hanto, "Deceased donors with a past history of malignancy: an organ procurement and transplantation network/united network for organ sharing update," *Transplantation*, vol. 84, no. 2, pp. 272–274, 2007.

[48] M. Santangelo, S. Spiezia, M. Clemente et al., "Immunodeficiency and multiple primary malignancies," in *Multiple Primary Malignancies*, A. Renda, Ed., Updates in Surgery, pp. 83–96, Springer, Milan, Italy, 2009.

[49] E. Taioli, P. Piselli, E. Arbustini et al., "Incidence of second primary cancer in transplanted patients," *Transplantation*, vol. 81, no. 7, pp. 982–985, 2006.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 604385, 15 pages
http://dx.doi.org/10.1155/2015/604385

Research Article

Mushroom β-Glucan May Immunomodulate the Tumor-Associated Macrophages in the Lewis Lung Carcinoma

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Received 14 June 2014; Revised 13 October 2014; Accepted 10 November 2014

Academic Editor: Swaleha Zubair

The present study showed that oral mushroom beta-glucan treatment significantly increased IFN-γ mRNA expression but significantly reduced COX-2 mRNA expression within the lung. For LLC tumor model, oral *Ganoderma lucidum* or *Antrodia camphorata* polysaccharides treatments significantly reduced TGF-β production in serum. In addition, IL-12 and IFN-γ mRNA expression were significantly increased, but IL-6, IL-10, COX-2, and TGF-β mRNA expression were substantially following oral mushroom polysaccharides treatments. The study highlights the efficacious effect of mushroom polysaccharides for ameliorating the immune suppression in the tumor microenvironment. Increased M1 phenotype of tumor-associated macrophages and attenuated M2 phenotype of tumor-associated macrophages could be achieved by ingesting mushroom polysaccharides.

1. Introduction

Tumor cells affect the surrounding cellular environment by promoting tumor growth and metastasis via establishment of a tumor microenvironment conducive to tumor development [1–5]. Tumor cells secrete inflammatory cytokines, such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10), that stimulate differentiation of regulatory T and Treg cells [6–10], as well as differentiation of tumor-associated macrophages (TAMs) into M2 macrophages, leading to host immune response and subsequent tumor cell evasion of this immune surveillance and attack, in turn enhancing tumor growth and metastasis [1, 11–17]. Various cytokines, chemokines, and growth factors are the primary elements in affecting the host antitumor ability and host evasion of tumor cells [3, 18]. Macrophages are the most important and abundant immune cells and there are primarily two types of macrophages based on function and differentiation: classically activated macrophage (M1 macrophage) and alternatively activated macrophage (M2 macrophage). M1 macrophages are characterized by tumor resistance, while M2 macrophages are characterized by tumor promotion [16, 19]. In mice models, macrophages present CD11b, F4/80, and colony-stimulating factor-1 receptor (CSF-1R), with F4/80 being the surface proteins for M1 and M2 macrophages [11, 20].

TAMs have the major role in the tumor microenvironment to bear immune inhibitory effect [20, 21]. Tumor cells and the surrounding stoma cells secrete cytokines and growth factors that stimulate TAMs and activate the various expressions, functions, receptor regulations, and secretions of chemokines [22, 23], including antitumor M1 macrophages and protumor M2 macrophages [16, 24–26]. Large amounts of transmitters, such as M-CSF, IL-6, IL-10, TGF-β, and COX-2, induce transformation of TAMs into M2 macrophages that secrete immune inhibitory chemokines with poorer antigen presenting and cytotoxic abilities, leading to tumor growth and metastasis [16, 21, 22, 27–34]. M2 macrophages and TAMs have protumor and immune inhibitory effects, secrete large amounts of IL-10, TGF-β, C-C motif chemokine ligand 17 (CCL17), and CCL22, attract noncytotoxic Treg and type II T-helper cells (TH2 cells) to aggregate in tumor

tissues, inhibit T-cell differentiation and functions, lower cytotoxic T-cell function, induce T-cell apoptosis, secrete CCL18, and attract naïve T cell [10, 16, 32, 35]. Immune modulatory and antitumor effects of mushroom beta-glucan have been noted by Ikekawa et al. in 1968 in the fruiting body extracts of *Lentinus edodes*, *Coriolus versicolor*, *Ganoderma tsugae*, *Flammulina velutipes,* and *Tricholoma matsutake* which have demonstrated significant antitumor activities towards transplanted tumor cells of sarcoma 180 [36–38]. Celecoxib is a COX-2 inhibitor and inhibits tumor growth by inhibiting synthesis of prostaglandin [39–46]. Nakanishi et al. noted that daily oral administration of celecoxib in tumor-bearing mice (colon cancer) resulted in enhanced secretion of IFN-γ by T cells and natural killer cells and altered the immune inhibitory effect in the tumor microenvironment, which in turn induced differentiation of macrophages into M1 macrophages and inhibited tumor growth [47]. We have discussed inflammation materials involved in the carcinogenesis [48]; furthermore in this study, we aim to investigate the mechanism in which mushroom beta-glucan modulates the TAM forward to the M1 macrophages and inhibits M2 macrophages, which in turn adequately inhibits tumor growth and metastasis in this examination.

2. Material and Methods

2.1. Experimental Protocol. A fixed dose of 1×10^6 cells/50 μL Lewis lung carcinoma cells (LLC1) was administered subcutaneously into the right inner thighs of 5 dependent C57BL/6JNarl mice (National Taiwan University Animal Reproduction and Research Center) for each group, followed by observation of tumor formation at the site of injection after two days. One day after the injection, mice were tube-fed with either twice-distilled water, celecoxib (Pfizer), or mushroom beta-glucan continuously for 12 days.

First, we examine whether the effect of *Antrodia camphorata* beta-glucan modulates the mice physiology response; the experiment group was divided into group 1: PC consisted of normal mice with subcutaneous injection of PBS, followed by oral feed of twice-distilled water; group 2: PB consisted of injection of PBS in normal mice, followed by oral feed of *Antrodia camphorata* beta-glucan. In the following experiment, we researched in the *Antrodia camphorata* beta-glucan involved in modulating the tumor microenvironment using the tumor-bearing mice. The experiment group was divided into group 3: TC consisted of tumor-bearing mice fed with twice-distilled water; group 4: TM consisted of mice fed with celecoxib; group 5: TB consisted of tumor-bearing mice fed with *Antrodia camphorata* beta-glucan; group 6: TX consisted of tumor-bearing mice fed with Ganoderma beta-glucan (Table 2). At day 14, mice were euthanized, and blood samples, tumor tissues, and lungs were collected or harvested. Tumor tissues were weighed, and samples from groups TM, TB, and TX were compared with that of group TC, by calculated tumor inhibitory rate, via ELISA to quantify serum TGF-β. RT-qPCR was used to quantify gene expression of IL-12, IFN-γ, GM-CSF, M-CSF, IL-6, IL-10, COX-2, and TGF-β in lung and tumor tissues. Flow cytometry was used to quantify the percentages of M1 and M2 macrophages in the lungs and tumor tissues.

2.2. Mushroom Polysaccharide. Our previous study has examined the safety assessment of mushroom beta-glucan [49]; moreover, in this experiment, mycelium of *Ganoderma lucidum* or *Antrodia camphorata* subcultured and maintained in sterile YM agar (0.02%) was used for the production of MBG. The manufacturing process was initiated by preparing a culture medium containing glucose, lactose, galactose, sucrose, mannose, and yeast extract. Mycelium of *Ganoderma lucidum* or *Antrodia camphorata* was then introduced into the sterile medium and cultured using a shaker incubator at temperatures ranging from 27 to 32°C for 3–5 weeks to achieve a full polymerization of MBG in the culture system. Subsequently, MBG from cultured mycelia was homogenized and disrupted using high speed homogenizer and ultrasonic vibration. The MBG solution was then filtered and concentrated using a ceramic membrane to strip most of the residual small molecules in the solution. The concentrated MBG was dried by lyophilization and then grinded into the powdered form. The sample was demonstrated to contain approximately 95% carbohydrate, 1% fat, 1% protein, 2% of ash, and 0.8% of water. Using Megazyme (Ireland) mushroom and yeast Beta-Glucan Kit, the crude extract was demonstrated to contain approximately 60–65% of MBG (MBG). The molecular weight of MBG was analyzed by high pressure liquid chromatographic (HPLC) using Shodex sugar KS series containing KS-G, KS-804, and KS-805 columns and detected using RI 2000 detector. Molecular weight was determined by referring to the standard cure using standard molecules including STDP-800 (molecular weight M.W. 8×10^5), STDP-400 (M.W. 4×10^5), STDP-200 (M.W. 2×10^5), STDP-100 (M.W. 1×10^5), and STDP-20 (M.W. 2×10^4). MBG was also processed for analysis of its glycosyl-linkage. The sample was premethylated, depolymerized, reduced, and acetylated. The resultant partially methylated alditol acetates (PMAAs) were then analyzed by gas chromatography-mass spectrometry (GC-MS) according to the procedures described by York et al. and Ciucanu and Kerek [50, 51].

Result from the HPLC analysis showed that MBG powder contained high molecular weight particles that ranged from 9.6 to 298 kDa. The result of GC-MS analysis showed that MBG powder contained 2-; 4-; and 6-; linked galactopyranosyl residues and 3-; 4-; 3,4-; 2,4-; 4,6-; and 3,4,6-linked glucopyranosyl residues.

2.3. Tumor-Bearing Mice. Lewis lung carcinoma cell (LLC, purchased from BCRC, Taiwan, BCRC #60050) was placed in 15 mL centrifuge tube with the cultured medium (DMEM, Sigma #D5648) and centrifuged at 200 ×g for five minutes. After removal of supernatant, cells were washed with PBS to further remove allergens in cell medium. Cells were then suspended in PBS culture medium at a concentration of 1×10^6 cells/50 μL and injected into the right inner thighs of the mice. Mice were treated, handled, and cared for following the NIH Guide, The Care and Use of Laboratory Animals. Tumor-bearing mice were euthanized prior to occurrence of cancer-associated symptoms that would limit the animal's

mobility or normal daily function. After the animals were euthanized, blood samples, tumor tissues, and lungs were collected or harvested.

2.4. Serum Concentration of TGF-β. Mouse TGF-β Platinum ELISA Kit (eBioscience) was used to quantify serum TGF-β. Experimental protocol published by Mouse TGF-βPlatinum ELISA Kit was followed, and samples were further analyzed with BioTek. Enzyme immunoassay analyzer (ELISA) was set at 450 nm.

2.5. Real-Time Polymerase Chain Reaction. RT-qPCR was used to analyze gene expressions of IL-12, IFN-γ, GM-CSF, M-CSF, IL-6, IL-10, COX-2, and TGF-β in the lungs, using β-actin as the reference gene. Experiments were conducted following the protocol published by SYBR Green Supermix Kits (Bio-Rad), using Bio-Rad CFX384 touch RT-PCR for analysis (Table 1).

2.6. Proportions of M1 and M2 Macrophages in the Lung and Tumor Cells

2.6.1. Centrifuge. Harvested lung and tumor tissues were cut into small pieces and placed in the culture medium and incubated with 5 mL PBS (containing 0.1 mg/mL collagenase (SIGMA)) at 37°C for 30 minutes, followed by passing of the ground tissues through steel mesh with 70 μm. The resulting cell suspension fluid was added into 5 mL Histopaque 1080 (SIGNMA) centrifuge tube and centrifuged for 30 minutes at 4°C, 400 ×g. After removing the supernatant and several rounds of washings with PBS to clear the Histopaque, FACS buffer was added to the cell suspension fluid to obtain final cell counts. Cell suspension fluid concentration was readjusted to 1×10^7 cells/mL and maintained at 4°C for the experiments.

2.6.2. Surface Marker Staining. 100 μL of suspension fluid was added to 0.25 μg APC anti-mouse F4/80 antibody (BioLegend), 1.0 μg PE anti-mouse CD86 antibody (BioLegend), and 0.125 μg FITC anti-mouse CD206 antibody (BioLegend), respectively. Homogenized solutions were placed in the dark at 4°C for 30 minutes, followed by addition of 200 μL FACS buffer, and centrifuged at 4°C, 300 ×g for 5 minutes. After removal of supernatant, the cellular solutions were washed with FACS buffer, followed by addition of 1 mL FACS buffer to resuspend cellular samples. Finally, cellular aggregates were broken up and analyzed with BD FACSCanto II, APC anti-mouse F4/80 antibody-specific M1 macrophage ($F4/80^+$), APC anti-mouse F4/80 antibody, and FITC anti-mouse CD206 antibody-specific M2 macrophage ($F4/80^+$, $CD206^+$). FlowJo software was used to analyze the percentage of macrophages in the lung tissues and the proportions of M1 and M2 macrophages in the lung and tumor tissue samples.

Percentage of macrophages: macrophages ($F4/80^+$)/cells.

Percentage of M1 macrophages: M1 ($F4/80^+$, $CD86^+$)/macrophages ($F4/80^+$).

Percentage of M2 macrophages: M2 ($F4/80^+$, $CD206^+$)/macrophages ($F4/80^+$).

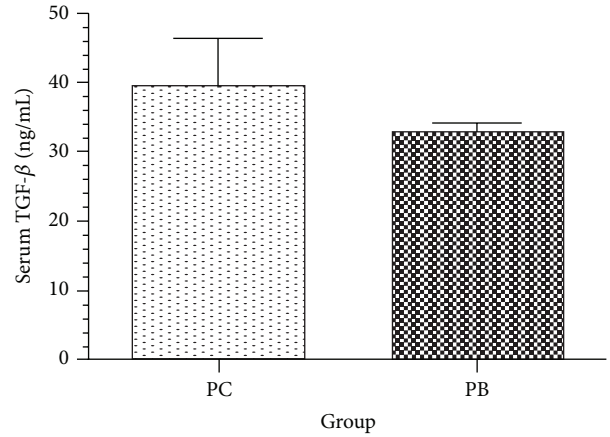


Figure 1: Effect of *Antrodia camphorata* beta-glucan on the amount of serum TGF-β. Normal mice were fed with either twice-distilled water or *Antrodia camphorata* beta-glucan daily and euthanized after 12 days. Blood samples were then collected ($n = 5$). *Group PC (control): fed with twice-distilled water; group PB (experiment): fed with *Antrodia camphorata* beta-glucan.

2.7. Statistical Analysis. Analysis was conducted using SPSS 17.0 software. One-way analysis of variance, one-way ANOVA, and Scheffe's method were conducted, with significance set at $P < 0.05$.

3. Results

3.1. Physiologic Effects of Antrodia camphorata Beta-Glucan on Normal Mice

3.1.1. Effect of Antrodia camphorata Beta-Glucan on Serum TGF-β Quantity in Normal Mice. Serum TGF-β quantity in PC group (control) was at 39.59 ± 5.645 ng/mL and compared to group PB (experiment) at 32.8 ± 1.879 ng/mL. There is no significant difference between the two groups ($P > 0.05$). Daily oral intake of *Antrodia camphorata* beta-glucan does not alter serum TGF-β in normal mice (Figure 1).

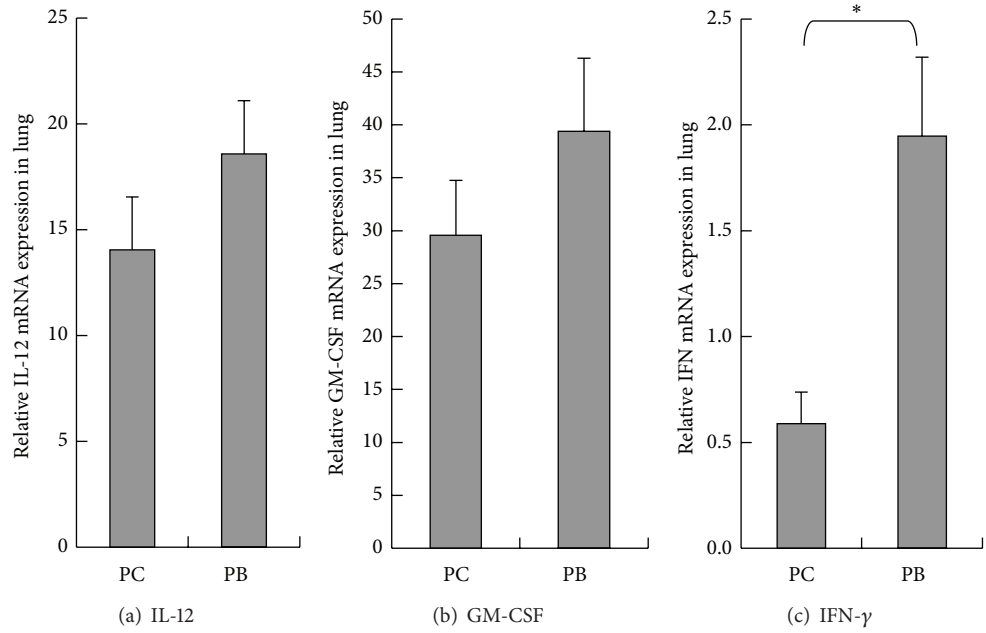
3.1.2. Effect of Antrodia camphorata Beta-Glucan on Quantities of Lung Cytokines and Gene Expression of Growth Factors in Normal Mice. As results shown in Figure 2(a), the amounts of IL-12 gene expression in the lungs of normal mice are as follows: group PB is at 0.0019 ± 0.00025, and group PC is lower at 0.0014 ± 0.00025. There is no statistical significant difference between groups PC and PB ($P > 0.05$). As results shown in Figure 2(b), the amounts of GM-CSF gene expression in the lungs of normal mice are as follows: group PB is at 39.3786 ± 6.90311,, and group PC is at 29.5757 ± 5.17426. There is no statistical significant difference between groups PC and PB ($P > 0.05$). As results shown in Figure 2(c), the amounts of IFN-γ gene expression in the lungs of normal mice are as follows: group PB is at 1.9469 ± 0.37199, and group PC is at 0.5895 ± 0.14802. The quantity of IFN-γ gene expression of group PB is statistically significantly higher than that of group PC ($P < 0.05$). As results shown in Figure 3(a), the amounts of M-CSF gene expression in

Table 1: Primer sequence.

Target Gene	GenBank #	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
β-actin	BC138614.1	AATCGTGCGTGACATCAA	AGAAGGAAGGCTGGAAAA
IFN-γ	BC119063.1	TCTGAGACAATGAACGCTAC	TTCCACATCTATGCCACT
GM-CSF	BC116880.1	GAAGATATTCGAGCAGGGTC	GAAATCCGCATAGGTGGT
IL-12	M86671.1	TGAAAGGCTGGGTATCGG	GCTGGAACTCCCTCTGTA
COX-2	BC052900.1	ATGACTGCCCAACTCCCA	AACCCAGGTCCTCGCTTA
IL-6	BC138766.1	TGCCTTCTTGGGACTGAT	TTGCCATTGCACAACTCTTT
M-CSF	M21149.1	TTCTACAAGTGGAAGTGGAGG	AGAGGGACATTGACAAACG
IL-10	BC137844.1	TTTCAAACAAAGGACCAG	GGATCATTTCCGATAAGG
TGF-β	M13177.1	GGCGGTGCTCGCTTTGTA	TTTCTCATAGATGGCGTTGTT

Table 2: Figure out the parameter detected in the presented study.

	Normal mice		Tumor-bearing mice			
	PC (distilled water)	PB (fed with Ganoderma beta-glucan)	TC (fed with distilled water)	TM (fed with celecoxib)	TB (fed with Ganoderma beta-glucan)	TX (fed with Ganoderma beta-glucan)
TGF-β (serum)	—	—	—	↓	↓	↓
IL-12 (lung)	—	—	—	—	—	—
IL-12 (tumor)	✗	✗	—	—	↑	↑
GM-CSF (lung)	—	—	—	—	—	—
GM-CSF (tumor)	✗	✗	—	—	—	—
IFN-γ (lung)	—	↑	—	—	—	—
IFN-γ (tumor)	✗	✗	—	↑	↑	↑
IL-10 (lung)	—	—	—	—	—	—
IL-10 (tumor)	✗	✗	—	↓	↓	↓
M-CSF (lung)	—	—	—	—	↓	↓
M-CSF (tumor)	✗	✗	—	↓	—	—
TGF-β (lung)	—	—	—	—	—	—
TGF-β (tumor)	✗	✗	—	↓	↓	↓
IL-6 (lung)	—	—	—	—	—	—
IL-6 (tumor)	✗	✗	—	↓	↓	↓
COX-2 (lung)	—	↓	—	—	—	—
COX-2 (tumor)	✗	✗	—	↓	↓	↓



(a) IL-12 (b) GM-CSF (c) IFN-γ

Figure 2: Effect of *Antrodia camphorata* beta-glucan on the amount of gene expressions of IL-12, GM-CSF, and IFN-γ in the lungs. Normal mice were fed with either twice-distilled water or *Antrodia camphorata* beta-glucan daily and euthanized after 12 days. Lungs were then harvested ($n = 5$). RT-qPCR was used to analyze gene expressions of IL-12, GM-CSF, and IFN-γ in the lungs. β-actin was used as the reference gene. (a) Amount of IL-12 gene expression. (b) Amount of GM-CSF gene expression. (c) Amount of IFN-γ gene expression. *Group PC: fed with twice-distilled water; group PB: fed with *Antrodia camphorata* beta-glucan.

the lungs of normal mice are as follows: group PC is higher at 0.5285 ± 0.07916, and group PB is lower at 0.5012 ± 0.04078. There is no statistical significant difference between groups PC and PB. As results shown in Figure 3(b), the amounts of IL-6 gene expression in the lungs of normal mice are as follows: group PB is higher at 0.1297 ± 0.03755, and group PC is lower at 0.1208 ± 0.03685. There is no statistical significant difference between groups PC and PB. As results shown in Figure 3(c), the amounts of IL-10 gene expression in the lungs of normal mice are as follows: group PB is higher at 0.0036 ± 0.00005, and group PC is lower at 0.0035 ± 0.00107. There is no statistical significant difference between groups PC and PB. As results shown in Figure 3(d), the amounts of COX-2 gene expression in the lungs of normal mice are as follows: group PC is higher at 0.0038 ± 0.00087, and group PB is lower at 0.0021 ± 0.00057. The quantity of COX-2 gene expression of group PB is statistically significantly lower than PC group ($P < 0.05$). As results shown in Figure 3(e), the amounts of TGF-β gene expression in the lungs of normal mice are as follows: group PC is higher at 2.1539 ± 0.5294, and group PB is lower at 1.6817 ± 0.07353. There is no statistical significant difference between groups PC and PB. Results shown in Figure 4(a) demonstrated higher group PC value at 36.32 ± 3.458% and lower group PB value at 35 ± 7.291%. There is no statistical significant difference between groups PC and PB in the lung M1 macrophage percentages. As results shown in Figure 4(b), percentages of M1 macrophages in the lung tissues of normal mice are higher in PB group at 4.41 ± 0.956% and lower in group PC at 3.33 ± 0.668%. There is no statistical significant difference between groups PC and PB. As results shown in Figure 4(c) for the percentages of M2 macrophage in the lung tissues, group PC is higher at 30.28 ± 1.612%, and group PB is lower at 25.86 ± 3.95%. There is no statistical significant difference between groups PC and PB.

3.2. Effect of Mushroom Beta-Glucan on Tumor-Bearing Mice

3.2.1. Effect of Mushroom Beta-Glucan on Serum TGF-β Quantity in Tumor-Bearing Mice. As results shown in Figure 5, amount of serum TGF-β in tumor-bearing mice is shown to be higher in group TC at 49.8 ± 12.454 ng/mL, followed by group TB at 33.64 ± 4.045 ng/mL and group TM at 28.45 ± 6.274 ng/mL, and lowest in group TX at 26.33 ± 5.901 ng/mL. Groups TM, TB, and TX show significantly lower serum TGF-β amount than group TC ($P < 0.05$). However, there are no statistically significant differences between groups TB, TX, and TM. Results show that daily oral intake of celecoxib or *Antrodia camphorata* beta-glucan in tumor-bearing mice lowers the amount of serum TGF-β in tumor-bearing mice.

3.2.2. Effect of Mushroom Beta-Glucan on Quantities of Lung Cytokines and Gene Expression of Growth Factors in Tumor-Bearing Mice. As results shown in Figure 6(a), the amounts of IL-12 gene expression in the lungs of tumor-bearing mice are the highest in group TB at 0.0048 ± 0.0023, followed by group TM at 0.0027 ± 0.00121 and group GX at 0.0024 ± 0.0007, and the lowest in group TC at 0.0018 ± 0.00056. There are no statistically significant differences between groups TM, TB, and TX. As results shown in Figure 6(b), the amounts of GM-CSF gene expression in the lungs of tumor-bearing mice are shown to be the highest in group TB at 40.4202 ± 16.8, followed by group TX at 29.9667 ± 3.67509 and group TM at 28.7425 ± 4.58706, and the lowest in group TC

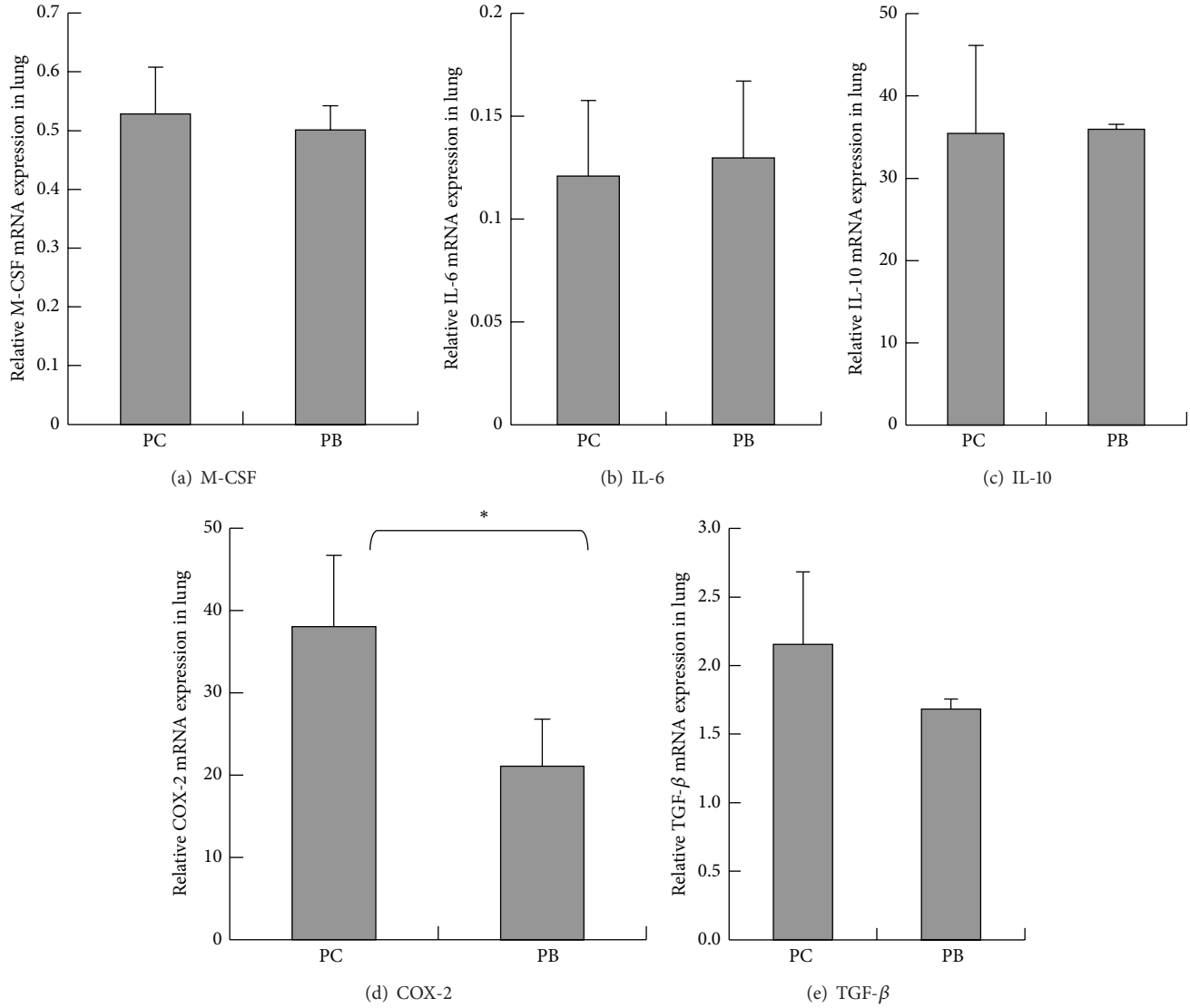


Figure 3: Effect of *Antrodia camphorata* beta-glucan on the amount of gene expressions of M-CSF, IL-6, IL-10, COX-2, and TGF-β in the lungs. Normal mice were fed with distilled water, *Antrodia camphorata* beta-glucan, or Ganoderma beta-glucan daily and euthanized after 12 days. Lungs were then harvested ($n = 5$). RT-qPCR was used to quantify the gene expressions of M-CSF, IL-6, IL-10, COX-2, and TGF-β in the lungs. β-actin was used as the reference gene. (a) Amount of M-CSF gene expression. (b) Amount of IL-6 gene expression. (c) Amount of IL-10 gene expression. (d) Amount of COX-2 gene expression. (e) Amount of TGF-β gene expression. *Group PC: fed with twice-distilled water; group PB: fed with *Antrodia camphorata* beta-glucan.

at 23.7612 ± 7.77548. There are no statistically significant differences between groups TM, TB, TX, and TC. As results shown in Figure 6(c), the amounts of IFN-γ gene expression in the lungs of tumor-bearing mice are the highest in group TM at 2.6666 ± 2.10062, followed by group TB at 1.8043 ± 0.31818 and group TC at 1.2801 ± 0.28564, and the lowest in group TX at 1.1816 ± 0.17898. There are no statistically significant differences between groups TM, TB, TX, and TC. As results shown in Figure 7(a), the amounts of M-CSF gene expression in the lungs of tumor-bearing mice are the highest in group TC at 0.4965 ± 0.1044, followed by group TM at 0.4098 ± 0.0458 and group TX at 0.3341 ± 0.02674, and the lowest in group TB at 0.2865 ± 0.06564. The amounts of M-CSF gene expression in the lungs of tumor-bearing mice are statistically significantly lower in groups TB and TX when compared to group TM but not statistically significantly different when compared to groups TC and TM. As results shown in Figure 7(b), the amounts of IL-6 gene expression in the lungs of tumor-bearing mice are the highest in group TC at 0.1788 ± 0.06732, followed by group TX at 0.1505 ± 0.05891 and group TB at 0.1199 ± 0.02936, and the lowest in group TM at 0.0705 ± 0.04733. There are no statistically significant differences between groups TM, TB, TX, and TC. As result shown in Figure 7(c), the amounts of IL-10 gene expression in the lungs of tumor-bearing mice are the highest in group TM at 0.0079 ± 0.00559, followed by group TC at

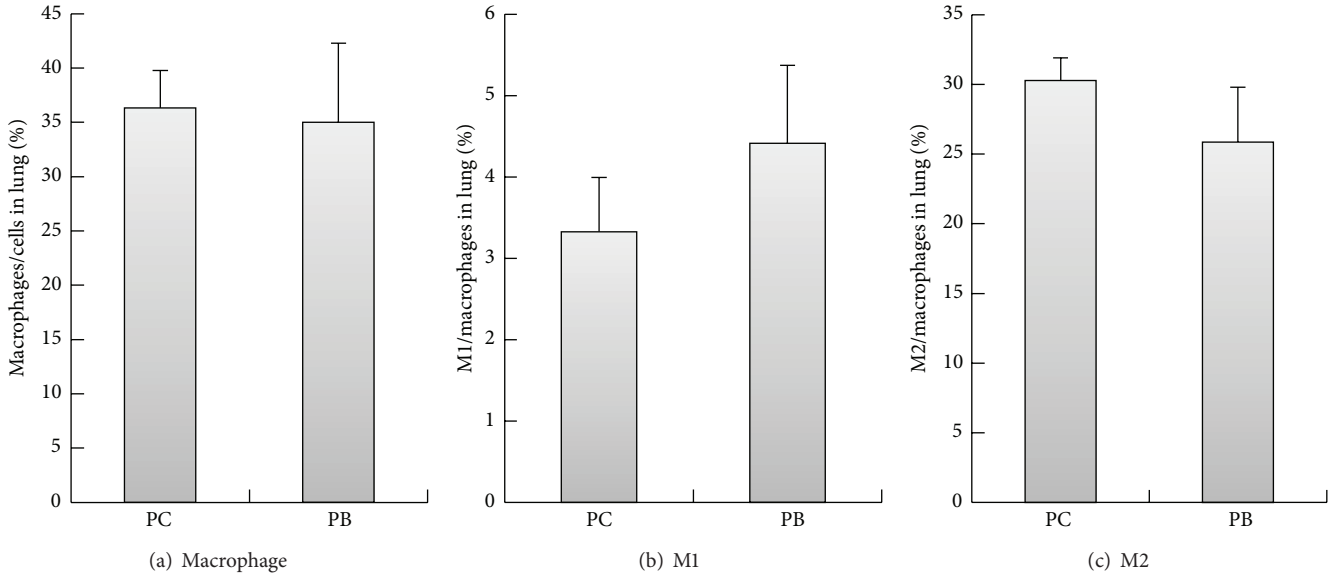


Figure 4: Effect of *Antrodia camphorata* beta-glucan on the percentages of macrophages and proportions of M1 and M2 macrophages. Normal mice were fed with distilled water or *Antrodia camphorata* beta-glucan daily and euthanized after 12 days. Lungs were then harvested ($n = 5$). Flow cytometer was used to analyze the percentages of M1 and M2 macrophages in the lungs. (a) Percentages of macrophages. (b) Percentages of M1 macrophages. (c) Percentages of M2 macrophages. *Group PC: fed with twice-distilled water; group PB: fed with *Antrodia camphorata* beta-glucan.

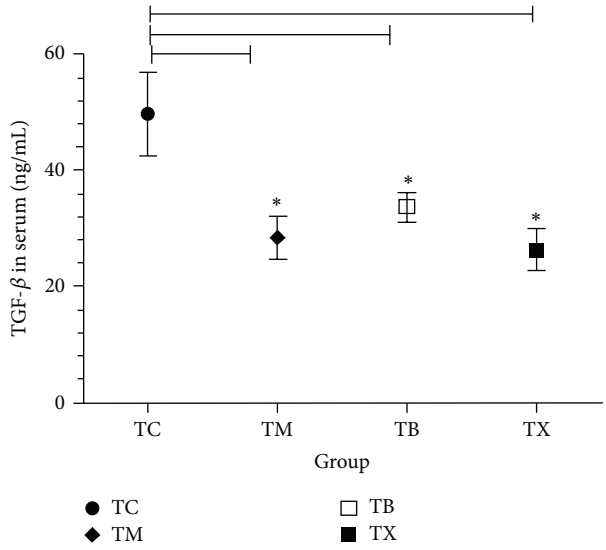
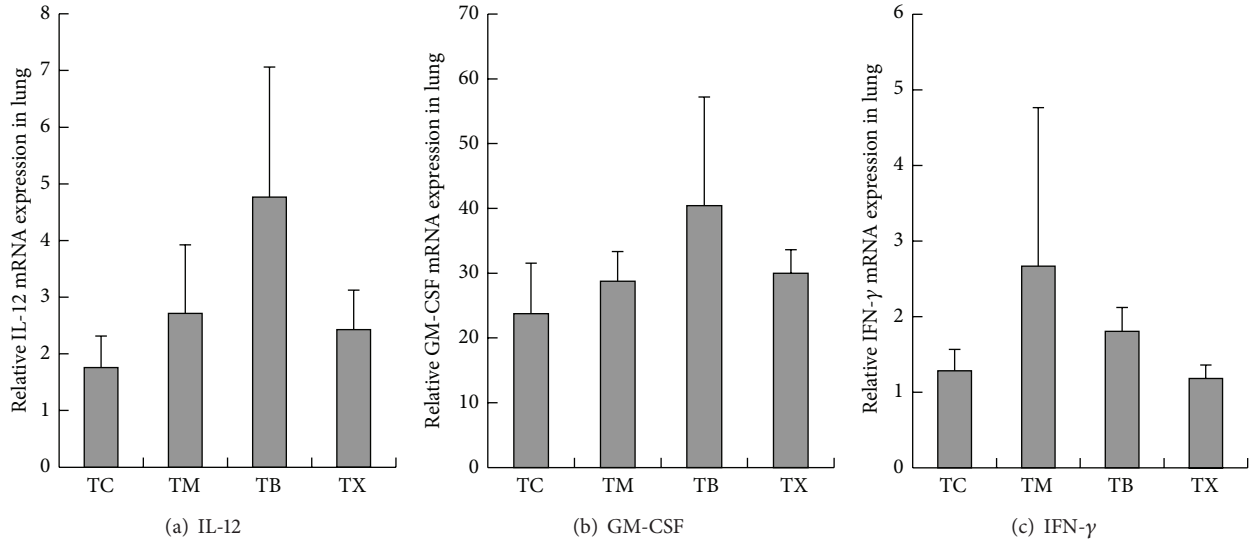


Figure 5: Effect of *Antrodia camphorata* beta-glucan on the amount of serum TGF-β in tumor-bearing mice. Tumor-bearing mice were fed with either twice-distilled water, celecoxib, *Antrodia camphorata* beta-glucan, or Ganoderma beta-glucan daily and euthanized after 12 days. Blood samples were then collected ($n = 5$). TGF-β Platinum ELISA Kit was used to quantify amount of serum TGF-β. *Group TC: fed with twice-distilled water; group TM: fed with celecoxib; group TB: fed with *Antrodia camphorata* beta-glucan; group TX: fed with Ganoderma beta-glucan.

0.0072 ± 0.00153 and group TB at 0.0033 ± 0.00246, and the lowest in group TX at 0.0026 ± 0.0003. As results shown in Figure 7(d), the amounts of COX-2 gene expression in the lungs of tumor-bearing mice are the highest in group TC at 0.0048 ± 0.00127, followed by group TM at 0.0039 ± 0.00108 and group TB at 0.0031 ± 0.00141, and the lowest in group TX at 0.0028 ± 0.00128. There are no statistically significant differences between groups TM, TB, TX, and TC. As results shown in Figure 7(e), the amounts of TGF-β gene expression in the lungs of tumor-bearing mice are the highest in group TC at 2.058 ± 0.31498, followed by group TM at 1.9589 ± 0.43123 and group TX at 1.9361 ± 0.36897, and the lowest in group TB at 1.9032 ± 0.49164. There are no statistically significant differences between groups TM, TB, TX, and TC.



(a) IL-12 (b) GM-CSF (c) IFN-γ

Figure 6: Effect of *Antrodia camphorata* beta-glucan on the gene expressions of IL-12, GM-CSF, and IFN-γ in the lungs of tumor-bearing mice. Tumor-bearing mice were fed with either twice-distilled water, celecoxib, *Antrodia camphorata* beta-glucan, or Ganoderma beta-glucan daily and euthanized after 12 days. Lungs were then harvested ($n = 5$). RT-qPCR was used to quantify the amount of gene expressions of IL-12, GM-CSF, and IFN-γ. *Group TC: fed with twice-distilled water; group TM: fed with celecoxib; group TB: fed with *Antrodia camphorata* beta-glucan; group TX: fed with Ganoderma beta-glucan.

Composite results show that daily oral intake of mushroom beta-glucan in tumor-bearing mice can lower the amounts of M-CSF gene expression in the lungs but does not affect the amounts of IL-12, GM-CSF, IFN-γ, IL-6, IL-10, COX-2, and TGF-β in the lungs.

3.2.3. Effect of Mushroom Beta-Glucan on Lung Macrophages and Proportions of M1 and M2 Macrophages in Tumor-Bearing Mice. As results shown in Figure 8(a), the percentages of macrophages in lung tissues of tumor-bearing mice are the highest in group TX at 43.94 ± 5.396%, followed by group TM at 38.15 ± 3.385% and group TB at 36.66 ± 7.19%, and the lowest in group TC at 36.56 ± 2.753%. There are no statistically significant differences between groups TM, TB, TX, and TC. As results shown in Figure 8(b), the percentages of M1 macrophages in lung tissues of tumor-bearing mice are the highest in group TX at 5.64 ± 0.734%, followed by group TB at 4.77 ± 1.364% and group TM at 4.66 ± 0.493%, and the lowest in group TC at 4.41 ± 1.142%. There are no statistically significant differences between groups TM, TB, TX, and TC. As results shown in Figure 8(c), the percentages of M2 macrophages in lung tissues of tumor-bearing mice are the highest in group TM at 31.17 ± 9.989%, followed by group TB at 28.98 ± 2.766% and group TC at 24.54 ± 2.621%, and the lowest in group TX at 22.73 ± 2.538%. There are no statistically significant differences between groups TM, TB, TX, and TC.

3.2.4. Effect of Mushroom Beta-Glucan on Tumor Tissue Cytokines and Gene Expression of Growth Factors in Tumor-Bearing Mice. As results shown in Figure 9(a), the amounts of IL-12 gene expression in tumor tissues of tumor-bearing mice are the highest in group TX at 0.005 ± 0.0025, followed by group TB at 0.0053 ± 0.00059 and group TC at 0.0023 ± 0.00044, and the lowest in group TM at 0.0016 ± 0.0004. The amounts of IL-12 gene expression in tumor tissues are statistically significantly higher in groups TB and TX when compared to groups TC and TM ($P < 0.05$). However, there is no statistical difference between groups TM and TC. As results shown in Figure 9(b), the amounts of GM-CSF gene expression in tumor tissues of tumor-bearing mice are the highest in group TX at 3.3869±2.38866, followed by group TB at 3.1262 ± 1.58598 and group TC at 2.377 ± 0.76023, and the lowest in group TM at 1.4979 ± 0.99286. There are no statistical differences between groups TM, TB, TX, and TC. As results shown in Figure 9(c), the amounts of IFN-γ gene expression in tumor tissues of tumor-bearing mice are the highest in group TX at 4.755 ± 1.37064, followed by group TB at 2.2302 ± 0.97283 and group TM at 2.1372 ± 0.46061, and the lowest in group TC at 0.4663 ± 0.16811. The amounts of IFN-γ gene expression in tumor tissues of tumor-bearing mice are statistically significantly higher in groups TM, TB, and TX when compared to group TC ($P < 0.05$), and group TX was statistically significantly higher in group TX than TM ($P < 0.05$). As results shown in Figure 10(a), the amounts of M-CSF gene expression in tumor tissues of tumor-bearing mice are the highest in group TC at 2.1827±0.59147, followed by group TB at 1.5069 ± 0.39195 and group TX at 1.1425 ± 0.71354, and the lowest in group TM at 0.6412 ± 0.2704. The amounts M-CSF gene expression in tumor tissues of tumor-bearing mice are statistically significantly lower in group TM when compared to group TC ($P < 0.05$). However, there are no statistical differences between groups TB, TX, and TC. As results shown in Figure 10(b), the amounts of IL-6 gene expression in tumor tissues of tumor-bearing mice are the highest in group TC at 1.0398 ± 0.24445, followed by group TB at 0.3408 ± 0.03372 and group TM at 0.2742 ± 0.06911, and the lowest in group TX at 0.2463 ± 0.17439. The amounts

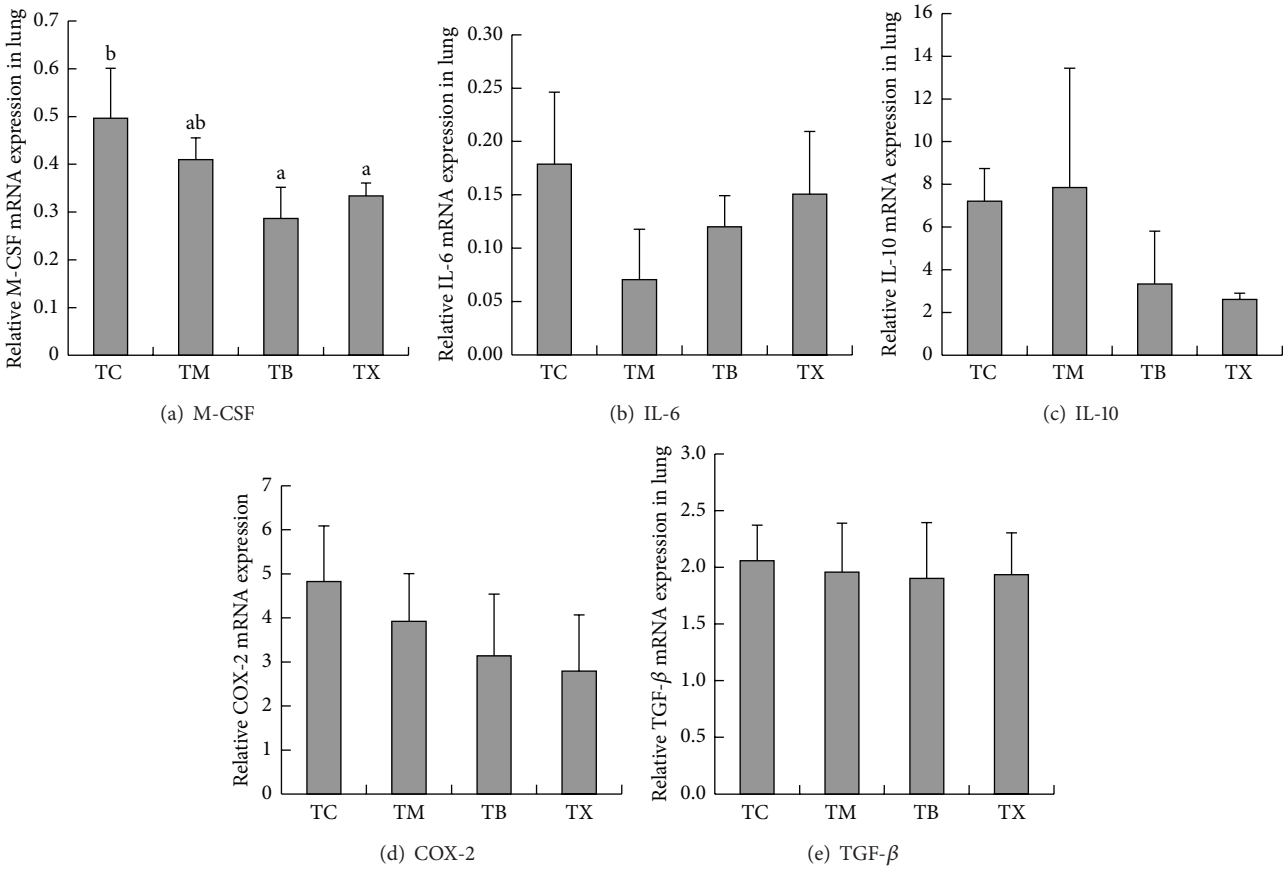


Figure 7: Effect of Ganoderma beta-glucan on the gene expressions of M-CSF, IL-6, IL-10, COX-2, and TGF-β in the lungs of tumor-bearing mice. Tumor-bearing mice were fed with either twice-distilled water, celecoxib, *Antrodia camphorata* beta-glucan, or Ganoderma beta-glucan daily and euthanized after 12 days. Lungs were then harvested ($n = 5$). RT-qPCR was used to quantify the amount of gene expressions of M-CSF, IL-6, IL-10, COX-2, and TGF-β in the lungs. β-actin was used as the reference gene. (a) Amount of M-CSF gene expression. (b) Amount of IL-6 gene expression. (c) Amount of IL-10 gene expression. (d) Amount of COX-2 gene expression. (e) Amount of TGF-β gene expression. *Group TC: fed with twice-distilled water; TM: fed with celecoxib; group TB: fed with *Antrodia camphorata* beta-glucan; group TX: fed with Ganoderma beta-glucan.

IL-6 gene expression in tumor tissues of tumor-bearing mice are statistically significantly lower in groups TM, TB, and TX when compared to group TC ($P < 0.05$). However, there are no statistical differences between groups TB, TX, and TM. As results shown in Figure 10(c), the amounts of IL-10 gene expression in tumor tissues of tumor-bearing mice were demonstrated to be the highest in group TC at 0.0332 ± 0.01199, followed by group TB at 0.0096 ± 0.0042 and group TM at 0.0085 ± 0.00505, and lowest in group TX at 0.0084 ± 0.01108. The amounts IL-10 gene expression in tumor tissues of tumor-bearing mice are statistically significantly lower in groups TM, TB, and TX when compared to group TC ($P < 0.05$). However, there are no statistical differences between groups TB, TX, and TM. As results shown in Figure 10(d), the amounts of COX-2 gene expression in tumor tissues of tumor-bearing mice are the highest in group TC at 0.1975 ± 0.05064, followed by group TB at 0.0742 ± 0.00202 and group TX at 0.0527 ± 0.03357, and the lowest in group TM at 0.045 ± 0.02347. The amounts COX-2 gene expression in tumor tissues of tumor-bearing mice are statistically significantly lower in groups TM, TB, and TX when compared to group TC ($P < 0.05$). However, there are no statistical differences between groups TB, TX, and TM. As results shown in Figure 10(e), the amounts of TGF-β gene expression in tumor tissues of tumor-bearing mice are the highest in group TC at 2.8931 ± 0.18312, followed by group TM at 1.2829 ± 0.13795 and group TX at 1.1744 ± 0.8451, and the lowest in group TB at 1.1555 ± 0.66985. The amounts of TGF-β gene expression in tumor tissues of tumor-bearing mice are statistically significantly lower in groups TM, TB, and TX when compared to group TC ($P < 0.05$). However, there are no statistical differences between groups TB, TX, and TM. Composite results show that daily oral intake of celecoxib in tumor-bearing mice can lower the amounts of M-CSF, IL-6, IL-10, COX-2, and TGF-β gene expression in tumors of tumor-bearing mice. Daily oral intake of mushroom beta-glucan in tumor-bearing mice increases the amounts of IL-12 and IFN-γ gene expression and lowers the gene expression of IL-6, IL-10, COX-2, and TGF-β in tumor tissues of tumor-bearing mice.

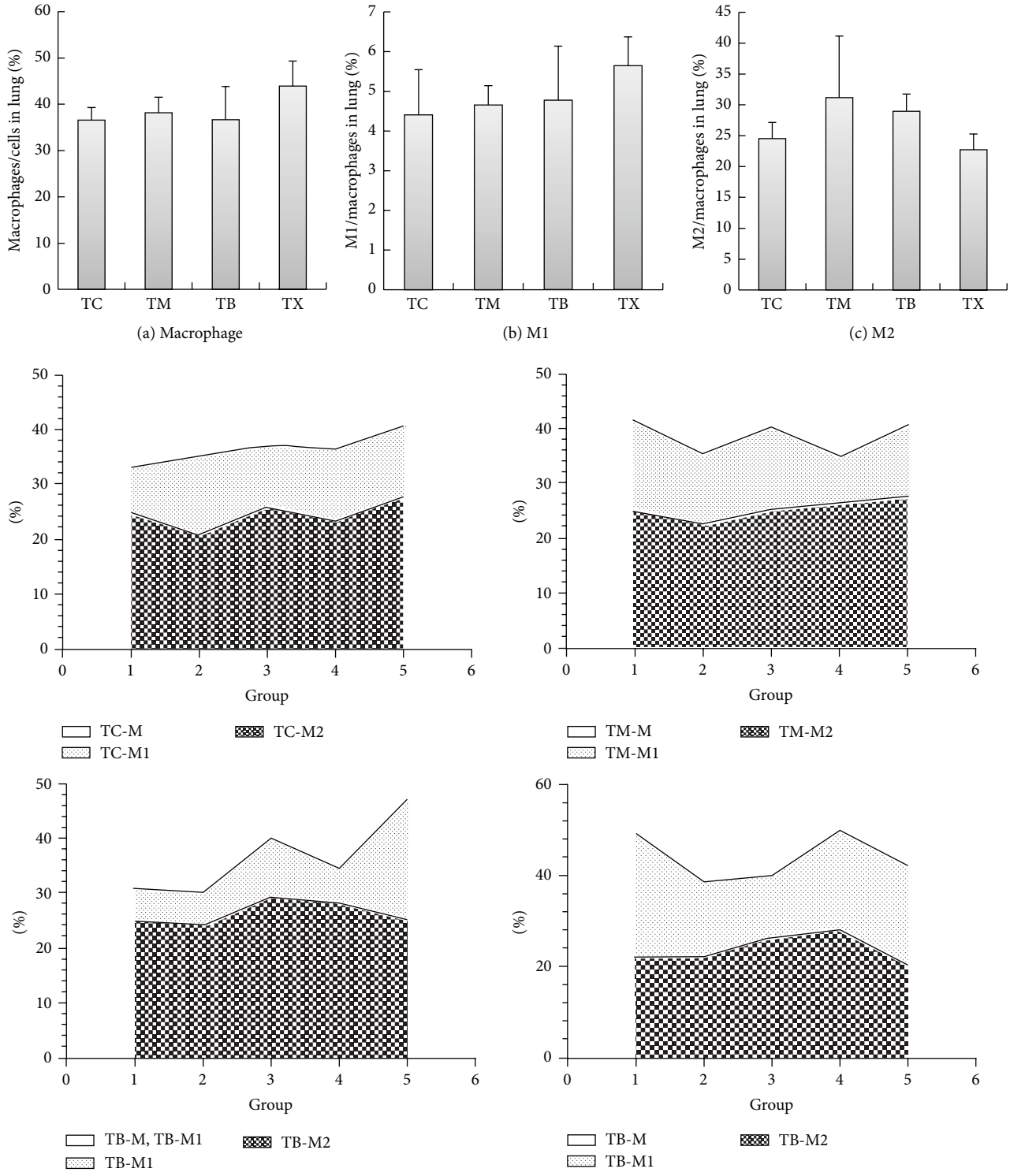
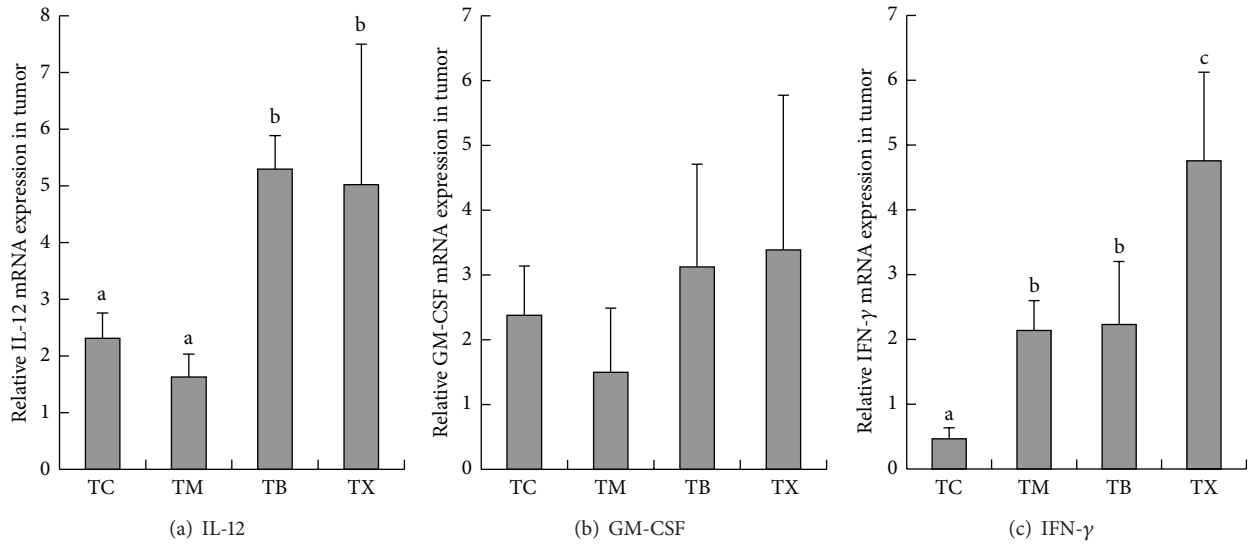


Figure 8: Effect of *Antrodia camphorata* beta-glucan on the percentages of macrophages and proportions of M1 and M2 macrophages in the lungs of tumor-bearing mice. Tumor-bearing mice were fed with either twice-distilled water, celecoxib, *Antrodia camphorata* beta-glucan, or Ganoderma beta-glucan daily and euthanized after 12 days. Lungs were then harvested ($n = 5$). Flow cytometer was used to analyze the percentages of macrophages and proportions of M1 and M2 macrophages in the lungs. (a) Percentages of macrophages. (b) Percentages of M1 macrophages. (c) Percentages of M2 macrophages. *Group TC: fed with twice-distilled water; TM: fed with celecoxib; group TB: fed with *Antrodia camphorata* beta-glucan; group TX: fed with Ganoderma beta-glucan.



(a) IL-12 (b) GM-CSF (c) IFN-γ

Figure 9: Effect of *Antrodia camphorata* beta-glucan on the amount of gene expressions of IL-12, GM-CSF, and IFN-γ in the tumor microenvironment of tumor-bearing mice. Tumor-bearing mice were fed with either twice-distilled water, celecoxib, *Antrodia camphorata* beta-glucan, or Ganoderma beta-glucan daily and euthanized after 12 days. Tumor tissues were then harvested ($n = 5$). RT-qPCR was used to quantify gene expressions of IL-12, GM-CSF, and IFN-γ in the tumor microenvironment. β-actin was used as the reference gene. *Group TC: fed with twice-distilled water; TM: fed with celecoxib; group TB: fed with *Antrodia camphorata* beta-glucan; group TX: fed with Ganoderma beta-glucan.

3.2.5. Effect of Mushroom Beta-Glucan on TAMs and Proportions of M1 and M2 Macrophages in Tumor Tissue in Tumor-Bearing Mice. As results shown in Figure 11(a), the percentages of macrophages in tumors of tumor-bearing mice are the highest in group TM at 31.23 ± 7.056%, followed by group TC at 29.64 ± 5.186% and group TB at 27.84 ± 7.739%, and the lowest in group TX at 26.46 ± 3.546%. There are no statistical differences between groups TM, TB, TX, and TC. As results shown in Figure 11(b), the percentages of M1 macrophages in tumors of tumor-bearing mice are the highest in group TX at 6.02 ± 0.759%, followed by group TM at 5.38 ± 2.168% and group TB at 3.29 ± 0.262%, and the lowest in group TC at 2.63 ± 0.412%. The percentages of M1 macrophages in tumors of tumor-bearing mice are statistically significantly higher in groups TM and TX when compared to group TC ($P < 0.05$). However, there is no statistical difference between groups TB and TC. As results shown in Figure 11(c), the percentages of M2 macrophages in tumors of tumor-bearing mice are the highest in group TC at 49.85 ± 3.297%, followed by group TB at 41.27 ± 5.689% and group TX at 34.83 ± 5.254%, and the lowest in group TM at 30.4±9.496%. The percentages of M2 macrophages in tumors of tumor-bearing mice are statistically significantly lower in groups TM and TX when compared to group TC ($P < 0.05$). However, there is no statistical difference between groups TB and TC. Composite results show that daily oral intake of celecoxib or mushroom beta-glucan in tumor-bearing mice can increase the percentage of M1 macrophages and lower the percentage of M2 macrophages in tumor-bearing mice.

4. Discussions

Nakanishi et al. found that celecoxib can alter the immune inhibitory effects of the tumor microenvironment by promoting transformation of TAMs into M1 macrophages, leading to inhibited tumor growth [47]. In our study, control group consisted of mice fed with celecoxib. After subcutaneous injection of LLC1 tumor cells and tumor development at the injection site, tumor-bearing mice were tube-fed with distilled water (group TC), celecoxib (group TM), *Antrodia camphorata*-derived beta-glucan (group TB), or *Ganoderma lucidum*-derived beta-glucan (group TX), respectively, for 12 consecutive days, and tumor sizes were recorded. Our study found that oral intake of celecoxib slowed tumor growth by 48.15%. In 1968, Ikekawa et al. found that the fruiting body extracts from *Lentinus edodes*, *Trametes versicolor*, *Ganoderma tsugae*, *Flammulina velutipes,* and *Tricholoma matsutake* demonstrated significant antitumor activities towards transplanted tumor cells of sarcoma 180 [36, 37, 52, 53].

In previous studies, *Antrodia camphorata*-derived beta-glucan has demonstrated inhibitory effects on tumor growth for sarcoma 37, sarcoma 180, Ehrlich ascites sarcoma, Yoshida sarcoma, and LLC1 transplanted tumor growth [54]. Daily intake of *Antrodia camphorata*-derived beta-glucan for 18 consecutive days has been demonstrated to slow tumor growth and reduce the rate of metastasis [55]. Cytotoxic T-cells activity and tumor occurrence rate were observed. Results showed that daily oral intake of *Grifola frondosa*-derived beta glucan or Lentinan can enhance cytotoxic T-cells activity and decrease tumor occurrence rate [56]. Additionally, they found that the addition of conditioned medium with tumor cells into the progenitor cells of dendritic cells can further inhibit maturation of dendritic cells and lower the antigen presenting capability of the dendritic cells [57]. Tumor cells were found to secrete M-CSF, inhibiting dendritic and T-cell differentiation and antitumor abilities [1, 57–59]. Our studies found that daily oral intake of

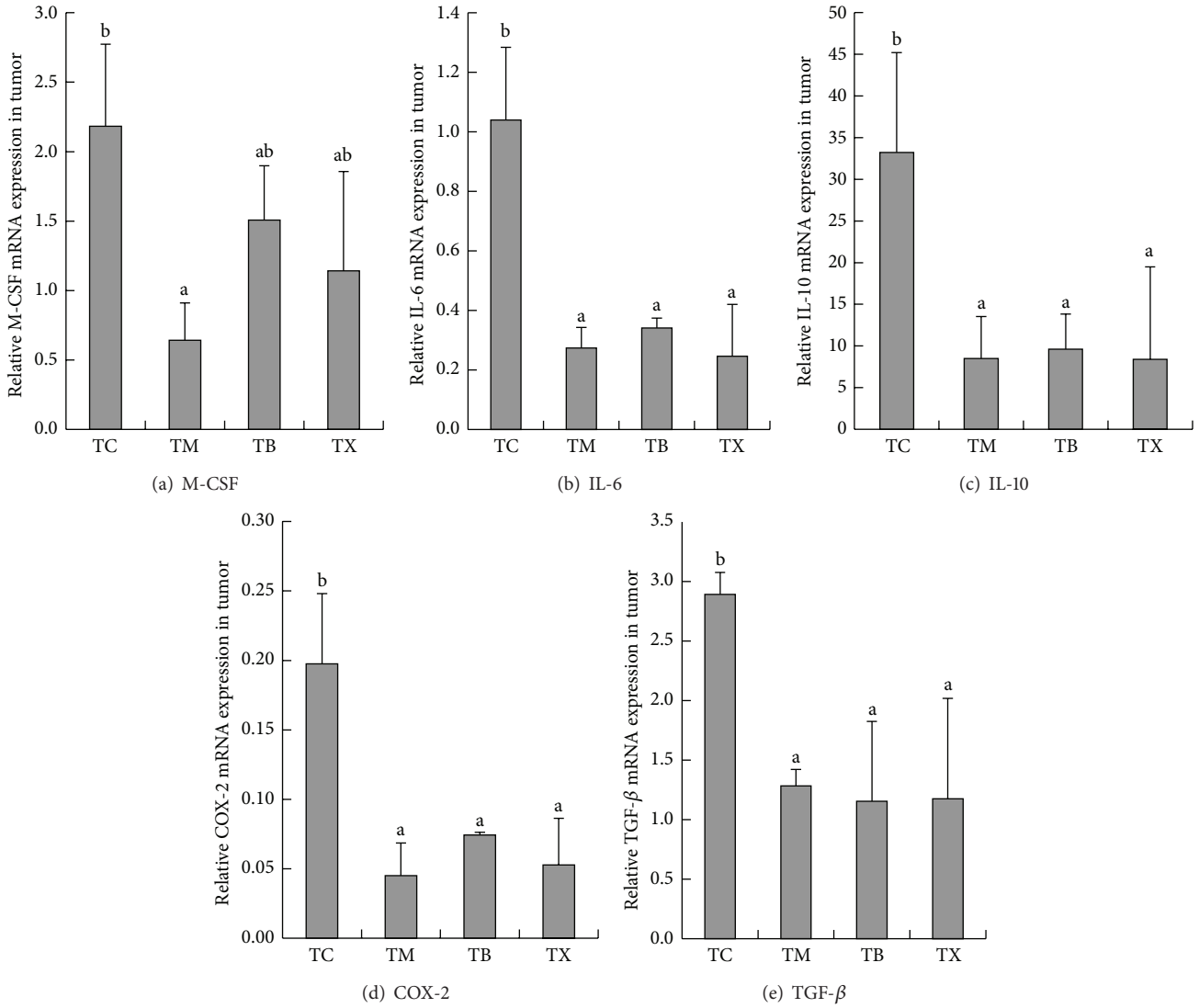
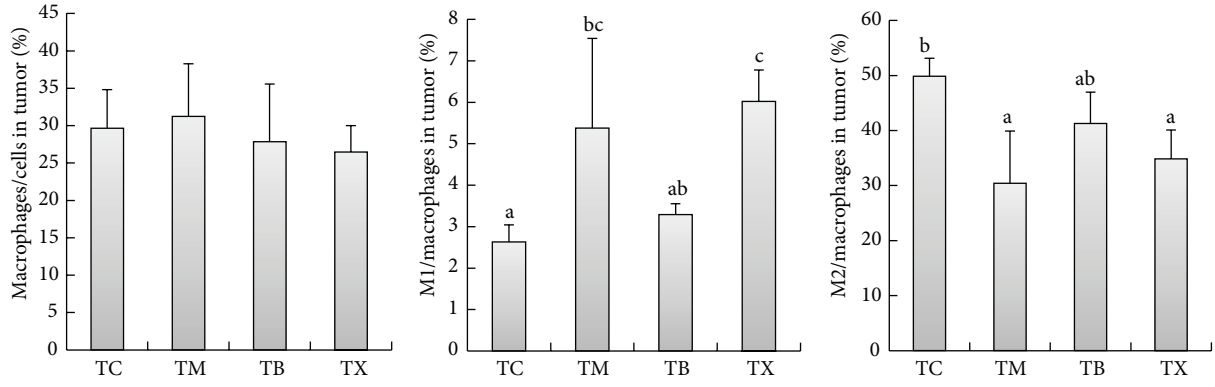


Figure 10: Effect of *Antrodia camphorata* beta-glucan on the amount of gene expressions of M-CSF, IL-6, IL-10, COX-2, and TGF-β in the tumor microenvironment of tumor-bearing mice. Tumor-bearing mice were fed with either twice-distilled water, celecoxib, *Antrodia camphorata* beta-glucan, or Ganoderma beta-glucan daily and euthanized after 12 days. Tumor tissues were then harvested ($n = 5$). RT-qPCR was used to quantify gene expressions of M-CSF, IL-6, IL-10, COX-2, and TGF-β. β-actin was used as the reference gene. (a) Amount of M-CSF gene expression. (b) Amount of IL-6 gene expression. (c) Amount of IL-10 gene expression. (d) Amount of COX-2 gene expression. (e) Amount of TGF-β gene expression. *Group TC: fed with twice-distilled water; TM: fed with celecoxib; group TB: fed with *Antrodia camphorata* beta-glucan; group TX: fed with Ganoderma beta-glucan.

mushroom beta-glucan from *Antrodia camphorata* or *Ganoderma lucidum* in tumor-bearing mice can reduce the amount of M-CSF gene expression in the lungs and that daily oral intake of celecoxib in tumor-bearing mice can reduce the amount of M-CSF gene expression in the tumor tissues.

The presented research has indicated that anticancer drugs are generally plagued by toxic manifestations at doses necessary for control of various forms of cancer; in order to alternate the side effect of the anticancer drug, some antioxidants and immunomodulators such as tuftsin [60, 61], picroliv [62], and medical mushroom [63, 64] have also been applied to impart significant antitumor activity presumably by nonspecific activation of the host immune system [65]. In this presented research, we exactly to examine the polysaccharide from medical mushroom to apply in the cancer therapy, and the data shown that the observation of the inflammation and toxicity response not to be significantly presented while feeding with the polysaccharide in the normal (control) group, but the function of anticancer by immunomodulation was to be observed in the tumor-bearing mice. We preliminarily conclude that daily oral intake of mushroom beta-glucan from *Antrodia camphorata* and *Ganoderma lucidum* in tumor-bearing mice can reduce the amount of M-CSF gene expression in the lungs. Daily oral intake of mushroom beta-glucan from *Antrodia camphorata* and *Ganoderma lucidum* in tumor-bearing mice can reduce



(a) TAM (b) M1 (c) M2

Figure 11: Effect of *Antrodia camphorata* beta-glucan on the percentages of macrophages and proportions of M1 and M2 macrophages in the tumor microenvironment of tumor-bearing mice. Tumor-bearing mice were fed with distilled water, celecoxib, *Antrodia camphorata* beta-glucan, or Ganoderma beta-glucan daily and euthanized after 12 days. Tumor tissue samples were then harvested ($n = 5$). Flow cytometer was used to analyze percentages of macrophages and proportions of M1 and M2 macrophages in the tumor microenvironment. (a) Percentages of macrophages in the tumor. (b) Percentages of M1 macrophages in the tumor. (c) Percentages of M2 macrophages in the tumor. *Group TC: fed with twice-distilled water; group TM: fed with celecoxib; group TB: fed with *Antrodia camphorata* beta-glucan; group TX: fed with Ganoderma beta-glucan.

the amount of M-CSF gene expression and in turn enhance differentiation of dendritic cells and their antigen presenting ability. Daily oral intake of celecoxib in tumor-bearing mice can lower the amount of gene expression of M-CSF by the tumor tissues, enhance differentiation of dendritic cell and T cells, and in turn reduce the immune-inhibitory effect of the tumor environment and inhibit the immune inhibitory effect of the tumor environment, further inhibiting tumor growth. In the tumor environment, the amounts of M1 and M2 macrophages are not equal [66]. It is currently known that tumor environment contains large amount of transmitters such as M-CSF, IL-6, IL-10, TGF-β, and COX-2 that induces tumor megakaryocytes to differentiate into M2 macrophages, which, in addition to having poorer antigen-presenting and cytotoxic abilities, also secretes factors that inhibit immune cells, resulting in enhanced immune inhibitory effect of the tumor environment [16, 21, 22, 27–34]. M2 macrophage in the tumor-bearing mice enhances tumor growth and immune inhibitory effects. They also secrete cytokines, such as IL-10 and TGF-β, in high quantities, that attract noncytotoxic Treg-cells and type 2 helper T cells to congregate in tumor tissues, which in turn inhibit the differentiation and normal functions of T cells, including their cytotoxic ability, and further lead to T-cells apoptosis [16, 32, 35, 67, 68].

Our study found that daily oral intake of celecoxib or mushroom beta-glucan from *Antrodia camphorata* can decrease the gene expression of IL-6, IL-10, COX-2, and TGF-β and further decreases the proportion of M2 macrophages in tumor-bearing mice. Based on these results, oral intake of celecoxib or mushroom beta-glucan from *Antrodia camphorata* can decrease the gene expression of IL-6, IL-10, COX-2, and TGF-β and further decreases the proportion of M2 macrophages in tumor-bearing mice, as well as decreasing the secretion of cytokines, such as IL-10 and TGF-β that decreases the immune inhibitory effect in the tumor environment.

5. Conclusion

Oral intake of mushroom beta-glucan in tumor-bearing mice demonstrated an increase in the gene expression of IL-12 and IFN-γ in tumor tissues and a decrease in serum TGF-β concentration and gene expressions of IL-6, IL-10, COX-2, and TGF-β in the tumor microenvironment. Our study found that mushroom beta-glucan can reduce the immune inhibitory effects of the tumor microenvironment in the host. Alteration of the tumor microenvironment promotes transformation of TAMs into M1 macrophages and reduces the transformation of TAMs into M2 macrophages.

Conflict of Interests

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this paper.

Authors' Contribution

Wan-Jhen Wang and Yu-Sheng Wu contributed equally to this work.

References

[1] J. A. Joyce and J. W. Pollard, "Microenvironmental regulation of metastasis," *Nature Reviews Cancer*, vol. 9, no. 4, pp. 239–252, 2009.

[2] L. M. Coussens and Z. Werb, "Inflammation and cancer," *Nature*, vol. 420, no. 6917, pp. 860–867, 2002.

[3] G. Baronzio, G. Fiorentini, and C. R. Cogle, *Cancer Microenvironment and Therapeutic Implications*, Springer, Berlin, Germany, 2009.

[4] F. Xing, J. Saidou, and K. Watabe, "Cancer associated fibroblasts (CAFs) in tumor microenvironment," *Frontiers in Bioscience*, vol. 15, no. 1, pp. 166–179, 2010.

[5] A. K. Nandi, S. Samanta, S. Maity et al., "Antioxidant and immunostimulant β-glucan from edible mushroom *Russula albonigra* (Krombh.) Fr," *Carbohydrate Polymers*, vol. 99, pp. 774–782, 2014.

[6] G. Castello, S. Scala, G. Palmieri, S. A. Curley, and F. Izzo, "HCV-related hepatocellular carcinoma: from chronic inflammation to cancer," *Clinical Immunology*, vol. 134, no. 3, pp. 237–250, 2010.

[7] C. A. Janeway, M. Walport, and P. Travers, *Immunobiology: The Immune System in Health and Disease*, Garland Science, 2005.

[8] S. Samanta, A. K. Nandi, I. K. Sen et al., "Structural characterization of an immunoenhancing glucan isolated from a mushroom *Macrolepiota dolichaula*," *International Journal of Biological Macromolecules*, vol. 61, pp. 89–96, 2013.

[9] A. K. Nandi, I. K. Sen, S. Samanta et al., "Glucan from hot aqueous extract of an ectomycorrhizal edible mushroom, *Russula albonigra* (Krombh.) Fr.: structural characterization and study of immunoenhancing properties," *Carbohydrate Research*, vol. 363, pp. 43–50, 2012.

[10] F. R. Smiderle, G. Alquini, M. Z. Tadra-Sfeir, M. Iacomini, H. J. Wichers, and L. J. L. D. van Griensven, "*Agaricus bisporus* and *Agaricus brasiliensis* $(1 \rightarrow 6)$-β-d-glucans show immunostimulatory activity on human THP-1 derived macrophages," *Carbohydrate Polymers*, vol. 94, no. 1, pp. 91–99, 2013.

[11] D. I. Gabrilovich and A. A. Hurwitz, *Tumor-Induced Immune Suppression*, Springer, 2008.

[12] W. Zou, "Immunosuppressive networks in the tumour environment and their therapeutic relevance," *Nature Reviews Cancer*, vol. 5, no. 4, pp. 263–274, 2005.

[13] G. P. Dunn, A. T. Bruce, H. Ikeda, L. J. Old, and R. D. Schreiber, "Cancer immunoediting: from immunosurveillance to tumor escape," *Nature Immunology*, vol. 3, no. 11, pp. 991–998, 2002.

[14] G. P. Dunn, L. J. Old, and R. D. Schreiber, "The immunobiology of cancer immunosurveillance and immunoediting," *Immunity*, vol. 21, no. 2, pp. 137–148, 2004.

[15] B.-Z. Qian and J. W. Pollard, "Macrophage diversity enhances tumor progression and metastasis," *Cell*, vol. 141, no. 1, pp. 39–51, 2010.

[16] D. W. Siemann, *Tumor Microenvironment*, Wiley, 2011.

[17] Y. Wang, J. Fang, X. Ni et al., "Inducement of cytokine release by GFPBW2, a novel polysaccharide from fruit bodies of *Grifola frondosa*, through dectin-1 in macrophages," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 47, pp. 11400–11409, 2013.

[18] N. Mach, S. Gillessen, S. B. Wilson, C. Sheehan, M. Mihm, and G. Dranoff, "Differences in dendritic cells stimulated *in vivo* by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand," *Cancer Research*, vol. 60, no. 12, pp. 3239–3246, 2000.

[19] S. K. Biswas and A. Mantovani, "Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm," *Nature Immunology*, vol. 11, no. 10, pp. 889–896, 2010.

[20] B. Ruffell, N. I. Affara, and L. M. Coussens, "Differential macrophage programming in the tumor microenvironment," *Trends in Immunology*, vol. 33, no. 3, pp. 119–126, 2012.

[21] R. A. Flavell, S. Sanjabi, S. H. Wrzesinski, and P. Licona-Limón, "The polarization of immune cells in the tumour environment by TGFβ," *Nature Reviews Immunology*, vol. 10, no. 8, pp. 554–567, 2010.

[22] L. Bingle, N. J. Brown, and C. E. Lewis, "The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies," *The Journal of Pathology*, vol. 196, no. 3, pp. 254–265, 2002.

[23] A. Mantovani, A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati, "The chemokine system in diverse forms of macrophage activation and polarization," *Trends in Immunology*, vol. 25, no. 12, pp. 677–686, 2004.

[24] A. Mantovani and A. Sica, "Macrophages, innate immunity and cancer: balance, tolerance, and diversity," *Current Opinion in Immunology*, vol. 22, no. 2, pp. 231–237, 2010.

[25] A. Mantovani, A. Sica, P. Allavena, C. Garlanda, and M. Locati, "Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation," *Human Immunology*, vol. 70, no. 5, pp. 325–330, 2009.

[26] C. Steidl, T. Lee, S. P. Shah et al., "Tumor-associated macrophages and survival in classic Hodgkin's lymphoma," *The New England Journal of Medicine*, vol. 362, no. 10, pp. 875–885, 2010.

[27] K. D. Elgert, D. G. Alleva, and D. W. Mullins, "Tumor-induced immune dysfunction: the macrophage connection," *Journal of Leukocyte Biology*, vol. 64, no. 3, pp. 275–290, 1998.

[28] C. Sunderkotter, M. Goebeler, K. Schulze-Osthoff, R. Bhardwaj, and C. Sorg, "Macrophage-derived angiogenesis factors," *Pharmacology and Therapeutics*, vol. 51, no. 2, pp. 195–216, 1991.

[29] E. Giraudo, M. Inoue, and D. Hanahan, "An amino-bisphosphonate targets MMP-9—expressing macrophages and angiogenesis to impair cervical carcinogenesis," *The Journal of Clinical Investigation*, vol. 114, no. 5, pp. 623–633, 2004.

[30] A. Ben-Baruch, "Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators," *Seminars in Cancer Biology*, vol. 16, no. 1, pp. 38–52, 2006.

[31] M. Mitsuhashi, J. Liu, S. Cao, X. Shi, and X. Ma, "Regulation of interleukin-12 gene expression and its anti-tumor activities by prostaglandin E2 derived from mammary carcinomas," *Journal of Leukocyte Biology*, vol. 76, no. 2, pp. 322–332, 2004.

[32] F. O. Martinez, L. Helming, and S. Gordon, "Alternative activation of macrophages: an immunologic functional perspective," *Annual Review of Immunology*, vol. 27, pp. 451–483, 2009.

[33] J. W. Pollard, "Tumour-educated macrophages promote tumour progression and metastasis," *Nature Reviews Cancer*, vol. 4, no. 1, pp. 71–78, 2004.

[34] A. Mantovani, S. Sozzani, M. Locati, P. Allavena, and A. Sica, "Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes," *Trends in Immunology*, vol. 23, no. 11, pp. 549–555, 2002.

[35] G. Solinas, G. Germano, A. Mantovani, and P. Allavena, "Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation," *Journal of Leukocyte Biology*, vol. 86, no. 5, pp. 1065–1073, 2009.

[36] T. Ikekawa, "Enokitake, *Flammulina velutipes*: host-mediated antitumor polysaccharides," *Food Reviews International*, vol. 11, pp. 203–206, 1995.

[37] T. Ikekawa, N. Uehara, Y. Maeda, M. Nakanishi, and F. Fukuoka, "Antitumor activity of aqueous extracts of edible mushrooms.," *Cancer Research*, vol. 29, no. 3, pp. 734–735, 1969.

[38] X. Xu, C. Pan, L. Zhang, and H. Ashida, "Immunomodulatory β-glucan from Lentinus edodes activates mitogen-activated protein kinases and nuclear factor-κB in murine RAW 264.7 macrophages," *The Journal of Biological Chemistry*, vol. 286, no. 36, pp. 31194–31198, 2011.

[39] C. T. Dang, A. J. Dannenberg, K. Subbaramaiah et al., "Phase II study of celecoxib and trastuzumab in metastatic breast cancer patients who have progressed after prior trastuzumab-based treatments," *Clinical Cancer Research*, vol. 10, no. 12 I, pp. 4062–4067, 2004.

[40] V. A. Levin, P. Giglio, V. K. Puduvalli et al., "Combination chemotherapy with 13-cis-retinoic acid and celecoxib in the treatment of glioblastoma multiforme," *Journal of Neuro-Oncology*, vol. 78, no. 1, pp. 85–90, 2006.

[41] N. K. Altorki, R. S. Keresztes, J. L. Port et al., "Celecoxib, a selective cyclo-oxygenase-2 inhibitor, enhances the response to preoperative paclitaxel and carboplatin in early-stage non-small-cell lung cancer," *Journal of Clinical Oncology*, vol. 21, no. 14, pp. 2645–2650, 2003.

[42] H. Gogas, A. Polyzos, I. Stavrinidis et al., "Temozolomide in combination with celecoxib in patients with advanced melanoma. A phase II study of the hellenic cooperative oncology group," *Journal of Clinical Oncology*, vol. 20, pp. 420–425, 2006.

[43] H. M. Prince, L. Mileshkin, A. Roberts et al., "A multicenter phase II trial of thalidomide and celecoxib for patients with relapsed and refractory multiple myeloma," *Clinical Cancer Research*, vol. 11, no. 15, pp. 5504–5514, 2005.

[44] R. Buckstein, R. S. Kerbel, Y. Snaked et al., "High-dose celecoxib and metronomic "low-dose" cyclophosphamide is an effective and safe therapy in patients with relapsed and refractory aggressive histology non-Hodgkin's lymphoma," *Clinical Cancer Research*, vol. 12, no. 17, pp. 5190–5198, 2006.

[45] V. Ferrari, F. Valcamonico, V. Amoroso et al., "Gemcitabine plus celecoxib (GECO) in advanced pancreatic cancer: a phase II trial," *Cancer Chemotherapy and Pharmacology*, vol. 57, no. 2, pp. 185–190, 2006.

[46] L. J. Wirth, R. I. Haddad, N. I. Lindeman et al., "Phase I study of gefitinib plus celecoxib in recurrent or metastatic squamous cell carcinoma of the head and neck," *Journal of Clinical Oncology*, vol. 23, no. 28, pp. 6976–6981, 2005.

[47] Y. Nakanishi, M. Nakatsuji, H. Seno et al., "COX-2 inhibition alters the phenotype of tumor-associated macrophages from M2 to M1 in $Apc^{Min/+}$ mouse polyps," *Carcinogenesis*, vol. 32, no. 9, pp. 1333–1339, 2011.

[48] Y.-S. Wu and S.-N. Chen, "Inflammation and carcinogenesis," *Journal of Cancer Therapy*, vol. 4, pp. 1449–1451, 2013.

[49] S. N. Chen, F. H. Nan, J. F. Wu, C. L. Lu, and M. G. Soni, "Safety assessment of mushroom β-glucan: Subchronic toxicity in rodents and mutagenicity studies," *Food and Chemical Toxicology*, vol. 49, no. 11, pp. 2890–2898, 2011.

[50] W. S. York, A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim, "Isolation and characterization of plant cell walls and cell wall components," *Methods in Enzymology*, vol. 118, pp. 3–40, 1986.

[51] I. Ciucanu and F. Kerek, "A simple and rapid method for the permethylation of carbohydrates," *Carbohydrate Research*, vol. 131, no. 2, pp. 209–217, 1984.

[52] K. Maity, S. Samanta, S. K. Bhanja et al., "An immunostimulating water insoluble β-glucan of an edible hybrid mushroom: isolation and characterization," *Fitoterapia*, vol. 84, no. 1, pp. 15–21, 2013.

[53] S. K. Bhanja, C. K. Nandan, S. Mandal et al., "Isolation and characterization of the immunostimulating β-glucans of an edible mushroom *Termitomyces robustus* var," *Carbohydrate Research*, vol. 357, pp. 83–89, 2012.

[54] S. Wasser, "Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides," *Applied Microbiology and Biotechnology*, vol. 60, no. 3, pp. 258–274, 2003.

[55] T. Inomata, G. B. Goodman, C. J. Fryer et al., "Immune reaction induced by X-rays and pions and its stimulation by schizophyllan (SPG)," *British Journal of Cancer*, vol. 27, pp. S122–S125, 1996.

[56] H. Nanba and K. Kubo, "Effect of Maitake D-fraction on cancer prevention," *Annals of the New York Academy of Sciences*, vol. 833, pp. 204–207, 1997.

[57] C. Menetrier-Caux, G. Montmain, M. C. Dieu, C. Bain, M. C. Favrot, and J. Y. Blay, "Inhibition of the differentiation of dendritic cells from $CD34^+$ progenitors by tumor cells: Role of interleukin-6 and macrophage colony- stimulating factor," *Blood*, vol. 92, no. 12, pp. 4778–4791, 1998.

[58] E. Y. Lin, V. Gouon-Evans, A. V. Nguyen, and J. W. Pollard, "The macrophage growth factor CSF-1 in mammary gland development and tumor progression," *Journal of Mammary Gland Biology and Neoplasia*, vol. 7, no. 2, pp. 147–162, 2002.

[59] C. E. Lewis and J. W. Pollard, "Distinct role of macrophages in different tumor microenvironments," *Cancer Research*, vol. 66, no. 2, pp. 605–612, 2006.

[60] A. Khan, A. A. Khan, V. Dwivedi, M. G. Ahmad, S. Hakeem, and M. Owais, "Tuftsin augments antitumor efficacy of liposomized etoposide against fibrosarcoma in Swiss albino mice," *Molecular Medicine*, vol. 13, no. 5-6, pp. 266–276, 2007.

[61] M. A. Khan, S. M. Faisal, and O. Mohammad, "Safety, efficacy and pharmacokinetics of tuftsin-loaded nystatin liposomes in murine model," *Journal of Drug Targeting*, vol. 14, no. 4, pp. 233–241, 2006.

[62] V. Dwivedi, A. Khan, A. Vasco et al., "Immunomodulator effect of picroliv and its potential in treatment against resistant *Plasmodium yoelii* (MDR) infection in mice," *Pharmaceutical Research*, vol. 25, no. 10, pp. 2312–2319, 2008.

[63] W.-Y. Ni, M.-F. Wu, N.-C. Liao et al., "Extract of medicinal mushroom *Agaricus blazei* Murill enhances the non-specific and adaptive immune activities in BALB/c mice," *In Vivo*, vol. 27, no. 6, pp. 779–786, 2013.

[64] M. Degen, B. Alexander, M. Choudhury, M. Eshghi, and S. Konno, "Alternative therapeutic approach to renal-cell carcinoma: induction of apoptosis with combination of vitamin K_3 and D-fraction," *Journal of Endourology*, vol. 27, no. 12, pp. 1499–1503, 2013.

[65] M. Farazuddin, B. Dua, Q. Zia, A. A. Khan, B. Joshi, and M. Owais, "Chemotherapeutic potential of curcumin-bearing microcells against hepatocellular carcinoma in model animals," *International Journal of Nanomedicine*, vol. 9, pp. 1139–1152, 2014.

[66] E. S. Ch'ng, H. Jaafar, and S. E. Tuan Sharif, "Breast tumor angiogenesis and tumor-associated macrophages: histopathologist's perspective," *Pathology Research International*, vol. 2011, Article ID 572706, 13 pages, 2011.

[67] T. J. Standiford, R. Kuick, U. Bhan, J. Chen, M. Newstead, and V. G. Keshamouni, "TGF-β-induced IRAK-M expression in tumor-associated macrophages regulates lung tumor growth," *Oncogene*, vol. 30, no. 21, pp. 2475–2484, 2011.

[68] K. Murphy, P. Travers, and M. Walport, *Immunobiology*, Graland Science, 7th edition, 2008.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 820813, 11 pages
http://dx.doi.org/10.1155/2015/820813

Review Article

Immunotherapy for Bone and Soft Tissue Sarcomas

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Received 25 July 2014; Accepted 13 November 2014

Academic Editor: Mohammad Owais

Although multimodal therapies including surgery, chemotherapy, and radiotherapy have improved clinical outcomes of patients with bone and soft tissue sarcomas, the prognosis of patients has plateaued over these 20 years. Immunotherapies have shown the effectiveness for several types of advanced tumors. Immunotherapies, such as cytokine therapies, vaccinations, and adoptive cell transfers, have also been investigated for bone and soft tissue sarcomas. Cytokine therapies with interleukin-2 or interferons have limited efficacy because of their cytotoxicities. Liposomal muramyl tripeptide phosphatidylethanolamine (L-MTP-PE), an activator of the innate immune system, has been approved as adjuvant therapeutics in combination with conventional chemotherapy in Europe, which has improved the 5-year overall survival of patients. Vaccinations and transfer of T cells transduced to express chimeric antigen receptors have shown some efficacy for sarcomas. Ipilimumab and nivolumab are monoclonal antibodies designed to inhibit immune checkpoint mechanisms. These antibodies have recently been shown to be effective for patients with melanoma and also investigated for patients with sarcomas. In this review, we provide an overview of various trials of immunotherapies for bone and soft tissue sarcomas, and discuss their potential as adjuvant therapies in combination with conventional therapies.

1. Introduction

Sarcomas are malignant tumors of mesenchymal origin, including bones, muscles, fat, nerves, and blood vessels. According to the Surveillance Epidemiology and End Results (SEER) database, prevalence of sarcoma accounts for nearly 21% of all pediatric solid malignant tumors and less than 1% of all adult solid malignant tumors [1]. It was estimated that approximately 11,400 Americans would be diagnosed with soft tissue sarcomas and 3,000 with bone sarcoma in 2013 [2]. Based on the survival data obtained from the National Cancer Data Base of the American College of Surgeons, the relative 5-year survival rate is approximately 66% for patients with bone and soft tissue sarcomas, 53.9% for osteosarcomas ($n = 8{,}104$), 75.2% for chondrosarcoma ($n = 6{,}476$), and 50.6% for Ewing's sarcomas ($n = 3{,}225$) [3]. According to the classification by the World Health Organization, the group of bone and soft tissue sarcomas includes more than 100 histological subtypes [4]. The prognosis of patients with bone and soft tissue sarcomas is associated with histological diagnoses [5]. Standard treatment modalities include surgical resection, chemotherapy, and often radiotherapy [6–8]. Despite these multimodality therapies, survival rates have not been improved over recent 20 years [9]. Therefore, new effective treatment over conventional therapy is urgently needed.

Historically, Coley reported a case of unresectable small-cell sarcoma of the neck in 1891. The sarcoma completely regressed after a severe episode of erysipelas. He reported

that a systemic response against erysipelas influenced the patient's tumor [10]. The mechanism by which erysipelas caused tumor regression was unclear at that time. However, it is now understood that the activation of innate immunity through Toll-like receptors (TLRs) by erysipelas followed by activation of acquired immunity specific to sarcoma may contribute to the underlying mechanism [11]. Thus, the case described by Coley was the first to demonstrate that the immune system is involved in the spontaneous regression of sarcomas. Over the past 100 years, his work had encouraged many scientists to work on cancer immunology, in an attempt to find a cure for cancers [12, 13].

The dissection of the molecular mechanisms of innate and acquired immunity has enabled medical doctors and scientists to apply various cancer immunotherapies such as vaccines, antibodies, adjuvants, and cell therapies [29–31]. Utilizing modern cancer immunotherapies for patients with sarcomas began in the 1980s as a cytokine therapy [32, 33], and more recently antigen-specific cancer vaccines and/or cell therapies have been developed [34, 35].

2. Overview of Cancer Immunology

2.1. Immune System Overview. Knowledge about the immune system is essential for understanding the principles underpinning cancer immunotherapy. There are two types of immune responses against microbes: called innate and adaptive immunity [36]. Innate immunity, whose main components are phagocytic cells (neutrophils and macrophages) and natural killer cells, provides the initial defense against invading microbes during infection [37, 38]. Small molecular proteins called cytokines mediate many activities of the cells involved in innate immunity. In addition to cytokines, pattern recognition molecules such as TLRs expressed on dendritic cells (DCs) and macrophages play critical roles in the activation of innate immunity. These components also have a role in communicating with acquired (adaptive) immunity [39, 40]. The key components of adaptive immunity, following the initial innate immunity, are T and B lymphocytes. The lymphocytes play a central role in eliminating infectious pathogens, virus infected cells, and cancer cells and also in generating antigen-specific memory cells [37].

Adaptive immunity consists of humoral and cell-mediated immunity. T lymphocytes recognize short peptides as antigens presented by major histocompatibility complexes (MHCs) on the cell surface of DCs [41, 42]. CD8 and CD4 T cells recognize antigen in the context of MHC class I and class II molecules, respectively [43, 44]. Primed and activated T cells differentiate into mature effector cells while undergoing clonal expansion. The effector CD8 T cells recognize virus infected cells and tumor cells and eliminate them from the body. The differentiation of naïve CD8 T cells into effector and memory CD8 T cells is mediated by the "help" of CD4 T cells or by a stimulation of TLRs of DC [43–45]. "Help" means signals occurring within DCs whose CD40 interacts with CD40L of CD4 T cells to express large amounts of CD80/86 to interact with CD28 of CD8 T cells [46–48]. Signals from either CD40 or TLRs activate DCs, and this process then initiates the activation of naïve CD8 T cells following antigen recognition [49].

DCs, B cells, and macrophages are professional antigen-presenting cells (APCs) [50, 51]. Among them, DCs are the most effective APCs [51, 52]. For example, B cells and macrophages present endogenous and internalized exogenous antigens with MHC class I and class II molecules [53], respectively. Therefore, B cells and macrophages can only activate CD4 T cells when they internalize extracellular antigens [54]. On the other hand, DCs are able to process both endogenous and exogenous antigens with MHC class I molecules to activate CD8 T cells. This is referred to as cross-presentation and is essential in fighting against virus infected cells and tumor cells [55–57].

2.2. Tumor Immunology and Immune Checkpoint. Tumor antigens recognized by the immune system are categorized into cancer testis antigens (CTAs), melanocyte differentiation antigens, mutated proteins, overexpressed proteins, and viral antigens [58] (Figure 1). Several types of CTAs have been identified in patients with sarcomas (Table 1). Because tumor antigens are potential targets that induce cytotoxic immune responses [59], many clinical trials have utilized tumor antigens as vaccines for decades. The results, however, are limited and the desired therapeutic effect is not achieved [60, 61].

Although antitumor immunity is induced in patients with cancer vaccines, recent advancements in cancer immunity have revealed the presence of immune-inhibitory mechanisms, referred to as immune checkpoints [62], in the draining of lymph nodes and tumor sites. CTLA-4, a protein receptor expressed on T cells, downregulates T cell activation [63]. The structure of CTLA-4 is similar to CD28, with a T cell costimulatory receptor. Immune inhibition is caused by the competition between CD28 and CTLA-4 to bind CD80/86 on DCs [64]. Regulatory T cells (Tregs) that define $CD4^+CD25^+Foxp3^+$ T cells highly express CTLA-4 and suppress the activation of cytotoxic lymphocytes [65]. The inhibition of activated T cells via CTLA-4 occurs particularly within draining lymph nodes [66]. Programmed cell death protein 1 (PD1) is also an immune checkpoint receptor expressed on T cells, particularly cytotoxic lymphocytes [67, 68]. Tumor cells upregulate the expression of PD-ligand 1(PD-L1), and the interaction of PD1 with PD-L1 downregulates the function of T cells within the tumor microenvironment [69, 70]. The immune checkpoint is therefore considered to be an important therapeutic target. Anti-CTLA-4 and anti-PD1 antibodies have been introduced for clinical use in some cancers [71]. In addition to CTLA4 and PD-1, there are similar cell surface molecules of activated effector T cells, such as Tim-3 and LAG3, that suppress tumor immunity [72]. Inflammation in the tumor microenvironment induces STAT3 activation within tumors and Tregs. In contrast, STAT3 in certain tumors is constitutively activated by genetic alterations [73, 74]. STAT3 activation leads tumor cells and Tregs to express molecules that are related to immune checkpoints, such as PD-L1, and eventually inhibit T cell function [75, 76].

Table 1: Cancer testis antigens in bone and soft tissue sarcomas.

Sarcoma subtypes	Expression of cancer testis antigens						
	NY-ESO	LAGE	MAGE-A3	MAGE-A4	MAGE-A9	PRAME	SSX-2
Bone sarcomas							
Osteosarcoma [14]	+	+	+	+			
Ewing's sarcoma [14]	+	+	+	+			
Chondrosarcoma [14]	+	+	+	+			
Soft tissue sarcomas							
Synovial sarcoma [15]	+	+	+	+	+	+	+
Malignant fibrous histiocytoma, pleomorphic spindle cell sarcoma [15]	+	+	+			+	+
Liposarcoma [15]	+	+	+	+	+		+
Leiomyosarcoma [15]		+	+	+	+	+	

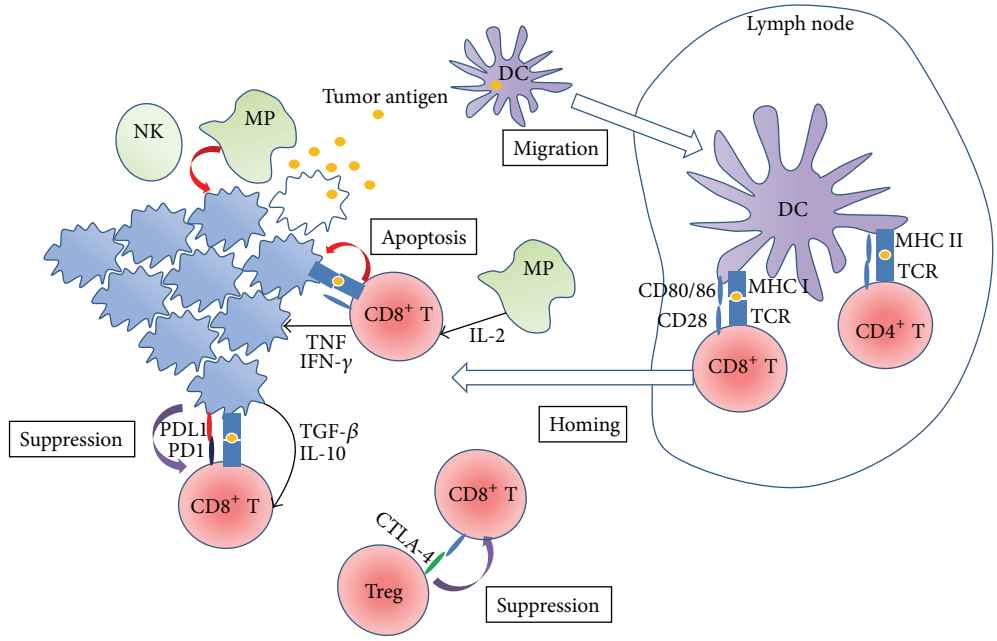

Figure 1: An overview of tumor immunology. Tumor cells are initially attacked by the innate immune system. DCs capture tumor antigens at the tumor site and migrate to the tumor draining lymph nodes. DCs present the tumor antigen to T cells within the lymph node. Antigen-specific CD4 and CD8 T cells are stimulated by DCs. After stimulation, T cells differentiate into effector cells and activate at the tumor site. Effector CD8 T cells kill tumor cells, although their function is regulated by the immune checkpoint mechanism. NK: natural killer cell; MP: macrophage; DC: dendritic cell.

3. Outcomes of Clinical Trials for Bone and Soft Tissue Sarcomas

Treatments for bone and soft tissue sarcomas include surgery, chemotherapy, and radiotherapy. To date, clinical results of combined therapies have been more successful than those of surgical approaches. However, as described above, the prognosis of bone and soft tissue sarcomas has plateaued since the 1990s. In these recent years, immunotherapies are expected to further improve the prognosis of patients, and several clinical trials have been performed (Tables 2 and 3).

3.1. Cytokine Therapies. Cytokines are proteins that regulate the immune system. Interleukin-2 (IL-2) and interferons (IFNs) have been used in the immunotherapy for sarcomas [77], and clinical results are evident. IL-2 leads to the activation and expansion of CD4 and CD8 T cells [78]. Rosenberg et al. established a tumor regression model involving recombinant IL-2 injection for murine melanoma and sarcomas [32]. Then, several studies described the effectiveness of high-dose IL-2 therapy for patients with metastatic melanomas [79, 80]. Therefore, recombinant IL-2 was administered to patients with bone and soft tissue sarcomas [16]. Schwinger et al. reported a positive clinical result using a high-dose IL-2 treatment in two patients with Ewing's sarcomas and four patients with metastatic osteosarcomas. Patients had already been treated with surgery (1–5 times), chemotherapy (7–43 cycles), and radiation therapy (for patients with Ewing's sarcoma). Although one patient with metastatic osteosarcoma progressed during the treatment period, two patients with

Table 2: Clinical trials stimulating innate immunity against bone and soft tissue sarcomas.

Agent	Number of patients	Diagnosis	Treatment	Follow-up	Clinical result
IL-2 [16]	6	Osteosarcoma, Ewing's sarcoma	$6–12 \times 10^6$ IU/m^2 for 5 days by every 3 weeks	7–71 months	Complete response (CR): 5 Progressive disease (PD): 5
IFNs [17]	3	Osteosarcoma	$2.5–5 \times 10^6$ IU/mL twice or thrice weekly	6–8 months	CR: 2 PD: 1
IFN-α2 [18]	20	Osteosarcoma, fibrosarcoma, chondrosarcoma, and malignant fibrous histiocytoma	5×10^7 IU/m^2 thrice weekly	1–3 months	Partial response (PR): 3
IFN-α [19]	89	Osteosarcoma	Cohort 1 (70 patients); 3×10^6 IU daily for a month Cohort 2 (19 patients); 3×10^6 IU daily for 3–5 years	10 years	Metastatic free survival: 39% Sarcoma specific survival: 43%
IFN-β [20]	158	Osteosarcoma (COSS-80)	1×10^5 IU/kg for 22 weeks	30 months	Disease-free survival +IFN: 77% −IFN: 73% (N.S.)
Pegylated IFN-α2b [21]	715	Osteosarcoma (EURAMOS-1)	Methotrexate, adriamycin, and cisplatin (MAP) +/−IFN (0.5–1.0 μg/kg/wk) for 2 years	Median follow-up 3.1 years	Event-free survival +IFN: 77% −IFN: 74% (N.S.)
L-MTP-PE [22]	662	Osteosarcoma (INT 0133)	MAP alone, MAP + L-MTP-PE, MAP + ifosfamide, MAP + ifosfamide + L-MTP-PE	6 years	Overall survival +L-MTP-PE: 78% −L-MTP-PE: 70% Event free survival No significant difference

osteosarcoma achieved complete responses with a median follow-up time of 28 months (range: 11–36 months). However, all patients experienced adverse effects such as fatigue, anorexia, diarrhea, nausea, vomiting, and high-grade fever. Two patients could not undergo IL-2 therapy [16]. Furthermore, the other initial study reported treatment related death caused in 1-2% of patients [81]. Consequently, it limited the administration of high-dose IL-2 therapy for its adverse effect [81, 82].

The use of IFN-α as an adjuvant therapy was initiated at the Karolinska Hospital in 1971 [19]. The Karolinska Hospital group reported that 10-year results of adjuvant IFN-α therapy. The clinical outcome was improved by introducing adjuvant IFN-α therapy. The metastasis-free survival rate was 39% and the sarcoma-free survival rate was 43% in adjuvant IFN therapy group. These clinical results were better than the group of surgiral therapy only (15–20%) [83]. COSS-80 study investigated the effectiveness of use of adjuvant chemotherapy with IFN [20]. The 30-month disease-free survival rate of the IFN arm was 77% and that of non-IFN arm 73%. However, there was no significant difference between two groups; EURAMOS-1 study, a recent study in Europe, investigated the efficacy of the use of adjuvant chemotherapy with pegylated-IFNα-2b [21]. In the interim statement, the median follow-up time in EURAMOS-1 study was 3.1 years. The event-free survival rate was 77% in the group with chemotherapy and IFN and 73% in the group without IFN [19]. This difference was also not significant. These observations suggest that conventional chemotherapy with IFN improves the prognosis of bone and soft tissue sarcomas to some extent.

3.2. Mifamurtide. Mifamurtide, liposomal muramyl tripeptide phosphatidylethanolamine (L-MTP-PE), is a new agent that is a synthetic analog of a muramyl dipeptide (MDP) [22]. Although its pharmacological behavior is similar to that of MDP, L-MTP-PE has a longer half-life than MDP [84]. The intracellular pattern recognition molecule NOD2 detects MDP and enhances NF-κB signaling [85]. Therefore, recognition of L-MTP-PE by NOD2 stimulates the production of IL-1β, IL-6, and TNF-α via the activation of NF-κB signaling in monocytes and macrophages [86, 87].

The efficacy of L-MTP-PE treatment for osteosarcomas has been examined in dogs. Dogs with postoperative

Table 3: Clinical trials stimulating adaptive immunity against bone and soft tissue sarcomas.

Agent	Number of patients	Diagnosis	Treatment	Immune response	Clinical result
Autologous tumor cells [23]	23	Sarcoma	Total 1.0×10^7 cells	Delayed-type hypersensitivity (DTH) positive: 8 patients	Median survival DTH responder: 16.6 months Nonresponder: 8.2 months
Tumor translocation breakpoint specific peptide-pulsed DCs [24]	52	Ewing's sarcoma, rhabdomyosarcoma	Total $4.2–143.0 \times 10^6$ cells	39% with immune response to the translocation breakpoint, 25% with response to E7-specific	Overall survival Vaccination: 43% Control: 31%
Tumor-specific synthetic peptides or tumor lysates pulsed DCs [25]	5	Ewing's sarcoma, synovial sarcoma, neuroblastoma	$2–15 \times 10^6$ pulsed DCs injected 6–8 times	DTH positive: 1 patient	CR: 1 (77 months) PD: 4 (2–27 months)
A 9-mer peptide from SYT-SSX fusion site [26]	21	Synovial sarcoma	0.1 or 1.0 mg peptide +/− adjuvant 6 times at 14-day interval	Tetramer positive CD8: 7 patients	Stable disease (SD): 1/9 peptide alone 6/12 vaccine with adjuvant
Anti-CTLA-4 antibody [27]	6	Synovial sarcoma (expressed NY-ESO-1)	Ipilimumab 3 mg/kg every 3 weeks for 3 cycles	DTH: all patients negative	Time to progression 0.47–2.1 months (median 1.85), overall survival time 0.77–19.7 months (median 8.75)
T cell receptor- (TCR-) transduced T cells (NY-ESO-1 specific) [28]	6	Synovial sarcoma (expressed NY-ESO-1)	TCR-transduced T cells +720,000 IU/kg of IL-2	Tetramer positive CD8: 5 patients	PR: 4 PD: 2

osteosarcomas were treated by intravenous L-MTP-PE injections. The median survival time of dogs treated by L-MTP-PE (222 days) was longer than that of nontreated dogs (77 days) [88]. In human, intergroup study 0133 (INT 0133) began in 1993. 662 patients with osteosarcoma were recruited in this study. The aim of the study was to evaluate the efficacy of supplementation with ifosfamide (IFO) and L-MTP-PE in basic adjuvant chemotherapy (cisplatin, doxorubicin, and high-dose methotrexate (MAP)). Patients were randomly assigned to receive MAP alone, MAP + IFO, MAP + L-MTP-PE, and MAP + IFO + L-MTP-PE. It was observed that the addition of L-MTP-PE to chemotherapy improved the six-year overall survival rate from 70% to 78% ($P = 0.03$). The hazard ratio for overall survival with the addition of MTP was 0.71 (95% CI: 0.52–0.96) [22, 89]. Therefore, L-MTP-PE has been approved in Europe for the treatment of osteosarcoma with chemotherapy. However, it has not been approved by FDA in the United States [87].

3.3. Vaccines. Multiple clinical trials using vaccines that target whole cells, lysates, proteins, and peptides have been investigated in patients with sarcomas [90–92]. Vaccines are combined with costimulatory adjuvants such as GM-CSF or IL-2 to enhance the immune response [93]. Therapeutic tumor vaccines are presented as antigen epitopes on MHC molecules by APCs. Tumor antigen specific T cells are activated by APCs. The aim of cancer vaccines is to stimulate the patient's own immune system to eliminate the tumor [94].

Autologous sarcoma cell lysates can be used as a vaccine in patients with sarcomas. A clinical study was performed to treat patients using their autologous tumor cell lysate as vaccines [23]. The study recruited 86 patients with sarcomas and tryed to establish short-term cell lines in vitro. 25 patients, who had an established tumor cell line, were injected with the tumor lysate vaccine. Before vaccine treatment, patients were screened to ensure they were not positive for delayed-type hypersensitivity (DTH) to irradiated tumor cells. After treatment, eight patients became positive for DTH. The median survival time of patients who became positive for DTH (16.6 months) was eight months longer than that of DTH-negative patients (8.2 months). However, objective responses were not recorded [23]. In the result, tumor lysate vaccines improved the survival time, but tumor regression disappeared.

Autologous DCs that are pulsed ex vivo with tumor cell lysate can stimulate host antitumor immunity [95, 96]. Adjuvant therapies using tumor lysate-pulsed DCs were investigated for children with solid tumors including bone

and soft tissue sarcomas. After tumor lysate-pulsed DC transfer, 70% of patients changed positively in the DTH test. This study resulted in one patient achieving complete remission and in five patients, the disease stabilized during the follow-up period of 16–30 months [97].

Tumor specific or overexpressed peptides are possible for therapeutic targets for antigen-specific immunotherapy [98, 99]. Bone and soft tissue sarcomas can have specific gene mutations and express mutated proteins [100]. Synovial sarcomas are known to have chromosomal translocation and synthesize the SYT-SSX mutated protein [101]. Kawaguchi et al. treated patients who had synovial sarcomas with SYT-SSX fusion gene-derived peptides [102]. The study enrolled 21 patients, who were injected subcutaneously with the 9 mer peptide with or without incomplete Freund's adjuvant (IFA) and IFN-α. Nine patients were injected with the peptide alone, and later in the study, 12 patients were injected the peptide with IFA and IFN-α. After treatment, in seven patients, the peptide tetramer-positive CD8 T cells appeared in PBMCs. With regard to the clinical result, in six patients, the disease stabilized during vaccination; however, in other patients, the disease progressed [26].

Tumor antigen-specific peptide pulsed DCs can stimulate peptide specific T cells 150 times more efficient than peptide alone [103]. Tumor-specific peptide pulsed DCs have been administered for immunotherapy against sarcoma, leukemia, and glioma [104]. 30 patients with Ewing's sarcomas and alveolar rhabdomyosarcoma were enrolled in a study for consolidative therapy. Patients were separated into three cohorts that received different dose of IL-2 (high, low, and none). Monocyte-derived DCs were cultured with tumor-derived breakpoint peptides (EWS-FLI1, EWS-FLI2, and PAX3/FKHR), and the E7 peptide was used as control [24]. After treatment, 39% of patients generated immune responses to the vaccinating peptide. The five-year overall survival of the immunotherapy group was 43% and that of the no-immunotherapy group was 31% [24]. Further, this treatment showed no severe adverse effect. For these reasons, vaccines from tumor cell lysate or tumor specific peptide can activate adaptive immune response against tumors. Antigen-specific peptide pulsed DCs can also enhance immune response. Vaccine therapies have validity for bone and soft tissue sarcomas.

CTAs are expressed only in germ line cells in humans; however, they are also expressed in various tumors [105]. More than 40 antigens have been identified [105]. For example, NY-ESO-1 is expressed in many osteosarcomas, leiomyosarcomas, and synovial sarcomas and LAGE-1 is expressed in liposarcomas, leiomyosarcomas, and synovial sarcomas (Table 1) [106]. MAGE-A3 was administered to patients with stage III/IV melanoma [107]. The effectiveness of MAGE-A3 against non-small-cell lung cancer (NSCLC) was reported in a phase II clinical trial [108, 109]. Thus CTAs have a potential to be immunotherapeutic targets against bone and soft tissue sarcomas.

3.4. Adoptive Cell Transfer. Adoptive cell transfer therapy is considered to provide large number of tumor reactive CD8T cells that secrete high levels of cytokines, IFNγ, TNFα, and IL-2 [110]. Tumor infiltrating lymphocytes (TILs) include tumor reactive CD8T cells. Antigen-specific T cells were sorted from patients. T cells were expanded and stimulated ex vivo. After ex vivo treatment, activated effector T cells were transferred to patients [110]. A small study examined six patients with synovial sarcomas or metastatic melanomas expressing NY-ESO-1. For inducing tumor lysis, T cell receptor (TCR) gene-modified T cells redirected towards NY-ESO-1 were generated [28]. Modified TCR displayed T cells were expanded with IL-2 ex vivo and then transferred to patients [111]. Two patients with melanoma showed complete regression, and 1 patient with synovial sarcoma showed disease stabilization for 18 months. Some types of adoptive cell transfer therapies are ongoing for patients with sarcomas, including autologous DC transport therapy for soft tissue sarcomas (NCT01347034) and hematopoietic cell transplantation and natural killer cell transport therapies for Ewing's sarcomas and rhabdomyosarcomas (NCT02100891).

3.5. Immune Checkpoint Blockade. Immune checkpoint blockade is likely to advance anticancer immunology. Ipilimumab, a fully human monoclonal antibody (IgG1), blocks CTLA-4 and promotes antitumor immunity [112]. Patients with metastatic melanomas treated with ipilimumab showed improved overall survival (from 6.4 months to 10.0 months) [113]. Six patients with advanced synovial sarcoma enrolled in a phase II study were treated with ipilimumab. The overall survival time ranged from 0.77 to 19.7 months (median: 8.75 months). Immunological responses after the treatment were different in each patient, and three patients showed an enhanced titer of CT24 (an uncharacterized CTA). All sarcomas expressed NY-ESO-1; however, NY-ESO-1 titers did not show any remarkable change [114].

Another immune checkpoint blockade agent is a human monoclonal anti-PD-1 antibody, called nivolumab [115]. Nivolumab has demonstrated efficacy against several types of cancers including melanoma, NSCLC, prostate cancer, renal cell carcinoma, and colorectal cancer [116]. The reported clinical outcomes of nivolumab therapies include a cumulative response rate of 18% among patients with NSCLC, 28% among patients with melanoma, and 27% among patients with renal cell carcinoma [116]. Furthermore, a phase I trial of nivolumab combined with ipilimumab enrolled 53 patients with advanced melanoma. This trial reported that 53% of patients experienced grade 3 or 4 adverse effects related to the therapy and 53% of patients had an objective response. Among patients treated with ipilimumab as a control, 20% had an objective response [117]. Thus, immune checkpoint blockade agents demonstrate efficacy in some types of tumors; however, further information is required to confirm the effectiveness of the immune blockade agents ipilimumab and nivolumab for bone and soft tissue sarcomas.

4. Conclusion and Future Directions

Conventional treatment for bone and soft tissue sarcomas consists of surgical resection, chemotherapy, and radiotherapy. However, clinical outcomes by these therapeutic modalities have not significantly improved in recent decades.

Under these circumstances, immunotherapy is expected to be a new therapeutic option for treatment. Cytokine therapies were initially regarded as a form of immunotherapy; however, their effectiveness was limited because of their toxicities. Only IFN-α-2 is used for maintenance therapy. Although L-MTP-PE induces antitumor effects via macrophage activation, the FDA has not approved its use because of the limited effectiveness. In Europe, L-MTP-PE efficacy has been confirmed in an international multicenter study. Vaccine therapy using tumor lysates or lysate-derived DCs has been investigated only in small-scale studies and in nonsarcoma patients. CTA peptide and fusion protein peptide therapies are expected to be novel sarcoma-effective vaccines. Addition of L-MTP-PE as an adjuvant may improve the vaccine therapy outcome. Novel microparticle-based drug delivery systems, such as microemulsion, nanoemulsion, nanoparticles, liposomes, and others, can load many kinds of various drugs and improve the drug delivery to target sites [118–121]. It has been reported that these systems improve the efficacy of vaccine and reduce adverse effects of cytokines [122–124]. Tuftsin, a tetrapeptide (Thr-Lys-Pro-Arg) fraction of immunoglobulin G molecule, binds to neutrophils and macrophages [125–127]. Tuftsin stimulates their phagocytic activity and enhances expression of nitric oxide synthase in macrophages. It has been demonstrated that tuftsin improves the efficacy of antibiotics against protozoan, bacterial, and fungal infections. Besides, tuftsin-bearing liposomized etoposide enhanced the therapeutic efficacy in murine fibrosarcoma models [128].

Immune checkpoint mechanism inhibits CD8 T cell function in tumor microenvironment [129]. Although immune checkpoint blockade molecules, anti-CTLA-4 antibody and anti-PD-1 antibody, have not been proven currently to have the effectiveness, there is too little information to decide efficacy of ipilimumab and nivolumab in sarcomas. Thus, immune checkpoint blockade medicines should be evaluated in the future. Adoptive cell transfer approaches are also the subject of new sarcoma treatment trials. Overall, these trials and successes suggest that immunotherapy is moving to the forefront of therapy for bone and soft tissue sarcomas.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The authors acknowledge a grant-in-aid for Scientific Research on Applying Health Technology from the Ministry of Health, Labor and Welfare of Japan, and a grant-in-aid for Health and Labour Sciences Research Expenses for Commission, Applied Research for Innovative Treatment of Cancer from the Ministry of Health, Labour and Welfare (H26-084).

References

[1] Z. Burningham, M. Hashibe, L. Spector et al., "The epidemiology of sarcoma," *Clinical Sarcoma Research*, vol. 2, no. 1, p. 14, 2012.

[2] National Cancer Institute, *A Snapshot of Sarcoma*, 2013.

[3] T. A. Damron, W. G. Ward, and A. Stewart, "Osteosarcoma, chondrosarcoma, and Ewing's sarcoma: national cancer data base report," *Clinical Orthopaedics and Related Research*, no. 459, pp. 40–47, 2007.

[4] L. A. Doyle, "Sarcoma classification: an update based on the 2013 world health organization classification of tumors of soft tissue and bone," *Cancer*, vol. 120, no. 12, pp. 1763–1774, 2014.

[5] A. Italiano, A. le Cesne, J. Mendiboure et al., "Prognostic factors and impact of adjuvant treatments on local and metastatic relapse of soft-tissue sarcoma patients in the competing risks setting," *Cancer*, vol. 120, no. 21, pp. 3361–3369, 2014.

[6] M. A. Tucker, G. J. D'Angio, J. D. Boice Jr. et al., "Bone sarcomas linked to radiotherapy and chemotherapy in children," *The New England Journal of Medicine*, vol. 317, no. 10, pp. 588–593, 1987.

[7] A. N. van Geel, U. Pastorino, K. W. Jauch et al., "Surgical treatment of lung metastases: the European organization for research and treatment of cancer-soft tissue and bone sarcoma group study of 255 patients," *Cancer*, vol. 77, no. 4, pp. 675–682, 1996.

[8] S. S. Bielack, B. Kempf-Bielack, G. Delling et al., "Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols," *Journal of Clinical Oncology*, vol. 20, no. 3, pp. 776–790, 2002.

[9] L. Mulder Renée, M. Paulides, T. Langer, L. C. Kremer, and E. C. van Dalen, "Cyclophosphamide versus ifosfamide for paediatric and young adult bone and soft tissue sarcoma patients," *Cochrane Database of Systematic Reviews*, vol. 17, no. 2, Article ID CD006300, 2012.

[10] W. B. Coley, "II. Contribution to the knowledge of sarcoma," *Annals of Surgery*, vol. 14, no. 3, pp. 199–220, 1891.

[11] J. Kluwe, A. Mencin, and R. F. Schwabe, "Toll-like receptors, wound healing, and carcinogenesis," *Journal of Molecular Medicine*, vol. 87, no. 2, pp. 125–138, 2009.

[12] F. Balkwill, "Tumour necrosis factor and cancer," *Nature Reviews Cancer*, vol. 9, no. 5, pp. 361–371, 2009.

[13] R. L. Modlin, "Innate immunity: ignored for decades, but not forgotten," *Journal of Investigative Dermatology*, vol. 132, no. 3, pp. 882–886, 2012.

[14] J. F. M. Jacobs, F. Brasseur, C. A. Hulsbergen-van de Kaa et al., "Cancer-germline gene expression in pediatric solid tumors using quantitative real-time PCR," *International Journal of Cancer*, vol. 120, no. 1, pp. 67–74, 2007.

[15] K. M. Skubitz, S. Pambuccian, J. C. Carlos, and A. P. N. Skubitz, "Identification of heterogeneity among soft tissue sarcomas by gene expression profiles from different tumors," *Journal of Translational Medicine*, vol. 6, article 23, 2008.

[16] W. Schwinger, V. Klass, M. Benesch et al., "Feasibility of high-dose interleukin-2 in heavily pretreated pediatric cancer patients," *Annals of Oncology*, vol. 16, no. 7, pp. 1199–1206, 2005.

[17] H. Ito, K. Murakami, T. Yanagawa et al., "Effect of human leukocyte interferon on the metastatic lung tumor of osteosarcoma: case reports," *Cancer*, vol. 46, no. 7, pp. 1562–1565, 1980.

[18] J. H. Edmonson, H. J. Long, S. Frytak, W. A. Smithson, and L. M. Itri, "Phase II study of recombinant alfa-2a interferon in patients with advanced bone sarcomas," *Cancer Treatment Reports*, vol. 71, no. 7-8, pp. 747–748, 1987.

[19] C. R. Müller, S. Smeland, H. C. F. Bauer, G. Sæter, and H. Strander, "Interferon-α as the only adjuvant treatment in high-grade osteosarcoma: long term results of the Karolinska

Hospital series," *Acta Oncologica*, vol. 44, no. 5, pp. 475–480, 2005.

[20] K. Winkler, G. Beron, R. Kotz et al., "Neoadjuvant chemotherapy for osteogenic sarcoma: Results of a cooperative German/Austrian study," *Journal of Clinical Oncology*, vol. 2, no. 6, pp. 617–624, 1984.

[21] S. S. Bielack, S. Smeland, J. Whelan et al., "MAP plus maintenance pegylated interferon alpha-2b (MAPIfn) versus MAP alone in patients with resectable high-grade osteosarcoma and good histologic response to preoperative MAP: first results of the EURAMOS-1 "good response" randomization," *Journal of Clinical Oncology*, vol. 31, no. 18, 2013.

[22] L. Kager, U. Potschger, and S. Bielack, "Review of mifamurtide in the treatment of patients with osteosarcoma," *Therapeutics and Clinical Risk Management*, vol. 6, no. 279–286, 2010.

[23] R. Dillman, N. Barth, S. Selvan et al., "Phase I/II trial of autologous tumor cell line-derived vaccines for recurrent or metastatic sarcomas," *Cancer Biotherapy and Radiopharmaceuticals*, vol. 19, no. 5, pp. 581–588, 2004.

[24] C. L. Mackall, E. H. Rhee, E. J. Read et al., "A pilot study of consolidative immunotherapy in patients with high-risk pediatric sarcomas," *Clinical Cancer Research*, vol. 14, no. 15, pp. 4850–4858, 2008.

[25] A. Suminoe, A. Matsuzaki, H. Hattori, Y. Koga, and T. Hara, "Immunotherapy with autologous dendritic cells and tumor antigens for children with refractory malignant solid tumors," *Pediatric Transplantation*, vol. 13, no. 6, pp. 746–753, 2009.

[26] S. Kawaguchi, T. Tsukahara, K. Ida et al., "SYT-SSX breakpoint peptide vaccines in patients with synovial sarcoma: a study from the Japanese Musculoskeletal Oncology Group," *Cancer Science*, vol. 103, no. 9, pp. 1625–1630, 2012.

[27] R. G. Maki, A. A. Jungbluth, S. Gnjatic et al., "A pilot study of anti-CTLA4 antibody ipilimumab in patients with synovial sarcoma," *Sarcoma*, vol. 2013, Article ID 168145, 8 pages, 2013.

[28] P. F. Robbins, R. A. Morgan, S. A. Feldman et al., "Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1," *Journal of Clinical Oncology*, vol. 29, no. 7, pp. 917–924, 2011.

[29] G. Schuler, B. Schuler-Thurner, and R. M. Steinman, "The use of dendritic cells in cancer immunotherapy," *Current Opinion in Immunology*, vol. 15, no. 2, pp. 138–147, 2003.

[30] L. Gattinoni, D. J. Powell Jr., S. A. Rosenberg, and N. P. Restifo, "Adoptive immunotherapy for cancer: building on success," *Nature Reviews Immunology*, vol. 6, no. 5, pp. 383–393, 2006.

[31] S. A. Rosenberg, N. P. Restifo, J. C. Yang, R. A. Morgan, and M. E. Dudley, "Adoptive cell transfer: a clinical path to effective cancer immunotherapy," *Nature Reviews Cancer*, vol. 8, no. 4, pp. 299–308, 2008.

[32] S. A. Rosenberg, J. J. Mule, P. J. Spiess, C. M. Reichert, and S. L. Schwarz, "Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2," *The Journal of Experimental Medicine*, vol. 161, no. 5, pp. 1169–1188, 1985.

[33] W. H. West, K. W. Tauer, J. R. Yannelli et al., "Constant-infusion recombinant interleukin-2 in adoptive immunotherapy of advanced cancer," *The New England Journal of Medicine*, vol. 316, no. 15, pp. 898–905, 1987.

[34] S. A. Rosenberg, P. Spiess, and R. Lafreniere, "A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes," *Science*, vol. 233, no. 4770, pp. 1318–1321, 1986.

[35] S. A. Rosenberg, J. C. Yang, and N. P. Restifo, "Cancer immunotherapy: moving beyond current vaccines," *Nature Medicine*, vol. 10, no. 9, pp. 909–915, 2004.

[36] K. E. de Visser, A. Eichten, and L. M. Coussens, "Paradoxical roles of the immune system during cancer development," *Nature Reviews Cancer*, vol. 6, no. 1, pp. 24–37, 2006.

[37] R. Medzhitov and C. A. Janeway Jr., "Innate immunity: the virtues of a nonclonal system of recognition," *Cell*, vol. 91, no. 3, pp. 295–298, 1997.

[38] C. A. Janeway Jr. and R. Medzhitov, "Innate immune recognition," *Annual Review of Immunology*, vol. 20, no. 1, pp. 197–216, 2002.

[39] T. Kawai and S. Akira, "TLR signaling," *Cell Death & Differentiation*, vol. 13, no. 5, pp. 816–825, 2006.

[40] K. Takeda and S. Akira, "TLR signaling pathways," *Seminars in Immunology*, vol. 16, no. 1, pp. 3–9, 2004.

[41] H. R. MacDonald and M. Nabholz, "T-cell activation," *Annual Review of Cell Biology*, vol. 2, pp. 231–253, 1986.

[42] J. E. Smith-Garvin, G. A. Koretzky, and M. S. Jordan, "T cell activation," *Annual Review of Immunology*, vol. 27, pp. 591–619, 2009.

[43] A. Grakoui, S. K. Bromley, C. Sumen et al., "The immunological synapse: a molecular machine controlling T cell activation," *Science*, vol. 285, no. 5425, pp. 221–227, 1999.

[44] S. M. Kaech, E. J. Wherry, and R. Ahmed, "Effector and memory T-cell differentiation: implications for vaccine development," *Nature Reviews Immunology*, vol. 2, no. 4, pp. 251–262, 2002.

[45] S. M. Kaech, S. Hemby, E. Kersh, and R. Ahmed, "Molecular and functional profiling of memory CD8 T cell differentiation," *Cell*, vol. 111, no. 6, pp. 837–851, 2002.

[46] D. Mauri, T. Wyss-Coray, H. Gallati, and W. J. Pichler, "Antigen-presenting T cells induce the development of cytotoxic $CD4^+$ T cells. I. Involvement of the CD80-CD28 adhesion molecules," *The Journal of Immunology*, vol. 155, no. 1, pp. 118–127, 1995.

[47] I. S. Grewal and R. A. Flavell, "A central role of CD40 ligand in the regulation of $CD4^+$ T-cell responses," *Immunology Today*, vol. 17, no. 9, pp. 410–414, 1996.

[48] S. R. M. Bennett, F. R. Carbone, F. Karamalis, R. A. Flaveli, J. F. A. P. Miller, and W. R. Heath, "Help for cytotoxic-T-cell responses is mediated by CD40 signalling," *Nature*, vol. 393, no. 6684, pp. 478–480, 1998.

[49] K. Murali-Krishna, J. D. Altman, M. Suresh et al., "Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection," *Immunity*, vol. 8, no. 2, pp. 177–187, 1998.

[50] R. M. Steinman, "The dendritic cell system and its role in immunogenicity," *Annual Review of Immunology*, vol. 9, pp. 271–296, 1991.

[51] J. Banchereau and R. M. Steinman, "Dendritic cells and the control of immunity," *Nature*, vol. 392, no. 6673, pp. 245–252, 1998.

[52] M. L. Albert, B. Sauter, and N. Bhardwaj, "Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLS," *Nature*, vol. 392, no. 6671, pp. 86–89, 1998.

[53] M. Corr, A. E. Slanetz, L. F. Boyd et al., "T cell receptor-MHC class I peptide interactions: affinity, kinetics, and specificity," *Science*, vol. 265, no. 5174, pp. 946–949, 1994.

[54] P. R. Scholl, P. R. Scholl, R. S. Geha, and R. S. Geha, "MHC class II signaling in B-cell activation," *Immunology Today*, vol. 15, no. 9, pp. 418–422, 1994.

[55] W. R. Heath and F. R. Carbone, "Cross-presentation, dendritic cells, tolerance and immunity," *Annual Review of Immunology*, vol. 19, no. 1, pp. 47–64, 2001.

[56] H. Udono, T. Yamano, Y. Kawabata, M. Ueda, and K. Yui, "Generation of cytotoxic T lymphocytes by MHC class I ligands fused to heat shock cognate protein 70," *International Immunology*, vol. 13, no. 10, pp. 1233–1242, 2001.

[57] A. L. Ackerman and P. Cresswell, "Cellular mechanisms governing cross-presentation of exogenous antigens," *Nature Immunology*, vol. 5, no. 7, pp. 678–684, 2004.

[58] M. Chiriva-Internati, A. Pandey, R. Saba et al., "Cancer testis antigens: a novel target in lung cancer," *International Reviews of Immunology*, vol. 31, no. 5, pp. 321–343, 2012.

[59] E. Stockert, E. Jäger, Y.-T. Chen et al., "A survey of the humoral immune response of cancer patients to a panel of human tumor antigens," *Journal of Experimental Medicine*, vol. 187, no. 8, pp. 1349–1354, 1998.

[60] C. Coppin, F. Porzsolt, J. Kumpf, A. Coldman, and T. Wilt, "Immunotherapy for advanced renal cell cancer," *Cochrane Database of Systematic Reviews*, 2004.

[61] A. D. Sasse, E. C. Sasse, L. G. O. Clark, L. Ulloa, and O. A. C. Clark, "Chemoimmunotherapy versus chemotherapy for metastatic malignant melanoma," *Cochrane Database of Systematic Reviews*, Article ID CD005413, 2007.

[62] G. A. Rabinovich, D. Gabrilovich, and E. M. Sotomayor, "Immunosuppressive strategies that are mediated by tumor cells," *Annual Review of Immunology*, vol. 25, pp. 267–296, 2007.

[63] T. L. Walunas, D. J. Lenschow, C. Y. Bakker et al., "CTLA-4 can function as a negative regulator of T cell activation," *Immunity*, vol. 1, no. 5, pp. 405–413, 1994.

[64] P. S. Linsley, J. L. Greene, W. Brady et al., "Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors," *Immunity*, vol. 1, no. 9, pp. 793–801, 1994.

[65] K. Wing, Y. Onishi, P. Prieto-Martin et al., "CTLA-4 control over Foxp3$^+$ regulatory T cell function," *Science*, vol. 322, no. 5899, pp. 271–275, 2008.

[66] E. R. Kearney, T. L. Walunas, R. W. Karr et al., "Antigen-dependent clonal expansion of a trace population of antigen-specific CD4$^+$ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4," *The Journal of Immunology*, vol. 155, no. 3, pp. 1032–1036, 1995.

[67] D. L. Barber, E. J. Wherry, D. Masopust et al., "Restoring function in exhausted CD8 T cells during chronic viral infection," *Nature*, vol. 439, no. 7077, pp. 682–687, 2006.

[68] C. Petrovas, J. P. Casazza, J. M. Brenchley et al., "PD-1 is a regulator of virus-specific CD8$^+$ T cell survival in HIV infection," *The Journal of Experimental Medicine*, vol. 203, no. 10, pp. 2281–2292, 2006.

[69] K. Sakuishi, L. Apetoh, J. M. Sullivan, B. R. Blazar, V. K. Kuchroo, and A. C. Anderson, "Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity," *Journal of Experimental Medicine*, vol. 207, no. 10, pp. 2187–2194, 2010.

[70] Y. Latchman, C. R. Wood, T. Chernova et al., "PD-L2 is a second ligand for PD-1 and inhibits T cell activation," *Nature Immunology*, vol. 2, no. 3, pp. 261–268, 2001.

[71] M. K. Callahan and J. D. Wolchok, "At the bedside: CTLA-4- and PD-1-blocking antibodies in cancer immunotherapy," *Journal of Leukocyte Biology*, vol. 94, no. 1, pp. 41–53, 2013.

[72] D. M. Pardoll, "The blockade of immune checkpoints in cancer immunotherapy," *Nature Reviews Cancer*, vol. 12, no. 4, pp. 252–264, 2012.

[73] A. Chaudhry, D. Rudra, P. Treuting et al., "CD4$^+$ regulatory T cells control TH17 responses in a stat3-dependent manner," *Science*, vol. 326, no. 5955, pp. 986–991, 2009.

[74] J. R. Brahmer, S. S. Tykodi, L. Q. M. Chow et al., "Safety and activity of anti-PD-L1 antibody in patients with advanced cancer," *The New England Journal of Medicine*, vol. 366, no. 26, pp. 2455–2465, 2012.

[75] Y. Iwai, M. Ishida, Y. Tanaka, T. Okazaki, T. Honjo, and N. Minato, "Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 19, pp. 12293–12297, 2002.

[76] L. M. Francisco, V. H. Salinas, K. E. Brown et al., "PD-L1 regulates the development, maintenance, and function of induced regulatory T cells," *Journal of Experimental Medicine*, vol. 206, no. 13, pp. 3015–3029, 2009.

[77] S. Lee and K. Margolin, "Cytokines in cancer immunotherapy," *Cancers*, vol. 3, no. 4, pp. 3856–3893, 2011.

[78] J.-P. Shaw, P. J. Utz, D. B. Durand, J. J. Toole, E. A. Emmel, and G. R. Crabtree, "Indentification of a putative regulator of early T cell activation genes," *Science*, vol. 241, no. 4862, pp. 202–205, 1988.

[79] M. T. Lotze, A. E. Chang, C. A. Seipp, C. Simpson, J. T. Vetto, and S. A. Rosenberg, "High-dose recombinant interleukin 2 in the treatment of patients with disseminated cancer. Responses, treatment-related morbidity, and histologic findings," *The Journal of the American Medical Association*, vol. 256, no. 22, pp. 3117–3124, 1986.

[80] M. B. Atkins, M. T. Lotze, J. P. Dutcher et al., "High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993," *Journal of Clinical Oncology*, vol. 17, no. 7, pp. 2105–2116, 1999.

[81] R. N. Schwartz, L. Stover, and J. Dutcher, "Managing toxicities of high-dose interleukin-2," *Oncology*, vol. 16, no. 11, pp. 11–20, 2002.

[82] D. J. Schwartzentruber, "Guidelines for the safe administration of high-dose interleukin-2," *Journal of Immunotherapy*, vol. 24, no. 4, pp. 287–293, 2001.

[83] M. P. Link, A. M. Goorin, A. W. Miser et al., "The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity," *The New England Journal of Medicine*, vol. 314, no. 25, pp. 1600–1606, 1986.

[84] K. Ando, K. Mori, N. Corradini, F. Redini, and D. Heymann, "Mifamurtide for the treatment of nonmetastatic osteosarcoma," *Expert Opinion on Pharmacotherapy*, vol. 12, no. 2, pp. 285–292, 2011.

[85] P. M. Anderson, M. Tomaras, and K. McConnell, "Mifamurtide in osteosarcoma-a practical review," *Drugs of Today*, vol. 46, no. 5, pp. 327–337, 2010.

[86] K. Geddes, J. G. Magalhães, and S. E. Girardin, "Unleashing the therapeutic potential of NOD-like receptors," *Nature Reviews Drug Discovery*, vol. 8, no. 6, pp. 465–479, 2009.

[87] J. E. Frampton, P. M. Anderson, A. J. Chou et al., "Mifamurtide: a review of its use in the treatment of osteosarcoma," *Pediatric Drugs*, vol. 12, no. 3, pp. 141–153, 2010.

[88] E. G. MacEwen, I. D. Kurzman, R. C. Rosenthal et al., "Therapy for osteosarcoma in dogs with intravenous injection of liposome-encapsulated muramyl tripeptide," *Journal of the National Cancer Institute*, vol. 81, no. 12, pp. 935–938, 1989.

[89] P. A. Meyers, C. L. Schwartz, M. D. Krailo et al., "Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival—a report from the children's oncology group," *Journal of Clinical Oncology*, vol. 26, no. 4, pp. 633–638, 2008.

[90] M. Ayyoub, R. N. Taub, M. L. Keohan et al., "The frequent expression of cancer/testis antigens provides opportunities for immunotherapeutic targeting of sarcoma," *Cancer Immunity*, vol. 4, no. 7, 2004.

[91] R. Takahashi, Y. Ishibashi, K. Hiraoka et al., "Phase II study of personalized peptide vaccination for refractory bone and soft tissue sarcoma patients," *Cancer Science*, vol. 104, no. 10, pp. 1285–1294, 2013.

[92] S. E. Finkelstein, M. Fishman, A. P. Conley, D. Gabrilovich, S. Antonia, and A. Chiappori, "Cellular immunotherapy for soft tissue sarcomas," *Immunotherapy*, vol. 4, no. 3, pp. 283–290, 2012.

[93] C. Harrison, "Vaccines: nanorings boost vaccine adjuvant effects," *Nature Reviews Drug Discovery*, vol. 13, no. 7, pp. 496–496, 2014.

[94] M. Vergati, C. Intrivici, N.-Y. Huen, J. Schlom, and K. Y. Tsang, "Strategies for cancer vaccine development," *Journal of Biomedicine and Biotechnology*, vol. 2010, Article ID 596432, 13 pages, 2010.

[95] F. J. Hsu, C. Benike, F. Fagnoni et al., "Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells," *Nature Medicine*, vol. 2, no. 1, pp. 52–58, 1996.

[96] C. M. Celluzzi, J. I. Mayordomo, W. J. Storkus, M. T. Lotze, and L. D. Falo Jr., "Peptide-pulsed dendritic cells induce antigen-specific, CTL-mediated protective tumor immunity," *Journal of Experimental Medicine*, vol. 183, no. 1, pp. 283–287, 1996.

[97] J. D. Geiger, R. J. Hutchinson, L. F. Hohenkirk et al., "Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cells and mediate tumor regression," *Cancer Research*, vol. 61, no. 23, pp. 8513–8519, 2001.

[98] H. L. Chen and D. P. Carbone, "p53 as a target for anti-cancer immunotherapy," *Molecular Medicine Today*, vol. 3, no. 4, pp. 160–167, 1997.

[99] S. Gnjatic, H. Nishikawa, A. A. Jungbluth et al., "NY-ESO-1: review of an immunogenic tumor antigen," *Advances in Cancer Research*, vol. 95, pp. 1–30, 2006.

[100] S. A. Rosenberg, "A new era for cancer immunotherapy based on the genes that encode cancer antigens," *Immunity*, vol. 10, no. 3, pp. 281–287, 1999.

[101] A. Kawai, J. Woodruff, J. H. Healey, M. F. Brennan, C. R. Antonescu, and M. Ladanyi, "*SYT-SSX* gene fusion as a determinant of morphology and prognosis in synovial sarcoma," *The New England Journal of Medicine*, vol. 338, no. 3, pp. 153–160, 1998.

[102] S. Kawaguchi, T. Wada, K. Ida et al., "Phase I vaccination trial of SYT-SSX junction peptide in patients with disseminated synovial sarcoma," *Journal of Translational Medicine*, vol. 3, article 1, 2005.

[103] L. He, H. Feng, A. Raymond et al., "Dendritic-cell-peptide immunization provides immunoprotection against *bcr-abl*-positive leukemia in mice," *Cancer Immunology, Immunotherapy*, vol. 50, no. 1, pp. 31–40, 2001.

[104] R. Yamanaka, N. Yajima, T. Abe et al., "Dendritic cell-based glioma immunotherapy (review)," *International Journal of Oncology*, vol. 23, no. 1, pp. 5–15, 2003.

[105] A. J. G. Simpson, O. L. Caballero, A. Jungbluth, Y.-T. Chen, and L. J. Old, "Cancer/testis antigens, gametogenesis and cancer," *Nature Reviews Cancer*, vol. 5, no. 8, pp. 615–625, 2005.

[106] R. L. Jones, S. M. Pollack, E. T. Loggers, E. T. Rodler, and C. Yee, "Immune-based therapies for sarcoma," *Sarcoma*, vol. 2011, Article ID 438940, 7 pages, 2011.

[107] C. Roeder, B. Schuler-Thurner, S. Berchtold et al., "MAGE-A3 is a frequent tumor antigen of metastasized melanoma," *Archives of Dermatological Research*, vol. 296, no. 7, pp. 314–319, 2005.

[108] W. Sienel, C. Varwerk, A. Linder et al., "Melanoma associated antigen (MAGE)-A3 expression in Stages I and II non-small cell lung cancer: results of a multi-center study," *European Journal of Cardio-Thoracic Surgery*, vol. 25, no. 1, pp. 131–134, 2004.

[109] O. L. Caballero and Y.-T. Chen, "Cancer/testis (CT) antigens: potential targets for immunotherapy," *Cancer Science*, vol. 100, no. 11, pp. 2014–2021, 2009.

[110] M. E. Dudley and S. A. Rosenberg, "Adoptive-cell-transfer therapy for the treatment of patients with cancer," *Nature Reviews Cancer*, vol. 3, no. 9, pp. 666–675, 2003.

[111] D. W. Lee, D. M. Barrett, C. Mackall, R. Orentas, and S. A. Grupp, "The future is now: chimeric antigen receptors as new targeted therapies for childhood cancer," *Clinical Cancer Research*, vol. 18, no. 10, pp. 2780–2790, 2012.

[112] J. Weber, "Review: anti-CTLA-4 antibody ipilimumab: case studies of clinical response and immune-related adverse events," *The Oncologist*, vol. 12, no. 7, pp. 864–872, 2007.

[113] F. S. Hodi, S. J. O'Day, D. F. McDermott et al., "Improved survival with ipilimumab in patients with metastatic melanoma," *The New England Journal of Medicine*, vol. 363, no. 8, pp. 711–723, 2010.

[114] K. Wiater, T. Witaj, J. Mackiewicz et al., "Efficacy and safety of ipilimumab therapy in patients with metastatic melanoma: a retrospective multicenter analysis," *Contemporary Oncology*, vol. 17, no. 3, pp. 257–262, 2013.

[115] E. D. Deeks, "Nivolumab: a review of its use in patients with malignant melanoma," *Drugs*, vol. 74, no. 11, pp. 1233–1239, 2014.

[116] S. L. Topalian, F. S. Hodi, J. R. Brahmer et al., "Safety, activity, and immune correlates of anti-PD-1 antibody in cancer," *The New England Journal of Medicine*, vol. 366, no. 26, pp. 2443–2454, 2012.

[117] J. D. Wolchok, H. Kluger, M. K. Callahan et al., "Nivolumab plus Ipilimumab in advanced melanoma," *The New England Journal of Medicine*, vol. 369, no. 2, pp. 122–133, 2013.

[118] M. Farazuddinm, B. Sharma, A. A. Khan, B. Joshi, and M. Owais, "Anticancer effcacy of perillyl alcohol-bearing PLGA microparticles," *International Journal of Nanomedicine*, vol. 7, pp. 35–47, 2012.

[119] A. Khan, Y. Shukla, N. Kalra et al., "Potential of diallyl sulfide bearing pH-sensitive liposomes in chemoprevention against DMBA-induced skin papilloma," *Molecular Medicine*, vol. 13, no. 7-8, pp. 443–451, 2007.

[120] M. Farazuddin, B. Dua, Q. Zia, A. A. Khan, B. Joshi, and M. Owais, "Chemotherapeutic potential of curcumin-bearing microcells against hepatocellular carcinoma in model animals," *International Journal of Nanomedicine*, vol. 9, no. 1, pp. 1139–1152, 2014.

[121] A. Chauhan, S. Zubair, A. Nadeem, S. A. Ansari, M. Y. Ansari, and O. Mohammad, "Escheriosome-mediated cytosolic

delivery of PLK1-specific siRNA: potential in treatment of liver cancer in BALB/c mice," *Nanomedicine*, vol. 9, no. 4, pp. 407–420, 2014.

[122] A. Chauhan, S. Zubair, S. Tufail et al., "Fungus-mediated biological synthesis of gold nanoparticles: potential in detection of liver cancer," *International Journal of Nanomedicine*, vol. 6, pp. 2305–2319, 2011.

[123] H. Singha, A. I. Mallick, C. Jana, N. Fatima, M. Owais, and P. Chaudhuri, "Co-immunization with interlukin-18 enhances the protective efficacy of liposomes encapsulated recombinant Cu-Zn superoxide dismutase protein against Brucella abortus," *Vaccine*, vol. 29, no. 29-30, pp. 4720–4727, 2011.

[124] M. A. Khan, A. Aljarbou, A. Khan, and M. Owais, "Immune stimulating and therapeutic potential of tuftsin-incorporated nystatin liposomes against *Cryptococcus neoformans* in leukopenic BALB/C mice," *FEMS Immunology and Medical Microbiology*, vol. 66, no. 1, pp. 88–97, 2012.

[125] M. A. Khan, A. Khan, and M. Owais, "Prophylactic use of liposomized tuftsin enhances the susceptibility of *Candida albicans* to fluconazole in leukopenic mice," *FEMS Immunology and Medical Microbiology*, vol. 46, no. 1, pp. 63–69, 2006.

[126] M. A. Khan and M. Owais, "Toxicity, stability and pharmacokinetics of amphotericin B in immunomodulator tuftsin-bearing liposomes in a murine model," *Journal of Antimicrobial Chemotherapy*, vol. 58, no. 1, pp. 125–132, 2006.

[127] M. A. Khan, S. M. Faisal, and O. Mohammad, "Safety, efficacy and pharmacokinetics of tuftsin-loaded nystatin liposomes in murine model," *Journal of Drug Targeting*, vol. 14, no. 4, pp. 233–241, 2006.

[128] A. Khan, A. A. Khan, V. Dwivedi, M. G. Ahmad, S. Hakeem, and M. Owais, "Tuftsin augments antitumor efficacy of liposomized etoposide against fibrosarcoma in Swiss albino mice," *Molecular Medicine*, vol. 13, no. 5-6, pp. 266–276, 2007.

[129] J. Crespo, H. Sun, T. H. Welling, Z. Tian, and W. Zou, "T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment," *Current Opinion in Immunology*, vol. 25, no. 2, pp. 214–221, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 717530, 12 pages
http://dx.doi.org/10.1155/2015/717530

Review Article

Dendritic Cell-Based Immunotherapy Treatment for Glioblastoma Multiforme

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Received 25 September 2014; Accepted 8 January 2015

Academic Editor: Yung-Fu Chang

Glioblastoma multiforme (GBM) is the most malignant glioma and patients diagnosed with this disease had poor outcomes even treated with the combination of conventional treatment (surgery, chemotherapy, and radiation). Dendritic cells (DCs) are the most powerful antigen presenting cells and DC-based vaccination has the potential to target and eliminate GBM cells and enhance the responses of these cells to the existing therapies with minimal damage to the healthy tissues around them. It can enhance recognition of GBM cells by the patients' immune system and activate vast, potent, and long-lasting immune reactions to eliminate them. Therefore, this therapy can prolong the survival of GBM patients and has wide and bright future in the treatment of GBM. Also, the efficacy of this therapy can be strengthened in several ways at some degree: the manipulation of immune regulatory components or costimulatory molecules on DCs; the appropriate choices of antigens for loading to enhance the effectiveness of the therapy; regulation of positive regulators or negative regulators in GBM microenvironment.

1. Introduction

Glioblastoma multiforme (GBM) is the most malignant glioma, accounting for 60–70% of all gliomas [1]; 88% of all GBM patients die within 3 years [2]. Complete surgical resection is difficult to perform due to tumor infiltration into the brain parenchyma and eventual tumor relapse [3]. The median survival time (MS) is 12–15 months with conventional treatment (surgery, chemotherapy, and radiation) for primary GBM patients [1], and 3–6 months for recurrent GBM patients [4].

Therefore, new treatment options are needed to improve patient outcomes for this unmet clinical need. Immunotherapy may be a successful treatment option with the advantage of high tumor-specific targeting [5]. Several reagents have recently gained the Food and Drug Administration (FDA) approval and have demonstrated clinical benefit [6, 7]. The focus of immunotherapy vaccines is based upon the concept that antigen presenting cells (APCs) can effectively be loaded with tumor derived antigens that will accelerate tumor eradication within *in vivo* settings [8]. Dendritic cells (DCs) are the most powerful human APCs and DC-based vaccines have the potential to improve clinical outcomes by enhancing GBM cell responses to existing therapy and/or stimulating innate immune responses with minimal toxicity. Ultimately, vaccination should enhance recognition of GBM cells by the patients' immune system and increase activity of tumor-infiltrating lymphocytes (TILs) against them [9], creating potent, long-lasting tumor-specific T lymphocytes. Within the context of this paper, we review DC-based vaccination for GBM patients as shown in Algorithm 1.

2. Dendritic Cells (DCs)

DCs are at minimum, large, granular lymphocytes with high cell surface markers: major histocompatibility complex (MHC) class I molecules, MHC class II molecules, and CD86, all of which can help identify DCs from other myeloid lineage cells [10]. They recognize and capture antigens in their immature state and then migrate to lymphoid organs where they present processed peptides (derived from captured antigens)

(1) Dendritic cells (DCs)
  (1.1) Selection of DC subpopulations
  (1.2) DC differentiation
  (1.3) Manipulation of co-stimulatory and co-inhibitory signals via DCs
    (1.3.1) Positive regulators
    (1.3.2) Negative regulators
(2) Loading antigens
  (2.1) Glioblastoma-associated antigens (GAAs) and glioblastoma specific antigens (GSAs)
  (2.2) Glioma stem cell (GSC) antigens
(3) Regulation of the GBM microenvironment
  (3.1) Positive regulators
  (3.2) Negative regulators

Algorithm 1: Classification scheme of approaches to strengthen the efficacy of DC vaccines in the treatment of glioblastoma.

to T cells in the context of MHC I or II [11, 12] and therefore induce tumor antigen-specific immune responses. They also display various characteristics in immune regulatory systems that balance the complex system of inflammatory and inhibitory immune reactions in the tumor microenvironment [3]. Therefore, they are involved in aspects of both innate and adaptive immune systems and can modulate immune functions, reverse immune suppression, and decrease tumor immune tolerance and therefore terminate low immunoreactivity in tumor patients [13].

2.1. Selection of DC Subpopulations. DCs can be divided into two distinct subtypes, types 1 and 2. Type 1 polarizing DC (DC1) subsets are associated with antitumor immunity as they direct effector T cell responses to the helper T cell 1 (Th1) phenotype, whereas the DC2 subset is vital for antitumor immunity against extracellular antigens (Figure 1). DC1 polarization induces abundant production of interleukin (IL)-12p70 heterodimer and IL-23, secretion of chemokine MIP-1, and expression of Delta-4 Notch ligand [14]. Products induced by DC1 are associated with chemoattraction and activation of Th1-type $CD4^+$ and $CD8^+$ T cells. Moreover, IL-12p70 is critical for the sensitization of high-avidity T cells which recognize and kill tumor targets directly [3, 14–16]. Therefore, the choice of DC1 may be inviting.

2.2. DC Differentiation. DC differentiation from bone marrow (BM) precursors can be induced by granulocyte macrophage colony-stimulating factor (GM-CSF) or FMS-like tyrosine kinase-3 ligand (Flt3L) (Figure 1). GM-CSF expands both DC1 and DC2 subsets, yielding more DC2 than DC1 cells, whereas Flt3L preferentially expands the DC1 subset. Both Flt3L and GM-CSF increase naive and memory T cells in mice, but memory $CD4^+$ and $CD8^+$ T cells are increased more by Flt3L compared to GM-CSF. GM-CSF increases the frequency of both Th1 and helper T cell 2 (Th2) cells, and Flt3L mainly increases Th1 cell frequency. DC1 isolated from Flt3L-injected mice had more IL-12p40 than IL-10, compared to DC2 [17, 18] (Table 1).

When BM cells were cultured with GM-CSF, followed by interferon (IFN)-γ, IFN-α, IL-4 and polyinosinic-polycytidylic acid (polyI:C), the proportion and function of the DC1 subset in GM-CSF-treated progenitor cells were increased. Such α-type-1 polarized DCs produced more IL-12 compared to the normal DC1 subset and they were more resistant to the immunosuppressive environment created by regulatory T cells (Tregs) [16]. Also α-type-1 polarized DC vaccines loaded with GBM antigens could effectively control GBM relapse by inducing Th1 and cytotoxic lymphocyte (CTL) responses and suppressing accumulation of Tregs in Draining Lymph Nodes (DLNs) in mouse models [15]. When mouse BM cells are cultured with Flt3L, followed by IL-6 stimulation, $CD34^+$ progenitor cells are expanded and then differentiated into DCs [19].

Table 1: Comparison between GM-CSF and Flt3L in culturing DCs. GM-CSF: granulocyte macrophage colony-stimulating factor, Flt3L: FMS-like tyrosine kinase-3 ligand, DC: dendritic cell, DC1: type 1 polarizing DC, DC2: type 2 polarizing DC, Th1: helper T cell 1, and Th2: helper T cell 2.

	GM-CSF	Flt3L
DC subsets expending [17]	DC1 < DC2	DC1 > DC2
T cells expanding [17]		More memory $CD4^+$, $CD8^+$ T cells
Helper T cells expanding [17]	Both Th1 and Th2	Mainly Th1

DCs conditioned from GM-CSF and DCs conditioned from Flt3L have different properties, and cell population admixtures may be best for DC preparations [3]. When Flt3L and GM-CSF were combined, DC infiltration into mouse tumors was inhibited and Tregs were activated, thereby promoted tumor tolerance [19]. And the combined cytokine regimen seems to increase the number of tumor-infiltrating dendritic cells (TIDCs) that can induce antigen-specific $CD8^+$ T cells but also $CD4^+$ Tregs that may neutralize the antitumor activity of the $CD8^+$ T cells in situ [20]. But the method culturing DCs from humans by using GM-CSF and Flt3L remains to be explored.

2.3. Manipulation of Costimulatory and Coinhibitory Signals via DCs. Many costimulatory and coinhibitory molecules found on DCs function differently in varying immune

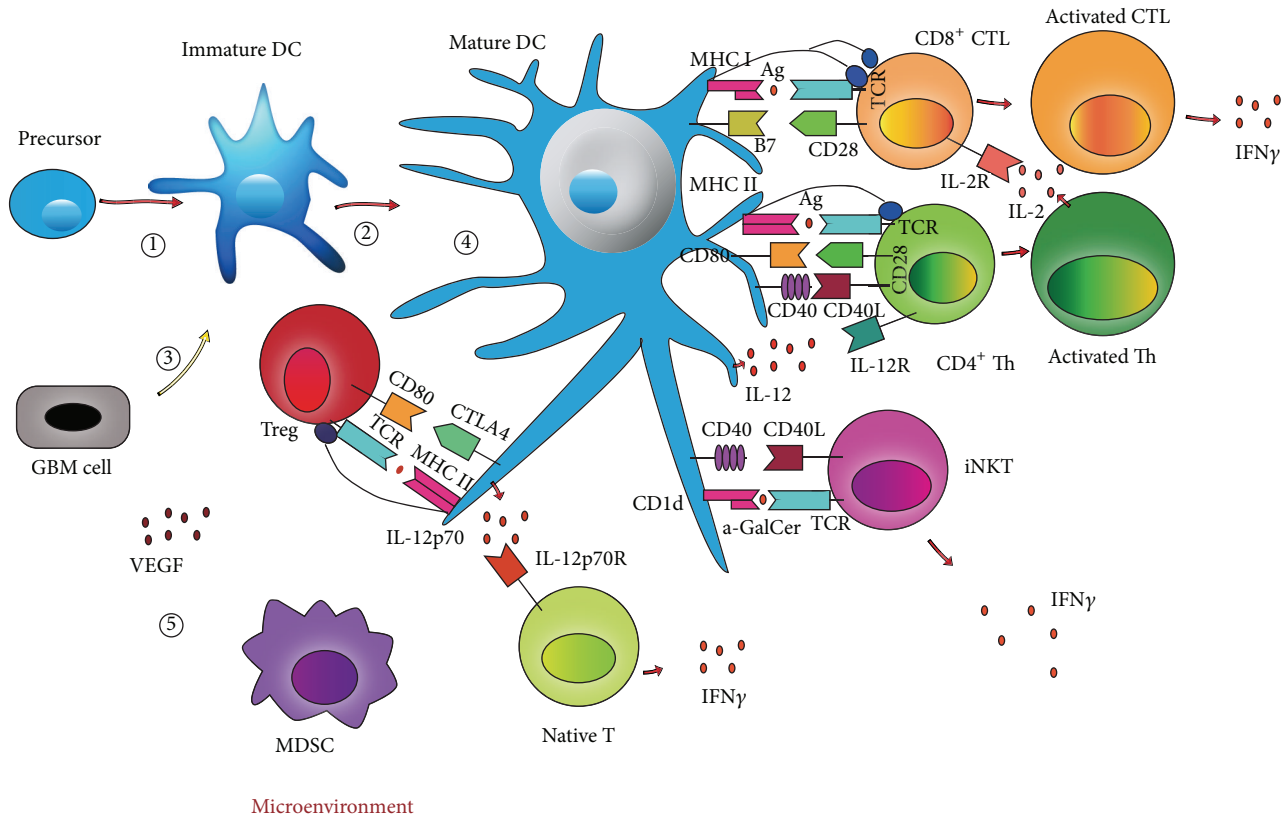

Figure 1: Dendritic cell (DC)-based vaccination immunotherapeutic strategies for glioblastoma multiforme (GBM). Bone-marrow derived precursors are differentiated into DCs by Flt3L or GM-CSF. DCs can be divided into two distinct subtypes, types 1 and 2. They act differently and have synergistic effects in antitumor immunity. They can be loaded with GBM antigens derived from RNA, DNA, proteins, peptides, lysates, glioma stem cells antigens, apoptotic cells or fusion. They recognize and capture antigens, then they present processed peptides (derived from captured antigens) to T cells in the context of major histocompatibility complex (MHC) class I or II (signal 1). Then pulse tumor-loaded DCs with maturation stimuli to increase the expression of costimulatory molecules such as CD80 (signal 2) and the secretion of proinflammatory cytokines such as IL-12 (signal 3). Then CD4+ helper T cells secrete IL-2 to stimulate CD8+ cytotoxic T cells which then secrete IFN-γ and exhibit cytolytic immune responses against GBM cells. Upregulating costimulatory signals or suppressing coinhibitory signals can strengthen the efficacy of DC vaccines. Manipulation of these signals includes: TLR agonists, CD40 ligand, CD70, tumor necrosis factor receptor superfamily-member 4 (TNFRSF4) ligandDi, iNKTs agonists, and silencing A20 or SOCS1 by siRNA et al. Moreover, regulation of GBM microenvironment also can enhance the efficacy of DC vaccines. These regulation includes: the addition of some leukocytes and cytokines, Treg depletion, MDSCs inhibition, and VEGF inhibition et al. Ag: antigen, CTL: cytotoxic T-cell, CTLA-4: cytotoxic T-lymphocyte antigen 4, DC: dendtiric cell, DC1: type 1 polarizing DC, DC2: type 2 polarizing DC, Flt3L: fms-like tyrosine kinase 3 ligand, GM-CSF: glanulocyte monocyte-colony stimulating factor, IFN: interferon, IL: interleukin, iNKTs: Invariant natural killer T cells, MDSC: myeloid-derived suppressor cell, MHC: major histocompatibility class, siRNA: small interfering RNA, SOCS1: suppressor of cytokine signaling 1, TCR: T cell receptor, Th: helper T cells, TLR: Toll-like receptor, Treg: regulatory T cell, VEGF: vascular endothelial growth factor. ① Differentiation: GM-CSF/Flt3L. ② Selection of subpopulation: DC1/DC2. ③ Antigen loading: RNA, DNA, proteins, peptides, lysates, glioma stem cell antigens, fusion, and apoptotic cells. ④ Manipulation signals in DCs: TLR agonists, CD40 ligand, CD70, TNFRSF4 ligandDi, iNKTs agonists, silencing A20 or SOCS1 by siRNA. ⑤ Regulation of GBM microenvironment: the manipulation of some leukocytes and cytokines, Treg depletion, MDSCs inhibition, and VEGF inhibition.

response situations. Upregulating costimulatory signals or suppressing coinhibitory signals can strengthen the efficacy of DC vaccines (Figure 1).

The presence of immunosuppressive conditioning (such as IL-10 or IL-27) or costimulatory molecule expression insufficiency (B7-1) or proinflammatory cytokine secretion (IL-12) can induce tolerogenic DCs, which express coinhibitory molecules and secrete immunosuppressive cytokines, subsequently inducing tolerance. Tolerogenic DCs can secret soluble factors which attract Tregs to the tumor microenvironment. These factors include chemokines CCL17 and CCL12, which bind to CCR4 and CCR8 receptors on Tregs [21]. Blockade of CCL17 and CCL22 can reduce Tregs migration to the tumor microenvironment, sustaining sufficient antitumor immunity [3].

2.3.1. Positive Regulators. Costimulatory molecules are important for the induction of immune responses. Robust T cell responses not only need a signal induced through T cell receptors via recognition of antigenic peptide MHC molecules on DCs, but also call for signals provided by interactions of costimulatory ligands on T cells and their

receptors on DCs [3, 22, 23]. Antigen-specific T cells become anergic in the absence of costimulatory molecule interactions [24]. Therefore, the therapeutic immunity of DC vaccines can be strengthened by upregulating the costimulatory molecules [25].

Costimulatory molecules belong to two major families: the B7/CD28 family and the tumor necrosis factor (TNF)/TNF receptor family. B7/CD28 family members are involved in initiation of cell-mediated immune responses, while TNF/TNF receptor family members are involved in the later phases of T-cell activation. B7 molecules expressed on DCs include CD80 (B7-1), CD86 (B7-2) [25], inducible costimulator (ICOS) ligand (B7-H2) [26], programmed death 1 ligand (PD-L1 or B7-H1), PD-L2 (B7-DC), B7-H3 [27], and B7-H4 [28]. TNF/TNF receptors include CD27, 4-1BB (CD137), tumor necrosis factor receptor superfamily-member 4 (TNFRSF4), tumor necrosis factor ligand superfamily-member 14 (TNFSF14), and glucocorticoid-induced tumor necrosis factor receptor (GITR) [29–31]. B7-1 and B7-2 bind two surface molecules on T cells, the stimulatory receptor CD28 and the inhibitory receptor CTLA-4 (CD152). The engagement of CTLA-4 by B7-1 or B7-2 downregulates immune responses thereby leading to immune tolerance and profound autoimmunity driven by self-reactive T cells that are converse to the engagement of CD28 which promotes T cell activation [27]. Therefore, to strengthen the antitumor immune responses, blockade of signaling transduced through CTLA-4 is essential in addition to upregulation of B7-1 and B7-2 by immunostimulants [32].

Expression of costimulatory molecules in DC vaccines can be increased by the pulse of some agents for maturation [3]. These agents include Toll-like receptor (TLR) agonists, CD40 ligand, CD70, TNFRSF4 ligand, calcium ionophores, and GITR ligand [3, 14, 33]. TLR agonists include follistatin-like 1 (FSL-1) and macrophage-activating lipopeptide 2 KDa (MALP2; TLR2/6 agonist), Pam3Cys (TLR1/2 agonist), polyI:C (TLR3 agonist), lipopolysaccharides (LPS) and monophosphoryl lipid A (MPL-A; TLR4 agonists), imiquimod and class B CpG oligodeoxynucleotide (CpG; TLR9 agonist), and R848 (TLR7 agonists) which inconsistently stimulate immune responses. For example, TLR1/2 and TLR3 agonists can induce responses from DC1, while TLR3/4 + TLR7/9 agonists mainly induce responses from DC2 [34]. Utilization of TLR agonists could enhance survival and trafficking of DCs *in situ* as well as prime tumor antigen-specific T lymphocytes [35]. TLRs are widely expressed in immune cells and tumor cells in which expressed preferentially [36] and the expression of TLRs on immune or GBM cells in the GBM microenvironment affects the therapeutic effect of TLR agonists. GL261 cells express TLR2, TLR3, and TLR4 and can increase MHC I expression and induce IL-6 secretion in respond to the corresponding TLR ligands [36]. When DCs are activated by TLR signals, they upregulate costimulatory molecules; secrete immunomodulatory cytokines (IL-12), and increase antigen procession and presentation to B and T lymphocytes. Intratumoral injection of TLR1/2 or TLR7 agonists produced a survival benefit, and TLR9 agonists had the best therapeutic effect for GL261 glioma cells, compared to less effective stimulation by TLR3 and TLR4 agonists alone [36]. Combining synergistic immunostimulants can elevate immune responses against GBM. For example, combining CD40 and TLR ligands significantly suppresses tumor growth in mice with melanoma [37].

Invariant natural killer T cells (iNKTs) are a subset of T cells that recognize glycolipid antigens bound to CD1d (a MHC Class I-like molecule highly expressed on DCs) by semi-invariant $\alpha\beta$ T-cell receptors [38]. They license DCs to initiate adaptive immune responses via CD40-CD40 ligand interactions between DCs and iNKTs. The potent synthetic iNKTs agonist α-galactosylceramide (α-GalCer) can promote T-cell responses to DC vaccines. DCs acquire α-GalCer and present it to CD1d molecules, and then DCs rapidly express immunostimulatory factors such as CD40 upon interaction with iNKTs, inducing enhanced capacity to drive conventional T-cell responses [39] (Figure 1).

2.3.2. Negative Regulators. Many negative regulators suppress immune responses and the blockage of these molecules may offer promise for increasing therapeutic efficacy of DC-based vaccination. PD-L1, PD-L2, and B7-H4 are costimulatory molecules which downregulate T-cell immune responses [28, 40]. Many GBM patients have aberrant expression of PD-L1 which correlates with a poor prognosis [22]. Blockade of PD-L1 and PD-L2 on DCs by an antibody can improve proliferation and cytokine production of $CD4^+$ T cells [41].

DCs also express molecules that may suppress antigen presentation or activation and function of T cells. The knockdown expression of these molecules by small interfering RNA (siRNA) can increase antitumor immunity mediated by DCs. These suppressive molecules include: zinc finger protein A20 (A20; a negative regulator of TLR and the TNF receptor signal pathway which stimulates T-cell mediated responses) [42], and the suppressor of cytokine signaling 1 (SOCS1; a negative regulator signaling through IFN-Γ, IL-2, IL-6, or IL-12, stimulators in T-cell expansion) [43]. Silencing A20 or SOCS1 in antigen-loaded DCs by siRNA caused DCs to activate a large amount of effector T cells and this was correlated to tumor growth inhibition in mice [3].

DC-derived immunoglobulin receptor 2 (DIgR2) and Notch ligands are surface molecules which direct suppressive effects on T cells and they are targets for increasing therapeutic efficacy of DC vaccines. When Delta1, a Notch ligand, is silenced by siRNA, cytokines secreted by $CD4^+$ T cells were increased in response to polyclonal T cell receptor activation [44] (Table 2).

3. Loading Antigens

The effectiveness of uptaking and loading GBM antigens on MHC complexes of DCs and expansion of DC subgroups which prime naïve T cells affect the therapeutic efficacy of DC vaccines. Thus, it is important to choose appropriate antigens for loading (Figure 1).

3.1. Glioblastoma-Associated Antigens (GAAs) and Glioblastoma Specific Antigens (GSAs). GBM antigens include GAAs and GSAs. Antigens loaded on DCs include RNA, DNA,

Table 2: Positive and negative regulators on DCs. TNF: tumor necrosis factor, ICOS: inducible costimulator, TNFRSF4: tumor necrosis factor receptor superfamily-member 4, TNFSF14: tumor necrosis factor ligand superfamily-member 14, GITR: glucocorticoid-induced tumor necrosis factor receptor, PD-L1: programmed death 1 ligand, A20: zinc finger protein A20, SOCS1: the suppressor of cytokine signaling 1, and DIgR2: DC-derived immunoglobulin receptor 2.

Positive regulators		Negative regulators
B7/CD28 family	TNF/TNF receptor family	
CD80 (B7-1) [25] CD86 (B7-2) [25] B7-H2 (ICOS ligand) [26] B7-H3 [27]	CD27 [30] TNFRSF4 [31] CD137 (4-1 BB) [30] TNFSF14 [29] GITR [30]	PD-L1 (B7-H1) [22] PD-L2 (B7-DC) [40] B7-H4 [28] A20 [42] SOCS1 [43] DIgR2 [44] Notch ligands [44]

proteins, peptides and lysates, or fusion and apoptotic cells [45]. Many identified GAAs been used for DC vaccination—antigen isolated from immunoselected melanoma-2 (AIM-2) [46], the α-2 chain of the IL-13 receptor (IL-13Rα2 chain) [47], human epidermal growth factor receptor 2 (HER2) [46], Ephrin type-A receptor 2 (EphA2) [48], gp100 [49], tenascin [50], survivin [51], melanoma antigen (MAGE)-1 [52], MAGE-3 [49], chitinase 3-like 1 (CHI3L1) [53], Wilms Tumor 1 Protein (WT-1) [54], SRY-related HMG-box gene (SOX)-11 and cytomegalovirus (CMV) antigens [55]. These have been over-expressed in GBMs and could initiate immune responses [56]. GAAs should be selected according to the human leukocyte antigen (HLA) genotype in each patient for HLA restriction in GBM [3]. However, GAAs often react weakly due to coexpression on normal tissues and subsequent host immunotolerance [55].

For GSAs, epidermal growth factor receptor variant III (EGFRvIII) is the only GSA targeted for GBM vaccination. It is present as a tumor-specific cell surface protein in 30–40% of GBM patients [57], and is absent on normal tissues, enhancing tumorigenicity [55]. In GBMs expressing EGFRvIII, DC vaccination can improve median progression-free survival (PFS) and median overall survival (OS) with minimal toxicity. When an anti-EGFRvIII DC vaccine was added to the standard therapy, patients had an increased PFS from 6.3 months to 14.2 months and improved OS from 15 to 26 months. Some vaccinated patients had serologic evidence of an anti-EGFRvIII humoral response to compete EGFRvIII-expressing GBM cells, and the median OS was 47.7 months in these patients compared to 22.8 months for vaccinated patients who did not develop serologic evidence of a humoral response. For patients who developed recurrent GBM after vaccination, pathological tissue demonstrated that recurrent GBM had lost EGFRvIII expression [58].

One patient newly diagnosed with GBM had obvious cytokine changes (related to DC vaccination) in IL-6, TNF-α, and IL-10 after receiving a GBM lysate-pulsed DC vaccination. Although cytokines declined after the first vaccine dose, IL-6 remained undetectable after all three doses, suggesting a potential antitumor immunological response [59].

CMV can modulate the malignant phenotype in GBMs, therefore DC vaccines pulsed with CMV antigens can be used to reduce GBM malignancy. After receiving a CMV peptide-pulsed DC vaccination, MS has been prolonged to 21 months in GBM patients [60].

Different GBM antigens offer varied efficacy of DC-based vaccination. Research to compare therapeutic efficacy of GAA peptide-loaded and autologous tumor lysate (ATL)-loaded DC vaccination in malignant glioma patients (most GBM patients) indicated that ATL-DC vaccination had greater feasibility for treatment and decreased fractions of activated natural killer (NK) cell populations (which were associated with prolonged survival in this trial), compared to GAA-DC vaccination [61].

Meta-analyses indicate that vaccination with whole-tumor antigens induced greater clinical responses than vaccination with defined tumor antigens for GAA expression heterogeneity [62, 63]. Single peptide vaccines can result in poor identification of specific GBM antigens for the escape of peptide-deficient variants; in consideration of the heterogeneous properties of GBM cells [64], most clinical trials with DC vaccination for GBM use whole-tumor lysates as sources of GAAs instead of artificially-synthesized peptides [3]. Genetic modification of DCs for antigen loading may also be an appropriate strategy, allowing multi-epitope presentation of full-length GAAs without HLA restrictions [43].

Whole GBM lysates can be generated from irradiation (apoptosis) or freeze-thawing (necrosis) of GBM cells. Lysates from apoptotic bodies increased the immunogenicity of GBM cells and enhanced GAA delivery to DCs more effectively than necrosis lysates [24]. However, loading DCs with apoptotic bodies of GBMs can increase risks in induction of tolerogenic DCs via the cyclooxygenase-2 (COX2) pathway [65]. DCs loaded with purified autophagosomes from autophagic tumor cells induced tumor-specific immune responses [66], and autophagy regulated selective release of high-mobility group B1 (HMGB1), which acted as an endogenous pattern recognition receptor (PRR) to induce DC maturation [67]. Therefore, autophagic tumor lysates and autophagosomes may be prudent choices for DC vaccines [66].

3.2. Glioma Stem Cell (GSC) Antigens. GSCs, a subpopulation that makes up 10–70% of the total cell population with GBMs, are closely related to GBM occurrence, progression, metastasis, recurrence, drug resistance and immune evasion [55, 68]. They possess higher immunogenicity compared with other tumor cells and can drive stronger immune responses [69]. Nestin (a type VI intermediate filament protein), CD15 and CD133, which exhibit different levels of expression in GBM cells, can be used as cell surface markers to isolate and characterize GSCs [70]. Nestin is broadly expressed in cancer stem cells (CSCs) from various malignancies, such as bladder, head and neck, ovarian, pancreatic, prostate, testicular, and uterine cancers [71–75]. CD15 is extensively expressed in thyroid, colorectal, lung, gastric, liver, nasopharynx, bladder cancer cells, while CD133 is broadly expressed in liver,

lung, prostate, cerebral, colon, melanoma cancer cells as conventional CSC antigens [76].

GSCs can secrete several immunosuppressive cytokines associated with recruitment and polarization of microglia/macrophages, and they include: soluble colony-stimulating factor-1 (sCSF-1), transforming growth factor-β1 (TGF-β1) and macrophage inhibitory cytokine-1 (MIC-1). Moreover, conditioned media from GSCs polarize microglia/macrophages to an M2 phenotype, inhibit phagocytosis of microglia/macrophages, induce secretion of immunosuppressive cytokines (IL-10 and TGF-β1), and inhibit T cell proliferation [77, 78].

The resistance of GBM to radiotherapy and chemotherapy may be mediated by GSCs which have more active DNA repair mechanisms [79] and highly express multi-drug resistance genes [80], can be enriched via neurosphere culture conditions and contribute to local immunosuppression in the GBM microenvironment [9, 55, 68, 77]. Vaccine studies using lysates from CSCs revealed that superior protective immunity compared to lysates from whole tumors in mice [81, 82]. Therefore, GSCs antigens may be ideal for vaccination whether such a choice is more effective than other antigens [9, 55], and DCs loaded with GSC antigens may stimulate T cells to produce tumor-specific cytotoxicity against GBM cells [83].

Several vaccination in rodent orthotopic GBM models with DC loaded with GSC antigens, have been reported to induce immune-reactivity and a survival benefit [69, 81]. In DCs stimulated with lysates from GSCs, expression of DC surface molecules (including CD80, CD86, CD11C and MHC II) is upregulated more compared with DCs loaded with normal antigens and these more effectively stimulate naive T cells to form tumor-specific cytotoxic T cells that kill glioma cells cultured *in vitro* [83]. Thus, GSCs contain unknown antigens with strong immunogenicity that can be recognized by DCs, which need more researches (Table 3).

Table 3: Antigens for loading on DCs. AIM-2: antigen isolated from immunoselected melanoma-2, IL-13Rα2 chain: the α-2 chain of the IL-13 receptor, HER2: human epidermal growth factor receptor 2, EphA2: Ephrin type-A receptor 2, MAGE: melanoma antigen, CHI3L1: chitinase 3-like 1, WT-1: Wilms Tumor 1 Protein, SOX: SRY-related HMG-box gene, CMV: cytomegalovirus, EGFR: epidermal growth factor receptor, and EGFRvIII: epidermal growth factor receptor variant III.

GAAs	GSAs	GSC antigens
AIM-2 [46], IL-13Rα2 chain [47], HER2 [46], EphA2 [48], gp100 [49], tenascin [50], surviving [51], MAGE-1 [52], MAGE-3 [49], CHI3L1 [53], WT-1 [54], SOX11 [55], CMV antigens [55]	EGFRvIII [57]	EGFR [81], SOX2 [81]

4. Regulation of the GBM Microenvironment

GBM cell immunogenicity depends on the microenvironment in which the cells grow. Many cytokines and other cells have unique roles in the GBM microenvironment, and some cause immune suppression. Thus, DC vaccination not only requires reduction of tumor load (tumor resection) as much as possible, but also calls for the regulation of the GBM microenvironment which including the addition of some positive regulators and the elimination of GBM-induced immune suppression [84] (Figure 1).

4.1. Positive Regulators. There are some positive regulators in the GBM microenvironment. The presence of some lymphocytes (such as $CD8^+$ T cells [85], $CD4^+$ T cells [86], and NK cells [87]), cytokines (such as type I IFN (IFN-α and IFN-β), and IL-12p70) in Th1-polarized microenvironments, can prime and activate antitumor cytotoxic and memory T cell responses [3].

Adoptively transferred tumor-specific T cells-especially those expressing chimeric antigen receptors (CARs)-enhanced immunity in preclinical studies, targeting several GBM antigens including: EGFRvIII, IL-13Ra2 chain, HER2, and CMV antigens [55, 88]. Research to study such efficacy by adding these cells to DC vaccines is forthcoming.

DCs can secrete cytokines and chemokines vital for immune polarization and recruitment of lymphocyte populations. Cytokine treatment is a powerful tool to induce robust anti-GBM cytotoxic and memory T-cell responses post-vaccination. IL-12p70 derived from DCs can stimulate IFN-γ production in naive T cells, promoting Th1 responses that overcome immune tolerance against tumor cells [3]. When Th1 cytokines such as IL-2 were pulsed to Flt3L-mediated gene therapy in a refractory rat model, therapeutic efficacy was strengthened by augmenting cytotoxic T lymphocyte responses and $CD8^+$ T cell mediated immunological memory [89]. Increased responsiveness of $CD8^+$ T lymphocytes to IL-2 was related to long-term survival of greater than 2 years post-vaccination in GBM patients. This technique activated the JAK-STAT signal pathways, causing phosphorylation of STAT-5 via cytokine receptors located on the T cell membrane [90]. Dimers then formed from the phosphorylated STATs (pSTAT) and trans-located into the nucleus to initiate gene transcription programs. Perhaps GBM induces immune suppression against IL-2 signals in T cells, and then DC vaccines counteract immune suppression [91]. STAT-5 is required for IL-2-induced cell cycle progression in T cells and the recruitment of antibody-induced T cells into tumor tissues [92]. Enhanced sensitivity to IL-2 signals and increased frequency of pSTAT-5 upregulate the clinical responsiveness of IL-2-primed $CD8^+$ T cells to intracranial tumors [93], and STAT5-deficient mice had altered NK cell function and decreased T and B cell proliferation in response to chemokines [94]. Adding recombinant IL-12 to lysates or RNA loaded DCs also can strengthen protective immunity against intracranial gliomas [95] and combining DC vaccination with IFN-β gene therapy benefits survival [96]. Long-term survival and specific cytotoxic T lymphocyte activity was induced when IFN-α was delivered in sequential pulses to DC vaccines in a mouse glioma model [97]. Thus, local proinflammatory cytokine production post-vaccination can affect the generation of effector memory $CD8^+$ T lymphocyte

populations [91], thereby ulteriorly influence the efficacy of DC vaccination. Since circulating cytokines demonstrate little relation to intracranial immune responses [59], intratumoral injection of immunostimulatory cytokines can prolong the survival of DCs administered subcutaneously.

4.2. Negative Regulators. Inhibitory cytokines as well as suppressive cell populations secreted by GBM cells and the existence of vascular endothelial growth factor (VEGF) in the GBM microenvironment are negative regulators in the immunity targeting GBMs.

Inhibitory cytokines such as transforming growth factor (TGF)-β in the GBM microenvironment prevent the immune response from translating into clinical efficacy [98, 99]. Substances that interfere with the TGF–β signal pathways have been tested in early clinical trials including inactivating antibodies (fresolimumab) and antisense oligonucleotides (trabedersen) [64, 100, 101], which may provide a solution to the infiltration of TGF-β and enhance antitumor immunity of DC-based vaccination.

Immune regulatory components such as Tregs or myeloid-derived suppressor cells (MDSCs) in the GBM microenvironmentcan cause immune tolerance. Tregs are a subpopulation of $CD4^+$ T lymphocytes [102]. Infiltration with Tregs is associated with glioma progression [103] and inhibiting $CD4^+$ T cells, $CD8^+$ T cells, DCs, and NK cells hinders a successful immune response [104–106]. Decreased post/pre-vaccination Tregs ratios were reported to be related to prolonged survival in glioma patients [61], so preventing or reversing these components through inhibition can enhance antitumor immunity of DC-based vaccination [3]. Depleting Tregs via antibody treatment to modulate the tumor microenvironment [99] can permit the generation of effective antitumor responses [104, 105] and these substances are attractive when combining with DC vaccination [64]. Tregs constitutively express the high affinity IL-2 receptor CD25, the transcription factor Forkhead box protein 3 (Foxp3) and the B7 ligand CTLA4 [102], all of which can be target for the depletion of Tregs. Immune responses were significantly enhanced after DC vaccination in GBM patients who received CD25 mAb blockade (daclizumab) and temozolomide chemotherapy [107]. However, CD25 is not a specific marker for Tregs. In a large glioma model, the depletion of Tregs by using blockade of CD25 strategy inhibited the clonal expansion of tumor specific T cells and decreased the efficacy of DC vaccines [108].

Foxp3 is a more specific marker expressed by Tregs in human GBM as compared to CD25 and it may be a target for Treg depletion. Foxp3 also suppresses IFN-γ and IL-2 secretion from $CD4^+$ T cells [109]. However, Foxp3 is intranuclear and cannot be depleted easily with immunoglobulins [110]. Delivery of inhibitors to NF-κB combined with immunogene therapy using Flt3L and thymidine kinase (TK) can suppress $Foxp3^+$ Tregs and product Th1 cytokines in the tumor microenvironment [89]. Another option exists for eliminating Tregs is anticytotoxic T-lymphocyte antigen 4 (CTLA4) antibodies. Ipilimumab, a monoclonal antibody targeting CTLA4, will soon be approved by FDA for advanced melanoma [32], including those with CNS metastases. This may be feasible for use in immunotherapy for GBM [55]. One study suggested that Treg depletion (using a CD25-targetting strategy that interfered with the clonal expansion of tumor antigen specific T lymphocytes) inhibited the efficiency of DC-based immunotherapy in a glioma model [108]. Therefore, the efficacy of Tregs depletion in GBM needs further researches.

MDSCs are also negative regulators for antitumor immunity. Although coculture of normal human monocytes with glioma cells *in vitro* acquired MDSC-like properties [111], GBM patients had increased MDSCs ($CD33^+$ $HLA\text{-}DR^-$) in peripheral blood compared to normal donors. MDSCs isolated from peripheral blood monocytes (PBMs) significantly restored T-cell function [112]. Thus, MDSCs are related to GBM tolerance and can be combined with DC vaccines. COX-2 inhibition (celecoxib) or anti-Gri antibody can block the development of MDSCs ($CD11b^+$ Gri^+) as well as CCL2-mediated accumulation in the GBM microenvironment and delay glioma development in a murine model [113, 114]. Moreover, accumulating evidence suggests that several chemotherapeutic agents (gemcitabine, docetaxel, 5-fluorouracil, and sunitinib malate, a receptor tyrosine kinase inhibitor) could reverse immune suppression mediated by MDSCs in mouse tumor models. Other compounds such as polyphenol E or all-trans-retinoic acid also can decrease MDSCs in mice and humans [3].

Vascular endothelial growth factor (VEGF) is another negative regulator which contributes to the immunosuppressive ability of GBMs. It can inhibit the maturation of DCs and antigen presentation, induce apoptosis of $CD8^+$ T cells, enhance Treg activity and diminish the infiltration of T cells in GBM endothelium. The inhibition of VEGF can block VEGF mediated angiogenesis of GBM and also suppress the growth of GSC-derived tumor cells [115]. Bevacizumab, a humanized monoclonal antibody which blocks VEGF mediated angiogenesis of GBM. This is an approved therapy for recurrent GBM by FDA and has been shown to be efficacious in newly diagnosed GBM patients in phase III clinical trials [55]. VEGF Trap such as aflibercept is also one of VEGF-targeting drugs. It has greater affinity for VEGF compared to anti-VEGF monoclonal antibodies and has improved survival as well as enhanced the activity of radiation therapy in preclinical studies. Meanwhile, GBM expresses VEGF receptors (VEGFRs) which may promote tumor growth. Therapies target VEGFRs by suppressing activation signaling of VEGFR can also effect. The activation of VEGFRs can be inhibited by blocking the tyrosine kinase activation site of VEGFR with tyrosine kinase inhibitors or blocking the ligand binding site of VEGFR with monoclonal antibodies or peptides. Several VEGFR tyrosine kinase inhibitors such as cediranib have induced powerful antiangiogenic and antitumor activity in preclinical GBM models. Considering these molecules also inhibit other relevant receptors, they may also increase toxicity [115] (Table 4).

5. DC-Based Vaccination

Once extracted from humans, DCs can be exposed to antigens expressed by GBM cells, and stimulated to take up,

Table 4: Regulators in the GBM microenvironment. GBM: glioblastoma multiforme, NK: natural killer; IFN: interferon; IL: interleukin, Tregs: regulatory T cells, MDSCs: myeloid-derived suppressor cells, TGF: transforming growth factor, and VEGF: vascular endothelial growth factor.

Positive regulators		Negative regulators	
Cells	Cytokines	Cells	Cytokines
$CD8^+$ T cells [85] $CD4^+$ T cells [86] NK cells [87]	IFN-α [97] IFN-β [96] IL-12p70 [3] IL-2 [89] IL-12 [95]	Tregs [103] MDSCs [112]	TGF-β [98] VEGF [115]

process, and display these antigens as peptides on their cell surface in the context of MHC class I or II molecules. These cells can then be infused back into patients as a vaccine therapy. T cells can be activated by recognizing MHC class I or II molecules via TCRs. Vaccines also induce cross-stimulation of $CD8^+$ (cytotoxic) T-cell (CTL) responses, as well as Th1 and Th2 pathways by stimulating differentiation of naïve $CD4^+$ T cells into helper T effectors [116, 117], which may be more effective than stimulating immunity using MHC I restricted peptides only [59]. Stimulated $CD8^+$ CTLs secrete IFN-γ and have potent cytolytic activity against GBM cells now recognized by the host's immune system [3, 9], whereby they recognize and destroy GBM cells via peptides derived from GAAs of MHC class I molecules. Meanwhile, activated $CD4^+$ T cells recognize peptides in the complex of MHC class II molecules and improve the capacity of DCs to induce CTLs via interaction between CD40 ligands on activated $CD4^+$ T cells and CD40 on DCs. Moreover, $CD4^+$ T cells maintain and expand CTLs by secreting cytokines such as IL-12. Not only can DCs elicit T-cell responses, but also they can improve the immunomodulatory and cytotoxic potential of NK and natural killer T (NKT) cells [5], both of which are also involved in the elimination of GBM. Furthermore, they also mediate tumor-directed cytotoxicity directly [9, 56] (Figure 1). In brief, DC vaccines can active patients' immune systems and strengthen the immune responses against GBM cells.

6. Summary

As a basic immunotherapy, DC vaccination is critical for initiating and boosting anti-GBM immunity; it has obvious complementarity with traditional treatments in promoting cross-presentation of antigens and long term immunologic memory, and it can prevent the recurrence and metastases of GBM [10]. Therefore, the combination of DC-based vaccination with traditional modalities may offer promise for novel GBM treatments [3]. Besides, the antitumor immunity of DC vaccination can be strengthened by inhibiting inhibitory signals or components; upregulating stimulatory molecules or signals; choosing specific antigens for loading; and regulating the GBM microenvironment. However, DC vaccines have had several limitations so far which include a surgical requirement, a several-week delay for vaccine generation, the possibility of immune overload due to excessive antigen exposure, and potential autoimmune reactions which due to vaccine contamination with normal host [55].

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Liu Yang and Geng Guo contributed equally to this work.

Acknowledgments

This work was supported by The National Natural Science Foundation of China (no. 81201991), The Basic Research Program of Shanxi Province of China (no. 2012021035-5), The Program for the Outstanding Innovative Teams of Higher Learning Institutions of Shanxi, and The Scientific Research Subject of Shanxi Provincial Health Department (no. 201301066).

References

[1] P. Y. Wen and S. Kesari, "Malignant gliomas in adults," *New England Journal of Medicine*, vol. 359, no. 5, pp. 492–507, 2008.

[2] I. Jovcevska, N. Kocevar, and R. Komel, "Glioma and glioblastoma—how much do we (not) know?" *Molecular Clinical Oncology*, vol. 1, no. 6, pp. 935–941, 2013.

[3] Y. Mineharu, M. G. Castro, P. R. Lowenstein, N. Sakai, and S. Miyamoto, "Dendritic Cell-based Immunotherapy for glioma: multiple regimens and implications in clinical trials," *Neurologia Medico-Chirurgica*, vol. 53, no. 11, pp. 741–754, 2013.

[4] M. L. Gruber and W. P. Buster, "Temozolomide in combination with irinotecan for treatment of recurrent malignant glioma," *American Journal of Clinical Oncology*, vol. 27, no. 1, pp. 33–38, 2004.

[5] M.-Y. Yang, P. M. Zetler, R. M. Prins, H. Khan-Farooqi, and L. M. Liau, "Immunotherapy for patients with malignant glioma: from theoretical principles to clinical applications," *Expert Review of Neurotherapeutics*, vol. 6, no. 10, pp. 1481–1494, 2006.

[6] M. A. Cheever and C. S. Higano, "PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine," *Clinical Cancer Research*, vol. 17, no. 11, pp. 3520–3526, 2011.

[7] K. Traynor, "Ipilimumab approved for metastatic melanoma," *The American Journal of Health-System Pharmacy*, vol. 68, no. 9, p. 768, 2011.

[8] A. Bregy, T. M. Wong, A. H. Shah, J. M. Goldberg, and R. J. Komotar, "Active immunotherapy using dendritic cells in the treatment of glioblastoma multiforme," *Cancer Treatment Reviews*, vol. 39, no. 8, pp. 891–907, 2013.

[9] J. C. Marsh, J. Goldfarb, T. D. Shafman, and A. Z. Diaz, "Current status of immunotherapy and gene therapy for high-grade gliomas," *Cancer Control*, vol. 20, no. 1, pp. 43–48, 2013.

[10] L. H. Butterfield, "Dendritic cells in cancer immunotherapy clinical trials: are we making progress?" *Frontiers in Immunology*, vol. 4, article 454, 2013.

[11] E. S. Trombetta and I. Mellman, "Cell biology of antigen processing in vitro and in vivo," *Annual Review of Immunology*, vol. 23, pp. 975–1028, 2005.

[12] L. Cohn and L. Delamarre, "Dendritic cell-targeted vaccines," *Frontiers in Immunology*, vol. 5, article 255, 2014.

[13] X. Jie, L. Hua, W. Jiang, F. Feng, G. Feng, and Z. Hua, "Clinical application of a dendritic cell vaccine raised against heat-shocked glioblastoma," *Cell Biochemistry and Biophysics*, vol. 62, no. 1, pp. 91–99, 2012.

[14] G. Napolitani, A. Rinaldi, F. Bertoni, F. Sallusto, and A. Lanzavecchia, "Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells," *Nature Immunology*, vol. 6, no. 8, pp. 769–776, 2005.

[15] M. Fujita, X. Zhu, R. Ueda et al., "Effective immunotherapy against murine gliomas using type 1 polarizing dendritic cells—significant roles of CXCL10," *Cancer Research*, vol. 69, no. 4, pp. 1587–1595, 2009.

[16] R. B. Mailliard, A. Wankowicz-Kalinska, Q. Cai et al., "Alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity," *Cancer Research*, vol. 64, no. 17, pp. 5934–5937, 2004.

[17] P. Parajuli, R. L. Mosley, V. Pisarev et al., "Flt3 ligand and granulocyte-macrophage colony-stimulating factor preferentially expand and stimulate different dendritic and T-cell subsets," *Experimental Hematology*, vol. 29, no. 10, pp. 1185–1193, 2001.

[18] B. J. Weigel, N. Nath, P. A. Taylor et al., "Comparative analysis of murine marrow-derived dendritic cells generated by Flt3L or GM-CSF/IL-4 and matured with immune stimulatory agents on the in vivo induction of antileukemia responses," *Blood*, vol. 100, no. 12, pp. 4169–4176, 2002.

[19] P. A. Cohen, G. K. Koski, B. J. Czerniecki et al., "STAT3-And STAT5-dependent pathways competitively regulate the pan-differentiation of CD34 pos cells into tumor-competent dendritic cells," *Blood*, vol. 112, no. 5, pp. 1832–1843, 2008.

[20] A. Berhanu, J. Huang, S. M. Alber, S. C. Watkins, and W. J. Storkus, "Combinational FLt3 ligand and granulocyte macrophage colony-stimulating factor treatment promotes enhanced tumor infiltration by dendritic cells and antitumor $CD8^+$ T-cell cross-priming but is ineffective as a therapy," *Cancer Research*, vol. 66, no. 9, pp. 4895–4903, 2006.

[21] A. Iellem, M. Mariani, R. Lang et al., "Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells," *The Journal of Experimental Medicine*, vol. 194, no. 6, pp. 847–853, 2001.

[22] D. Capece, D. Verzella, M. Fischietti, F. Zazzeroni, and E. Alesse, "Targeting costimulatory molecules to improve antitumor immunity," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 926321, 17 pages, 2012.

[23] K. J. Lafferty, H. S. Warren, and J. A. Woolnough, "A mediator acting as a costimulator for the development of cytotoxic responses in vitro," *Advances in Experimental Medicine and Biology*, vol. 114, pp. 497–501, 1979.

[24] Y. Mineharu, G. D. King, A. G. Muhammad et al., "Engineering the brain tumor microenvironment enhances the efficacy of dendritic cell vaccination: implications for clinical trial design," *Clinical Cancer Research*, vol. 17, no. 14, pp. 4705–4718, 2011.

[25] G. Driessens, J. Kline, and T. F. Gajewski, "Costimulatory and coinhibitory receptors in anti-tumor immunity," *Immunological Reviews*, vol. 229, no. 1, pp. 126–144, 2009.

[26] L. Strauss, C. Bergmann, M. J. Szczepanski, S. Lang, J. M. Kirkwood, and T. L. Whiteside, "Expression of ICOS on human melanoma-infiltrating $CD4^+CD25^{high}Foxp3^+$ T regulatory cells: implications and impact on tumor-mediated immune suppression," *Journal of Immunology*, vol. 180, no. 5, pp. 2967–2980, 2008.

[27] R. J. Greenwald, G. J. Freeman, and A. H. Sharpe, "The B7 family revisited," *Annual Review of Immunology*, vol. 23, pp. 515–548, 2005.

[28] G. L. Sica, I. H. Choi, G. Zhu et al., "B7-H4, a molecule of the B7 family, negatively regulates T cell immunity," *Immunity*, vol. 18, no. 6, pp. 849–861, 2003.

[29] M. L. del Rio, C. L. Lucas, L. Buhler, G. Rayat, and J. I. Rodriguez-Barbosa, "HVEM/LIGHT/BTLA/CD160 cosignaling pathways as targets for immune regulation," *Journal of Leukocyte Biology*, vol. 87, no. 2, pp. 223–235, 2010.

[30] T. H. Watts, "TNF/TNFR family members in costimulation of T cell responses," *Annual Review of Immunology*, vol. 23, pp. 23–68, 2005.

[31] M. Croft, "Costimulation of T cells by OX40, 4-1BB, and CD27," *Cytokine and Growth Factor Reviews*, vol. 14, no. 3-4, pp. 265–273, 2003.

[32] F. S. Hodi, S. J. O'Day, D. F. McDermott et al., "Improved survival with ipilimumab in patients with metastatic melanoma," *The New England Journal of Medicine*, vol. 363, no. 8, pp. 711–723, 2010.

[33] L. Bonifaz, D. Bonnyay, K. Mahnke, M. Rivera, M. C. Nussenzweig, and R. M. Steinman, "Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral $CD8^+$ T cell tolerance," *The Journal of Experimental Medicine*, vol. 196, no. 12, pp. 1627–1638, 2002.

[34] S. N. Lim, S. Kuhn, E. Hyde, and F. Ronchese, "Combined TLR stimulation with Pam3Cys and Poly I: C enhances Flt3-ligand dendritic cell activation for tumor immunotherapy," *Journal of Immunotherapy*, vol. 35, no. 9, pp. 670–679, 2012.

[35] R. M. Prins, N. Craft, K. W. Bruhn et al., "The TLR-7 agonist, imiquimod, enhances dendritic cell survival and promotes tumor antigen-specific T cell priming: Relation to central nervous system antitumor immunity," *Journal of Immunology*, vol. 176, no. 1, pp. 157–164, 2006.

[36] O. M. Grauer, J. W. Molling, E. Bennink et al., "TLR ligands in the local treatment of established intracerebral murine gliomas," *Journal of Immunology*, vol. 181, no. 10, pp. 6720–6729, 2008.

[37] G. W. Stone, S. Barzee, V. Snarsky et al., "Nanoparticle-delivered multimeric soluble CD40L DNA combined with toll-like receptor agonists as a treatment for melanoma," *PLoS ONE*, vol. 4, no. 10, Article ID e7334, 2009.

[38] F. Calabi, J. M. Jarvis, L. Martin, and C. Milstein, "Two classes of CD1 genes," *European Journal of Immunology*, vol. 19, no. 2, pp. 285–292, 1989.

[39] M. K. Hunn and I. F. Hermans, "Exploiting invariant NKT cells to promote T-cell responses to cancer vaccines," *OncoImmunology*, vol. 2, no. 4, Article ID e23789, 2013.

[40] Y. Latchman, C. R. Wood, T. Chernova et al., "PD-L2 is a second ligand for PD-1 and inhibits T cell activation," *Nature Immunology*, vol. 2, no. 3, pp. 261–268, 2001.

[41] J. A. Brown, D. M. Dorfman, F.-R. Ma et al., "Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production," *The Journal of Immunology*, vol. 170, no. 3, pp. 1257–1266, 2003.

[42] D. L. Boone, E. E. Turer, E. G. Lee et al., "The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses," *Nature Immunology*, vol. 5, no. 10, pp. 1052–1060, 2004.

[43] J. E. Boudreau, A. Bonehill, K. Thielemans, and Y. Wan, "Engineering dendritic cells to enhance cancer immunotherapy," *Molecular Therapy*, vol. 19, no. 5, pp. 841–853, 2011.

[44] Y. Stallwood, E. Briend, K. M. Ray et al., "Small interfering RNA-mediated knockdown of notch ligands in primary $CD4^+$ T cells and dendritic cells enhances cytokine production," *The Journal of Immunology*, vol. 177, no. 2, pp. 885–895, 2006.

[45] J.-H. Gu and G. Li, "Dendritic cell-based immunotherapy for malignant glioma," *Neuroscience Bulletin*, vol. 24, no. 1, pp. 39–44, 2008.

[46] S. Phuphanich, C. J. Wheeler, J. D. Rudnick et al., "Phase i trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma," *Cancer Immunology, Immunotherapy*, vol. 62, no. 1, pp. 125–135, 2013.

[47] F. Okano, W. J. Storkus, W. H. Chambers, I. F. Pollack, and H. Okada, "Identification of a novel HLA-A^*0201-restricted, cytotoxic T lymphocyte epitope in a human glioma-associated antigen, interleukin 13 receptor α2 chain," *Clinical Cancer Research*, vol. 8, no. 9, pp. 2851–2855, 2002.

[48] M. Hatano, J. Eguchi, T. Tatsumi et al., "EphA2 as a glioma-associated antigen: a novel target for glioma vaccines," *Neoplasia*, vol. 7, no. 8, pp. 717–722, 2005.

[49] S. Saikali, T. Avril, B. Collet et al., "Expression of nine tumour antigens in a series of human glioblastoma multiforme: interest of EGFRvIII, IL-13Ralpha2, gp100 and TRP-2 for immunotherapy," *Journal of Neuro-Oncology*, vol. 81, no. 2, pp. 139–148, 2007.

[50] N. Brösicke, F. K. H. van Landeghem, B. Scheffler, and A. Faissner, "Tenascin-C is expressed by human glioma in vivo and shows a strong association with tumor blood vessels," *Cell and Tissue Research*, vol. 354, no. 2, pp. 409–430, 2013.

[51] H. Guo, Y. Wang, T. Song et al., "Silencing of survivin using YM155 inhibits invasion and suppresses proliferation in glioma cells," *Cell Biochemistry and Biophysics*, 2014.

[52] G. Liu, H. Ying, G. Zeng, C. J. Wheeler, K. L. Black, and J. S. Yu, "HER-2, gp100, and MAGE-1 are expressed in human glioblastoma and recognized by cytotoxic T cells," *Cancer Research*, vol. 64, no. 14, pp. 4980–4986, 2004.

[53] C. L. Nutt, R. A. Betensky, M. A. Brower, T. T. Batchelor, D. N. Louis, and A. O. Stemmer-Rachamimov, "YKL-40 is a differential diagnostic marker for histologic subtypes of high-grade gliomas," *Clinical Cancer Research*, vol. 11, no. 6, pp. 2258–2264, 2005.

[54] Y. Chiba, M. Kinoshita, Y. Okita et al., "Use of 11C-methionine PET parametric response map for monitoring WT1 immunotherapy response in recurrent malignant glioma," *Journal of Neurosurgery*, vol. 116, no. 4, pp. 835–842, 2012.

[55] D. A. Reardon, K. W. Wucherpfennig, G. Freeman et al., "An update on vaccine therapy and other immunotherapeutic approaches for glioblastoma," *Expert Review of Vaccines*, vol. 12, no. 6, pp. 597–615, 2013.

[56] X. Xu, F. Stockhammer, and M. Schmitt, "Cellular-based immunotherapies for patients with glioblastoma multiforme," *Clinical and Developmental Immunology*, vol. 2012, Article ID 764213, 15 pages, 2012.

[57] A. J. Wong, J. M. Ruppert, S. H. Bigner et al., "Structural alterations of the epidermal growth factor receptor gene in human gliomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 7, pp. 2965–2969, 1992.

[58] J. H. Sampson, A. B. Heimberger, G. E. Archer et al., "Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma," *Journal of Clinical Oncology*, vol. 28, no. 31, pp. 4722–4729, 2010.

[59] J. L. Lasky III, E. H. Panosyan, A. Plant et al., "Autologous tumor lysate-pulsed dendritic cell immunotherapy for pediatric patients with newly diagnosed or recurrent high-grade gliomas," *Anticancer Research*, vol. 33, no. 5, pp. 2047–2056, 2013.

[60] K. Dziurzynski, S. M. Chang, A. B. Heimberger et al., "Consensus on the role of human cytomegalovirus in glioblastoma," *Neuro-Oncology*, vol. 14, no. 3, pp. 246–255, 2012.

[61] R. M. Prins, X. Wang, H. Soto et al., "Comparison of glioma-associated antigen peptide-loaded versus autologous tumor lysate-loaded dendritic cell vaccination in malignant glioma patients," *Journal of Immunotherapy*, vol. 36, no. 2, pp. 152–157, 2013.

[62] M. A. Neller, J. A. López, and C. W. Schmidt, "Antigens for cancer immunotherapy," *Seminars in Immunology*, vol. 20, no. 5, pp. 286–295, 2008.

[63] S. Nava, M. Dossena, S. Pogliani et al., "An optimized method for manufacturing a clinical scale dendritic cell-based vaccine for the treatment of glioblastoma," *PLoS ONE*, vol. 7, no. 12, Article ID e52301, 2012.

[64] M. Eyrich, J. Rachor, S. C. Schreiber, M. Wolfl, and P. G. Schlegel, "Dendritic cell vaccination in pediatric Gliomas: lessons learnt and future perspectives," *Frontiers in Pediatrics*, vol. 1, article 12, 2013.

[65] Y. Akasaki, G. Liu, N. H. C. Chung, M. Ehtesham, K. L. Black, and J. S. Yu, "Induction of a $CD4^+$ T regulatory type 1 response by cyclooxygenase-2-overexpressing glioma," *The Journal of Immunology*, vol. 173, no. 7, pp. 4352–4359, 2004.

[66] Y. Li, L.-X. Wang, G. Yang, F. Hao, W. J. Urba, and H.-M. Hu, "Efficient cross-presentation depends on autophagy in tumor cells," *Cancer Research*, vol. 68, no. 17, pp. 6889–6895, 2008.

[67] J. Thorburn, H. Horita, J. Redzic, K. Hansen, A. E. Frankel, and A. Thorburn, "Autophagy regulates selective HMGB1 release in tumor cells that are destined to die," *Cell Death and Differentiation*, vol. 16, no. 1, pp. 175–183, 2009.

[68] L. Prestegarden and P. Ø. Enger, "Cancer stem cells in the central nervous system—a critical review," *Cancer Research*, vol. 70, no. 21, pp. 8255–8258, 2010.

[69] S. Pellegatta, P. L. Poliani, D. Corno et al., "Neurospheres enriched in cancer stem-like cells are highly effective in eliciting a dendritic cell-mediated immune response against malignant gliomas," *Cancer Research*, vol. 66, no. 21, pp. 10247–10252, 2006.

[70] X. Jin, J.-E. Jung, S. Beck, and H. Kim, "Cell surface Nestin is a biomarker for glioma stem cells," *Biochemical and Biophysical Research Communications*, vol. 433, no. 4, pp. 496–501, 2013.

[71] S. K. Singh, I. D. Clarke, T. Hide, and P. B. Dirks, "Cancer stem cells in nervous system tumors," *Oncogene*, vol. 23, no. 43, pp. 7267–7273, 2004.

[72] S. Kasper, "Exploring the origins of the normal prostate and prostate cancer stem cell," *Stem Cell Reviews*, vol. 4, no. 3, pp. 193–201, 2008.

[73] A. Bentivegna, D. Conconi, E. Panzeri et al., "Biological heterogeneity of putative bladder cancer stem-like cell populations from human bladder transitional cell carcinoma samples," *Cancer Science*, vol. 101, no. 2, pp. 416–424, 2010.

[74] K. Okuno, S. Ohta, H. Kato, T. Taga, K. Sugita, and Y. Takeuchi, "Expression of neural stem cell markers in malignant rhabdoid tumor cell lines," *Oncology Reports*, vol. 23, no. 2, pp. 485–492, 2010.

[75] A. Jimeno, G. Feldmann, A. Suárez-Gauthier et al., "A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development," *Molecular Cancer Therapeutics*, vol. 8, no. 2, pp. 310–314, 2009.

[76] D. Corbeil, K. Röper, A. Hellwig et al., "The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions," *The Journal of Biological Chemistry*, vol. 275, no. 8, pp. 5512–5520, 2000.

[77] A. Wu, J. Wei, L.-Y. Kong et al., "Glioma cancer stem cells induce immunosuppressive macrophages/microglia," *Neuro-Oncology*, vol. 12, no. 11, pp. 1113–1125, 2010.

[78] T. di Tomaso, S. Mazzoleni, E. Wang et al., "Immunobiological characterization of cancer stem cells isolated from glioblastoma patients," *Clinical Cancer Research*, vol. 16, no. 3, pp. 800–813, 2010.

[79] S. Bao, Q. Wu, R. E. McLendon et al., "Glioma stem cells promote radioresistance by preferential activation of the DNA damage response," *Nature*, vol. 444, no. 7120, pp. 756–760, 2006.

[80] A.-M. Bleau, D. Hambardzumyan, T. Ozawa et al., "PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells," *Cell Stem Cell*, vol. 4, no. 3, pp. 226–235, 2009.

[81] Q. Xu, G. Liu, X. Yuan et al., "Antigen-specific T-cell response from dendritic cell vaccination using cancer stem-like cell-associated antigens," *Stem Cells*, vol. 27, no. 8, pp. 1734–1740, 2009.

[82] A. J. Ghods, D. Irvin, G. Liu et al., "Spheres isolated from 9L gliosarcoma rat cell line possess chemoresistant and aggressive cancer stem-like cells," *Stem Cells*, vol. 25, no. 7, pp. 1645–1653, 2007.

[83] B. Ji, Q. Chen, B. Liu et al., "Glioma stem cell-targeted dendritic cells as a tumor vaccine against malignant glioma," *Yonsei Medical Journal*, vol. 54, no. 1, pp. 92–100, 2013.

[84] M. A. Hatiboglu, J. Wei, A. S. G. Wu, and A. B. Heimberger, "Immune therapeutic targeting of glioma cancer stem cells," *Targeted Oncology*, vol. 5, no. 3, pp. 217–227, 2010.

[85] P. Kalinski, Y. Nakamura, P. Watchmaker, A. Giermasz, R. Muthuswamy, and R. B. Mailliard, "Helper roles of NK and $CD8^+$ T cells in the induction of tumor immunity. Polarized dendritic cells as cancer vaccines," *Immunologic Research*, vol. 36, no. 1–3, pp. 137–146, 2006.

[86] S. Koido, Y. Enomoto, V. Apostolopoulos, and J. Gong, "Tumor regression by CD4 T-cells primed with dendritic/tumor fusion cell vaccines," *Anticancer Research*, vol. 34, no. 8, pp. 3917–3924, 2014.

[87] J. A. Wargo, L. Y. Schumacher, B. Comin-Anduix et al., "Natural killer cells play a critical role in the immune response following immunization with melanoma-antigen-engineered dendritic cells," *Cancer Gene Therapy*, vol. 12, no. 6, pp. 516–527, 2005.

[88] R. M. Prins, C. J. Shu, C. G. Radu et al., "Anti-tumor activity and trafficking of self, tumor-specific T cells against tumors located in the brain," *Cancer Immunology, Immunotherapy*, vol. 57, no. 9, pp. 1279–1289, 2008.

[89] Y. Mineharu, A. G. Muhammad, K. Yagiz et al., "Gene therapy-mediated reprogramming tumor infiltrating T cells using IL-2 and inhibiting NF-kappaB signaling improves the efficacy of immunotherapy in a brain cancer model," *Neurotherapeutics*, vol. 9, no. 4, pp. 827–843, 2012.

[90] J. N. Ihle and I. M. Kerr, "Jaks and Stats in signaling by the cytokine receptor superfamily," *Trends in Genetics*, vol. 11, no. 2, pp. 69–74, 1995.

[91] R. G. Everson, R. M. Jin, X. Wang et al., "Cytokine responsiveness of $CD8^+$ T cells is a reproducible biomarker for the clinical efficacy of dendritic cell vaccination in glioblastoma patients," *Journal for ImmunoTherapy of Cancer*, vol. 2, article 10, 2014.

[92] R. Moriggl, D. J. Topham, S. Teglund et al., "Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells," *Immunity*, vol. 10, no. 2, pp. 249–259, 1999.

[93] D. N. Lisiero, H. Soto, L. M. Liau, and R. M. Prins, "Enhanced sensitivity to IL-2 signaling regulates the clinical responsiveness of IL-12-primed $CD8^+$ T cells in a melanoma model," *Journal of Immunology*, vol. 186, no. 9, pp. 5068–5077, 2011.

[94] S. Akira, "Functional roles of STAT family proteins: lessons from knockout mice," *Stem Cells*, vol. 17, no. 3, pp. 138–146, 1999.

[95] O. Insug, G. Ku, H. C. J. Ertl, and M. Blaszczyk-Thurin, "A dendritic cell vaccine induces protective immunity to intracranial growth of glioma," *Anticancer Research*, vol. 22, no. 2A, pp. 613–621, 2002.

[96] R. Saito, M. Mizuno, N. Nakahara et al., "Vaccination with tumor cell lysate-pulsed dendritic cells augments the effect of *IFN-β* gene therapy for malignant glioma in an experimental mouse intracranial glioma," *International Journal of Cancer*, vol. 111, no. 5, pp. 777–782, 2004.

[97] H. Okada, P. Kalinski, R. Ueda et al., "Induction of $CD8^+$ T-cell responses against novel glioma-associated antigen peptides and clinical activity by vaccinations with α-type 1 polarized dendritic cells and polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose in patients with recurrent malignant glioma," *Journal of Clinical Oncology*, vol. 29, no. 3, pp. 330–336, 2011.

[98] C. Siepl, S. Bodmer, K. Frei et al., "The glioblastoma-derived T cell suppressor factor/transforming growth factor-β2 inhibits T cell growth without affecting the interaction of interleukin 2 with its receptor," *European Journal of Immunology*, vol. 18, no. 4, pp. 593–600, 1988.

[99] W. Maes, G. G. Rosas, B. Verbinnen et al., "DC vaccination with anti-CD25 treatment leads to long-term immunity against experimental glioma," *Neuro-Oncology*, vol. 11, no. 5, pp. 529–542, 2009.

[100] H. Trachtman, F. C. Fervenza, D. S. Gipson et al., "A phase 1, single-dose study of fresolimumab, an anti-TGF-B antibody, in treatment-resistant primary focal segmental glomerulosclerosis," *Kidney International*, vol. 79, no. 11, pp. 1236–1243, 2011.

[101] U. Bogdahn, P. Hau, G. Stockhammer et al., "Targeted therapy for high-grade glioma with the TGF-β2 inhibitor trabedersen: results of a randomized and controlled phase IIb study," *Neuro-Oncology*, vol. 13, no. 1, pp. 132–142, 2011.

[102] Y. Qu and Y. Zhao, "Regulatory $CD4^+CD25^+$ T-cells are controlled by multiple pathways at multiple levels," *International Reviews of Immunology*, vol. 26, no. 3-4, pp. 145–160, 2007.

[103] O. M. Grauer, S. Nierkens, E. Bennink et al., "CD4+FoxP3+ regulatory T cells gradually accumulate in gliomas during tumor growth and efficiently suppress antiglionia immune responses in vivo," *International Journal of Cancer*, vol. 121, no. 1, pp. 95–105, 2007.

[104] O. M. Grauer, R. P. M. Sutmuller, W. van Maren et al., "Elimination of regulatory T cells is essential for an effective

vaccination with tumor lysate-pulsed dendritic cells in a murine glioma model," *International Journal of Cancer*, vol. 122, no. 8, pp. 1794–1802, 2008.

[105] M. A. Morse, A. C. Hobeika, T. Osada et al., "Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines," *Blood*, vol. 112, no. 3, pp. 610–618, 2008.

[106] A. A. Thomas, M. S. Ernstoff, and C. E. Fadul, "Immunotherapy for the treatment of glioblastoma," *Cancer Journal*, vol. 18, no. 1, pp. 59–68, 2012.

[107] D. A. Mitchell, X. Cui, R. J. Schmittling et al., "Monoclonal antibody blockade of IL-2 receptor α during lymphopenia selectively depletes regulatory T cells in mice and humans," *Blood*, vol. 118, no. 11, pp. 3003–3012, 2011.

[108] J. F. Curtin, M. Candolfi, T. M. Fakhouri et al., "Treg depletion inhibits efficacy of cancer immunotherapy: implications for clinical trials," *PLoS ONE*, vol. 3, no. 4, Article ID e1983, 2008.

[109] E. Bettelli, M. Dastrange, and M. Oukka, "Foxp3 interacts with nuclear factor of activated T cells and NF-kappaB to repress cytokine gene expression and effector functions of T helper cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 14, pp. 5138–5143, 2005.

[110] J. H. Park, J. S. Ko, Y. Shin et al., "Intranuclear interactomic inhibition of FoxP3 suppresses functions of Treg cells," *Biochemical and Biophysical Research Communications*, vol. 452, no. 1, pp. 1–7, 2014.

[111] J. C. Rodrigues, G. C. Gonzalez, L. Zhang et al., "Normal human monocytes exposed to glioma cells acquire myeloid-derived suppressor cell-like properties," *Neuro-Oncology*, vol. 12, no. 4, pp. 351–365, 2010.

[112] B. Raychaudhuri, P. R. J. Ireland, J. Ko et al., "Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma," *Neuro-Oncology*, vol. 13, no. 6, pp. 591–599, 2011.

[113] H. Zhang, M. Tian, C. Xiu, Y. Wang, and G. Tang, "Enhancement of antitumor activity by combination of tumor lysate-pulsed dendritic cells and celecoxib in a rat glioma model," *Oncology Research*, vol. 20, no. 10, pp. 447–455, 2013.

[114] M. Fujita, G. Kohanbash, W. Fellows-Mayle et al., "COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells," *Cancer Research*, vol. 71, no. 7, pp. 2664–2674, 2011.

[115] D. A. Reardon, S. Turner, K. B. Peters et al., "A review of VEGF/VEGFR-targeted therapeutics for recurrent glioblastoma," *Journal of the National Comprehensive Cancer Network*, vol. 9, no. 4, pp. 414–427, 2011.

[116] W. Kim and L. M. Liau, "Dendritic cell vaccines for brain tumors," *Neurosurgery Clinics of North America*, vol. 21, no. 1, pp. 139–157, 2010.

[117] K. L. Knutson and M. L. Disis, "Tumor antigen-specific T helper cells in cancer immunity and immunotherapy," *Cancer Immunology, Immunotherapy*, vol. 54, no. 8, pp. 721–728, 2005.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 207684, 12 pages
http://dx.doi.org/10.1155/2015/207684

Research Article

miR-451a Inhibited Cell Proliferation and Enhanced Tamoxifen Sensitive in Breast Cancer via Macrophage Migration Inhibitory Factor

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Received 22 July 2014; Revised 27 October 2014; Accepted 5 November 2014

Academic Editor: Mohammad Owais

This study aims to investigate the regulative effects of microRNA-451a (miR-451a) on cell proliferation and sensitivity to tamoxifen in breast cancer cells. In cell culture experiments, the lentiviral vectors of pHBLV-miR-451a and pHBLV-miR-451a sponge were constructed and used to transfect MCF-7 and LCC2 cells. The transfection efficiency was tested by fluorescent observation, and cell lines with stable over- or downregulated expression of miR-451a were established. The expression of miR-451a and the target gene macrophage migration inhibitory factor (MIF) were detected by real-time reverse transcriptase polymerase chain reaction and/or western blot. Moreover, MTT assay, colony formation, and Transwell invasion assays were also performed. Data showed that the recombinant lentiviral vectors were constructed correctly, and the virus titer was 1×10^8 CFU/mL. The stable transfected cells were obtained. Overexpression of miR-451a downregulated MIF expression in mRNA and protein levels and inhibited cell proliferation, colony formation, and invasion of breast cancer cells. Downregulation of miR-451a upregulated MIF expression and increased breast cancer cell growth, invasion, and tamoxifen sensitivity. In summary, the miR-451a/MIF pathway may play important roles in the biological properties of breast cancer cells and may be a potential therapeutic target for breast cancer.

1. Introduction

MicroRNAs (miRNAs) are noncoding small RNAs (19–25 ribonucleotides) that can regulate gene expression at transcriptional and posttranscriptional levels by binding to the 3′-untranslated regions (3′UTRs) of target mRNA [1]. miRNAs have been reported to be involved in a range of biological processes, including cell proliferation and apoptosis. Altered miRNAs expressions were likely to contribute to human diseases including cancers [2]. To date more than 900 miRNAs have been identified, but the functions and mechanisms of many of them in cancers remain to be determined [3]. miR-451a is located on chromosome 17q11.2, a region that is amplified in some types of carcinomas [4]. Previous studies have demonstrated that miR-451a inhibited cell growth and proliferation and enhanced the activity of anticancer drugs [5, 6].

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that is involved in carcinogenic transformation and cancer development. The MIF levels are increased in a number of cancers including breast cancer and contribute to the survival and homeostasis control of cancer cells [7].

Breast carcinoma is the most frequent malignant neoplasm diagnosed in women, and approximately 70% of this cancer expresses the estrogen receptor (ER). Tamoxifen is

the most common and effective treatment for patients with ERα-positive breast cancers, which significantly reduces disease progression and is associated with preferable impact on survival of patients [8]. However, almost 50% of patients with advanced breast cancer do not respond to tamoxifen treatment. Furthermore, a lot of patients treated with tamoxifen suffer relapses, even when the initial responses are positive [9, 10]. It has been reported that altered expression of specific miRNAs is responsible for tamoxifen resistance, which can be used to predict the outcomes and responses to treatment in breast cancer patients [11]. Furthermore, the acquisition of tamoxifen resistance in breast cancer cells was accompanied by a significant increase in the cells' invasive ability. In order to explore the functions of miR-451a in breast cancer cells, we established recombinant lentiviral vectors with over- or downexpression of miR-451a, and the vectors were used to transfect MCF-7 and LCC2 breast cancer cells. Functional studies showed that miR-451a downregulated MIF expression, inhibited cell proliferation and invasion, and increased tamoxifen sensitivity of these breast cancer cells.

2. Materials and Methods

2.1. Construction of Transfer Vectors pHBLV-miR-451a and pHBLV-miR-451a Sponge. We used the lentiviral vectors of pHBLV, pSPAX2, and pMD2G, which were a transfer vector, packaging plasmid, and envelope plasmid, respectively. For lentivirus construction, the sequence of mature miR-451a (5′-AAACCGUUACCAUUAC-UGAGUU-3′) was obtained from miR-Base (http://www.mirbase.org/). The pre-miR-451a and miR-451a sponge oligonucleotides were chemically synthesized by Sunny Biotech Co., Ltd., Shanghai, China, and the primers were as follows: pre-miR-451a F: 5′-ACAGGATCCCTGGAGCCTGACAAGG-3′ (EcoRI restriction site is underlined points), pre-miR-451a R: 5′-ATTGAATTCAAAAAAGCCCCCA-CCCCTGCCTT-3′ (BamHI restriction site is underlined points), miR-451a sponge F: ACAGGATCCAACTCA-GTAATGGTAACGGTTTGCTAGAACTCAGTAATGG-TAACGGTTTGCTAGAACTCAGTA, and miR-451a sponge R: ATCGAATTCAAACC-GTTACCATTACTGAGTTC-TAGCAAACCGTTACCATTACTGAGTTCTAGCAAA-CCGTT. Then the recovered PCR products with precursor sequence for miR-451a self-complementary miR-451a sponge were inserted into pHBLV-U6-ZsGreen-Puro lentiviral vectors (Hanbio, Shanghai, China). After the pre-miR-451a and miR-451a sponge lentiviral-based vectors were transformed to DH5α cells, antibiotic-resistant colonies were selected on LB-ampicillin agar plates. The plasmid containing the target gene was verified by PCR, double digestion, and DNA sequencing.

2.2. Cell Lines and Culture. Human embryonic kidney-(HEK-) 293T cells and human breast cancer cell lines (MCF-7 and LCC2 cells) were obtained from Shanghai Institute of Cell Biology (CAS, China) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT), 100 kU/L^{-1} penicillin, and 100 mg/L^{-1} streptomycin at 37°C in a humidified incubator with 5% CO_2. The tamoxifen-resistant cells LCC2 were cultured in the presence of 10^{-12} M 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich, St. Louis, MO, USA) to maintain the resistant properties.

2.3. Packaging and Purification of Lentiviral Particles. The logarithmic of HEK-293T cells were trypsinized, and the cell density was adjusted to 1.5×10^6 cells/mL with complete medium. The cells were reseeded into T75 cell culture flasks and cultured for 24 h. The cells were 80%–90% confluent on the day of transfection. The recombinant vectors encoding miR-451a or miR-451a sponge and packaging plasmids (pSPAX2 and pMD2G) were cotransfected into HEK-293T cells with LipoFiter (Hanbio, Shanghai, China). After 6 h transfection, the cell culture medium was replaced by fresh complete DMEM. The expression of ZsGreen was determined after 24 h transfection, and the culture supernatant was collected after 48 h transfection and centrifuged at 4,000 ×g for 10 min to remove any cell debris sedimentation. Then the supernatant was filtered through a 0.22 μm cellulose acetate filter and centrifuged at 72,000 ×g for 120 min at 4°C to harvest the high-titer lentivirus containing miR-451a or miR-451a sponge gene. The lentivirus without transgene was produced in the same way and used as negative control.

2.4. Determination of Virus Titer. The HEK-293T cells were seeded into 96-well plates at 1.0×10^3 cells per well. After 24 h, the media were replaced with serial diluted virus solution, and the cells were cultivated under the conditions of 5% CO_2 and 37°C. After 72 h, the number of fluorescent cells and total cells were counted under microscope. The titer of virus was acquired using the appropriate dilution.

2.5. Virus Transfection and Selection of Stable Transfected Cell Line. Logarithmic phases of MCF-7 and LCC2 cells were cultured at 1×10^5 cells per well in 24-well plates and divided into blank control, Lv-negative control Lv-miR-451a, and Lv-miR-451a sponge. After 24 h, the mixture of appropriate volume of virus stock solution and cell culture medium was added to cells. After 24 h incubation, the virus solution was removed and replaced with complete medium. To establish stable transfected cell lines, 72 h after transduction, puromycin was added to the medium at the concentration of 5 μg/mL. After antibiotic selection for 3 weeks, the blank control cells were completely dead, and the corresponding stable transfected cell lines were obtained.

2.6. Detection of miR-451a and MIF Gene Expression by Real-Time RT-PCR. Total RNAs from MCF-7 and LCC2 Cells were prepared using the Trizol reagent (Invitrogen, Carlsbad, CA) after the viral transduction. RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada) was used for the reverse transcription of total RNA (0.1 μg) according to the manufacturer's instructions. Real-time PCR was performed using the SYBR Green Master Mix (Takara Bio Inc.). Primers were used as follows: miR-451a (F: 5′-ACACTCCAGCTGGGAA-ACCGTTACCATTACT-3′; R:

5′-CTGGTGTCGTGGAGTCGGCAA-3′), U6 (F: 5′-CTCGCTTCGGCAGCACA-3′; R: 5′-AACGCTTCACGAATTTGCGT-3′), MIF (F: 5′-GCAGAACCGCTCCTACAGCA-3′; R: 5′-GGCTCT-TAGGCGAAGGTGGA-3′), and β-actin (F: 5-′CCTGGCACCCAGCACAAT-3′; R: 5′-GGGCCGGACTCGTCATACT-3′). All the reactions were performed in triplicate using the ABI Prism 7500 real-time PCR system (Applied Biosystem, Foster City, CA, USA). The data were expressed as $2^{-\Delta\Delta Ct}$.

2.7. Cell Proliferation Assay. Cell proliferation was measured by MTT assay. MCF-7 and LCC2 cells transfected with scrambled oligonucleotide, miR-451a, or miR-451a sponge were seeded into 96-well plates (6×10^3 cells/well) and treated with 4-OHT at different concentrations for 48 h. 20 μL MTT (5 mg/mL) was added into each well and incubated for 4 h. After the media were removed, 150 μL DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added to each well to dissolve the formazan crystal. The absorbance was measured at 490 nm with a microplate reader (Bio-Rad, Hercules CA). The concentration of 4-OHT yielding 50% growth inhibition (IC_{50}) was calculated using medium effect algorithm with three independent experiments [12].

To draw the cell growth curve, MCF-7 and LCC2 cells were seeded into 96-well plates (3×10^3 cells/well); after transfection, MTT assay was performed to detect cell survival at Days 1, 2, 3, 4, 5, 6, and 7, and optical density (OD) was measured at 490 nm.

2.8. Colony Formation Assay. MCF-7 and LCC2 cells were plated at a low density (5×10^3 cells per 10 cm plate) after transfection and incubated for 14 days at 37°C and 5% CO_2 in a humidified atmosphere. Colonies were fixed with 90% ethanol, stained with crystal violet, and counted under inverted microscope.

2.9. Transwell Invasive and Wound Healing Assay. Transwell chambers (Corning) with 8 μm pore size were coated with Matrigel (BD Bioscience) on the upper surface. 200 μL MCF-7 and LCC2 cells suspension with the recombinant lentiviral vector was plated into the upper chambers, and the medium with 10% FBS was piped into the lower Transwell chamber. After 24 h, the cells that had invaded through the membrane to the lower chambers were fixed with 90% ethanol and stained and counted under inverted microscope (400x).

Cells were seeded into 6-well plate about 24 h. When the cells grew to over 90% confluence, they were wounded by scratching with a pipette tip and washed with PBS for three times. Cells were refreshed with 2.5% FBS and photographed after 24 h and 48 h incubation at 37°C.

2.10. Western Blot. Cells transfected with lentiviral vectors were lysed with lysis buffer at 4°C for 30 min. The protein concentration was measured by the Bio-Rad assay system (Bio-Rad Hercules, CA, USA). The same amount of proteins (50 μg) was separated on 9% SDS-PAGE and transferred to PVDF membrane (Bio-Rad Hercules, CA, USA). The membrane was blocked by 5% nonfat milk at room temperature for 1.5 h, incubated with rabbit monoclonal anti-MIF antibody (1 : 1000, Abcam) at 4°C overnight, and then washed with PBST followed by incubation with the second antibody (goat anti-rabbit IgG, 1 : 2000, Boster, Wuhan, China) at room temperature for 1.5 h. The signal was detected using Chemiluminescence Kit (Millipore Corporation, Billerica, MA, USA). The intensity of the bands was quantitated using the Image Gel Documentation System, and data were presented as the relative pixel density normalized to GAPDH [13].

2.11. Statistical Analysis. The data were presented as mean ± SD. Experimental data were analyzed by one-way analysis of variance (ANOVA), and statistical analysis was performed with SPSS 16.0 software. Significance was defined by $P < 0.05$.

3. Results

3.1. PCR Amplification and Sequencing of Pre-miR-451a and miR-451a Sponge Fragments. DNA fragments of pre-miR-451a and miR-451a sponge were successfully amplified by PCR. Electrophoresis showed the specific bands of pre-miR-451a and miR-451a at 243 bp and 88 bp, respectively (Figures 1(a) and 1(b)). The sequences of pre-miR-451a and miR-451a sponge were also analyzed (Figures 1(c) and 1(d)).

3.2. Lentivirus Packaging and Transduction of MCF-7 and LCC2 Cells. HEK-293T cells were cotransfected with the transfer plasmid, pHBLV-transgenes, the envelope plasmid (pMD2G), and the packaging plasmids (pSPAX2). The virus titer was calculated by the formula virus titer = $3 \times 10^4 \times 12\% \times 30 \times 10^3 = 1.08 \times 10^8$ PFU/mL. ZsGreen was expressed after MCF-7 and LCC2 cells were transduced with the lentivirus. After selection for 3 weeks by puromycin, we obtained stable transfected cell lines of MCF-7 and LCC2 cells. All the MCF-7 cells with ZsGreen expression were observed under microscope (Figure 2), and LCC2 cells were also observed (data not shown).

3.3. The Expression of miR-451a and MIF before and after Transfection in MCF-7 and LCC2 Cells. We performed real-time RT-PCR to test the expression of miR-451a and MIF. The expression of miR-451a was significantly lower, but the mRNA expression of MIF was significantly higher in LCC2 cells compared with MCF-7 cells (Figure 3(a)). After cell transfection, the expression of miR-451a was significantly increased in Lv-miR-451a group, whereas it significantly decreased in Lv-miR-451a sponge group compared with the Lv-miR-451a NC ($P < 0.05$) in both of MCF-7 and LCC2 cells (Figure 3(b)). Thus, we established the stable transfected cell lines of MCF-7-miR-451a, MCF-7-miR-451a sponge, LCC2-miR-451a, and LCC2-miR-451a sponge. Furthermore, overexpression of miR-451a significantly decreased the MIF mRNA expression. In contrast, downregulation of miR-451a significantly increased MIF mRNA expression (Figure 3).

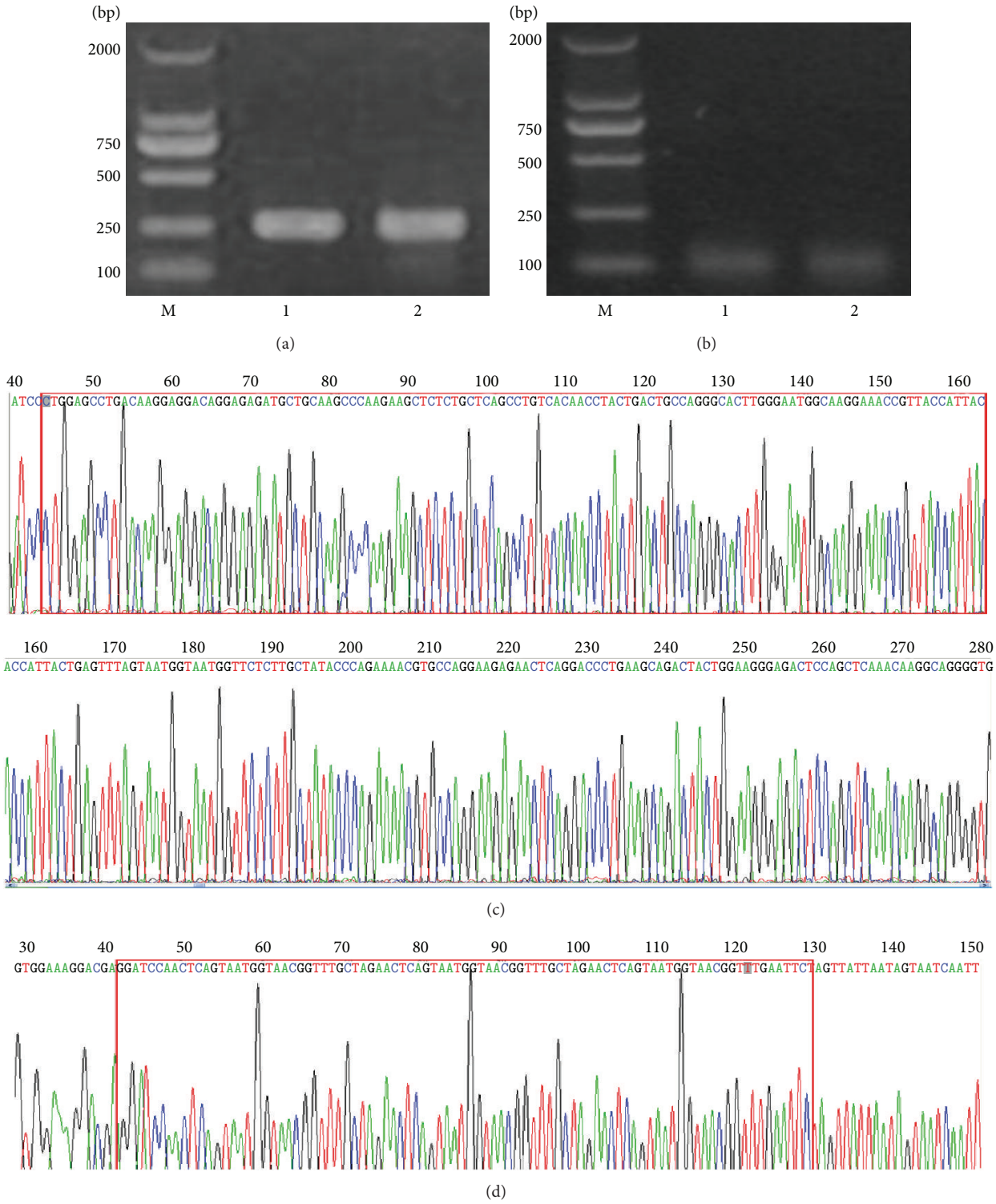

Figure 1: Electrophoresis of PCR products and sequencing analysis of pre-miR-451a and miR-451a sponge. (a) and (b) Electrophoresis indicated the specific bands of pre-miR-451a and miR-451a at 243 bp and 88 bp; (c) and (d) sequencing results indicated that the pre-miR-451a and miR-451a sponge sequences were correct.

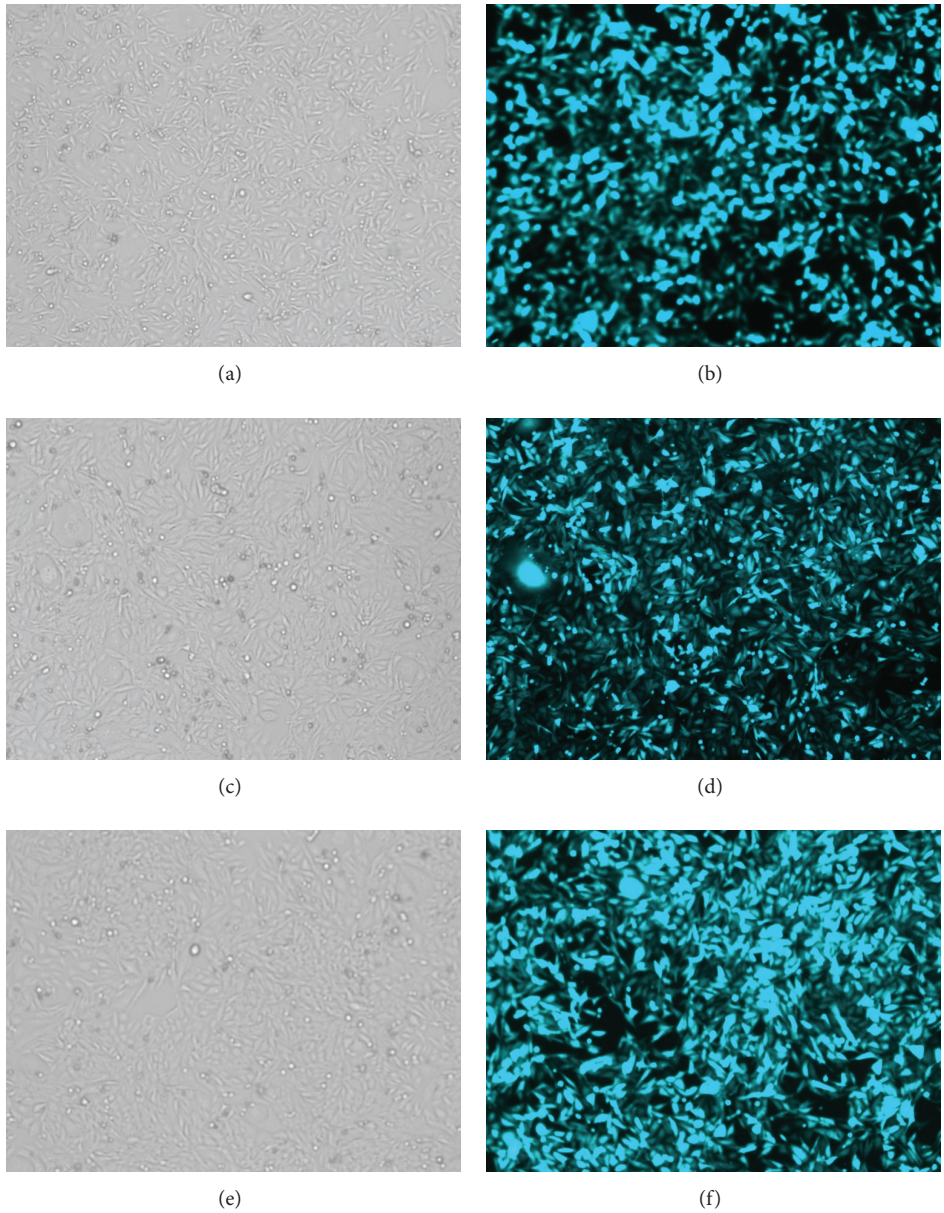

Figure 2: MCF-7 cells transduced by lentivirus after puromycin selection (×200). (a), (c), and (e) Light microscopy of Lv-miR-451a NC, Lv-miR-451a, and Lv-miR-451a sponge; (b), (d), and (f) fluorescent microscopy of Lv-miR-451a NC, Lv-miR-451a, and Lv-miR-451a sponge. All the cells with ZsGreen expression and the stable cell lines were obtained (note: NC means negative control; Lv means lentivirus).

3.4. miR-451a Suppressed MCF-7 and LCC2 Cell Proliferation and Colony Formation. MTT assay showed that the cell proliferation of MCF-7 (Figure 4(a)) and LCC2 cells (Figure 4(b)) transfected with Lv-miR-451a was significantly decreased compared with negative control (NC). Colony formation assay showed that the proliferation and colony numbers of the cells transfected with Lv-miR-451a were significantly decreased compared to negative control (NC) (Figure 4(c)). In contrast, the proliferation and colony formation were significantly increased in MCF-7 and LCC2 cells transfected with Lv-miR-451a sponge compared with Lv-miR-451a NC.

3.5. miR-451a Suppressed the Invasion Ability of MCF-7 and LCC2 Cells. We first compared the invasion ability of MCF-7 and LCC2 cells at baseline and found that LCC2 cells were more aggressive than MCF-7 (Figure 5(a)). Next we measured the invasion ability of the cells after transfection with Lv-miR-451a, Lv-miR-451a sponge, or Lv-miR-451a NC. The results showed that transfection with Lv-miR-451a significantly inhibited the invasive ability in both of MCF-7 and LCC2 cells compared to Lv-miR-451a NC transfected cells (Figure 5(b)). On the other hand, the invasion ability of the cells transfected with Lv-miR-451a sponge was significantly

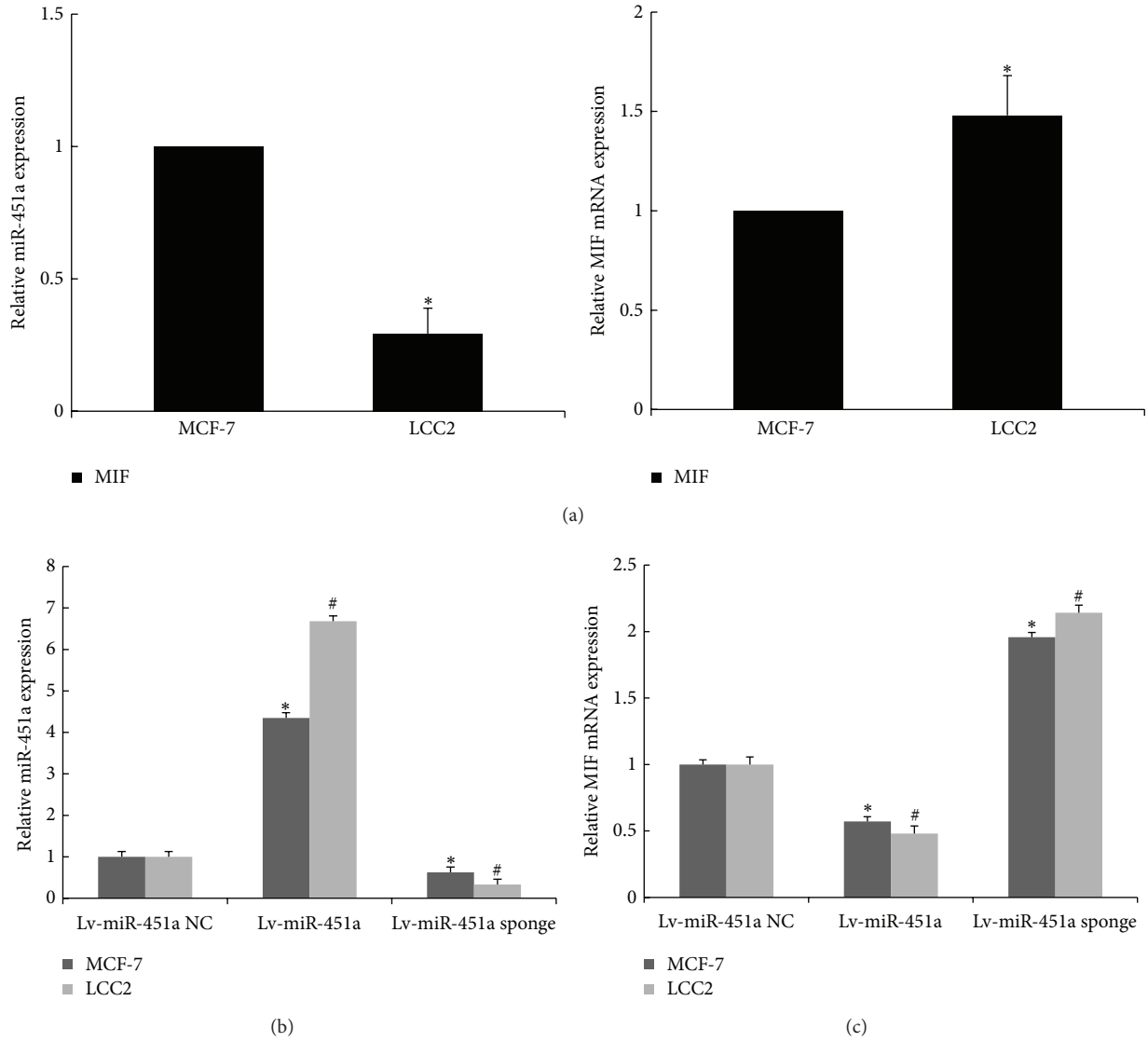

Figure 3: The expressions of miR-451a and MIF were examined by real-time PCR. (a) total RNA extracted from the cell lines of MCF-7 and LCC2 was subjected to real-time PCR with miR-451a-specific or MIF-specific primers. The miR-451a expression or MIF mRNA expression was normalized to U6 or GAPDH expression. $^{*}P < 0.05$ versus MCF-7 cell; (b) total RNA extracted from the stable cell lines of MCF-7 and LCC2 was subjected to real-time PCR with miR-451a-specific primers. The induction of miR-451a expression was normalized to U6 expression; (c) total RNA extracted from these cells was subjected to real-time PCR with MIF-specific primers. The induction of MIF mRNA expression was normalized to GAPDH expression. $^{*}P < 0.05$ versus Lv-miR-451a NC (MCF-7); $^{\#}P < 0.05$ versus Lv-miR-451a NC (LCC2) (note: NC means negative control; Lv means lentivirus).

enhanced compared with those transfected with Lv-miR-451a NC (Figure 5(b)).

3.6. Ectopic Expression of miR-451a Partially Increased the 4-OHT Sensitivity of MCF-7 and LCC2 Cells. To understand whether the transfected cells have different responses to 4-OHT, the cells were treated with different concentrations of 4-OHT and cell survival rate was measured. The inhibition rate of cellular survival was significantly increased and the IC_{50} value significantly decreased in both of MCF-7 and LCC2 cells transfected with Lv-miR-451a compared to Lv-miR-451a NC transfected cells. However, the inhibition rate of cellular survival was much lower and IC_{50} was higher in cells transfected with Lv-miR-451a sponge compared with those transfected with Lv-miR-451a NC (Figure 6). All these results confirmed that cells transfected with Lv-miR-451a would restore their sensitivity to 4-OHT, whereas the cells transfected with Lv-miR-451a sponge acquired the drug resistance.

3.7. Ectopic Expression of miR-451a Regulated MIF Expression in MCF-7 and LCC2 Cells. Western blot was used to detect MIF expression, and the results showed that MIF protein level was significantly decreased in both MCF-7 and LCC2 cells

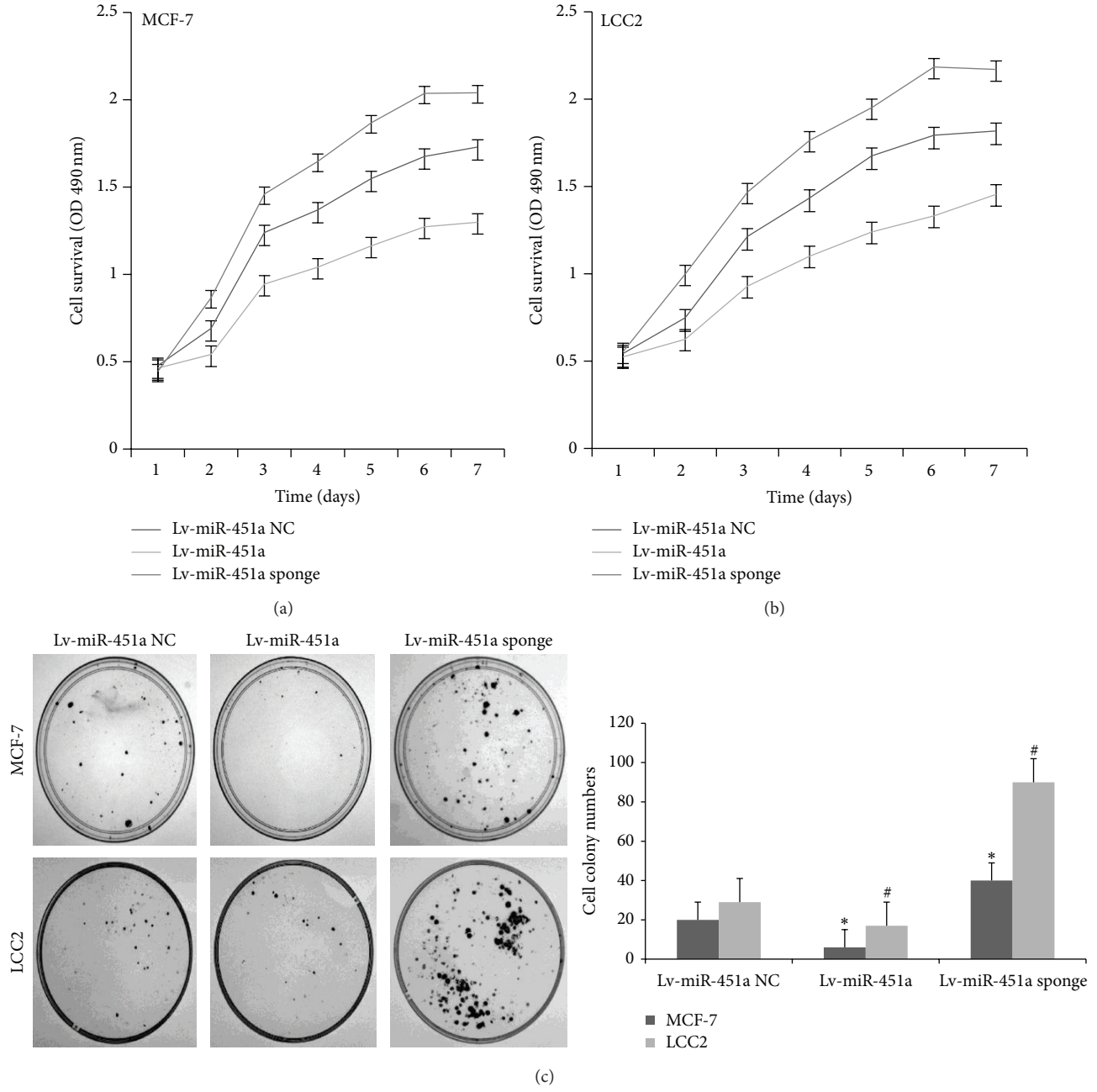

Figure 4: Effect of miR-451a and miR-451a sponge on cell proliferation and colony formation of MCF-7 and LCC2 cells. Cell proliferation was examined by MTT assay for MCF-7 cells (a) and LCC2 cells (b) transfected with Lv-miR-451a, Lv-miR-451a sponge, or Lv-miR-451a NC. (c) Colony formation was examined for MCF-7 cells and LCC2 cells transfected with Lv-miR-451a, Lv-miR-451a sponge, or Lv-miR-451a NC. $^{*}P < 0.05$ versus Lv-miR-451a NC (MCF-7); $^{\#}P < 0.05$ versus Lv-miR-451a NC (LCC2) (note: NC means negative control; Lv means lentivirus).

transfected with Lv-miR-451a and significantly increased in cells transfected with Lv-miR-451a sponge compared with Lv-miR-451a NC and control group. There was no significant difference between Lv-miR-451a NC and control groups ($P > 0.05$) (Figure 7).

3.8. MIF Regulated the Cells Invasion and Tamoxifen Sensitivity of LCC2 Cells. In order to determine whether MIF could regulate the breast cancer cells invasion and tamoxifen sensitivity, we transfected LCC2 cells with siMIF and siNC (negative control) and found that siMIF significantly reduced the cell invasion and motility. MTT assay results also showed that siMIF significantly increased the cell survival inhibition rate of 4-OHT, consequently decreased the IC_{50} value, and partially restored the tamoxifen sensitivity of LCC2 cells (Figure 8).

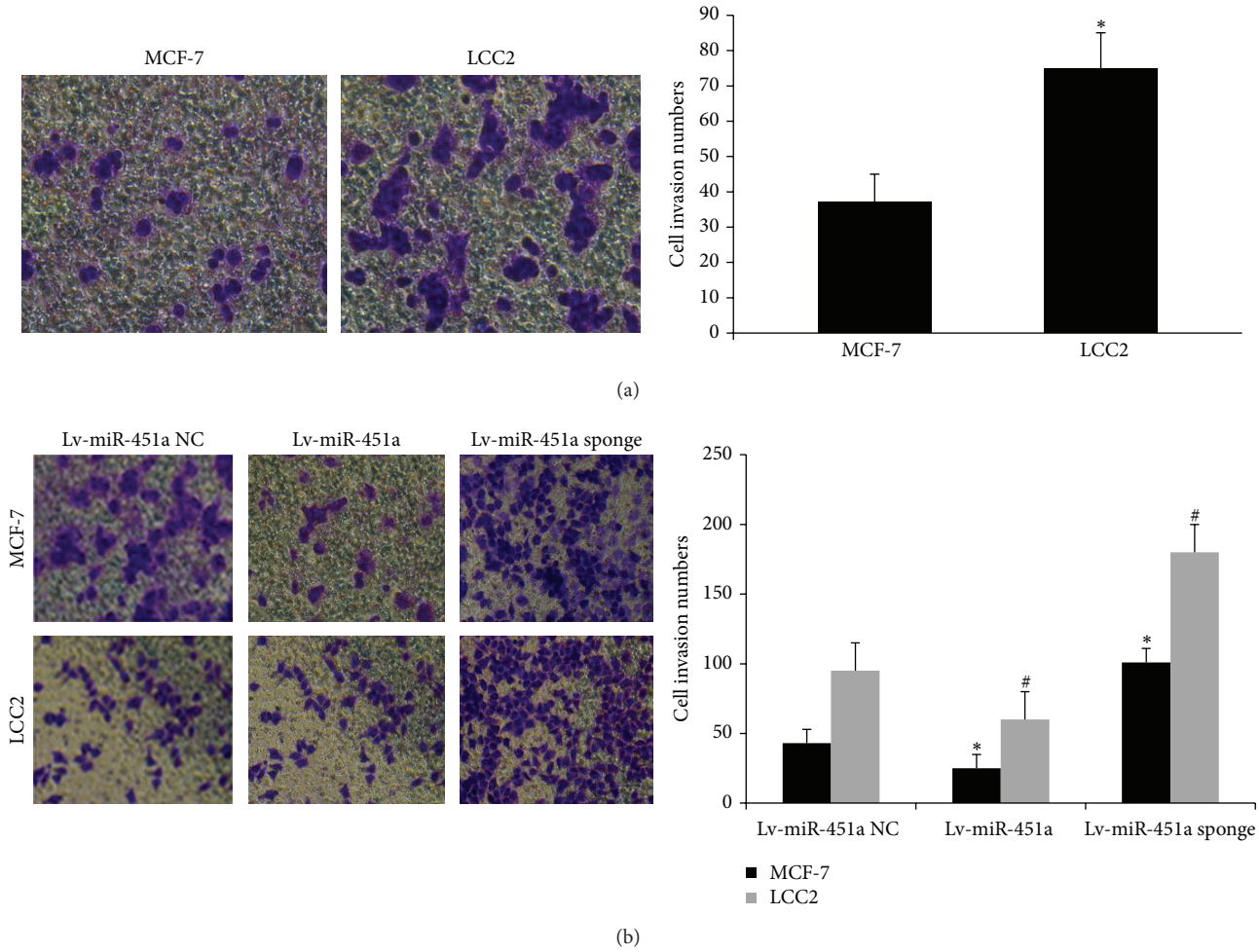

Figure 5: Effects of miR-451a and miR-451a sponge on cell invasion ability. (a) The invasion ability of MCF-7 and LCC2 cells. $^{*}P < 0.05$ versus MCF-7 cell; (b) representative images of the invasion of MCF-7 and LCC2 cells transfected with Lv-miR-451a sponge or Lv-miR-451a. $^{*}P < 0.05$ versus Lv-miR-451a NC (MCF-7); $^{\#}P < 0.05$ versus Lv-miR-451a NC (LCC2) (note: NC means negative control; Lv means lentivirus).

4. Discussion

Gene transfection is a process in which nucleic acids are transported into cells and maintain their biological functions in the cells. At present, plasmid vectors and virus vectors are widely used as gene carriers in various studies to explore the roles of miRNAs in the regulation of gene expression, signal transduction, and gene therapy research. Lentiviral vector is designed based on the transformation of HIV-1 production and can be used for both dividing and nondividing cells with the advantages of integrating large exogenous gene into the host chromosome, stable expression, small immune response, and safety [14]. In this study, we established lentiviral expression vectors of Lv-miR-451a and Lv-miR-451a sponge and therefore built a foundation for efficient and stable transfection of MCF-7 and LCC2 cells.

Recently, miRNAs have been demonstrated to play important roles in maintaining normal cellular functions, and the dysregulation of miRNAs could lead to the initiation and progression of tumors [15, 16]. miR-451 (also named as miR-451a) was downregulated in several tumors, such as breast cancer, lung cancer, and gastric cancer [5, 17, 18]. Furthermore, the abnormal expression of miR-451a was involved in the functions of cancer cells, including cell proliferation and survival, migration, and invasion [17, 18]. Each miRNA has hundreds of target genes and has the ability to harbor the target sequence in their 3′-untranslated region (UTR) segment to the seed region of the miRNA [1]. Several targets of miR-451a have been identified, and MIF was verified as one of them using luciferase reporter gene assay in previous study [18]. A number of studies showed that MIF expression was upregulated in various tumors, which can promote cell proliferation and invasion and correlate with worse survival prognosis [19]. However, only a few reports have been presented regarding the MIF function in breast cancer. In order to have a better understanding of the function of miR-451a/MIF pathway in breast cancer, in this study we first detected the miR-451a and MIF expression

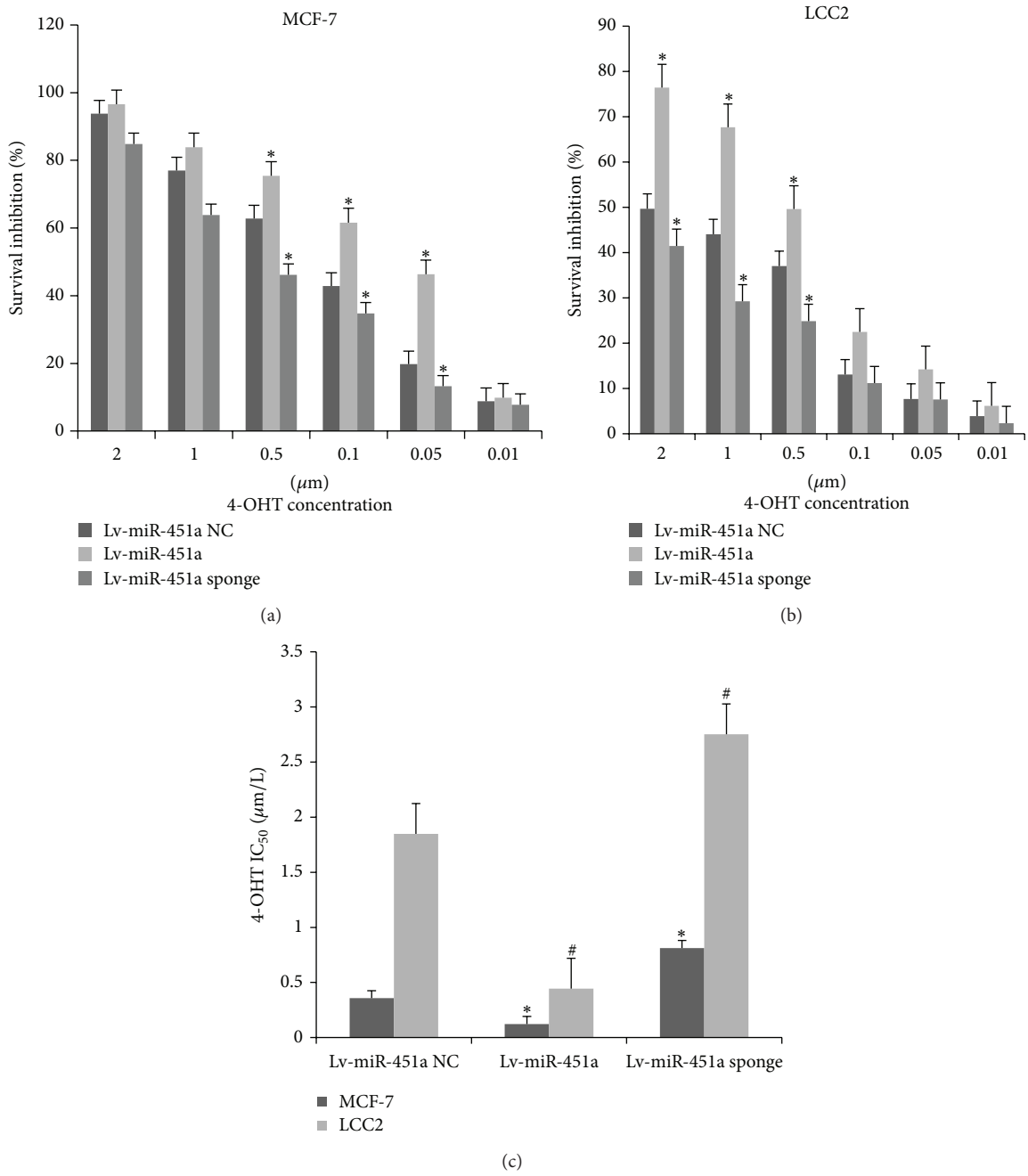

Figure 6: Effects of miR-451a and miR-451a sponge on cell survival and 4-OHT sensitivity in breast cancer cells. (a-b) MTT assay was performed to test cell survival inhibition rate in MCF-7 (a) and LCC2 (b) cells after being transfected with Lv-miR-451a or Lv-miR-451a sponge or Lv-miR-451a NC and treated with different concentrations of 4-OHT; (c) IC_{50} value of 4-OHT was determined after cells were transfected with Lv-miR-451a or Lv-miR-451a sponge or Lv-miR-451a NC. $^{*}P < 0.05$ versus Lv-miR-451a NC (MCF-7); $^{\#}P < 0.05$ versus Lv-miR-451a NC (LCC2) (note: NC means negative control; Lv means lentivirus).

in MCF-7 and LCC2 cells. We found that miR-451a was decreased but MIF was increased in LCC2 cells compared to MCF-7, which were negative correlation, and LCC2 cells were more aggressive than MCF-7. We next established and verified the stable over- or downregulated expression of miR-451a by transducing Lv-miR-451a or Lv-miR-451a sponge into MCF-7 and LCC2 cell lines. Consequentially, up- or downregulation of miR-451a decreased or increased MIF mRNA and protein expression in these cells, respectively. Furthermore, we found that the cell proliferation and colony formation were suppressed by miR-451a upregulation but increased by miR-451a downregulation. Moreover, miR-451a overexpression inhibited the cell invasive capacity, and the opposite results were found in the cells with downexpression of miR-451a. Our findings are consistent with previous studies in nasopharyngeal carcinoma [20]. Several studies

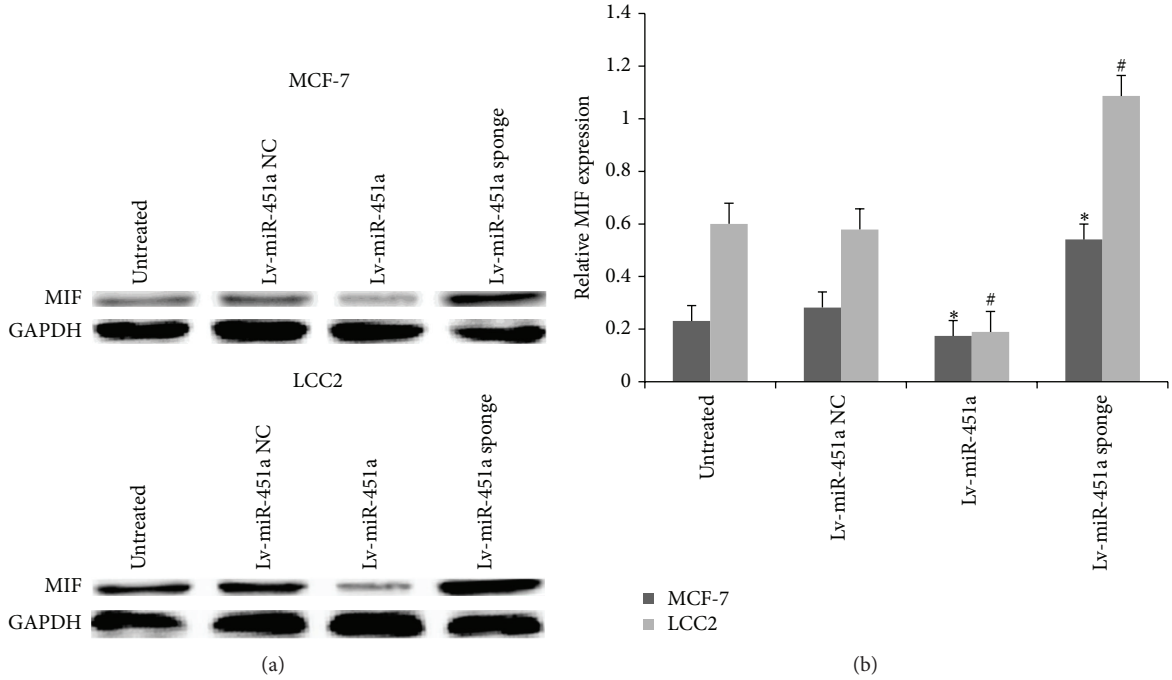

Figure 7: Expression of MIF in each group. The MCF-7 and LCC2 cells were transfected with Lv-miR-451a or Lv-miR-451a sponge or Lv-miR-451a NC. Total lysates were prepared and subjected to western blot with anti-MIF antibody. $^{\#}P < 0.05$ versus Lv-miR-451a NC or control (MCF-7); $^{*}P < 0.05$ versus Lv-miR-451a NC or control (LCC2) (note: NC means negative control; Lv means lentivirus).

also indicated that MIF was indirectly involved in promoting cancer growth and progression, while depleting endogenous MIF caused a >40% reduction in cancer cell growth in colon cancer [21–23].

The use of most of the anticancer drugs, including tamoxifen, is limited due to the drug resistance by breast cancer cells, which is a serious problem in treating breast cancer patients with positive ERα expression, as evidences showed that the expression of estrogen receptor (ERα) determined the response of breast cancer cells to tamoxifen [24–26]. The relation between MIF expression level and ER/PR status has been reported, which indicated that JAB1 was a binding partner of MIF and interacted with ER and PR [27]. The altered expression of miRNAs was involved in tamoxifen resistance development and predicted the therapeutic response in breast cancer patients [11]. Previous studies suggested that downregulation of miR-451a might be the mechanism of developing resistance to tamoxifen therapy [5]. Our study showed that miR-451a overexpression in MCF-7 and LCC2 cells enhanced the cell survival inhibition rate and restored the sensitivity to tamoxifen to some extent based on the changes of IC_{50} value. miR-451a may play an important role in chemotherapy resistance as well, and upregulation of miR-451a enhanced the cisplatin and doxorubicin effectiveness [28]. In addition, our study indicated that miR-451a could negatively regulate the MIF protein expression, which is consistent with the finding in gastric cancer [18]. These results suggested that upregulation of MIF induced by downregulation of miR-451a may be one of the underlying mechanisms of tamoxifen resistance in breast cancer. In this study, we used siRNA to study MIF function according to previous studies [29] and found that siMIF significantly reduced the cell invasion and motility, also decreased the IC_{50} value, and partially restored the tamoxifen sensitivity of LCC2 cells. In this sense, our study demonstrated that the inverse correlation between miR-451a and MIF might play important roles in breast cancer. This is the first report to show that miR-451a can suppress cell invasive ability and enhance tamoxifen sensitivity in breast cancer cells.

In summary, in the current study we constructed the lentiviral vectors of miR-451a and established stable transfected cell lines. The functional studies indicated that miR-451a, as a tumor suppressor, had important functions in breast cancer progression through MIF pathway. Further in vitro and in vivo studies are warranted to explore the molecular mechanisms of miR-451a/MIF pathway in breast cancer.

Conflict of Interests

No potential conflict of interests is relevant to this paper disclosed.

Authors' Contribution

Zhenru Liu and Tianyu Miao contributed equally to this paper.

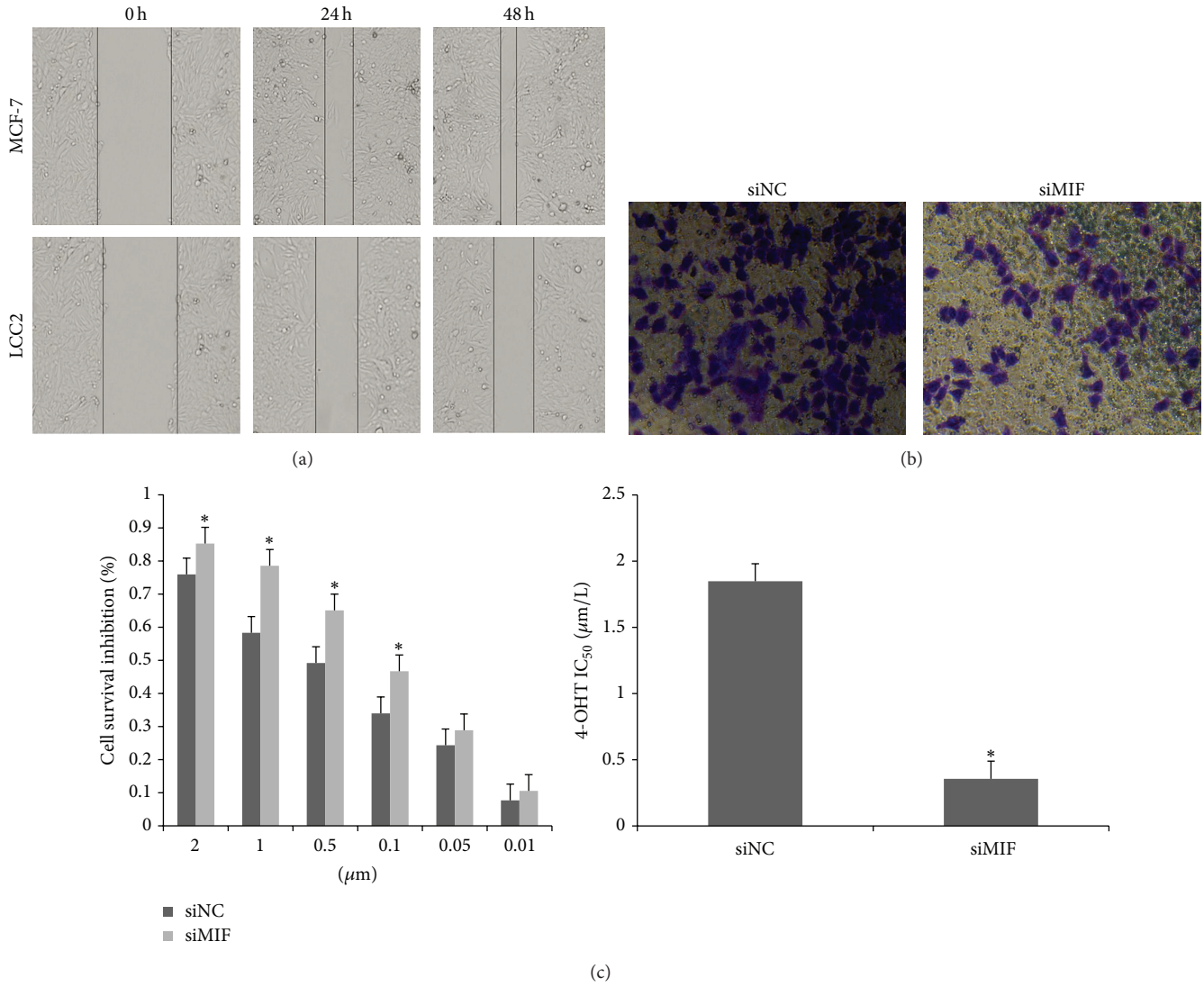

Figure 8: MIF was involved in breast cancer cells invasion and tamoxifen sensitivity. (a) After wounding cells were cultured for another 24 h or 48 h, siMIF increased the area of unhealed cell wound size; (b) LCC2 cells were seeded into a Matrigel-coated membrane and cultured for 24 h; siMIF reduced the number of invading cells compared to the siNC; (c) MTT assay indicated that siMIF significantly increased the cell survival inhibition rate in LCC2 cells compared to siNC. The IC_{50} value of 4-OHT was examined after the cells were treated with different concentrations of 4-OHT. The results showed that siMIF significantly reduced the IC_{50} of 4-OHT compared to siNC. $^{*}P < 0.05$ versus siNC.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 81170606) and the Ph.D. Programs Foundation of Ministry of Education of China (no. 20120181110087).

References

[1] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[2] N. Othman and N. H. Nagoor, "The role of microRNAs in the regulation of apoptosis in lung cancer and its application in cancer treatment," *BioMed Research International*, vol. 2014, Article ID 318030, 19 pages, 2014.

[3] S. Griffiths-Jones, H. K. Saini, S. Van Dongen, and A. J. Enright, "miRBase: tools for microRNA genomics," *Nucleic Acids Research*, vol. 36, no. 1, pp. D154–D158, 2008.

[4] E. H. Mahlamaki, M. Barlund, M. Tanner et al., "Frequent amplification of 8q24, 11q, 17q, and 20q-specific genes in pancreatic cancer," *Genes Chromosomes and Cancer*, vol. 35, no. 4, pp. 353–358, 2002.

[5] A. Bergamaschi and B. S. Katzenellenbogen, "Tamoxifen down-regulation of miR-451 increases 14-3-3ζ and promotes breast cancer cell survival and endocrine resistance," *Oncogene*, vol. 31, no. 1, pp. 39–47, 2012.

[6] O. Kovalchuk, J. Filkowski, J. Meservy et al., "Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin," *Molecular Cancer Therapeutics*, vol. 7, no. 7, pp. 2152–2159, 2008.

[7] H. Bando, G. Matsumoto, M. Bando et al., "Expression of macrophage migration inhibitory factor in human breast cancer: association with nodal spread," *Japanese Journal of Cancer Research*, vol. 93, no. 4, pp. 389–396, 2002.

[8] R. García-Becerra, N. Santos, L. Díaz, and J. Camacho, "Mechanisms of resistance to endocrine therapy in breast cancer: focus on signaling pathways, miRNAs and genetically based resistance," *International Journal of Molecular Sciences*, vol. 14, no. 1, pp. 108–145, 2013.

[9] M. Farazuddin, B. Dua, Q. Zia, A. A. Khan, B. Joshi, and M. Owais, "Chemotherapeutic potential of curcumin-bearing microcells against hepatocellular carcinoma in model animals," *International Journal of Nanomedicine*, vol. 9, no. 1, pp. 1139–1152, 2014.

[10] W. J. Gradishar, "Tamoxifen—what next?" *The Oncologist*, vol. 9, no. 4, pp. 378–384, 2004.

[11] M. V. Iorio, P. Casalini, C. Piovan, L. Braccioli, and E. Tagliabue, "Breast cancer and microRNAs: therapeutic impact," *Breast*, vol. 20, supplement 3, pp. S63–S70, 2011.

[12] A. Chauhan, S. Zubair, A. Sherwani, and M. Owais, "Aloe vera induced biomimetic assemblage of nucleobase into nanosized particles," *PLoS ONE*, vol. 7, no. 3, Article ID e32049, 2012.

[13] A. Khan, Y. Shukla, N. Kalra et al., "Potential of diallyl sulfide bearing pH-sensitive liposomes in chemoprevention against DMBA-induced skin papilloma," *Molecular Medicine*, vol. 13, no. 7-8, pp. 443–451, 2007.

[14] A. Horiguchi, R. Zheng, O. B. Goodman et al., "Lentiviral vector neutral endopeptidase gene transfer suppresses prostate cancer tumor growth," *Cancer Gene Therapy*, vol. 14, no. 6, pp. 583–589, 2007.

[15] G. A. Calin and C. M. Croce, "MicroRNA-cancer connection: the beginning of a new tale," *Cancer Research*, vol. 66, no. 15, pp. 7390–7394, 2006.

[16] A. Esquela-Kerscher and F. J. Slack, "Oncomirs—microRNAs with a role in cancer," *Nature Reviews Cancer*, vol. 6, no. 4, pp. 259–269, 2006.

[17] R. Wang, Z.-X. Wang, J.-S. Yang, X. Pan, W. De, and L.-B. Chen, "MicroRNA-451 functions as a tumor suppressor in human non-small cell lung cancer by targeting ras-related protein 14 (RAB14)," *Oncogene*, vol. 30, no. 23, pp. 2644–2658, 2011.

[18] E. Bandres, N. Bitarte, F. Arias et al., "microRNA-451 regulates macrophage migration inhibitory factor production and proliferation of gastrointestinal cancer cells," *Clinical Cancer Research*, vol. 15, no. 7, pp. 2281–2290, 2009.

[19] M. Ferrero-Poüs, K. Hacène, C. Bouchet, V. Le Doussal, M. Tubiana-Hulin, and F. Spyratos, "Relationship between c-erbB-2 and other tumor characteristics in breast cancer prognosis," *Clinical Cancer Research*, vol. 6, no. 12, pp. 4745–4754, 2000.

[20] N. Liu, N. Jiang, R. Guo et al., "MiR-451 inhibits cell growth and invasion by targeting MIF and is associated with survival in nasopharyngeal carcinoma," *Molecular Cancer*, vol. 12, no. 1, article 123, 2013.

[21] K. L. Meyer-Siegler, E. C. Leifheit, and P. L. Vera, "Inhibition of macrophage migration inhibitory factor decreases proliferation and cytokine expression in bladder cancer cells," *BMC Cancer*, vol. 4, article 34, 2004.

[22] T. Hagemann, S. C. Robinson, R. G. Thompson, K. Charles, H. Kulbe, and F. R. Balkwill, "Ovarian cancer cell-derived migration inhibitory factor enhances tumor growth, progression, and angiogenesis," *Molecular Cancer Therapeutics*, vol. 6, no. 7, pp. 1993–2002, 2007.

[23] N. Takahashi, "Involvement of macrophage migration inhibitory factor (MIF) in the mechanism of tumor cell growth," *Molecular Medicine*, vol. 4, no. 11, pp. 707–714, 1998.

[24] M. Farazuddinm, B. Sharma, A. A. Khan, B. Joshi, and M. Owais, "Anticancer effcacy of perillyl alcohol-bearing PLGA microparticles," *International Journal of Nanomedicine*, vol. 7, pp. 35–47, 2012.

[25] A. Khan, A. A. Khan, V. Dwivedi, M. G. Ahmad, S. Hakeem, and M. Owais, "Tuftsin augments antitumor efficacy of liposomized etoposide against fibrosarcoma in Swiss albino mice," *Molecular Medicine*, vol. 13, no. 5-6, pp. 266–276, 2007.

[26] J. A. Vendrell, K. E. Robertson, P. Ravel et al., "A candidate molecular signature associated with tamoxifen failure in primary breast cancer," *Breast Cancer Research*, vol. 10, no. 5, article R88, 2008.

[27] A. Chauchereau, M. Georgiakaki, M. Perrin-Wolff, E. Milgrom, and H. Loosfelt, "JAB1 interacts with both the progesterone receptor and SRC-1," *Journal of Biological Chemistry*, vol. 275, no. 12, pp. 8540–8548, 2000.

[28] H.-B. Bian, X. Pan, J.-S. Yang, Z.-X. Wang, and W. De, "Upregulation of microRNA-451 increases cisplatin sensitivity of non-small cell lung cancer cell line (A549)," *Journal of Experimental and Clinical Cancer Research*, vol. 30, no. 1, article 20, 2011.

[29] A. Chauhan, S. Zubair, A. Nadeem, S. A. Ansari, M. Y. Ansari, and O. Mohammad, "Escheriosome-mediated cytosolic delivery of PLK1-specific siRNA: potential in treatment of liver cancer in BALB/c mice," *Nanomedicine*, vol. 9, no. 4, pp. 407–420, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 432479, 5 pages
http://dx.doi.org/10.1155/2015/432479

Research Article

Intratumoral Heterogeneity of MAGE-C1/CT7 and MAGE-C2/CT10 Expression in Mucosal Melanoma

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Received 25 July 2014; Accepted 10 December 2014

Academic Editor: Anshu Agrawal

Mucosal melanoma is a rare disease, which differs from its cutaneous counterpart genetically and for its clinical behaviour. Moreover this is a heterogeneous disease based on the tissue of origin. As CT7 and CT10 are highly expressed in cutaneous melanoma and are immunogenic in this disease, we analysed their expression throughout the different subtypes of mucosal melanoma and tumor development. We detected a frequent expression of CT7 in primaries and corresponding metastases (55%) as well as for CT10 (30%). This expression resulted to be heterogeneous in the same tumor specimen and moreover influenced by the tissue of origin. Our results support the role of these antigens in immunotherapy for mucosal melanoma.

1. Introduction

Cancer-testis (CT) antigen (Ags) represent a family of proteins widely studied in the field of cancer immunotherapy because of their restrictive expression pattern and immunogenicity in cancer patients [1]. In normal tissues, the expression of CT antigen is restricted to germ line tissues (namely, placenta, ovaries, and testis), which express small amounts of HLA molecules, making CT antigen no more recognisable from the immune system. For this reason, CTAgs represent ideal candidates for vaccination strategies in cancer. Although expressed in several cancers, their role in tumorigenesis is however unclear. Of interest is that the aberrant expression of germ line genes in cancer reflects the activation of a program which is silenced in somatic cells; moreover, it is very well known that the gain of genes is one of the driving forces of tumorigenesis [2]. This has been also postulated for different CT antigen, expressed diffusely in malignant tissues and frequently coexpressed as the consequence of a common promoter demethylation [3, 4]. Among the CT antigen, the MAGE family is one of the most extensively investigated antigen so far, with documented expression in several cancers [2]. Between these, MAGE-C1/CT7 (from now on CT7) and MAGE-C2/CT10 (from now on CT10) are highly expressed in cutaneous melanoma (CM), represent strong prognostic markers [5], and spontaneously induce a specific cellular immune response in melanoma patients [6, 7].

Different from the cutaneous counterpart are mucosal melanomas (MM). These are unrelated to ultraviolet light exposure, develop at later age and more frequently at advanced stage, are characterised by a high risk of local recurrence, and develop more often distant metastases. Genetically they differ from the cutaneous counterpart for the rare presence of *Braf* mutations. Of interest is also that MM are a heterogeneous disease and, based on the tissue of origin, genetic differences have been detected as for *c-kit* mutations,

which were found in almost half of genital melanoma and nonsinonasal melanoma [8–10].

Due to clinical and genetic differences between CM and MM and heterogeneity of MM, we aimed at evaluating the expression of CT7 and CT10 in MM and their presence throughout the different subtypes and tumor development.

2. Materials and Methods

2.1. Patients' Population. 54 melanoma samples from 33 patients were analysed. From these, 33 out of 54 were primary melanomas of which 12 derived from a gynaecological localization (vulva and vagina), 1 from the anus, 3 from the conjunctiva, and 17 from the sinonasal region (of these, 3 cases were melanoma in situ). From 21/33 patients matching samples were available as follows: primary and metachronous recurrence in 14 cases (8 local and 6 distant) and 7 cases with primary and synchronous lymphonodal metastases. Description of samples and matching recurrence or metastases is reported in Table S1 (see Table S1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2015/432479). Tumor specimens were retrieved from the archives of the Institute for Surgical Pathology Zurich (University Hospital Zurich) and the Department of Dermatology of the University Umberto I, Rome, Italy, between 1996 and 2012. All cases were reviewed from an experienced pathologist (DM). Approval for the use of melanoma tissue was obtained from the official ethical authorities of the Canton Zurich (StV 16-2007, Amendment, 2014). All patients provided a written informed consent in accordance with the Declaration of Helsinki.

2.2. Immunohistochemistry. Tissue sections of 2.0 μm were cut, mounted on glass slides, deparaffinised, rehydrated, and stained with hematoxylin-eosin using standard histological techniques. Heavily pigmented melanomas are difficult for immunohistochemical interpretation; therefore, in order to avoid false positive results the slides were bleached before using immunohistochemistry as previously described [11].

For immunohistochemical staining, the Ventana Benchmark automated staining system and Ventana reagents were used (Ventana Medical Systems, Tucson, AZ). Immunohistochemistry was performed as recently described [12, 13]. Primary antibodies against CT7 (clone CT7-33, Dako Cytomation, Dilution 1 : 80, Glostrup, Denmark) and CT10 clone LX-CT10.5, Dilution 1 : 100 [14] were used. Immunohistochemical analysis for CT7 and CT10 was evaluated based on the percentage of positive cells and defined as positive if at least 5% of tumor cells were positive for each of the two antigen [15, 16].

2.3. c-Kit Mutation Analysis. From 28 melanomas deriving from 19 patients *c-KIT* mutational analysis was known. These data are available from our previous study [10].

2.4. Statistical Analysis. Correlations between primary melanomas and their metastases for CT10 or CT7 expression were analyzed using Spearman's rank correlation. CT7 and CT10 expression were compared between different localisation groups using the Mann-Whitney U test. P values below 0.05 were considered as significant. IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL) was used for statistical analyses. GraphPad Prism 5 was used for Boxplots and Graphs.

3. Results

3.1. MAGE-C1/CT7 and MAGE-C2/CT10 Expression in Mucosal Melanoma. As previously described [5], we found CT7 expression in the nucleus, cytoplasm, or both compartments in both primary melanoma and metastases in 30 out of 54 (55%) melanoma lesions (Figure 1(a)). There was a significant difference of CT7 expression between sinonasal and gynecological melanoma ($P = 0.002$). Twenty-five out of 32 (78%) sinonasal melanomas stained positive for CT7 in contrast to only 3 out of 17 (18%) of gynecological melanoma samples (Figure 1(b)). CT7 expression was also detected in 1 out of 2 anal melanoma lesions and 1 out of 3 melanomas of the conjunctiva (Table S1(a)).

CT10 expression was detected in 16 of 54 melanomas (30%); of these 13 showed a nuclear staining and 3 a combined nuclear and cytoplasmic expression. In contrast to CT7, we found no significant difference in CT10 expression between sinonasal (30%) and gynecological (26%) melanoma (for details, see Table S1(b)). CT10 expression was also detected in 2 out of 2 anal melanoma lesions and none from the conjunctiva (Table S1(b)).

Coexpression of CT7 and CT10 was detected in 6 out of 33 (18%) primaries and 5 out of 21 (24%) metastases (Figures 1(d) and 1(f)).

3.2. Correlation of MAGE-C1/CT7 and MAGE-C2/CT10 Expression in Primary Melanoma and Its Recurrence. Analysis of primary tumors and corresponding recurrence (21 cases) showed a significant correlation of CT7 expression in primary MM and the recurrence ($P = 0.001$; Spearman's correlation coefficient 0,7) as well as of CT10 ($P = 0.01$; Spearman's correlation coefficient 0,6).

Analysing the matched samples of sinonasal melanomas in detail, only one patient showed CT7 expression in the primary tumor and negative recurrence. In all other patients, if CT7 expression was present in the primary tumor, this was also detected in the recurrence; moreover in five cases an increase of CT7 expression was detected in the recurrence (Figures 1(a), 1(c), and 1(e)). For CT10, 2 patients showed an increased expression in the recurrence, 2 showed a stable expression, and 3 cases, positive in the primary lesion, had negative recurrences (Figures 2(a) and 2(b)).

4. Discussion

Mucosal melanoma is a rare disease with aggressive features and, due to occult localization, the diagnosis most frequently occurs in the late stages. This disease differs from the cutaneous counterparts for its biology and genetic features as is the case of BRAF, which is often mutated in cutaneous

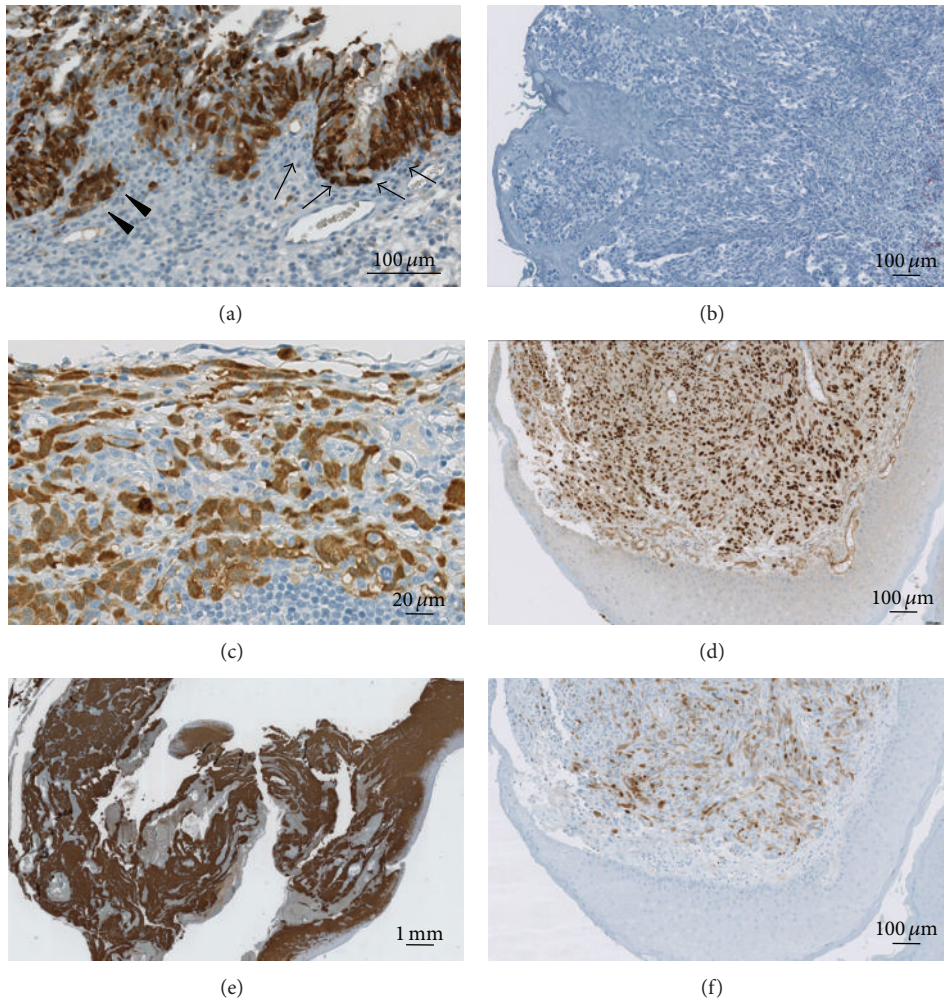

Figure 1: CT7 and CT10 expression in melanoma: primary sinonasal melanoma with positivity for CT7 in the in situ (arrow) and invasive part (arrow head (a)). Maintained CT7 expression in the corresponding metastases (c) and local recurrence (e). Negativity for CT7 in a vaginal melanoma (b). Nuclear positivity for CT10 (d) and cytoplasmic positivity for CT7 (f) on the same anal melanoma.

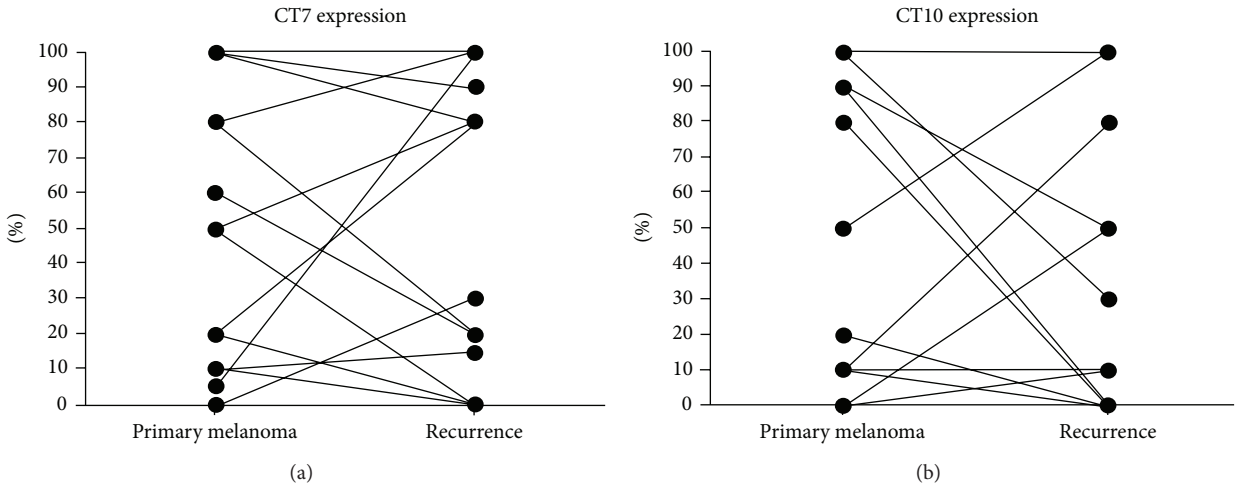

Figure 2: Percentage of CT7 (a) and CT10 (b) melanoma cells in the primary melanoma and corresponding recurrence.

melanoma and rarely in MM [8, 17]. Moreover, MM does not represent a unique tumor entity but differs due to alternative ontogenesis [8, 18] as, for example, mutations of *KIT* are more often detected in gynecological MM rather than sinonasal differing also clinically in distinct forms, a unilocular and multilocular subtype [19]. Moreover, from a clinical perspective, a major event in MM compared to CM is also the more frequent local recurrences due to the anatomical challenges of a radical resection. For these reasons we focused our interest in MM in order to evaluate the immunogenicity of this rare disease. Indeed, immunotherapy plays a major role in melanoma [20] with extraordinary responses in patients treated with immune checkpoint inhibitors and we have previously demonstrated the spontaneous immunogenicity of cutaneous melanoma by the frequent expression on CT7 and CT10 and spontaneous immune responses to CT7 in these patients. In this study, we analysed the expression of CT7 and CT10 in 54 MM samples and detected a frequent expression of CT7 in primaries and corresponding metastases (55%) as well as of CT10 (30%). This expression resulted to be heterogeneous in the same tumor specimen. This result is very intriguing, because although heterogeneity of melanoma is very well known, MAGE antigen are commonly considered to be coexpressed upon activation of a common promoter [21], especially for CT7 and CT10, which are located next to each other on the X chromosome and share more than 50% of sequence-homology.

This result suggests a possible modulation mechanism by which tumors can escape the immune recognition by switch-on and -off of protein that might also be involved in tumorigenesis and formation of metastases [12, 22–25]. Of importance is that CT7 expression, compared to other vaccine-targets for melanoma [26], does not get lost during tumor progression, stressing the potential role of this antigen for therapeutic purposes and monitoring of immune responses at all stages of disease. To this, we have previously demonstrated and characterized a CT7-specific cellular immune response in melanoma patients [7]. One of these patients was affected by sinonasal melanoma and his tumor sample is included in the current cohort of cases, demonstrating the immunogenicity of CT7 also in mucosal melanoma.

Of interest is that CT7 might represent an ideal candidate for vaccination especially in sinonasal melanomas, as its expression, compared to the gynecological ones, occurs in about 78% of samples compared to the gynecological ones, where its expression is detected in 18% of lesions. This is of major interest as our study demonstrates that CT7 expression is influenced by the tissue of origin and might be modulated by events related to ontogenesis.

In order to get new insights on the mechanism of expression of these genes we intended to define the correlation between CT7 and CT10 expression and the presence of activating KIT mutations; this is based on the frequent activation of the *KIT* gene in MM and the finding that an activated *KIT* may allow MAGE gene expression in mast cell leukemia [27]. In contrast to mast cell leukemia we found no clear-cut correlation between *KIT* mutation and MAGE gene expression: mutational analysis for *KIT* was found in 5/28 available samples and CT7 expression was detected in none and CT10 in 1 of 5 (20%) of these cases (primaries and metastasis). However, due to the very limited number of *KIT* activation positive cases, the relation between *KIT* activation and CT7 and CT10 expression, respectively, in mucosal melanoma is not yet clear and has to be analysed in a larger cohort of patients.

Taken together our findings demonstrate CT7 and CT10 as good therapeutic targets for vaccination strategies and monitoring of immune responses for patients with mucosal, especially sinonasal, melanoma. However, as failure to cancer vaccines due to tumor-associated immunosuppression might occur, possible combinatorial treatments with immune-checkpoint inhibitors would be ideal for future clinical studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] M. J. Scanlan, A. J. G. Simpson, and L. J. Old, "The cancer/testis genes: review, standardization, and commentary," *Cancer Immunity*, vol. 4, p. 1, 2004.

[2] A. J. G. Simpson, O. L. Caballero, A. Jungbluth, Y.-T. Chen, and L. J. Old, "Cancer/testis antigens, gametogenesis and cancer," *Nature Reviews Cancer*, vol. 5, no. 8, pp. 615–625, 2005.

[3] L. Sigalotti, E. Fratta, S. Coral et al., "Intratumor heterogeneity of cancer/testis antigens expression in human cutaneous melanoma is methylation-regulated and functionally reverted by 5-Aza-2′-deoxycytidine," *Cancer Research*, vol. 64, no. 24, pp. 9167–9171, 2004.

[4] C. de Smet, C. Lurquin, B. Lethé, V. Martelange, and T. Boon, "DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter," *Molecular and Cellular Biology*, vol. 19, no. 11, pp. 7327–7335, 1999.

[5] A. Curioni-Fontecedro, N. Nuber, D. Mihic-Probst et al., "Expression of MAGE-C1/CT7 and MAGE-C2/CT10 predicts lymph node metastasis in melanoma patients," *PLoS ONE*, vol. 6, no. 6, Article ID e21418, 2011.

[6] D. Godelaine, J. Carrasco, F. Brasseur et al., "A new tumor-specific antigen encoded by *MAGE-C2* and presented to cytolytic T lymphocytes by HLA-B44," *Cancer Immunology, Immunotherapy*, vol. 56, no. 6, pp. 753–759, 2007.

[7] N. Nuber, A. Curioni-Fontecedro, C. Matter et al., "Fine analysis of spontaneous MAGE-C1/CT7-specific immunity in melanoma patients," *Proceedings of the National Academy of Sciences of the United States*, vol. 107, no. 34, pp. 15187–15192, 2010.

[8] A. Zebary, M. Jangard, K. Omholt, B. Ragnarsson-Olding, and J. Hansson, "KIT, NRAS and BRAF mutations in sinonasal mucosal melanoma: a study of 56 cases," *British Journal of Cancer*, vol. 109, no. 3, pp. 559–564, 2013.

[9] T. N. Roth, C. Gengler, G. F. Huber, and D. Holzmann, "Outcome of sinonasal melanoma: clinical experience and review of the literature," *Head & Neck*, vol. 32, no. 10, pp. 1385–1392, 2010.

[10] N. L. Schoenewolf, C. Bull, B. Belloni et al., "Sinonasal, genital and acrolentiginous melanomas show distinct characteristics of

KIT expression and mutations," *European Journal of Cancer*, vol. 48, no. 12, pp. 1842–1852, 2012.

[11] C. J. Kligora, K. P. Fair, M. S. Clem, and J. W. Patterson, "A comparison of melanin bleaching and azure blue counterstaining in the immunohistochemical diagnosis of malignant melanoma," *Modern Pathology*, vol. 12, no. 12, pp. 1143–1147, 1999.

[12] M. Tinguely, B. Jenni, A. Knights et al., "MAGE-C1/CT-7 expression in plasma cell myeloma: sub-cellular localization impacts on clinical outcome," *Cancer Science*, vol. 99, no. 4, pp. 720–725, 2008.

[13] M.-O. Riener, P. J. Wild, C. Soll et al., "Frequent expression of the novel cancer testis antigen MAGE-C2/CT-10 in hepatocellular carcinoma," *International Journal of Cancer*, vol. 124, no. 2, pp. 352–357, 2009.

[14] R. Zhuang, Y. Zhu, L. Fang et al., "Generation of monoclonal antibodies to cancer/testis (CT) antigen CT10/MAGE-C2," *Cancer Immunity*, vol. 6, p. 7, 2006.

[15] A. A. Jungbluth, Y. T. Chen, K. J. Busam et al., "CT7 (MAGE-C1) antigen expression in normal and neoplastic tissues," *International Journal of Cancer*, vol. 99, no. 6, pp. 839–845, 2002.

[16] M. Luftl, G. Schuler, and A. A. Jungbluth, "Melanoma or not? Cancer testis antigens may help," *British Journal of Dermatology*, vol. 151, pp. 1213–1218, 2004.

[17] M. A. Postow, O. Hamid, and R. D. Carvajal, "Mucosal melanoma: pathogenesis, clinical behavior, and management," *Current Oncology Reports*, vol. 14, no. 5, pp. 441–448, 2012.

[18] N. L. Schoenewolf, R. Dummer, D. Mihic-Probst et al., "Detecting BRAF mutations in formalin-fixed melanoma: experiences with twostate-of-the-art techniques," *Case Reports in Oncology*, vol. 5, no. 2, pp. 280–289, 2012.

[19] O. Stanimirov Rossi, D. Vital, M. B. Soyka, T. N. Roth, G. F. Huber, and D. Holzmann, "Multilocular sinonasal malignant melanoma: a poor prognostic subgroup?" *European Archives of Oto-Rhino-Laryngology*, 2014.

[20] O. Hamid, C. Robert, A. Daud et al., "Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma," *The New England Journal of Medicine*, vol. 369, pp. 134–144, 2013.

[21] S. Bhan, S. S. Negi, C. Shao et al., "BORIS binding to the promoters of cancer testis antigens, *MAGEA2*, *MAGEA3*, and *MAGEA4*, is associated with their transcriptional activation in lung cancer," *Clinical Cancer Research*, vol. 17, no. 13, pp. 4267–4276, 2011.

[22] M. V. Dhodapkar, K. Osman, J. Teruya-Feldstein et al., "Expression of cancer/testis (CT) antigens MAGE-A1, MAGE-A3, MAGE-A4, CT-7, and NY-ESO-1 in malignant gammopathies is heterogeneous and correlates with site, stage and risk status of disease," *Cancer Immunology*, vol. 3, p. 9, 2003.

[23] C. Barrow, J. Browning, D. MacGregor et al., "Tumor antigen expression in melanoma varies according to antigen and stage," *Clinical Cancer Research*, vol. 12, no. 3 I, pp. 764–771, 2006.

[24] A. A. Jungbluth, S. Ely, M. DiLiberto et al., "The cancer-testis antigens CT7 (MAGE-C1) and MAGE-A3/6 are commonly expressed in multiple myeloma and correlate with plasma-cell proliferation," *Blood*, vol. 106, no. 1, pp. 167–174, 2005.

[25] J. R. Peng, H. S. Chen, D. C. Mou et al., "Expression of cancer/testis (CT) antigens in Chinese hepatocellular carcinoma and its correlation with clinical parameters," *Cancer Letters*, vol. 219, no. 2, pp. 223–232, 2005.

[26] D. Lenggenhager, A. Curioni-Fontecedro, M. Storz et al., "An aggressive hypoxia related subpopulation of melanoma cells is TRP-2 negative," *Translational Oncology*, vol. 7, no. 2, pp. 206–212, 2014.

[27] B. Yang, J. Wu, N. Maddodi, Y. Ma, V. Setaluri, and B. J. Longley, "Epigenetic control of *MAGE* gene expression by the KIT tyrosine kinase," *Journal of Investigative Dermatology*, vol. 127, no. 9, pp. 2123–2128, 2007.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 367354, 8 pages
http://dx.doi.org/10.1155/2015/367354

Review Article

Immunotherapy in Metastatic Renal Cell Carcinoma: A Comprehensive Review

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Received 27 November 2014; Accepted 4 March 2015

Academic Editor: Aziz A. Chentoufi

Localized renal cell carcinoma (RCC) is often curable by surgery alone. However, metastatic RCC is generally incurable. In the 1990s, immunotherapy in the form of cytokines was the mainstay of treatment for metastatic RCC. However, responses were seen in only a minority of highly selected patients with substantial treatment-related toxicities. The advent of targeted agents such as vascular endothelial growth factor tyrosine kinase inhibitors VEGF-TKIs and mammalian target of rapamycin (mTOR) inhibitors led to a change in this paradigm due to improved response rates and progression-free survival, a better safety profile, and the convenience of oral administration. However, most patients ultimately progress with about 12% being alive at 5 years. In contrast, durable responses lasting 10 years or more are noted in a minority of those treated with cytokines. More recently, an improved overall survival with newer forms of immunotherapy in other malignancies (such as melanoma and prostate cancer) has led to a resurgence of interest in immune therapies in metastatic RCC. In this review we discuss the rationale for immunotherapy and recent developments in immunotherapeutic strategies for treating metastatic RCC.

1. Introduction

Renal cell cancer (RCC) is the sixth most common malignancy in men and the eighth most common malignancy in women in the United States. The incidence of RCC rose by 1.6% per year between 2002 and 2011 with 63,920 new cases and 13,860 deaths anticipated in 2014 [1]. More than a decade ago, immunotherapy with cytokines was the standard treatment for metastatic RCC (mRCC). Subsequently, targeted agents such as vascular endothelial growth factor tyrosine kinase inhibitors (VEGF-TKIs) and inhibitors of mammalian target of rapamycin (mTOR) showed significantly improved responses and progression-free survival (PFS). These agents were also relatively well tolerated, thereby changing the treatment paradigm for metastatic RCC. Comparison of disease specific survival for de novo metastatic RCC between 1992–2004 (pretargeted therapy) and 2005–2009 (era of targeted therapies) showed an improvement from 13 months to 16 months ($P < 0.0001$) [2]. Upon further risk stratification, sequential use of VEGF-TKIs may yield median survivals of 43, 22, and 7.3 months in favorable-, intermediate-, and poor-risk groups, respectively [3]. Yet only 12% of patients with metastatic RCC are alive at five years, with the majority eventually developing treatment resistance and disease progression. In contrast, cytokine therapy with high-dose interleukin 2 may achieve a complete response in 7–10% of cases with some persisting beyond 10 years [4], thereby "curing" a subset of patients of their disease. However, no significant improvement in overall survival occurs and severe toxicities limit their clinical utility. In the last few years, new immunotherapeutic targets have been identified with reports of durable responses, improved overall survival, and better tolerability. In this review we discuss the rationale for immunotherapy, current status of cytokine therapy, status of biomarkers to improve patient selection, and recent advances in immunotherapy for metastatic RCC. For the purpose of this review, mRCC refers to clear cell histology only.

Table 1: Proposed mechanisms of tumor mediated immune evasion.

Effects of tumor mediated immune evasion on T cells	Molecular mechanisms underlying tumor effects on T cells
Direct deletion of immune effector cells	Expression of death inducing ligand (Fas)
	Secretion of immunosuppressive cytokines: IL-10, TGFβ
Direct tolerization of tumor reactive T cells	Cross presentation of tumor antigens by bone marrow APCs
	B7-H1 expression by tumor and induction of T cell apoptosis
Inhibition of T cell activation or induction of anergy	Lack of expression of costimulatory molecules (CD 28 on T cells, B7 ligands on APCs)
	Overexpression of inhibitory costimulatory molecules CTLA-4, PD-1, and PD-1 ligands

IL: interleukin, TGF: tissue growth factor, APCs: antigen-presenting cells, B7-H1: B7-homolog 1, CTLA-4: cytotoxic T lymphocyte antigen, PD1: programmed death-1, PD-L1: programmed death-ligand 1, and VEGF: vascular endothelial growth factor.

2. Rationale for Immunotherapy in Renal Cell Cancer

Reports of spontaneous regressions, prolonged disease stability, and late relapses after nephrectomy suggest an inherent role of immune mechanisms in the natural history of RCC [5–8]. In keeping with these anecdotal reports, diffuse tumor infiltration with T cells, natural killer (NK) cells, dendritic cells (DCs), and macrophages have been described in RCC [9–11], but the precise role of each cell type is not well understood. Yet, most tumors are not eliminated by immune effector cells, possibly because of the incompletely understood mechanisms of immune tolerance. Most antigens expressed by tumor cells are merely overexpressed normal self-antigens. Moreover, tumor cells act as poor antigen-presenting cells. Thus, the repertoire of cytotoxic T cells (CTLs) in the host that recognize the tumor antigens as foreign is probably small. Mapara and Sykes [12] comprehensively reviewed the basic principles of immune tolerance to tumors as summarized in the context of RCC in Table 1.

The ensuing sections discuss the past and current developments to overcome tumor mediated immune evasion in metastatic RCC. Broadly, these include (1) T cell modulation, for example, with cytokines and immune checkpoint inhibitors, (2) adoptive cellular immunotherapy, and (3) vaccination.

3. Immunotherapy for Renal Cell Cancer: Past and Current Developments

3.1. T Cell Modulation

3.1.1. The Current Status of Cytokine Therapy in Metastatic RCC. The two principal cytokines with proven efficacy in metastatic renal cancer are interferon-alpha (INF-α) and high-dose interleukin 2 (IL2). IL2 is a potent stimulator of T cell proliferation and differentiation, while INF-α has antiangiogenic effects, promoting antigen presentation and dendritic cell maturation [13]. However, their exact mechanism of action is unknown.

High-dose IL2 was approved for mRCC in 1992. Long-term follow-up of 255 patients with mRCC enrolled in seven phase II clinical trials of high-dose IL2 reported objective responses in 15% including complete responses (CR) in 7% of patients. IL2 was administered at 600,000 IU/kg for 14 doses or at 720,000 IU/kg for 12 doses every 8 hours per treatment week. For the complete responders the median duration of response was at least 80 months (range 7–>131 months). Median survival time for all 255 patients remained 16.3 months as of 2000 [4]. In addition to reversible toxicities, a 3-4% treatment related mortality was a deterrent to widespread use. Efforts to minimize toxicity while improving response rates with IL2 have included dose reductions, schedule changes, combination of interferon and sorafenib, and chemotherapy which either did not improve response rates significantly or improved responses at the cost of increased toxicity [14–18]. The combination of sorafenib and bevacizumab with cytokines has been used with some success in renal cell cancer. However, these combinations do not appear to produce more durable responses than cytokines alone [19–24]. Clinical benefit and durable CRs following high-dose IL2 administration were also recently reported after prior use of TKIs [25].

Several retrospective studies have evaluated predictors of efficacy or resistance to cytokines and proposed various clinical, serological, and histologic biomarkers. Risk models developed in accordance with these biomarkers are listed in Table 2. In addition to these models, a correlation between response to cytokines and serum levels of VEGF and fibronectin has also been suggested [26].

The cytokine working group undertook the "SELECT" trial [30, 31] to prospectively evaluate whether the available risk stratification tools and biomarkers were predictive of response to high-dose IL2. Of the models shown in Table 2, ISM or MSKCC scores were unable to improve selection criteria. No responses were seen in the high UCLA SANI risk group and non-clear-cell RCC. Interestingly, response (including durable response lasting more than 3 years) was positively associated with tumor expression of PD-L1 or B7-H1 (programmed death ligand) by IHC staining [32].

Despite clinical benefit in a minority of patients the durability of responses seen with high-dose IL2 is yet to be surpassed by currently available VEGF-TKIs in mRCC [33]. However toxicity remains a concern and there is a lack of robust tools to predict benefit in an individual patient. Efforts to maximize clinical benefit with better tolerated therapy have led to renewed interest in developing "targeted immunotherapy."

Table 2: Risk stratification models in cytokine treated metastatic RCC.

Risk models	Model factors	Outcomes
MSKCC [27]	KPS <80% LDH 1.5x ULN Hemoglobin < LLN Corrected calcium > ULN Interval from diagnosis to treatment of <1 year	**Median OS (months)** Favorable: 30 Intermediate: 14 Poor: 5
UCLA SANI [28]	Lymph Node status Constitutional symptoms Location of metastasis Sarcomatoid histology TSH	**5-year OS (%) and ORR (%)** Low risk: 41 and 43 Intermediate: 19 and 27 High: 0 and 15
ISM [29]	Histology: clear cell with alveolar features absence of papillary or granular features CA-9 expression by IHC	Good risk accounted for 96% of responding patients and 56% of nonresponding patients

KPS: Karnofsky performance status, ULN: upper limit of normal, LLL: lower limit of normal, ISM: integrated selection model, ORR: overall response rate, CA-9: carbonic anhydrase-9, and IHC: immunohistochemistry.

3.1.2. Immune Checkpoint Inhibitors. Checkpoint receptors (CPRs) on cytotoxic T lymphocytes (CTLs) block costimulatory signals at various stages of immune activation after ligand binding. This results in T cell anergy and immunosuppression. Blocking these CPRs appears to improve the ability of CTLs to mount and sustain an effective T cell response. Cytotoxic T lymphocyte antigen (CTLA-4) is a CPR on T cells that ligates to B7 molecules (CD80 and CD86) on antigen-presenting cells (APCs) and inhibits T cell proliferation as well as function. Programmed death-1 (PD-1) is another T cell receptor which is expressed on activated, antigen-exhausted T cells and binds to its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), thereby inducing anergy. While PD-1 is expressed primarily on mononuclear cell infiltrates, PD-L (PD-L1 being the predominant ligand) is expressed by tumor cells. Among the solid tumors, PD-L1 expression has largely been demonstrated in melanoma, non-small-cell lung cancer, and RCC cells and correlates with poor outcomes when treated with existing systemic therapies [34, 35]. Blocking the PD-1 pathway enhances immune responses by stimulating effector T cells in the tumor and its microenvironment. Alternatively, it may also decrease the number or suppressive activity of regulatory T cells [36] (Figure 1).

(A) Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) Antibody. Ipilimumab, a monoclonal antibody directed against CTLA-4, was the first drug that was shown to produce a survival benefit in advanced melanoma [38]. In a single institution phase II study of ipilimumab in metastatic RCC, 5 of 40 responses were noted in the higher dose group (3 mg/kg every 3 weeks) compared to 1 of 21 responses in the lower dose group (3 mg/kg followed by 1 mg/kg every 3 weeks). Interestingly a significant association was observed between autoimmune events and tumor regression (30% with AE versus 0% without AE). Though all responses were partial, patients who had failed IL2 also responded [39]. To our knowledge, there are currently no other studies of single agent ipilimumab in RCC and the ongoing trials are evaluating the efficacy of combining ipilimumab with PD-1 blockade in RCC [40]. A phase III study of ipilimumab and the anti-PD-1 antibody nivolumab versus sunitinib is currently recruiting patients with previously untreated advanced or metastatic RCC (CheckMate 214).

Although a phase I study of another CTLA-4-directed monoclonal antibody, tremelimumab in combination with sunitinib, showed RR of 43% in metastatic RCC the combination was not recommended for further investigation due to rapid onset renal failure noted in a subset of patients [41].

(B) Programmed Death-1 Inhibitors (PD-1). Nivolumab (previously BMS 936558 and MDX-1106) is a fully humanized PD-1 blocking antibody. Promising responses, some durable, have been reported in phase I and II studies in melanoma, non-small-cell lung cancer [42, 43], and, more recently, renal cell cancer.

A phase I study of nivolumab in patients with relapsed or refractory solid tumors demonstrated its safety and clinical efficacy as a single infusion of 0.3, 1, 3, or 10 mg/kg [42]. Subsequently, nivolumab was tested in a larger study with 296 patients that included 34 patients with metastatic renal cell carcinoma (most had received two or more prior regimens). Objective responses occurred in 4 of 17 patients (24%) treated with a dose of 1.0 mg/kg and in 5 of 16 (31%) treated with 10.0 mg per kilogram. Of the responding patients, more than 50% had responses lasting a year or more with one being a complete response (6%). Nine patients (27%) had stable disease beyond 24 weeks. Infusion reactions which were mostly grades I and II were managed by glucocorticoids and antihistamines. Most of the other adverse events which included rash, hypothyroidism, hepatitis, nausea, adrenal insufficiency, diarrhea, and vitiligo were grades I-II. Hypopituitarism was observed in less than 1%. Grades III-IV immune mediated toxicities specifically pneumonitis were observed in 14/296 patients [44]. Results of the phase II study assessing the efficacy of nivolumab at three dose levels (0.3, 2, or 10 mg/kg IV every 3 weeks) in 168 patients with previously treated mRCC were recently presented at ASCO 2014 [45]. No dose-response relationship for PFS was observed and a

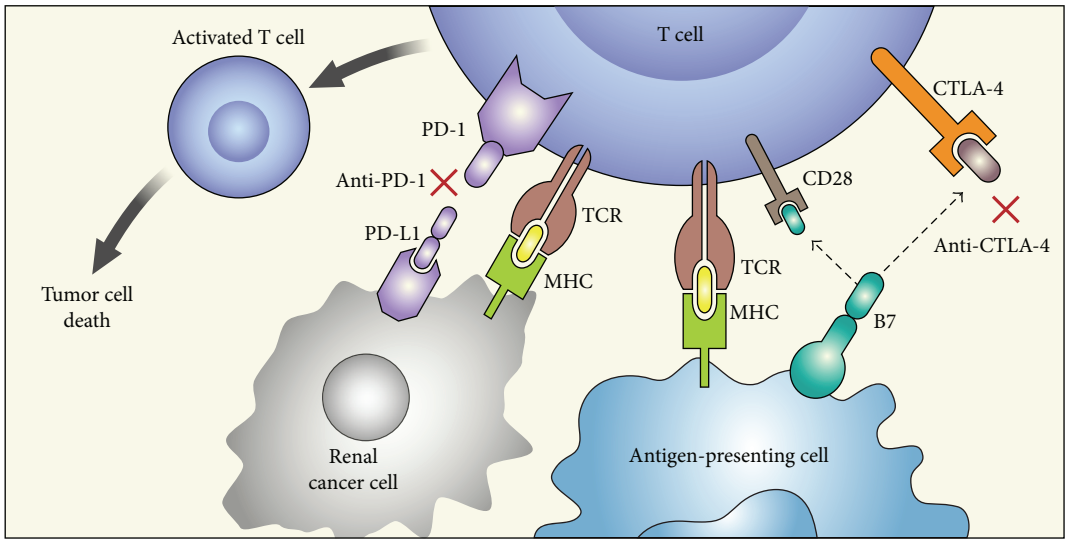

Figure 1: Mechanism of action of immune checkpoint inhibitors. PD-1 is expressed on activated T cells and when it binds to its ligand PD-L1 on tumor cells leads to T cell exhaustion. CTLA-4 competes with CD28 (costimulatory T cell molecule) for B7 ligands (CD80 and CD86 that are not shown in the figure) and upon activation decreases T cell proliferation as well as activity. Blockade of CTLA-4 (by anti-CTLA-4) and PD-1 (anti-PD-1) or PD-L1 stimulates effector T cells to produce antitumor responses. Adapted by permission from Macmillan Publishers Ltd. [37], copyright (Jan 2014). PD-1: programmed death-1, PD-L1: programmed death-ligand 1, MHC: major histocompatibility complex, TCR: T cell receptor, and CTLA-4: cytotoxic T lymphocyte antigen.

response rate of at least 20% was observed at all doses. Despite an unimpressive PFS, responses were durable and persisted for about 2 years. Improved OS was noted with doses of 2 and 10 mg/kg (25.5 and 24.7 months, resp., versus 18.2 months for 0.3 mg/kg).

With the demonstration of safety and antitumor activity of PD-1 blockade, ongoing trials are evaluating combinations of agents with activity in RCC. VEGF-TKIs may augment the antitumor efficacy of PD-1 blockade by reducing the percentage of tumor infiltrating regulatory T cells and enhancing the activity of CTLs [46–48]. Combinations of nivolumab with sunitinib or pazopanib [49], bevacizumab (NCT02210117), and the anti-CTLA-4 antibody ipilimumab [40] are currently undergoing clinical testing. Results of the phase I study evaluating the combination of nivolumab with sunitinib or pazopanib in previously treated mRCC were presented at ASCO 2014 [49]. Overall response rate was 52% with sunitinib and 45% with pazopanib. Dose limiting liver toxicity was noted in the pazopanib arm leading to its closure. PFS at 24 weeks was 78%, which however is comparable to sunitinib alone in the first-line treatment of metastatic RCC [50]. In another study, the combination of nivolumab and ipilimumab showed a response rate of 45% [40] with an acceptable safety profile. Durability of responses with these combinations should be assessed in phase III studies.

Other PD-1 and PD-L1 inhibitors such as pembrolizumab (MK-3475) and pidilizumab (CT-011) are also under evaluation (Table 3).

As results of efficacy, tolerability, and durability of responses with immune checkpoint inhibitors (specifically with PD-1) emerge, efforts to guide patient selection are also underway. In this context, PD-L1 expression has been proposed as a potential biomarker of response. It was recently shown that responses across multiple cancer types (including RCC) were observed in tumors expressing high levels of PD-L1, especially when PD-L1 was expressed by tumor-infiltrating immune cells [51]. In the phase II study of single agent nivolumab for previously treated RCC, 31% responses were seen in PD-L1 positive tumors compared to 18% in PD-L1 negative RCC [45]. However, in the combination studies of nivolumab with ipilimumab, sunitinib, or pazopanib, a significant proportion of patients with PD-L1 negative tumors also responded to the treatments. Thus, the precise role of PD-1/PD-L1 expression as a biomarker is yet to be defined.

3.2. Adoptive Cellular Immunotherapy. Adoptive cellular immunotherapy (ACI) entails in vitro expansion of immune effectors (autologous or allogeneic lymphocytes) with antitumor activity and reinfusing them into the tumor bearing host. First described in RCC in 1992, ACI has thereafter been evaluated in several clinical studies with or without cytokines [52–55]. Conflicting data on efficacy, significant cost, and a labor intensive process of preparation has limited the pace of development of ACI in RCC.

3.3. Vaccine Therapy. Vaccines carry tumor antigens on a vehicle that may be a cell, peptide, or a vector. They are designed to enhance innate or adaptive immunity depending on the antigen and vehicle. Examples include autologous tumor cell vaccines, dendritic cell (DC) based vaccines, and peptide based vaccines. Results from ongoing trials of DC vaccines in RCC are the most promising and are discussed here.

Table 3: Programmed death (PD-1 and PD-L1) inhibitors in various phases of development.

Agent	Description	Target	Phase of development	Being tested in RCC	Trial identifier
BMS 936558/MDX-1106/nivolumab	Human IgG monoclonal Ab	PD-1	I, II, and III	Yes	NCT01472081 NCT01354431 NCT01668784 NCT02210117 NCT02231749
MK-3475/pembrolizumab	Human IgG4 monoclonal Ab	PD-1	I and II	Yes	NCT01704287 NCT02318771 NCT02212730 NCT02133742 NCT01295827 NCT02089685 NCT02014636
CT-011*/pidilizumab	Human IgG1 monoclonal Ab	PD-1	II	Yes	NCT01441765
MPDL3280A	Monoclonal Ab	PD-L1	I and II	Yes	NCT01375842** NCT01633970
BMS-936559/MDX1105-01	Human IgG4 monoclonal Ab	PD-L1	I	Yes	NCT00729664
AMP-224	B7-DC/IgG1 fusion protein	PD-1	I	Yes	NCT01352884

Ab: antibody, DC: dendritic cell, PD: programmed death, and RCC: renal cell cancer. * PD-1 blockade alone or in combination with the dendritic cell (DC)/renal cell carcinoma (RCC) fusion cell vaccination. ** Phase II comparing MPDL3280A monotherapy or in combination with bevacizumab versus sunitinib in patients with previously untreated locally advanced or metastatic RCC.

The efficacy and success of sipuleucel T in metastatic prostate cancer [56] prompted the evaluation of dendritic cell (DC) vaccines in metastatic renal cell cancer. DCs play a critical role in producing antitumor immunity. Although mature DCs are potent stimulators of CTLs and natural killer cells (NKCs), immature DCs may tolerize the T cells and decrease their antitumor responses [12]. In vivo, DCs are often inefficient APCs; hence peptide vaccines that rely on DCs may not induce a strong enough antitumor immune response. To constitute these vaccines DCs are allowed to undergo maturation ex vivo in the presence of tumor antigens and then infuse into the tumor bearing host. Phase I studies of vaccines containing DCs transfected with tumor RNA or pulsed with tumor lysate found them to be safe and effective in RCC either alone [57–59] or in combination with cytokines [60, 61].

The most compelling evidence for the efficacy of dendritic cell vaccines came from the phase II study of AGS-003 with sunitinib in de novo metastatic RCC. Updated results were presented in the 2014 Annual Meeting of American Society of Clinical Oncology [50, 62]. The production of AGS-003 is a multistep process and starts with leukapheresis to collect DCs from the tumor bearing host. AGS-003 is manufactured by transfecting the autologous DCs with patient-specific RCC tissue amplified RNA and synthetic-truncated human CD40 ligand RNA, which has the potential to stimulate the immune system. The vaccine is then reintroduced into the patient intradermally, eliciting a highly specific CTL response through the initiation of a signaling cascade that causes the secretion of the cytokine IL-12. In this study, 21 patients with newly diagnosed unfavorable-risk (time from diagnosis to treatment of less than 1 year) mRCC received sunitinib plus AGS-003. The median PFS was 11.2 months and the median OS was 30.2 months. 52% patients survived beyond 30 months, 23% of them still alive after 5 years. When responses were analyzed by baseline Heng risk status [3], patients in the intermediate-risk group ($n = 11$) had an OS of 57 months and poor-risk patients ($n = 10$) had OS of 9.1 months (ranged up to 56.3 months). The absolute change in CD8+CD28+ memory T cells directly and significantly correlated with prolonged OS and PFS. This was a marked improvement from a median OS of 22.5 months for intermediate-risk patients and 7.8 months for poor-risk patients for patients treated with VEGF-TKIs [63]. No additive toxicity other than grades I and II infusion site reactions was noted.

The rationale for combining vaccine therapy with sunitinib comes from the observed favorable effects of VEGF-TKIs on reversing immunosuppression by decreasing Tregs and myeloid derived suppressor cells in the tumor microenvironment [64]. The promising results from the phase II trial have prompted an ongoing phase III study of this combination (NCT01582672/ADAPT). The ADAPT clinical study is a randomized trial, where the experimental arm would receive a combination of AGS-003 and a first-line targeted therapy, starting with sunitinib. The comparator arm would receive standard treatment beginning with sunitinib alone. After 6 weeks of targeted therapy beginning with sunitinib, patients will receive 8 doses of AGS-003 during the first year and for those continuing to benefit after the first year of treatment, booster doses of AGS-003 will be given every 3 months thereafter, in combination with standard targeted therapy. The primary end point of the trial is overall survival.

Despite the constraints of cost and the multistep constitution process, their relative safety and early results showing unprecedented outcomes in mRCC warrant continued evaluation of therapeutic vaccines in phase III studies.

4. Conclusion

Renewed interest in reprogramming the immune system to improve the outlook for metastatic RCC has led to evaluation of several immune checkpoint inhibitors and vaccination strategies in multiple ongoing trials. The OS reported with the PD-1 inhibitor nivolumab in previously treated mRCC has already exceeded the median OS reported with IL2 in the first-line treatment of mRCC. Manageable toxicities and wider applicability add to its appeal. Clinical benefit over and above that with TKIs is yet to be proven as is the durability of responses comparable to IL2. Combinations of PD 1 inhibition with VEGF-TKIs and CTLA-4 inhibitors have shown significantly higher response rates, though the safety of these combinations is in question. Meaningful clinical benefit has been observed with these checkpoint inhibitors in heavily pretreated patients with mRCC which has significant implications. The 5-year follow-up results from the phase II study of AGS-003 in combination with sunitinib have shown an unprecedented survival in mRCC regardless of the risk category. Results of the phase III study are eagerly awaited. In our opinion, addition of these newer modulators of immunity to the available treatments for management of mRCC (VEGF-TKIs, mTOR inhibitors, and surgical cytoreduction) may significantly alter the long-term outcomes in mRCC. It would be of significant clinical interest to simultaneously evaluate appropriate treatment sequencing and tools to improve on patient selection. Until long-term data on the durability of treatment responses are available, IL2 may still be considered in a small group of otherwise healthy patients with mRCC who have a low disease burden. However, when appropriate, participation in clinical trials evaluating immune modulation must be encouraged.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] R. Siegel, J. Ma, Z. Zou, and A. Jemal, "Cancer statistics, 2014," *CA: A Cancer Journal for Clinicians*, vol. 64, no. 1, pp. 9–29, 2014.

[2] S. K. Pal, R. A. Nelson, and N. Vogelzang, "Disease-specific survival in de novo metastatic renal cell carcinoma in the cytokine and targeted therapy era," *PLoS ONE*, vol. 8, no. 5, Article ID e63341, 2013.

[3] D. Y. Heng, W. Xie, M. M. Regan et al., "External validation and comparison with other models of the international metastatic renal-cell carcinoma database consortium prognostic model: a population-based study," *The Lancet Oncology*, vol. 14, no. 2, pp. 141–148, 2013.

[4] R. I. Fisher, S. A. Rosenberg, and G. Fyfe, "Long-term survival update for high-dose recombinant interleukin-2 in patients with renal cell carcinoma," *Cancer Journal from Scientific American*, vol. 6, supplement 1, pp. S55–S57, 2000.

[5] M. M. Elhilali, M. Gleave, Y. Fradet et al., "Placebo-associated remissions in a multicentre, randomized, double-blind trial of interferon γ-1b for the treatment of metastatic renal cell carcinoma. The Canadian Urologic Oncology Group," *BJU International*, vol. 86, no. 6, pp. 613–618, 2000.

[6] R. T. Oliver, "Surveillance as a possible option for management of metastatic renal cell carcinoma," *Seminars in Urology*, vol. 7, no. 3, pp. 149–152, 1989.

[7] N. J. Vogelzang, E. R. Priest, and L. Borden, "Spontaneous regression of histologically proved pulmonary metastases from renal cell carcinoma: a case with 5-year followup," *Journal of Urology*, vol. 148, no. 4, pp. 1247–1248, 1992.

[8] R. J. Papac, "Spontaneous regression of cancer," *Cancer Treatment Reviews*, vol. 22, no. 6, pp. 395–423, 1996.

[9] O. Nakano, M. Sato, Y. Naito et al., "Proliferative activity of intratumoral $CD8^+$ T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity," *Cancer Research*, vol. 61, no. 13, pp. 5132–5136, 2001.

[10] J. P. Alexander, S. Kudoh, K. A. Melsop et al., "T-cells infiltrating renal cell carcinoma display a poor proliferative response even though they can produce interleukin 2 and express interleukin 2 receptors," *Cancer Research*, vol. 53, no. 6, pp. 1380–1387, 1993.

[11] Y. Komohara, H. Hasita, K. Ohnishi et al., "Macrophage infiltration and its prognostic relevance in clear cell renal cell carcinoma," *Cancer Science*, vol. 102, no. 7, pp. 1424–1431, 2011.

[12] M. Y. Mapara and M. Sykes, "Tolerance and cancer: mechanisms of tumor evasion and strategies for breaking tolerance," *Journal of Clinical Oncology*, vol. 22, no. 6, pp. 1136–1151, 2004.

[13] M. Fishman and J. Seigne, "Immunotherapy of metastatic renal cell cancer," *Cancer Control*, vol. 9, no. 4, pp. 293–304, 2002.

[14] J. C. Yang, R. M. Sherry, S. M. Steinberg et al., "Randomized study of high-dose and low-dose interleukin-2 in patients with metastatic renal cancer," *Journal of Clinical Oncology*, vol. 21, no. 16, pp. 3127–3132, 2003.

[15] J. A. Klapper, S. G. Downey, F. O. Smith et al., "High-dose interleukin-2 for the treatment of metastatic renal cell carcinoma: a retrospective analysis of response and survival in patients treated in the Surgery Branch at the National Cancer Institute between 1986 and 2006," *Cancer*, vol. 113, no. 2, pp. 293–301, 2008.

[16] D. F. McDermott, "Randomized phase III trial of high-dose interleukin-2 versus subcutaneous interleukin-2 and interferon in patients with metastatic renal cell carcinoma," *Journal of Clinical Oncology*, vol. 23, no. 1, pp. 133–141, 2005.

[17] G. Procopio, E. Verzoni, S. Bracarda et al., "Overall survival for sorafenib plus interleukin-2 compared with sorafenib alone in metastatic renal cell carcinoma (mRCC): final results of the ROSORC trial," *Annals of Oncology*, vol. 24, no. 12, pp. 2967–2971, 2013.

[18] A. Lindemann, K. Hoeffken, R. E. Schmidt et al., "A multicenter trial of interleukin-2 and low-dose cyclophosphamide in highly chemotherapy-resistant malignancies," *Cancer Treatment Reviews*, vol. 16, pp. 53–57, 1989.

[19] B. I. Rini, S. Halabi, J. E. Rosenberg et al., "Bevacizumab plus interferon alfa compared with interferon alfa monotherapy in patients with metastatic renal cell carcinoma: CALGB 90206," *Journal of Clinical Oncology*, vol. 26, no. 33, pp. 5422–5428, 2008.

[20] U. B. Dandamudi, M. Ghebremichael, J. A. Sosman et al., "A phase II study of bevacizumab and high-dose interleukin-2 in patients with metastatic renal cell carcinoma: a cytokine Working Group (CWG) study," *Journal of Immunotherapy*, vol. 36, no. 9, pp. 490–495, 2013.

[21] C. W. Ryan, B. H. Goldman, P. N. Lara Jr. et al., "Sorafenib with interferon alfa-2b as first-line treatment of advanced renal carcinoma: a phase II study of the southwest oncology group," *Journal of Clinical Oncology*, vol. 25, no. 22, pp. 3296–3301, 2007.

[22] J. A. Gollob, W. K. Rathmell, T. M. Richmond et al., "Phase II trial of sorafenib plus interferon alfa-2b as first- or second-line therapy in patients with metastatic renal cell cancer," *Journal of Clinical Oncology*, vol. 25, no. 22, pp. 3288–3295, 2007.

[23] B. Escudier, C. Szczylik, T. E. Hutson et al., "Randomized phase II trial of first-line treatment with sorafenib versus interferon Alfa-2a in patients with metastatic renal cell carcinoma," *Journal of Clinical Oncology*, vol. 27, no. 8, pp. 1280–1289, 2009.

[24] E. Jonasch, P. Corn, L. C. Pagliaro et al., "Upfront, randomized, phase 2 trial of sorafenib versus sorafenib and low-dose interferon alfa in patients with advanced renal cell carcinoma: clinical and biomarker analysis," *Cancer*, vol. 116, no. 1, pp. 57–65, 2010.

[25] E. T. Lam, M. K. Wong, N. Agarwal et al., "Retrospective analysis of the safety and efficacy of high-dose interleukin-2 after prior tyrosine kinase inhibitor therapy in patients with advanced renal cell carcinoma," *Journal of Immunotherapy*, vol. 37, no. 7, pp. 360–365, 2014.

[26] M. Sabatino, S. Kim-Schulze, M. C. Panelli et al., "Serum vascular endothelial growth factor and fibronectin predict clinical response to high-dose interleukin-2 therapy," *Journal of Clinical Oncology*, vol. 27, no. 16, pp. 2645–2652, 2009.

[27] R. J. Motzer, J. Bacik, B. A. Murphy, P. Russo, and M. Mazumdar, "Interferon-alfa as a comparative treatment for clinical trials of new therapies against advanced renal cell carcinoma," *Journal of Clinical Oncology*, vol. 20, no. 1, pp. 289–296, 2002.

[28] B. C. Leibovich, K.-R. Han, M. H. T. Bui et al., "Scoring algorithm to predict survival after nephrectomy and immunotherapy in patients with metastatic renal cell carcinoma: a stratification tool for prospective clinical trials," *Cancer*, vol. 98, no. 12, pp. 2566–2575, 2003.

[29] M. Atkins, M. Regan, D. McDermott et al., "Carbonic anhydrase IX expression predicts outcome of interleukin 2 therapy for renal cancer," *Clinical Cancer Research*, vol. 11, no. 10, pp. 3714–3721, 2005.

[30] J. M. Clement and D. F. McDermott, "The high-dose aldesleukin (IL-2) 'select' trial: a trial designed to prospectively validate predictive models of response to high-dose IL-2 treatment in patients with metastatic renal cell carcinoma," *Clinical Genitourinary Cancer*, vol. 7, no. 2, pp. E7–E9, 2009.

[31] D. F. McDermott, M. S. Ghebremichael, S. Signoretti et al., "The high-dose aldesleukin (HD IL-2) 'SELECT' trial in patients with metastatic renal cell carcinoma (mRCC)," *Journal of Clinical Oncology*, vol. 28, no. 15, 2010.

[32] J. M. Clement and D. F. McDermott, "The high-dose aldesleukin (IL-2) 'select' trial: a trial designed to prospectively validate predictive models of response to high-dose IL-2 treatment in patients with metastatic renal cell carcinoma," *Clinical Cancer Research*, vol. 7, no. 2, pp. E7–E9, 2009.

[33] L. Albiges, S. Oudard, S. Negrier et al., "Complete remission with tyrosine kinase inhibitors in renal cell carcinoma," *Journal of Clinical Oncology*, vol. 30, no. 5, pp. 482–487, 2012.

[34] R. H. Thompson, S. M. Kuntz, B. C. Leibovich et al., "Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up," *Cancer Research*, vol. 66, no. 7, pp. 3381–3385, 2006.

[35] J. M. Taube, A. Klein, J. R. Brahmer et al., "Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy," *Clinical Cancer Research*, vol. 20, no. 19, pp. 5064–5074, 2014.

[36] D. M. Pardoll, "The blockade of immune checkpoints in cancer immunotherapy," *Nature Reviews Cancer*, vol. 12, no. 4, pp. 252–264, 2012.

[37] C. G. Drake, E. J. Lipson, and J. R. Brahmer, "Breathing new life into immunotherapy: review of melanoma, lung and kidney cancer," *Nature Reviews Clinical Oncology*, vol. 11, no. 1, pp. 24–37, 2014.

[38] F. S. Hodi, S. J. O'Day, D. F. McDermott et al., "Improved survival with ipilimumab in patients with metastatic melanoma," *The New England Journal of Medicine*, vol. 363, no. 8, pp. 711–723, 2010.

[39] J. C. Yang, M. Hughes, U. Kammula et al., "Ipilimumab (anti-CTLA4 antibody) causes regression of metastatic renal cell cancer associated with enteritis and hypophysitis," *Journal of Immunotherapy*, vol. 30, no. 8, pp. 825–830, 2007.

[40] H. J. Hammers, "Phase I study of nivolumab in combination with ipilimumab in metastatic renal cell carcinoma (mRCC)," *Journal of Clinical Oncology*, vol. 32, supplement, article 5S, 2014, ASCO Annual Meeting Abstract No. 4504.

[41] B. I. Rini, M. Stein, P. Shannon et al., "Phase 1 dose-escalation trial of tremelimumab plus sunitinib in patients with metastatic renal cell carcinoma," *Cancer*, vol. 117, no. 4, pp. 758–767, 2011.

[42] J. R. Brahmer, C. G. Drake, I. Wollner et al., "Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates," *Journal of Clinical Oncology*, vol. 28, no. 19, pp. 3167–3175, 2010.

[43] S. L. Topalian, M. Sznol, D. F. McDermott et al., "Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab.," *Journal of Clinical Oncology*, vol. 32, no. 10, pp. 1020–1030, 2014.

[44] S. L. Topalian, F. S. Hodi, J. R. Brahmer et al., "Safety, activity, and immune correlates of anti-PD-1 antibody in cancer," *The New England Journal of Medicine*, vol. 366, no. 26, pp. 2443–2454, 2012.

[45] R. J. Motzer, B. I. Rini, D. F. McDermott et al., "Nivolumab for metastatic renal cell carcinoma: results of a randomized phase II trial," *Journal of Clinical Oncology*, 2014.

[46] O. Adotevi, H. Pere, P. Ravel et al., "A decrease of regulatory T cells correlates with overall survival after sunitinib-based antiangiogenic therapy in metastatic renal cancer patients," *Journal of Immunotherapy*, vol. 33, no. 9, pp. 991–998, 2010.

[47] I. M. E. Desar, J. F. M. Jacobs, C. A. Hulsbergen-VandeKaa et al., "Sorafenib reduces the percentage of tumour infiltrating regulatory T cells in renal cell carcinoma patients," *International Journal of Cancer*, vol. 129, no. 2, pp. 507–512, 2011.

[48] Y. Huang, Y. Wang, Y. Li, K. Guo, and Y. He, "Role of sorafenib and sunitinib in the induction of expressions of NKG2D ligands in nasopharyngeal carcinoma with high expression of ABCG2," *Journal of Cancer Research and Clinical Oncology*, vol. 137, no. 5, pp. 829–837, 2011.

[49] A. Amin, "Nivolumab (anti-PD-1; BMS-936558, ONO-4538) in combination with sunitinib or pazopanib in patients (pts) with

metastatic renal cell carcinoma (mRCC)," *Journal of Clinical Oncology*, vol. 32, supplement, abstract 5010, no. 5s, 2014.

[50] A. Amin, A. Z. Dudek, T. F. Logan et al., "Long term survival in unfavorable-risk mRCC patients treated with a combination of autologous immunotherapy (AGS-003) plus sunitinib," *Journal of Clinical Oncology*, vol. 32, supplement, p. 5s, 2014, (abstract 4524).

[51] R. S. Herbst, J.-C. Soria, M. Kowanetz et al., "Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients," *Nature*, vol. 515, no. 7528, pp. 563–567, 2014.

[52] R. J. Krane, G. A. Carpinito, S. D. Ross, P. T. Lavin, and M. E. Osband, "Treatment of metastatic renal cell carcinoma with autolymphocyte therapy. Low toxicity outpatient approach to adoptive immunotherapy without use of in vivo interleukin-2," *Urology*, vol. 35, no. 5, pp. 417–422, 1990.

[53] T. M. Law, R. J. Motzer, M. Mazumdar et al., "Phase III randomized trial of interleukin-2 with or without lymphokine-activated killer cells in the treatment of patients with advanced renal cell carcinoma," *Cancer*, vol. 76, no. 5, pp. 824–832, 1995.

[54] R. A. Figlin, J. A. Thompson, R. M. Bukowski et al., "Multicenter, randomized, phase III trial of $CD8^+$ tumor-infiltrating lymphocytes in combination with recombinant interleukin-2 in metastatic renal cell carcinoma," *Journal of Clinical Oncology*, vol. 17, no. 8, pp. 2521–2529, 1999.

[55] L. Liu, W. Zhang, X. Qi et al., "Randomized study of autologous cytokine-induced killer cell immunotherapy in metastatic renal carcinoma," *Clinical Cancer Research*, vol. 18, no. 6, pp. 1751–1759, 2012.

[56] P. W. Kantoff, C. S. Higano, N. D. Shore et al., "Sipuleucel-T immunotherapy for castration-resistant prostate cancer," *The New England Journal of Medicine*, vol. 363, no. 5, pp. 411–422, 2010.

[57] Z. Su, J. Dannull, A. Heiser et al., "Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells," *Cancer Research*, vol. 63, no. 9, pp. 2127–2133, 2003.

[58] J. H. Kim, Y. Lee, Y.-S. Bae et al., "Phase I/II study of immunotherapy using autologous tumor lysate-pulsed dendritic cells in patients with metastatic renal cell carcinoma," *Clinical Immunology*, vol. 125, no. 3, pp. 257–267, 2007.

[59] A. Berntsen, R. Trepiakas, L. Wenandy et al., "Therapeutic dendritic cell vaccination of patients with metastatic renal cell carcinoma: a clinical phase 1/2 trial," *Journal of Immunotherapy*, vol. 31, no. 8, pp. 771–780, 2008.

[60] T. Schwaab, A. Schwarzer, B. Wolf et al., "Clinical and immunologic effects of intranodal autologous tumor lysate-dendritic cell vaccine with aldesleukin (interleukin 2) and IFN-α2a therapy in metastatic renal cell carcinoma patients," *Clinical Cancer Research*, vol. 15, no. 15, pp. 4986–4992, 2009.

[61] A. Flörcken, J. Kopp, K. Movassaghi et al., "Allogeneic partially HLA-matched dendritic cells (DC) as a vaccine in metastatic renal cell cancer (mRCC): final analysis of a clinical phase I/II study," *Journal of Clinical Oncology*, vol. 30, supplement, abstract e15053, 2012.

[62] R. A. Figlin, "Phase II study combining personalized dendritic cell (DC)-based therapy, AGS-003, with Sunitinib in metastatic renal cell carcinoma (mRCC)," *Journal of Clinical Oncology*, vol. 30, supplement 5, 2012, Genitourinary Cancers Symposium, Abstract No. 348.

[63] R. J. Motzer, T. E. Hutson, L. McCann, K. Deen, and T. K. Choueiri, "Overall survival in renal-cell carcinoma with pazopanib versus sunitinib," *The New England Journal of Medicine*, vol. 370, no. 18, pp. 1769–1770, 2014.

[64] J. S. Ko, A. H. Zea, B. I. Rini et al., "Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients," *Clinical Cancer Research*, vol. 15, no. 6, pp. 2148–2157, 2009.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 261620, 12 pages
http://dx.doi.org/10.1155/2015/261620

Review Article

Effect and Molecular Mechanisms of Traditional Chinese Medicine on Regulating Tumor Immunosuppressive Microenvironment

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Received 24 July 2014; Accepted 16 December 2014

Academic Editor: Yung-Fu Chang

Traditional Chinese medicine (TCM) is an important complementary strategy for treating cancer in China. The mechanism is related to regulating the internal environment and remodeling the tumor immunosuppressive microenvironment (TIM). Herein we illustrate how TIM is reformed and its protumor activity on promoting tumor cell proliferation, angiogenesis and lymphangiogenesis, tumor invasion, and the oncogenicity of cancer stem cells. Furthermore we summarize the effects and mechanism of TCM on regulating TIM via enhancing antitumor immune responses (e.g., regulating the expression of MHC molecules and Fas/FasL, attenuating cancerigenic ability of cancer stem cells) and remolding immunosuppressive cells (e.g., reversing immune phenotypes of T lymphocytes and tumor associated macrophages, promoting dendritic cells mature, restraining myeloid derived suppressor cells function, and regulating Th1/Th2 factors). We also reveal the bidirectional and multitargeting functions of TCM on regulating TIM. Hopefully, it provides new theoretical basis for TCM clinical practice in cancer treatment and prevention.

1. Introduction

Chronic inflammation and immune suppression are the two core characteristics of the tumor microenvironment. It has been proven that chronic inflammation plays an important role in tumorigenesis and development; for instance inflammatory large intestine disease leads to colorectal cancer, H. Pylori infection breeds gastric cancer, and hepatitis B and C virus infection causes hepatocellular carcinoma (HCC). However, immune cells in tumor microenvironment promote tumor progression on the other hand: they constitute tumor immunosuppressive microenvironment (TIM) and alleviate tumor immune escape and tumorigenesis. Harmful stimulating factors such as hypoxia, acid environment, hyperosmosis, and inflammatory cytokines in tumor microenvironment facilitate the formation of TIM. According to the theory of tumor immunoediting, in TIM released tumor cells and immunosuppressive factors remodel the phenotype of immune cells, which decreases its antitumor function; meanwhile, remodeled immune cells "resculpture" tumor cells and make them become of low immunogenicity and might favor immune escape of tumor cells [1, 2]. Besides these, immune suppressive cells in TIM also bring out angiogenesis and lymphangiogenesis, playing a vital role in tumor development and metastasis. Thus, TIM is considered as a novel target for cancer treatment.

Traditional Chinese medicine (TCM) is a very important tumor treatment strategy in China [3]. It is accepted that TCM can reduce the toxicity of chemotherapy and radiotherapy, enhance the antitumor effect of these therapies, alleviate tumor-induced clinical symptoms and cancer pains, and prolong the survival time of postoperational and advanced stage cancer patients [4]. Though the effect mechanism of TCM is not very clear, increasing data has shown that it may relate

with its action on regulating tumor immune environment, the novel target according to the theory of immunoediting [5, 6]. In this paper, we will introduce the formation and function of TIM and summarize recent researches of TCM on regulating it.

2. Tumor and Its Matrix Cells Constitute Tumor Immunosuppressive Microenvironment

Tumor microenvironment is a complex compound, including tumor cells, stromal cells, extracellular matrix, cellular factors, and chemokines. The microenvironment plays a pivotal role in the process of cancer development and metastasis. Downregulating the immune function and combined with extracellular matrix proteins and matrix-degrading enzymes, stromal cells (e.g., angiogenic cells, immune cells, and tumor associated fibroblasts) and cell factors they produced encompass tumor cells and form the tumor immunosuppressive microenvironment [7].

In TIM, tumor cells do not express classical MHC I molecules but express nonclassical MHC molecules, leading to the immunosuppression and tumor progression [8]. Meanwhile, tumor cells express a variety of immunosuppressive factors, such as IL-10, IL-6, and TGF-β, reversing immune cells to immunosuppressive phenotypes, for example, regulatory T cells, regulatory B cells, tumor associated macrophages (TAMs), regulatory dendritic cells, and myeloid derived suppressor cells (MDSCs). These immunosuppressive cells interact with each other and increasingly proliferate and express immunosuppressive and protumor factors. Moreover, they consume proinflammatory factors (IL-2, TNF-α), promote T cells apoptosis, and reduce the antitumor activity of NK cells. They produce matrix metalloproteinases (MMPs) and vascular endothelial growth factors (VEGF) as well, promoting angiogenesis and tumor invasion [9–12]. All these tumorigenesis responses give negative feedback to immunocytes, breeding a vicious circle for tumor treating.

3. Immune Suppressive Cells in TIM Promote Tumorigenesis and Progression

3.1. Promoting Tumor Cell Proliferation. Studies have proven the close relationship between TIM and tumorigenesis in different kinds of cancer diseases [13, 14]. When turning to basic researches, Fritz et al. [15] found that macrophages produce IGF-1 which directly stimulates neoplastic proliferation through Erk and Akt activation. Munari et al. [16] demonstrated that gastric lymphoma-infiltrating macrophages highly produced APRIL, which was regarded as a novel cytokine crucial in sustaining B cell proliferation and causing a mucosa associated lymphoid tissue B cell lymphoma proliferation. When coculturing with breast cancer cells, IL-4-activated macrophage transported microRNAs from itself to breast cancer cells, and one of microRNAs, miR-223, promoted the invasion of breast cancer cells via the Mef2c-β-catenin pathway [17]. These studies conclude and inside provide evidences that immune suppressive cells in TIM promote tumor cells multiplication.

3.2. Promoting Angiogenesis and Lymphangiogenesis. Tumor hematal and lymphatic vessels are important in tumor progression and serve as paths and tubes for nutrients and metabolites transportation, promoting tumor cells growth and metastasis. TIM plays a vital role in the process of angiogenesis and lymphangiogenesis. In tumor hypoxic microenvironment, tumor cells, tumor associated macrophages, dendritic cells, myeloid derived suppressor cells, and neutrophil cells secrete a variety of proangiogenesis and prolymphangiogenesis factors (e.g., VEGF, VEGF-C, MMP-9, TGF-β, and COX-2) [18, 19]. In recent years, studies also found a subtype of TAMs, called TIE2-expressing macrophages (TEM). TEMs overexpress angiopoietin-2 (ANG2) and mediate a crosstalk with vascular endothelial cells via Ang2-TIE2 pathway to promote angiogenesis [20]. Moreover, TAMs could transdifferentiate to lymphatic endothelial cells and, as endothelial progenitor cells, take part in lymphangiogenesis in tumor tissues under certain conditions [21]. Thereby, TIM is directly involved in the formation of vessels in tumors.

3.3. Promoting Tumor Invasion. The invasive ability of tumor cells is the basis of tumor local invasion and distant metastasis, and prior to this is the remolding of the extracellular matrix. TAM, Th-17 cells, and DCs in TIM produce MMPs and TGF-β to dissolve extracellular collagens, so as to remold the extracellular matrix. TAMs used both the mesenchymal mode requiring MMPs and the amoeboid migration mode to infiltrate tumor cell spheroids and promote MMP-independent invasion by tumor cells [22]. Epithelial mesenchymal transition (EMT) is the morphological change process of tumor cells invasion and metastasis [23]. TAMs could mediate EMT of tumor cells and promote the tumor progression through the TLR4/IL-10 signaling pathway [24]. By activating TGF-β, EGF, and HGF signaling pathway, MDSCs induced EMT as well [25]. Hence, TIM makes it easier for tumor invasion via both affecting it straightly and mediating EMT.

3.4. Promoting the Oncogenicity of Cancer Stem Cell. Cancer stem-like cell (CSC) is a hotspot of the tumor research in recent years. It is widely thought that CSC is a tumorigenesis and metastasis "seed cell" and the "springhead" of tumor immune escape. CSC has a continuous self-renewal and proliferation ability, and it differentiates incompletely, resisting radiation and chemotherapy and producing immune suppressive factors. Losing normal exogenous apoptosis signaling pathways, CSC strongly shows the resistance to apoptosis functions [26, 27]. Immune suppressive cells in TIM also participate in regulating CSC function. Yang et al. [28] found that TAMs mediate tumorigenesis through regulation of breast CSCs and promote CSC-like phenotypes in murine breast cancer cells by upregulating their expression of Sox-2. TAM markers are associated with cancer stem cell marker in oral squamous cell carcinoma [29]. MDSCs had similar features that triggered microRNA101 expression in

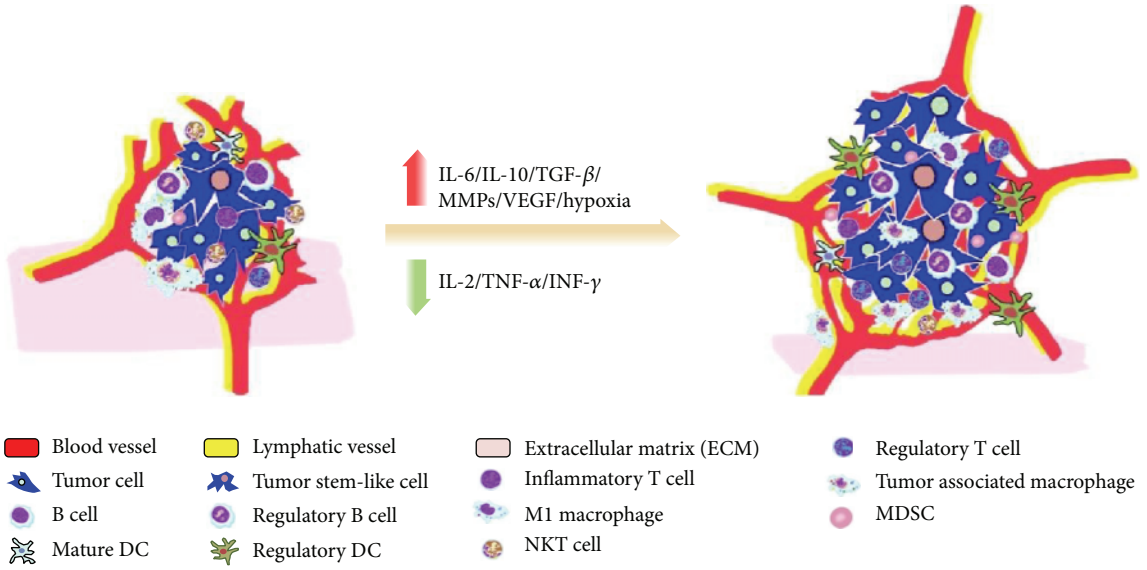

Figure 1: Tumor immunosuppressive microenvironment promotes tumorigenesis and progression.

cancer cells and increased cancer cell stemness and increasing metastatic and tumorigenic potential targeted stem cell core genes [30]. In conclusion, TIM enhances CSC progression in mutual effect (Figure 1).

The hypoxia environment brings TIM increasingly producing immune suppressive and angiogenesis factors (e.g., IL-6, IL-10, TGF-β, MMPs, and VEGF) and downregulating inflammatory cytokines such as IL-2, TNF-α, and INF-gamma, promoting immune suppressive cells proliferation and CSCs growth and inhibiting antitumor cells. Thus, TIM leads to tumorigenesis, angiogenesis, lymphangiogenesis, and tumor ECM remolding and causes tumor progression and metastasis.

4. TCM Enhances Tumor Immune Responses

4.1. TCM Promotes Classic MHC Molecules Expression. MHC, also called human leukocyte antigen (HLA) in human tissues, is the important immunological recognition molecule in the process of tumor immune response. Classic MHC molecule can be divided into two subgroups: MHC class I and MHC class II. MHC I have a vital role in presenting tumor antigens to T cell receptor. After identifying the tumor antigen peptide and MHC class I molecule, the cytotoxic T cell (CTL) is activated and launched a series of cytolysis reactions to kill tumor cells. MHC II presents tumor antigens to $CD4^+$T helper cells, leading to cellular mediated immune response. However, both immune and malignant cells in the tumor microenvironment do not express typical MHC molecules. They downregulate MHC class I expression, overexpress nonclassical HLA such as HLA-G, HLA-E, and HLA-F, which have been recently proven to be correlated with poor clinical outcome, and escape from T and NK cell-mediated recognition [8, 31].

Studies have shown that TCM can upregulate MHC molecules in tumor microenvironment. Li et al. [32] explored the effect of Invigorating Spleen and Detoxification Decoction (ISD) (Radix Codonopsis, *Poria*, Rhizoma Atractylodis Macrocephalae, Radix Glycyrrhizae, Radix Bupleuri, Rhizoma Curcumae, and *Herba Scutellariae barbatae*) on MHC molecules in the rat liver cancer tissue and found that ISD could enhance the expression of MHC I and MHC II and prolong the rat survival time. Besides the effects on tumor cells, TCM could also increase MHC expression in immune cells. *Anoectochilus formosanus* is a medicinal herb in Asia and extracts of *A. formosanus* have been reported to possess antitumor activities. Kuan et al. [33] found that *A. formosanus* could stimulate the MHC II expression. In vitro experiment showed Fei Liu Ping Extractum (FLP) (Radix Panacis Quinquefolii, *Cordyceps*, Radix Astragali, Radix Codonopsis, Radix Glehniae, Radix Ophiopogonis, Herba Agrimoniae, *Polygonum bistorta* L., *Thlaspi arvense* Linn., *Hedyotis diffusa*, Semen Juglandis, Semen *Armeniacae amarum*, and Bulbus Fritillariae Cirrhosae) could upregulate the expression of MHC II of dendritic cells and improve the body function of antitumor immunological surveillance [34]. These results suggest that TCM could enhance tumor antigen-presenting ability by upregulating classic MHC expression in both tumor and immune cells.

4.2. TCM Induces Tumor Cells Apoptosis via Fas/FasL Pathway. Fas and its natural ligand FasL are molecules expressed on cellular membranes. The activation of Fas/FasL pathway plays an important role in cell apoptosis. Physiologically, cytotoxic T lymphocytes ($Fas^{low}FasL^{high}$) express FasL and combine with the Fas expressed by target cells ($Fas^{high}FasL^{low}$), resulting in the trimerization and activation of the Fas receptor, and then mediate target cells apoptosis.

Unlike normal target cells, tumor cells express nonfunctional Fas or low quantity of Fas; meanwhile overexpression of FasL promotes tumor cells immune escape by preventing the combination with cytotoxic T cells and induces CTL apoptosis [35].

Our previous study showed that TCM herbal medicine Yang Wei Kang Liu Granule (YWKL) (Radix Astragali, Radix Ginseng, *Hedyotis diffusa*, Yunnan Manyleaf Paris Rhizome, Radix Notoginseng, Radix Paeoniae Rubra, and Hematoxylon) could increase FasL expression and downregulate Fas expression in T lymphocytes of gastric cancer patients, which indicates YWKLF may inhibit cancer by inducing apoptosis [36]. Our subsequent studies established MGC-803 stomach cancer cell model in vitro and showed FasL mRNA in MGC-803 declined significantly after treatment by YWKL [37]. These mean YWKL may enhance cancer cells' sensitivity to immune response cells like CTL and lead to tumor cells apoptosis by regulating Fas/FasL pathway.

4.3. TCM Attenuates Oncogenicity of CSCs. Cancer stem-like cells (CSCs) are a more malignant composition in tumor tissues inhibiting immune response. Researches have demonstrated CSCs take part in carcinogenesis and therapy resistance in kinds of tumors [38–41]. CSCs rarely express immune recognition molecules such as HLA-DR and costimulatory molecules such as CD80 and CD86. Furthermore, laboratory researches show CSC tumor spheres lowly express Fas and highly express membrane complement regulatory proteins and FoxP3 [42]. All these findings indicate CSCs prevent antitumor immune responses and promote immunosuppressive microenvironment formation. Thus, it is rational to target CSCs to reverse tumor immune suppression.

Some medicinal herbs have been proved to have anti-CSC ability. Chang et al. [43] cultured the hMG63-derived cancer stem cells in permissive microenvironments for stem cell differentiation and found Bufalin inhibited the proliferation and sphere formation of cancer stem cells. Zhang et al. [44] found that Huaier (*Trametes robiniophila* Murr.) aqueous extract had significant efficacy on inhibiting spheroid formation ($P < 0.05$) and reduced the aldehyde dehydrogenase (ALDH) positive cell population in colorectal primary cancer cells ($P < 0.05$). Further study revealed Huaier extract downregulated the Wnt/β-catenin pathway, which is one of the critical pathways demonstrated to mediate the self-renewal of CSCs. Honokiol is an extract from medicinal herb Cortex Magnoliae Officinalis. Research showed that it can thwart tumor growth [45]. Recently, Ponnurangam et al. [46] found the ability of Honokiol to enhance the sensitivity of colon CSCs to ionizing radiation is by inhibiting the γ-secretase complex and the Notch signaling pathway. These findings indicate that Chinese medicine may reduce CSCs and weaken tumor immune resistance as well (Figure 2).

While being treated by Chinese medicine, the percentage of CSCs is decreased; Classic MHC and Fas molecules are expressed more on tumor cytomembranes, which lead to tumor cells having lower malignant degree and easier to be recognized and killed by the immune system.

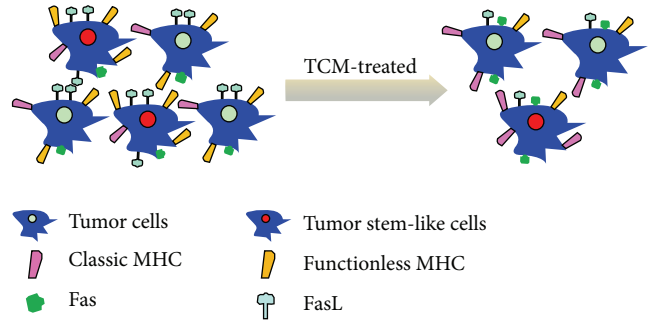

Figure 2: TCM enhances tumor cells immune responses.

5. TCM Reverses the Immunosuppressive Phenotype and Regulates the Antitumor Functions of Immune Cells

Immune cells show various functions and phenotypes and play multiple roles in antitumor immunity and protumorigenesis, which are determined and influenced by tumor immune microenvironment. TCM herbs indicate a biphasic regulation on tumor cells phenotype to enhance antitumor immune responses: increasing proinflammatory phenotypic antitumor activity while reversing and remolding the suppressive function (phenotype) of immunocytes (Figure 3).

5.1. TCM Enhances T lymphocyte Antitumor Abilities. T lymphocytes are notably involved in tumor adaptive immune cells but also have been found participating in tumor immune suppression in different subtypes [47]. T cells express specific recognition and signal transduction related to TCR/CD3 complex molecules. Based on their function and phenotypes, T cells are divided into three main subtypes: cytotoxic T cells ($CD3^+CD8^+$), helper T cells ($CD3^+CD4^+$), and regulatory T cells ($CD3^+CD4^+CD25^+$). $CD4^+$T cells produce IL-2 and IL-15 which enhance the activation of $CD8^+$T cells, maintain the activity of CTL, and also activate innate immune cells such as natural kill cells and dendritic cells. Meanwhile, part of $CD4^+$T cells has the ability to kill tumor cells directly. $CD8^+$T cells release perforin and particle enzymes to kill and dissolve tumor cells and lead to target cells apoptosis through Fas/FasL pathway. $CD4^+CD25^+$T cell (Treg) is an immune regulating subtype which is regarded to play immunosuppressive role in tumor microenvironment. Tregs produce quantity of immune suppressive factors, for example, IL-10 and TGF-β, compete with response T cells for consuming IL-2, affect metabolism of other T cells, and downregulate the stimulation of dendritic cells [48]. Thus, it is the key to regulating tumor immune microenvironment via enhancing antitumor efficacy of T cells and inhibiting Treg cell and its function.

It is notable that TCM may adjust immune function by targeting T cells. Feiyanning Decoction (FYN) (Radix Astragali, Rhizoma Atractylodis Macrocephalae, Pseudobulbus Cremastrae seu Pleiones, *Salvia Chinensis* Benth., *Paris polyphylla*, Corium Bufonis, Nidus Vespae, Rhizoma Polygonati, Fructus Corni, Herba Epimedii, Fructus Ligustri Lucidi,

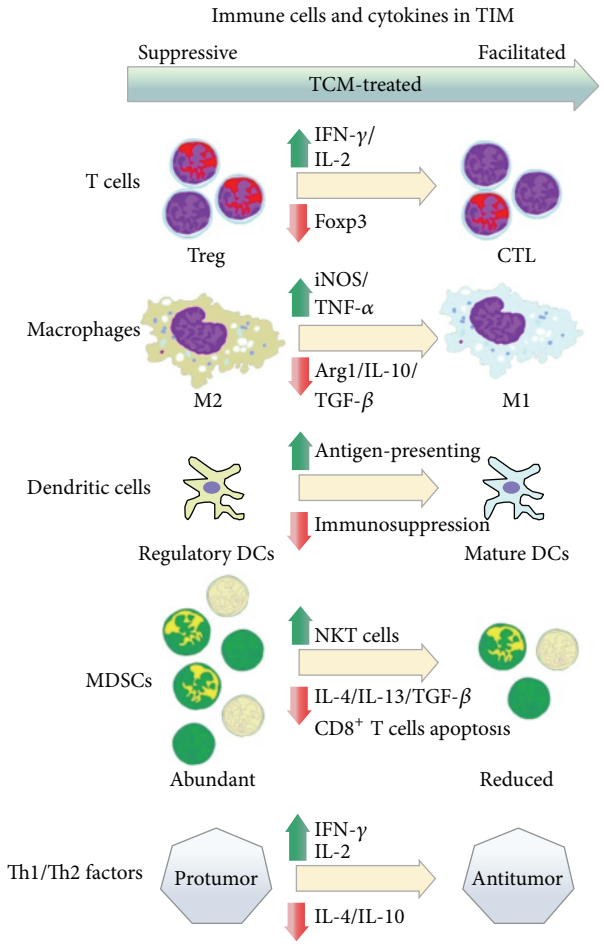

Figure 3: Molecular mechanism of TCM on regulating immune cells in TIM.

and *Ganoderma*) is an antitumor compound prescription of TCM, which has been proven effective in the clinical research [49]. Recent study in Lewis lung carcinoma bearing mice model showed FYN's effect on Tregs. It was found that the numbers of $CD4^+CD25^+$ regulatory T cells in spleen, thymus, and tumor were lower in the FYN group than in the model group ($P < 0.05$). The expression of Foxp3 mRNA in spleen, thymus, and tumor was also significantly downregulated in the FYN group [50]. There are other studies that showed main ingredients of FYN such as Astragaloside significantly increased IL-2 and IFN-γ secretion of T cells and promoted T cells immune activity [51].

5.2. TCM Regulates M1/M2 Phenotypes of TAMs. Tumor associated macrophages (TAMs) derived from peripheral circulating monocytes occupy about 30%–50% of the total tumor stroma cells. After being recruited to the tumor microenvironment, TAMs differentiate into two polarized phenotypes, that is, the classic activation polarization (M1 phenotype) and the alternative activation polarization (M2 phenotype). Induced by IFN-γ, IL-6, and so forth, M1 macrophages display proinflammatory, antigen-presenting, and antitumor effects, through release of soluble enzyme, TNF, and IFN and activation of T cell immune responses to inhibit tumor cells. However, M2 macrophages, activated by IL-10, IL-13, and so forth, have an immune regulating and suppressive role via multiple ways to promote tumor progression. Recent studies indicated TAMs tend to M2 protumor phenotype through stimulating tumor cell proliferation, inhibiting tumor immune microenvironment, promoting matrix remodeling, and accelerating angiogenesis and lymphangiogenesis [52, 53]. Thus, TAMs are associated with tumor progression and metastasis.

Researchers reported TCM herbs could inhibit macrophages' inflammatory effect [54], and there are certain studies that showed TCM could switch the phenotype of TAMs from M2 to M1 during tumor progression. *Schisandra* polysaccharide (SCPP11) is an extract ingredient from herb medicine *Schisandra chinensis*, which has been used in TCM for centuries and proved to have antitumor activities. Recent study results showed that SCPP11 could increase the pinocytic activity of peritoneal macrophages in CTX-induced immunosuppression mice. Moreover, SCPP11 significantly increased immunoglobulin levels and cytokines levels in vivo and induced RAW264.7 cells (a monocyte/macrophage cell line of mice for in vitro experiments) to secrete cytokines in vitro and RAW264.7 cells pretreated with SCPP11 significantly inhibited the proliferation of HepG-2 cells, via promoting the expression of both iNOS protein and iNOS and TNF-α mRNA [55]. These results showed that SCPP11 could enhance the antitumor effect of macrophages. Other researchers found five extracts (UM01, QH11, BNQM, GNCC, and DCXC) of *Cordyceps sinensis* could significantly increase cell proliferation and NO production of RAW264.7 cells [56]. Zhang et al. found that *Ganoderma atrum* polysaccharide (PSG-1) increased macrophage phagocytosis and the levels of cytokines and nitride oxide through TLR4-mediated NF-κB and MAPK signaling pathways in S180 tumor bearing mice model [57]. Shenqi Fuzheng Injection (Radix Codonopsis, Radix Astragali) was indicated enhancing peritoneal macrophage phagocytosis in immunosuppressed mice as well [58]. Our studies found similar results that TCM decoction Fuzheng Jiedu Formula (FZJD) (Radix Astragali, Radix Codonopsis, Rhizoma Atractylodis Macrocephalae, Radix Polygoni Multiflori, Fructus Lycii, Yunnan Manyleaf Paris Rhizome, *Hedyotis diffusa*, and *Actinidia arguta* Planch.), including Radix Codonopsis and Radix Astragali, not only promoted macrophage phagocytosis but also enhanced M1/M2 and anti-/protumor factors of TAMs in tumor microenvironment. Specifically, FZJD reduced IL-10 and TGF-β expression and raised the ratio of iNOS/Arg1 which represents the M1/M2 proportion. All the findings above illustrate Chinese medicinal herbs could inhibit tumor cells by promoting macrophages antitumor function and reversing TAM phenotype M2 to M1.

5.3. TCM Enhancing Antigen-Presenting Function of Dendritic Cells (DCs). Dendritic cells (DCs) are professional antigen-presenting cells, which activate initial T cell by presenting and delivering antigens and mediate acquired tumor immune cytotoxicity. However, recent studies found a subset of DCs in

the tumor microenvironment inducing immunosuppression called regulatory DCs or tumor associated DCs (TADCs). These TADCs have a low ability to present antigen, induce T cells differentiating to Treg subtype, and consequently impair T cell-mediated tumor killing effects. Furthermore, TADCs lead to a decreasing ratio of Th cells and effective T cells apoptosis through a way such as reactive oxygen species, the indoleamine 2, 3-2 oxidase (IDO), and releasing immune suppressive factors [59]. TADCs are also involved in tumor angiogenesis, tumor cell proliferation, and invasion [60]. Thus, we can see from above that DCs have a similar polarization like macrophages (M1/M2). Regulating dendritic cells and enhancing their antigen-presenting function could be a possible way for TCM antitumor effects.

Zhang and Liu found that TCM with function of supplementing Pi and nourishing Shen could improve the inflammatory function of DCs in patients with chronic hepatitis B [61]. Our laboratory group explored the possible influence of TCM drug FLP on regulating DCs in peripheral blood, spleen, and tumor in mice with transplanted Lewis lung cancer. We found that the percentages of DCs (per thousand) in tumor bearing mice were increased to 2.55 ± 0.29 in peripheral blood and 2.70 ± 0.63 in spleen ($P < 0.01$) after FLP treatment [62]. Further studies showed FLP promoted DCs maturity, reversed DCs regulatory (immunosuppressive) phenotype, and increased DCs membrane MHC II, CD80, CD83, CD86, and CD40 expression. FLP also promoted the IL-12 secretion of DCs and enhanced the function of DC-LPAK tumor killing way [34, 63]. These results indicate TCM may have regulating effect on DCs.

5.4. TCM Restrains Myeloid Derived Suppressor Cells (MDSCs). Myeloid derived suppressor cells (MDSCs) are a special kind of cell population which play essential role in malignant tumor immune suppression. The main immune inhibition effects of MDSCs reflect on suppressing T cells related immune responses and inducing T cell apoptosis, promoting inflammatory mediated tumor recurrence and metastasis. MDSCs are a group of heterogeneous cells, including immature macrophages, dendritic cells, and granulocytes. Pathological stimulations like malignant tumor and inflammation block immature bone marrow cells differentiation into mature cells, leading to the expansion of MDSCs and contributing to the negative regulation of tumor immune response. MDSCs facilitate angiogenesis by releasing VEGF, MMPs, TGF-β, and so forth and inhibiting T cell-mediated tumor acquired immune responses with overexpression of Arg1, iNOS, Ros, and so forth. The increasing IL-10 productivity of MDSCs restricts macrophages and DCs antitumor function as well [64].

Since 2007, we established the collaboration with the National Cancer Institute (NCI) Molecular Immunoregulation Laboratory and carried out series studies on the immune regulation effect of TCM. The results showed TCM herbs Radix Ginseng and Radix Notoginseng had a certain inhibitory effect on MDSCs phenotype and protumor functions [65]. Our recent studies found that the levels of MDSCs in breast cancer patient peripheral blood are positively associated with tumor progression and TCM decoction Shugan Jianpi Formula (Radix Bupleuri, Radix Paeoniae Alba, Spica Prunellae, Radix Curcumae, Holboellia Fargesii Reaub, Radix Astragali, Radix Notoginseng, and Radix Glycyrrhizae) (SGJP) had an inhibition function in MDSCs proliferation and could prevent MDSCs induced IL-4, IL-13, and TGF-β expression and $CD8^+$T cells apoptosis. Meanwhile, SGJP enhanced and regulated inflammatory functions of NKT cells, which were associated with MDSCs regulation [66]. These above were addressed by Jak-Stats signal pathways and indicated TCM herbs affecting MDSCs.

5.5. TCM Regulates Th1/Th2 Immune Factors Secretion. Th1/Th2 immune factors also have a polarization of promoting antitumor immunocompetence and tumorigenesis ability. Th1 factors like IL-2, IL-6, TNF-α, and INF-γ, are linked to proinflammation, cytotoxic and cytophagic enhancement, and other antitumor functions, while Th2 factors, for example, IL-4, IL-10, and TGF-β, play a suppressive role in tumor immune microenvironment and promote tumor recurrence and metastasis.

Wei et al. [67, 68] found that TCM herb Radix Astragali (AG) and tetramethylpyrazine (TTMP) extracted from a medicinal herb *Ligusticum chuanxiong* could reverse predominance of Th2 cytokines in lung cancer patients. AG enhanced the levels of Th1 cytokine (IFN-γ and IL-2) as well as its transcript factor (T-bet) expression in culture supernatant and reduced those of Th2 cytokines in cultured peripheral blood mononuclear cells (PBMC) of lung cancer patients. TTMP could enhance supernatant concentration and gene expression levels of IFN-γ, IL-2, and T-bet but reduce those of type 2 cytokines (e.g., IL-4, IL-10). These results demonstrated that AG and TTMP could reverse the type 2 dominant status, which might offer an alternative therapeutic regime for lung cancer patients. Other studies of TCM compound decoctions, containing *Astragalus* composition, showed a similar result as well. Fuzheng Yiliu Decoction (FYD) (Ginseng, *Astragalus, Ganoderma lucidum, Angelica sinensis*, and *Lycium chinense*) remarkably inhibited proliferation and induced apoptosis of hepatoma cells at least by promoting the production of IL-2 and TNF-α in vivo [69]. Wang and Chen [70] observed the effect of Aidi injection (*Mylabris*, Radix Ginseng, Radix Astragali, and Radix et Caulis Acanthopanacis Senticosi) on peripheral blood expression of Th1/Th2 transcription factors and cytokines in patients with esophageal squamous cell carcinoma (ESCC) during radiotherapy. They found a Th2 toward drift phenomenon that, compared with the healthy control group, the expressions of Th1 type transcription factor T-bet and cytokines IFN-γ, IL-2 in ESCC patients were significantly lower, while expressions of Th2 type transcription factor GATA-3 and cytokines IL-4, IL-10 were significantly higher. When combined with intravenous dripping of Aidi injection during radiotherapy, the Th2 toward drift was inhibited. These results demonstrated that TCM herbs might reverse the Th2 predominant status, which is a probable alternative therapeutic regime in future.

Suppressive phenotypes of immune cells are regulated and reversed by TCM treating. Concretely, inflammatory T cells and NKT cells increasingly proliferate in contrast to the reducing quantity of regulatory T cells and MDSCs. Suppressive macrophages and DCs change their functions to antitumor effects, such as M2 to M1 phenotype reversing and DCs maturation. All these alterations are influenced and accompanied by increasing inflammatory factors expression and immune suppressive cytokines decreasing.

6. Difference between TCM and Modern Western Medicine in Cancer Immunotherapy

6.1. TCM Has Multitarget and Bidirectional Immunoregulation Effect. Based on the preliminary studies, the multitarget function of TCM compound on regulating TIM is assigned to its multicomponents. First, the compound is usually composed of more than one herb, and the herb often consists of various ingredients and each ingredient always contains different kinds of chemicals. There are some certain chemicals extracted from TCM herbs that are found to have immune regulating function: specifically, *Ganoderma lucidum* polysaccharides (GLPS), a kind of effective ingredient extracted from *Ganoderma lucidum* (*G. lucidum*), which has long been prescribed to prevent and treat various human diseases, particularly in China, Japan, and Korea. Some researches showed that GLPS had a potential role in cancer treatment; further studies suggested that the antitumor activities of GLPS are mediated by its immunoregulation effect. GLPS might act on immune-related cells as well as immune cells such as B lymphocytes, T lymphocytes, dendritic cells, macrophages, and natural killer cells [71]. *Solanum nigrum* Linn., another effective TCM herb in cancer treatment, has been proven to have antitumor activity by enhancing the $CD4^+/CD8^+$ ratio of the T lymphocyte subpopulation. Razali et al. [72] named SN-ppF3 from *Solanum nigrum* Linn. polysaccharide that could significantly induce phagocytosis activity and stimulated the production of TNF-α and IL-6 and nitric oxide synthase expression of RAW264.7 cells.

Besides the ingredient found directly from the herbs, the postmetabolized ingredients of TCM compound may also play an immunoregulating role. Bae et al. [73] found that, after oral administration of BST204, a purified ginseng dry extract containing ginsenosides such as Rg1 and Rh1, only the S epimers, S-Rh2 and S-Rg3, could be determined in rat plasma. Studies also found that the oral bioavailability of ginsenoside Rg1 is very low in rats and ginsenosides might be metabolized by intestinal microflora before absorption into blood [74, 75]. These indicated that some ingredients in TCM herbs might be effective after being metabolized or some of the chemicals inactive in vitro could play roles in organisms after being metabolized. Continuing in depth studies, metabolite of ginsenosides compound K was found in suppressing the activation of the NF-κB pathway and attenuating metastatic growth of hepatocellular carcinoma [76]. Another study showed that Rh2 metabolic modification ginsenoside *aglycone protopanaxadiol* (aPPD) activated in treating prostate cancer combined with docetaxel and was more effective than its prototype [77]. TCM herbs such as ginseng have been proved to have immune enhancement, and it is a possible hypothesis that their metabolites modulate immune microenvironments.

As mentioned above, due to its complicated ingredients TCM compound has a multitarget effect. It might act directly on various immune cells, while it can also play a role through regulating immune factors in different immune signal pathways [78]. Another important feature of TCM is its bidirectional effect. For instance, Radix Astragali has effects on both neoplasms (immune enhancement) and rheumatoid arthritis (immune suppression) [79, 80]. In addition, TIM brings out abnormal expression of signal pathways and cytokines; meanwhile the ratio of function-opposite immune cell phenotypes such as M1/M2 macrophages is also imbalanced in TIM, which is called disharmony according to TCM theory. TCM always affect multiple targets, and they could appropriately reverse an unbalanced state (immunologic derangement in TIM) to a relative harmony state.

6.2. Western Medicine Acts as Guided Missiles in Cancer Immunotherapy. The characteristic of Western medicine is focused on definite targets that means Western medicine has specific targets relatively visualized and detectable, just like a guided missile hit a target with a minimal error. These immunotherapies are guided into the following fields: (1) monoclonal antibodies against either tumor cells (bevacizumab [81], cixutumumab [82], rituximab [83], etc.) or immunosuppressive factors (tremelimumab [84], nivolumab [85], etc.); (2) tumor vaccines based on polypeptide (MAGE-A3 [86], etc.), DCs (belagenpumatucel-L [87], etc.), or other targets vaccines; (3) immune cells adoptive treatment, such as CIK [88]; (4) soluble tumor virus gene therapy [89], such as GC0070 and GL-ONC1. These indicate the convinced evidences and targets of Western medicine immunotherapy, but there are some problems and side effects, such as the dermatologic events associated with cixutumumab [90].

To make up for deficiencies of Western medicine specificity, it is feasible to introduce TCM in modern immunotherapies, such as the immune enhancement and side effects remission [91, 92]. Above all, it is benefit to patients that we combine the Western medicine (specificity) with TCM (multitarget and bidirectional regulating) in immunotherapies, and there is a promising field waiting us to study and explore.

7. Summary

TCM plays an important role in cancer treatment in China via diversity ways (Figure 4). The formation and dynamic changes of TIM are a multifaceted process and are including multiple targets, different types of cells, and various signal pathways. Here we maintain that the TCM, both monomer and compound, has a certain effect on regulating TIM, and ways and points the TCM is affecting are diverse. More specifically, TCM herbs influenced not only tumor cells, but immune cells and cytokines as well as signal pathways

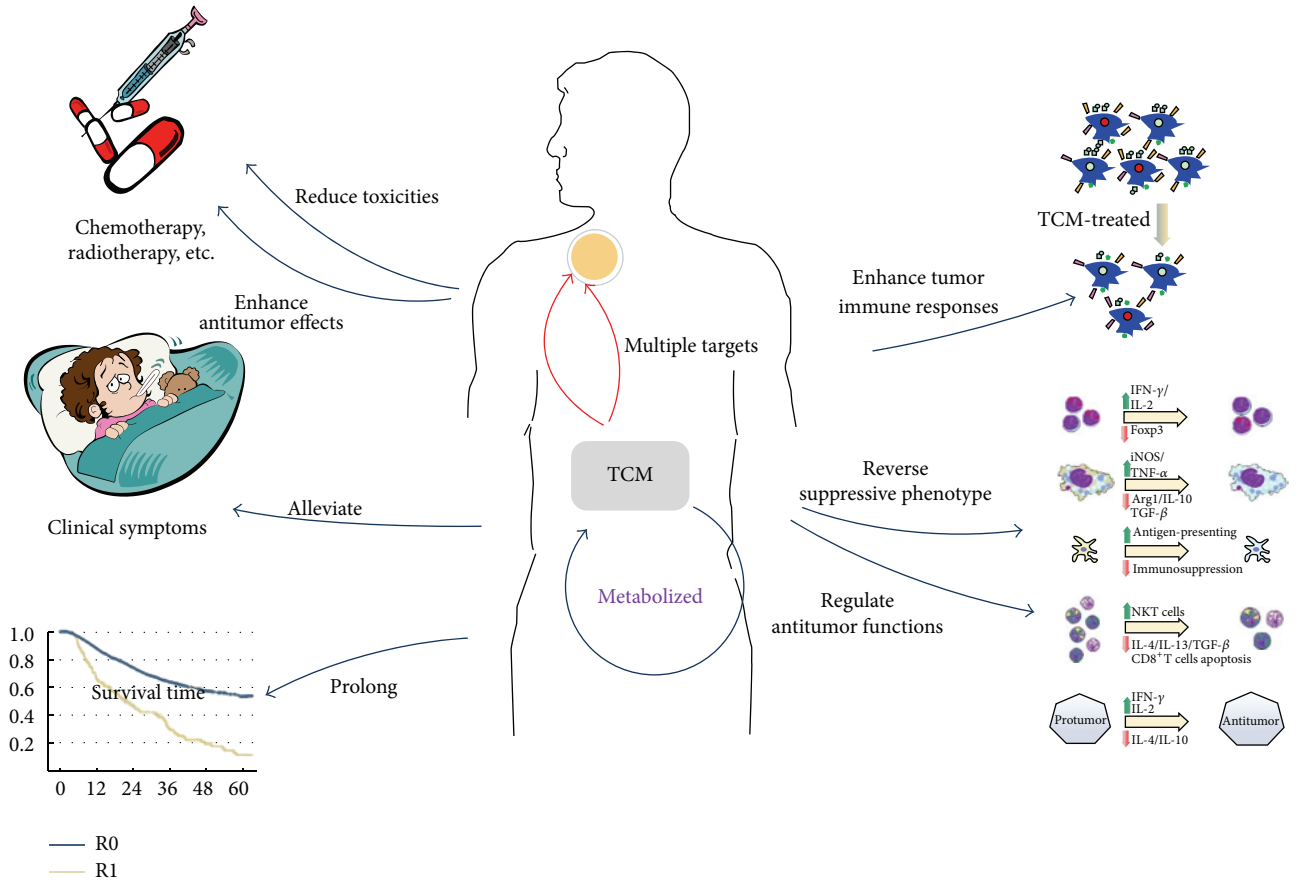

Figure 4: General functions and mechanisms of TCM on tumor therapies.

[93, 94]. For the further analysis of these TCM herbs, we can see Radix Codonopsis, Radix Ginseng, Radix Astragali Preparata, Rhizoma Atractylodis Macrocephalae, Yunnan Manyleaf Paris Rhizome, *Hedyotis diffusa*, and so forth exist in kinds of TCM compound decoctions, which have different roles in regulating TIM; that is to say some of TCM herbs may have multiple effect on TIM. Treatment based on syndrome (ZHENG) differentiation is the characteristic and treatment guide of TCM. Studies showed tumor microenvironment differentiated under different TCM ZHENG models and had a relationship with treatment response to herbal medicine [95]. TCM herbs are cataloged according to their effect on certain TCM ZHENGs as Radix Codonopsis, Radix Ginseng, Radix Astragali Preparata, and Rhizoma Atractylodis Macrocephalae are tonified medicines to treat Qi deficiency ZHENG while Yunnan Manyleaf Paris Rhizome and *Hedyotis diffusa* relieve heat-toxin ZHENG in TCM pharmacology. TCM herbs are also considered to have dual-direction regulating effects on TIM as enhancing tumor cells antigenic responses and immune cells antitumor abilities while inhibiting the tumorigenesis functions. Above all, it is worth a further study to figure out the relationship and biochemical mechanism of different catalogs of TCM herbs in regulating TIM. With the rapid development of immunology, TCM effect on regulating TIM will play a more important role in tumor complementary and alternative therapies.

It has been accepted in China that TCM can reduce the toxicity and enhance the antitumor effect of chemotherapy and radiotherapy, alleviate tumor-induced clinical symptoms, and prolong the survival time of postoperational and advanced stage cancer patients [4]. Meanwhile, TCM regulates TIM by enhancing tumor immune responses, reverses suppressive phenotype of immune cells, and promotes their antitumor functions.

Abbreviations

ANG: Angiopoietin
APRIL: A proliferation inducing ligand
COX-2: Cyclooxygenase-2
CSC: Cancer stem-like cell
DC: Dendritic cell
EGF: Epidermal growth factor
EMT: Epithelial mesenchymal transition
Erk: Extracellular regulated protein kinases
HCC: Hepatocellular carcinoma
HGF: Hepatocyte growth factor
HLA: Human leukocyte antigen
IGF-1: Insulin-like growth factor-1
IL: Interleukin
INF-γ: Gamma interferon
MDSC: Myeloid derived suppressor cell

MHC: Major histocompatibility complex
MMP: Matrix metalloproteinase
TAM: Tumor associated macrophage
TCM: Traditional Chinese medicine
TGF-β: Transforming growth factor-beta
TIM: Tumor immunosuppressive microenvironment
TNF-α: Tumor necrosis factor-alpha
VEGF: Vascular endothelial growth factor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work was supported by National Natural Science Foundation of China (nos. 81072802, 81273946, and 81473463).

References

[1] G. P. Dunn, A. T. Bruce, H. Ikeda, L. J. Old, and R. D. Schreiber, "Cancer immunoediting: from immunosurveillance to tumor escape," *Nature Immunology*, vol. 3, no. 11, pp. 991–998, 2002.

[2] G. P. Dunn, L. J. Old, and R. D. Schreiber, "The three Es of cancer immunoediting," *Annual Review of Immunology*, vol. 22, pp. 329–360, 2004.

[3] J. Li and H.-S. Lin, "Integrative medicine: a characteristic China model for cancer treatment," *Chinese Journal of Integrative Medicine*, vol. 17, no. 4, pp. 243–245, 2011.

[4] J. Li, L. Li, R. Liu, and H.-S. Lin, "Establishing Chinese medicine characteristic tumor response evaluation system is the key to promote internationalization of Chinese medicine oncology," *Chinese Journal of Integrative Medicine*, vol. 18, no. 10, pp. 730–736, 2012.

[5] L. Xu, J.-M. Feng, J.-X. Li et al., "Tanshinone-1 induces tumor cell killing, enhanced by inhibition of secondary activation of signaling networks," *Cell Death & Disease*, vol. 4, article e905, 2013.

[6] Y. Jia, Q. Guan, Y. Guo, and C. Du, "Reduction of inflammatory hyperplasia in the intestine in colon cancer-prone mice by water-extract of *Cistanche deserticola*," *Phytotherapy Research*, vol. 26, no. 6, pp. 812–819, 2012.

[7] M. Egeblad, E. S. Nakasone, and Z. Werb, "Tumors as organs: complex tissues that interface with the entire organism," *Developmental Cell*, vol. 18, no. 6, pp. 884–901, 2010.

[8] G. Kochan, D. Escors, K. Breckpot, and D. Guerrero-Setas, "Role of non-classical MHC class I molecules in cancer immunosuppression," *OncoImmunology*, vol. 2, no. 11, Article ID e26491, 2013.

[9] C. Fremd, F. Schuetz, C. Sohn, P. Beckhove, and C. Domschke, "B cell-regulated immune responses in tumor models and cancer patients," *OncoImmunology*, vol. 2, no. 7, Article ID e25443, 2013.

[10] D. Lindau, P. Gielen, M. Kroesen, P. Wesseling, and G. J. Adema, "The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells," *Immunology*, vol. 138, no. 2, pp. 105–115, 2013.

[11] S. Ostrand-Rosenberg, P. Sinha, D. W. Beury, and V. K. Clements, "Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression," *Seminars in Cancer Biology*, vol. 22, no. 4, pp. 275–281, 2012.

[12] G. Landskron, M. de La Fuente, P. Thuwajit, C. Thuwajit, and M. A. Hermoso, "Chronic inflammation and cytokines in the tumor microenvironment," *Journal of Immunology Research*, vol. 2014, Article ID 149185, 19 pages, 2014.

[13] H. W. Chung and J.-B. Lim, "Role of the tumor microenvironment in the pathogenesis of gastric carcinoma," *World Journal of Gastroenterology*, vol. 20, no. 7, pp. 1667–1680, 2014.

[14] H. Bolouri, "Network dynamics in the tumor microenvironment," *Seminars in Cancer Biology*, vol. 30, pp. 52–59, 2015.

[15] J. M. Fritz, L. D. Dwyer-Nield, and A. M. Malkinson, "Stimulation of neoplastic mouse lung cell proliferation by alveolar macrophage-derived, insulin-like growth factor-1 can be blocked by inhibiting MEK and PI3K activation," *Molecular Cancer*, vol. 10, article 1476, 2011.

[16] F. Munari, S. Lonardi, M. A. Cassatella et al., "Tumor-associated macrophages as major source of APRIL in gastric MALT lymphoma," *Blood*, vol. 117, no. 24, pp. 6612–6616, 2011.

[17] M. Yang, J. Chen, F. Su et al., "Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells," *Molecular Cancer*, vol. 10, article 117, 2011.

[18] S. E. J. Chambers, C. L. O'Neill, T. M. O'Doherty, R. J. Medina, and A. W. Stitt, "The role of immune-related myeloid cells in angiogenesis," *Immunobiology*, vol. 218, no. 11, pp. 1370–1375, 2013.

[19] B. Zhang, G. Yao, Y. Zhang, J. Gao, B. Yang, and Z. Rao, "M2-Polarized tumor-associated macrophages are associated with poor prognoses resulting from accelerated lymphangiogenesis in lung adenocarcinoma," *Clinics*, vol. 66, no. 11, pp. 1879–1886, 2011.

[20] M. de Palma and L. Naldini, "Angiopoietin-2 TIEs up macrophages in tumor angiogenesis," *Clinical Cancer Research*, vol. 17, no. 16, pp. 5226–5232, 2011.

[21] S. Ran and K. E. Montgomery, "Macrophage-mediated lymphangiogenesis: the emerging role of macrophages as lymphatic endothelial progenitors," *Cancers*, vol. 4, no. 3, pp. 618–657, 2012.

[22] R. Guiet, E. van Goethem, C. Cougoule et al., "The process of macrophage migration promotes matrix metalloproteinase-independent invasion by tumor cells," *The Journal of Immunology*, vol. 187, no. 7, pp. 3806–3814, 2011.

[23] T. Tsuji, S. Ibaragi, and G.-F. Hu, "Epithelial-mesenchymal transition and cell cooperativity in metastasis," *Cancer Research*, vol. 69, no. 18, pp. 7135–7139, 2009.

[24] C.-Y. Liu, J.-Y. Xu, X.-Y. Shi et al., "M2-polarized tumor-associated macrophages promoted epithelial—mesenchymal transition in pancreatic cancer cells, partially through TLR4/IL-10 signaling pathway," *Laboratory Investigation*, vol. 93, no. 7, pp. 844–854, 2013.

[25] B. Toh, X. Wang, J. Keeble et al., "Mesenchymal transition and dissemination of cancer cells is driven by myeloid-derived suppressor cells infiltrating the primary tumor," *PLoS Biology*, vol. 9, no. 9, Article ID e1001162, 2011.

[26] F. Iovino, S. Meraviglia, M. Spina et al., "Immunotherapy targeting colon cancer stem cells," *Immunotherapy*, vol. 3, no. 1, pp. 97–106, 2011.

[27] C. P. Beier, P. Kumar, K. Meyer et al., "The cancer stem cell subtype determines immune infiltration of Glioblastoma," *Stem Cells and Development*, vol. 21, no. 15, pp. 2753–2761, 2012.

[28] J. Yang, D. Liao, C. Chen et al., "Tumor-associated macrophages regulate murine breast cancer stem cells through a novel paracrine egfr/stat3/sox-2 signaling pathway," *Stem Cells*, vol. 31, no. 2, pp. 248–258, 2013.

[29] K.-F. He, L. Zhang, C.-F. Huang et al., "CD163+ tumor-associated macrophages correlated with poor prognosis and cancer stem cells in oral squamous cell carcinoma," *BioMed Research International*, vol. 2014, Article ID 838632, 9 pages, 2014.

[30] T. X. Cui, I. Kryczek, L. Zhao et al., "Myeloid-derived suppressor cells enhance stemness of cancer cells by inducing microRNA101 and suppressing the corepressor CTBP2," *Immunity*, vol. 39, no. 3, pp. 611–621, 2013.

[31] J. Bukur, S. Jasinski, and B. Seliger, "The role of classical and non-classical HLA class I antigens in human tumors," *Seminars in Cancer Biology*, vol. 22, no. 4, pp. 350–358, 2012.

[32] Y.-L. Li, B.-G. Sun, T. Xiang, Z.-X. Chen, and S.-J. Zhang, "Effect of invigorating spleen and detoxification decoction on MHCI/MHCII in spleen-deficiency liver cancer rats survival," *Journal of Chinese Medicinal Materials*, vol. 37, no. 3, pp. 456–462, 2014.

[33] Y.-C. Kuan, W.-T. Lee, C.-L. Hung, C. Yang, and F. Sheu, "Investigating the function of a novel protein from *Anoectochilus formosanus* which induced macrophage differentiation through TLR4-mediated NF-κB activation," *International Immunopharmacology*, vol. 14, no. 1, pp. 114–120, 2012.

[34] H. Zheng, B. Piao, H. Lin, L. Xiong, and B. Hua, "Influence of Feiliuping Extractum and its modified formulas on function of dendritic cells," *Journal of Beijing University of Traditional Chinese Medicine*, vol. 30, no. 8, pp. 525–528,549,577, 2007.

[35] M. Villa-Morales and J. Fernández-Piqueras, "Targeting the Fas/FasL signaling pathway in cancer therapy," *Expert Opinion on Therapeutic Targets*, vol. 16, no. 1, pp. 85–101, 2012.

[36] J. Li, G. Z. Sun, X. Qi, Y. X. Pei, H. F. Yan, and Y. H. He, "Effect of 'YangWeiKangLiu' granules on peripheral blood T-lymphocyte rDNA transcription activity and Fas expression in gastric cancer metastasis," *Chinese Journal of Integrative Medicine*, vol. 8, no. 4, pp. 253–255, 2002.

[37] J. Li, G.-Z. Sun, H.-S. Lin et al., "The herb medicine formula 'Yang Wei Kang Liu' improves the survival of late stage gastric cancer patients and induces the apoptosis of human gastric cancer cell line through Fas/Fas ligand and Bax/Bcl-2 pathways," *International Immunopharmacology*, vol. 8, no. 9, pp. 1196–1206, 2008.

[38] K. Li, Z. Dan, and Y.-Q. Nie, "Gastric cancer stem cells in gastric carcinogenesis, progression, prevention and treatment," *World Journal of Gastroenterology*, vol. 20, no. 18, pp. 5420–5426, 2014.

[39] F. Mannello, "Understanding breast cancer stem cell heterogeneity: time to move on to a new research paradigm," *BMC Medicine*, vol. 11, article 169, 2013.

[40] T. Szafarowski and M. J. Szczepanski, "Cancer stem cells in head and neck squamous cell carcinoma," *Otolaryngologia Polska*, vol. 68, no. 3, pp. 105–111, 2014.

[41] J. Pasquier and A. Rafii, "Role of the microenvironment in ovarian cancer stem cell maintenance," *BioMed Research International*, vol. 2013, Article ID 630782, 10 pages, 2013.

[42] R. Xie, H. Long, and B. Zhu, "Cancer stem cell and tumor immunity," *Immunological Journal*, vol. 26, no. 10, pp. 909–912, 2010.

[43] Y. Chang, Y. Zhao, H. Zhan, X. Wei, T. Liu, and B. Zheng, "Bufalin inhibits the differentiation and proliferation of human osteosarcoma cell line hMG63-derived cancer stem cells," *Tumor Biology*, vol. 35, no. 2, pp. 1075–1082, 2014.

[44] T. Zhang, K. Wang, J. Zhang et al., "Huaier aqueous extract inhibits colorectal cancer stem cell growth partially via down-regulation of the Wnt/β-catenin pathway," *Oncology Letters*, vol. 5, no. 4, pp. 1171–1176, 2013.

[45] H. C. Pan, D. W. Lai, K. H. Lan et al., "Honokiol thwarts gastric tumor growth and peritoneal dissemination by inhibiting Tpl2 in an orthotopic model," *Carcinogenesis*, vol. 34, no. 11, pp. 2568–2579, 2013.

[46] S. Ponnurangam, J. M. V. Mammen, S. Ramalingam et al., "Honokiol in combination with radiation targets notch signaling to inhibit colon cancer stem cells," *Molecular Cancer Therapeutics*, vol. 11, no. 4, pp. 963–972, 2012.

[47] M. I. Carvalho, I. Pires, J. Prada, and F. L. Queiroga, "A role for T-lymphocytes in human breast cancer and in canine mammary tumors," *BioMed Research International*, vol. 2014, Article ID 130894, 11 pages, 2014.

[48] T. F. Gajewski, H. Schreiber, and Y.-X. Fu, "Innate and adaptive immune cells in the tumor microenvironment," *Nature Immunology*, vol. 14, no. 10, pp. 1014–1022, 2013.

[49] Y. Liu, "Antineoplastic synergistic party and lung YanNing party combined with chemotherapy treatment of advanced lung adenocarcinoma random parallel control study," *Journal of Practical Traditional Chinese Internal Medicine*, vol. 27, no. 6, pp. 111–113, 2013.

[50] J. Guo, J.-Y. Wang, Z. Zheng, Q. Wang, and C.-S. Dong, "Effects of chinese herbal medicine Feiyanning Decoction on the ratio of CD4+ CD25+ regulatory T cells and expression of transcription factor Foxp3 in mice bearing lewis lung carcinoma," *Journal of Chinese Integrative Medicine*, vol. 10, no. 5, pp. 584–590, 2012.

[51] C.-P. Wan, L.-X. Gao, L.-F. Hou et al., "Astragaloside II triggers T cell activation through regulation of CD45 protein tyrosine phosphatase activity," *Acta Pharmacologica Sinica*, vol. 34, no. 4, pp. 522–530, 2013.

[52] D. Capece, M. Fischietti, D. Verzella et al., "The inflammatory microenvironment in hepatocellular carcinoma: a pivotal role for tumor-associated macrophages," *BioMed Research International*, vol. 2013, Article ID 187204, 15 pages, 2013.

[53] Q.-J. Guo and J. Li, "The role of tumor-associated macrophages in remodeling tumor immune microenvironment," *Tumor*, vol. 33, no. 10, pp. 922–927, 2013.

[54] G. Chen, K.-K. Li, C.-H. Fung et al., "Er-Miao-San, a traditional herbal formula containing Rhizoma Atractylodis and Cortex Phellodendri inhibits inflammatory mediators in LPS-stimulated RAW264.7 macrophages through inhibition of NF-κB pathway and MAPKs activation," *Journal of Ethnopharmacology*, vol. 154, no. 3, pp. 711–718, 2014.

[55] T. Zhao, Y. Feng, J. Li et al., "*Schisandra* polysaccharide evokes immunomodulatory activity through TLR 4-mediated activation of macrophages," *International Journal of Biological Macromolecules*, vol. 65, pp. 33–40, 2014.

[56] L.-Z. Meng, B.-Q. Lin, B. Wang et al., "Mycelia extracts of fungal strains isolated from *Cordyceps sinensis* differently enhance the function of RAW 264.7 macrophages," *Journal of Ethnopharmacology*, vol. 148, no. 3, pp. 818–825, 2013.

[57] S. Zhang, S. Nie, D. Huang, J. Huang, Y. Wang, and M. Xie, "Polysaccharide from Ganoderma atrum evokes antitumor activity via Toll-like receptor 4-mediated NF-κB and mitogen-activated protein kinase signaling pathways," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 15, pp. 3676–3682, 2013.

[58] J. Wang, X. Tong, P. Li, H. Cao, and W. Su, "Immuno-enhancement effects of Shenqi Fuzheng Injection on cyclophosphamide-induced immunosuppression in Balb/c mice," *Journal of Ethnopharmacology*, vol. 139, no. 3, pp. 788–795, 2012.

[59] B. Seliger and C. Massa, "The dark side of dendritic cells: development and exploitation of tolerogenic activity that favor tumor outgrowth and immune escape," *Frontiers in Immunology*, vol. 4, article 419, 2013.

[60] Y. Ma, G. V. Shurin, D. W. Gutkin, and M. R. Shurin, "Tumor associated regulatory dendritic cells," *Seminars in Cancer Biology*, vol. 22, no. 4, pp. 298–306, 2012.

[61] Y.-H. Zhang and Y.-H. Liu, "Influence of TCM therapy for supplementing Pi and nourishing Shen on dendritic cell function in patients with chronic hepatitis B treated by lamivudine," *Chinese Journal of Integrative Medicine*, vol. 15, no. 1, pp. 60–62, 2009.

[62] H.-G. Zheng, B.-K. Piao, and B.-J. Hua, "Effect of feiliuping ointment and its disassembled prescriptions on dendritic cell of mice with transplanted Lewis lung cancer," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 30, no. 12, pp. 1288–1291, 2010.

[63] Y. M. Zhou, B. K. Piao, H. G. Zheng et al., "Influence on DC stimulating LPAK anti-tumor activity by feiliuping extract and its decomposed recipe," *Cancer Research on Prevention and Treatment*, vol. 40, no. 1, pp. 3–6, 2013.

[64] Y. S. Khaled, B. J. Ammori, and E. Elkord, "Myeloid-derived suppressor cells in cancer: recent progress and prospects," *Immunology and Cell Biology*, vol. 91, no. 8, pp. 493–502, 2013.

[65] J. Li, X. Chen, H. S. Lin, J. J. Oppenheim, and O. M. Z. Howard, "Inhibitory effect of Sheng Qi Formula (SQF) on Gr-1$^+$ CD11b$^+$ myeloid immunosuppressor cells (MIC) in the 4T1 murine mammary cancer model," *AACR Annual Meeting Abstract*, vol. 2008, article 5336, 9th AACR Annual Meeting, San Diego, Calif, USA, April 2008, 2008.

[66] M. Taniguchi, K. I. Seino, and T. Nakayama, "The NKT cell system: bridging innate and acquired immunity," *Nature Immunology*, vol. 4, no. 12, pp. 1164–1165, 2003.

[67] H. Wei, R. Sun, W. Xiao et al., "Traditional Chinese medicine Astragalus reverses predominance of Th2 cytokines and their up-stream transcript factors in lung cancer patients," *Oncology Reports*, vol. 10, no. 5, pp. 1507–1512, 2003.

[68] H. Wei, R. Sun, W. Xiao et al., "Type two cytokines predominance of human lung cancer and its reverse by traditional Chinese medicine TTMP," *Cellular & Molecular Immunology.*, vol. 1, no. 1, pp. 63–70, 2004.

[69] X. Z. Chen, Z. Y. Cao, L. M. Liao, Z. Z. Liu, and J. Du, "Application of serum pharmacology in evaluating the antitumor effect of FuzhengYiliu Decoction from Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 20, no. 6, pp. 450–455, 2014.

[70] Q. Wang and D. Y. Chen, "Effect of Aidi injection on peripheral blood expression of Th1/Th2 transcription factors and cytokines in patients with esophageal squamous cell carcinoma during radiotherapy," *Zhongguo Zhong Xi Yi Jie He ZaZhi*, vol. 29, no. 5, pp. 394–397, 2009.

[71] Z. Xu, X. Chen, Z. Zhong, L. Chen, and Y. Wang, "*Ganoderma lucidum* polysaccharides: immunomodulation and potential anti-tumor activities," *The American Journal of Chinese Medicine*, vol. 39, no. 1, pp. 15–27, 2011.

[72] F. N. Razali, A. Ismail, N. Z. Abidin, and A. S. Shuib, "Stimulatory effects of polysaccharide fraction from *Solanum nigrum* on RAW 264.7 murine macrophage cells," *PLoS ONE*, vol. 9, no. 10, Article ID e108988, 2014.

[73] S. H. Bae, J. B. Park, Y. F. Zheng et al., "Pharmacokinetics and tissue distribution of ginsenoside Rh2 and Rg3 epimers after oral administration of BST204, a purified ginseng dry extract, in rats," *Xenobiotica*, vol. 44, no. 12, pp. 1099–1107, 2014.

[74] Z. Y. Tan, W. N. Xiong, X. Z. Huang, and J. Q. Liang, "Pharmacokinetics and bioavailability of ginsenoside Rg1 in rats," *Zhong Yao Cai*, vol. 36, no. 7, pp. 1121–1123, 2013.

[75] D. Peng, H. Wang, C. Qu, L. Xie, S. M. Wicks, and J. Xie, "Ginsenoside Re: its chemistry, metabolism and pharmacokinetics," *Chinese Medicine*, vol. 7, article 2, 2012.

[76] Y. Ming, Z. Chen, L. Chen et al., "Ginsenoside compound K attenuates metastatic growth of hepatocellular carcinoma, which is associated with the translocation of nuclear factor-κB p65 and reduction of matrix metalloproteinase-2/9," *Planta Medica*, vol. 77, no. 5, pp. 428–433, 2011.

[77] A. G. Musende, A. Eberding, W. Jia, E. Ramsay, M. B. Bally, and E. T. Guns, "Rh2 or its aglycone aPPD in combination with docetaxel for treatment of prostate cancer," *Prostate*, vol. 70, no. 13, pp. 1437–1447, 2010.

[78] W. Huihui, C. Yulong, and M. Junhua, "Molecular mechanism of immunity regulated by TCM in tumor patients," *China Journal of Chinese Medicine*, vol. 29, no. 197, pp. 1405–1408, 2014.

[79] B. Wang and M. Z. Chen, "Astragaloside IV possesses antiarthritic effect by preventing interleukin 1β-induced joint inflammation and cartilage damage," *Archives of Pharmacal Research*, vol. 37, no. 6, pp. 793–802, 2014.

[80] S. Sun, K. Zheng, H. Zhao et al., "Regulatory effect of astragalus polysaccharides on intestinal intraepithelial $\gamma\delta$T cells of tumor bearing mice," *Molecules*, vol. 19, no. 9, pp. 15224–15236, 2014.

[81] M. R. Stockler, F. Hilpert, M. Friedlander et al., "Patient-reported outcome results from the open-label phase III AURELIA trial evaluating bevacizumab-containing therapy for platinum-resistant ovarian cancer," *Journal of Clinical Oncology*, vol. 32, no. 13, pp. 1309–1316, 2014.

[82] S. Malempati, B. Weigel, A. M. Ingle et al., "Phase I/II trial and pharmacokinetic study of cixutumumab in pediatric patients with refractory solid tumors and Ewing sarcoma: a report from the Children's Oncology Group," *Journal of Clinical Oncology*, vol. 30, no. 3, pp. 256–262, 2012.

[83] T. J. Molina, D. Canioni, C. Copie-Bergman et al., "Young patients with non-germinal center B-cell-like diffuse large B-cell lymphoma benefit from intensified chemotherapy with ACVBP plus rituximab compared with CHOP plus rituximab: analysis of data from the Groupe d'Etudes des Lymphomes de l'Adulte/Lymphoma Study Association phase III trial LNH 03-2B," *Journal of Clinical Oncology*, vol. 32, no. 35, pp. 3996–4003, 2014.

[84] A. Ribas, R. Kefford, M. A. Marshall et al., "Phase III randomized clinical trial comparing tremelimumab with standard-of-care chemotherapy in patients with advanced melanoma," *Journal of Clinical Oncology*, vol. 31, no. 5, pp. 616–622, 2013.

[85] S. L. Topalian, M. Sznol, D. F. McDermott et al., "Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab," *Journal of Clinical Oncology*, vol. 32, no. 10, pp. 1020–1030, 2014.

[86] J. Vansteenkiste, M. Zielinski, A. Linder et al., "Adjuvant MAGE-A3 immunotherapy in resected non-small-cell lung cancer: phase II randomized study results," *Journal of Clinical Oncology*, vol. 31, no. 19, pp. 2396–2403, 2013.

[87] J. R. Brahmer, "Harnessing the immune system for the treatment of non-small-cell lung cancer," *Journal of Clinical Oncology*, vol. 31, no. 8, pp. 1021–1028, 2013.

[88] X. Yu, H. Zhao, L. Liu et al., "A randomized phase II study of autologous cytokine-induced killer cells in treatment of

hepatocelluar carcinoma," *Journal of Clinical Immunology*, vol. 34, no. 2, pp. 194–203, 2014.

[89] M. J. Atherton and B. D. Lichty, "Evolution of oncolytic viruses: novel strategies for cancer treatment," *Immunotherapy*, vol. 5, no. 11, pp. 1191–1206, 2013.

[90] M. Daher, M. E. Lacouture, D. Rathkopf et al., "Case series of dermatologic events associated with the insulin-like growth factor receptor 1 inhibitor cixutumumab," *Journal of Clinical Oncology*, vol. 29, no. 21, pp. e638–e640, 2011.

[91] M. Liu, H. Tan, X. Zhang et al., "Hematopoietic effects and mechanisms of *Fufang E'jiao Jiang* on radiotherapy and chemotherapy-induced myelosuppressed mice," *Journal of Ethnopharmacology*, vol. 152, no. 3, pp. 575–584, 2014.

[92] C. Zhao, J. Chen, B. Yu et al., "Effect of modified Taohongsiwu decoction on patients with chemotherapy-induced hand-foot syndrome," *Journal of Traditional Chinese Medicine*, vol. 34, no. 1, pp. 10–14, 2014.

[93] B. C. He, J. L. Gao, B. Q. Zhang et al., "Tetrandrine inhibits Wnt/β-catenin signaling and suppresses tumor growth of human colorectal cancer," *Molecular Pharmacology*, vol. 79, no. 2, pp. 211–219, 2011.

[94] W. C. S. Cho and K. N. Leung, "*In vitro* and *in vivo* anti-tumor effects of *Astragalus membranaceus*," *Cancer Letters*, vol. 252, no. 1, pp. 43–54, 2007.

[95] Z. Chen, L. Y. Chen, P. Wang, H. Y. Dai, S. Gao, and K. Wang, "Tumor microenvironment varies under different TCM *ZHENG* models and correlates with treatment response to herbal medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 635702, 10 pages, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 869547, 14 pages
http://dx.doi.org/10.1155/2015/869547

Review Article

"Adherent" versus Other Isolation Strategies for Expanding Purified, Potent, and Activated Human NK Cells for Cancer Immunotherapy

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Received 25 August 2014; Accepted 7 November 2014

Academic Editor: Mohammad Owais

Natural killer (NK) cells have long been hypothesized to play a central role in the development of new immunotherapies to combat a variety of cancers due to their intrinsic ability to lyse tumor cells. For the past several decades, various isolation and expansion methods have been developed to harness the full antitumor potential of NK cells. These protocols have varied greatly between laboratories and several have been optimized for large-scale clinical use despite associated complexity and high cost. Here, we present a simple method of "adherent" enrichment and expansion of NK cells, developed using both healthy donors' and cancer patients' peripheral blood mononuclear cells (PBMCs), and compare its effectiveness with various published protocols to highlight the pros and cons of their use in adoptive cell therapy. By building upon the concepts and data presented, future research can be adapted to provide simple, cost-effective, reproducible, and translatable procedures for personalized treatment with NK cells.

1. Introduction

Natural killer (NK) cells are innate immune cells that comprise 5–20% of peripheral blood mononuclear cells (PBMCs) [1]. As their name suggests, NK cells have an intrinsic ability to spontaneously lyse virally infected and cancerous cells, a function that is normally mediated by a balance of activating receptors (e.g., NKG2D) and inhibitory receptors (killer immunoglobulin-like receptors (KIR), NKG2A) [2]. The activation signals are triggered by receptors like NKG2D, which recognize stress ligands like MICA and MICB on potential target cells, and CD16, which binds to the Fc portion of IgG antibodies to initiate antibody-dependent cellular cytotoxicity (ADCC) of a target cell. Conversely, inhibitory signals triggered by KIR are capable of thwarting this activation when bound to self-MHC molecules on the target cell [3]. This prevents NK cells from lysing the body's own cells and allows effective targeting of virally infected or tumor cells, which commonly downregulate MHC as an immune escape mechanism [4]. There are two subsets of NK cells in the blood based on phenotype and function. They are $CD56^{bright}CD16^{dim}$, which tend to play an immunoregulatory role releasing cytokines like IFN-γ, TNF-β, and GM-CSF [5], and $CD56^{dim}CD16^{bright}$, which are more cytotoxic and express higher levels of CD16 [6]. When activated, these NK cells alter their receptor expression and produce higher amounts of perforin and granzyme as well as upregulating receptors involved with dendritic cell crosstalk [7].

NK cells are derived from the common lymphoid progenitor, along with T and B cells [8]. They are traditionally considered innate immune cells since they utilize germline-encoded receptors, mentioned above, to elicit their effector functions rather than undergo genetic rearrangement of immensely diverse antigen receptors. Emerging evidence, however, suggests that NK cells are more like the adaptive immune system than previously thought, which could provide even more advantages for their use in immunotherapy

[9]. They are capable of undergoing a selection process to prevent autoreactivity [10], survive for long periods of time *in vivo* [11], and undergo robust memory-like responses upon a secondary challenge with antigen [12–14].

The antitumor effects of NK cells have long been realized in *in vitro* and *in vivo* studies [15]. More recent *in vivo* studies have further elucidated NK cells' role in tumor immunity, showing that, upon depletion of NK cells using NK1.1 antibody, mice had impaired rejection of tumor cells and enhanced tumor metastasis [16]. In addition, the presence of NK cell receptor ligands and lack of MHC Class I expression on target cells have been shown to play a role in tumor rejection [17, 18]. Importantly, this NK cell-mediated tumor rejection can also induce the development of tumor-specific T cells, capable of rejecting a secondary tumor challenge [19]. However, several immunosuppressive events can occur during tumor development that can inhibit NK cell cytotoxic function and affect tumor rejection. For example, tumor-associated NK cells are shown to downregulate activating receptors and become less cytotoxic [20, 21] and downregulation of essential chemokines like CCL2 can prevent NK cell trafficking to the site of a developing tumor [22, 23]. There is also evidence that suggests that some genetic mutations that affect NK cell activation result in an increased risk to develop cancer [24].

Due to NK cells intrinsic antitumor activity, they have long been hypothesized to play a central role in new cancer immunotherapies [25]. Administration of tumor-specific antibodies like rituximab (anti-CD20) and trastuzumab (anti-Her2/neu) is thought to rely on NK cell-mediated ADCC for their antitumor activity [3, 26]. Further approaches are attempting to specifically boost NK cell ADCC, like the use of bispecific killer cell engagers (BiKEs), which initiate CD16-mediated tumor-specific lysis [27, 28]. The main focus of NK cell immunotherapy has been developing protocols to isolate NK cells from patients' peripheral blood, expand activated NK cells to large numbers, and return them to the patient in the hope of a potent antitumor effect [29–31]. Multiple approaches have been investigated to develop NK cell adoptive cell therapy using various isolation and expansion methods. For all protocols, patient's PBMCs must first be isolated through leukapheresis, a density gradient using Ficoll-Paque solution, or another Good Manufacturing Practice- (GMP-) compliant method. Further NK cell isolation can be achieved using magnetic cell sorting (MACS) to separate $CD3^{-}CD56^{+}$ cells from PBMCs [32] or T cell depletion using anti-CD3 antibody (OKT-3) [33]. Expansion and activation of NK cells has been achieved using various cytokines, namely, IL-2 [34], or using "feeder cells" that selectively activate NK cells through cell-cell contact [35, 36]. Once expanded, these NK cells have been tested to treat a wide variety of cancers, including both liquid and solid tumors [37, 38]. However, the development of GMP-compliant procedures that ensure consistent isolation and expansion of NK cells that retain their potency as well as the safety of the final product for the patient remains a major hurdle [21].

In the present study, we compare a simple and cost-effective method of "adherent" enrichment and expansion of NK cells, aimed at being directly translatable to the clinic, developed in our laboratory with various published NK cell enrichment and expansion methods by considering NK cell purity, potency, expansion, experimental procedure, cost, and the ease of translation to the clinic. These protocols have been divided into four categories based on their expansion methods: no or brief *in vitro* stimulation, cytokines, feeder cells, and, lastly, our "adherent" enrichment and expansion of NK cells.

2. No or Brief *In Vitro* Stimulation

Since it is difficult to isolate a large number of NK cells from the peripheral blood, studies have investigated the direct injection of freshly isolated or overnight stimulated NK cells. Miller et al. stimulated MACS CD3-depleted PBMCs overnight in IL-2 supplemented media [39]. This product was generated from PBMCs of haploidentical donors and contained an average of 40% NK cells. Forty-three patients were tested. Five out of nineteen AML patients, that received more intense preconditioning with cyclophosphamide and fludarabine, achieved a complete remission and survival of infused NK cells. To show survival/expansion of the NK cells, the authors used RT-PCR. They also removed expanded NK cells after 14 days during the more intense preconditioning and showed they were capable of lysing K-562 cells.

Rubnitz et al. investigated the use of haploidentical NK cells to prevent relapse of AML patients in first complete remission. Patients were preconditioned with cyclophosphamide and fludarabine followed by infusion of KIR-HLA mismatched NK cells and 6-day IL-2 administration. Engraftment was safe and successful and all ten patients remained in complete remission after two years [40]. Curti et al. treated thirteen AML patients with MACS-purified $CD56^{+}$ NK cells from KIR-HLA mismatched donors that were not stimulated *in vitro* [41]. These authors also preconditioned the patient with cyclophosphamide and fludarabine followed by infusion of 2.74×10^{6} cells/kg (product contained both NK and NK-T cells) and IL-2 dose administration. One out of five patients with active disease and two patients in molecular relapse achieved a transient complete response. Three of six patients that were in a complete remission before receiving NK cells were still in remission at the time this work was published. This treatment was also considered safe and feasible.

Stern et al. performed a two-center phase II trial treating sixteen patients with infusions of purified NK cells after a haploidentical stem cell transplant [42]. NK cells were isolated using a two-step CliniMACS procedure that depleted $CD3^{+}$ cells and then positively selected $CD56^{+}$ cells. This product was cryopreserved until its use. Four of sixteen patients were alive and still in remission at the time this work was published. However, this result is similar to historical controls and therefore, the NK cells had no apparent effect on relapse. As described, most of these studies involve patients who were in remission from hematopoietic cancers and used some preconditioning or stem cell transplant along with exogenous IL-2. In addition, they used healthy donor derived NK cells that may be more potent antitumor effector cells compared to patient's NK cells. However, this approach is

unable to generate large numbers of these NK cells due to the low percentage of NK cells in the peripheral blood especially in cancer patients [21].

3. Cytokines

3.1. IL-2. The antitumor effects of IL-2 and IL-2 treated lymphocytes have been extensively studied. Particularly, lymphokine-activated killer (LAK) cells have been generated *in vitro* by treating human PBMCs with 200 U/mL IL-2 for several days. This LAK population is highly cytotoxic against tumor cells and has been investigated in preclinical mouse models and clinical trials since the 1980s [43]. Further study of LAK cells showed that although NK cells ($CD3^-CD56^+$) comprised only a small percentage of the population, they were the most potent cytotoxic subset [44]. The ability of IL-2 to expand NK cells and enhance their cytotoxic function has also been studied since the 1980s [45–47]; however, IL-2 will also stimulate T cells. In particular, T regulatory cells (T_{regs}) express high levels of the IL-2 receptor (CD25) and can limit the availability of IL-2 to NK cells [48]. For this reason, MACS purification or anti-CD3 depleting antibody is typically used to enhance NK cell purity. This has been effective at expanding NK cells but can cause IL-2 dependence of NK cells as well as toxicity in patients when the cytokine is given at high doses [49].

In 2001, Carlens et al. achieved an NK expansion with relatively high purity using IL-2 and OKT-3 (anti-CD3 antibody) [34]. OKT-3 has been shown to deplete T cells by binding to and internalizing CD3 [50]. By using IL-2 supplemented Cellgro SCGM media with 5% human serum and OKT-3, a 193-fold expansion of 55% pure NK cells was observed after 21 days, as determined by $CD3^-CD56^+$ cells. This initial proof-of-principle study performed in healthy donors was further verified using PBMCs from multiple myeloma (MM) patients. This study, published by the same group, now demonstrated a 1,625-fold expansion of patient-derived NK cells with 65% purity [30]. The expanded NK cells showed increased cytotoxicity against MM target cells and higher percentage of these NK cells underwent degranulation, as determined by CD107a staining. Importantly, these cells were not cytotoxic against normal host cells. This approach also uses all GMP-compliant components. In the initial clinical trial investigating this method, Barkholt et al. expanded $CD56^+$ cells (NK and NK-T cells) from PBMCs of haploidentical donors as described above [51]. Each patient in the trial had received an allogeneic stem cell transplant. After a 20-day expansion, $CD3^-CD56^+$ cells expanded an average of 6,000-fold and made up ~35% of the cell product. These cells were infused to five patients with various cancers, followed by two further infusions of cryopreserved cells. Only one patient, who had hepatocellular carcinoma, had a detectable response determined by decreased serum α-fetoprotein levels. This approach was also deemed safe albeit with less percent of NK cells. More recently, a large-scale protocol has been adapted to use closed-system bioreactors [52]. By automating this process in a closed system, the ability to undergo clinical trials may now be more feasible.

Expanding NK cells with IL-2 alone would be ideal for the translation of NK cell expansion protocols because GMP-compliant recombinant human IL-2 is widely available and relatively inexpensive. However, the nonspecific properties of IL-2 also cause expansion of contaminating T cells. If expanded PBMCs are from a donor rather than the patient being treated, stringent isolation methods are required to ensure a purified NK cell product to prevent contaminating T cells from causing GVHD in the recipient. These isolation methods can further complicate GMP-compliant protocols, particularly when performing large-scale expansions.

3.2. IL-15. Due to the difficulty in achieving large numbers of pure NK cells using only IL-2, other cytokines like IL-7 [53], IL-21 [29], and IFN-γ [33] have been investigated to improve NK expansion and potency. Namely, IL-15 has been a major cytokine used to upregulate NK cell activating receptors and induce proliferation [54]. Studies suggest that IL-15 may not help with proliferation but may preserve the viability of NK cells, achieving a better yield [55]. However, more recent studies suggest IL-15 is not required for survival of human NK cells and does not augment or support T_{regs} *in vivo* [56]. We also observed similar enhanced viability of NK cells with higher percent lysis of K-562 cells when combining IL-2 and IL-15 in our method of "adherent" enrichment and expansion of NK cells as described below (data not shown; see Section 5).

Klingemann and Martinson showed successful expansion after MACS positive selection of $CD56^+$ cells. The use of IL-2 or a combination of IL-2 and IL-15 was compared over a 14-day period under GMP conditions [32]. Although similar expansions occurred using both culture conditions, the combination of cytokines allowed NK cells to lyse a higher percentage of target cells at a 1 : 1 E : T ratio. Starting with a population of 60% $CD3^-CD56^+$ and 30% $CD3^+CD56^+$ after MACS selection, the authors note that this expansion varied greatly and $CD3^+CD56^+$ cells (NK-T cells) expanded 2-3 times as much as $CD3^-CD56^+$ cells (NK cells) although exact percentages were not shown.

Suck et al. published a comparison of IL-2 and IL-15 supplemented CellGro SCGM using GMP-compliant conditions to expand NK cells within a LAK population [57]. OKT-3 was included in the media throughout the first five days to deplete the T cells. Data showed superior long-term NK cell expansion and cytotoxic ability using IL-15 compared to IL-2. Over a 4-week period, an average of 2,320-fold NK cell expansion was observed compared to 1,084-fold using IL-2. The purity of NK cells was 40%–50% after a 15-day culture. In addition, both activating and inhibitory receptors were upregulated in the IL-15 expanded NK cells but not the IL-2 expanded NK cells. This expansion was also modified to use Baxter Lifecell culture bags, showing the reproducibility of this protocol in a closed-system.

While some success has been documented in expanding NK cells with cytokines other than IL-2, a consensus cytokine or method has not emerged to demonstrate a clear advantage. In some cases, combination with IL-2 is needed and when the expansion is compared with IL-2 alone, only a slight benefit is observed. In addition, it would be difficult to test

these protocols in clinical trials. Most cytokines, like GMP-compliant IL-15, are much more expensive and less common than IL-2, whereas other cytokines, like IL-21, shown to increase cytotoxicity and cytokine production in NK cells, are not yet available for clinical use [58].

4. Feeder Cells

4.1. Tumor Cell Lines as Feeder Cells. For more than 20 years, feeder cells have also been considered a potential strategy to expand and activate NK cells [59]. By using cells capable of being lysed by NK cells, like K-562, or those capable of providing crosstalk, like PBMCs [60], activation signaling can be provided to NK cells to stimulate growth and cytotoxic activity. These feeder cells are normally irradiated before coculture to inhibit growth. Genetic modification has been used in many cases to express NK cell activation signals, like 4-1BBL and membrane bound IL-15 (mbIL-15). Most protocols use these feeder cells in addition to IL-2 to achieve dramatic NK expansions.

In 2002, Harada et al. successfully used a Wilms tumor cell line, HFWT, as feeder cells for natural killer cell expansion [35]. HWFT is an NK-sensitive, adherent cell line with downregulated MHC class I expression. On day 0, 1×10^6 healthy donor PBMCs were seeded at a 10 : 1 E : T with HFWT target cells in IL-2 supplemented RHAMα media. This method obtained a 401-fold expansion with 77% purity of $CD3^-CD56^+$ cells over a 21-day procedure. This approach also produced better expansion and purity than unmodified K-562 cells. These expanded cells were cytotoxic against live HFWT cells as well as a gliosarcoma cell line. NK cells could also be expanded from a patient and were shown to be cytotoxic against autologous brain tumor cells. Also, a final experiment confirmed the need for cell-cell contact between feeder cells and NK cells to initiate activation. This was performed using cell inserts to prevent direct interaction of feeder and PBMCs in the same well. Only 5% of cells above the filter were NK cells compared to 78% NK cells in contact with the feeder cells, showing that direct cell contact is necessary for activation. This protocol was tested in a clinical trial by the same group in Japan to treat grade-3 and grade-4 glioma patients [61]. Autologous NK cells were isolated from patients with an average purity of 82.2%. In addition, adjuvant low-dose IFN-β was administered weekly. Three partial responses and two mixed responses occurred. In a recent follow-up article, the authors mention that this remains a potentially effective therapy but they are investigating vaccine strategies, in part due to the high costs of *ex vivo* expansion [62].

In 2005, Imai et al. genetically modified K-562 cells to express mbIL-15 and 4-1BBL using retroviral transduction [63]. The authors mention several reasons for this approach: NK cells have previously been stimulated with K-562 cells, modified K-562 cells have successfully expanded $CD8^+$ T cells, and IL-15 is more potent when presented to NK cells in a membrane bound form [64]. Optimal NK cell number and purity were obtained when culturing with K-562 cells that expressed both IL-15 and 4-1BBL in IL-2 supplemented RPMI-1640 media. Additionally, the transduction of an anti-CD19 chimeric antigen receptor gave NK cells the ability to overcome tolerance of B cell leukemia. Ultimately, NK cells expanded to >1,000-fold after a 21-day protocol. The resulting NK cells were highly cytotoxic against patient leukemia cells. In later work, this method was directly compared to IL-2, IL-15, and IL-21 in a 7-day treatment of PBMCs [29]. Here, NK cells expanded with feeder cells yielded larger number of cells and exhibited higher cytotoxicity against target cells. Next, a large-scale expansion protocol was optimized expanding NK cells with K-562-mbIL15-4-1BBL feeder cells in VueLife bags using IL-2 supplemented Cellgro SCGM media [29]. This approach, named the Natural Killer Cell Activation and Expansion System (NKAES), was geared towards treating acute myeloid leukemia (AML) and expanded cells were shown to lyse tumors cells both *in vitro* and *in vivo*. Also, the authors established a master cell bank using GMP guidelines that included safety measures to ensure no cell growth and no viability of K-562 cells after NK expansion. After seven days, the NK cells had expanded between 33- and 141-fold with 83.1% purity. Efficacy of these expanded cells to treat solid tumors like Ewing sarcoma, rhabdomyosarcoma, neuroblastoma, and osteosarcoma was shown by demonstrating NK cell lysis of cell lines both *in vitro* and *in vivo* [37]. This extensively studied expansion method supports the potential to treat both solid and liquid tumors as well as expand genetically modified NK cells for targeting specific cancers [65].

Several other feeder cell protocols have also been successful expanding NK cells. Denman et al. used irradiated K-562 cells expressing membrane bound IL-21 and IL-2 supplemented media to achieve a mean 47,967-fold expansion of NK cells in 21 days [66]. This can be performed starting with PBMCs or purified NK cells in tissue culture flasks. More recently, this expansion method was shown to generate expanded NK cells from PBMCs from children with neuroblastoma [67]. These expanded NK cells were 83% $CD3^-CD56^+$ cells and were cytotoxic against neuroblastoma cell lines *in vitro* and *in vivo*. Cytotoxicity was increased with the addition of an anti-GD2 antibody. Also, these cells could be cryopreserved and maintain their antitumor activities.

Dowell et al. introduced an ovarian carcinoma cell line (OVCAR) modified to express 4-1BBL and IL-12 using an adenoviral vector [68]. These feeder cells were cocultured with nonadherent PBMCs in IL-2 supplemented media to achieve a 29.7-fold expansion that yielded 70–90% $CD3^-CD56^+$ cells in 7 days and this approach supported 21-day NK cell culture. A phenotypic analysis of the expanded NK cells showed they were mainly $CD56^{bright}CD16^-$ NK cells and lysed K-562 and OVCAR cell lines.

Berg et al. demonstrated an NK expansion method using an Epstein-Barr virus-transformed lymphoblastoid cell line (EBV-LCL) feeder cells, a cell line shown to be safe for clinical trials [31]. The protocol begins with 2×10^8 NK cells purified using the CliniMACS system, selecting $CD3^-CD56^+$ PBMCs. It requires only one round of stimulation with EBV-LCL feeder cells and continued culture with IL-2 supplemented *X-VIVO* 20 media in Baxter Lifecell bags. This results in

up to a 850-fold expansion of 98% pure $CD3^-CD56^+$ cells after a 2-3-week culture [31, 55]. These expanded NK cells will be tested in combination with bortezomib, an FDA approved proteasome inhibitor that has been shown to sensitize tumor cells to TRAIL-dependent NK cell lysis. One potential roadblock could be obtaining the initial 2×10^8 NK cells required to begin this procedure due to the low percentage of NK cells in PBMCs, especially in cancer patients. Also, the expanded NK cells were shown to have IL-2 dependence. When IL-2 was removed from the media or cells were frozen, NK cells became inactivated. Although restoration of the activated NK cell phenotype could be restored by readministering IL-2, this might require administration of IL-2 to the patient. Finally, the authors note that IL-2 dependence could potentially prevent attempts to freeze cells to use for multiple administrations. Preliminary results of a phase I dose escalation study treating chromic lymphoid leukemia (CLL) and other metastatic tumors indicate that administering 1×10^8 NK cells/kg is safe and tolerated. At the time of this report, seven out of fourteen patients had stable disease, two patients had a 30% decrease in serum tumor markers, and one minor response of a metastatic kidney cancer patient was noticed [69].

4.2. PBMCs as Feeder Cells. Another strategy shown to expand NK cells is the use of irradiated PBMCs as feeder cells. In 2002, Luhm et al. published a GMP-compliant NK cell expansion method using irradiated PBMCs, IL-2, and IL-15 in a closed system [70]. NK cells were isolated using negative MACS selection and feeder cells were generated by irradiating PBMCs after 3–5 days in culture. Using this approach, the authors achieved an 80- to 200-fold expansion of NK cells that were 75% $CD3^-CD56^+CD16^+$ and highly cytotoxic against various tumor cell lines. A similar protocol was used by Siegler et al. to achieve single-KIR^+ NK cells using CliniMACS [71]. After a 268.3-fold expansion with 99% $CD3^-CD56^+$ cells over 19 days using irradiated PBMCs with IL-2, IL-15, and OKT-3, these expanded NK cells were highly cytotoxic against primary human AML blasts both *in vitro* and in an NSG xenograft mouse model. Several other preclinical papers use this approach and demonstrate the ability of PBMCs to act as feeder cells, expanding autologous or allogeneic NK cells [36, 72, 73].

Parkhurst et al. treated eight patients with metastatic melanoma or renal cell carcinoma after lymphodepletion [74]. NK cells were expanded from MACS CD3-depleted PBMCs cultured with irradiated autologous PBMCs, OKT-3, and IL-2 over 21 days. Patients received an average of 4.7×10^{10} cells containing 96% $CD3^-CD56^+$ cells. These cells were highly cytotoxic *in vitro* and were deemed safe *in vivo*. However, no clinical responses were observed despite persistence of these cells in the patients.

5. "Adherent" Enrichment and Expansion of NK Cells

Some human NK cells have been shown to become "adherent" upon IL-2 activation [75–77]. These adherent NK cells (A-NK) were characterized to possess higher cytotoxicity against a variety of hematopoietic and nonhematopoietic (solid) tumors *in vitro* and in experimental *ex vivo* models than activated nonadherent NK cells (NA-NK) [78–80]. However, these studies did not attempt to enrich and expand the "adherent" enriched NK cells. Here, we report a novel proof-of-principle, cost effective, and simple technology of "adherent" enrichment and subsequent expansion of NK cells for adaptive cell therapy applications utilizing ionomycin and IL-2 priming/activation of NK cells in PBMCs. Costimulatory cell contact-dependent factors as well as a primary mitogenic stimulus are required to jump-start optimal proliferation of resting NK cells [81, 82]. Thus, we used IL-2 to prime the resting NK cells and ionomycin to administer essential costimulatory early signals that bypass the requirement of cell contact-dependent mechanism [83, 84]. Cryopreserved PBMCs from healthy donors and metastatic cancer patients (blood samples from patients were obtained using Thomas Jefferson University Institutional Review Board (IRB) approved protocol) were thawed, washed, counted, and checked for viability (Countess, Life Technologies) and primed with ionomycin (0.75 μg/mL Sigma) and 1000 U/mL IL-2 (Proleukin, Prometheus Laboratories Inc.) in complete SCGM medium (CellGenix) containing 10% Human AB Serum (HABS; Gemini), 1x Glutamax (Gibco by Life Technologies), and 25 U/mL penicillin and 25 mg/mL streptomycin (Cellgro) and cultured in tissue culture-treated flask (Corning) overnight at 37°C incubator with 5% CO_2. On day 1, floating cells and ionomycin were removed by washing the flask 2-3 times with warm IMDM (Gibco by Life Technologies). The left over adherent cells in the flask were cultured with fresh complete SCGM and 1,000 U/mL IL-2. On days 3 and 5, equal amount of fresh medium was added and the concentration of IL-2 was adjusted to 500 U/mL IL-2. On day 7, adherent enriched and expanded cells were harvested, counted, and checked for percent viability and put back in culture at $1–1.5 \times 10^6$ cells/mL in complete SCGM with 500 U/mL IL-2. Every 2-3 days, an equal amount of fresh medium was added and IL-2 concentration was adjusted to 500 U/mL IL-2 throughout the duration of the two-week culture. At the end of the culture period, expanded cells were harvested using HBSS (Corning Cellgro) with 1% HABS and 0.25 M EDTA (Invitrogen by Life Technologies), washed, counted, and checked for viability. In addition, flow cytometry was performed for determining phenotype of cells present in the culture (NK, T, and NK-T cells) using anti-CD3 and CD56 antibodies, expression of CD16 and CD62L, and cytotoxicity against K-562 cells using CFSE/7AAD cytotoxicity assay kit (Abnoa).

In three preliminary experiments using our "adherent" enrichment and expansion protocol, NK cells from 25×10^6 PBMCs of each healthy donor, #3, #4, and #21, yielded $68.3\% \pm 16.5\%$, $15.8\% \pm 0.9\%$, and $89.0\% \pm 6.2\%$ NK cells with a final expanded NK cell number of $92.5 \pm 2.8 \times 10^6$, $18.6 \pm 0.5 \times 10^6$, and $40.0 \pm 0.1 \times 10^6$, respectively. Based on this, experiments were set out to assess the feasibility of enriching and expanding NK cells from PBMCs of solid metastatic cancer patients. As shown in Table 1(a), nine cancer patients' PBMCs (median 24.6×10^6 cells) were primed overnight.

The resulting adherent cells were then enriched and expanded for two weeks, NK cell purity was 82%–98% (median 88%) in eight out of nine cases and 64% in one case (Figure 1(a)). The median percent viability was 92% with median viable total expanded cells of 64×10^6. Within the total cells, the median NK cells were 57×10^6 cells. Based on availability of patient samples, expansions were repeated with PBMCs from patient donors, CH, RS, WN, and MT, and NK cell purity of 67%, 98%, 93%, and 83% was obtained, respectively. Due to enhanced purity of NK cells from cancer patients' PBMCs, healthy donor PBMCs were used to further test and improve the "adherent" NK cells enrichment methodology particularly focusing on the removal of nonadherent cells effectively on day 1 to enhance the purity and number of NK cells (Table 1(b)). In the small-scale attempts, six healthy donors' PBMCs (median 26.2×10^6 cells) were primed overnight. The resulting adherent cells were then enriched and expanded for two weeks. This improved NK cell purity of median 95% was achieved in five out of six cases and it was 70% in one case (Figure 1(b)). The median percent viability was 93% with median viable total expanded cells of 232×10^6. Within the total cells, the median NK cells were 220×10^6 cells. To note, compared with initial preliminary experiments with healthy donor #4 and donor #21, we obtained much greater NK cell purity and yield using the improved washing step on day 1 as shown in the Table 1(b).

Large-scale expansion was also successful using our methodology. Initial 40 million PBMCs of healthy donor SS* (Table 1(b)) yielded 580 million enriched NK cells with a purity of 99% NK cells after 14 days. Using another healthy donor #21*, 90 million PBMCs were primed and then "adherent" enriched and expanded; the cell yield was 450×10^6 cells with a purity of 92% NK cells in two weeks (Table 1(b)). To note, in a previous experiment testing of 82×10^6 PBMCs from donor #21 yielded 389×10^6 NK cells with a purity of 69% (with 16% T cells and 17% NK-T cells) and a viability of 94% (data not shown). Notably, as shown in Table 1(b) for the same donor (*21), the purity and yield of NK cells were greatly enhanced using the improved day 1 washing step, which seems to be critical in obtaining enhanced NK cell purity using the current "adherent" enriched protocol. Importantly, effective removal of floating cells increased not only the purity of NK cells but also the number of NK cells within the expanded total number of cells.

Although the numbers and the composition of adherent cells in the flask on day 1 were not determined in each case, a separate experiment suggests that about 40% of the total NK cells (~10% of total PBMCs) were recovered in the adherent population which expanded to yield the final expanded NK cells. Others reported only a small portion (4–30%) is capable of responding to IL-2 stimulation by adherence [77, 79]. The cell composition of the combined floating and adherent cells was determined by flow cytometry using anti-CD3 and anti-CD56 antibodies after overnight stimulation with IL-2 and ionomycin. It contained 25% NK cells, 43% T cells, 14% NK-T cells, and 18% negative cells. In the floating cells, 15% were NK cells, 57% were NK cells, 16% were NK-T cells, and 13% were negative cells. To note, due to small-scale attempt, we could not obtain sufficient number of adherent cells to carry out the flow cytometry analysis. Based on the above cell composition, we recommend to start with median 220×10^6 "healthy" donor PBMCs in culture to achieve about 2×10^9 NK cells with median purity of 95%, in about two weeks. For "cancer" patients' PBMCs, we recommend median 870×10^6 PBMCs to achieve 2×10^9 NK cells due to the lower starting percentage of NK cells in majority of the patients' PBMCs (Table 1(a)). However, using the improved washing step to remove the nonadherent cells on day 1 as shown in Table 1(b), the starting number of PBMCs can be significantly reduced. These recommendations will be further verified in the future experiments. In addition, it is to note that advantage of our methodology lies on obtaining potent activated A-NK cells which will expectedly reduce the number of required cells to transfer into patients compared to total activated mixed NK cells (adherent and nonadherent NK cells) with potentially reduced potency of NK cells. The expanded cells from both cancer patients and healthy donor PBMCs exhibited cytotoxicity against K-562 cells after expansion for 14 days (Figures 1(a) and 1(b)). In general, the lesser purity of NK cells showed decreased percent killing at various E : T cells tested. In one case, we tested the cytotoxicity of expanded NK cells against allogeneic human uveal melanoma cells and observed potent cytotoxicity (data not shown).

CD16 (Fcγ receptor III) is expressed on NK cells involved in facilitating ADCC by binding to the Fc portion of various antibodies and is directly involved in the lysis of some virus-infected cells and tumor cells [85]. Within our enrichment and expansion method, CD16 was widely expressed on NK cells (>80%; data not shown) and was unchanged after *ex vivo* enrichment and expansion of NK cells. However, the change in the MFI of CD16 on expanded NK cells (defined as >30% variation of MFI) from cancer patient donor PBMCs revealed CD16 MFI lower in three, higher in two, and the same in four cases compared to level on NK cells in PBMCs (Table 2). A different pattern was observed in expanded NK cells from healthy donor PBMCs with higher MFI of CD16 in five and the same level in three cases, and none had lower CD16 MFI. Previous studies have shown that NK cell activation by cytokines and target cell stimulation led to marked decreases in CD16, potentially mediated by a disintegrin and metalloproteinase-17 (ADAM-17) upon activation (as well as loss of CD62L; Table 3) [86]. However, we have seen a mixed pattern of expression of CD16 levels, which may be attributed to the culture conditions and also donor variation. In this regard, metalloproteinase inhibitors could be included during *ex vivo* enrichment and expansion of "adherent" NK cells to enhance the level of CD16 [87].

CD62L (L-Selectin) on NK cells plays an important role in its recruitment from the circulation into the bone marrow and secondary lymphoid tissues which after restimulation can accomplish multiple effector tasks [88, 89]. We assessed the change in the expression of CD62L in terms of percent of positive cells and MFI of NK cells within the expanded cells compared to its expression on corresponding NK cells in the PBMCs of healthy donors. Expanded NK cells were of median 30% $CD62L^+$ from small-scale enrichment and expansion,

Table 1: Cell count, viability, phenotype distribution, and fold expansion of "adherent" enriched and expanded cells from PBMCs of (a) cancer patients and (b) healthy donors.

(a)

Donor	PBMCs			Enriched and expanded cells on day 7		Enriched and expanded cells on day 14					
	Viable cell count	Percent viability	Percent NK cells ($CD3^-CD56^+$)	Viable cell count	Percent viability	Total viable cell count	Viable NK cell count	Percent NK cells ($CD3^-CD56^+$)	Percent T cells ($CD3^+CD56^-$)	Percent NK-T cells ($CD3^+CD56^+$)	Percent viability
SB	20.0×10^6	82	30.5	14.0×10^6	88	72.0×10^6	63.3×10^6	87.9	9.2	1.5	92
CH	32.0×10^6	87	5.4	9.9×10^6	78	108.0×10^6	69.3×10^6	**64.2**	**22.1**	**12.6**	93
TP	10.0×10^6	90	7.9	5.6×10^6	58	36.0×10^6	30.8×10^6	85.5	12.2	1.6	86
RS	24.0×10^6	84	8.8	12.0×10^6	93	136.0×10^6	111.1×10^6	**81.7**	**15.8**	**1.5**	95
JB	24.6×10^6	87	14.3	16.8×10^6	84	64.0×10^6	56.6×10^6	88.4	4.1	7.0	91
WN	25.0×10^6	92	25.4	9.4×10^6	89	70.0×10^6	66.7×10^6	95.3	3.2	1.0	92
GW	23.5×10^6	94	6.4	12.0×10^6	92	30.0×10^6	27.2×10^6	90.6	6.9	0.5	88
MT	29.5×10^6	91	14.3	22.0×10^6	82	54.0×10^6	47.0×10^6	87.0	5.5	6.4	95
LS	25.0×10^6	87	4.0	13.0×10^6	92	40.0×10^6	39.2×10^6	**98.0**	**0.8**	**1.0**	91

(b)

Donor	PBMCs			Enriched and expanded cells on day 7		Enriched and expanded cells on day 14					
	Viable cell count	Percent viability	Percent NK cells ($CD3^-CD56^+$)	Viable cell count	Percent viability	Total viable cell count	Viable NK cell count	Percent NK cells ($CD3^-CD56^+$)	Percent T cells ($CD3^+CD56^-$)	Percent NK-T cells ($CD3^+CD56^+$)	Percent viability
2	26.4×10^6	80	13.1	42.0×10^6	79	216.0×10^6	203.5×10^6	94.2	4.2	0.8	84
JL	27.0×10^6	84	17.2	19.0×10^6	94	180.0×10^6	152.6×10^6	**84.8**	**8.1**	**6.1**	94
4	28.0×10^6	77	5.7	50.0×10^6	95	409.2×10^6	285.6×10^6	**69.8**	**14.5**	**15.4**	97
AS	20.0×10^6	79	17.0	17.4×10^6	93	320.0×10^6	304.6×10^6	95.2	1.3	2.8	90
SS	25.0×10^6	86	15.7	21.0×10^6	93	201.6×10^6	197.4×10^6	97.9	0.8	0.5	91
*SS	40.0×10^6	85	13.9	48.0×10^6	95	580.0×10^6	573.0×10^6	**98.8**	**0.6**	**0.4**	93
21	26.0×10^6	70	19.8	19.2×10^6	85	248.0×10^6	235.4×10^6	94.9	2.5	2.4	94
*21	96.0×10^6	91	19.8	75.0×10^6	90	451.1×10^6	415.0×10^6	92.0	2.6	4.7	86

*Large-scale expansion.

Bold numbers represent low, medium and high percent expanded NK cells with respective presence of T cells and NK-T cells.

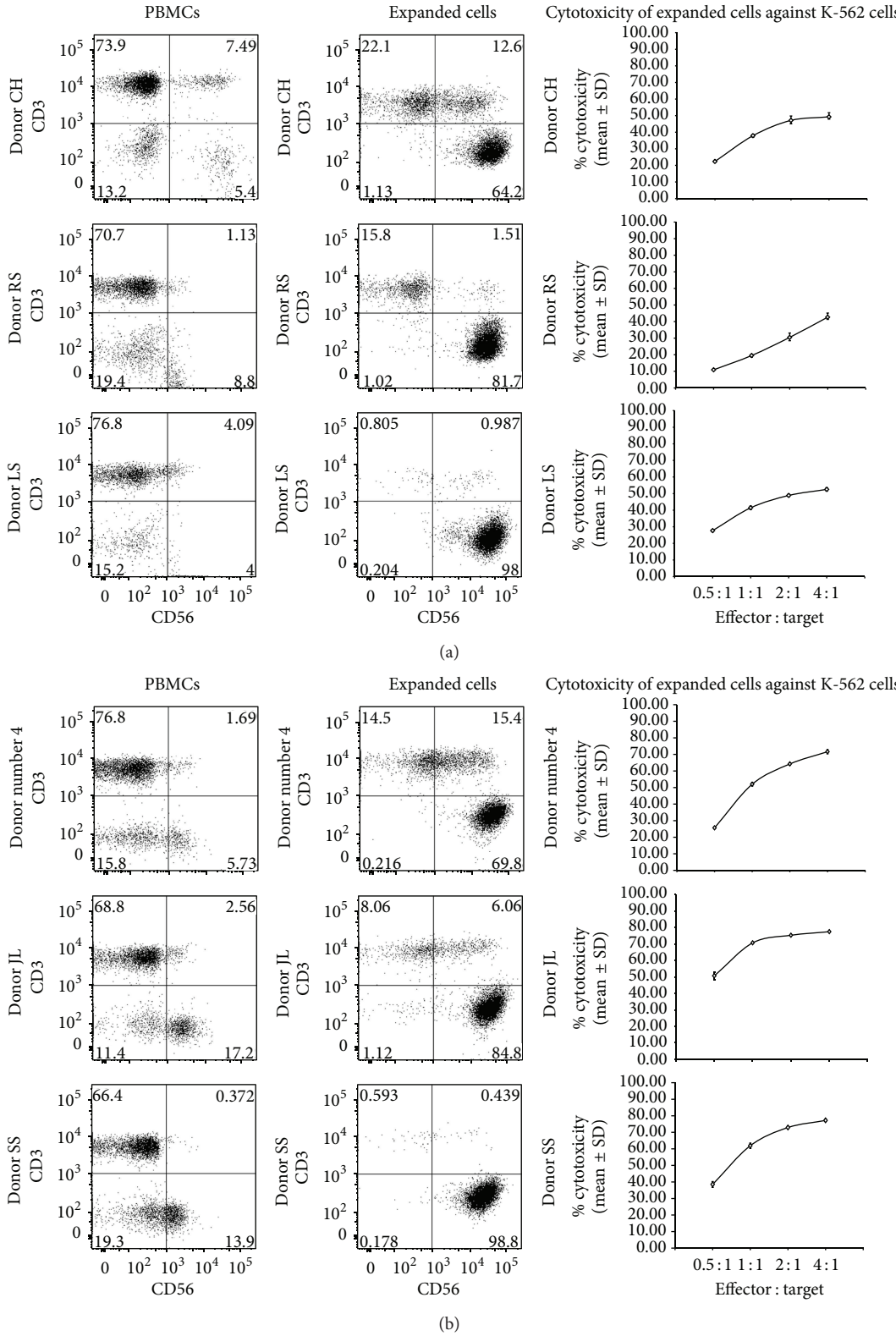

Figure 1: Select (low, medium, and high percent expanded NK cells; Refer to bold cases in Tables 1(a) and 1(b)) flow cytometry dot blots of phenotype of (a) cancer patient and (b) healthy donor PBMCs and corresponding expanded cells, and cytotoxicity of expanded cells against K-562 cells. PBMC populations and two-week expanded cells were stained by incubating with Pacific Blue conjugated anti-CD3 (Clone HIT3a, Biolegend) and Phycoerythrin (PE/Cy7) conjugated anti-CD56 antibodies (Clone MEM-188, Biolegend) and fixed in 2% paraformaldehyde. For all flow cytometry experiments, appropriate IgG isotype controls were used to assess nonspecific staining. Cells were analyzed using a BD LSRII FACS flow cytometer and the data was processed using FlowJo Flow Cytometry Analysis Software (TreeStar Inc.). Cytotoxicity of expanded cells against CFSE-labeled K-562 cells was carried out at various E : T using 7-AAD/CFSE cell-mediated cytotoxicity assay kit (Abnoa).

Table 2: Change in the MFI of CD16 on $CD3^-CD56^+$ cells of PBMCs and "adherent" enriched and expanded cells. Analysis of CD16 expression on $CD3^-CD56^+$ cells of PBMCs and "adherent" enriched and expanded cells. PBMC populations and two-week expanded cells were stained with Pacific Blue conjugated anti-CD3 (Clone HIT3a, Biolegend), Phycoerythrin-Cyanine 7 (PE/Cy7) conjugated anti-CD56 (Clone MEM-188, Biolegend), and FITC conjugated anti-CD16 (Clone 3G8, Biolegend) antibodies and fixed in 2% paraformaldehyde. For all flow cytometry experiments, appropriate IgG isotype controls were used to assess nonspecific staining. Cells were analyzed using a BD LSRII flow cytometer (BD Biosciences) and the data was analyzed using FlowJo Flow Cytometry Analysis Software (TreeStar Inc.).

Donor	Percent NK cells in PBMCs	Percent NK cell purity on day 14	CD16 MFI of NK cells in PBMCs	CD16 MFI of day 14 NK cells	Change in $CD16^+$ NK cell MFI after enrichment and expansion for 14 days
Cancer patient donor PBMCs					
SB	30.5	87.9	22,746	11,512	49.4% lower
CH	5.4	64.2	13,600	24,478	80.0% higher
TP	7.9	85.5	10,198	10,833	6.2% higher
RS	8.8	81.7	15,150	10,545	30.4% lower
JB	14.3	88.4	10,644	8,191	23.0% lower
WN	25.4	95.3	11,369	19,061	67.7% higher
GW	6.4	90.6	14,215	3,221	77.3% lower
MT	14.3	87.0	9,624	12,104	25.8% higher
LS	4.0	98.0	10,698	10,833	1.2% higher
Healthy donor PBMCs					
2	13.1	94.2	25,808	36,272	40.5% higher
JL	17.2	84.8	14,687	19,563	33.2% higher
4	5.7	69.8	13,828	22,281	61.1% higher
AS	17.0	95.2	24,726	22,696	8.2% lower
SS	15.7	97.9	14,761	21,258	44.0% higher
*SS	13.9	98.8	14,613	20,062	37.3% higher
21	19.8	94.9	11,484	14,322	24.7% higher
*21	19.8	92.0	11,484	10,732	6.5% lower

*Large-scale expansion.

Table 3: Change in the percent and MFI of CD62L on $CD3^-CD56^+$ cells from healthy donor PBMCs after enrichment and expansion. Analysis of CD62L expression on $CD3^-CD56^+$ cells before and after enrichment and expansion. PBMC populations and two-week expanded cells were stained with Pacific Blue conjugated anti-CD3 (Clone HIT3a, Biolegend), Phycoerythrin-Cyanine 7 (PE/Cy7) conjugated anti-CD56 (Clone MEM-188, Biolegend), and Allophycocyanin (APC) conjugated anti-CD62L (Clone P3.6.2.8.1, eBioscience) antibodies and fixed in 2% paraformaldehyde. For all flow cytometry experiments, appropriate IgG isotype controls were used to assess nonspecific staining. Cells were analyzed using a BD LSRII flow cytometer (BD Biosciences) and the data was analyzed using FlowJo Flow Cytometry Analysis Software (TreeStar Inc.).

Donor	In PBMCs			In expanded NK cells on day 14			Culture-induced change	
	Percent NK cells	Percent $CD62L^+$ NK cells	CD62L MFI of NK cells	Percent NK cell purity	Percent $CD62L^+$ NK cells	MFI of CD62L	Change in $CD62L^+$ NK cells after expansion	Change in $CD62L^+$ NK cell MFI after expansion
2	13.1	30.8	5,647	94.2	24.8	4,383	19.5% lower	22.4% lower
JL	17.2	17.3	5,689	84.8	35.3	4,394	104.0% higher	32.8% lower
4	5.7	14.4	2,846	69.8	25.7	3,579	78.5% higher	25.8% higher
AS	17.0	68.5	13,316	95.2	62.9	9,119	8.2% lower	31.5% lower
SS	15.7	69.0	6,672	97.9	34.9	3,373	49.4% lower	49.4% lower
*SS	13.9	65.7	6,927	98.8	50.1	5,891	23.7% lower	15.0% lower
21	19.8	36.0	5,891	94.9	14.7	2,953	59.2% lower	49.1% lower
*21	19.8	36.0	5,891	92.0	23.6	2,537	34.4% lower	56.9% lower

*Large-scale expansion.

Table 4: Advantages and disadvantages of methods of NK cell isolation and expansion.

NK cell isolation approach			NK cell expansion approach		
Method	Advantages	Disadvantages	Method	Advantages	Disadvantages
CliniMACS	(i) Allows a highly purified NK cell product (ii) Clinical-grade separation method approved for GMP use	(i) Complicated protocol and increased costs (ii) Two selections may be necessary (CD3 depletion/CD56 positive selection) (iii) Positive selection by anti-CD56 antibody binding could affect NK cell activation	No or brief stimulation	(i) Does not require long-term cell culture (ii) Minimal costs of cytokines and reagents (iii) Individual clinical trials have been able to treat a large number of patients due to minimal labor	(i) Require large number of starting PBMCs (ii) Low percentage and cell number of NK cells (iii) Minimal reactivation of NK cells that may be preventing antitumor response
OKT-3	(i) Widely used antibody to eliminate $CD3^+$ cells from culture (ii) GMP-grade antibody is available (iii) Eliminates need for expensive MACS procedure and simplifies the process	(i) Percent of NK cells in final culture varies (ii) Antibody needed for several days, while T cells are present and they could compete with NK cells for cytokines, limiting their effect	Cytokines	(i) GMP-grade IL-2 is widely available and affordable, allowing effective translation (ii) Capable of generating highly activated NK cells from patients (iii) Large-scale expansions have been successful and reproducible	(i) Purity of final NK cell product has traditionally been suboptimal (ii) Low final NK cell number compared to feeder cell expansion (iii) Cytokines other than IL-2 are more expensive and most are not GMP-grade yet.
Adherent selection	(i) Minimal cost needed (ii) Simple and reproducible (iii) Overnight selection, allowing quick elimination of T cells and preventing T cells from taking up cytokines/media (iv) Upon enrichment IL-2 is used for expansion without any additional feeder cells	(i) Adherent culture flasks are needed instead of culture bags (ii) Large-scale enrichment and expansion need to be tested using patient PBMCs reproducing similar NK cell purity with healthy donor PBMCs	Feeder cells	(i) Highest number of final NK cells has been achieved using this approach (ii) Ability to present various activating molecules to NK cells by genetically modifying feeder cells (iii) Some protocols require no further isolation	(i) GMP-grade feeder cells must be developed and maintained (ii) Most protocols also require IL-2 (iii) Final product must be ensured to be deficient of all feeder cells before returning to the patient

Note. The table above summarizes the pros and cons of choosing a particular NK cell expansion method in NK cell isolation and NK cell expansion. Considerations for this table include NK cell purity of final product, NK cell viability, reproducibility, cost-effectiveness, GMP protocols, and clinical application.

expressing percentages consistent with starting NK cells within PBMCs of six donors of median 36% $CD62L^+$. After *ex vivo* enrichment and expansion, $CD62L^+$ NK cells were higher in two, lower in three, and the same in three cases compared to percent $CD62L^+$ NK cells in PBMCs. However, change in the MFI of CD62L on expanded NK cells (defined as >30% variation of MFI after expansion) was lower in five and remained the same in three cases. Considering such reduction of CD62L expression on the expanded adherent NK cells, nicotinamide (NAM) that is shown to substantially increase CD62L expression on NK cells could be tested with these cells [90, 91]. To note, we did not assess the CD62L expression on NK cells of cancer patients, which can be assessed in future experiments.

6. Conclusions

As highlighted in Table 4, among the various approaches of NK cell isolation and expansion, tested in the clinic, the use of feeder cells has been most effective in selectively expanding NK cells from healthy donor and patient's PBMCs. More consistent and higher cell numbers have been achieved compared to previous protocols using cytokines alone. However, the addition of feeder cells into a protocol further complicates the translation of these methods. The majority of these protocols use cytokines, and some required CliniMACS isolation methods to achieve optimal NK expansion. The introduction of these variables can result in greatly increased costs and more regulation to comply with when conducting a clinical trial which makes it difficult to move on to multicenter trials. The "adherent" enrichment and expansion of NK cells we describe offer a simple, cost-effective, reproducible, and translatable procedure with a high purity and potency of NK cells compared to other methodologies for personalized adoptive cell therapy. Future work with this method could easily incorporate agents that modify the phenotype of NK cells that could favor homing and enhance multiple facets of effector functions [90–92]. In addition, this method can also

be tested in large scale with patient PBMCs for translating into clinical application.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Senthamil R. Selvan conceived the idea of "adherent" enrichment and expansion of NK cells, designed and carried out experiments, acquired and analyzed the data, and prepared and critically revised the paper. John P. Dowling carried out certain experiments and extensive literature search and drafted and critically revised the paper. Both authors approved the final version to be published and agreed with all aspects of the work.

Acknowledgments

The authors thank Dr. Neal Flomenberg, Dr. William Kelly, Dr. Michael Mastrangelo, Dr. Takami Sato, Dr. Jianqing Lin, Dr. Ashwin Sama, and Dr. Jianke Zhang (Thomas Jefferson University, Philadelphia, PA) for their support with this study, Natalya Kopylova (Thomas Jefferson University, Philadelphia, PA) for technical assistance, and Colleen Katen (Thomas Jefferson University, Philadelphia, PA) for editorial corrections. The study was supported by research fund from Department of Medical Oncology, Thomas Jefferson University, Philadelphia, PA.

References

[1] D. R. Shook and D. Campana, "Natural killer cell engineering for cellular therapy of cancer," *Tissue Antigens*, vol. 78, no. 6, pp. 409–415, 2011.

[2] K. S. Campbell and J. Hasegawa, "Natural killer cell biology: an update and future directions," *Journal of Allergy and Clinical Immunology*, vol. 132, no. 3, pp. 536–544, 2013.

[3] A. K. Purdy and K. S. Campbell, "Natural killer cells and cancer: regulation by the killer cell ig-like receptors (KIR)," *Cancer Biology and Therapy*, vol. 8, no. 23, pp. 2211–2220, 2009.

[4] R. D. Schreiber, L. J. Old, and M. J. Smyth, "Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion," *Science*, vol. 331, no. 6024, pp. 1565–1570, 2011.

[5] M. A. Cooper, T. A. Fehniger, S. C. Turner et al., "Human natural killer cells: a unique innate immunoregulatory role for the CD56bright subset," *Blood*, vol. 97, no. 10, pp. 3146–3151, 2001.

[6] M. A. Cooper, T. A. Fehniger, and M. A. Caligiuri, "The biology of human natural killer-cell subsets," *Trends in Immunology*, vol. 22, no. 11, pp. 633–640, 2001.

[7] K. U. Park, P. Jin, M. Sabatino et al., "Gene expression analysis of ex vivo expanded and freshly isolated NK cells from cancer patients," *Journal of Immunotherapy*, vol. 33, no. 9, pp. 945–955, 2010.

[8] J. C. Sun and L. L. Lanier, "NK cell development, homeostasis and function: parallels with $CD8^+$ T cells," *Nature Reviews Immunology*, vol. 11, no. 10, pp. 645–657, 2011.

[9] A. Marcus and D. H. Raulet, "Evidence for natural killer cell memory," *Current Biology*, vol. 23, no. 17, pp. R817–R820, 2013.

[10] M. T. Orr and L. L. Lanier, "Natural killer cell education and tolerance," *Cell*, vol. 142, no. 6, pp. 847–856, 2010.

[11] J. C. Sun, J. N. Beilke, N. A. Bezman, and L. L. Lanier, "Homeostatic proliferation generates long-lived natural killer cells that respond against viral infection," *Journal of Experimental Medicine*, vol. 208, no. 2, pp. 357–368, 2011.

[12] J. G. O'Leary, M. Goodarzi, D. L. Drayton, and U. H. von Andrian, "T cell- and B cell-independent adaptive immunity mediated by natural killer cells," *Nature Immunology*, vol. 7, no. 5, pp. 507–516, 2006.

[13] J. C. Sun, J. N. Beilke, and L. L. Lanier, "Adaptive immune features of natural killer cells," *Nature*, vol. 457, pp. 557–561, 2009.

[14] M. A. Cooper, J. M. Elliott, P. A. Keyel, L. Yang, J. A. Carrero, and W. M. Yokoyama, "Cytokine-induced memory-like natural killer cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 6, pp. 1915–1919, 2009.

[15] G. Trinchieri, "Biology of natural killer cells," *Advances in Immunology*, vol. 47, pp. 187–376, 1989.

[16] S. Kim, K. Iizuka, H. L. Aguila, I. L. Weissman, and W. M. Yokoyama, "*In Vivo* natural killer cell activities revealed by natural killer cell- deficient mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 6, pp. 2731–2736, 2000.

[17] A. Diefenbach, E. R. Jensen, A. M. Jamieson, and D. H. Raulet, "Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity," *Nature*, vol. 413, no. 6852, pp. 165–171, 2001.

[18] A. Cerwenka, J. L. Baron, and L. L. Lanier, "Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 20, pp. 11521–11526, 2001.

[19] J. M. Kelly, P. K. Darcy, J. L. Markby et al., "Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection," *Nature Immunology*, vol. 3, no. 1, pp. 83–90, 2002.

[20] A. Jewett and H.-C. Tseng, "Tumor induced inactivation of natural killer cell cytotoxic function; implication in growth, expansion and differentiation of cancer stem cells," *Journal of Cancer*, vol. 2, no. 1, pp. 443–457, 2011.

[21] J. Mordoh, E. M. Levy, and M. P. Roberti, "Natural killer cells in human cancer: from biological functions to clinical applications," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 676198, 11 pages, 2011.

[22] A. A. Maghazachi, "Role of chemokines in the biology of natural killer cells," *Current Topics in Microbiology and Immunology*, vol. 341, no. 1, pp. 37–58, 2010.

[23] A. Iannello, T. W. Thompson, M. Ardolino, S. W. Lowe, and D. H. Raulet, "p53-dependent chemokine production by senescent tumor cells supports NKG2D-dependent tumor elimination by natural killer cells," *Journal of Experimental Medicine*, vol. 210, no. 10, pp. 2057–2069, 2013.

[24] S. M. Wood, H.-G. Ljunggren, and Y. T. Bryceson, "Insights into NK cell biology from human genetics and disease associations," *Cellular and Molecular Life Sciences*, vol. 68, no. 21, pp. 3479–3493, 2011.

[25] K. Malmberg, Y. Bryceson, M. Carlsten et al., "NK cell-mediated targeting of human cancer and possibilities for new means of immunotherapy," *Cancer Immunology, Immunotherapy*, vol. 57, no. 10, 2008.

[26] U. J. E. Seidel, P. Schlegel, and P. Lang, "Natural killer cell mediated antibody-dependent cellular cytotoxicity in tumor immunotherapy with therapeutic antibodies," *Frontiers in Immunology*, vol. 4, article 76, Article ID Article 76, 2013.

[27] J. S. Miller, "Therapeutic applications: natural killer cells in the clinic," *Hematology—American Society of Hematology. Education Program*, vol. 2013, pp. 247–253, 2013.

[28] M. K. Gleason, J. A. Ross, E. D. Warlick et al., "CD16xCD33 bispecific killer cell engager (BiKE) activates NK cells against primary MDS and MDSC CD33+ targets," *Blood*, vol. 123, pp. 3016–3026, 2014.

[29] H. Fujisaki, H. Kakuda, N. Shimasaki et al., "Expansion of highly cytotoxic human natural killer cells for cancer cell therapy," *Cancer Research*, vol. 69, no. 9, pp. 4010–4017, 2009.

[30] E. Alici, T. Sutlu, B. Björkstrand et al., "Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components," *Blood*, vol. 111, no. 6, pp. 3155–3162, 2008.

[31] M. Berg, A. Lundqvist, P. McCoy Jr. et al., "Clinical-grade ex vivo-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells," *Cytotherapy*, vol. 11, no. 3, pp. 341–355, 2009.

[32] H.-G. Klingemann and J. Martinson, "*Ex vivo* expansion of natural killer cells for clinical applications," *Cytotherapy*, vol. 6, no. 1, pp. 15–22, 2004.

[33] G. Bonanno, P. Iudicone, A. Mariotti et al., "Thymoglobulin, interferon-γ and interleukin-2 efficiently expand cytokine-induced killer (CIK) cells in clinical-grade cultures," *Journal of Translational Medicine*, vol. 8, article 129, 2010.

[34] S. Carlens, M. Gilljam, B. J. Chambers et al., "A new method for in vitro expansion of cytotoxic human $CD3^{-}CD56^{+}$ natural killer cells," *Human Immunology*, vol. 62, no. 10, pp. 1092–1098, 2001.

[35] H. Harada, K. Saijo, S. Watanabe et al., "Selective expansion of human natural killer cells from peripheral blood mononuclear cells by the cell line, HFWT," *Japanese Journal of Cancer Research*, vol. 93, no. 3, pp. 313–319, 2002.

[36] E.-K. Kim, Y.-O. Ahn, S. Kim, T. M. Kim, B. Keam, and D. S. Heo, "Ex vivo activation and expansion of natural killer cells from patients with advanced cancer with feeder cells from healthy volunteers," *Cytotherapy*, vol. 15, no. 2, pp. 231–241, 2013.

[37] D. Cho, D. R. Shook, N. Shimasaki, Y.-H. Chang, H. Fujisaki, and D. Campana, "Cytotoxicity of activated natural killer cells against pediatric solid tumors," *Clinical Cancer Research*, vol. 16, no. 15, pp. 3901–3909, 2010.

[38] T. Oikawa, K. Kawai, I. Ishiwata, T. Ohno, and H. Akaza, "Induction of potent antitumour natural-killer cells from peripheral blood of patients with advanced prostate cancer," *BJU International*, vol. 92, no. 9, pp. 1009–1015, 2003.

[39] J. S. Miller, Y. Soignier, A. Panoskaltsis-Mortari et al., "Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer," *Blood*, vol. 105, no. 8, pp. 3051–3057, 2005.

[40] J. E. Rubnitz, H. Inaba, R. C. Ribeiro et al., "NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia," *Journal of Clinical Oncology*, vol. 28, no. 6, pp. 955–959, 2010.

[41] A. Curti, L. Ruggeri, A. D'Addio et al., "Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients," *Blood*, vol. 118, no. 12, pp. 3273–3279, 2011.

[42] M. Stern, J. R. Passweg, S. Meyer-Monard et al., "Pre-emptive immunotherapy with purified natural killer cells after haploidentical SCT: a prospective phase II study in two centers," *Bone Marrow Transplantation*, vol. 48, no. 3, pp. 433–438, 2013.

[43] J. R. T. Monson, C. W. Ramsden, G. R. Giles, T. G. Brennan, and P. J. Guillou, "Lymphokine activated killer (LAK) cells in patients with gastrointestinal cancer," *Gut*, vol. 28, no. 11, pp. 1420–1425, 1987.

[44] I. G. H. Schmidt-Wolf, P. Lefterova, B. A. Mehta et al., "Phenotypic characterization and identification of effector cells involved in tumor cell recognition of cytokine-induced killer cells," *Experimental Hematology*, vol. 21, no. 13, pp. 1673–1679, 1993.

[45] C. S. Henney, K. Kuribayashi, D. E. Kern, and S. Gillis, "Interleukin-2 augments natural killer cell activity," *Nature*, vol. 291, no. 5813, pp. 335–338, 1981.

[46] D. Kabelitz, H. Kirchner, D. Armerding, and H. Wagner, "Recombinant interleukin 2 rapidly augments human natural killer cell activity," *Cellular Immunology*, vol. 93, no. 1, pp. 38–45, 1985.

[47] E. Lotzová, C. A. Savary, and R. B. Herberman, "Induction of NK cell activity against fresh human leukemia in culture with interleukin 2," *Journal of Immunology*, vol. 138, no. 8, pp. 2718–2727, 1987.

[48] G. Gasteiger, S. Hemmers, M. A. Firth et al., "IL-2-dependent tuning of NK cell sensitivity for target cells is controlled by regulatory T cells," *The Journal of Experimental Medicine*, vol. 210, no. 6, pp. 1167–1178, 2013.

[49] G. Suck and M. B. C. Koh, "Emerging natural killer cell immunotherapies: large-scale ex vivo production of highly potent anticancer effectors," *Hematology/Oncology and Stem Cell Therapy*, vol. 3, no. 3, pp. 135–142, 2010.

[50] D. J. Norman, "Mechanisms of action and overview of OKT3," *Therapeutic Drug Monitoring*, vol. 17, no. 6, pp. 615–620, 1995.

[51] L. Barkholt, E. Alici, R. Conrad et al., "Safety analysis of *ex vivo*-expanded NK and NK-like T cells administered to cancer patients: a phase I clinical study," *Immunotherapy*, vol. 1, no. 5, pp. 753–764, 2009.

[52] T. Sutlu, B. Stellan, M. Gilljam et al., "Clinical-grade, large-scale, feeder-free expansion of highly active human natural killer cells for adoptive immunotherapy using an automated bioreactor," *Cytotherapy*, vol. 12, no. 8, pp. 1044–1055, 2010.

[53] V. Decot, L. Voillard, V. Latger-Cannard et al., "Natural-killer cell amplification for adoptive leukemia relapse immunotherapy:comparison of three cytokines, IL-2, IL-15, or IL-7 and impact on NKG2D, KIR2DL1, and KIR2DL2 expression," *Experimental Hematology*, vol. 38, no. 5, pp. 351–362, 2010.

[54] J. Zhang, R. Sun, H. Wei, and Z. Tian, "Characterization of interleukin-15 gene-modified human natural killer cells: implications for adoptive cellular immunotherapy," *Haematologica*, vol. 89, no. 3, pp. 338–347, 2004.

[55] R. W. Childs and M. Berg, "Bringing natural killer cells to the clinic: ex vivo manipulation," *Hematology—American Society of Hematology. Education Program*, vol. 2013, pp. 234–246, 2013.

[56] H. Lebrec, M. J. Horner, K. S. Gorski et al., "Homeostasis of human NK cells is not IL-15 dependent," *The Journal of Immunology*, vol. 191, no. 11, pp. 5551–5558, 2013.

[57] G. Suck, V. Y. S. Oei, Y. C. Linn et al., "Interleukin-15 supports generation of highly potent clinical-grade natural killer cells in

long-term cultures for targeting hematological malignancies," *Experimental Hematology*, vol. 39, no. 9, pp. 904–914, 2011.

[58] Y.-K. Park, D.-J. Shin, D. Cho et al., "Interleukin-21 increases direct cytotoxicity and IFN-γ production of Ex Vivo expanded nk cells towards breast cancer cells," *Anticancer Research*, vol. 32, no. 3, pp. 839–846, 2012.

[59] H. Rabinowich, P. Sedlmayr, R. B. Herberman, and T. L. Whiteside, "Increased proliferation, lytic activity, and purity of human natural killer cells cocultured with mitogen-activated feeder cells," *Cellular Immunology*, vol. 135, no. 2, pp. 454–470, 1991.

[60] A. Malhotra and A. Shanker, "NK cells: immune cross-talk and therapeutic implications," *Immunotherapy*, vol. 3, no. 10, pp. 1143–1166, 2011.

[61] E. Ishikawa, K. Tsuboi, K. Saijo et al., "Autologous natural killer cell therapy for human recurrent malignant glioma," *Anticancer Research*, vol. 24, no. 3 B, pp. 1861–1871, 2004.

[62] E. Ishikawa, S. Takano, T. Ohno, and K. Tsuboi, "Adoptive cell transfer therapy for malignant gliomas," *Advances in Experimental Medicine and Biology*, vol. 746, pp. 109–120, 2012.

[63] C. Imai, S. Iwamoto, and D. Campana, "Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells," *Blood*, vol. 106, no. 1, pp. 376–383, 2005.

[64] H. Kobayashi, S. Dubois, N. Sato et al., "Role of trans-cellular IL-15 presentation in the activation of NK cell-mediated killing, which leads to enhanced tumor immunosurveillance," *Blood*, vol. 105, no. 2, pp. 721–727, 2005.

[65] N. Shimasaki, H. Fujisaki, D. Cho et al., "A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies," *Cytotherapy*, vol. 14, no. 7, pp. 830–840, 2012.

[66] C. J. Denman, V. V. Senyukov, S. S. Somanchi et al., "Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells," *PLoS ONE*, vol. 7, no. 1, Article ID e30264, 2012.

[67] Y. Liu, H.-W. Wu, M. A. Sheard et al., "Growth and activation of natural killer cells *Ex Vivo* from children with neuroblastoma for adoptive cell therapy," *Clinical Cancer Research*, vol. 19, no. 8, pp. 2132–2143, 2013.

[68] A. C. Dowell, K. A. Oldham, R. I. Bhatt, S. P. Lee, and P. F. Searle, "Long-term proliferation of functional human NK cells, with conversion of CD56 dim NK cells to a CD56 bright phenotype, induced by carcinoma cells co-expressing 4-1BBL and IL-12," *Cancer Immunology, Immunotherapy*, vol. 61, no. 5, pp. 615–628, 2012.

[69] A. Lundqvist, M. Berg, A. Smith, and R. W. Childs, "Bortezomib treatment to potentiate the anti-tumor immunity of ex-vivo expanded adoptively infused autologous natural killer cells," *Journal of Cancer*, vol. 2, no. 1, pp. 383–385, 2011.

[70] J. Luhm, J.-M. Brand, P. Koritke, M. Höppner, H. Kirchner, and C. Frohn, "Large-scale generation of natural killer lymphocytes for clinical application," *Journal of Hematotherapy and Stem Cell Research*, vol. 11, no. 4, pp. 651–657, 2002.

[71] U. Siegler, S. Meyer-Monard, S. Jrger et al., "Good manufacturing practice-compliant cell sorting and large-scale expansion of single KIR-positive alloreactive human natural killer cells for multiple infusions to leukemia patients," *Cytotherapy*, vol. 12, no. 6, pp. 750–763, 2010.

[72] C. J. Voskens, R. Watanabe, S. Rollins, D. Campana, K. Hasumi, and D. L. Mann, "Ex-vivo expanded human NK cells express activating receptors that mediate cytotoxicity of allogeneic and autologous cancer cell lines by direct recognition and antibody directed cellular cytotoxicity," *Journal of Experimental and Clinical Cancer Research*, vol. 29, no. 1, article 134, 2010.

[73] O. Lim, Y. Lee, H. Chung et al., "GMP-compliant, large-scale expanded allogeneic natural killer cells have potent cytolytic activity against cancer cells *In Vitro* and *In Vivo*," *PLoS ONE*, vol. 8, no. 1, Article ID e53611, 2013.

[74] M. R. Parkhurst, J. P. Riley, M. E. Dudley, and S. A. Rosenberg, "Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression," *Clinical Cancer Research*, vol. 17, no. 19, pp. 6287–6297, 2011.

[75] R. J. Melder, E. R. Walker, R. B. Herberman, and T. L. Whiteside, "Surface characteristics, morphology, and ultrastructure of human adherent lymphokine-activated killer cells," *Journal of Leukocyte Biology*, vol. 48, no. 2, pp. 163–173, 1990.

[76] R. J. Melder, E. Walker, R. B. Herberman, and T. L. Whiteside, "Adhesion characteristics of human interleukin 2-activated natural killer cells," *Cellular Immunology*, vol. 132, no. 1, pp. 177–192, 1991.

[77] N. L. Vujanovic, H. Rabinowich, Y. J. Lee, L. Jost, R. B. Herberman, and T. L. Whiteside, "Distinct phenotypic and functional chracteristics of human natural killer cells obtained by rapid interleukin 2-induced adherence to plastic," *Cellular Immunology*, vol. 151, no. 1, pp. 133–157, 1993.

[78] C. Jemma, A. Cignetti, M. Geuna, P. Caretto, S. Vai, and G. Forni, "In vitro and in vivo comparison of the activity of human lymphokine-activated killer (LAK) cells and adherent LAK cells," *Journal of Immunotherapy*, vol. 10, no. 3, pp. 189–199, 1991.

[79] N. L. Vujanovic, S. Yasumura, H. Hirabayashi et al., "Antitumor activities of subsets of human IL-2-activated natural killer cells in solid tissues," *The Journal of Immunology*, vol. 154, no. 1, pp. 281–289, 1995.

[80] T. L. Whiteside, M.-W. Sung, S. Nagashima, K. Chikamatsu, K. Okada, and N. L. Vujanovic, "Human tumor antigen-specific T lymphocytes and interleukin-2-activated natural killer cells: comparisons of antitumor effects in vitro and in vivo," *Clinical Cancer Research*, vol. 4, no. 5, pp. 1135–1145, 1998.

[81] C. W. McCrady, F. Li, A. J. Grant, R. E. Merchant, and R. A. Carchman, "Alteration of human lymphokine-activated killer cell activity by manipulation of protein kinase C and cytosolic Ca^{2+}," *Cancer Research*, vol. 48, no. 3, pp. 635–640, 1988.

[82] M. J. Robertson, T. J. Manley, C. Donahue, H. Levine, and J. Ritz, "Costimulatory signals are required for optimal proliferation of human natural killer cells," *Journal of Immunology*, vol. 150, no. 5, pp. 1705–1714, 1993.

[83] M. J. Robertson, C. Cameron, S. Lazo, K. J. Cochran, S. D. Voss, and J. Ritz, "Costimulation of human natural killer cell proliferation: role of accessory cytokines and cell contact-dependent signals," *Natural Immunity*, vol. 15, no. 5, pp. 213–226, 1997.

[84] H. Jouishomme and D. Rigal, "Proliferation and activation of human mononuclear cells induced by ionomycin in a serum-free medium," *Thymus*, vol. 17, no. 3, pp. 137–146, 1991.

[85] O. Mandelboim, P. Malik, D. M. Davis, C. H. Jo, J. E. Boyson, and J. L. Strominger, "Human CD16 as a lysis receptor mediating direct natural killer cell cytotoxicity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 10, pp. 5640–5644, 1999.

[86] R. Romee, B. Foley, T. Lenvik et al., "NK cell CD16 surface expression and function is regulated by a disintegrin and

metalloprotease-17 (ADAM17)," *Blood*, vol. 121, no. 18, pp. 3599–3608, 2013.

[87] Q. Zhou, A. Gil-Krzewska, G. Peruzzi, and F. Borrego, "Matrix metalloproteinases inhibition promotes the polyfunctionality of human natural killer cells in therapeutic antibody-based anti-tumour immunotherapy," *Clinical and Experimental Immunology*, vol. 173, no. 1, pp. 131–139, 2013.

[88] M. Frey, N. B. Packianathan, T. A. Fehniger et al., "Differential expression and function of L-selectin on CD56(bright) and CD56(dim) natural killer cell subsets," *The Journal of Immunology*, vol. 161, no. 1, pp. 400–408, 1998.

[89] K. Juelke, M. Killig, M. Luetke-Eversloh et al., "CD62L expression identifies a unique subset of polyfunctional CD56 dim NK cells," *Blood*, vol. 116, no. 8, pp. 1299–1307, 2010.

[90] G. M. Frei, N. Persi, C. Lador et al., "Nicotinamide , a form of vitamin B_3, promotes expansion of natural killer cells that display increased in vivo survival and cytotoxic activity," *Blood (ASH Annual Meeting Abstracts)*, vol. 118, p. 4035, 2011.

[91] M. Berg, T. Peled, R. N. Reger et al., "Improved homing to bone marrow, spleen and lung of adoptively infused NK cells expanded ex vivo with the small molecule nicotinamide using feeder-free conditions," *Blood (ASH Annual Meeting Abstracts)*, vol. 122, p. 897, 2013.

[92] S. S. Somanchi, V. V. Senyukov, C. J. Denman, and D. A. Lee, "Expansion, purification, and functional assessment of human peripheral blood NK cells," *Journal of Visualized Experiments*, no. 48, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 865101, 9 pages
http://dx.doi.org/10.1155/2015/865101

Review Article

Which Metrics Are Appropriate to Describe the Value of New Cancer Therapies?

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Received 7 July 2014; Accepted 31 October 2014

Academic Editor: Swaleha Zubair

Patients with certain cancers are treated with curative intent, but for others the results are less favorable and different therapeutic approaches are needed. Early data suggest that new therapies, which modulate immune responses to cancers, may have potential for long-term survival in a proportion of cases. Therefore, it is timely to consider whether metrics generally used to describe the medical value of therapies for patients with common solid tumors remain appropriate for therapies with curative potential. Literature reviews were conducted to define how various stakeholders describe cure in oncology and to identify the endpoints used in clinical trials for selected solid tumors. The results showed that "cure" is described using various terms that can be divided broadly into lack of disease progression, eradication of cancerous cells, and survival. The review of trial endpoints showed frequent use of median overall survival (OS) and progression- and response-related endpoints. Because these endpoints were mainly described in the context of chemotherapies that are not generally curative, they may not adequately capture outcomes of new therapeutic modalities with potential for long-term survival. More appropriate endpoints may include mean OS, cure fraction, and OS rate at landmark time points.

1. Introduction

The intentions and expectations of cancer therapy differ substantially depending on the type of malignancy and its extent. There are patients with cancer whose treatment can be curative, meaning that these patients no longer have active malignancy and will eventually die of something other than the cancer. In these cases, despite the different biology of the cancers and their treatment, what "cure" actually means is similar for all. Examples of curative cancer therapies include the surgical resection or radical irradiation of early epithelial cancers; adjuvant chemotherapy for breast and colon cancer; systemic therapy for childhood tumors such as acute lymphoblastic leukemia, osteosarcoma, or neuroblastoma; and systemic therapies in adults for germ cell tumors, some lymphomas, and acute leukemias. However, for most patients with advanced solid tumors, treatment is not given with curative intent, rather with the expectation of improving survival by some time and/or alleviating symptoms. In a review of new drug approvals by the United States Food and Drug Administration (FDA) between July 2005 and December 2007, approval was based on an effect on overall survival (OS) for only 10 of the 53 agents approved [1]. Consequently, the description and assessment of cure have not been central to the reporting of results in the treatment of these patients [2]. The generally poor prognosis for patients with most types of advanced solid tumors continues to drive research to identify and develop new therapeutic approaches that offer the potential for long-term survival. The ultimate goal is to achieve a durable survival benefit that allows patients to be considered "cured."

The concept that autologous immune surveillance fails during the development of cancer and that the restoration

of this recognition might produce therapeutic benefit has long been investigated [4]. The possible feasibility of this in practice has been recognized with the recent development of active immunotherapies designed to modulate the patient's immune system directly, thereby overcoming the mechanisms by which tumors evade recognition and elimination. Examples include ipilimumab, a cytotoxic T-lymphocyte antigen-4 [CTLA-4] immune checkpoint inhibitor, as well as nivolumab and pembrolizumab, programmed death-1 [PD-1] immune checkpoint inhibitors [5, 6]. These forms of treatment appear to act quite differently to conventional cytotoxic drugs or small molecule enzyme inhibitors, in that a proportion of the patients treated derive long-term benefit with prolonged suppression of the malignancy and no evidence of regrowth despite several years of follow-up [3, 7–10]. This is in-keeping with the known mechanisms of immunity, which generally confers almost life-long protection in the case of infectious diseases.

The recognition that there may be new modalities of cancer treatment, immunotherapy in particular, with the potential for long-term survival and possibly cure warrants a review of whether the metrics normally used to evaluate new compounds for advanced solid tumors are appropriate and accurately capture the important treatment outcomes [2, 5]. Against this background, it is helpful to consider how "cure" is currently described and assessed for patients with cancer.

2. Terms Used to Describe Curative Cancer Treatments

A review of literature from four sources (journal articles, conference and congress proceedings, health technology assessment [HTA] reports, and selected websites) published in English between January 1, 2007, and November 14, 2012, was conducted to identify "cure" definitions. To verify the appropriate choice and use of search terms, the search was validated against two relevant articles [11, 12]. Additionally, it was evaluated whether the definition varied according to the stakeholder or group describing it (i.e., academics, healthcare professionals, patients and patient interest groups, or payers). The literature search strategy was designed pragmatically to prioritize the identification of key information in a very large literature base. Notably, the review did not identify any published reports exploring the definition of cure broadly across oncology. Please refer to Supplementary Material 1 for detailed search methodology, limitations, outcomes, and results (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/865101).

The literature review identified 3,932 documents, of which 169 were included following screening. The final sample included journal articles ($n = 83$), HTA documents ($n = 7$), conference abstracts ($n = 56$), and documents from patients/patient advocacy organizations ($n = 18$) and health care professionals' ($n = 5$) websites. Briefly, the results of the literature review showed that, across the materials evaluated, cure could be described using one or more of the following broad categories, with different descriptions or metrics used for assessment within each category:

(1) *lack of disease progression*, including complete remission, disease-/event-/recurrence-/progression-free survival, functional cure, and time to treatment failure,

(2) *eradication of cancerous cells*, including terms related to the removal of cancerous cells (by various therapeutic modalities, radiation therapy, and killing of cancer cells),

(3) *survival*, including landmark OS rates (from 2 to 20 years), median OS, cure models, cure fraction, the patient dying from noncancer-related causes, and the mortality rate aligning with the general population; in this category, most groups discussed cure in the context of a follow-up period of about 5 years.

The results from this review revealed differences between groups' opinions on what describes a cancer "cure." Healthcare professionals and academics placed a larger emphasis on disease progression and survival, while patients and payers focused on the successful eradication of cancerous cells. The percentage of documents using survival, lack of disease progression, or lack of disease progression plus survival as cure definitions was 35%, 28%, and 20%, respectively, for healthcare professionals; 39%, 39%, and 0%, respectively, for academics; 22%, 0%, and 0%, respectively, for patients/patient advocacy organizations; and 0%, 43%, and 0%, respectively, for HTA agencies (see Supplementary Figure 2).

Moreover, many of the descriptions, particularly as used by clinicians, were associated with cure in the context of hematologic malignancies. Of the 60 documents identified for healthcare professionals that contained cure definitions, 43 (72%) were in relation to hematologic malignancies compared with 15 (25%) for solid tumors (two reports had generalized content). This is not surprising since relatively a few therapies for solid tumors offer the potential for long-term survival, while, for certain hematologic malignancies, treatments are given with curative intent [13, 14]. As reflected in the results, cure can be described in terms of survival, measured as a proportion of patients who die of causes other than their disease if the follow-up period is long enough, or as a surrogate such as the proportion of patients alive at a time point appropriate to the type of cancer (e.g., 5 years for rapidly recurring cancers or 10 to 20 years for those that typically relapse later). Another component to the description of cure is more functional, relating to the time during which patients remain free from cancer progression or the effects of cancer on their health.

The use of descriptions relating to the eradication of cancer cells, particularly in documents from patients and payers, is thought provoking (please refer to Supplementary Material 1, Table 1, and Figure 2). It can never be proven that all the tumor cells have been destroyed, but rather they simply do not manifest themselves, allowing the patient to live without overt cancer until death from another cause (therefore, they are "cured" of the original disease). It can be proposed that treatments that are given for a relatively short duration and result in a long-term survival with no evidence of tumor recurrence, such as surgical resection for early epithelial cancer or adjuvant therapy for breast cancer or

ipilimumab for metastatic melanoma, may completely eradicate the tumor, although this is not fully provable. In some hematologic malignancies, immunophenotypic or molecular techniques show that malignant cells persist in the absence of clinical signs or symptoms (termed minimal residual disease (MRD)), meaning that there is potential for tumor regrowth. For chronic myeloid leukemia, continued therapy with agents such as imatinib or dasatinib may be needed to maintain tumor control while treatment continues, despite the presence of such MRD [15]. In these settings, use of the terms operational or functional cure has become common [16]. In this context, describing cure as the eradication of cancer cells is neither measurable nor accurate. Although the results suggest that this is how certain groups describe cure, in clinical practice, curative cancer therapies may not align with that definition.

Understanding that cure is not always described or assessed in the same way and is not frequently used for advanced solid tumors since relatively a few therapies are given with curative intent, a further literature search was conducted to identify which endpoints are commonly used to assess treatments for patients with malignant melanoma, non-small cell lung cancer (NSCLC), and renal cell carcinoma (RCC) in clinical trial settings. This information was then used as a basis to address whether the commonly used endpoints would be appropriate to assess the medical value of therapies with the potential for long-term survival and how/whether these endpoints may support the notion of a "cure" in the way normally understood.

3. Endpoints Used to Assess Treatment Outcomes in Malignant Melanoma, NSCLC, and RCC

A literature review of information published in English between January 1, 2007, and December 31, 2012, was conducted to identify the clinical endpoints used in malignant melanoma, NSCLC, and RCC clinical trials and their frequency of use. These tumors were selected as they are the main types in which novel immunotherapies have been evaluated to date. Only endpoints directly referring to clinically measured outcomes were included, and not point estimates such as hazard ratio (HR) which are derived from data analysis. Two literature sources were searched: published systematic reviews of clinical trials and HTA reports. Additionally, documents identified during the first search that also provided metrics (in conjunction with cure definitions) were included in the results of this second search. Full details of the methodology and limitations are included in Supplementary Material 2. Again, this literature search strategy was designed pragmatically to prioritize the identification of key information in a very large literature base.

The literature search identified 2,951 documents, of which 146 were included in the final sample, comprising 92 clinical trial review papers and 54 HTA documents. The endpoints identified in the clinical trial reviews and HTA reports are shown in Tables 1 and 2, respectively. Overall, the most common endpoints reported were response rate, disease-free survival (DFS) or progression-free survival (PFS), median OS, recurrence rate, and quality of life. The endpoints used to assess clinical response were the most heterogeneous and, within this category, response rate or objective response rate (ORR) was the most frequently used. These endpoints were also used across all evaluated tumor types. Endpoints related to disease progression were relatively homogeneous, with PFS, DFS, recurrence-free survival, and time to progression (TTP) being the most frequently used. In the survival category, endpoints were fairly homogeneous with median OS used frequently across the tumor types. OS, in the majority of cases, median OS specifically, was the only "survival" endpoint reported for malignant melanoma and RCC, accounting for 82% and 83% of the "survival" endpoints reported in clinical trial review papers and HTA reports, respectively. Endpoints relating to recurrence and/or relapse were not found in HTA reports but were included in clinical trial review papers. In the review papers, local or overall recurrence rates were the most commonly used.

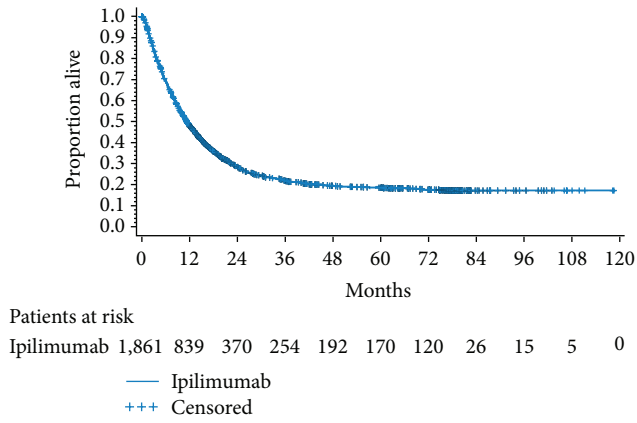


Figure 1: Pooled analysis of 1,861 ipilimumab-treated patients from 12 clinical trials [3]. Median overall survival was 11.4 months (95% CI: 10.7–12.1 months) and 3-year overall survival was 22% (95% CI: 20–24%).

When comparing the endpoints reported for the different tumor types, there were only minor differences (Figure 2). Survival- and response-related endpoints were more frequent in malignant melanoma, while disease progression and quality-of-life endpoints were more common in RCC.

Several methodological papers have provided recommendations for endpoints that should be considered as part of clinical trial design for malignant melanoma [17], NSCLC [18, 19], and RCC [20, 21] (summarized in Table 3). The recommendations made in these papers largely align with the results from the current literature.

4. Different Therapeutic Approaches May Require Different Clinical Value Metrics

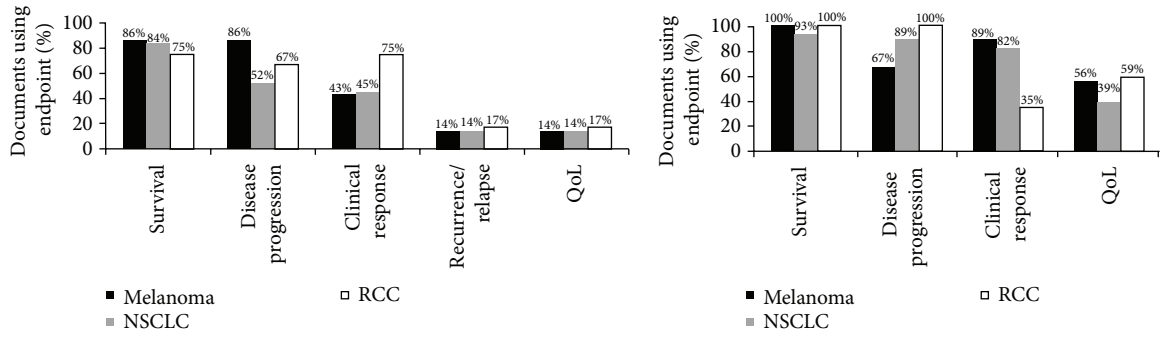
Many of the frequently used endpoints were developed primarily to evaluate the clinical activity of standard therapeutic modalities, such as cytotoxic chemotherapy, small-molecule

Table 1: Endpoints identified in clinical trial reviews.

Category[a]	Endpoint reported (% of category) (N = 266)			
	Included in >1 cancer type	Malignant melanoma only	NSCLC only	RCC only
Clinical response (n = 79)	Complication rate (26%) Response rate (23%) Local control/local tumor control (9%) Absolute benefit (8%) Disease control rate (3%) Complete response (1%)	Partial response (3%)	Time to progression (8%) Objective response rate (4%) Tumor response (3%) Symptom improvement (3%) 3-year freedom from local progression (1%) Length of hospital stay (1%) Radiological improvement (1%) Treatment failure rate (1%) Tumor progression (1%) Postoperative complication rate (1%)	Positive margin rate (1%) Surgery success rate (1%)
Disease progression (n = 66)	Progression-free survival (36%) Disease-free survival (32%) Recurrence-free survival (23%)	Distant metastasis-free survival (2%) Locoregional PFS (2%)	Asymptomatic survival (2%) Quality-adjusted PFS (2%)	
Survival (n = 87)	OS[b] (82%)		Drug related deaths (6%) 5-year survival (3%) Cause-specific mortality (3%) 1-year survival (2%) Treatment mortality/morbidity (2%) Perioperative morbidity (1%)	
Recurrence/relapse (n = 21)	Local recurrence rate (38%) Recurrence rate (overall) (14%)		Relapse (14%) Asymptomatic recurrence (5%) Distant recurrence (5%) Remission (5%) Symptom-free period (5%) Systemic recurrence (5%) Time to recurrence (5%) Time to relapse (5%)	
QoL (n = 13)	Health-related QoL (8%)	QoL (92%)		

[a]Review papers may include more than one endpoint category.

[b]Included review papers of clinical trials that did not consistently report whether the articles they referenced used median or mean OS. When mentioned, the majority of included trial review papers referred to median OS.



(a) Clinical trial reviews

(b) HTA reports

Figure 2: Clinical endpoints used for the different tumor types in (a) clinical trial review papers and (b) health technology assessment reports.

Table 2: Endpoints identified in health technology assessment reports.

Category[a]	Endpoint reported (% of category) $N = 232$			
	Included in >1 cancer type	Malignant melanoma only	NSCLC only	RCC only
Clinical response ($n = 79$)	Complete response (11%) Duration of response (13%) Objective response rate (13%) Overall response rate (12%) Partial response (10%) Objective tumor response (8%) Disease control rate (4%) Stable disease (4%) Time to response (3%) Response rates (1%)	Disease progression (1%) Near complete response (1%)	Tumor response (12%) Best tumor response (1%) Physical functioning (1%) Symptomatic improvement (1%) Time to tumor progression (1%) Time to worsening of patient reported outcomes (1%) Tolerance (1%)	
Disease progression ($n = 65$)	Progression-free survival (72%) Time to progression/progressive disease (15%)		Time to treatment failure (8%) Time to worsening of symptoms (3%)	Time to first event (2%)
Survival ($n = 63$)	Median OS (83%) 1-year survival (13%) 2-year survival (5%)			
QoL ($n = 25$)	QoL (68%) Health-related QoL (20%)		Disease-specific questionnaire (4%) Lung cancer symptom scale (4%)	Patient reported outcomes (4%)

[a]HTA reports may include more than one endpoint category.

Table 3: Recommended clinical endpoints for trials in patients with malignant melanoma, non-small cell lung cancer, or renal cell carcinoma.

Malignant melanoma [17]	NSCLC [18, 19]	RCC [20, 21]
Overall survival[a]	Disease stability	Median overall survival
Progression-free survival	Growth modulation index	Median progression-free survival
Quality of life	Median overall survival	Response rate
	Median progression-free survival	
	Time to progression	

[a]The article did not specify median or mean OS.

enzyme inhibitors, and radiation. Because of their mechanisms of action, these approaches often have immediate effects that may initially reduce tumor size, but, subsequently, the disease often progresses over time; for patients with advanced disease, they are not generally considered curative. In view of these potential clinical effects, median OS, PFS, and response rate are, in general, appropriate and accurate endpoints for these types of therapies [22].

The evaluation of new therapies with different mechanisms of action may require different or additional metrics to assess and describe value. For example, as discussed earlier, some data suggest that active immunotherapies have the potential for long-term survival, likely achieved through the restoration of durable antitumor immune responses, without necessarily being accompanied by rapid tumor shrinkage [3, 9, 10, 23, 24]. Therefore, different endpoints may be more appropriate for these therapies [17, 22].

4.1. Survival-Related Endpoints. OS is considered the gold standard for efficacy in solid tumor oncology clinical trials, and median OS is often quoted as the primary or secondary endpoint of interest. Median OS is often used because it allows survival to be estimated before all patients have experienced an event, that is, death, thereby allowing timely reporting of outcomes.

However, median OS may not be the best endpoint for therapies with potential for long-term benefit [17, 22, 25]. Consider the hypothetical survival curve with a therapy that results in long-term survival in a small proportion of patients versus one with a cytotoxic or targeted therapy that causes an initial, rapid reduction in tumor volume but also no or low prolonged benefit (Figure 3). Median OS is calculated as the point in time after diagnosis or initiation of treatment at which 50% of patients are still alive. However, this assessment may be insufficient for treatments that offer long-term benefit because it does not provide information pertaining to the

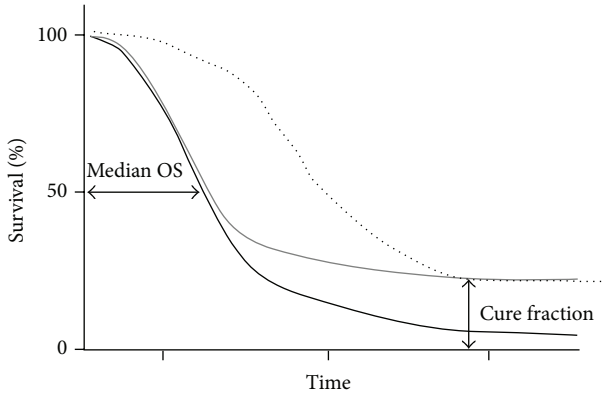


Figure 3: Hypothetical survival curves. The grey line represents an agent (potentially an immunotherapy) that results in long-term benefit in a proportion of patients, the black line represents standard of care (potentially a cytotoxic agent), and the dotted line shows all-cause mortality.

small proportion of patients who occupy the tail of the survival curve. As such, median OS is considered less suitable for survival curves that are skewed to the right since it does not differentiate the proportion of patients alive or dead after 50% of the patients have died [25]. Furthermore, while median OS provides a measure of when 50% of patients will die, it does not provide a true reflection of the survival time that may be expected from the patients who are alive after the median OS is reached. For example, in a phase 3 trial of ipilimumab (MDX010-020), the median OS of patients who received ipilimumab alone was 10.1 months ($n = 137$) compared with 6.4 months in the control arm ($n = 136$). However, long-term follow-up showed that 13 of the 53 patients (25%) in the ipilimumab group who were randomized ≥3 years before study survival cut-off date survived for 3 years or longer compared with 5 of 50 patients in the control group (10%), an outcome not well reflected by the median OS values [7, 23].

Because of the potential limitations of median OS in describing value of treatments where a proportion of patients experience durable survival, alternative or additional measures of survival may be required. A recent report suggested that combining a robust HR (less than 0.8) with a corresponding improvement in median OS (within a range of 2.5 to 6 months) may define a minimum clinical outcome that could serve as the starting point of a discussion about the medical value of a new treatment [26]. Additionally, other publications have provided examples of how median OS can be supplemented with mean OS as an additional endpoint of interest (as measured by the area under the Kaplan-Meier curve) [23, 25, 27–29]. Other alternatives to median OS are endpoints relating to landmark survival rates, for example at 2, 3, and 5 years after the start of therapy or whichever duration is appropriate to the type of therapy. Although long-term trial follow-up is required, landmark survival at up to 5 years after treatment has been reported with some agents for some solid tumors and hematologic malignancies [7, 8, 30, 31]. In the pooled analysis of ipilimumab-treated patients, the survival rate at 3 years was 22%, and this marked the start of an OS plateau that extended through at least 10 years in some patients (Figure 1). However, these data also reflect the fact that almost 80% of the patients had died within 3 years of treatment initiation [3].

Another potential metric for therapies with potential long-term survival is cure fraction (the proportion of patients who survive and no longer experience the excess mortality rate of the disease) [12]. As discussed above, a "cure" is defined here as a patient population that has the same chance (based on HR) of dying as a member of the general population, depending on the mechanism of action of the agent and the cancer type. This can be achieved through a relatively short course treatment or continual therapy allowing the cancer to be treated as a chronic disease. Standard survival analysis methods, like the Cox proportional hazards model, provide no direct estimate of the cure fraction. However, it may be appropriate to use cure-fraction models for survival data, if a proportion of patients may be cured by a treatment [32]. Such models have been explored for glioblastoma, colon cancer, and breast cancer [33–35].

Against the background of these alternative survival endpoints, reports on the approval of new oncologic drugs and associated documentation show increasing acceptance by HTA bodies of survival endpoints other than median OS [21, 36]. For example, in 2012, mean OS was accepted by the National Institute for Health and Care Excellence (NICE) in the United Kingdom as part of the submission for ipilimumab for previously treated advanced (unresectable or metastatic) melanoma [37]. Furthermore, some HTA bodies are consistently using mean OS in economic models and these models can relatively accurately predict the proportion of patients alive at specific time points. Typically, mean OS is calculated as the area under the Kaplan Meier curve (as an adjusted mean) [25].

4.2. Progression-Related Endpoints. Recently, the United States FDA and the European Medicines Agency (EMA) have shown willingness to accept other endpoints as surrogates for OS benefit; PFS and DFS are frequently used, as reflected by the literature review. These are attractive alternatives to median OS since they can be determined earlier, are less influenced by competing causes of death, and are not influenced by second-line therapies [22, 38]. Between July 2005 and December 2007, the FDA approved 44 new products; of these, four used DFS, three used PFS and OS, 11 used PFS or TTP, and 10 used ORR [1]. In Europe, the EMA updated their clinical trial guidelines in 2012 and currently accepts PFS and DFS as primary endpoints in oncology trials [36]. However, a correlation between PFS and OS has only been reported for a limited number of tumor types [22, 38].

PFS and DFS are appropriate for assessing the activity of agents likely to elicit rapid control of tumor growth, but they may be less suitable for therapies where tumor control may develop over time. In some patients receiving immunotherapies, their disease may apparently progress in size (as assessed using standard RECIST or WHO criteria) before there is evidence of disease stabilization, or there may

be progression in some lesions while others regress. Some patients may have prolonged disease stabilization, which may evolve over time to become a partial or even complete response, despite sometimes being preceded by progressive disease. These types of response patterns may reflect the time it can take the immunotherapy to modulate the immune system and achieve clinically effective tumor control, and how the immune system responds to an evolving tumor [39, 40]. However, this is not the case for all immunotherapies. Other data show that some patients treated with immunotherapies can have rapid, more conventional types of response [24].

Endpoints such as DFS or PFS may underestimate the activity of novel therapies if associated with prolonged stable disease or unconventional responses, even though these responses may translate into a prolonged survival benefit [39, 40]. As an example, in a phase 3 trial of sipuleucel-T, a therapeutic cancer vaccine, in patients with metastatic castration-resistant prostate cancer, treatment resulted in an improvement in median overall survival (25.8 months with sipuleucel-T versus 21.7 months with placebo; HR for death in the sipuleucel-T group, 0.78; 95% confidence interval [CI], and 0.62–0.98; $P = 0.03$). However, the median PFS was similar in the sipuleucel-T and placebo groups (3.7 months versus 3.6 months) [41]. Similarly, in a randomized phase 2 trial with another investigational cancer vaccine, PROSTVAC-VF, the vaccine improved median OS but not PFS [42].

4.3. Response-Related Endpoints. Response rate is another frequently used surrogate endpoint and has been the basis for drug approvals [1]. However, response-related endpoints may not be appropriate for treatments that do not always act by eliciting rapid shrinkages in tumor volume [22]. Data for immunotherapies show that although some patients attain a durable survival benefit, response rates may be lower than expected. This is because response rate, as assessed using standard criteria, may not be a good surrogate for OS if therapies have the potential for prolonged stable disease or other unconventional responses [39, 40]. In a phase 3 trial with ipilimumab in patients with advanced melanoma, the 2-year survival rate was 23.5% in patients who received ipilimumab alone, while the objective response rate was only 10.9% [23].

In addition to endpoints directly related to efficacy, health-related quality of life (HRQoL) is increasingly being recognized as an important endpoint in oncology clinical trials. In patients with advanced disease and a limited life expectancy, depending on the therapy being evaluated, survival alone may not be an appropriate endpoint and improving or maintaining HRQoL becomes a priority. The two most commonly used questionnaires for determining HRQoL in cancer patients are the European Organisation for the Research and Treatment of Cancer Quality of Life Questionnaire (EORTC-QLQ)-C39 and the Functional Assessment of Cancer Therapy-General (FACT-G) [43]. EMA clinical trial guidelines were updated in 2012 and suggest that HRQoL may be an informative endpoint, especially in the palliative setting [36].

5. Conclusions

With the emergence of immunotherapy and other new modalities, we are reassessing our expectations for the treatment of patients with advanced solid tumors. For certain patient populations, we are starting to see, either in practice or clinical trials, therapies with the potential for long-term survival and even cure. It is important to consider whether the commonly used endpoints, that is, median OS, PFS, DFS, and response rate, are appropriate to describe value, especially when the new agents have mechanisms of action that may translate into different clinical effects. On reviewing the activity observed with different active immunotherapies in clinical trials, it is clear that *median* OS, PFS, DFS, and response rate may not adequately capture the potential outcomes with these therapies and additional metrics may be needed.

More appropriate or additional metrics for therapies with the potential for long-term survival may include *mean* OS, cure fraction, and OS rate at landmark time points. Encouragingly, recent approvals suggest that agencies like the FDA and EMA are willing to accept additional metrics if they better characterize the agent's activity, although HTA bodies, such as NICE and the Institute for Quality and Efficiency in Health Care (IQWiG), may require different or additional metrics for reimbursement assessment. Furthermore, as the results of the literature review show, cure is a defined and utilized concept for cancers where potentially curative treatments are available. However, the description of cure is relatively heterogeneous and differs depending on the person or group. Because most advanced solid tumors are currently considered incurable with available therapies, the literature review showed that endpoints commonly used in clinical trials of malignant melanoma, NSCLC, and RCC are not well aligned with how cure is described in other cancers. In view of this, if we continue to see new therapies being developed with the potential for long-term survival, then efforts should be made to use the appropriate endpoints and the related set of value metrics that best describe the clinical and other outcomes of these new treatments.

Disclosure

Samuel Wagner is an employee of Bristol-Myers Squibb, the sponsor of the original research.

Conflict of Interests

Peter Johnson has received consultancy fees and speaking honoraria from Bristol-Myers Squibb. Samuel Wagner is an employee of Bristol-Myers Squibb, the sponsor of the original research. Wolfgang Greiner received financial compensation for participating in a Bristol-Myers Squibb advisory board.

Acknowledgments

The authors take full responsibility for the content of this publication and confirm that it reflects their viewpoint and

medical expertise. The authors also wish to acknowledge Rebecca Turner, StemScientific, funded by Bristol-Myers Squibb, for providing writing and editorial support. The authors received no financial compensation for authoring the paper. This work was supported by Bristol-Myers Squibb. Peter Johnson has received consultancy fees and speaking honoraria from Bristol-Myers Squibb. Wolfgang Greiner received financial compensation for participating in a Bristol-Myers Squibb advisory board.

References

[1] R. Sridhara, J. R. Johnson, R. Justice, P. Keegan, A. Chakravarty, and R. Pazdur, "Review of oncology and hematology drug product approvals at the US Food and Drug Administration between July 2005 and December 2007," *Journal of the National Cancer Institute*, vol. 102, no. 4, pp. 230–243, 2010.

[2] S. A. Rosenberg, "Raising the bar: the curative potential of human cancer immunotherapy," *Science Translational Medicine*, vol. 4, no. 127, Article ID 127ps8, 2012.

[3] D. Schadendorf, F. S. Hodi, C. Robert et al., "Pooled analysis of long-term survival data from phase II and phase III trials of ipilimumab in metastatic or locally advanced, unresectable melanoma," in *Proceedings of the European Cancer Congress (ECC '13)*, Abs 24LBA, 2013.

[4] J. M. Kirkwood, L. H. Butterfield, A. A. Tarhini, H. Zarour, P. Kalinski, and S. Ferrone, "Immunotherapy of cancer in 2012," *CA: A Cancer Journal for Clinicians*, vol. 62, no. 5, pp. 309–335, 2012.

[5] O. J. Finn, "Immuno-oncology: understanding the function and dysfunction of the immune system in cancer," *Annals of Oncology*, vol. 23, supplement 8, pp. viii6–viii9, 2012.

[6] J. D. Wolchok, F. S. Hodi, J. S. Weber et al., "Development of ipilimumab: a novel immunotherapeutic approach for the treatment of advanced melanoma," *Annals of the New York Academy of Sciences*, vol. 1291, no. 1, pp. 1–13, 2013.

[7] D. McDermott, J. Haanen, T. T. Chen, P. Lorigan, and S. O'Day, "Efficacy and safety of ipilimumab in metastatic melanoma patients surviving more than 2 years following treatment in a phase III trial (MDX010-20)," *Annals of Oncology*, vol. 24, no. 10, pp. 2694–2698, 2013.

[8] F. S. Hodi, S. L. Topalian, J. Brahmer et al., "Survival and long-term safety in patients with advanced solid tumors receiving nivolumab (anti-PD-1; BMS-936558; ONO-4538)," in *Proceedings of the European Cancer Congress (ECC '13)*, Abs 880P, 2013.

[9] O. Hamid, C. Robert, A. Daud et al., "Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma," *The New England Journal of Medicine*, vol. 369, no. 2, pp. 134–144, 2013.

[10] J. C. Soria, C. Cruz, R. Bahleda et al., "Clinical activity, safety and biomarkers of PD-L1 blockade in non-small cell lung cancer (NSCLC): additional analyses from a clinical study of the engineered antibody MPDL3280A (anti-PDL1)," in *Proceedings of the CC Congress*, Abstract 3408, 2013.

[11] D. N. Lakdawalla, J. A. Romley, Y. Sanchez, J. R. Maclean, J. R. Penrod, and T. Philipson, "How cancer patients value hope and the implications for cost-effectiveness assessments of high-cost cancer therapies," *Health Affairs*, vol. 31, no. 4, pp. 676–682, 2012.

[12] M. Othus, B. Barlogie, M. L. LeBlanc, and J. J. Crowley, "Cure models as a useful statistical tool for analyzing survival," *Clinical Cancer Research*, vol. 18, no. 14, pp. 3731–3736, 2012.

[13] J. V. Melo and D. M. Ross, "Minimal residual disease and discontinuation of therapy in chronic myeloid leukemia: can we aim at a cure?" in *ASH Education Program Book*, pp. 136–142, 2011.

[14] F.-X. Mahon, "Is going for cure in chronic myeloid leukemia possible and justifiable?" *Hematology/The Education Program of the American Society of Hematology*, vol. 2012, pp. 122–128, 2012.

[15] E. Paietta, "Assessing minimal residual disease (MRD) in leukemia: a changing definition and concept?" *Bone Marrow Transplantation*, vol. 29, no. 6, pp. 459–465, 2002.

[16] J. Goldman and M. Gordon, "Why do chronic myelogenous leukemia stem cells survive allogeneic stem cell transplantation or imatinib: does it really matter?" *Leukemia & Lymphoma*, vol. 47, no. 1, pp. 1–7, 2006.

[17] A. Ribas, P. Hersey, M. R. Middleton et al., "New challenges in endpoints for drug development in advanced melanoma," *Clinical Cancer Research*, vol. 18, no. 2, pp. 336–341, 2012.

[18] M. di Maio, C. Gallo, E. de Maio et al., "Methodological aspects of lung cancer clinical trials in the era of targeted agents," *Lung Cancer*, vol. 67, no. 2, pp. 127–135, 2010.

[19] C. A. Dooms and J. F. Vansteenkiste, "Treatment of patients with advanced non-small cell lung cancer," *American Journal of Cancer*, vol. 3, no. 5, pp. 281–290, 2004.

[20] T. E. Hutson, "Targeted therapies for the treatment of metastatic renal cell carcinoma: clinical evidence," *The Oncologist*, vol. 16, supplement 2, pp. 14–22, 2011.

[21] S. K. Pal and R. A. Figlin, "Targeted therapies for renal cell carcinoma: understanding their impact on survival," *Targeted Oncology*, vol. 5, no. 2, pp. 131–138, 2010.

[22] M. Bilusic and J. L. Gulley, "Endpoints, patient selection, and biomarkers in the design of clinical trials for cancer vaccines," *Cancer Immunology, Immunotherapy*, vol. 61, no. 1, pp. 109–117, 2012.

[23] F. S. Hodi, S. J. O'Day, D. F. McDermott et al., "Improved survival with ipilimumab in patients with metastatic melanoma," *The New England Journal of Medicine*, vol. 363, no. 8, pp. 711–723, 2010.

[24] S. L. Topalian, F. S. Hodi, J. R. Brahmer et al., "Safety, activity, and immune correlates of anti-PD-1 antibody in cancer," *The New England Journal of Medicine*, vol. 366, no. 26, pp. 2443–2454, 2012.

[25] A. Davies, A. Briggs, J. Schneider et al., "The ends justify the mean: outcome measures for estimating the value of new cancer therapies," *Health Outcomes Research in Medicine*, vol. 3, no. 1, pp. e25–e36, 2012.

[26] L. M. Ellis, D. S. Bernstein, E. E. Voest et al., "American Society of Clinical Oncology perspective: raising the bar for clinical trials by defining clinically meaningful outcomes," *Journal of Clinical Oncology*, vol. 32, no. 12, pp. 1277–1280, 2014.

[27] J. S. De Bono, S. Oudard, and M. Ozguroglu, "Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial," *The Lancet*, vol. 377, no. 9767, pp. 1147–1154, 2010.

[28] S. Oudard, A. Joulain, A. de Geer, and O. Sartor, "Cabazitaxel or mitoxantrone with prednisone in patients with metastatic castration-resistant prostate cancer (mCRPC) previously treated with docetaxel: estimating mean overall survival (OS) for health economic analyses from a phase III trial (TROPIC)," *Journal of Clinical Oncology*, vol. 29, supplement 7, ASCO, abstract 128, 2011.

[29] D. Y.-S. Kuo, S. V. Blank, P. J. Christos et al., "Paclitaxel plus oxaliplatin for recurrent or metastatic cervical cancer: a New York Cancer Consortium Study," *Gynecologic Oncology*, vol. 116, no. 3, pp. 442–446, 2010.

[30] J. F. S. Miguel, R. Schlag, N. K. Khuageva et al., "Persistent overall survival benefit and no increased risk of second malignancies with bortezomib-melphalan-prednisone versus melphalan-prednisone in patients with previously untreated multiple myeloma," *Journal of Clinical Oncology*, vol. 31, no. 4, pp. 448–455, 2013.

[31] A. Lopez-Chavez, T. Young, S. Fages et al., "Bevacizumab maintenance in patients with advanced non-small-cell lung cancer, clinical patterns, and outcomes in the eastern cooperative oncology group 4599 study: results of an exploratory analysis," *Journal of Thoracic Oncology*, vol. 7, no. 11, pp. 1707–1712, 2012.

[32] P. C. Lambert, J. R. Thompson, C. L. Weston, and P. W. Dickman, "Estimating and modeling the cure fraction in population-based cancer survival analysis," *Biostatistics*, vol. 8, no. 3, pp. 576–594, 2007.

[33] N. R. Smoll, K. Schaller, and O. P. Gautschi, "The cure fraction of glioblastoma multiforme," *Neuroepidemiology*, vol. 39, no. 1, pp. 63–69, 2012.

[34] P. C. Lambert, P. W. Dickman, P. Österlund, T. Andersson, R. Sankila, and B. Glimelius, "Temporal trends in the proportion cured for cancer of the colon and rectum: a population-based study using data from the Finnish Cancer Registry," *International Journal of Cancer*, vol. 121, no. 9, pp. 2052–2059, 2007.

[35] L. Huang, K. A. Johnson, A. B. Mariotto, J. J. Dignam, and E. J. Feuer, "Population-based survival-cure analysis of ER-negative breast cancer," *Breast Cancer Research and Treatment*, vol. 123, no. 1, pp. 257–264, 2010.

[36] European Medicines Agency Oncology Working Party, "Guideline on the evaluation of anticancer medicinal products in man," 2014, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/01/WC500137128.pdf.

[37] National Institute for Health and Clinical Excellence Final Appraisal Determination, "Ipilimumab for previously treated advanced (unresectable or metastatic) melanoma," December 2013, http://www.nice.org.uk/nicemedia/live/12092/61322/61322.pdf.

[38] B. Sherrill, J. A. Kaye, R. Sandin, J. C. Cappelleri, and C. Chen, "Review of meta-analyses evaluating surrogate endpoints for overall survival in oncology," *OncoTargets and Therapy*, vol. 5, pp. 287–296, 2012.

[39] J. D. Wolchok, A. Hoos, S. O'Day et al., "Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria," *Clinical Cancer Research*, vol. 15, no. 23, pp. 7412–7420, 2009.

[40] A. Hoos, "Evolution of end points for cancer immunotherapy trials," *Annals of Oncology*, vol. 23, supplement 8, Article ID mds263, pp. viii47–viii52, 2012.

[41] P. W. Kantoff, C. S. Higano, N. D. Shore et al., "Sipuleucel-T immunotherapy for castration-resistant prostate cancer," *The New England Journal of Medicine*, vol. 363, no. 5, pp. 411–422, 2010.

[42] P. W. Kantoff, T. J. Schuetz, B. A. Blumenstein et al., "Overall survival analysis of a phase II randomized controlled trial of a poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer," *Journal of Clinical Oncology*, vol. 28, no. 7, pp. 1099–1105, 2010.

[43] G. Bedard, L. Zeng, H. Lam et al., "Meaningful change in oncology quality-of-life instruments: a systematic literature review," *Expert Review of Pharmacoeconomics & Outcomes Research*, vol. 12, no. 4, pp. 475–483, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 362542, 9 pages
http://dx.doi.org/10.1155/2015/362542

Research Article

Polymorphisms of *NFκB1* and *IκBα* and Their Synergistic Effect on Nasopharyngeal Carcinoma Susceptibility

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Received 5 June 2014; Revised 23 September 2014; Accepted 23 September 2014

Academic Editor: Yung-Fu Chang

Nasopharyngeal carcinoma (NPC) is a multifactoral and polygenic disease with high prevalence in Southeast Asia and Southern China. Environmental factors and genetic susceptibility play important roles in NPC pathogenesis. In the present study, we tested the hypothesis that single nucleotide polymorphisms (SNPs) in nuclear factor-kappa B (*NFκB*) and its inhibitor (*IκBα*) conferred consistent risks for NPC. Four putatively functional SNPs (*NFκB1*: rs28362491del>ins ATTG; *NFκB2*: rs12769316G>A; *IκBα*: rs2233406C>T and rs696G>A) were analyzed to evaluate their associations with NPC risk in total 1590 NPC cases and 1979 cancer-free controls. We found that the rs28362491 insATTG variants (ins/del + ins/ins) in *NFκB1* conferred an increased risk of NPC (odds ratio [OR] = 1.30, 95% confidence interval [CI] = 1.09–1.55, and $P = 2.80 \times 10^{-3}$) compared with the del/del homozygous genotype. The rs696AA variant in *IκBα* had an increased risk of NPC (OR = 1.41, 95% CI = 1.20–1.66, and $P = 2.28 \times 10^{-5}$) by decreasing *IκBα* expression due to the modulation of microRNA hsa-miR-449a. Furthermore, both adverse genotypes of *NFκB*/*IκBα* and their interaction also exerted an increased risk on NPC. Taken together, Our findings indicated that genetic variants in *NFκB1* (rs28362491del>ins ATTG) and *IκBα* (rs696G>A) and their synergistic effect might contribute to NPC predisposition.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy of the head and neck that originates from the epithelial lining of the nasopharynx [1]. There were an estimated 84,400 incident cases of NPC and 51,600 deaths in 2008, representing about 0.7% of the global cancer burden [2]. NPC is rare in most parts of the world but is a leading malignancy in Southeast Asia and Southern China, with high incidence rate (40 per 100,000 person-years) [3, 4]. This distinctively geographic and ethnic distribution of NPC indicates that NPC is a malignancy with complex etiology involving both genetic and environmental factors [5].

Accumulating researches have revealed several well-established risk factors for NPC, such as Epstein-Barr virus (EBV) infection [6], certain dietary factors [7], and family history of cancer [8]. Studies have demonstrated that EBV is involved in direct carcinogenesis by triggering various cellular responses including the activation of inflammation [9, 10]. As a crucial inflammatory mediator, nuclear factor kappa-B (*NFκB*) and its endogenous inhibitors *NFκBI* (*IκB*) provide a critical mechanistic link between inflammation and tumor [11–14]. It has been reported that many signal transduction pathways, originating from a wide multifarious cellular stimuli, converge on the *NFκB*/*IκB* complex playing an essential role in cell angiogenesis, cell adhesion, proliferation, antiapoptosis, and repressing immune response [15]. Furthermore, the abnormalities of *NFκB* signaling pathway provide the cells with the production of growth factors as well as resistance to apoptotic and genotoxic insults, contributing to multiple carcinogenesis processes including tumor initiation, promotion, invasion, and metastasis [16, 17].

NFκB1 and *NFκB2* are two major forms of the *NFκB* family in human [18], and they can be inactivated by the most common protein of IκB family, NFkB inhibitor α (*IκBα*) [19]. Since *NFκB* is responsible for the regulation of many other genes in disease progression, variants in the genes coding for the *NFκB* and *IκB* proteins could be potentially related to disease development. Previous studies have identified several single nucleotide polymorphisms (SNPs) in *NFκB1*/*NFκB2* and *IκBα* to be associated with a great variety of diseases including inflammatory disorder and cancer [20–23]. However, mechanisms behind how specific polymorphisms of these various genes associate with NPC are still unclearly known. So we hypothesized that the SNPs in *NFκB*/*IκBα* genes may influence the NPC susceptibility.

In the present study, we analyzed the associations between four putatively functional SNPs (rs28362491del>ins ATTG in *NFκB1*; rs12769316G>A in *NFκB2*; rs2233406C>T and rs696 G>A in *IκBα*) and NPC risk in 906 NPC patients and 1072 age and sex frequency-matched controls in southern Chinese, and then validated the remarkable findings with 684 NPC patients and 907 controls in eastern Chinese. Biochemical assays were further performed to identify the biological effects of these polymorphisms.

2. Material and Methods

2.1. Study Subjects. Two independent hospital-based case-control studies including a southern Chinese population as a discovery set and an eastern Chinese population as a validation set were previously described briefly [24]. In the discovery set, 906 cases and 1072 cancer-free controls were recruited from April 2002 to June 2010 in Guangzhou city. In the validation set, 684 NPC and 907 healthy controls were consecutively recruited from March 2001 to May 2009 in Suzhou city. After their provision of written informed consent, each participant was scheduled for an interview to provide information on smoking status, and other factors with a structured questionnaire and to donate 5 mL peripheral blood. The definitions of the smoking status, pack-years smoked, alcohol use, and the family history of cancer have been described elsewhere [25–27]. Moreover, the EBV infection status and tumor stages of cases were obtained from the medical records. The study was approved by the institutional review boards of Guangzhou Medical University and Soochow University.

2.2. SNP Selection and Genotyping. Several SNPs located at *NFκB* or *IκBα* gene have been identified in previous reports. Among them, four polymorphisms [i.e., rs28362491del>ins ATTG of *NFκB1*; rs12769316 G>A of *NFκB2*; rs2233406C>T and rs696G>A of *IκBα*] were putatively functional and reported to be associated with various human diseases [21, 22, 28–31]. Furthermore, no other SNPs except these four polymorphisms located in predicted 3000 bp promoter region, coding region, and 3′-untranslated region (3′-UTR) of *NFκB* or *IκBα* gene were common with minor allele frequency (MAF) > 5% in Chinese based on HapMap public database. Therefore, these four polymorphisms were selected in our study.

The genomic DNA of each subject was extracted from 2 mL whole blood using the DNA Blood Mini Kit (Tiangen, China) according to the manufacturer's instructions and the final concentrations of all DNA samples were normalized to 20 ng/ul with a good purity (OD_{260}/OD_{280} = 1.8~2.0). TaqMan allelic discrimination assay, performing in the ABI PRISM 7500 Sequence Detection Systems (Applied Biosystems, Foster City, CA), was used to detect genotypes of the chosen SNPs. The genotypes were automatically determined by Sequence Detection Systems software 2.0.1 (Applied Biosystems; Supplementary Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/362542). Primers and probes were designed by Primer Express 3.0 software (Applied Biosystems) and synthesized by Shanghai GeneCore Biotechnologies (Shanghai, China) as shown in Supplementary Table S1. Approximately 10% of the samples were also randomly selected for a blinded regenotyping and 60 samples for sequencing, and the results were in 100% agreement (Supplementary Figure S1).

Because the biological effect of the rs28362491 del>ins ATTG variants has been evaluated elsewhere [20], here we focused on the functional effect of rs696 G>A polymorphism on NPC risk.

2.3. Construction of Reporter Plasmids. As a significant association was later observed for rs696G>A and NPC risk, we then constructed two reporter plasmids containing rs696 G or A allele to determine whether this polymorphism had any effect on its gene expression. The rs696G allele reporter was constructed by amplifying the 296 bp 3′-UTR of *IκBα* (+1 nt to +296 nt downstream to the translation stop site TGA) from subjects with homozygous rs696GG genotype using the forward primer: 5′-CCG ctcgag CGC AAA GGG GCT GAA AGA-3′ and reverse primer: 5′-ATA AGA AT gcggccgc ATA AAA TGT GGT CCT TCC ATG-3′, including the *XhoI* and *NotI* restriction sites. The amplified fragments and Psi-CHECK2 basic vector with renilla and firefly luciferase gene sequences (Promega, Madison, WI, USA) were then cocleaved by using *XhoI* and *NotI* (New England, BioLabs) and then ligated by T4 DNA ligase (New England, BioLabs) to product rs696G allele reporter gene (Figure 1(a)). The rs696A allele reporter gene was then obtained from the "G" construct by site-directed mutagenesis using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All reporter constructs were sequenced to confirm the sequence, orientation and integrity of each insert.

2.4. RNA Interference, Transient Transfections and Luciferase Assays. Two human NPC cell lines CNE-1 and CNE-2 purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were conducted *in vitro* luciferase assays described previously [25, 26]. Because the bioinformatics analysis (http://www.targetscan.org/ and http://microrna.sanger.ac.uk/) showed that the rs696G>A would change the binding of the microRNA miR-449a and

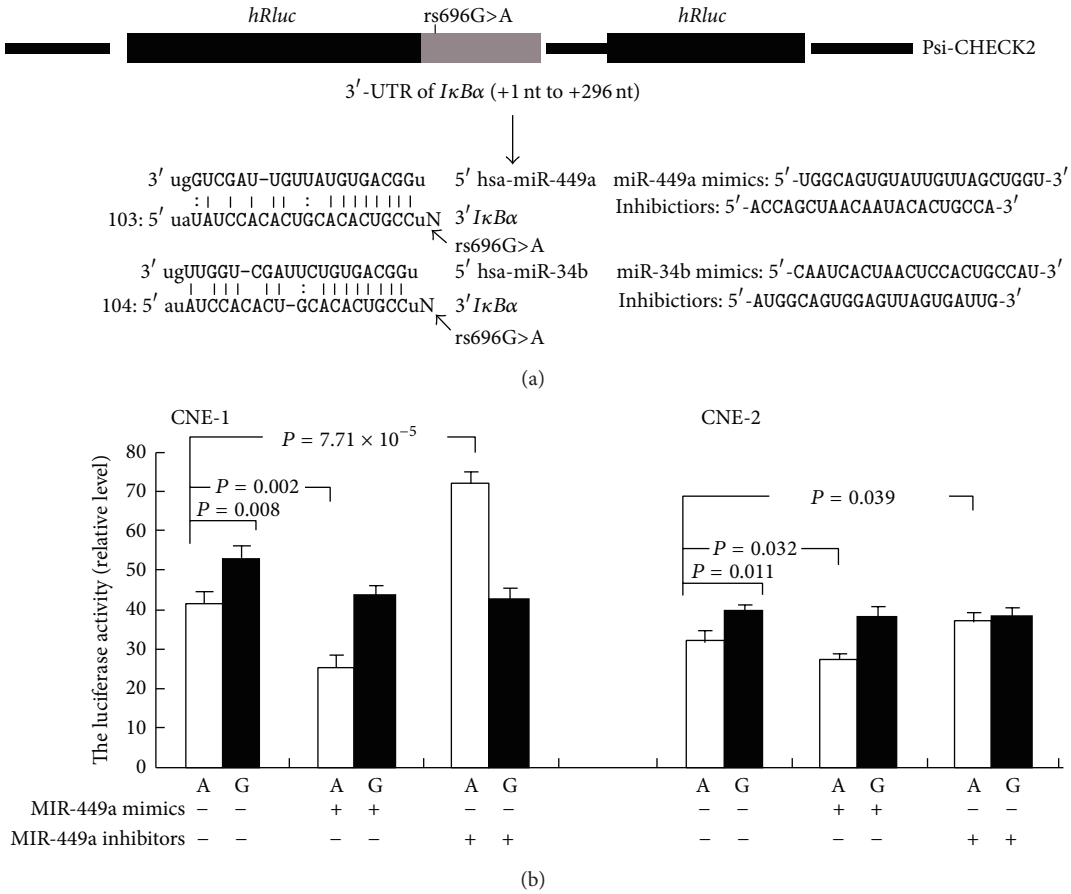

Figure 1: Effects of the rs696G>A polymorphism and treatment with microRNAs on *IκBα* transcriptional activity in different cell lines. (a). Schematic of the reporter gene construct with a 296 bp 3′-UTR of *IκBα* (+1 nt to 296 nt downstream to the translation stop site TGA) including rs696G>A polymorphism and a putative target site of miR-449a and miR-34b highly conserved in the *IκBα* mRNA 3′-UTR. (b). Luciferase expression of the two constructs in CNE-1 and CNE-2 cells. The renilla luciferase activity of each construct was normalized against the internal control of firefly luciferase. Columns, mean from three independent experiments; bars, SD; and Student's *t* test were used to test the differences in the expression levels of different constructs.

miR-34b, we then executed the RNA interference assay to show their effect interacted with this polymorphism. The cells were cultured in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) with 10% fetal bovine serum (Gibco-BRL) and penicillin (100 units/mL)/streptomycin (100 μg/mL) at a 37°C in the presence of 5% CO_2. CNE-1 and CNE-2 were seeded onto 24-well plates with 1×10^5 cells per well and cultured for 24 h. The cells were then transiently transfected with 1.5 μg of reporter plasmids (G or A allele) alone or co-transfected with or without microRNA mimics or inhibitors synthesized by GenePharma Co (Shanghai, China) using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The activities of *IκBα*-Psi-CHECK2 reporter with renilla luciferase and the internal standard with firefly luciferase was then measured by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) in followed 14–16 hours. Independent triplicate experiments were done for each plasmid construct.

2.5. Statistical Analysis. Chi-square test or Student's *t* test was used to assess the difference in the distributions of demographic characteristics and genotypes between cases and controls. The association between each SNP and cancer risk was estimated using an unconditional logistic regression model with adjustments for surrounding factors. A multiplicative interaction was suggested to detect the possible gene-environment or gene-gene interactions [25]. Homogeneity test was performed with Breslow-Day test. The statistical power was calculated by the PS Software [32]. Student's *t* test was also used to examine the difference in levels of luciferase reporter gene expression between different constructs. All tests were two-sided by using the SAS software (version 9.3; SAS Institute, Cary, NC, USA) and $P < 0.05$ was considered statistically significant.

3. Results

3.1. NFκB/IκBα Genotypes and NPC Risk. The distributions of demographic characteristics for all participants were described previously [24] and presented in Supplementary Table S2. Briefly, no significant deviations were observed in age, sex, and family history of cancer between cases and controls. However, other factors including smoking, drinking status, and EBV infection were significantly different ($P <$

0.05 for all). Furthermore, the homogeneity test revealed that the frequency distributions of drinking sand EBV infection status were not homogeneous between the two populations ($P < 0.05$), reflecting slightly different lifestyle among two populations.

The observed genotype frequencies of all SNPs were in agreement with the Hardy-Weinberg equilibrium in controls ($P > 0.05$ for all) as shown in Table 1. In discovery set, we found that the rs28362491ins variant genotypes (ins/ins + ins/del) of *NFκB1* conferred a 1.28-fold increased risk of NPC compared with del/del genotype (odds ratio [OR] = 1.28; 95% confidence interval [CI] = 1.01–1.63; $P = 0.011$) in a dominant genetic model, which is best fitted to criteria of the smallest AIC value. The individuals carrying rs696AA genotype exerted a 1.46-fold increased NPC risk compared to those with rs696G (GG + GA) genotypes (OR = 1.46; 95% CI = 1.17–1.82; $P = 7.4 \times 10^{-4}$) under a recessive genetic model. However, for other polymorphisms, no significant associations with NPC susceptibility were observed.

The above remarkable findings were confirmed from the validation set. As a consistent harmful role of *NFκB1* rs28362491del/ins and *IκBα* rs696G>A polymorphism for NPC risk, individuals carrying rs28362491ins variant genotypes had a 1.31-fold increased cancer risk compared with del/del genotype (OR = 1.31; 95% CI = 1.02–1.70; $P = 0.009$), and the rs696AA adverse genotype present a 1.38-fold risk of NPC compared to rs696 (GG + GA) genotypes (OR = 1.38; 95% CI = 1.09–1.75; $P = 0.007$). We then merged the two sets to increase the study power because the associations of the adverse genotype in the two datasets were homogeneous ($P = 0.941$ for rs28362491del>ins ATTG and $P = 0.938$ for rs696G>A). We found that the rs28362491ins variant genotypes of *NFκB1* had a 30% excess risk of NPC (OR = 1.30; 95% CI = 1.09–1.55; $P = 2.80 \times 10^{-3}$) compared to del/del genotype. Similarly, the rs696AA variant genotype conferred a 1.41-fold increased cancer risk (OR = 1.41; 95% CI = 1.20–1.66; $P = 2.28 \times 10^{-5}$).

We further explored the combined adverse genotypes of these two polymorphisms on the NPC risk. We defined rs28362491ins variant genotypes (ins/del + ins/ins) and rs696AA genotype as risk genotypes. The carriers of rs28362491del/del and rs696GG/AG have zero risk genotype, the carriers of ins/del (ins/ins) and rs696GG/AG or del/del and rs696AA have one risk genotype, and the ins/del (ins/ins) and rs696AA carriers have two risk genotypes. We found that the number of risk genotypes had consistently significant associations with NPC risk in the discovery set, the validation set, and the merged set. Compared to the zero risk genotype, the individuals carrying risk genotypes conferred an increased risk of NPC in a dose-depend ent manner in the pooled populations (OR = 1.25; 95% CI = 1.02–1.52; $P = 0.029$ for one risk genotype; OR = 1.82; 95% CI = 1.44–2.31; $P = 6.34 \times 10^{-7}$ for two risk genotypes; $P_{\text{trend}} = 2.98 \times 10^{-7}$).

3.2. Stratification Analysis and NFκB1-IκBα Interaction on NPC Risk. As shown in Table 2, the significant dose-effect of number of adverse genotypes on NPC risk were observed in all the subgroups. In addition, the potential gene-gene interaction of rs28362491del/ins and rs696G>A polymorphism on the risk of NPC was also investigated. We found that the individuals carrying rs696AA variant genotype conferred a more prominent adverse role on the risk of NPC compared to those with rs696GG/AG genotypes, while accompanied with rs28362491del/ins unfavorable genotype (OR = 1.38; 95% CI = 1.09–1.74; $P = 0.007$) or with rs28362491ins/ins adverse variant (OR = 1.53; 95% CI = 1.17–2.02; $P = 0.002$). Moreover, a significant positive interaction between the variations of *NFκB1* and *IκBα* on NPC risk was also observed ($P = 2.25 \times 10^{-6}$, shown in Table 3).

3.3. Luciferase Activity Assay. As visualized in Figure 1(b), luciferase assays showed that the transcription activity of the reporter gene which integrated the *IκBα* 3′-UTR with rs696A allele was suppressed more efficiently than that with G allele both in CNE-1 and CNE-2 cell (P value is 0.008 and 0.011, resp.).

The miR-449a mimics could further reduce the reporter genes' activity with rs696A allele ($P = 0.002$), and the miR-449a inhibitor reversed and upregulated reporter genes' activity ($P = 7.71 \times 10^{-5}$). However, the miR-34b failed to exhibit any effect on the reporter genes either with rs696A or G allele ($P > 0.05$ for all, data not shown). Taken together, these results indicated that hsa-miR-449a but not hsa-miR-34b specially binds to rs696A allele of the *IκBα* 3′-UTR and thus suppresses the expression of the *IκBα* gene *in vitro*.

4. Discussion

In the present hospital-based retrospective study, we found that the rs28362491ins ATTG variants of *NFκB1* conferred an increased risk of NPC, and the rs696AA variant of *IκBα* contributed an increased risk of NPC by decreasing *IκBα* expression under the modulation of hsa-miR-449a but not hsa-miR-34b. Both unfavorable genotypes of *NFκB1* and *IκBα* and their interaction exerted an effect on increasing NPC risk. To the best of our knowledge, this is the first study to investigate the genetic variants in *NFκB1* and *IκBα* on the risk of NPC.

Various studies have demonstrated that *NFkB1* and *IκBα* play a critical role in complicated human pathologies by regulating downstream genes involved in the immune response, cell proliferation, apoptosis, and senescence in addition to tumorigenesis [33, 34]. As a vital role in LMP1-mediated signal transduction, upregulated expression or overactivation of *NFκB1* has been reported to promote the NPC initiation [35]. Meanwhile, *IκBα*, which functions to suppress the effect of *NFκB1*, has been elucidated to be inactive or downregulated during various stimuli induced *NFκB* activation progresses and, in consequence, loses its protective role for human disease [36, 37]. Taking into consideration of the vital function on carcinogenesis and tumor progression by *NFκB/IκBα* manipulations, whether variations within the *NFκB* and its inhibitory protein *IκBα* could potentially

Table 1: Distribution of genotypes of *NFκB*/*IκBα* and associations with the risk of NPC.

Genotypes/alleles	Discovery set			Validation set			Merged set		
	Case *n* (%)	Controls[a] *n* (%)	Adjusted OR (95% CI)[b]	Case *n* (%)	Controls[a] *n* (%)	Adjusted OR (95% CI)[b]	Case *n* (%)	Controls[a] *n* (%)	Adjusted OR (95% CI)[b]
Total number of subjects	906	1072		684	907		1590	1979	
Total number of alleles	1812	2144		1368	1814		3180	3958	
NFκB1:									
rs28362491 del/ins									
del/del	152 (16.8)	224 (20.9)	1.00 (ref.)	117 (17.1)	195 (21.5)	1.00 (ref.)	269 (16.9)	419 (21.2)	1.00 (ref.)
del/ins	438 (48.3)	512 (47.8)	1.22 (0.94–1.56)	331 (48.4)	438 (48.3)	1.25 (0.95–1.64)	769 (49.4)	950 (48.0)	**1.24 (1.03–1.49)**
ins/ins	316 (34.9)	336 (31.3)	**1.39 (1.06–1.82)**	236 (34.5)	274 (30.2)	**1.42 (1.06–1.90)**	552 (34.7)	610 (30.8)	**1.41 (1.16–1.71)**
Trend test *P* value			**0.012**			**0.010**			**0.001**
Dominant model[c]									
ins/ins + del/ins versus del/del	754 (83.2)	848 (79.1)	**1.28 (1.01–1.63)**	567 (82.9)	712 (78.5)	**1.31 (1.02–1.70)**	1321 (83.1)	1560 (78.8)	**1.30 (1.09–1.55)**
NFκB2:									
rs12769316 G>A									
GG	485 (53.5)	599 (55.9)	1.00 (ref.)						
AG	347 (38.3)	391 (36.5)	1.06 (0.87–1.29)						
AA	74 (8.2)	82 (7.6)	1.13 (0.80–1.60)						
Trend test *P* value			0.320						
IκBα:									
rs2233406 C>T									
CC	701 (77.4)	813 (75.8)	1.00 (ref.)						
TC	188 (20.7)	244 (22.8)	0.90 (0.72–1.13)						
TT	17 (1.9)	15 (1.4)	1.54 (0.74–3.19)						
Trend test *P* value			0.618						
IκBα:									
rs696 G>A									
GG	256 (28.3)	317 (29.6)	1.00 (ref.)	182 (26.6)	255 (28.1)	1.00 (ref.)	438 (27.5)	572 (28.9)	1.00 (ref.)
AG	415 (45.8)	540 (50.4)	0.94 (0.76–1.17)	318 (46.5)	463 (51.1)	0.94 (0.74–1.20)	733 (46.1)	1003 (50.7)	0.94 (0.80–1.11)
AA	235 (25.9)	215 (20.1)	**1.40 (1.08–1.82)**	184 (26.9)	189 (20.8)	**1.33 (1.01–1.76)**	419 (26.4)	404 (20.4)	**1.37 (1.13–1.65)**
Trend test *P* value			**0.021**			**0.035**			**0.003**
Recessive model[c]									
AA versus GG + AA	235 (25.9)	215 (20.1)	**1.46 (1.17–1.82)**	184 (26.9)	188 (20.7)	**1.38 (1.09–1.75)**	419 (26.4)	403 (20.3)	**1.41 (1.20–1.66)**
Combined *NFκB1*/*IκBα* genotypes									
Number of risk genotypes[d]									
0	105 (11.6)	183 (17.1)	1.00 (ref.)	99 (14.5)	148 (16.3)	1.00 (ref.)	204 (12.8)	331 (16.7)	1.00 (ref.)
1	613 (67.7)	715 (66.7)	**1.48 (1.12–1.94)**	419 (61.3)	617 (68.0)	1.05 (0.76–1.34)	1032 (64.9)	1332 (67.3)	**1.25 (1.02–1.52)**
2	188 (20.7)	174 (16.2)	**1.93 (1.39–2.69)**	166 (24.3)	142 (15.7)	**1.71 (1.21–2.41)**	354 (22.2)	316 (16.0)	**1.82 (1.44–2.31)**
Trend test *P* value			**3.93×10^{-4}**			**9.42×10^{-5}**			**2.98×10^{-7}**

[a]The observed genotype frequencies among the control subjects were in agreement with the Hardy-Weinberg equilibrium ($p^2 + 2pq + q^2 = 1$) in the control subjects of both sets ($P > 0.05$ for all).

[b]Adjusted in a logistic regression model that included age, sex, smoking status, alcohol use, family history of cancer, and dataset.

[c]Akaike information criterion (AIC) value.

[d]Genotype combinations of two polymorphisms in the *NFκB1* and *IκBα*: ins variant genotypes rs28362491 (ins/del + ins/ins) and rs696AA genotype are defined as risk genotypes.

Table 2: Stratification analysis of the number of risk genotypes in *NFκB1/IκBα* by selected variables in NPC patients and controls.

	Patients ($n = 1590$)			Controls ($n = 1979$)			Adjusted OR (95% CI)[a]			
	0[b] *n* (%)	1[b] *n* (%)	2[b] *n* (%)	0[b] *n* (%)	1[b] *n* (%)	2[b] *n* (%)	0[b]	1[b]	2[b]	P_{trend}[c]
Age (years)										
<50	86 (13.0)	425 (64.2)	151 (22.8)	145 (17.0)	578 (67.6)	132 (15.4)	1.00 (ref.)	1.27 (0.94–1.72)	**1.88 (1.31–2.71)**	**3.47×10^{-4}**
≥50	118 (12.7)	607 (65.4)	203 (21.9)	186 (16.5)	754 (67.1)	184 (16.4)	1.00 (ref.)	1.25 (0.97–1.63)	**1.83 (1.34–2.51)**	**2.56×10^{-4}**
Sex										
Male	135 (11.9)	743 (65.7)	253 (22.3)	232 (16.5)	946 (67.4)	226 (16.1)	1.00 (ref.)	1.35 (1.07–1.71)	**1.98 (1.49–2.63)**	**1.57×10^{-6}**
Female	69 (15.0)	289 (63.0)	101 (22.0)	99 (17.2)	386 (67.1)	90 (15.7)	1.00 (ref.)	1.06 (0.77–1.56)	**1.60 (1.07–2.20)**	**1.32×10^{-6}**
Family history of cancer										
YES	12 (7.3)	106 (64.2)	47 (28.5)	27 (15.8)	120 (70.2)	24 (14.0)	1.00 (ref.)	1.74 (0.82–3.71)	**3.92 (1.63–9.39)**	**9.60×10^{-4}**
NO	192 (13.5)	926 (65.0)	307 (21.5)	304 (16.8)	1212 (67.0)	292 (16.2)	1.00 (ref.)	1.22 (1.00–1.50)	**1.68 (1.31–2.15)**	**2.41×10^{-5}**
Smoking status										
Ever	98 (11.5)	579 (67.7)	178 (20.8)	158 (16.9)	630 (67.2)	149 (15.9)	1.00 (ref.)	1.50 (1.13–1.99)	**1.95 (1.39–2.76)**	**1.33×10^{-4}**
Never	106 (14.4)	453 (61.6)	176 (24.0)	173 (16.6)	702 (67.4)	167 (16.0)	1.00 (ref.)	1.05 (0.80–1.38)	**1.70 (1.22–2.37)**	**8.41×10^{-4}**
Drinking status										
Ever	103 (13.2)	497 (63.8)	179 (23.0)	98 (16.2)	415 (68.5)	93 (15.3)	1.00 (ref.)	1.13 (0.83–1.53)	**1.80 (1.23–2.62)**	**1.36×10^{-3}**
Never	101 (12.4)	535 (66.0)	175 (21.6)	233 (17.0)	917 (66.8)	223 (16.2)	1.00 (ref.)	1.35 (1.04–1.74)	**1.81 (1.33–2.47)**	**1.31×10^{-4}**
EBV infection										
Positive	161 (13.0)	791 (63.8)	287 (23.2)	57 (17.0)	222 (66.1)	57 (16.9)	1.00 (ref.)	1.27 (0.91–1.79)	**1.74 (1.14–2.66)**	**8.91×10^{-3}**
Negative	43 (12.2)	241 (68.7)	67 (19.1)	274 (16.7)	1110 (67.6)	259 (15.8)	1.00 (ref.)	1.39 (0.97–1.98)	**1.69 (1.10–2.58)**	**0.017**
Stages										
I	8 (10.3)	52 (66.7)	18 (23.1)	331 (16.7)	1332 (67.3)	316 (16.0)	1.00 (ref.)	1.55 (0.80–3.43)	**2.35 (1.02–5.46)**	**0.039**
II	51 (12.1)	279 (66.3)	91 (21.6)				1.00 (ref.)	1.35 (0.98–1.88)	**1.81 (1.27–2.73)**	**8.58×10^{-4}**
III	90 (13.0)	451 (65.1)	152 (21.9)				1.00 (ref.)	1.25 (0.96–1.62)	**1.79 (1.32–2.44)**	**1.35×10^{-4}**
IV	58 (14.6)	239 (60.1)	101 (25.3)				1.00 (ref.)	1.03 (0.73–1.38)	**1.84 (1.28–2.64)**	**2.97×10^{-4}**

[a]Compared with zero risk genotype, ORs were adjusted in a logistic regression model that included age, sex, smoking status, drinking status, and family history of cancer.

[b]Genotype combinations of the two polymorphisms in the *NFκB1* and *IκBα*: ins variant genotypes rs28362491 (ins/del + ins/ins) and rs696AA genotype are defined as risk genotypes: i.e., the carriers of rs28362491 del/del and rs696 GG/AG have zero risk genotype; the carriers of ins/del (ins/ins) and rs696 GG/AG, or del/del and rs696AA have one risk genotype; and the ins/del (ins/ins) and rs696AA carriers have two risk genotypes.

[c]Trend test for NPC risk, with number of risk genotypes in each stratum.

Table 3: Interaction analysis between the variations of *NFκB1* and *IκBα* on NPC risk.

	Cases (n = 1590) *IκBα*: rs696 G>A		Controls (n = 1979) *IκBα*: rs696 G>A		Crude OR (95% CI)[a]	Adjusted OR (95% CI)[a]	P_{inter}[b]
	GG + AG n (%)	AA n (%)	GG + AG n (%)	AA n (%)	AA versus GG + AG	AA versus GG + AG	
NFκB1:							
rs28362491 del/ins							
del/del	204 (75.8)	65 (24.2)	331 (79.0)	88 (21.0)	1.20 (0.83–1.73)	1.24 (0.85–1.80)	
del/ins	575 (74.8)	194 (25.2)	762 (80.2)	188 (19.8)	1.37 (1.09–1.72)	**1.38 (1.09–1.74)**	
ins/ins	392 (71.0)	160 (29.0)	482 (79.0)	124 (21.0)	1.54 (1.18–2.01)	**1.53 (1.17–2.02)**	
Combined genotypes							$\mathbf{2.25 \times 10^{-6}}$
del/ins + ins/ins	967 (73.2)	354 (26.8)	1244 (79.7)	316 (20.3)	1.44 (1.21–1.71)	**1.45 (1.22–1.74)**	

[a]ORs were adjusted for age, sex and smoking status, and alcohol use, family history of cancer in a logistic regression model.
[b]P value of test for the multiplicative interaction between *rs696* G>A genotypes and rs28362491 del/ins genotypes on cancer risk in logistic regression models.

influence the function of *NFκB* and in turn facilitate tumor development were noteworthy.

Several evidences have been evaluated that *NFκB* and *IκBα* polymorphisms were associated with a series of cancer types including bladder cancer [38], colorectal cancer [39], and lung cancer [40]. Previous studies have provided the testification of rs28362491ins ATTG variants relatively increased the *NFκB1* gene expression and thus promoted the susceptibility of human disease [20, 41]. And another two studies of 479 gastric cancer cases and 880 controls in Japanese [42] and 1001 sporadic colorectal cancer patients and 1005 cancer-free controls in Chinese [22] also displayed harmful role of rs28362491ins variants for gastric cancer and CRC risk. Furthermore, rs696G>A polymorphism in the 3′-UTR of *IκBα* showed an increased risk of developing CRC in Chinese population [39]. In the current study, we received a consistent result as the rs28362491ins variants contributed an unfavorable effect on NPC susceptibility. Likewise, we also found that rs696G>A polymorphism in the 3′-UTR of *IκBα* gene conferred an increased risk of NPC. It is well known that microRNA could cause mRNA cleavage or translational suppression via imperfect binding to the 3′-UTR of target genes. According to the bioinformatics analysis, we found that the rs696G>A would change the potential bindings for the microRNAs miR-449a and miR-34b. We then performed a luciferase assay *in vitro* and the results indicated that the rs696A allele strengthened the binding capacity of miR-449a but not miR-34b, to the 3′-UTR of *IκBα* gene, which in turn inhibited the *IκBα* transcriptional activities. Correspondingly, recent literatures have identified the content that variations located in microRNA binding sites could affect miRNA-target recognition efficiency and gene expression and thus potentially to be associated with cancers [43]. This reconciles with our findings that the miR-449a could specially regulate the activities of *IκBα* genes with rs696A but not G allele and thus influence the risk of NPC.

We further analyzed the combined effect of the *NFκB1* and *IκBα* polymorphisms and their possible interaction on NPC risk. We found the adverse number of genotypes of *NFκB1*/*IκBα* offered an increased risk for NPC in a dose-dependent manner. Meanwhile, a significant interaction of *NFκB1* and *IκBα* variations on NPC risk was also observed. As previous biological mechanism indicated that rs696G>A SNP in *IκBα* could depress its expression; in contrast, rs28362491ins variants may relatively increase the *NFκB1* gene expression. It is reasonable about the fact that rs696G>A polymorphism might abolish the suppression of *IκBα* to *NFκB1* and upexpression *NFκB1* caused by rs28362491ins variants; these alterations in turn facilitated the carcinogenesis of NPC, which is potently supported our findings as gene-gene interaction of *NFκB1* and *IκBα* polymorphisms contributing a detrimental role on NPC risk.

As a hospital-based case-control study, some limitations in current study such as information bias would be ineluctable. However, with the fairly large sample size and two study populations, we have achieved high statistical powers (87.7% for *NFκB1* and 99.0% for *IκBα*) of the associations between *NFκB1*/*IκBα* polymorphism and NPC risk, and biological experiments also confirmed these significant associations. In addition, for the gene-gene interaction, we further analyzed the false-positive report probability (FPRP) and found that, under the assumption of a 0.0001 prior probability and a 1.50 prior OR as suggested by Wacholder et al. [44], the FPRP for the observed interaction of rs28362491ins variants and rs696A variant genotypes on NPC risk yielded a value of 0.027, which is lower than the preset FPRP-level criterion 0.20, indicating that our finding is noteworthy.

5. Conclusion

In conclusion, this preliminary study indicated that both *NFκB1* and *IκBα* polymorphisms were associated with NPC risk. Remarkable interaction between these two SNPs on the risk of NPC was also observed. These findings suggest that polymorphisms of *NFκB1* and *IκBα* may contribute a synergistic effect on NPC susceptibility in Chinese population. Validations with larger population-based studies in different ethnic groups and further biological assays are warranted to confirm our findings.

Conflict of Interests

The authors have declared no conflict of interests.

Authors' Contribution

Y. Liu and F. Qiu contributed equally to this work.

Acknowledgments

This study was supported by the National Natural Scientific Foundation of China Grants 30671813, 30872178, 81072366, 81273149, and 81473040 (J. Lu), and partly by 81001278, 81171895, 81472630 (Y. Zhou), 81402753 (L. Yang), 81102159 (L. Zhang), 81102061 (Q. Jiang), and Guangdong Provincial High Level Experts Grants 2010-79 (J. Lu). The authors thank Dr. Bohang Zeng, Dr. Zhanhong Xie, and Ms. Wanmin Zeng for their assistance in recruiting the subjects.

References

[1] J.-P. Liu, L. Cassar, A. Pinto, and H. Li, "Mechanisms of cell immortalization mediated by EB viral activation of telomerase in nasopharyngeal carcinoma," *Cell Research*, vol. 16, no. 10, pp. 809–817, 2006.

[2] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA: A Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[3] W.-H. Jia, Q.-H. Huang, J. Liao et al., "Trends in incidence and mortality of nasopharyngeal carcinoma over a 20–25 year period (1978/1983–2002) in Sihui and Cangwu counties in Southern China," *BMC Cancer*, vol. 6, article 178, 2006.

[4] M. C. Yu and J.-M. Yuan, "Epidemiology of nasopharyngeal carcinoma," *Seminars in Cancer Biology*, vol. 12, no. 6, pp. 421–429, 2002.

[5] E. T. Chang and H.-O. Adami, "The enigmatic epidemiology of nasopharyngeal carcinoma," *Cancer Epidemiology Biomarkers and Prevention*, vol. 15, no. 10, pp. 1765–1777, 2006.

[6] M. A. Vasef, A. Ferlito, and L. M. Weiss, "Nasopharyngeal carcinoma, with emphasis on its relationship to Epstein-Barr virus," *Annals of Otology, Rhinology and Laryngology*, vol. 106, no. 4, pp. 348–356, 1997.

[7] R. W. Armstrong, P. B. Imrey, M. S. Lye, M. J. Armstrong, M. C. Yu, and S. Sani, "Nasopharyngeal carcinoma in Malaysian Chinese: salted fish and other dietary exposures," *International Journal of Cancer*, vol. 77, no. 2, pp. 228–235, 1998.

[8] X. Ji, W. Zhang, C. Xie, B. Wang, G. Zhang, and F. Zhou, "Nasopharyngeal carcinoma risk by histologic type in central China: impact of smoking, alcohol and family history," *International Journal of Cancer*, vol. 129, no. 3, pp. 724–732, 2011.

[9] H. Zheng, L. L. Li, D. S. Hu, X. Y. Deng, and Y. Cao, "Role of Epstein-Barr virus encoded latent membrane protein 1 in the carcinogenesis of nasopharyngeal carcinoma," *Cellular & Molecular Immunology*, vol. 4, no. 3, pp. 185–196, 2007.

[10] J. L. Arbiser, "Implications of Epstein-Barr Virus (EBV)-induced carcinogenesis on cutaneous inflammation and carcinogenesis: evidence of recurring patterns of angiogenesis and signal transduction," *Journal of Investigative Dermatology*, vol. 124, no. 5, pp. xi–xii, 2005.

[11] F. R. Greten, L. Eckmann, T. F. Greten et al., "IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer," *Cell*, vol. 118, no. 3, pp. 285–296, 2004.

[12] E. Pikarsky, R. M. Porat, I. Stein et al., "NF-κB functions as a tumour promoter in inflammation-associated cancer," *Nature*, vol. 431, no. 7007, pp. 461–466, 2004.

[13] M. Karin, "Nuclear factor-κB in cancer development and progression," *Nature*, vol. 441, no. 7092, pp. 431–436, 2006.

[14] M. Karin, "NF-kappaB as a critical link between inflammation and cancer," *Cold Spring Harbor Perspectives in Biology*, vol. 1, no. 5, Article ID a000141, 2009.

[15] B. B. Aggarwal, Y. Takada, S. Shishodia et al., "Nuclear transcription factor NF-kappa B: role in biology and medicine," *Indian Journal of Experimental Biology*, vol. 42, no. 4, pp. 341–353, 2004.

[16] M. M. Chaturvedi, B. Sung, V. R. Yadav, R. Kannappan, and B. B. Aggarwal, "NF-κB addiction and its role in cancer: one size does not fit all," *Oncogene*, vol. 30, no. 14, pp. 1615–1630, 2011.

[17] M. Karin and F. R. Greten, "NF-κB: linking inflammation and immunity to cancer development and progression," *Nature Reviews Immunology*, vol. 5, no. 10, pp. 749–759, 2005.

[18] F. Chen, V. Castranova, X. Shi, and L. M. Demers, "New insights into the role of nuclear factor-κB, a ubiquitous transcription factor in the initiation of diseases," *Clinical Chemistry*, vol. 45, no. 1, pp. 7–17, 1999.

[19] M. S. Hayden, A. P. West, and S. Ghosh, "SnapShot: NF-κB signaling pathways," *Cell*, vol. 127, no. 6, pp. 1286.e1–1286.e2, 2006.

[20] A. S. Karban, T. Okazaki, C. I. M. Panhuysen et al., "Functional annotation of a novel NFKB1 promoter polymorphism that increases risk for ulcerative colitis," *Human Molecular Genetics*, vol. 13, no. 1, pp. 35–45, 2004.

[21] Y. He, H. Zhang, J. Yin et al., "IkappaBalpha gene promoter polymorphisms are associated with hepatocarcinogenesis in patients infected with hepatitis B virus genotype C," *Carcinogenesis*, vol. 30, no. 11, pp. 1916–1922, 2009.

[22] S. Song, D. Chen, J. Lu et al., "NFkappaB1 and NFkappaBIA polymorphisms are associated with increased risk for sporadic colorectal cancer in a southern Chinese population," *PLoS ONE*, vol. 6, no. 6, Article ID e21726, 2011.

[23] D. Huang, L. Yang, Y. Liu et al., "Functional polymorphisms in NFκB1/IκBα predict risks of chronic obstructive pulmonary disease and lung cancer in Chinese," *Human Genetics*, vol. 132, no. 4, pp. 451–460, 2013.

[24] L. Yang, B. Liu, F. Qiu et al., "The effect of functional MAPKAPK2 copy number variation CNV-30450 on elevating nasopharyngeal carcinoma risk is modulated by EBV infection," *Carcinogenesis*, vol. 35, no. 1, pp. 46–52, 2014.

[25] J. Lu, L. Yang, H. Zhao et al., "The polymorphism and haplotypes of *PIN1* gene are associated with the risk of lung cancer in southern and eastern chinese populations," *Human Mutation*, vol. 32, no. 11, pp. 1299–1308, 2011.

[26] B. Liu, D. Chen, L. Yang et al., "A functional variant (−1304T>G) in the MKK4 promoter contributes to a decreased risk of lung cancer by increasing the promoter activity," *Carcinogenesis*, vol. 31, no. 8, pp. 1405–1411, 2010.

[27] L. Yang, Y. Li, M. Cheng et al., "A functional polymorphism at microRNA-629-binding site in the 3′-untranslated region of nbs1 gene confers an increased risk of lung cancer in southern and eastern chinese population," *Carcinogenesis*, vol. 33, no. 2, pp. 338–347, 2012.

[28] V. Sampath, M. Le, L. Lane et al., "The *NFKB1* (g.-24519delATTG) variant is associated with necrotizing enterocolitis (NEC) in premature infants," *Journal of Surgical Research*, vol. 169, no. 1, pp. e51–e57, 2011.

[29] M. Marcos, I. Pastor, R. González-Sarmiento, and F. J. Laso, "A functional polymorphism of the NFKB1 gene increases the risk for alcoholic liver cirrhosis in patients with alcohol dependence," *Alcoholism: Clinical and Experimental Research*, vol. 33, no. 11, pp. 1857–1862, 2009.

[30] Y. H. Hung, C. C. Wu, and T. T. Ou, "IκBα promoter polymorphisms in patients with Behçet's disease," *Disease Markers*, vol. 28, no. 1, pp. 55–62, 2010.

[31] P. Zhang, Q. Wei, X. Li et al., "A functional insertion/deletion polymorphism in the promoter region of the NFKB1 gene increases susceptibility for prostate cancer," *Cancer Genetics and Cytogenetics*, vol. 191, no. 2, pp. 73–77, 2009.

[32] W. D. Dupont and W. D. Plummer Jr., "Power and sample size calculations. A review and computer program," *Controlled Clinical Trials*, vol. 11, no. 2, pp. 116–128, 1990.

[33] L. L. Yates and D. C. Górecki, "The nuclear factor-kappaB (NF-κB): from a versatile transcription factor to a ubiquitous therapeutic target," *Acta Biochimica Polonica*, vol. 53, no. 4, pp. 651–662, 2006.

[34] R. O. Escarcega, "The transcription factor NF-κB in human diseases," *Revista Médica del Instituto Mexicano del Seguro Social*, vol. 48, no. 1, pp. 55–60, 2010.

[35] C.-C. Chen, H.-P. Liu, M. Chao et al., "NF-κB-mediated transcriptional upregulation of TNFAIP2 by the Epstein-Barr virus oncoprotein, LMP1, promotes cell motility in nasopharyngeal carcinoma," *Oncogene*, vol. 33, pp. 3648–3659, 2014.

[36] H. J. Kim, K. W. Kim, B. P. Yu, and H. Y. Chung, "The effect of age on cyclooxygenase-2 gene expression: NF-kappaB activation and IkappaBalpha degradation," *Free Radical Biology & Medicine*, vol. 28, no. 5, pp. 683–692, 2000.

[37] H. Abe, C. N. Hayes, H. Ochi et al., "Inverse association of IL28B genotype and liver mRNA expression of genes promoting or suppressing antiviral state," *Journal of Medical Virology*, vol. 83, no. 9, pp. 1597–1607, 2011.

[38] K. Riemann, L. Becker, H. Struwe, H. Rübben, A. Eisenhardt, and W. Siffert, "Insertion/deletion polymorphism in the promoter of NFKB1 as a potential molecular marker for the risk of recurrence in superficial bladder cancer," *International Journal of Clinical Pharmacology and Therapeutics*, vol. 45, no. 8, pp. 423–430, 2007.

[39] J. Gao, D. Pfeifer, L.-J. He et al., "Association of NFKBIA polymorphism with colorectal cancer risk and prognosis in Swedish and Chinese populations," *Scandinavian Journal of Gastroenterology*, vol. 42, no. 3, pp. 345–350, 2007.

[40] D. Huang, L. Yang, Y. Liu et al., "Functional polymorphisms in NFκB1/IκBα predict risks of chronic obstructive pulmonary disease and lung cancer in Chinese," *Human Genetics*, vol. 132, no. 4, pp. 451–460, 2013.

[41] P. Li, J. Gu, X. Yang et al., "Functional promoter-94 ins/del ATTG polymorphism in *NFKB1* gene is associated with bladder cancer risk in a Chinese population," *PLoS ONE*, vol. 8, no. 8, Article ID e71604, 2013.

[42] T. Arisawa, T. Tahara, H. Shiroeda et al., "Functional promoter polymorphisms of NFKB1 influence susceptibility to the diffuse type of gastric cancer," *Oncology Reports*, vol. 30, no. 6, pp. 3013–3019, 2013.

[43] Z. Yu, Z. Li, N. Jolicoeur et al., "Aberrant allele frequencies of the SNPs located in microRNA target sites are potentially associated with human cancers," *Nucleic Acids Research*, vol. 35, no. 13, pp. 4535–4541, 2007.

[44] S. Wacholder, S. Chanock, M. Garcia-Closas, L. El Ghormli, and N. Rothman, "Assessing the probability that a positive report is false: an approach for molecular epidemiology studies," *Journal of the National Cancer Institute*, vol. 96, no. 6, pp. 434–442, 2004.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 347867, 10 pages
http://dx.doi.org/10.1155/2015/347867

Research Article

Expression of *FOXP3*, *CD14*, and *ARG1* in Neuroblastoma Tumor Tissue from High-Risk Patients Predicts Event-Free and Overall Survival

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Received 10 July 2014; Revised 12 January 2015; Accepted 14 January 2015

Academic Editor: Mohammad Owais

The prognosis of children with metastatic neuroblastoma (NB) > 18 months at diagnosis is dismal. Since the immune status of the tumor microenvironment could play a role in the history of disease, we evaluated the expression of *CD45*, *CD14*, *ARG1*, *CD163*, *CD4*, *FOXP3*, *Perforin-1* (*PRF1*), *Granzyme B (GRMB)*, and *IL-10* mRNAs in primary tumors at diagnosis from children with metastatic NB and tested whether the transcript levels are significantly associated to event-free and overall survival (EFS and OS, resp.). Children with high expression of *CD14*, *ARG1* and *FOXP3* mRNA in their primary tumors had significantly better EFS. Elevated expression of *CD14*, and *FOXP3* mRNA was significantly associated to better OS. *CD14* mRNA expression levels significantly correlated to all markers, with the exception of *CD4*. Strong positive correlations were found between *PRF1* and *CD163*, as well as between *PFR1* and *FOXP3*. It is worth noting that the combination of high levels of *CD14*, *FOXP3*, and *ARG1* mRNAs identified a small group of patients with excellent EFS and OS, whereas low levels of *CD14* were sufficient to identify patients with dismal survival. Thus, the immune status of the primary tumors of high-risk NB patients may influence the natural history of this pediatric cancer.

1. Introduction

Neuroblastoma (NB) is a pediatric neuroectodermal solid tumor with a heterogeneous clinical behavior [1]. Despite intensive multimodal therapy, patients presenting with metastatic disease, that is, stage 4 according to INSS [2] or stage M according to INRG-SS [3], aged more than 18 months at diagnosis have dismal survival rate. The search for powerful prognostic markers for this subset of patients is aimed to identify the cases that can be cured by standard therapy and the ultra-high-risk cases that need to be enrolled in new experimental trials.

Recently, the presence of high levels of NB-related molecular markers in bone marrow (BM) and peripheral blood (PB) samples at diagnosis has been shown to be highly predictive of event free survival (EFS) and overall survival (OS) [4]. However, a prognostic marker in the primary tumor could be helpful to improve patients' stratification. Unfortunately, although several gene expressions profiling studies of primary tumor specimens have identified prognostic signatures [5–12], so far none of the latter has a predictive power within the subset of stage 4 patients aged > 18 months at diagnosis.

It is increasingly evident that the tumor microenvironment plays an important role in driving the fate of antitumor

response (see [13] for a review). Indeed, several membrane-bound or soluble factors produced by normal and neoplastic cells in the tumor microenvironment may downregulate the antitumor immune response and greatly influence the natural history of cancer. Recently, specific gene signatures related to a successful immune response and to tumor rejection processes have been related to tumor outcome in different tumors [14].

In human NB, information on the presence and activity of specific subsets of immune suppressive cells and soluble factors in the primary tumors is scanty [15]. Facchetti et al. [16] showed that NB primary tumors show different degrees of lymphocyte infiltration, but no correlation with survival was found. A gene expression study performed on primary tumors [12] suggested a negative role for myeloid-derived suppressor cells in the prognosis of metastatic NB patients. Recently, the same authors [17, 18] demonstrated that the inclusion of 5 inflammation related genes increased the predictive power of the gene signature, since tumors from high-risk NB patients present a greater infiltration of CD163+, M2-type, tumor associated macrophages (TAMs). Interestingly, NKT cells can be instructed to selectively kill these TAMs [18].

An important immune suppressive role is ascribed to CD4+CD25^{high}FoxP3+ T cells, also termed Treg cells. No difference in their number was found in PB samples from a small cohort of low- and high-risk patients [19]. However, Tilak et al. have recently shown that the frequency of Treg in PB samples was higher in NB patients than in healthy children and that frequency was reduced after chemotherapy [20]. Nevertheless, the analysis of Treg in several tumor types indicated that FoxP3+ expression may be related to CD4+ T-cell activation rather than to an immune suppressive phenotype [21, 22]. This finding has important clinical implications since depletion of CD4+CD25^{high}FoxP3+ cells has been proposed as a tool to enhance tumor responses. Indeed, Carlson and coworkers [23] recently demonstrated that NB tumor-infiltrating CD4+ T cells can be activated in the tumor milieu, but not in the periphery. Gowda et al. [19] surprisingly found that the immunosuppressive cytokine IL-10, produced by Treg, T regulatory type 1 (Tr1) cells [24], and cells of the innate immunity, such as NK and macrophages, was elevated in PB of patients with low-risk NB, suggesting a protective role of innate immunity.

To gain insight into the role of different immune cell populations in the natural history of metastatic NB, we have evaluated the mRNA expression of the following molecular markers in 41 primary tumors at diagnosis: *CD45*: all leukocytes, *CD14*: monocyte-macrophages, *ARG1*: activated macrophages, *CD163*: M2 TAM, *CD4*: T helper cells, *FOXP3*: Treg, *Perforin-1 (PRF1)*, and *Granzyme B (GRMB)*: cytotoxic T lymphocytes and activated NK cells. In addition we evaluated the mRNA expression of the immune suppressive cytokine *IL-10*. We then tested whether expression of these genes significantly correlated to survival.

Table 1: Patients' characteristics.

	N	%
Age at diagnosis		
<18 months	0	
>18 months	41	*100*
Sex		
Female	17	*41.5*
Male	24	*58.5*
MYCN status		
Amplified	13	*31.7*
Not amplified	28	*68.3*
Primary tumor site		
Adrenal	25	*61.0*
Thorax	3	*7.3*
Abdomen	13	*31.7*
Metastatic sites		
Bone Marrow	13	*31.7*
Bone Marrow + bone	24	*58.6*
Pleura	2	*4.9*
Bone	1	*2.4*
Other	1	*2.4*
Relapse		
No	15	*36.6*
Yes	26	*63.4*
Outcome		
Alive	16	*39.0*
Dead of disease	25	*61.0*

2. Materials and Methods

2.1. Patients and Tumors. Patients included in the study were diagnosed in Italy with stage 4 NB between December 1992 and October 2006. Disease staging [2] was made at the referring oncology center and centrally reviewed at the Gaslini Institute. The median age at diagnosis was 3.4 years (range 1.5–6.3) and the median follow-up was 46.7 months (range 0.3–172.4). Patients were treated according to protocols NB-92, NB-95, and NB-97, which include induction therapy followed by myeloablative chemotherapy with autologous stem cell transplantation for consolidation. Survival rates for these protocols have been demonstrated to be similar [25] and are in line with the expected survival rate for patients with metastatic NB aged > 18 months at diagnosis [3]. Follow-up data at January 2014 were retrieved from the Italian Neuroblastoma Registry (INBR) [25]. Patients' characteristics are reported in Table 1.

After histological diagnosis, an aliquot of the primary tumor surgically resected at diagnosis was centralized at the Gaslini Institute and stored at −80°C until RNA was extracted. Only tumors with neoplastic cell content higher than 80% were included.

The study was approved by the Institutions' Ethical Committees and all analyses were performed according to the Helsinki declaration.

2.2. RNA Extraction and RT-qPCR Analysis. Total RNA was extracted from primary tumors as previously described [26]. One hundred ng of total RNA was reverse transcribed and then amplified for each molecular marker in duplicate by qPCR, using the following assays from Life Technology (Life Technologies Europe BV, Monza, Italy): *CD45*: Hs00365634_g1, *CD14*: Hs02621496_s1, *CD163*: Hs00174705_m1, *ARG1*: Hs00968979_m1, *CD4*: Hs01058407_m1, *FOXP3*: Hs00203958_m1, *IL10*: Hs00961622_m1, *PRF1*: Hs00169473_m1, *GZMB*: Hs01554355_m1, and primers and probe for β2-microglobulin (*B2M*) [27]. The level of expression of each marker was normalized to the expression of *B2M*, according to the delta Ct method [28] and results were reported as $2^{-\text{delta Ct}}$. All markers were tested on a panel of 10 NB cell lines and none of these markers was expressed by the tumor cells themselves. Moreover, to exclude DNA contamination the cDNA obtained in the absence of reverse transcriptase was included in each qPCR assay. Water was also run as negative control.

2.3. Statistical Analysis. The Wilcoxon-Mann-Whitney test was used to compare median values, and the Spearman ρ coefficient was used to assess correlation between variables. Event-free and overall survival (EFS and OS, resp.) analyses were performed according to the Kaplan-Meier method and compared by the log-rank test. A P value < 0.05 was considered as statistically significant. Analyses were made using the Prism software (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Expression of Molecular Markers for Different Immune Cell Populations in Primary NB Tumors. We evaluated the expression of *CD45*, *CD14*, *CD163*, *ARG1*, *CD4*, *FOXP3*, *PRF1*, *GMZB*, and *IL10* mRNAs in primary tumor samples taken at diagnosis from 41 patients with metastatic NB >18 months at diagnosis. After normalization to *B2M* expression, the median expression value was used to stratify patients and correlation with EFS and OS was tested by the log rank test. High-risk patients with high expression of *CD14*, *ARG1*, and *FOXP3* mRNA in their primary tumors had a significantly better EFS (Figures 1(b), 1(d), and 1(f), $P = 0.0083$, $P = 0.0482$, and $P = 0.0024$, resp.). In addition, a trend toward a better survival was seen for patients with high expression of *CD45* and *PRF1* (Figures 1(a) and 1(g), resp.).

The OS was significantly better for patients with high *CD14* and *FOXP3* RNA expression in their primary tumors at diagnosis (Figures 2(b) and 2(f), $P = 0.0008$ and $P = 0.0022$, resp.). It is worth noting that all the patients with *CD14* expression below the median died of disease. Overall survival was also better for patients that had high *CD45* and *PRF1* expression (Figures 2(a) and 2(g), $P = 0.0266$ and $P = 0.0486$, resp.).

Levels of *CD163*, *CD4*, *GZMB*, and *IL10* mRNA in primary tumors of stage 4 NB patients were never associated to different EFS or OS (Figures 1 and 2, resp.).

3.2. Correlation of Expression Levels of Molecular Markers. We then analyzed potential correlation in the expression of the molecular markers. *CD14* mRNA expression levels significantly correlated to all the other markers (Figures 3(a) to 3(f)), with the exception of *CD4* (not shown). Surprisingly, strong positive correlations were found between *PRF1* and *CD163*, as well as between *PFR1* and *FOXP3* (Figures 3(g) and 3(h), resp.). Positive correlations were also found between *IL10* and *ARG1*, between *IL10* and *CD4* (Figures 3(j) and 3(k), resp.), and between *CD163* and *ARG1* (Figure 3(l)).

3.3. Predictive Power of Molecular Markers. Based on the results of survival analyses and correlation studies, we tested whether the combination of markers had a higher predictive power than a single marker. As shown in Figure 4, the combination of high levels of *CD14*, *FOXP3*, and *ARG1* expression strongly predicted good EFS and OS. However, low levels of *CD14* remained the best predictor of a dismal survival (Figure 2(b)).

3.4. Analysis of a Public NB Tumor Gene Expression Profiling Dataset. We checked in a public gene expression profiling dataset of 40 stage 4 NB tumors (R2: Genomics Analysis and Visualization Platform: http://hgserver1.amc.nl/cgi-bin/r2/main.cgi, tumor neuroblastoma, Versteeg) significant associations between the expression levels of the studied molecular markers and EFS and OS. It is important to note that the public dataset reports microarray data and that Kaplan-Meyer plot is given for each probe according to ROC analysis. Despite the difference in the type of data and analysis, the significant association of high *FOXP3* mRNA levels with better EFS and OS was confirmed (Figure 5). No significant association was found for *CD14* and *ARG1* (not shown).

4. Discussion

The prognostic role of molecular analysis of immune cells and of the immunosuppressive cytokine *IL10* has been evaluated in primary tumors from 41 children with metastatic NB aged more than 18 months at diagnosis. The results indicate that high level of *CD14*, *ARG1*, and *FOXP3* mRNA expression in primary NB tumor significantly correlated to a better survival. Each of these markers had predictive power; however, the combined use of the three markers improved survival prediction and allowed to identify patients that can be cured by standard therapy. Furthermore, the association of *FOXP3* mRNA levels with different EFS and OS was confirmed in a gene expression dataset of 40 stage 4 NB tumors analyzed by microarray. The discrepancy in the predictive power of *CD14* and *ARG1* observed by RT-qPCR and microarray analysis may relate to the different sensitivity/specificity of the oligonucleotide probes used. Moreover, in the Versteeg's database the tumor cell content required for inclusion was

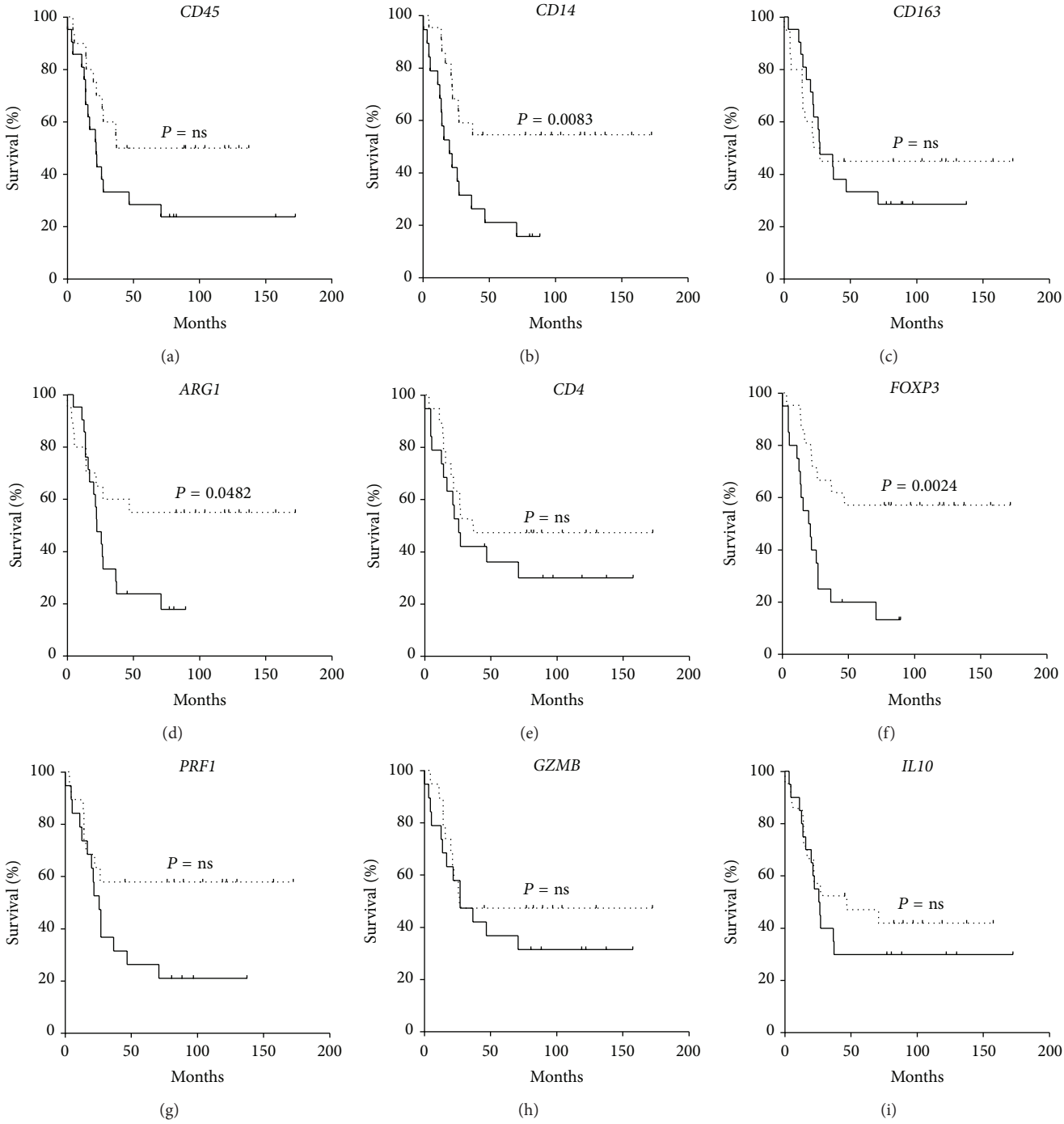


Figure 1: Kaplan-Meyer plots of EFS of stage 4 patients stratified according to the level of mRNA expression below (continuous line) or above (dotted line) the median of *CD45* (a), *CD14* (b), *CD163* (c), *ARG1* (d), *CD4* (e), *FOXP3* (f), *PRF1* (g), *GZMB* (h), and *IL10* (i).

lower and patients < 18 months could not be excluded. Thus, our findings need to be confirmed by qPCR in a prospective study with a greater cohort of stage 4 NB patients > 18 months.

The demonstration that *FOXP3* expression positively associated to a better EFS and OS of high-risk NB patients is in agreement with previous reports in head and neck and colorectal cancer patients [29, 30]. It has been suggested that activation state or suppressive functions of the infiltrating T cells greatly depend on the specific microenvironment present in the anatomical site [21]. *FOXP3* expression did not correlate to *CD4* expression but correlated to *PFR1* mRNA levels, further supporting the tenet that *FOXP3* mRNA expression in NB tumors was an indicator of effector T-cell activation rather than of immunosuppressive Treg cells [21, 22, 31–33]. Since *FOXP3* expression correlated to *CD14* expression, the presence of macrophages or dendritic cells

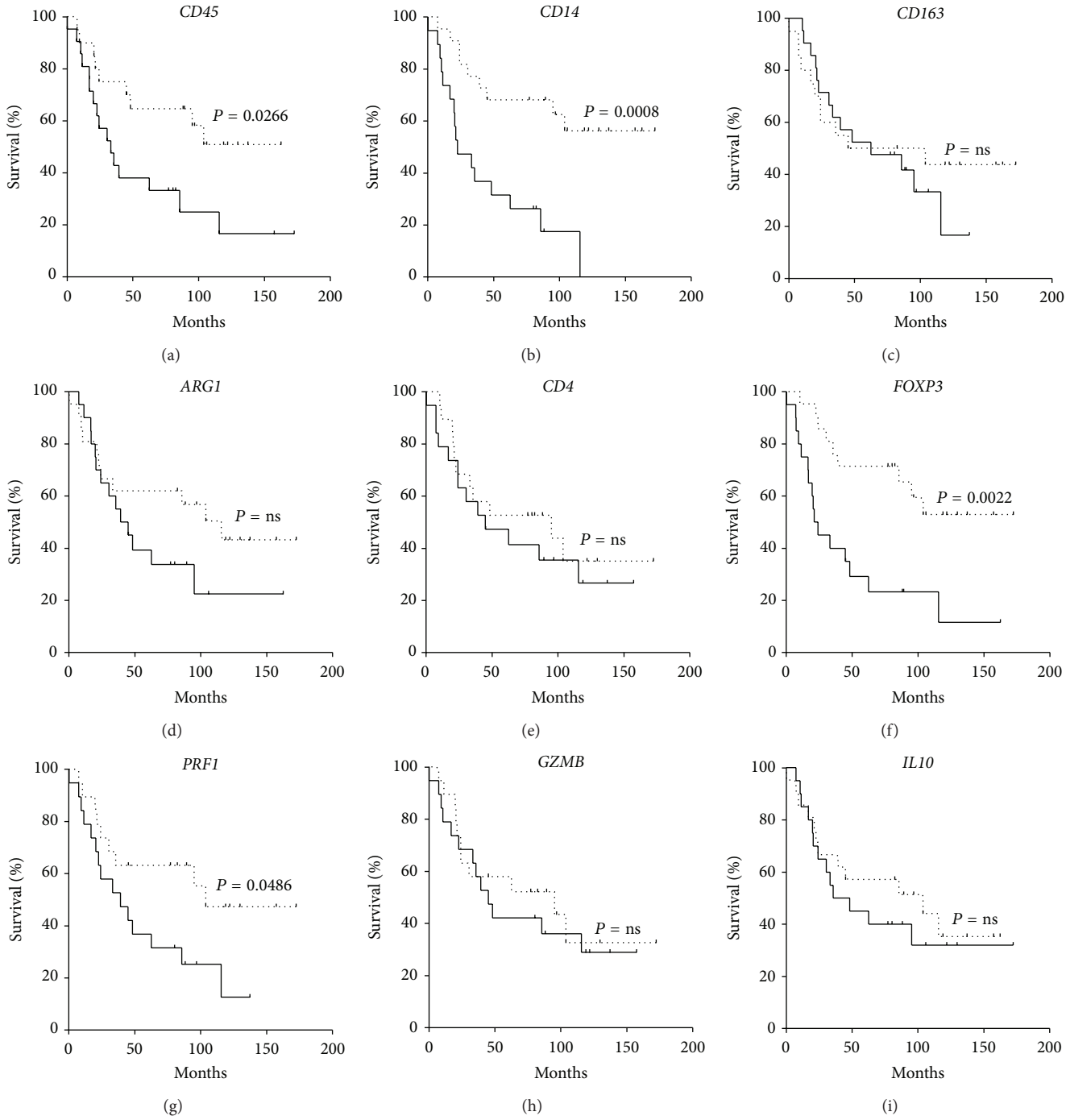


Figure 2: Kaplan-Meyer plots of OS of stage 4 patients stratified according to the level of mRNA expression below (continuous line) or above (dotted line) the median of *CD45* (a), *CD14* (b), *CD163* (c), *ARG1* (d), *CD4* (e), *FOXP3* (f), *PRF1* (g), *GZMB* (h), and *IL10* (i).

expressing *CD14* may be responsible for the activation of effector T cells, which in turn limited tumor progression. It is of note that *CD14* expression levels, but not those of the TAM marker *CD163*, significantly associated to different survival, suggesting that CD14+ cells other than TAMs may favor the induction of an effective immune response.

Regarding the role of FoxP3-expressing cells, in murine syngeneic NB models, CD4+FoxP3+ Treg cells increase in secondary lymphoid organs of NB-bearing mice and their depletion increased the effects of immunotherapy and of hematopoietic stem cell transplantation [34–37]. The apparent discrepancy between syngeneic murine models and human NB may relate to a different expression of FoxP3 in activated effector T cells in mouse and human [33]. In addition, human NB cells express low, if any, levels of HLA class I molecules [38]. This defect may hamper their

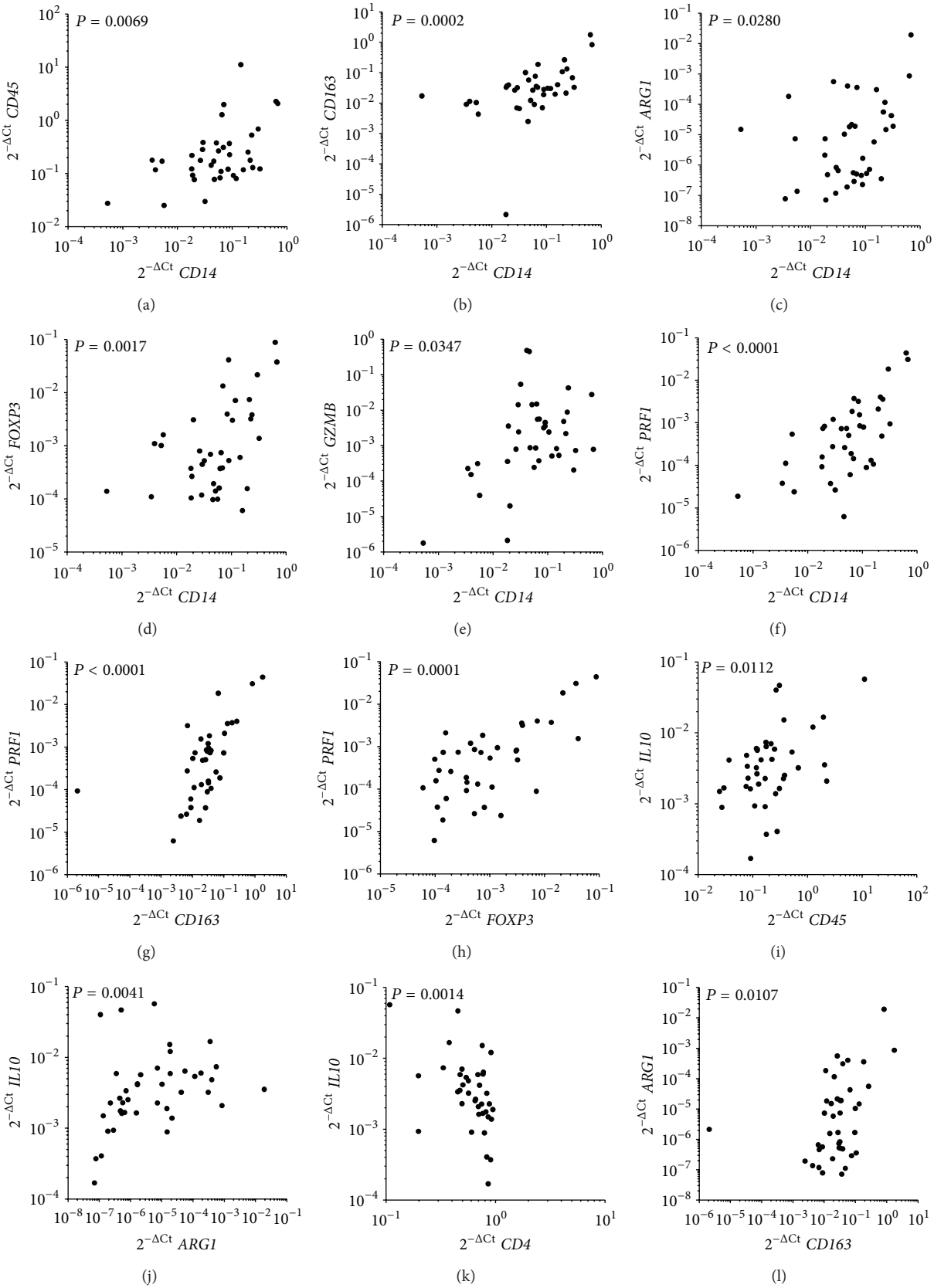


Figure 3: Significant r Spearman's correlations between *CD14* and *CD45* (a), *CD163* (b), *ARG1* (c), *FOXP3* (d), *GZMB* (e), and *PRF1* (f); between *PRF1* and *CD163* (g) and *FOXP3* (h); between *IL10* and *CD45* (i), *ARG1* (j), and *CD4* (k); and between *CD163* and *ARG1* (l).

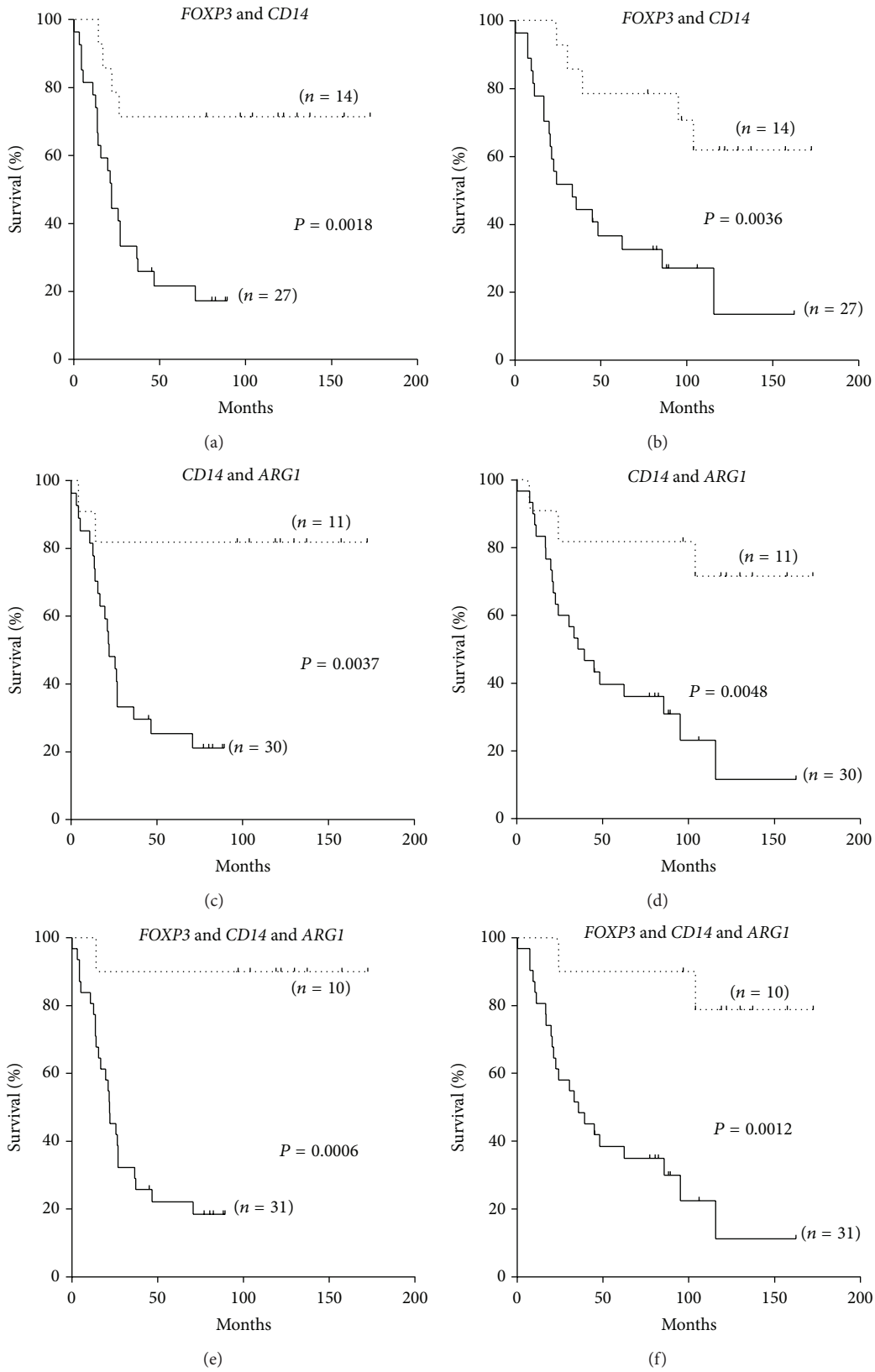
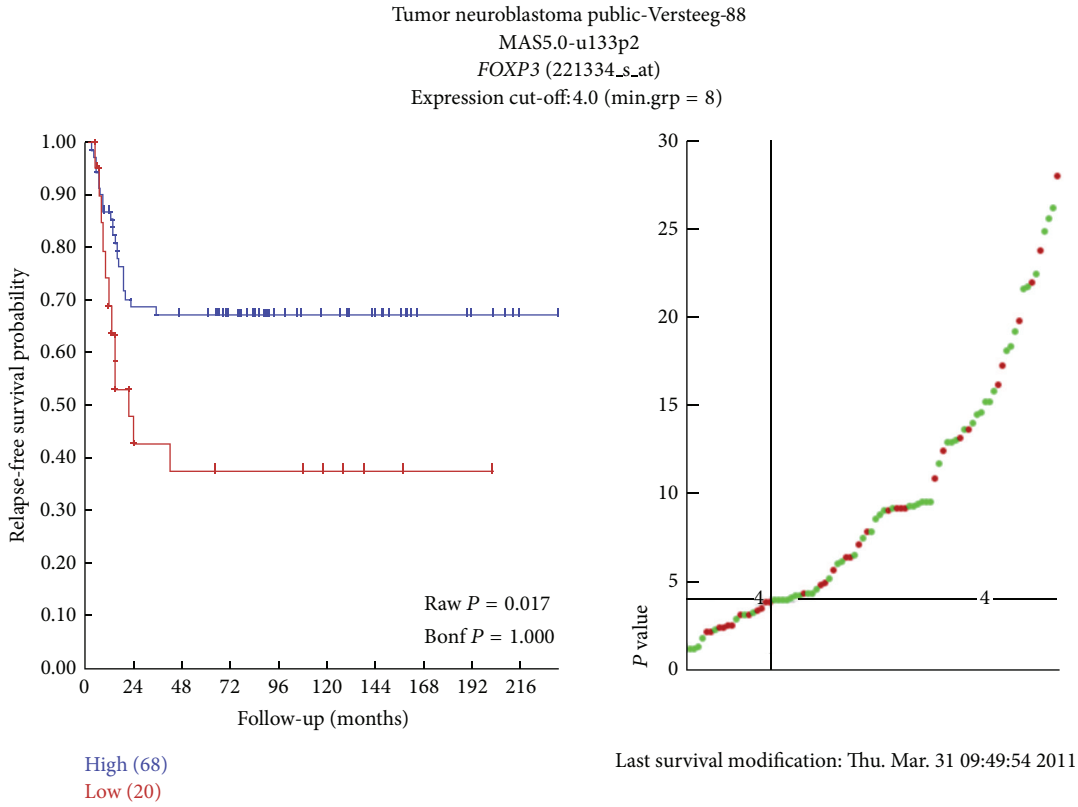
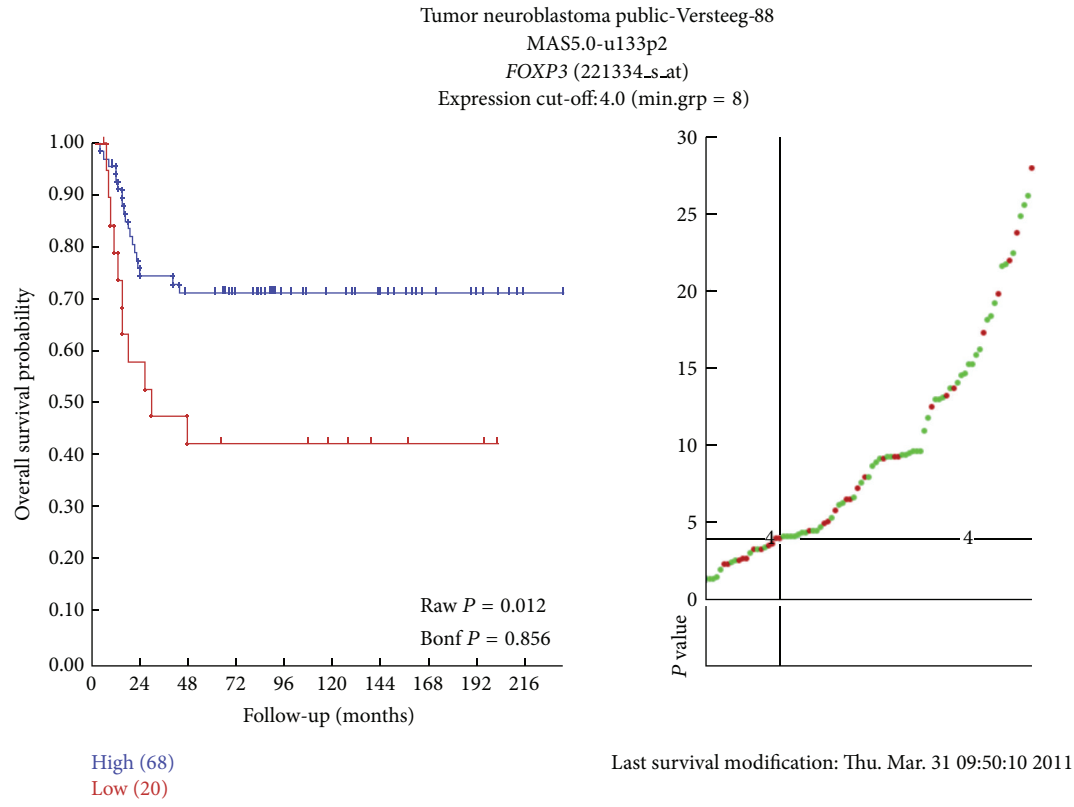


Figure 4: Kaplan-Meyer plots of EFS (left panels) and OS (right panels) of stage 4 patients stratified according to the level of mRNA expression below (continuous line) or above (dotted line) the median for *FOXP3* and *CD14* ((a) and (b)), *CD14* and *ARG1* ((c) and (d)), and for *FOXP3*, *CD14*, and *ARG1* ((e) and (f)). The number of patients in each curve is given in brackets.



(a)



(b)

Figure 5: Kaplan-Meyer plots of EFS (a) and OS (b) of stage 4 patients from the public NB database stratified according to the level of *FOXP3* mRNA expression below or above the ROC determined cut-off.

recognition by CD8+ T cells but on the other hand may facilitate NK cell-mediated killing [39]. Nonetheless, HLA class I molecule expression can be restored in human NB cells by IFN-γ [40], produced by activated T and NK cells, allowing NB cell recognition by CTLs. It is important to note that NKT cells have been involved in the immune response to human NB [18] and that activated NKT cells may express FoxP3 [41]. Therefore a possible role of activated NKT cells cannot be excluded. Finally, since low expression of all tested molecular markers, although not significantly, associated to a worse survival, the possibility that all infiltrating immune cells may play a role in improving antitumor responses needs to be considered.

Taken together, our findings suggest that if the primary tumor of stage 4 NB patients >18 months is infiltrated by FoxP3-expressing effector T or NKT cells, an effective antitumor response may take place and cooperate with standard therapy to increase survival.

5. Conclusions

High expression of *FOXP3*, *CD14*, and *ARG1* mRNA in the primary tumors of high-risk NB patients was predictive of better survival, suggesting that the immune status of the tumor may influence the natural history of this pediatric cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Sara Stigliani and Michela Croce equally contributed to the work.

Acknowledgments

This study was supported by Fondazione Italiana per la Lotta al Neuroblastoma (to Maria Valeria Corrias and Michela Croce), by AIRC IG13518 (to Silvano Ferrini), and by Ministero Della Salute (Ricerca Corrente and Cinque per mille to Vito Pistoia and Silvano Ferrini). Sara Stigliani is a recipient of Airc fellowship, Barbara Carlini and Michela Croce are recpients of Fondazione Italiana per la lotta al neuroblastoma fellowships. The excellent data management of INRB by Giovanni Erminio is deeply acknowledged.

References

[1] N.-K. V. Cheung and M. A. Dyer, "Neuroblastoma: developmental biology, cancer genomics and immunotherapy," *Nature Reviews Cancer*, vol. 13, no. 6, pp. 397–411, 2013.

[2] G. M. Brodeur, J. Pritchard, F. Berthold et al., "Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment," *Journal of Clinical Oncology*, vol. 11, no. 8, pp. 1466–1477, 1993.

[3] S. L. Cohn, A. D. J. Pearson, W. B. London et al., "The International Neuroblastoma Risk Group (INRG) classification system: an INRG task force report," *Journal of Clinical Oncology*, vol. 27, no. 2, pp. 289–297, 2009.

[4] V. F. Viprey, W. M. Gregory, M. V. Corrias et al., "Neuroblastoma mRNAs predict outcome in children with stage 4 neuroblastoma: a European HR-NBL1/SIOPEN study," *Journal of Clinical Oncology*, vol. 32, no. 10, pp. 1074–1083, 2014.

[5] J. Vermeulen, K. de Preter, A. Naranjo et al., "Predicting outcomes for children with neuroblastoma using a multigene-expression signature: a retrospective SIOPEN/COG/GPOH study," *The Lancet Oncology*, vol. 10, no. 7, pp. 663–671, 2009.

[6] J. Takita, M. Ishii, S. Tsutsumi et al., "Gene expression profiling and identification of novel prognostic marker genes in neuroblastoma," *Genes Chromosomes and Cancer*, vol. 40, no. 2, pp. 120–132, 2004.

[7] A. Schramm, J. H. Schulte, L. Klein-Hitpass et al., "Prediction of clinical outcome and biological characterization of neuroblastoma by expression profiling," *Oncogene*, vol. 24, no. 53, pp. 7902–7912, 2005.

[8] M. Ohira, S. Oba, Y. Nakamura et al., "Expression profiling using a tumor-specific cDNA microarray predicts the prognosis of intermediate risk neuroblastomas," *Cancer Cell*, vol. 7, no. 4, pp. 337–350, 2005.

[9] A. Oberthuer, B. Hero, F. Berthold et al., "Prognostic impact of gene expression-based classification for neuroblastoma," *Journal of Clinical Oncology*, vol. 28, no. 21, pp. 3506–3515, 2010.

[10] E. Hiyama, K. Hiyama, H. Yamaoka, T. Sueda, C. P. Reynolds, and T. Yokoyama, "Expression profiling of favorable and unfavorable neuroblastomas," *Pediatric Surgery International*, vol. 20, no. 1, pp. 33–38, 2004.

[11] Q.-R. Chen, Y. K. Song, J. S. Wei et al., "An integrated cross-platform prognosis study on neuroblastoma patients," *Genomics*, vol. 92, no. 4, pp. 195–203, 2008.

[12] S. Asgharzadeh, R. Pique-Regi, R. Sposto et al., "Prognostic significance of gene expression profiles of metastatic neuroblastomas lacking MYCN gene amplification," *Journal of the National Cancer Institute*, vol. 98, no. 17, pp. 1193–1203, 2006.

[13] D. Lindau, P. Gielen, M. Kroesen, P. Wesseling, and G. J. Adema, "The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells," *Immunology*, vol. 138, no. 2, pp. 105–115, 2013.

[14] J. Galon, H. K. Angell, D. Bedognetti, and F. M. Marincola, "The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures," *Immunity*, vol. 39, no. 1, pp. 11–26, 2013.

[15] R. C. Seeger, "Immunology and immunotherapy of neuroblastoma," *Seminars in Cancer Biology*, vol. 21, no. 4, pp. 229–237, 2011.

[16] P. Facchetti, I. Prigione, F. Ghiotto, P. Tasso, A. Garaventa, and V. Pistoia, "Functional and molecular characterization of tumour-infiltrating lymphocytes and clones thereof from a major-histocompatibility-complex-negative human tumour: neuroblastoma," *Cancer Immunology, Immunotherapy*, vol. 42, no. 3, pp. 170–178, 1996.

[17] S. Asgharzadeh, J. A. Salo, L. Ji et al., "Clinical significance of tumor-associated inflammatory cells in metastatic neuroblastoma," *Journal of Clinical Oncology*, vol. 30, no. 28, pp. 3525–3532, 2012.

[18] L. Song, S. Asgharzadeh, J. Salo et al., "Vα24-invariant NKT cells mediate antitumor activity via killing of tumor-associated

macrophages," *The Journal of Clinical Investigation*, vol. 119, no. 6, pp. 1524–1536, 2009.

[19] M. Gowda, K. Godder, M. Kmieciak et al., "Distinct signatures of the immune responses in low risk versus high risk neuroblastoma," *Journal of Translational Medicine*, vol. 9, no. 1, article 170, 2011.

[20] T. Tilak, S. Sherawat, S. Agarwala, R. Gupta, S. Vishnubhatla, and S. Bakhshi, "Circulating t-regulatory cells in neuroblastoma: a pilot prospective study," *Pediatric Hematology and Oncology*, vol. 31, no. 8, pp. 717–722, 2014.

[21] F. Martin, S. Ladoire, G. Mignot, L. Apetoh, and F. Ghiringhelli, "Human FOXP3 and cancer," *Oncogene*, vol. 29, no. 29, pp. 4121–4129, 2010.

[22] R. J. DeLeeuw, S. E. Kost, J. A. Kakal, and B. H. Nelson, "The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature," *Clinical Cancer Research*, vol. 18, no. 11, pp. 3022–3029, 2012.

[23] L. M. Carlson, A. de Geer, B. Sveinbjornsson et al., "The microenvironment of human neuroblastoma supports the activation of tumor-associated T lymphocytes," *OncoImmunology*, vol. 2, Article ID e23618, 2013.

[24] N. Gagliani, C. F. Magnani, S. Huber et al., "Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells," *Nature Medicine*, vol. 19, no. 6, pp. 739–746, 2013.

[25] R. Haupt, A. Garaventa, C. Gambini et al., "Improved survival of children with neuroblastoma between 1979 and 2005: a report of the Italian neuroblastoma registry," *Journal of Clinical Oncology*, vol. 28, no. 14, pp. 2331–2338, 2010.

[26] P. Scaruffi, S. Stigliani, S. Moretti et al., "Transcribed-ultra conserved region expression is associated with outcome in high-risk neuroblastoma," *BMC Cancer*, vol. 9, article 441, 2009.

[27] V. F. Viprey, M. V. Corrias, B. Kagedal et al., "Standardisation of operating procedures for the detection of minimal disease by QRT-PCR in children with neuroblastoma: quality assurance on behalf of SIOPEN-R-NET," *European Journal of Cancer*, vol. 43, no. 2, pp. 341–350, 2007.

[28] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[29] C. Badoual, S. Hans, J. Rodriguez et al., "Prognostic value of tumor-infiltrating $CD4^+$ T-cell subpopulations in head and neck cancers," *Clinical Cancer Research*, vol. 12, no. 2, pp. 465–472, 2006.

[30] P. Salama, M. Phillips, F. Grieu et al., "Tumor-infiltrating $FOXP3^+$ T regulatory cells show strong prognostic significance in colorectal cancer," *Journal of Clinical Oncology*, vol. 27, no. 2, pp. 186–192, 2009.

[31] M. Kmieciak, M. Gowda, L. Graham et al., "Human T cells express CD25 and Foxp3 upon activation and exhibit effector/memory phenotypes without any regulatory/suppressor function," *Journal of Translational Medicine*, vol. 7, article 89, 2009.

[32] J. Wang, A. Ioan-Facsinay, E. I. H. van der Voort, T. W. J. Huizinga, and R. E. M. Toes, "Transient expression of FOXP3 in human activated nonregulatory $CD4^+$ T cells," *European Journal of Immunology*, vol. 37, no. 1, pp. 129–138, 2007.

[33] S. E. Allan, S. Q. Crome, N. K. Crellin et al., "Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production," *International Immunology*, vol. 19, no. 4, pp. 345–354, 2007.

[34] V. Rigo, M. V. Corrias, A. M. Orengo et al., "Recombinant IL-21 and anti-CD4 antibodies cooperate in syngeneic neuroblastoma immunotherapy and mediate long-lasting immunity," *Cancer Immunology, Immunotherapy*, vol. 63, no. 5, pp. 501–511, 2014.

[35] M. Croce, M. V. Corrias, A. M. Orengo et al., "Transient depletion of $CD4^+$ T cells augments IL-21-based immunotherapy of disseminated neuroblastoma in syngeneic mice," *International Journal of Cancer*, vol. 127, no. 5, pp. 1141–1150, 2010.

[36] W. Jing, J. A. Gershan, and B. D. Johnson, "Depletion of CD4 T cells enhances immunotherapy for neuroblastoma after syngeneic HSCT but compromises development of antitumor immune memory," *Blood*, vol. 113, no. 18, pp. 4449–4457, 2009.

[37] W. Jing, X. Yan, W. H. D. Hallett, J. A. Gershan, and B. D. Johnson, "Depletion of $CD25^+$ T cells from hematopoietic stem cell grafts increases posttransplantation vaccine-induced immunity to neuroblastoma," *Blood*, vol. 117, no. 25, pp. 6952–6962, 2011.

[38] M. V. Corrias, M. Occhino, M. Croce et al., "Lack of HLA-class I antigens in human neuroblastoma cells: analysis of its relationship to TAP and tapasin expression," *Tissue Antigens*, vol. 57, no. 2, pp. 110–117, 2001.

[39] R. Castriconi, A. Dondero, M. V. Corrias et al., "Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction," *Cancer Research*, vol. 64, no. 24, pp. 9180–9184, 2004.

[40] M. Ponzoni, F. Guarnaccia, M. V. Corrias, and P. Cornaglia-Ferraris, "Uncoordinate induction and differential regulation of HLA class-I and class-II expression by γ-interferon in differentiating human neuroblastoma cells," *International Journal of Cancer*, vol. 55, no. 5, pp. 817–823, 1993.

[41] P. Engelmann, K. Farkas, J. Kis et al., "Characterization of human invariant natural killer T cells expressing FoxP3," *International Immunology*, vol. 23, no. 8, pp. 473–484, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 345413, 8 pages
http://dx.doi.org/10.1155/2015/345413

Research Article

The Effect of C-X-C Motif Chemokine 13 on Hepatocellular Carcinoma Associates with Wnt Signaling

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Received 1 October 2014; Accepted 4 January 2015

Academic Editor: Anshu Agrawal

Objects. To investigate the effect of CXCL13 (C-X-C motif chemokine 13) on hepatocellular carcinoma and clarify the potential mechanisms. *Methods*. 32 patients with hepatocellular carcinoma and 12 healthy controls were recruited for analyzing the expression of CXCL13 by RT-PCR (reverse transcription-polymerase chain reaction). ELISA (enzyme-linked immune-sorbent assay) was used to test the concentration of serum CXCL13. The interaction between CXCL13 and Wnt signaling was analyzed by western blot. In vitro PBMCs cultured with HepG2 supernatant, the levels of IL-12, IL4, IL-6, and IL-17, and four IgG subclasses were detected by ELISA. *Results*. The rate of high expression CXCL13 was 63.4% in advanced HCC patients, and the serum CXCL13 was also at a high level in stage IV HCC patients. Meanwhile CXCL13 level was positively correlated with serum ALT (Alanine Transaminase) and AST (Aspartate Aminotransferase). CXCL13 and Wnt/β-catenin signaling shared a positive feedback loop. Furthermore, CXCL13 could obviously promote the expressions of IL-12 and IL-17, and induce IgG4 secreted by B cells. *Conclusions*. The effect of CXCL13 on promoting liver cancer is related to the activation of Wnt/β-catenin pathway and the facilitation of IL-12, IL-17 and IgG4. CXCL13 plays an important role in the progression of HCC, and it may act as a potential target for the diagnosis and treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the most primary malignant tumor of the liver cells (with more than 750,000 new cases diagnosed every year worldwide) and the third most deadly tumor globally, following lung and stomach cancers [1]. Unlike other carcinomas, the mortality from most malignancies has decreased steadily in the last 20 years, however, that from liver cancer has increased significantly from 1990 to 2005, by as much as 50% in men [2]. Though the main risk factors for HCC development have been clearly identified, such as hepatitis B and C virus infection, alcohol abuse, and some chronic liver diseases [3, 4], there is still preliminary understanding of the key drivers of this malignancy. Another lethal problem of HCC is the symptoms of early-stage HCC which are often not apparent, so many patients are diagnosed at advanced stages [5, 6], leading to less effective therapies. What is more, though antivirals and vaccination could effectively decrease the incidence of HCC, there is not widely accepted chemopreventive strategy to limit development of HCC once cirrhosis is established [7, 8]. Limited treatment options highlight the need to clarify the mechanisms in HCC development and to identify early disease biomarkers and new therapy targets.

The molecular pathogenesis of HCC is relative to multiple influences such as tumor microenvironment [9, 10] and abnormal activation of some signaling pathways [11, 12]. Several clear evidences have confirmed that chemokine/chemokine receptor in the inflammatory interactions plays critical roles in tumorigenesis and metastasis.

The chemokines are a group of small (<15 kDa), soluble proteins that bind to their G-protein-coupled receptors to mediate different pro- and anti-inflammatory responses [13–15]. Based on the position of the key cysteine residues located in the N-terminal region, chemokines are subdivided into four families: CXC, CC, C, and CX3C chemokines, in which the X represents any amino acid [16]. Chemokines play an essential role in tumor progression, acting on endothelial, epithelial, and tumor cells. They are reported to sustain tumor cell growth, induce angiogenesis, and facilitate tumor escape through evasion of immune surveillance [17–19]. Several chemokines/chemokines receptors appear to be directly implicated in HCC. The CCL20-CCR6 axis may mediate the growth and progression of HCC through phosphorylation of MAPK [20, 21] and is associated with poor prognosis after resection [22, 23]. CCL5-CCR1 promotes metastasis and invasion of the HCC cell line Huh7 and CCL3-CCR1 contributes to the growth and progression of HCC, whereas CX3CL1-CX3CR1 axis is believed to be involved in HCC tumor growth inhibition [24]. CXCL12-CXCR4 axis plays a critical role in migration of tumor cells into metastatic sites in HCC [25].

CXCL13 (B-lymphocyte chemoattractant (BLC)), which is the only chemokine binding to its receptor CXCR5, is mainly secreted by follicular dendritic cells (FDCs), monocyte-like mature macrophages, and stromal cells in the B-cell area of the secondary lymphoid tissues [26, 27], where the B cells encounter the antigen and differentiate [28]. CXCL13 may contribute significantly to breast tumor formation and mediate the progress of prostate cancer through activating JNK and ERK pathways [29–31]. However, the effect of CXCL13 in HCC has not yet been clarified clearly.

In this study, we confirmed the high level CXCL13 was existed in both tissue and serum of advanced liver cancer patients. We also found the activation of Wnt/β-catenin signaling could increase the expressions of CXCL13 and its receptor CXCR5, while CXCL13 could stimulate the Wnt/β-catenin pathway. By culturing PBMCs with HepG2 culture supernatant, CXCL13 was identified to significantly induce the production of IL-12 and IL-17 secreted by T cells, and moreover, it could upregulate the concentration of IgG4 secreted by B cells. These results indicated that the effect of CXCL13 on promoting liver cancer development might be related to the activation of Wnt/β-catenin pathway and the facilitation of IL-12, IL-17 and IgG4. CXCL13 played an important role in the progression of HCC, and it might serve as a potential target for the diagnosis and treatment of HCC.

2. Materials and Methods

2.1. Patients. A total of 32 patients with new onset HCC and 12 gender- and age-matched health controls (HC) were recruited from the Hepatopancreatobiliary Department of the First Hospital of Jilin University (Changchun, China). To avoid the interference of other diseases, the participators with any autoimmune diseases, hepatitis virus infection, or those who had received drug therapies within the past 6 months were excluded from the study. Written informed consent was obtained from individual subjects and the experimental protocol was approved by the Ethical Committee of the First Hospital of Jilin University. Their demographic and clinical characteristics of these subjects are show in Table 1. The levels of serum AST (Aspartate Aminotransferase), ALT (Alanine Transaminase), and AFP (Alpha Fetal Protein) in participants were detected by Biochemistry Automatic Analyzer (Roche Diagnostics, Branchburg, USA).

Fresh liver tumor samples were collected immediately after surgical resection and stored in liquid nitrogen until further use. The corresponding serum samples were stored in −80°C freezer.

2.2. Isolation of PBMCs and Cell Culture In Vitro. Peripheral blood mononuclear cells (PBMCs) from venous blood samples (10 mL) of health controls were sorted by Ficoll-Paque (Amersham Biosciences, USA) density-gradient centrifugation. PBMCs were washed three times in Hanks balanced salt solution (Gibco, Canada), counted, and suspended in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen), containing 10% FBS (Gibco, USA) and penicillin streptomycin solution (Hyclone, USA). The liver cancer cell line HepG2 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured as mentioned above.

To analyze the effect of CXCL13 on the immune status of HCC, PBMCs cells (5×10^5 cells/well) were seeded in a 24-well plate (Corning, USA), culturing using the supernatant of HepG2 cells. After 72 h, the supernatant was taken and levels of cytokines and IgG subclasses were determined by ELISA. For the proliferation of T lymphocytes, PBMCs were initially cultured in a 24-well plate precoated with CD3/CD28 mAbs (1 μg/mL CD3 and 10 μg/mL CD28 resp., both from invitrogen, USA). To activate B cells, 3 μg/mL CpGB and 10 ng/mL IL-2 (both from R&D Systems, USA) were used to stimulate B cells for 72 h.

100 ng/mL DKK-1 (ACROBiosystems, USA) was obtained from PeproTech, using to generally block Wnt/β-catenin pathways, and 100 mM LiCl (Nacarai Tesque, Japan) acted as a stimulator of this pathway. Recombinant human CXCL13 (100 ng/mL) [31, 32] was from R&D, USA. Dexamethasone (0.2 mg/mL) was used to inhibit CXCL13 expression [33].

2.3. Enzyme-Linked Immune-Sorbent Assay (ELISA). According to the manufacturer's instructions, enzyme-linked immune-sorbent assay (ELISA) of serum and cell culture supernatant was performed on 96-well plates. The serum or supernatant levels of human CXCL13 and IL-12/4/6/17 were determined by ELISA kit from R&D, USA. The concentrations of IgG1, IgG2, IgG3, IgG4, and total IgG were determined by ELISA (Uscn Life Science, China) as described previously [34, 35]. The absorbance of the plates was read at 450 nm using an Automated Microplated Reader (Bio-Tek, USA).

2.4. Analysis of mRNA Levels by Reverse Transcription (RT) PCR. To test the mRNA expression in liver cancer tissues, total RNA from HCC tissues were extracted using TRIzol

Table 1: The clinical characteristics of subjects.

Parameters	Group A Stages I-II $n = 10$	Group B Stage III $n = 8$	Group C Stage IV $n = 14$	HC $n = 12$
Age (years)	52 (46–67)	48 (44–68)	54 (50–75)	49 (43–74)
Gender: female/male	7/3	6/2	12/2	11/1
AFP (ng/mL)	58.2* (21.7–83.3)	152.7* (10.4–325.4)	194.6* (6.3–810)	7.1 (1.3–11.1)
ALT (U/L)	67.3* (18–243)	152.7* (98.1–305.8)	215.2* (145–712.6)	24.2 (13.6–27.8)
AST (U/L)	93.1* (34–737)	183.4* (79.2–385.2)	371* (127.1–963.7)	22.4 (15.3–32.1)
Rate of high level CXCL13 (%)	20	37.5	64.3	—

Data shown are real case number or median (range).
Normal values: AFP: <25 ng/mL; ALT: <40 IU/L; AST: <40 IU/L.
HC: healthy control.
—: not available.
* $P < 0.05$ versus the HC.

(Invitrogen, USA) according to the manufacturer's instructions. RNA pellets were stored in sterile ribonuclease-free water. Reverse transcription was carried out using 1–3 μg total RNA, 0.5 μg oligo (dT), and Superscript II enzyme (Invitrogen, USA). The gene-specific primers for RT-PCR were listed as follows: CXCL13 forward 5′-CTGGTCAGCAGCCTCTCTC-3′, and reverse 5′-TTCTCAATACTTCCATCATTCTTT-3′; GAPDH forward 5′-CACCAACTGGGACGACAT-3′, and reverse 5′-ACAGCCTGGATAGCAACG-3′. The mRNA expression of interested gene in each sample was normalized against housekeeping gene.

2.5. Western Blotting. To illustrate the interaction between CXCL13 and Wnt pathway, HepG2 cells were plated in 6-well plates (3×10^6 cells/well). After 8 h treatment, cells were harvested and lysed on ice for 30 min in RIPA (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% TritonX-100, 1 mM EDTA, 5 mM NaF, 1 mM sodium vanadate, and protease inhibitors cocktail) buffer and protein extracts were quantitated. 20 μg of total protein was then subjected to 10–15% SDS-PAGE, electrophoresed and transferred to a nitrocellulose membrane. After using 5% nonfat milk in Tris-buffered saline for blocking, the membrane was washed and incubated with the indicated antibodies. The primary antibodies for β-catenin, phospho-GSK-3β (Ser9), GSK3β, CXCL13, CXCR5, and GAPDH were all from Santa Cruz, USA. The animal-matched horseradish peroxidase-conjugated secondary antibody was purchased from Santa Cruz as well.

2.6. Statistical Analysis. Data are expressed as median and range or individual mean values. The difference between the groups was analyzed by Mann-Whitney test using the Graphpad 5.0 software. The relationship between variables was evaluated using the Spearman rank correlation test. A two-side P value of <0.05 was considered statistically significant.

3. Results

3.1. Serum Level of CXCL13 Was Increased in Liver Cancer Tissues and Was Relative to the Development of HCC. As a potent chemokine mainly secreted by monocytes, lymphocytes and dendritic cells, CXCL13 could also be expressed in liver tissues as we tested. The mRNA level of CXCL13 was obviously higher in most of the stage IV liver cancer patients (63.4%) than that in health control as shown in Table 1. To confirm the serum level of CXCL13, we detected its concentration in stage I-II group: A, stage III group: B, stage IV group: C, and health controls: HC. The ELISA results indicated that serum CXCL13 was significantly higher in all HCC groups compared with HC. In particular, the concentration of CXCL13 in group C was much higher than that in group B, and likewise, CXCL13 in group B was higher than that in group A as shown in Figure 1(a). Furthermore, we also analyzed the relationship between CXCL13 and clinical features of liver cancer patients. A positive correlation was found between the concentrations of serum CXCL13 and ALT ($P < 0.0001$, $r^2 = 0.5009$) or AST ($P < 0.0001$, $r^2 = 0.4589$). No other obvious difference was observed in any of the clinical parameters (Figures 1(b) and 1(c)). Thus the serum CXCL13 level was associated with the progression of HCC, and it might be a potential biomarker for liver cancer progression.

3.2. The Mutual Promotion between CXCL13 and Wnt/β-Catenin Signaling. Though the regulatory role of CXCL13 has been proven to mediate the activation of JNK and MAPK pathways [30, 31] in prostate cancer invasion and migration, its modulating acts on Wnt/β-catenin signaling in HCC and the effect of Wnt/β-catenin pathway on CXCL13 was still obscure. In HepG2 cells, by treated with Wnt/β-catenin inhibitor DKK-1, we found decreased expressions of CXCL13 and CXCR5 compared with that in control, whereas upregulated levels of CXCL13 and CXCR5 were observed in stimulant LiCl treated group as shown in Figure 2(a). Furthermore, to investigate the influence of CXCL13 on this

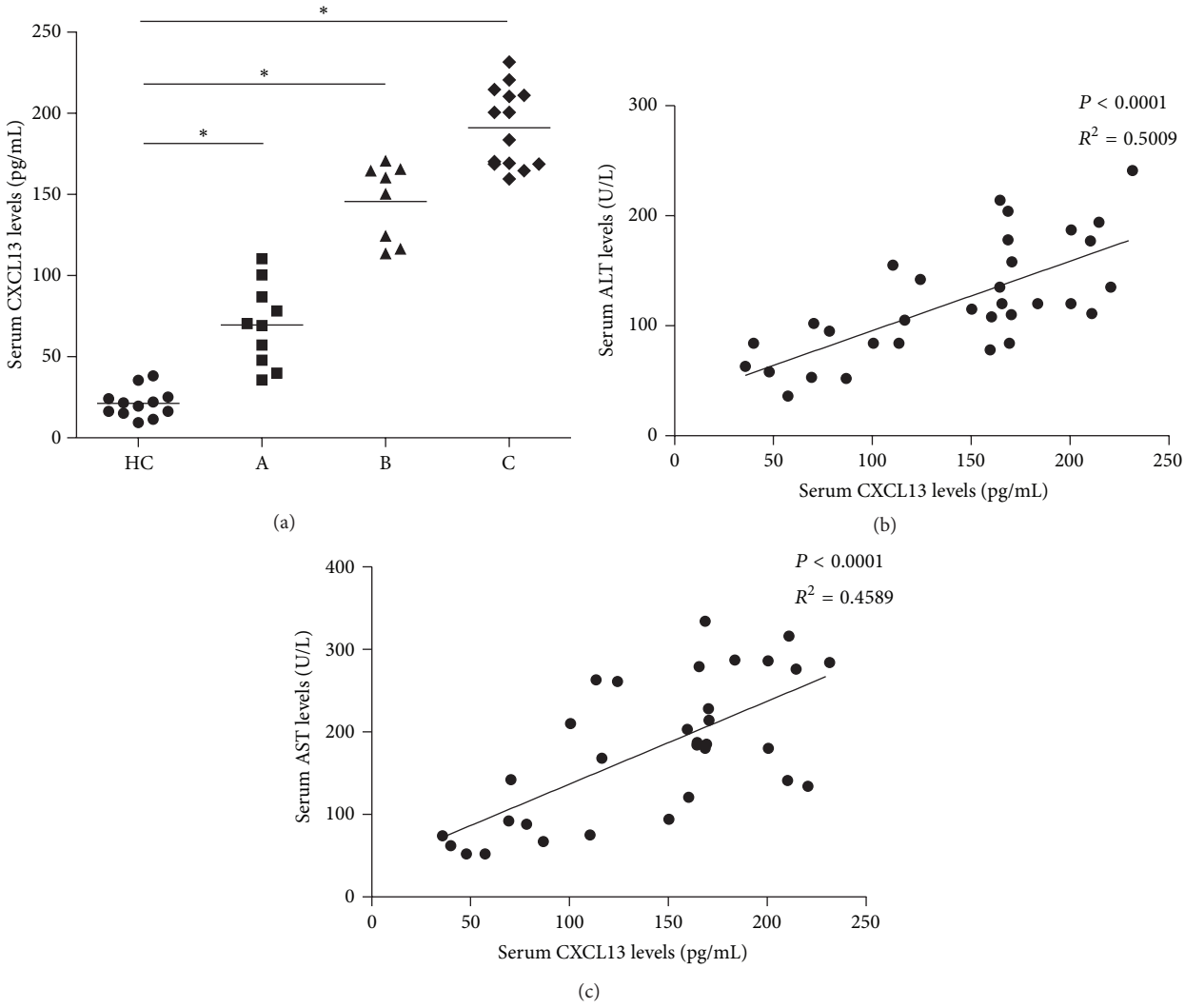

Figure 1: The concentration of serum CXCL13 in liver cancer patients. (a) The serum CXCL13 level in 32 HCC patients and 12 health controls detected by ELISA. CXCL13 level was much higher in stage IV HCC patients than that in other groups. There also existed statistical significance between serum CXCL13 in stage III and stages I-II group ($^*P < 0.05$). The horizontal lines indicate the median values for each group. (b-c) The potential correlations between CXCL13 and serum ALT and AST were analyzed by the Spearman correlation tests.

signaling, dexamethasone was added into the HepG2 cell medium to inhibit CXCL13. As we predicted, a decreased β-catenin and an increased p-GSK-3β expression were observed in the dexamethasone treated group, while inverse expressions of these two factors were founded in the CXCL13 stimulated group (Figure 2(b)). The results demonstrated there existed a positive relationship between CXCL13 and Wnt/β-catenin signaling in HCC, and this mutual interaction might promote the level of each other and led to the tumor microenvironment formation.

3.3. CXCL13 Upregulated the Concentrations of IL-12 and IL-17 in HCC. CXCL13 has been revealed to play important roles in the immune response such as regulating lymphocyte migration and promoting inflammation; thus we further detected the influence of CXCL13 on immune status relative interleukins—IL-12, IL-4, IL-6, and IL-17 in three groups: dexamethasone group: A, CXCL13 group: B, and PBMCs control group. Cytokines in PBMCs which were cultured with HepG2 supernatant were collected and measured by ELISA. The concentrations of IL-12 and IL-17 in supernatant were significantly increased in CXCL13 stimulated group, while decreased in dexamethasone treated group ($P < 0.05$, Figure 3). But the levels of IL-4 and IL-6 were not statistically significant. Thus we suspected the proinflammatory effect of CXCL13 which was potentially mediated by triggering the expressions of cytokines, IL-12 and IL-17, which presented for the activation of Th1 and Th17 cells, respectively.

3.4. CXCL13 Significantly Improved IgG4 Secreting by B Cells. Functionally, CXCL13 was initially acted as a selective chemoattractant for B lymphocytes and B helper T cells via its

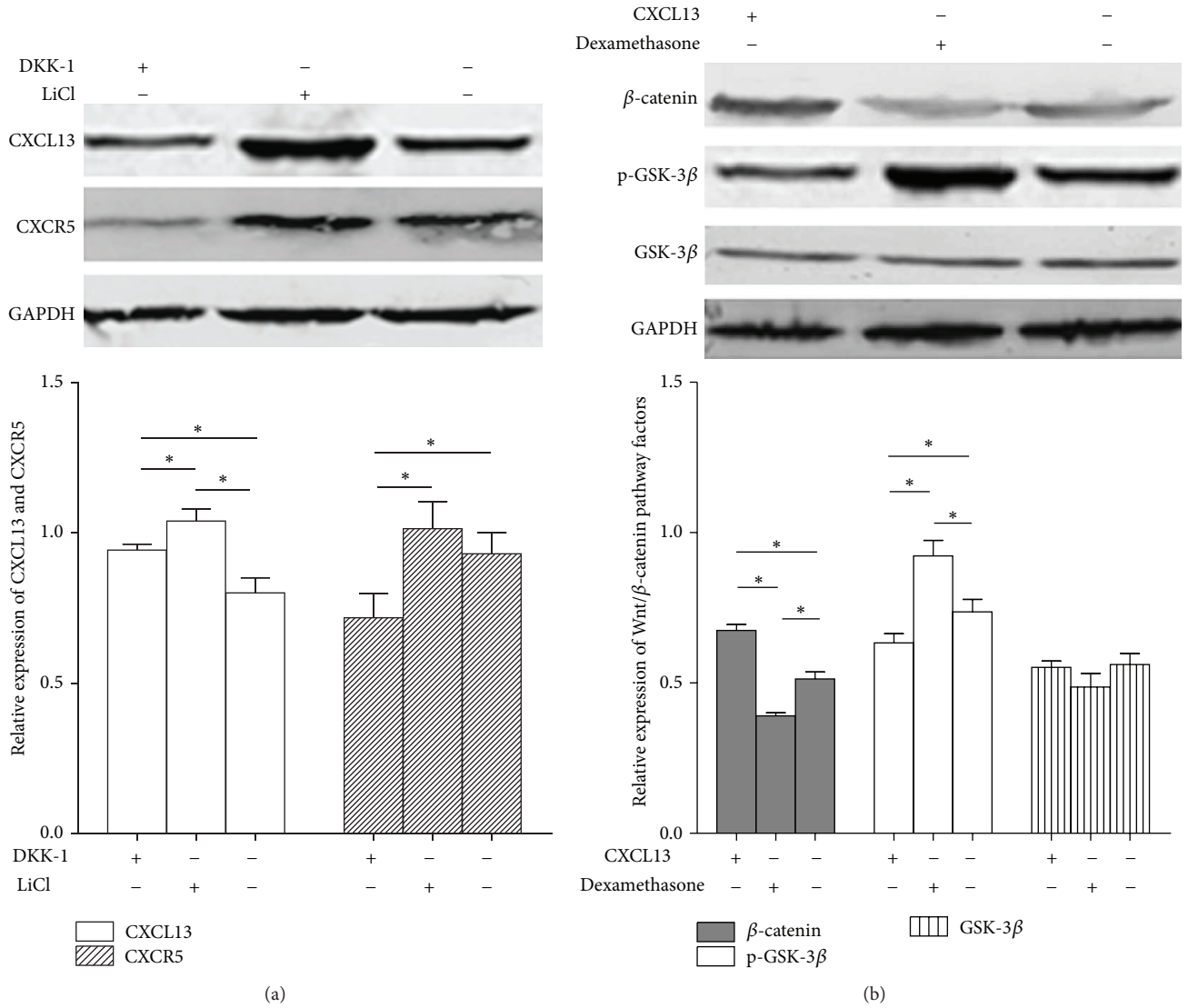

Figure 2: The interaction between CXCL13 and Wnt/β-catenin pathway. (a) The effect of Wnt/β-catenin signaling on CXCL13 and its receptor CXCR5 was analyzed by adding DKK-1 or LiCl to inhibit or stimulate the pathway, respectively. (b) The effect of CXCL13 on Wnt/β-catenin signaling. Dexamethasone was used to inhibit CXCL13. Gene expression levels were determined with GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as the reference. A positive feedback loop was observed between CXCL13 and Wnt/β-catenin signaling ($^*P < 0.05$). Experiments were performed in triplicate and repeated three times.

specific receptor CXCR5 and secreted by the stromal cells in the B-cell area of the secondary lymphoid tissues, where the B cells encounter the antigen and differentiate. Considering the influence of CXCL13 on promoting B-cell maturation, we next examined the proportions of IgG subclasses in PBMCs cultured with HepG2 supernatant. The groups divided as follows: dexamethasone group: A, CXCL13 group: B, and PBMCs with no additives which were set to be control group. After CpGB and IL-2 stimulating, we detected significantly increased IgG4/IgG total ratios in CXCL13 group as shown in Figure 4. These results indicated that CXCL13 could improve the polarized expression of IgG4 secreted by B cells. The high level of IgG4+ B cells may participate in the invasion and metastasis of liver cancer.

4. Discussion

The pathogenesis of HCC has been extensively analyzed to be closely associated with chronic inflammation which is mediated by inflammatory cytokines and chemotactic factors. In this study, our observation of elevated rate of high level CXCL13 in advanced liver cancer patients indicated a very close relation between CXCL13 and liver cancer. Further we proved that the concentration of serum CXCL13 was associated with HCC progression and positively correlated with serum ALT and AST. These findings implied the potential role of CXCL13 to be a biomarker for the pathogenesis of HCC.

The progression of HCC is a result of a complex cellular system with reciprocal signaling. Three regulatory networks,

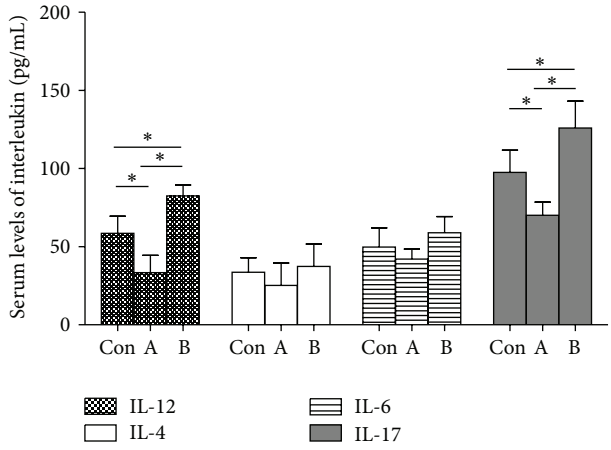

Figure 3: The influence of CXCL13 on T lymphocytes related inflammatory cytokines. PBMCs (5×10^5/well) were isolated from health volunteers and seeded in 24-well plate precoated with CD3/CD28 mAbs. By culturing with HepG2 supernatant for 72 h, the concentrations of IL-12, IL-4, IL-6, and IL-17 were detected by ELISA. Meanwhile, PBMCs cultured with no stimulus were set to be negative control. The levels of IL-12 and IL-17 were obviously promoted by CXCL13 ($^*P < 0.05$). Experiments were performed in triplicate and repeated three times.

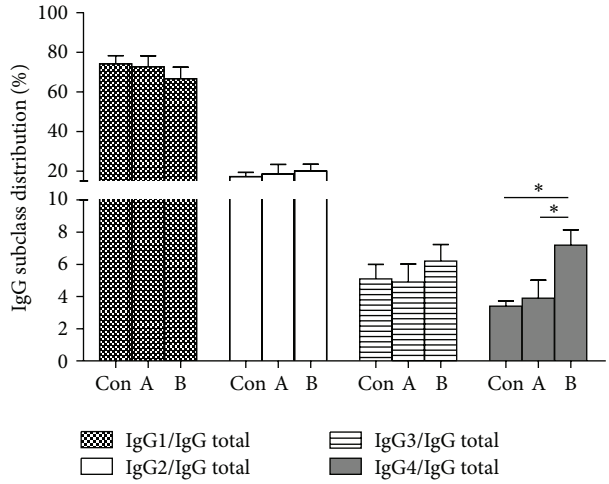

Figure 4: The effect of CXCL13 on B cells polarization. PBMCs (5×10^5/well) were cultured in 24-well plate and stimulated using CpGB and IL-2 for 72 h to obtain activated B cells. The concentrations of IgG1, IgG2, IgG3, IgG4, and IgG total were detected using ELISA kits. PBMCs cultured with no stimulus were set to be negative control. The ratio of IgG4/IgG was obviously higher than that in other groups, while the ratios of IgG1/IgG, IgG2/IgG, and IgG3/IgG showed no statistical significances ($^*P < 0.05$). Experiments were performed in triplicate and repeated three times.

PI3 K/Akt, ERK/MAPK, and JNK/c-Jun pathways, are confirmed to be dramatically induced by CXCL13 in prostate cancer [32]. Wnt/β-catenin signaling is also a very important pathway related to the tumor development. After Wnt binding to its receptor Frizzled (Fzd) and lipoprotein receptor-related protein 5/6 (LRP5/6), Axin/adenomatous polyposis coli (APC)/glycogen synthase kinase (GSK)3β complex is inactivated, which leads to the accumulation of β-catenin. Then β-catenin translocates to the nucleus and interacts with transcription factors of the T-cell factor (TCF) and lymphoid-enhancing factor (LEF) families, promoting the transcription of many oncogenic factors, such as c-MYC, cyclin D1, and VEGF [36, 37]. By preventing the Wnt signaling by its inhibitor Dkk-1 and stimulating this pathway by LiCl in HepG2 cells, we observed decreased levels of CXCL13 and its receptor CXCR5 in DKK-1 group, whereas increased expressions of these two factors was found in LiCl group. Likewise, Wnt/β-catenin transduction pathway was found to be stimulated by CXCL13 and blocked by dexamethasone as well. These data showed a positive relationship between CXCL13 and Wnt/β-catenin pathway in HCC and indicated that the mechanism of CXCL13 in promoting proinflammatory reaction might be mediated by the mutual feedback with Wnt/β-catenin signaling.

Considering CXCL13 plays a central role in the positioning, cooperation and activation of T and B cells within lymphoid and extralymphoid sites, especially in the differentiation of B cells into antibody-producing plasma cells [28], we also detected the effect of CXCL13 on interleukins secreted by T lymphocytes and IgG subclasses secreted by B lymphocytes. IL-12 is able to shift differentiation of CD4+ Th0 cells towards the Th1 phenotype [38], while IL-4 and IL-6 generally mediate the development of Th2 mediated diseases and regulate the Th1 responses by inhibition of interferon-γ (IFN-γ) production [39, 40]. IL-17 is a proinflammatory cytokine and required in vivo for the stabilization and proliferation of Th17 cells. It plays a very important role in chronic inflammatory state and stimulates the production of multiple chemokines, including CXCL1, CXCL8, CCL7, and CCL20 [41]. In PBMCs cultured with HepG2 supernatant, the expressions of IL-12 and IL-17 obviously showed an upregulation in CXCL13 group and a decreasing trend in dexamethasone group ($P < 0.05$), while minimal changes of IL-4 and IL-6 level were found in our experiment. The abnormally high production of IL-12 and IL-17, which presented Th1 and Th17 cell, respectively, indicated an immunological imbalance in CXCL13 stimulated group and that might be related to the HCC progression.

Compared with that in T lymphocytes, CXCL13 may have a crucial function in B lymphocytes as well. To clarify the effect of CXCL13 on B lymphocytes, we analyzed the proportions of IgG subclasses, which were reported to be associated with many cancers' progression [42, 43]. After stimulating B cells in vitro, we detected a higher IgG4/IgG total ratios in CXCL13 treated group compared with that in dexamethasone group or control. The ratios of IgG1, IgG2, and IgG3 showed no statistical significances in all groups. Our results suggested that CXCL13 might induce the imbalance of different subclasses secreted by B cells and mediated immunomodulatory activities in tumor microenvironment.

In conclusion, this study underlines the prognostic relevance of CXCL13 to HCC. We observed a considerable overexpression of CXCL13 in both liver cancer tissues and serum, and its level was positively correlated with serum ALT and AST. Furthermore, CXCL13 and Wnt/β-catenin

signaling shared a positive feedback loop. Subsequently, we also found CXCL13 might function through activating Th1 and Th17 reaction and inducing IgG4 production. Thus CXCL13 may act as a promising prognostic marker and a potential therapeutic target in HCC.

Conflict of Interests

The authors indicated no potential conflict of interests.

Authors' Contribution

Chunyan Li and Dong Kang made equal contributions to this study.

Acknowledgments

The authors are very grateful to Dr. Hai Zhang for clinical samples collection. This study was supported by grants from the Jilin Province Science and Technology Agency (no. 20110456).

References

[1] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA: A Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[2] L. Zender, A. Villanueva, V. Tovar, D. Sia, D. Y. Chiang, and J. M. Llovet, "Cancer gene discovery in hepatocellular carcinoma," *Journal of Hepatology*, vol. 52, no. 6, pp. 921–929, 2010.

[3] K. M. Mohandas, "Hepatitis B associated hepatocellular carcinoma: epidemiology, diagnosis and treatment," *Hepatitis B Annual*, vol. 1, no. 1, pp. 140–152, 2004.

[4] C. Bréchot, "Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms," *Gastroenterology*, vol. 127, no. 5, supplement 1, pp. S56–S61, 2004.

[5] T. Takayama, T. Sekine, M. Makuuchi et al., "Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial," *The Lancet*, vol. 356, no. 9232, pp. 802–807, 2000.

[6] A. G. Singal, M. Nehra, B. Adams-Huet et al., "Detection of hepatocellular carcinoma at advanced stages among patients in the HALT-C trial: where did surveillance fail?" *The American Journal of Gastroenterology*, vol. 108, no. 3, pp. 425–432, 2013.

[7] A. Villanueva and J. M. Llovet, "Targeted therapies for hepatocellular carcinoma," *Gastroenterology*, vol. 140, no. 5, pp. 1410–1426, 2011.

[8] R. Dhanasekaran, A. Limaye, and R. Cabrera, "Hepatocellular carcinoma: current trends in worldwide epidemiology, risk factors, diagnosis, and therapeutics," *Journal of Hepatic Medicine*, vol. 4, pp. 19–37, 2012.

[9] A. O'Garra and P. Vieira, "Regulatory T cells and mechanisms of immune system control," *Nature Medicine*, vol. 10, no. 8, pp. 801–805, 2004.

[10] R. K. Jain, "Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers," *Journal of Clinical Oncology*, vol. 31, no. 17, pp. 2205–2218, 2013.

[11] A. Moeini, H. Cornellà, and A. Villanueva, "Emerging signaling pathways in hepatocellular carcinoma," *Liver Cancer*, vol. 1, no. 2, pp. 83–93, 2012.

[12] S. Zheng, W. P. Tansey, S. W. Hiebert, and Z. Zhao, "Integrative network analysis identifies key genes and pathways in the progression of hepatitis C virus induced hepatocellular carcinoma," *BMC Medical Genomics*, vol. 4, article 62, 2011.

[13] T. Kakinuma and S. T. Hwang, "Chemokines, chemokine receptors, and cancer metastasis," *Journal of Leukocyte Biology*, vol. 79, no. 4, pp. 639–651, 2006.

[14] K. Franciszkiewicz, A. Boissonnas, M. Boutet, C. Combadière, and F. Mami-Chouaib, "Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response," *Cancer Research*, vol. 72, no. 24, pp. 6325–6332, 2012.

[15] B. Moser and P. Loetscher, "Lymphocyte traffic control by chemokines," *Nature Immunology*, vol. 2, no. 2, pp. 123–128, 2001.

[16] K. Bacon, M. Baggiolini, H. Broxmeyer et al., "Chemokine/chemokine receptor nomenclature," *Journal of Interferon and Cytokine Research*, vol. 22, no. 10, pp. 1067–1068, 2002.

[17] T. Tanaka, Z. Bai, Y. Srinoulprasert, B. Yang, H. Hayasaka, and M. Miyasaka, "Chemokines in tumor progression and metastasis," *Cancer Science*, vol. 96, no. 6, pp. 317–322, 2005.

[18] A. Zlotnik, "Chemokines in neoplastic progression," *Seminars in Cancer Biology*, vol. 14, no. 3, pp. 181–185, 2004.

[19] S. Zöchbauer-Müller, A. F. Gazdar, and J. D. Minna, "Molecular pathogenesis of lung cancer," *Annual Review of Physiology*, vol. 64, pp. 681–708, 2002.

[20] H. Fujii, Y. Itoh, K. Yamaguchi et al., "Chemokine CCL20 enhances the growth of HuH7 cells via phosphorylation of p44/42 MAPK in vitro," *Biochemical and Biophysical Research Communications*, vol. 322, no. 3, pp. 1052–1058, 2004.

[21] C. Rubie, V. O. Frick, M. Wagner et al., "Enhanced expression and clinical significance of CC-chemokine MIP-3α in hepatocellular carcinoma," *Scandinavian Journal of Immunology*, vol. 63, no. 6, pp. 468–477, 2006.

[22] H. Uchida, Y. Iwashita, A. Sasaki et al., "Chemokine receptor CCR6 as a prognostic factor after hepatic resection for hepatocellular carcinoma," *Journal of Gastroenterology and Hepatology*, vol. 21, no. 1, pp. 161–168, 2006.

[23] X. Ding, K. Wang, H. Wang et al., "High expression of CCL20 is associated with poor prognosis in patients with hepatocellular carcinoma after curative resection," *Journal of Gastrointestinal Surgery*, vol. 16, no. 4, pp. 828–836, 2012.

[24] F. Huang and X.-P. Geng, "Chemokines and hepatocellular carcinoma," *World Journal of Gastroenterology*, vol. 16, no. 15, pp. 1832–1836, 2010.

[25] G.-T. Lin, H.-F. Tseng, C.-H. Yang et al., "Combinational polymorphisms of seven CXCL12-related genes are protective against breast cancer in Taiwan," *OMICS*, vol. 13, no. 2, pp. 165–172, 2009.

[26] G. Aust, D. Sittig, L. Becherer et al., "The role of CXCR5 and its ligand CXCL13 in the compartmentalization of lymphocytes in thyroids affected by autoimmune thyroid diseases," *European Journal of Endocrinology*, vol. 150, no. 2, pp. 225–234, 2004.

[27] L. de Chaisemartin, J. Goc, D. Damotte et al., "Characterization of chemokines and adhesion molecules associated with T cell presence in tertiary lymphoid structures in human lung cancer," *Cancer Research*, vol. 71, no. 20, pp. 6391–6399, 2011.

[28] C. D. C. Allen, K. M. Ansel, C. Low et al., "Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5," *Nature Immunology*, vol. 5, no. 9, pp. 943–952, 2004.

[29] J. Panse, K. Friedrichs, A. Marx et al., "Chemokine CXCL13 is overexpressed in the tumour tissue and in the peripheral blood

of breast cancer patients," *British Journal of Cancer*, vol. 99, no. 6, pp. 930–938, 2008.

[30] Z. Zeng, Y. X. Shi, I. J. Samudio et al., "Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML," *Blood*, vol. 113, no. 24, pp. 6215–6224, 2009.

[31] C. P. El-Haibi, R. Singh, P. K. Sharma, S. Singh, and J. W. Lillard, "CXCL13 mediates prostate cancer cell proliferation through JNK signalling and invasion through ERK activation," *Cell Proliferation*, vol. 44, no. 4, pp. 311–319, 2011.

[32] C. P. El-Haibi, R. Singh, P. Gupta et al., "Antibody microarray analysis of signaling networks regulated by Cxcl13 and Cxcr5 in prostate cancer," *Journal of Proteomics and Bioinformatics*, vol. 5, no. 8, pp. 177–184, 2012.

[33] A. Meraouna, G. Cizeron-Clairac, R. Le Panse et al., "The chemokine CXCL13 is a key molecule in autoimmune myasthenia gravis," *Blood*, vol. 108, no. 2, pp. 432–440, 2006.

[34] P. Karagiannis, A. E. Gilbert, D. H. Josephs et al., "IgG4 subclass antibodies impair antitumor immunity in melanoma," *Journal of Clinical Investigation*, vol. 123, no. 4, pp. 1457–1474, 2013.

[35] A. E. Gilbert, P. Karagiannis, T. Dodev et al., "Monitoring the systemic human memory B cell compartment of melanoma patients for anti-tumor IgG antibodies," *PLoS ONE*, vol. 6, no. 4, Article ID e19330, 2011.

[36] C. Gao and Y.-G. Chen, "Dishevelled: the hub of Wnt signaling," *Cellular Signalling*, vol. 22, no. 5, pp. 717–727, 2010.

[37] A. J. Mikels and R. Nusse, "Purified Wnt5a protein activates or inhibits β-catenin-TCF signaling depending on receptor context," *PLoS Biology*, vol. 4, no. 4, pp. 570–582, 2006.

[38] R. Pappu, V. Ramirez-Carrozzi, and A. Sambandam, "The interleukin-17 cytokine family: critical players in host defence and inflammatory diseases," *Immunology*, vol. 134, no. 1, pp. 8–16, 2011.

[39] I. G. Luzina, A. D. Keegan, N. M. Heller, G. A. W. Rook, T. Shea-Donohue, and S. P. Atamas, "Regulation of inflammation by interleukin-4: a review of 'alternatives'," *Journal of Leukocyte Biology*, vol. 92, no. 4, pp. 753–764, 2012.

[40] I. H. Heijink, E. Vellenga, P. Borger, D. S. Postma, J. G. R. de Monchy, and H. F. Kauffman, "Interleukin-6 promotes the production of interleukin-4 and interleukin-5 by interleukin-2-dependent and -independent mechanisms in freshly isolated human T cells," *Immunology*, vol. 107, no. 3, pp. 316–324, 2002.

[41] R. M. Onishi and S. L. Gaffen, "Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease," *Immunology*, vol. 129, no. 3, pp. 311–321, 2010.

[42] C. Papadea and I. J. Check, "Human immunoglobulin G and immunoglobulin G subclasses: biochemical, genetic, and clinical aspects," *Critical Reviews in Clinical Laboratory Sciences*, vol. 27, no. 1, pp. 27–58, 1989.

[43] P. Karagiannis, A. E. Gilbert, D. H. Josephs et al., "IgG4 subclass antibodies impair antitumor immunity in melanoma," *Journal of Clinical Investigation*, vol. 123, no. 4, pp. 1457–1474, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 605478, 12 pages
http://dx.doi.org/10.1155/2015/605478

Review Article

Clinical Development of Immune Checkpoint Inhibitors

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Received 25 July 2014; Accepted 7 December 2014

Academic Editor: Swaleha Zubair

Recent progress in cancer immunotherapy has been remarkable. Most striking are the clinical development and approval of immunomodulators, also known as immune checkpoint inhibitors. These monoclonal antibodies (mAb) are directed to immune checkpoint molecules, which are expressed on immune cells and mediate signals to attenuate excessive immune reactions. Although mAbs targeting tumor associated antigens, such as anti-CD20 mAb and anti-Her2 mAb, directly recognize tumor cells and induce cell death, immune checkpoint inhibitors restore and augment the antitumor immune activities of cytotoxic T cells by blocking immune checkpoint molecules on T cells or their ligands on antigen presenting and tumor cells. Based on preclinical data, many clinical trials have demonstrated the acceptable safety profiles and efficacies of immune checkpoint inhibitors in a variety of cancers. The first in class approved immune checkpoint inhibitor is ipilimumab, an anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) mAb. Two pivotal phase III randomized controlled trials demonstrated a survival benefit in patients with metastatic melanoma. In 2011, the US Food and Drug Administration (FDA) approved ipilimumab for metastatic melanoma. Several clinical trials have since investigated new agents, alone and in combination, for various cancers. In this review, we discuss the current development status of and future challenges in utilizing immune checkpoint inhibitors.

1. Introduction

In this decade, remarkable progress has been made in the clinical application of cancer immunotherapies. Most notable is the emergence of immune checkpoint inhibitors. Large-scale clinical trials have shown their feasibility and efficacy for patients with advanced malignancies. The therapeutic targets, or "immune checkpoints," are also known as coinhibitory molecules or costimulatory molecules expressed on T cells.

As the name implies, costimulatory/inhibitory molecules mediate positive/negative signals that modify MHC-TCR (major histocompatibility complex-T-cell receptor) signaling pathways. These signals each regulate T-cell survival, proliferation, differentiation, or responsiveness to cognate antigens. The net effect depends on the balance among signals [1]. T-cell activation requires costimulatory signals. If they contact antigens without costimulatory ligands on antigen presenting cells (APCs), T cells remain inactivated in a state of anergy.

Coinhibitory molecules induce T-cell dysfunction (so called "T-cell exhaustion") or apoptosis. Employing this inhibitory pathway, the immune system can attenuate excessive immune reactions and ensure self-tolerance, which is important for maintaining immune homeostasis. These functions involve programmed cell death protein-1 (PD-1), programmed cell death-1 ligand-1/2 (PD-L1/2), cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin mucin-3 (TIM-3), and B and T lymphocyte attenuator (BTLA). Tumor cells harness

these suppressive effects as one of their "immunoediting" mechanisms [2]. As shown in recent clinical trials, immune checkpoint blockade with monoclonal antibody promotes endogenous antitumor activities of immune cells and achieves clinically significant benefits for cancer patients [3, 4].

In this review, we focus on the current development status of and future challenges in utilizing immune checkpoint inhibitors, especially CTLA-4, PD-1, and PD-L1.

2. Anti-CTLA-4 Antibody

CTLA-4 (also known as CD152) is a member of the CD28 family of receptors [21, 22]. CTLA-4 is inducibly expressed on the surfaces of activated conventional $CD4^+$ and $CD8^+$ T cells. CTLA-4 binds to ligands B7.1 (CD80) and B7.2 (CD86) on APCs, where it competes with costimulatory receptor CD28 to bind with shared ligands. As CTLA-4 binds with higher affinity than CD28, it reduces CD28-dependent costimulation. CTLA-4 also mediates direct inhibitory effects on the MHC-TCR pathway [23]. CTLA-4 recruits 2 phosphatases, SHP-2 and PP2A, to its intracellular YVKM domain. SHP-2 dephosphorylates the CD3ζ chain, attenuating the TCR signal. PP2A inhibits downstream Akt phosphorylation, further impairing TCR signaling. Furthermore, CTLA-4 is constitutively and highly expressed on $CD4^+CD25^+FOXP3^+$ regulatory T cells (T regs) and plays a role in their suppressive functions [24–26]. CTLA-4 knockout mice have a lethal autoimmune-like syndrome. Prominent infiltration of $CD4^+$ T cells is detected in multiple organs. Thus, CTLA-4 is considered to be indispensable for maintaining immune homeostasis.

In the tumor microenvironment, CTLA-4 suppresses antitumor immune activities. In animal models, it has been shown that CTLA-4 blockade leads to reactivation of the antitumor immune response and tumor shrinkage [27–29]. The mechanism of action has not yet been fully elucidated. Observations made to date suggest that anti-CTLA-4 antibodies function not only by blocking inhibitory signals from reaching effector T cells but also by depleting regulatory T cells in the tumor microenvironment [30, 31]. For use in humans, based on preclinical studies, two anti-CTLA-4 antibodies have been developed: ipilimumab (Bristol-Myers Squibb) and tremelimumab (Pfizer).

2.1. Ipilimumab. Ipilimumab is a fully humanized IgG1 monoclonal antibody that inhibits CTLA-4 [32, 33].

Early clinical trials evaluated ipilimumab in patients with a variety of malignancies, including melanoma, prostate cancer, renal cell carcinoma, and non-Hodgkin lymphoma [34–45]. Some of these studies combined ipilimumab with a peptide vaccine, chemotherapy, or IL-2. Based on preclinical data, ipilimumab was administered at a dose range of 0.1–20 mg/kg, employing single or multiple dosing schedules (every 3-4 weeks).

A phase I study evaluated a single 3 mg/kg dose of ipilimumab for patients with metastatic hormone-refractory prostate cancer. Two (14%) of 14 patients showed ≧50% decline in prostate specific antigen. One (7%) patient developed grade 3 rash/pruritus requiring systemic corticosteroid administration [36]. Another phase I trial combined ipilimumab (administered at 3 mg/kg every 3 weeks) with a glycoprotein (gp) 100 peptide vaccine for patients with metastatic melanoma. Three (21%) of 14 patients responded to this treatment, including 2 showing complete responses (CRs). Grade 3 to 4 immune-related adverse events (irAEs) occurred in 6 (43%) patients. These irAEs included dermatitis, enterocolitis, hepatitis, and hypophysitis [34]. On the whole, irAEs were mild and manageable with therapy discontinuation and/or appropriate treatments, including corticosteroids.

A phase II trial compared 3 doses (0.3, 3, or 10 mg/kg) administered every 3 weeks for a total of 4 doses. Eligible patients were permitted to receive reinduction therapy (at a dose of 10 mg/kg) or maintenance therapy (administered at the previously assigned dose level every 12 weeks). The overall response rate (ORR) in the 10 mg/kg arm was superior to those in the other arms (11.1% versus 4.2% versus 0.0%), but irAEs were also higher in the 10 mg/kg arm [43]. The optimal dosing and scheduling are as yet unknown. A phase III randomized trial (NCT01515189) is currently comparing 2 doses (3 mg/kg versus 10 mg/kg). No consensus has yet been reached on the relative significance of reinduction versus maintenance therapy [46, 47]. A prospective study comparing reinduction therapy versus the physician's choice of chemotherapy (NCT00495066) is currently underway.

Based on pivotal phase III randomized controlled trials (RCTs) showing survival benefit, ipilimumab was approved by the US Food and Drug Administration (FDA) for metastatic melanoma [5, 6]. In the landmark phase III trial for patients with previously treated metastatic melanoma, ipilimumab (administered at 3 mg/kg every 3 weeks for a total of 4 doses) with or without the gp 100 peptide vaccination was compared with the gp 100 peptide vaccine alone. Eligible patients were permitted to receive reinduction therapy. The median OSs in the ipilimumab-containing arms were significantly superior to that in the gp 100 alone arm (10.1 months in ipilimumab/gp 100, 10.0 months in ipilimumab alone, and 6.4 months in gp 100 alone, hazard ratio (HR) 0.68; $P < 0.001$). Grade 3 to 4 irAEs were seen in 10–15% of patients in the ipilimumab-containing arms, while 3% in the gp 100 alone arm experienced irAEs. There were 14 treatment-related deaths (2.1%), including 7 patients with irAEs [5]. Long-term follow-up analysis confirmed an approximately 20% survival rate for patients in the ipilimumab-containing arms. Safety profiles in long-term survivors were comparable among the 3 groups, and new onset irAEs after the last dose of ipilimumab were infrequent (8%; all grades) [48]. The other phase III trial compared ipilimumab (at 10 mg/kg every 3 weeks for 4 doses)/dacarbazine with dacarbazine/placebo, followed by maintenance therapy with ipilimumab or placebo administered every 12 weeks for eligible patients. Overall survival (OS) was significantly longer in the ipilimumab/dacarbazine arm (11.2 versus 9.1 months), and the higher survival rates were durable (47.3% versus 36.3% at 1 year, 28.5% versus 17.9% at 2 years, 20.8% versus 12.2% at 3 years, HR for death 0.72; $P < 0.001$). Grade 3 to 4 AEs were seen in more patients in the ipilimumab/dacarbazine arm (56.3% versus 27.5%;

$P < 0.001$). No drug-related deaths occurred among those in the ipilimumab/dacarbazine arm [6].

The analysis of the collected data from 12 previous clinical trials, which include 1861 ipilimumab-treated patients with advanced melanoma, demonstrated a median OS of 11.4 months and 3-year OS rate of 22%. The OS curve started to show plateau around year 3, which was independent of the dose of ipilimumab (3 or 10 mg/kg), therapy line (treatment-naïve or not), or use of maintenance therapy [49].

2.2. Tremelimumab. Tremelimumab is a human IgG2 monoclonal antibody that blocks CTLA-4 [50].

Early clinical trials on tremelimumab monotherapy showed response rates of 2–17%, and these responses were durable (>150 days) [51–57]. Based on preclinical and clinical data, the standard regimen is 15 mg/kg every 90 days. Most adverse events were mild and manageable. These adverse events included skin rash, diarrhea, and endocrine abnormalities.

A phase III study compared tremelimumab (15 mg/kg every 3 months) with chemotherapy (physician's choice) in patients with untreated advanced melanoma [7]. This study demonstrated no benefits in either ORR (10.7% versus 9.8%) or OS (12.6 mo versus 10.7 mo), but a superior response duration was seen (35.8 versus 13.7 months). This observation might be explained by patient selection bias (exclusion of patients with lactate dehydrogenase (LDH) >2x upper limit of normal), drug crossover (to ipilimumab) in the control arm, and even a potentially suboptimal dosing regimen. Tremelimumab is still being investigated for other tumors, both alone and as combination therapy (Table 1).

3. Anti-PD-1 Antibodies

Programmed cell death protein-1 (PD-1; also known as CD279), like CTLA-4, is a coinhibitory CD28-family molecule [22]. While CTLA-4 works in the early phase of naïve-T-cell activation, PD-1 functions mainly in the late phase, in which PD-1 induces exhaustion or anergy in effector T cells. Thus, PD-1 is considered to play an important role in chronic inflammation such as that associated with viral infection or tumor exposure [58]. PD-1 is expressed on activated T cells, T regs [59], activated B cells, NK cells, and monocytes. It binds to the B7-family ligands PD-L1 (programmed death ligand-1, B7-H1) and PD-L2 (programmed death ligand-2, B7-DC) on APCs. PD-1 has cytoplasmic domain motifs known as ITIM (immunoreceptor tyrosine-based inhibitory motif) and ITSM (immunoreceptor tyrosine-based switch motif) [23]. When these motifs are phosphorylated, they recruit two inhibitory phosphatases, SHP-1 and SHP-2 (SHP: SH2-containing-phosphatase). These phosphatases dephosphorylate the CD3ζ chain, decreasing TCR signaling. Although the inhibitory mechanisms of CTLA-4 and PD-1 have some similarity in terms of inhibiting Akt activation, CTLA-4 can also interfere with Akt independently via PP2A [23]. PD-1 knockout mice show a milder lupus-like syndrome than CTLA-4 knockout mice [60].

Tumor cells utilize the PD-1-PD-L1/2 pathway to evade immune-cell attack [61]. Blockade of this pathway was shown to restore and augment antitumor immune activities [62].

3.1. Nivolumab (BMS-936558/ONO-4538). Nivolumab is a fully humanized IgG4 monoclonal antibody that blocks PD-1 [62].

Phase I studies tested nivolumab in such cancers as melanoma, non-small cell carcinoma of the lung (NSCLC), ovarian cancer, and renal cell carcinoma. These studies showed response rates of approximately 20–30%, durable tumor regression (>1 year), and an acceptable safety profile, with Grade 3 to 4 irAEs developing in about 20% of patients [8, 9, 63–65]. In long-term follow-up of the phase I trial for advanced melanoma, median OS was 16.8 months and survival rates were 62% at 1 year and 43% at 2 years. The patients requiring discontinuation of treatment maintained their tumor responses for at least 16 months (16–56 months). Long-term safety profiles were acceptable and similar to those described in a previous report [8]. The preliminary results of a phase I study evaluating nivolumab (at 3 mg/kg q2w) for untreated advanced NSCLC were recently reported. The ORR was 30% with 2 complete remissions (CRs), as measured by RECIST. ORR and progression-free survival (PFS) correlated with PD-L1 positivity (67% versus 0% for ORR, 45.6 mo versus 36.1 mo for median PFS). AEs were generally manageable and grade 3 to 4 AEs occurred in 3 patients, including rash, increased transaminase, and hyperglycemia [66].

Recently the interim analysis report of a phase III study (NCT01721746), comparing nivolumab monotherapy (at 3 mg/kg q2w) with investigator's choice chemotherapy in ipilimumab-refractory advanced melanoma, was shown. The ORRs were 32% in the nivolumab arm and 11% in the control arm, with the median duration of response in the nivolumab arm not reached. Grade 3 to 4 drug-related AEs were less frequent in the nivolumab arm (9% versus 31%) [10]. Another phase III study (NCT01721772) compared nivolumab monotherapy (at 3 mg/kg q2w) with dacarbazine in 418 patients with previously untreated stage III or IV melanoma. This study was stopped ahead of schedule and unblinded after independent data monitoring committee found significant survival superiority in nivolumab over dacarbazine. The results from the double-blind part of the study before the stoppage showed that the OS rate at 1 year was significantly higher in the nivolumab arm (72.9% versus 42.1%, HR for death 0.42; $P < 0.001$), and the median PFS was also significantly longer in the nivolumab arm (5.1 versus 2.2 months, HR for death or progression 0.43; $P < 0.001$). Grade 3 to 4 drug-related AEs occurred in more patients in the dacarbazine arm (11.7% versus 17.6%). No drug-related deaths occurred in both arms [11]. A phase II study (NCT01927419) of nivolumab in combination with ipilimumab compared with ipilimumab alone for advanced melanoma is currently ongoing (recruitment has been completed).

In 2013, nivolumab received Fast Track designation for the treatment of NSCLC, melanoma, and renal cell carcinoma (RCC) from the FDA. In April 2014, a rolling submission to the FDA for nivolumab in third-line pretreated NSCLC was started. In May 2014, nivolumab received a Breakthrough

Table 1

Target molecule	Drug name	Phase	Status/NCT number	Disease	Number of patients	Study design	Response	Survival	Treatment-related adverse events (≧Gr3)	Reference
CTLA-4	Ipilimumab	III	Completed (NCT00094653)	Melanoma	676	Endpoint: safety/efficacy Ipi + gp100 versus Ipi versus gp100	Ipi + gp100: ORR 5.7%; SD 14.4%	Ipi + gp100 versus gp100: 10.1 versus 6.4 mos	Ipi + gp100: drug-related 17.4%; irAEs 10.2%; diarrhea 4.5%; fatigue 5.0%; dyspnea 3.7%; anemia 2.9%; endocrine abnl. 11%; AST↑ 0.5%; ALT↑ 0.3%	[5]
		III	Completed (NCT00324155)	Melanoma	502	Endpoint: efficacy Ipi + DTIC versus PBO + DITC	Ipi + DTIC: ORR 15.2%; SD 18.0%	Ipi + DTIC versus PBO + DTIC: 11.2 versus 9.1 mos	Ipi + DTIC: immune-related 41.7%; pruritus 2.0%; rash 1.2%; diarrhea 4.0%; colitis 6.1%; AST↑ 17.4%; ALT↑ 20.7%	[6]
	Tremelimumab	III	Completed (NCT00257205)	Melanoma	655	Endpoint: efficacy treme. versus chemo.	ORR 10.7%	Treme. versus chemo.: 12.6 versus 10.7 mos (NS)	52%; diarrhea/colitis 18%; fatigue 6%; rash 2%; pruritus 1%; dyspnea 3%; hypothalamus and pituitary disorders 1%; hepatitis 1%	[7]

Table 1: Continued.

Target molecule	Drug name	Phase	Status/NCT number	Disease	Number of patients	Study design	Response	Survival	Treatment-related adverse events (≧Gr3)	Reference
PD-1	Nivolumab (BMS-936558/ONO-4538)	I	Ongoing (not recruiting) (NCT00730639)	Melanoma	107	Endpoint: safety/efficacy 5 dosing regimens	ORR 30.8%; median duration of response 104 wks; SD (≧24 wks) 6.5%	OS 16.8 mos; PFS 3.7 mos	22.4%; fatigue 1.9%; diarrhea 1.9%; abdominal pain 1.9%; lymphopenia 2.8%	[8]
		I	Ongoing (not recruiting) (NCT01176461)	Melanoma	90	Endpoint: safety/efficacy 3 dosing regimens	ORR 25%; SD (≧24 wks) 21%	PFS (at 24 wks) 46%	5.6%; rash 2.2%; interstitial pneumonitis 2.2%	[9]
		III	Ongoing (not recruiting) (NCT 01721772)	Melanoma	370	Endpoint: efficacy Nivo. versus ICC	ORR 32% versus 11%	NA	9% versus 31%	[10]
		III	Completed (NCT01721772)	Melanoma	418	Endpoint: efficacy Nivo. versus dacarbazine	ORR 40.0% versus 13.9%	OS (at 1 yr) 72.9% versus 42.1%, median PFS 5.1 versus 2.2 mo	11.7% versus 17.6%; fatigue 0.5%; diarrhea 1.0%; rash 0.5%; vomiting 0.5%	[11]
	Pidilizumab (CT-011)	II	Completed (NCT01435369)	Melanoma	103	Endpoint: safety/efficacy 2 dosing regimens	ORR 5.9%	OS (at 1 yr): 64.5%	NA	[12]
		I	Ongoing (not recruiting) (NCT01295827)	Melanoma	135	Endpoint: safety/efficacy 3 dosing regimens	ORR 38% by RECIST and 37% by irRC	Median PFS >7 mos	13%; hypothyroidism 1%; diarrhea 1%; fatigue 1%; AST↑ 1%; renal failure 1%; rash 2%; pruritus 1%	[13]
	Pembrolizumab (MK-3475)	I	Ongoing (not recruiting) (NCT01295827)	Untreated NSCLC	57	Endpoint: safety/efficacy 3 dosing regimens	ORR 26% by RECIST and 47% by irRC	Median OS NR; OS at 1 yr 80%; median PFS 45.6%; PFS at 24 wks 70%	CK↑ 2%; pericardial effusion 2%; pneumonitis 2%; acute kidney injury 2%	[14]
		I	Ongoing (not recruiting) (NCT01848834)	Head and neck cancer	60	Endpoint: safety/efficacy single arm	ORR 19.6% in total, 20.0% in HPV+, and 19.4% in HPV−;	NA	Gr3–5 16.7%; Rash 3.3%	[15]
		I	Ongoing (not recruiting) (NCT01848834)	Gastric cancer	39	Endpoint: safety/efficacy single arm	ORR 30.2% by RECIST	NA	7.7%; hypoxia 2.6%; peripheral neuropathy 2.6%; pneumonia 2.6%	[16]

Table 1: Continued.

Target molecule	Drug name	Phase	Status/NCT number	Disease	Number of patients	Study design	Response	Survival	Treatment-related adverse events (≧Gr3)	Reference
	BMS-936559	I	Ongoing (not recruiting) (NCT00729664)	Melanoma	52	Endpoint: safety 4 dose levels	ORR 17%; SD (≧24 wks) 27%	PFS (at 24 wks) 42%	9%; fatigue 1%; infusion reaction 1%; lymphopenia 1%	[17]
				NSCLC	49		ORR 10%; SD (≧24 wks) 12%	PFS (at 24 wks) 31%		
				Ovarian cancer	17		ORR 6%; SD (≧24 wks) 18%	PFS (at 24 wks) 22%		
				Renal cell carcinoma	17		ORR 12%; SD (≧24 wks) 41%	PFS (at 24 wks) 53%		
PD-L1	MPDL3280A	I	Recruiting (NCT01375842)	Urothelial bladder cancer	68	Endpoint: safety/efficacy/ biomarker single arm	ORR: PD-L1 + 43% (at 6 wks) and 52% (at 12 wks); PD-L1 − 11% (at 6 wks);	NA	4%; no irAE	[18]
	MEDI4736	I	Recruiting (NCT01693562)	Advanced solid tumors	26 (as of Jan 2014)	Endpoint: safety/efficacy single arm	PR 15.4%; disease control rate (≧12 wks) 46%	NA	Any Gr 34%; Gr3/4 0%; no DLT; no MTD	[19]
	MSB0019718C	I	Recruiting (NCT01772004)	Refractory malignancies	27 (as of Jan 2014)	Endpoint: safety single arm	NA	NA	Treatment discontinuation 52.2% (8.7% for AEs); drug-related AEs 11.1%; DLT 3.7% (CPK↑, myositis, and myocarditis)	[20]

Abbreviations: NSCLC, non-small cell lung cancer; Ipi, Ipilimumab; gp100, glycoprotein 100 peptide vaccine; DITC, dacarbazine; PBO, placebo; ORR, objective response rate; PR, partial response; SD, stable disease; mo, month; wk, week; RECIST, response evaluation criteria in solid tumors; irRC, immune-related response criteria; HPV, human papillomavirus; NA, not available; NS, not significant; NR, not reached; OS, overall survival; PFS, progression-free survival; AE, adverse event; irAE, immune-related adverse event; Gr, Grade; abnl., abnormality; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CPK, creatine phosphokinase; DLT, dose limiting toxicity; MTD, maximum tolerance dose; ICC, investigator's choise chemotherapy.

Therapy designation for non-Hodgkin lymphoma from the FDA. In Japan, in July 2014, nivolumab received manufacturing and marketing approval for unresectable melanoma from the domestic regulator, the Ministry of Health Labor and Welfare, which made nivolumab the first in anti-PD-1 antibody to receive regulatory approval in the world.

3.2. Pidilizumab (CT-011). Pidilizumab (CT-011) is a humanized IgG-1κ monoclonal antibody that blocks PD-1. In animal models, an antitumor effect was achieved with BAT monoclonal antibody (a murine mAb developed against a membrane preparation of a Burkitt lymphoma cell line), from which pidilizumab is derived [67, 68].

In humans, the safety and tolerability of the single dose regimen were shown in a phase I study of patients with advanced hematologic malignancies [69]. No treatment-related toxicities occurred and the maximum tolerated dose was not identified in this trial (0.2–6 mg/kg).

Pidilizumab has been tested in phase II trials, as monotherapy for patients with diffuse large B-cell lymphoma after autologous hematopoietic stem-cell transplantation [70] and as combined therapy with rituximab for relapsed follicular lymphoma [71]. Both trials showed promising efficacies even in high-risk patients.

The results of a phase II trial in patients with pretreated advanced melanoma were recently reported. ORR was 5.9%, measured by immune-related response criteria (irRC), and the OS rate at 1 year was 64.5%. The patients who had been pretreated with ipilimumab (51% of patients) tended to experience a higher rate of immune-related stable disease (irSD) and longer PFS (2.8 mo versus 1.9 mo) [12].

3.3. Pembrolizumab (MK-3475, Formally Known as Lambrolizumab). Pembrolizumab (MK-3475) is a humanized monoclonal IgG-4κ antibody that blocks PD-1.

A phase I dose-escalation study evaluated three dose levels, 1 mg/kg, 3 mg/kg, and 10 mg/kg, administered every 2 weeks, in patients with multiple solid tumors [72]. All dose levels were found to be safe, and the maximum tolerated dose was not identified. Clinical responses were observed at all dose levels. Another phase I study tested 3 regimens (2 mg/kg every 3 weeks and 10 mg/kg every 2 or 3 weeks) in patients with advanced melanoma [13]. AEs were generally mild and grade 3 to 4 AEs were seen in 13% of patients. The ORRs ranged from 38% to 52%, in the biweekly 10 mg/kg cohort (measured by RECIST), showing no significant differences. These responses were durable, with the median PFS exceeding 7 months for all three regimens.

An ongoing phase II trial is now comparing 2 dose levels of pembrolizumab with investigator-choice chemotherapy in patients with previously treated advanced melanoma (NCT01704287). Another ongoing phase II trial is also evaluating 2 dose schedules of pembrolizumab (10 mg/kg q2w or q3w) compared with ipilimumab (3 mg/kg q3w) for advanced melanoma (NCT01866319).

In April 2013, pembrolizumab received the Breakthrough Therapy designation for advanced melanoma from the FDA. After being reviewed under the FDA's Accelerated Approval program, in September 2014, pembrolizumab received approval for treatment of patients with advanced melanoma by the FDA.

Besides melanoma, several early trials have showed the tolerability and antitumor effects of pembrolizumab in other tumors. The preliminary results of another phase I study evaluating pembrolizumab in untreated PD-L1-positive NSCLC were recently reported. The overall objective response rate was 25% (33% in the 2 mg/kg q3w, 20% in the 10 mg/kg q3w, and 31% in the 10 mg/kg q2w group), as measured by RECIST. AEs were generally mild and grade 3 to 4 AEs occurred in 3 patients, including pneumonitis requiring treatment discontinuation [14]. Another preliminary result was reported for the phase I trial of pembrolizumab as monotherapy, administered at 2 mg/kg every 2 weeks, to 60 patients with recurrent/metastatic head and neck cancers. Grade 3 to 4 drug-related AEs were reported in 16.7% of patients. The best ORR was 20% in all patients (assessed by RECIST 1.1). Efficacies were comparable between human papilloma virus- (HPV-) positive and HPV-negative patients (20.0% versus 19.4%) [15]. Another phase I study (NCT01848834) assessed pembrolizumab in the patients with previously treated advanced gastric cancer that expressed PD-L1. The enrolled 39 patients were treated with pembrolizumab at 10 mg/kg q2w. Median follow-up period was 6 months. Treatment-related AEs occurred in 24 patients (61.5%), and those of grade 3 to 5 occurred in 3 patients (pneumonitis, peripheral neuropathy, and hypoxia). ORR was 30.8% and disease control rate was 43.6%. Responses were mostly ongoing and the median response duration was not reached [16].

4. Anti-PD-L1 Antibodies

PD-L1 (also known as B7-H1 or CD274) and PD-L2 (also known as B7-DC or CD273) are inhibitory B7-family molecules that bind the PD-1 receptor. PD-L1 is inducibly expressed on a variety of hematopoietic and nonhematopoietic cells, including most human tumor cells and cells within the tumor microenvironment [61]. PD-L1 expression has been shown to correlate inversely with the clinical outcomes of some malignancies. PD-L2 is expressed on hematopoietic cells. PD-L1 knockout mice show infiltration of lymphocytes into nonlymphoid organs and exacerbation of preexisting autoimmune diseases [73, 74].

As mentioned above, the PD-1-PD-L1 axis is one of the main mechanisms by which cancer cells evade immune-cell attack [61]. Blockade of this pathway was shown to reinforce antitumor immune activities [62]. Because PD-L1 also interacts with CD80 [75, 76], anti-PD-L1 antibody might have optimal clinical potency against PD-1.

4.1. BMS-936559. BMS-936559 is a fully humanized IgG4 monoclonal anti-PD-L1 antibody. It inhibits the binding of PD-L1 to PD-1 and CD80. A phase I dose-escalation study evaluated BMS-936559 in 207 patients with selected cancers, including melanoma, NSCLC, ovarian cancer, and renal cell carcinoma. The study drug was administered at 4 dose levels (0.3–10 mg/kg) every 14 days, 3 times in each 6-week course for up to 16 cycles, when either CR or disease progression was confirmed. The ORRs were 6–17% and efficacy was durable

(>1 year in 8 of 16 patients who responded). Grade 3 to 4 irAEs, seen in 9% of the patients, were treatment-related in 5% [17].

4.2. MPDL3280A. MPDL3280A is a humanized IgG-1κ monoclonal anti-PD-L1 antibody. It is genetically engineered to modify the Fc domain, thereby impairing the antibody-dependent cellular cytotoxicity of PD-L1 expressing cells [77, 78].

A phase I trial of MPDL3280A as monotherapy for advanced melanoma achieved a response rate of 26% and PFS of 35% at 24 weeks. Grade 3 to 4 AEs were seen in 33% of patients [79]. The results of another phase I trial were recently reported. MPDL3280A was tested in patients with pretreated metastatic urothelial bladder cancer. ORR in PD-L1-positive patients was superior to that in PD-L1-negative patients (43% versus 11% at 6 weeks). ORR at 12 weeks was 52% in PD-L1-positive patients. Grade 3 to 4 AEs were seen in 4% of patients, with no irAEs [18]. The FDA has granted the Breakthrough Therapy designation to MPDL3280A.

4.3. MEDI4736. MEDI4736 is a humanized IgG-1κ monoclonal antibody that blocks PD-L1. MEDI4736 demonstrated tumor regression and improved survival in a mouse model.

A "first-time-in-human" phase I study evaluating the safety, tolerability, and pharmacokinetics of this agent in patients with advanced solid tumors is currently underway (NCT01693562). The interim report was recently presented. As of January 2014, 26 patients were receiving dose-escalation treatments and had been given a median of 5 (1–25) q2w and 4.5 (1–7) q3w doses of MEDI4736 across 6 cohorts (0.1–10 mg/kg q2w; 15 mg/kg q3w). No dose limiting toxicities (DLTs) or maximum tolerated dose was identified. Treatment-related AEs occurred in 34% of patients, but all were grade 1 to 2 and did not lead totreatment discontinuation. Four of the 26 patients showed partial responses (PRs). The rate (PR + stable disease $\geqq$ 12 weeks) was 46%. Clinical responses were durable, with 11 patients remaining in the study (2+ to 14.9+ months) [19]. Another phase I trial is now testing the combination of MEDI4736 plus tremelimumab (NCT01975831).

4.4. MSB0010718C. MSB0010718 is a fully humanized IgG1 monoclonal antibody directed to PD-L1. A phase I trial is currently testing MSB0010718 to assess its safety, tolerability, and pharmacokinetics in patients with refractory malignancies (NCT01772004). As of January 2014, 27 patients had been enrolled and were participating in a dose-escalation study (3 + 3 design; 1, 3, 10, and 20 mg/kg, q2w). Twenty-three patients had been followed for at least 4 weeks. Discontinuation of the treatment had been necessary in 12 patients (52.2%): 9 (39.1%) due to progression of disease, 2 (8.7%) for AEs, and 1 (4.3%) because the patient died. Grade 3 to 4 drug-related toxicities included laboratory abnormalities in 3 patients. One DLT was observed in 1 patient at dose level 4 (20 mg/kg): an irAE with creatine kinase elevation, myositis, and myocarditis [20].

5. Combination Therapy

Recent clinical trials have actively investigated the potential for synergistic effects by combining immune checkpoint inhibitors with other agents. The partner agents/therapies include other checkpoint agents, cytotoxic agents, anticancer vaccines, cytokines, and radiotherapy.

A phase I study evaluated combined therapy with ipilimumab plus nivolumab in patients with advanced melanoma [80]. The patients received ipilimumab once every 3 weeks for 4 doses and nivolumab once every 3 weeks for 8 doses concurrently. Then, eligible patients were permitted to receive both once every 12 weeks up to 8 doses. Grade 3 to 4 treatment-related AEs were seen in 53% of the concurrent-cohort patients but were mild and manageable. The maximum tolerated dose was 3 mg/kg of ipilimumab and 1 mg/kg of nivolumab, a dosing regimen at which 53% of patients showed responses. Recent follow-up surveys confirmed OS to be 94% at 1 year and 88% at 2 years in this cohort. An expansion cohort, with the patients receiving 3 mg/kg of ipilimumab and 1 mg/kg of nivolumab every 3 weeks for 4 doses and 1 mg/kg of nivolumab every 2 weeks until disease progression, is currently being evaluated in a phase II/III study [81]. A phase III trial (NCT01844505) evaluating this combination is currently ongoing (recruitment has been completed).

6. Biomarkers for Predicting Clinical Benefits and Adverse Reactions

Although immune checkpoint inhibitors have shown promising safety and efficacy, to date only a small proportion of patients have achieved long-term survival, with severe irAEs occurring on occasion. Biomarkers predicting clinical benefit may enable physicians to select individualized treatments for their patents and thereby maximize clinical benefits. Thus, there is an urgent need to identify "baseline (pretreatment)" biomarkers predicting responses or toxicities. Several biomarkers for examining T-cell proliferation or activation and other forms of antigen-specific immunity have been assessed in the context of immune checkpoint inhibitors.

Immunohistochemical PD-L1 expression in a tumor specimen is among the potential markers for PD-1-PD-L1-directed therapies. In a phase I study of nivolumab, though the data obtained are preliminary, an objective response was seen only in the patients who showed immunohistochemical PD-L1 expression in pretreatment tumor specimens [63]. These observations may support the strategy of selecting PD-L1-positive patients for therapy. However, PD-L1 expression on tumor cells is inducible and is susceptible to influences of the tumor microenvironment. Furthermore, technical advances in PD-L1 immunostaining are still needed. Also, the value of PD-L1 IHC staining as a predictive biomarker for combination therapy with nivolumab plus ipilimumab has yet to be validated [80]. As yet, the applicability and significance of PD-L1 expression as a baseline biomarker must be interpreted with caution and further prospective evaluations are needed, including the results of ongoing randomized

clinical trials that are prospectively evaluating PD-L1 IHC as a companion diagnostic platform (NCT01721746).

Another potential biomarker is pretreatment levels of monocytic myeloid-derived suppressor cells (m-MDSCs) [82, 83]. A recent retrospective study suggested higher pretreatment quantities of $Lin^{-}CD14^{+}HLA\text{-}DR^{low/-}$ m-MDSC to be associated with inferior OS in patients with metastatic melanoma treated with ipilimumab [83].

Recent genetic analysis using whole-exome sequencing showed the significance of somatic mutational load as predictive biomarker of clinical benefit in melanoma patients treated with CTLA-4 blockade. The neopeptide signature associated with clinical response was identified and predicted mutant peptides were verified to activate patient T cell *in vitro* [84].

Other potential predictive/prognostic biomarkers include the gene expression profiles obtained employing tumor biopsies [85, 86], CRP level [87], absolute lymphocyte and eosinophil counts [88], and LDH levels [89]. These possibilities await further research.

7. Conclusion

Immune checkpoint inhibitors have opened a new era of cancer immunotherapy. Since the FDA approval was obtained for the anti-CTLA-4 monoclonal antibody ipilimumab, several large-scale clinical trials have evaluated new agents both alone and in combinations with other conventional or new therapies. Future challenges include exploring new target molecules and immune cells, optimizing dosing regimens and combination therapies, validating the safety and efficacy of these novel treatment strategies in many other malignancies, establishing an immunomonitoring system to be applied during therapy, and identifying biomarkers predicting clinical responses and toxicities. Active, ongoing investigations are anticipated to provide further clinical benefits for patients with cancers that are currently refractory to treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] L. Chen and D. B. Flies, "Molecular mechanisms of T cell co-stimulation and co-inhibition," *Nature Reviews Immunology*, vol. 13, no. 4, pp. 227–242, 2013.

[2] R. D. Schreiber, L. J. Old, and M. J. Smyth, "Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion," *Science*, vol. 331, no. 6024, pp. 1565–1570, 2011.

[3] D. B. Page, M. A. Postow, M. K. Callahan, J. P. Allison, and J. D. Wolchok, "Immune modulation in cancer with antibodies," *Annual Review of Medicine*, vol. 65, pp. 185–202, 2014.

[4] D. M. Pardoll, "The blockade of immune checkpoints in cancer immunotherapy," *Nature Reviews Cancer*, vol. 12, no. 4, pp. 252–264, 2012.

[5] F. S. Hodi, S. J. O'Day, D. F. McDermott et al., "Improved survival with ipilimumab in patients with metastatic melanoma," *The New England Journal of Medicine*, vol. 363, no. 8, pp. 711–723, 2010.

[6] C. Robert, L. Thomas, I. Bondarenko et al., "Ipilimumab plus dacarbazine for previously untreated metastatic melanoma," *The New England Journal of Medicine*, vol. 364, no. 26, pp. 2517–2526, 2011.

[7] A. Ribas, R. Kefford, M. A. Marshall et al., "Phase III randomized clinical trial comparing tremelimumab with standard-of-care chemotherapy in patients with advanced melanoma," *Journal of Clinical Oncology*, vol. 31, no. 5, pp. 616–622, 2013.

[8] S. L. Topalian, M. Sznol, D. F. McDermott et al., "Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab," *Journal of Clinical Oncology*, vol. 32, no. 10, pp. 1020–1030, 2014.

[9] J. S. Weber, R. R. Kudchadkar, B. Yu et al., "Safety, efficacy, and biomarkers of nivolumab with vaccine in ipilimumab-refractory or -naive melanoma," *Journal of Clinical Oncology*, vol. 31, no. 34, pp. 4311–4318, 2013.

[10] J. S. Weber, S. D'Angelo, F. S. Hodi et al., "A phase 3 randomized, open-label study of nivolumab (anti-PD-1; BMS-936558; ONO-4538) versus investigator's choice chemotherapy (ICC) in patients with advanced melanoma after prior anti-CTLA-4 therapy," ESMO Meeting Abstracts, 2014.

[11] C. Robert, G. V. Long, B. Brady et al., "Nivolumab in previously untreated melanoma without BRAF mutation," *The New England Journal of Medicine*, 2014.

[12] M. B. Atkins, R. R. Kudchadkar, M. Sznol et al., "Phase 2, multicenter, safety and efficacy study of pidilizumab in patients with metastatic melanoma," in *Proceedings of the ASCO Annual Meeting*, vol. 32, p. 9001, 2014.

[13] O. Hamid, C. Robert, A. Daud et al., "Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma," *The New England Journal of Medicine*, vol. 369, no. 2, pp. 134–144, 2013.

[14] N. A. Rizvi, E. B. Garon, A. Patnaik et al., "Safety and clinical activity of MK-3475 as initial therapy in patients with advanced non-small cell lung cancer (NSCLC)," *Proceedings of the ASCO Meeting Abstracts*, vol. 32, p. 8007, 2014.

[15] T. Y. Seiwert, B. Burtness, J. Weiss et al., "A phase Ib study of MK-3475 in patients with human papillomavirus (HPV)-associated and non-HPV-associated head and neck (H/N) cancer," *ASCO Meeting Abstracts*, vol. 32, article 6011, 2014.

[16] K. Muro, Y. Bang, V. Shankaran et al., "A phase 1b study of pembrolizumab (Pembro; MK-3475) in patients (Pts) with advanced gastric cancer," ESMO Meeting Abstracts, 2014.

[17] J. R. Brahmer, S. S. Tykodi, L. Q. M. Chow et al., "Safety and activity of anti-PD-L1 antibody in patients with advanced cancer," *The New England Journal of Medicine*, vol. 366, no. 26, pp. 2455–2465, 2012.

[18] T. Powles, N. J. Vogelzang, G. D. Fine et al., "Inhibition of PD-L1 by MPDL3280A and clinical activity in pts with metastatic urothelial bladder cancer (UBC)," *ASCO Meeting Abstracts*, vol. 32, article 5011, 2014.

[19] J. Lutzky, S. J. Antonia, A. Blake-Haskins et al., "A phase 1 study of MEDI4736, an anti–PD-L1 antibody, in patients with advanced solid tumors," *ASCO Meeting Abstracts*, vol. 32, abstract 3001, 2014.

[20] C. R. Heery, G. H. O'Sullivan Coyne, R. A. Madan et al., "Phase I open-label, multiple ascending dose trial of MSB0010718C, an anti-PD-L1 monoclonal antibody, in advanced solid malignancies," in *Proceedings of the ASCO Meeting Abstracts*, vol. 32, p. 3064, 2014.

[21] C. E. Rudd, A. Taylor, and H. Schneider, "CD28 and CTLA-4 coreceptor expression and signal transduction," *Immunological Reviews*, vol. 229, no. 1, pp. 12–26, 2009.

[22] L. Chen, "Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity," *Nature Reviews Immunology*, vol. 4, no. 5, pp. 336–347, 2004.

[23] C. J. Nirschl and C. G. Drake, "Molecular pathways: coexpression of immune checkpoint molecules: Signaling pathways and implications for cancer immunotherapy," *Clinical Cancer Research*, vol. 19, no. 18, pp. 4917–4924, 2013.

[24] D. M. Sansom and L. S. K. Walker, "The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology," *Immunological Reviews*, vol. 212, pp. 131–148, 2006.

[25] K. Wing, Y. Onishi, P. Prieto-Martin et al., "CTLA-4 control over Foxp3$^+$ regulatory T cell function," *Science*, vol. 322, no. 5899, pp. 271–275, 2008.

[26] L. S. K. Walker, "Treg and CTLA-4: two intertwining pathways to immune tolerance," *Journal of Autoimmunity*, vol. 45, pp. 49–57, 2013.

[27] D. R. Leach, M. F. Krummel, and J. P. Allison, "Enhancement of antitumor immunity by CTLA-4 blockade," *Science*, vol. 271, no. 5256, pp. 1734–1736, 1996.

[28] A. van Elsas, A. A. Hurwitz, and J. P. Allison, "Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony- stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation," *Journal of Experimental Medicine*, vol. 190, no. 3, pp. 355–366, 1999.

[29] S. A. Quezada, T. R. Simpson, K. S. Peggs et al., "Tumor-reactive CD4$^+$ T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts," *The Journal of Experimental Medicine*, vol. 207, no. 3, pp. 637–650, 2010.

[30] K. S. Peggs, S. A. Quezada, C. A. Chambers, A. J. Korman, and J. P. Allison, "Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies," *The Journal of Experimental Medicine*, vol. 206, no. 8, pp. 1717–1725, 2009.

[31] T. R. Simpson, F. Li, W. Montalvo-Ortiz et al., "Fc-dependent depletion of tumor-infiltrating regulatory t cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma," *The Journal of Experimental Medicine*, vol. 210, no. 9, pp. 1695–1710, 2013.

[32] A. Hoos, R. Ibrahim, A. Korman et al., "Development of ipilimumab: contribution to a new paradigm for cancer immunotherapy," *Seminars in Oncology*, vol. 37, no. 5, pp. 533–546, 2010.

[33] E. J. Lipson and C. G. Drake, "Ipilimumab: an anti-CTLA-4 antibody for metastatic melanoma," *Clinical Cancer Research*, vol. 17, no. 22, pp. 6958–6962, 2011.

[34] G. Q. Phan, J. C. Yang, R. M. Sherry et al., "Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 14, pp. 8372–8377, 2003.

[35] A. V. Maker, G. Q. Phan, P. Attia et al., "Tumor regression and autoimmunity in patients treated with cytotoxic T lymphocyte-associated antigen 4 blockade and interleukin 2: a phase I/II study," *Annals of Surgical Oncology*, vol. 12, no. 12, pp. 1005–1016, 2005.

[36] E. J. Small, N. S. Tchekmedyian, B. I. Rini, L. Fong, I. Lowy, and J. P. Allison, "A pilot trial of CTLA-4 blockade with human anti-CTLA-4 in patients with hormone-refractory prostate cancer," *Clinical Cancer Research*, vol. 13, no. 6, pp. 1810–1815, 2007.

[37] S. M. Ansell, S. A. Hurvitz, P. A. Koenig et al., "Phase I study of ipilimumab, an anti-CTLA-4 monoclonal antibody, in patients with relapsed and refractory B-cell non-Hodgkin lymphoma," *Clinical Cancer Research*, vol. 15, no. 20, pp. 6446–6453, 2009.

[38] L. Fong, S. S. Kwek, S. O'Brien et al., "Potentiating endogenous antitumor immunity to prostate cancer through combination immunotherapy with CTLA4 blockade and GM-CSF," *Cancer Research*, vol. 69, no. 2, pp. 609–615, 2009.

[39] F. S. Hodi, M. Butler, D. A. Oble et al., "Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 8, pp. 3005–3010, 2008.

[40] J. C. Yang, M. Hughes, U. Kammula et al., "Ipilimumab (anti-CTLA4 antibody) causes regression of metastatic renal cell cancer associated with enteritis and hypophysitis," *Journal of Immunotherapy*, vol. 30, no. 8, pp. 825–830, 2007.

[41] J. Weber, J. A. Thompson, O. Hamid et al., "A randomized, double-blind, placebo-controlled, phase II study comparing the tolerability and efficacy of ipilimumab administered with or without prophylactic budesonide in patients with unresectable stage III or IV melanoma," *Clinical Cancer Research*, vol. 15, no. 17, pp. 5591–5598, 2009.

[42] S. J. O'Day, M. Maio, V. Chiarion-Sileni et al., "Efficacy and safety of ipilimumab monotherapy in patients with pretreated advanced melanoma: a multicenter single-arm phase II study," *Annals of Oncology*, vol. 21, no. 8, pp. 1712–1717, 2010.

[43] J. D. Wolchok, B. Neyns, G. Linette et al., "Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study," *The Lancet Oncology*, vol. 11, no. 2, pp. 155–164, 2010.

[44] E. M. Hersh, S. J. O'Day, J. Powderly et al., "A phase II multicenter study of ipilimumab with or without dacarbazine in chemotherapy-naïve patients with advanced melanoma," *Investigational New Drugs*, vol. 29, no. 3, pp. 489–498, 2011.

[45] K. Margolin, M. S. Ernstoff, O. Hamid et al., "Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial," *The Lancet Oncology*, vol. 13, no. 5, pp. 459–465, 2012.

[46] C. Robert, D. Schadendorf, M. Messina, F. S. Hodi, and S. O'Day, "Efficacy and safety of retreatment with ipilimumab in patients with pretreated advanced melanoma who progressed after initially achieving disease control," *Clinical Cancer Research*, vol. 19, no. 8, pp. 2232–2239, 2013.

[47] V. Chiarion-Sileni, J. Pigozzo, P. A. Ascierto et al., "Ipilimumab retreatment in patients with pretreated advanced melanoma: the expanded access programme in Italy," *British Journal of Cancer*, vol. 110, no. 7, pp. 1721–1726, 2014.

[48] D. McDermott, J. Haanen, T.-T. Chen, P. Lorigan, and S. O'Day, "Efficacy and safety of ipilimumab in metastatic melanoma patients surviving more than 2 years following treatment in a phase III trial (MDX010-20)," *Annals of Oncology*, vol. 24, no. 10, pp. 2694–2698, 2013.

[49] D. Schadendorf, C. Robert, J. S. Weber et al., "Pooled analysis of long-term survival data from phase II and phase III trials of ipilimumab in metastatic or locally advanced, unresectable melanoma," *European Journal of Cancer*, vol. 49, article S11, 2013.

[50] A. Ribas, D. C. Hanson, D. A. Noe et al., "Tremelimumab (CP-675,206), a cytotoxic T lymphocyte—associated antigen 4 blocking monoclonal antibody in clinical development for patients with cancer," *The Oncologist*, vol. 12, no. 7, pp. 873–883, 2007.

[51] A. Ribas, L. H. Camacho, G. Lopez-Berestein et al., "Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206," *Journal of Clinical Oncology*, vol. 23, no. 35, pp. 8968–8977, 2005.

[52] L. H. Camacho, S. Antonia, J. Sosman et al., "Phase I/II trial of tremelimumab in patients with metastatic melanoma," *Journal of Clinical Oncology*, vol. 27, no. 7, pp. 1075–1081, 2009.

[53] A. Ribas, B. Comin-Anduix, B. Chmielowski et al., "Dendritic cell vaccination combined with CTLA4 blockade in patients with metastatic melanoma," *Clinical Cancer Research*, vol. 15, no. 19, pp. 6267–6276, 2009.

[54] K. Y. Chung, I. Gore, L. Fong et al., "Phase II study of the anti-cytotoxic T-lymphocyte-associated antigen 4 monoclonal antibody, tremelimumab, in patients with refractory metastatic colorectal cancer," *Journal of Clinical Oncology*, vol. 28, no. 21, pp. 3485–3490, 2010.

[55] J. M. Kirkwood, P. Lorigan, P. Hersey et al., "Phase II trial of tremelimumab (CP-675,206) in patients with advanced refractory or relapsed melanoma," *Clinical Cancer Research*, vol. 16, no. 3, pp. 1042–1048, 2010.

[56] B. Sangro, C. Gomez-Martin, M. de la Mata et al., "A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C," *Journal of Hepatology*, vol. 59, no. 1, pp. 81–88, 2013.

[57] C. Ralph, E. Elkord, D. J. Burt et al., "Modulation of lymphocyte regulation for cancer therapy: a phase II trial of tremelimumab in advanced gastric and esophageal adenocarcinoma," *Clinical Cancer Research*, vol. 16, no. 5, pp. 1662–1672, 2010.

[58] D. L. Barber, E. J. Wherry, D. Masopust et al., "Restoring function in exhausted CD8 T cells during chronic viral infection," *Nature*, vol. 439, no. 7077, pp. 682–687, 2006.

[59] L. M. Francisco, V. H. Salinas, K. E. Brown et al., "PD-L1 regulates the development, maintenance, and function of induced regulatory T cells," *The Journal of Experimental Medicine*, vol. 206, no. 13, pp. 3015–3029, 2009.

[60] T. Okazaki, Y. Tanaka, R. Nishio et al., "Autoantibodies against cardiac troponin I are responsible for dilated cardiomyopathy in PD-1-deficient mice," *Nature Medicine*, vol. 9, no. 12, pp. 1477–1483, 2003.

[61] W. Zou and L. Chen, "Inhibitory B7-family molecules in the tumour microenvironment," *Nature Reviews Immunology*, vol. 8, no. 6, pp. 467–477, 2008.

[62] S. L. Topalian, C. G. Drake, and D. M. Pardoll, "Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity," *Current Opinion in Immunology*, vol. 24, no. 2, pp. 207–212, 2012.

[63] S. L. Topalian, F. S. Hodi, J. R. Brahmer et al., "Safety, activity, and immune correlates of anti-PD-1 antibody in cancer," *The New England Journal of Medicine*, vol. 366, no. 26, pp. 2443–2454, 2012.

[64] J. R. Brahmer, C. G. Drake, I. Wollner et al., "Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates," *Journal of Clinical Oncology*, vol. 28, no. 19, pp. 3167–3175, 2010.

[65] E. J. Lipson, W. H. Sharfman, C. G. Drake et al., "Durable cancer regression off-treatment and effective reinduction therapy with an anti-PD-1 antibody," *Clinical Cancer Research*, vol. 19, no. 2, pp. 462–468, 2013.

[66] S. N. Gettinger, F. A. Shepherd, S. J. Antonia et al., "First-line nivolumab (anti-PD-1; BMS-936558, ONO-4538) monotherapy in advanced NSCLC: Safety, efficacy, and correlation of outcomes with PD-L1 status," in *Proceedings of the ASCO Annual Meeting*, ASCO Meeting Abstracts 32:8024, 2014.

[67] B. Hardy, L. Indjiia, G. Rodionov, A. Raiter, and A. Inbal, "Treatment with BAT monoclonal antibody decreases tumor burden in a murine model of leukemia/lymphoma," *International Journal of Oncology*, vol. 19, no. 5, pp. 897–902, 2001.

[68] B. Hardy, Y. Niv, L. Fadaeev, and A. Raiter, "BAT mAb induces lymphopoiesis in nude mice," *International Immunology*, vol. 17, no. 5, pp. 615–619, 2005.

[69] R. Berger, R. Rotem-Yehudar, G. Slama et al., "Phase i safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies," *Clinical Cancer Research*, vol. 14, no. 10, pp. 3044–3051, 2008.

[70] P. Armand, A. Nagler, E. A. Weller et al., "Disabling immune tolerance by programmed death-1 blockade with pidilizumab after autologous hematopoietic stem-cell transplantation for diffuse large B-cell lymphoma: results of an international phase II trial," *Journal of Clinical Oncology*, vol. 31, no. 33, pp. 4199–4206, 2013.

[71] J. R. Westin, F. Chu, M. Zhang et al., "Safety and activity of PD1 blockade by pidilizumab in combination with rituximab in patients with relapsed follicular lymphoma: a single group, open-label, phase 2 trial," *The Lancet Oncology*, vol. 15, no. 1, pp. 69–77, 2014.

[72] A. Patnaik, S. P. Kang, A. W. Tolcher et al., "Phase I study of MK-3475 (anti-PD-1 monoclonal antibody) in patients with advanced solid tumors," in *Proceedings of the ASCO Annual Meeting*, abstract 2512, May 2012.

[73] H. Dong, G. Zhu, K. Tamada, D. B. Flies, J. M. A. van Deursen, and L. Chen, "B7-H1 determines accumulation and deletion of intrahepatic $CD8^+$ T lymphocytes," *Immunity*, vol. 20, no. 3, pp. 327–336, 2004.

[74] Y. E. Latchman, S. C. Liang, Y. Wu et al., "PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 29, pp. 10691–10696, 2004.

[75] M. J. Butte, M. E. Keir, T. B. Phamduy, A. H. Sharpe, and G. J. Freeman, "Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses," *Immunity*, vol. 27, no. 1, pp. 111–122, 2007.

[76] J.-J. Park, R. Omiya, Y. Matsumura et al., "B7-H1/CD80 interaction is required for the induction and maintenance of peripheral T-cell tolerance," *Blood*, vol. 116, no. 8, pp. 1291–1298, 2010.

[77] D. S. Chen, B. A. Irving, and F. S. Hodi, "Molecular pathways: next-generation immunotherapy-inhibiting programmed death-ligand 1 and programmed death-1," *Clinical Cancer Research*, vol. 18, no. 24, pp. 6580–6587, 2012.

[78] A. J. Furness, F. A. Vargas, K. S. Peggs, and S. A. Quezada, "Impact of tumour microenvironment and Fc receptors on the activity of immunomodulatory antibodies," *Trends in Immunology*, vol. 35, pp. 290–298, 2014.

[79] R. S. Herbst, M. S. Gordon, G. D. Fine et al., "A study of MPDL3280A, an engineered PD-L1 antibody in patients with locally advanced or metastatic tumors," *ASCO Meeting Abstracts*, vol. 31, p. 3000, 2013.

[80] J. D. Wolchok, H. Kluger, M. K. Callahan et al., "Nivolumab plus Ipilimumab in advanced melanoma," *The New England Journal of Medicine*, vol. 369, no. 2, pp. 122–133, 2013.

[81] M. Sznol, H. M. Kluger, M. K. Callahan et al., "Survival, response duration, and activity by BRAF mutation (MT) status of nivolumab (NIVO, anti-PD-1, BMS-936558, ONO-4538) and ipilimumab (IPI) concurrent therapy in advanced melanoma (MEL)," in *Proceedings of the ASCO Annual Meeting*, ASCO Meeting Abstracts 32:LBA9003, 2014.

[82] C. Meyer, L. Cagnon, C. M. Costa-Nunes et al., "Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab," *Cancer Immunology, Immunotherapy*, vol. 63, no. 3, pp. 247–257, 2014.

[83] S. Kitano, M. A. Postow, C. G. Ziegler et al., "Computational algorithm-driven evaluation of monocytic myeloid-derived suppressor cell frequency for prediction of clinical outcomes," *Cancer Immunology Research*, vol. 2, no. 8, pp. 812–821, 2014.

[84] A. Snyder, V. Makarov, T. Merghoub et al., "Genetic basis for clinical response to CTLA-4 blockade in melanoma," *The New England Journal of Medicine*, vol. 371, pp. 2189–2199, 2014.

[85] O. Hamid, H. Schmidt, A. Nissan et al., "A prospective phase II trial exploring the association between tumor microenvironment biomarkers and clinical activity of ipilimumab in advanced melanoma," *Journal of Translational Medicine*, vol. 9, no. 1, article 204, 2011.

[86] R.-R. Ji, S. D. Chasalow, L. Wang et al., "An immune-active tumor microenvironment favors clinical response to ipilimumab," *Cancer Immunology, Immunotherapy*, vol. 61, no. 7, pp. 1019–1031, 2012.

[87] S. Wilgenhof, S. D. Four, F. Vandenbroucke et al., "Single-center experience with ipilimumab in an expanded access program for patients with pretreated advanced melanoma," *Journal of Immunotherapy*, vol. 36, no. 3, pp. 215–222, 2013.

[88] J. Delyon, C. Mateus, D. Lefeuvre et al., "Experience in daily practice with ipilimumab for thetreatment of patients with metastatic melanoma: anearly increase in lymphocyte and eosinophil countsis associated with improved survival," *Annals of Oncology*, vol. 24, no. 6, pp. 1697–1703, 2013.

[89] S. Kelderman, B. Heemskerk, H. van Tinteren et al., "Lactate dehydrogenase as a selection criterion for ipilimumab treatment in metastatic melanoma," *Cancer Immunology, Immunotherapy*, vol. 63, no. 5, pp. 449–458, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 238431, 9 pages
http://dx.doi.org/10.1155/2015/238431

Review Article

Functional Alteration of Natural Killer Cells and Cytotoxic T Lymphocytes upon Asbestos Exposure and in Malignant Mesothelioma Patients

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Received 30 June 2014; Accepted 29 September 2014

Academic Editor: Mohammad Owais

Malignant mesothelioma is caused by exposure to asbestos, which is known to have carcinogenic effects. However, the development of mesothelioma takes a long period and results from a low or intermediate dose of exposure. These findings have motivated us to investigate the immunological effects of asbestos exposure and analyze immune functions of patients with mesothelioma and pleural plaque, a sign of exposure to asbestos. Here, we review our knowledge concerning natural killer (NK) cells and cytotoxic T lymphocytes (CTL). NK cells showed impaired cytotoxicity with altered expression of activating receptors upon exposure to asbestos, while induction of granzyme$^+$ cells in CD8$^+$ lymphocytes was suppressed by asbestos exposure. It is interesting that a decrease in NKp46, a representative activating receptor, is common between NK cells in PBMC culture with asbestos and those of mesothelioma patients. Moreover, it was observed that CD8$^+$ lymphocytes may be stimulated by some kind of "nonself" cells in plaque-positive individuals and in mesothelioma patients, whereas CTL in mesothelioma is impaired by poststimulation maintenance of cytotoxicity. These findings suggest that analysis of immunological parameters might contribute to the evaluation of health conditions of asbestos-exposed individuals and to a greater understanding of the pathology of malignant mesothelioma.

1. Introduction

Inhalation of naturally occurring particles and fibers causes not only pulmonary fibrosis following an inflammatory response, but also tumor and autoimmune diseases. To date, we have focused on and examined the effect of asbestos exposure on the functions of various kinds of immune competent cells. These studies confirmed that functional decreases in T helper (Th) cells, natural killer (NK) cells, and cytotoxic T lymphocytes (CTLs) were caused by exposure to asbestos, decreases that were also partly observed in patients with malignant mesothelioma, following examination of cell lines and primary cells cultured with asbestos and analyzing cells prepared from the peripheral blood of patients (Table 1) [1–8]. Recently, we have concentrated on the analysis of CTL function in individuals exposed to asbestos and in patients with malignant mesothelioma and found interesting similarities and differences between the groups [9]. These studies give us the opportunity to think in an integrated manner regarding alteration of tumor immunity, and the role played by NK cells and CTLs upon asbestos exposure and in mesothelioma patients. Therefore, here we first show

Table 1: The major part of our previous studies about immunological effects of asbestos exposure and analysis for immune functions of patients.

Analyses for	Asbestos in cultures or name of diseases	Results	References
(i) Natural killer (NK) cells			
Human NK cell line, YT-A1	Culture with chrysotile	Decreases in natural cytotoxicity, cell surface NKG2D, and 2B4 and phosphorylation of ERK	[5, 6]
Peripheral blood $CD56^+$ NK cells	Malignant mesothelioma	Low cytotoxicity, low expression of cell surface NKp46	[5]
Human NK cells in PBMC	Culture with chrysotile	Decrease in cell surface NKp46	[5]
(ii) T helper cells			
Human T cell line, MT-2	Culture with chrysotile	Resistance against asbestos-induced apoptosis, increases in secretion of IL-10 and expression of bcl-2 mRNA, decreases in secretion of IFN-γ, TNF-α, IL-6, and CXCL10, and surface expression and mRNA of CXCR3	[1, 2]
	Culture with crocidolite	Resistance against asbestos-induced apoptosis, increases in secretion of IL-10 and ratio of bcl-2/bax mRNAs, and decreases in secretion of IFN-γ and TNF-α	[4]
Peripheral blood $CD4^+$ T cells	Malignant mesothelioma	Very low expression of cell surface CXCR3, low IFN-γ mRNA, and high bcl-2 mRNA	[1, 3]
	Pleural plaque	Low expression of cell surface CXCR3	[3]
Isolated human $CD4^+$ T cells	Culture with chrysotile	Decreases in cell surface CXCR3 and intracellular IFN-γ	[3]
(iii) Cytotoxic T lymphocytes (CTL)			
Human $CD8^+$ T cells in mixed lymphocyte reaction	Culture with chrysotile	Decreases in allogeneic cytotoxicity and intracellular IFN-γ and granzyme B	[7]
Peripheral blood $CD8^+$ T cells	Malignant mesothelioma	High percentage of $perforin^+$ cells, stimulation-induced decrease in $perforin^+$ cells	[9]
	Pleural plaque	High percentage of $perforin^+$ cells	[9]

findings concerning NK cells and then investigate CTLs as found in a cell culture exposed to asbestos as well as in individuals exposed to asbestos and patients with malignant mesothelioma. Before discussing these subjects, we first describe the background of our studies, various aspects of asbestos, malignant mesothelioma, and the relationship between asbestos exposure and immune function.

2. Asbestos, Malignant Mesothelioma, and Immune Function

Asbestos is a kind of naturally occurring mineral fiber that has valuable physical and chemical characteristics including flexibility, as well as fire and heat resistance, which has resulted in the enormous use of asbestos globally. However, in the latter part of the 20th century many reports established that inhalation of asbestos causes malignant mesothelioma, which marked asbestos as one of the representative carcinogenic materials [10–16]. Malignant mesothelioma begins in mesothelial cells covering the inner surface of pleural, pericardial, and peritoneal cavities, as well as the tunica vaginalis, and pleural mesothelioma is the major condition [17]. Asbestos is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC), and it is thought that almost all cases of malignant mesothelioma are caused by exposure to asbestos. Asbestos causes cellular toxicity and mutagenicity and induces the generation of reactive oxygen species (ROS). In addition, it is known that the amounts of oxidized pyrimidine and alkalized nucleic acid base components correlate with the period of exposure to asbestos and that intratracheal instillation of asbestos induces an increase in the mutation frequency of lung DNA in rats [17–21]. However, the relationship between asbestos and malignant mesothelioma cannot be attributed to a "dose-dependent relationship," which is regarded as a general rule in toxicology. It is thought that malignant mesothelioma is caused by a relatively low or intermediate dose of asbestos exposure, whereas a high dose of exposure causes asbestosis [22]. It is also known that malignant mesothelioma occurs

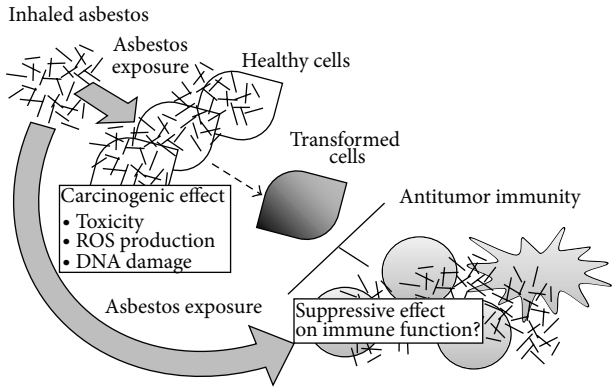


Figure 1: Possible effect of asbestos exposure on antitumor immunity. It is illustrated that there might be a suppressive effect of asbestos exposure on antitumor immunity in the pathology of tumor diseases caused by exposure to asbestos. It is well known that asbestos has a carcinogenic effect, but the development of malignant mesothelioma takes a long time after exposure to asbestos, suggesting the existence of effective antitumor immunity and subsequent impairment caused by asbestos exposure.

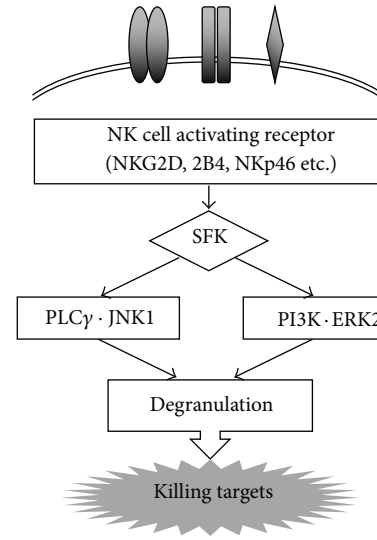


Figure 2: NK cell activating receptors and signal transduction leading to killing targets. NK cells recognize target cells by various kinds of activating and inhibitory receptors. Activation uses common machinery that induces cytotoxicity for targets. The bindings of activating receptors with each ligand transduce through the Src family kinase- (SFK-) dependent phosphoinositide-3 kinase (PI3K) → extracellular signal-regulated kinase 2 (ERK2) pathway and the phospholipase Cγ → c-Jun N-terminal kinase 1 (JNK1) pathway. Finally, degranulation is induced, by which perforin and granzymes in cytotoxic granules are released and work on target cells to induce apoptosis.

even in people exposed environmentally to asbestos. In addition, it takes about 40 years to develop malignant mesothelioma after the initial exposure to asbestos. These findings suggest that mesothelioma may not be caused just by the direct effect of asbestos on mesothelial cells, in which asbestos exposure might exert some kind of alternative effect on the body and allowed us to consider that one possible candidate is its effect on immune function (Figure 1). Inhaled asbestos reaches the pleural cavity through the trachea, bronchus, and pulmonary alveoli, and some asbestos arrive at the regional lymph nodes. Accumulation of asbestos in lymph nodes was observed in people exposed to asbestos nonoccupationally and occupationally [23, 24]. Dodson et al. reported that the total amount of asbestos in the lung was quite low, whereas most cases having asbestos in the lymph nodes showed larger amounts of asbestos in the nodes than in the lung [23, 24]. Thus, immune competent cells may have contact with asbestos not only in nonlymphoid tissue and the area of pulmonary parenchyma and pleural cavity with the inflammatory response, but also in lymphoid tissue of bronchoalveolar, mediastinal, and intercostal nodes, and so on.

3. Activating Receptors on NK Cells

Our study focused on and examined the expression of activating receptors on the surface of NK cells, which play a crucial role in recognition for target cells leading to induction of cytotoxicity. Instead of antigen-specific receptors such as the T cell receptor and immunoglobulin for T and B cells, NK cells express various kinds of activating and suppressive receptors [25, 26]. Suppressive and activating receptors on NK cells recognize MHC class I and the ligands derived from infected viruses and tumor cells to contribute to suppression and activation of cytotoxicity, respectively. Some of the killer cell immunoglobulin-like receptors (KIRs) and heterodimers CD94 and NKG2A or NKG2B are suppressive receptors. On the other hand, the homodimer of NKG2D, 2B4, which is a member of the signaling lymphocyte activation molecule (SLAM) family, and NKp46, a member of the natural cytotoxicity receptor (NCR) family, are receptors that play a role in the induction of cytotoxicity. It has been found that activating receptors employ the same pathway of intracellular signal transduction [27]. After binding of ligands, degranulation of cytotoxic granules including perforin and granzymes is induced through the Src family kinase- (SFK-) dependent phosphoinositide-3 kinase (PI3K) → extracellular signal-regulated kinase 2 (ERK2) pathway and the phospholipase Cγ → c-Jun N-terminal kinase 1 (JNK1) pathway. Perforin and granzymes released by degranulation cause target cells to undergo apoptosis (Figure 2).

4. Impaired Cytotoxicity and Altered Expression of Activating Receptors in an NK Cell Line Exposed to Asbestos

We began examining the effect of asbestos exposure on cell lines. The human NK cell line of YT-A1 was cultured with continuous exposure to chrysotile B (CB) asbestos at 5 μg/mL, named the YT-CB5 subline and was then examined periodically concerning cytotoxicity for K567 cells and expression of cell surface receptors, with results being

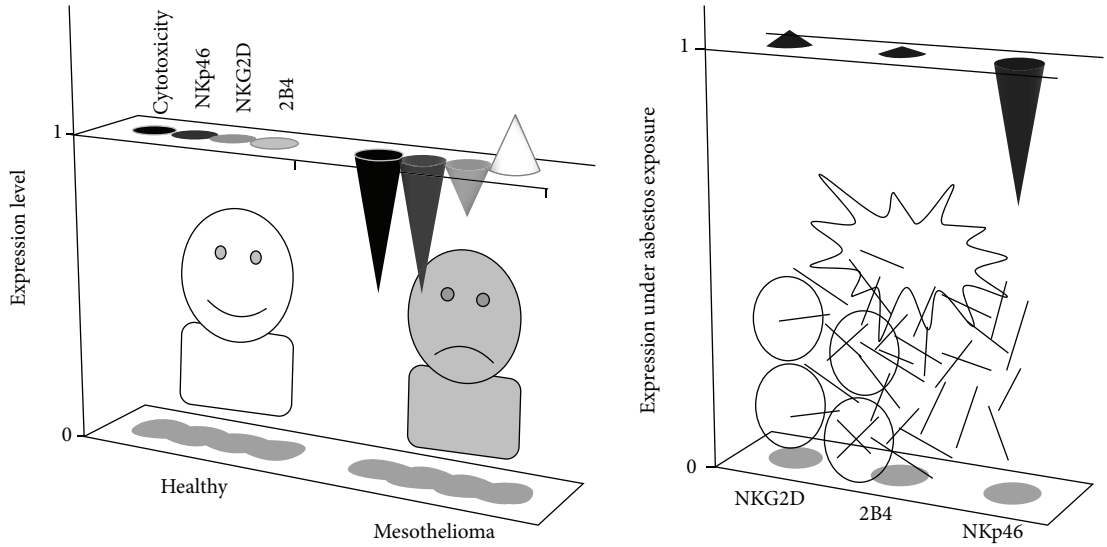


Figure 3: The characteristic decrease of NKp46 on NK cells shared by *in vitro* asbestos exposure and malignant mesothelioma patients. Peripheral blood NK cells in 7 patients with malignant mesothelioma showed decreased cell surface NKp46, but not NKG2D or 2B4, among activating receptors, compared with 10 healthy individuals, and it is interesting that this was also shown by NK cells in PBMCs cultured with asbestos. The relative alterations of expression level are shown.

compared to those of the control subline of YT-Org. Initially, the effect of asbestos exposure on viability of the cell line was checked and 5 μg/mL of CB was chosen and utilized as a concentration having no effect on cell growth and apoptosis. Although YT-CB5 showed a normal level of cytotoxicity comparable to YT-Org until 1 month after the start of culture, it showed impaired cytotoxicity after around 5 months [5]. In accord with the impaired cytotoxicity, YT-CB5 showed decreases in cell surface expression of NKG2D and 2B4, whereas NKG2A and CD94 showed no changes in expression. Although it is known that cytotoxicity against K562 cells is independent of 2B4, the decrease in 2B4-dependent cytotoxicity in YT-CB5 was confirmed by a reverse antibody-dependent cell-mediated cytotoxicity (ADCC) assay. Moreover, YT-CB5 showed the decrease in phosphorylation of ERK1/2 following incubation with K562 cells, and the SFK inhibitor of pp2 or the PI3K inhibitor of wortmannin caused the decrease in phosphor-ERK1/2 of YT-Org. In addition, YT-CB5 also showed a low level of phosphor-ERK1/2 under stimulation with antibody to NKG2D [6]. Thus, it was found that asbestos exposure causes impairment of cytotoxicity with altered expression of activating receptors in NK cells.

5. Decrease in NKp46 on NK Cells in Culture upon Asbestos Exposure and in Patients with Malignant Mesothelioma

After the study of the cell line exposed to asbestos, we examined the function of peripheral blood NK cells in patients with malignant mesothelioma. Peripheral blood mononuclear cells (PBMCs) prepared from peripheral blood were assayed for cytotoxicity against K562 cells and the expression level of activating receptors on the cell surface of NK cells, and results were compared between healthy and mesothelioma individuals. To evaluate the lytic activity of a given cell number, the cytotoxicity per 5000 NK cells was calculated from the percentage of NK cells in PBMCs. Mesothelioma patients showed lower cytotoxicity of NK cells than healthy individuals, and also exhibited alteration in expression of activating receptors in their NK cells, which differed from YT-CB5. The NK cells of mesothelioma patients exhibited a characteristic decrease in expression of NKp46, whereas NKG2D and 2B4 showed normal expression (Figure 3) [5]. PBMCs were then cultured in media supplemented with IL-2 and exposed to CB at 5 μg/mL for 7 days and assayed for the expression of activating receptors on the cell surface of NK cells. As shown by NK cells of mesothelioma patients, NK cells showed a decrease in NKp46 in the culture upon CB exposure, whereas NKG2D and 2B4 did not differ from the control culture (Figure 3). In addition, glass wool, which represents a man-made mineral fiber and a substitute for asbestos, did not cause such an alteration in expression of activating receptors, unlike CB asbestos. It was therefore interesting to discover that peripheral blood NK cells in mesothelioma patients showed a characteristic decrease in cell surface NKp46 with low cytotoxicity, similar to that of NK cells in the culture with asbestos, suggesting the possibility that impairment of NK cell function might be caused by inhaled asbestos and may be related to the pathology of malignant mesothelioma.

6. Relationship between Cytotoxicity, Expression of Activating Receptors, and Signal Transduction in NK Cells

To examine the relationship between low levels of cytotoxicity and activating receptors, peripheral blood NK cells were isolated from the PBMCs of healthy individuals and assayed

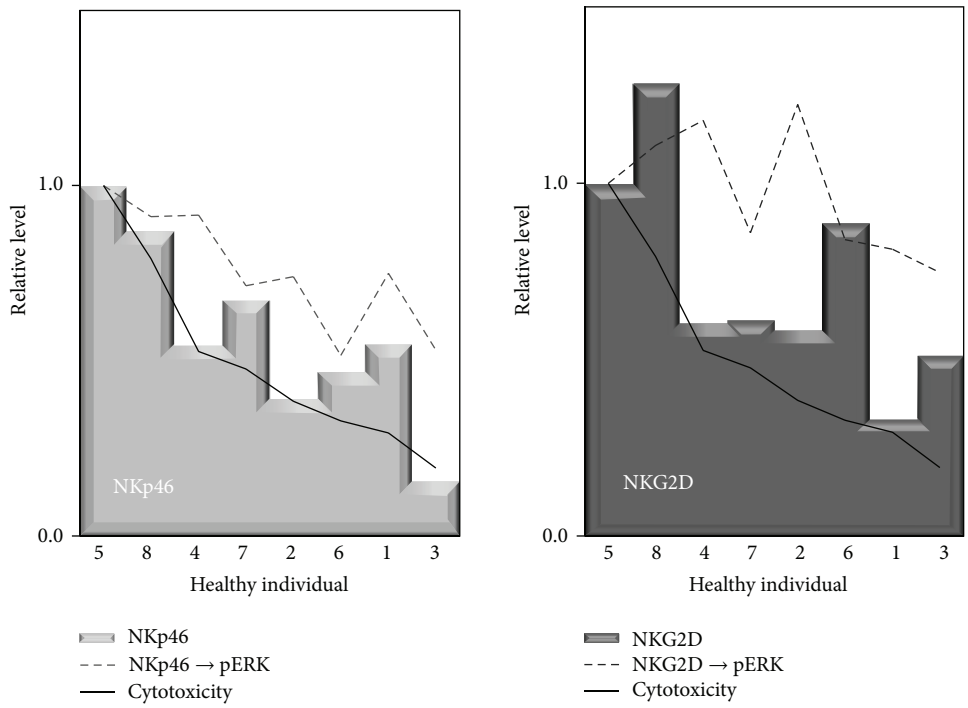


Figure 4: Relationship among cytotoxicity, expression of NKp46 or NK2D, and ERK phosphorylation. When the NK cells of healthy individuals were analyzed, an individual with high cytotoxicity showed high expression of NKp46 and high phosphorylation of ERK induced through NKp46, and there was also a similar relationship among cytotoxicity, NKG2D expression, and ERK phosphorylation. These findings suggest that low expression of NKp46 may be attributed to decreased cytotoxicity of NK cells in patients with malignant mesothelioma. The levels in each individual relative to an individual with the highest cytotoxicity are shown.

regarding cytotoxicity for K562 cells, cell surface expression of activating receptors, and phosphorylation of ERK1/2 after stimulation with antibodies to receptors, and results were compared between individuals. When individuals were put in descending order of cytotoxicity, an individual with high cytotoxicity showed high expression of NKp46 and NKG2D, whereas another with low cytotoxicity showed the opposite trend (Figure 4). In accord with this finding, an individual with high cytotoxicity showed a high level of phosphor-ERK1/2 following stimulation with antibodies to NKp46 or NKG2D, whereas another with low cytotoxicity showed a low level of phosphorylation of ERK1/2 [6]. In contrast, the expression level of 2B4 and the phosphorylation level of EKR1/2 following stimulation with 2B4 did not show such a relationship with cytotoxicity. These findings indicate that expression levels of NKG2D and NKp46 are related to the degree of cytotoxicity induced by stimulation with those receptors through signal transduction downstream of the receptors, suggesting that decreased cytotoxicity of NK cells in mesothelioma patients might be attributed to low expression of NKp46.

7. Cytotoxic T Lymphocytes and Inhalation Exposure to Asbestos

In antitumor immunity, $CD8^+$ T lymphocytes play a more crucial role in cytotoxicity against target cells in an antigen-specific manner, together with the natural cytotoxicity of NK cells [28]. CTLs also utilize the same tools to injure targets such as NK cells, in which perforin and granzymes are released from CTLs into an intercellular space and induce apoptosis of target cells [29]. However, $CD8^+$ T lymphocytes have to be selected clonally and proliferate and differentiate into functional CTLs in order to exert matured cytotoxicity for targets [30]. The differentiation of functional CTLs is a very complex event, in which various kinds of immune cells contribute to CTL differentiation. At first, dendritic cells transfer antigen to lymphoid organs as regional lymph nodes, where they or node-resident dendritic cells are ready to present antigen for T cells as antigen presenting cells (APC) [31]. Naïve $CD8^+$ T lymphocytes then migrate into the nodes and communicate with APC, and those having specificity for the antigen presented are chosen [32]. Moreover, $CD4^+$ T lymphocytes capable of recognizing the same antigen also have to migrate into the nodes and communicate with APC to lead to differentiation of functional CTLs [33]. Thus, the lymph node is a place where immune cells necessary for CTL differentiation meet and communicate with each other, while inhaled asbestos also migrates into regional nodes and accumulates there as mentioned above. These findings indicated that inhaled asbestos might affect differentiation of functional CTLs and motivated us to examine this possibility.

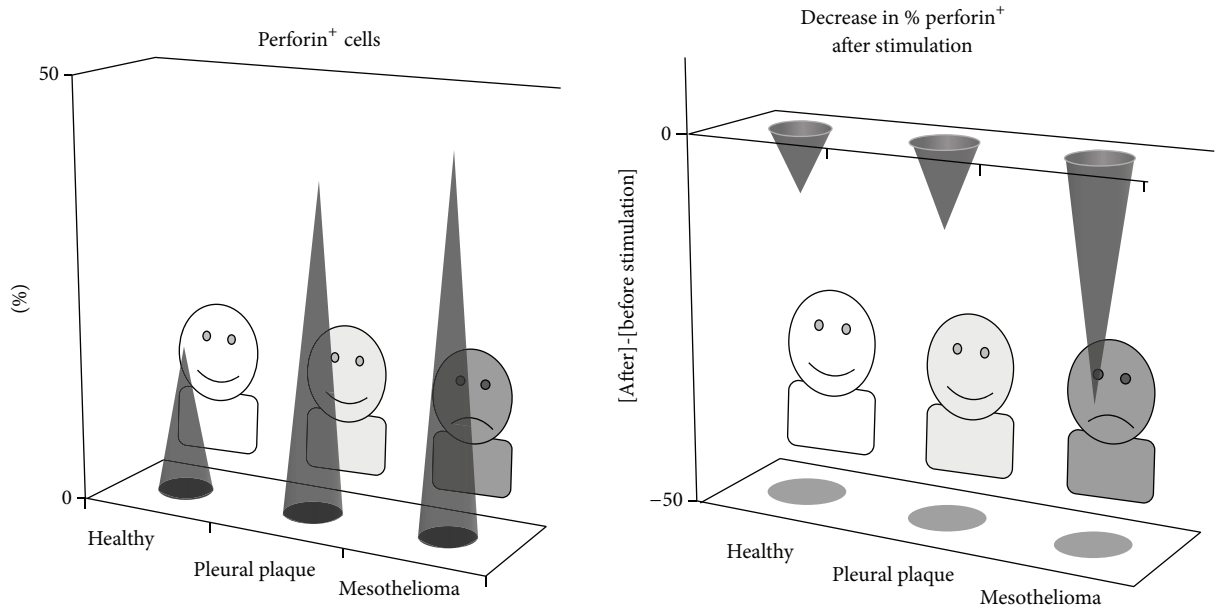


Figure 5: CTL function in patients with malignant mesothelioma and individuals positive for pleural plaque. Both 16 plaque-positive individuals and 14 mesothelioma patients showed a high percentage of perforin$^+$ cells in CD8$^+$ lymphocytes, compared with 16 healthy volunteers, whereas a decrease after stimulation was observed in mesothelioma. These findings indicate that CD8$^+$ lymphocytes are stimulated by some kind of "nonself" cells in both plaque-positive individuals and mesothelioma patients, and that poststimulation maintenance of cytotoxicity is impaired in mesothelioma.

8. Effect of Asbestos on Induction of Cytotoxic T Lymphocytes by Mixed Lymphocyte Reaction

We thus attempted to investigate the effect of asbestos on acquired immunity in the antitumor response using the mixed lymphocyte reaction (MLR), an experimental method to induce cell-mediated acquired immunity using an allogeneic set of PBMCs or lymphoid cells, and an easy tool to mimic *in vitro* induction of CTL function from naïve CD8$^+$ T cells. PBMCs were cultured with a stimulator of allogeneic PBMCs upon exposure to CB asbestos, and examined for the characteristics and allogeneic cytotoxicity of CD8$^+$ T cells [7]. CB exposure suppressed the increase in cell number of CD8$^+$ lymphocytes, which when sorted from the culture showed a decrease in cytotoxicity against allogeneic targets compared with those from the control culture. CD8$^+$ lymphocytes from the CB-exposed culture also showed low percentages of intracellular granzyme B and IFN-γ and cell surface CD25 and CD45RO, and a high percentage of CD45RA. In addition, suppressed cell proliferation of CD8$^+$ lymphocytes upon exposure to CB was also confirmed by the CFSE labeling method. In contrast, those lymphocytes did not differ in apoptosis from those of the control group. Moreover, the productions of TNF-α and IFN-γ in the supernatant from the CB-exposed culture were low, whereas IL-2 did not differ in production. These findings therefore indicate that the induction of CTL function in MLR was suppressed by asbestos, and it was found that asbestos exposure has the potential to exert a suppressive effect on CTL induction following antigen stimulation.

9. Functional Properties of CD8$^+$ Lymphocytes in Patients with Pleural Plaque and Malignant Mesothelioma

Malignant mesothelioma is attributed to asbestos exposure, which can be determined by examination for pleural plaque using image analyses involving X-ray and CT-scan methods. Pleural plaque is an objective sign of previous asbestos inhalation, and is known to be whitish, sharply circumscribed, fibrous, hyaline, sometimes calcified, forms patches involving parietal pleura, and is regarded as harmless [34]. Our recent analysis of peripheral blood CD8$^+$ lymphocytes in individuals positive for pleural plaque and patients with malignant mesothelioma revealed the similarities and differences between these groups. Individuals in the pleural plaque and malignant mesothelioma groups showed higher percentages of perforin$^+$ cells and CD45RA$^-$ cells in fresh CD8$^+$ lymphocytes than healthy individuals. However, patients in the mesothelioma group showed a decrease in perforin$^+$ cells following stimulation with PMA and ionomycin, whereas most of the healthy and plaque-positive individuals retained those cells after stimulation (Figure 5) [9]. The decrease in cells positive for intracellular perforin following stimulation might have been attributed to enhanced degranulation of cytotoxic granules, indicating increased cytotoxicity in mesothelioma, since degranulation is a process that releases perforin and granzymes, which act as factors to injure target cells. However, we confirmed that the CD8$^+$ lymphocytes did not show an increase of cell surface CD107a, a representative marker of degranulation, following stimulation. Thus, it was clarified that patients with malignant mesothelioma have

characteristics of impairment in stimulation-induced cytotoxicity of peripheral blood $CD8^+$ lymphocytes. Additionally, it is also important that they showed a similar alteration of function, namely, an increase in perforin$^+$ cells, compared to $CD8^+$ lymphocytes in plaque-positive individuals, which suggests that such a characteristic might be related with inhalation exposure to asbestos.

10. Significance of Our Study Results

As described above, our studies demonstrated that asbestos exposure has the potential to cause suppressed function of NK cells and CTLs. Malignant mesothelioma is caused by exposure to asbestos, but its development is limited by the parts that have been exposed to asbestos, suggesting the existence of effective antitumor immunity against transformed cells at an initial phase in the body of individuals exposed to asbestos. In addition, it is well known that asbestos-exposed individuals take a very long time to develop malignant mesothelioma after exposure, suggesting that antitumor immunity fought transformed cells until the individual began to suffer from malignant mesothelioma. These findings highlight the importance of the monitoring and intervention of immune function in asbestos-exposed people.

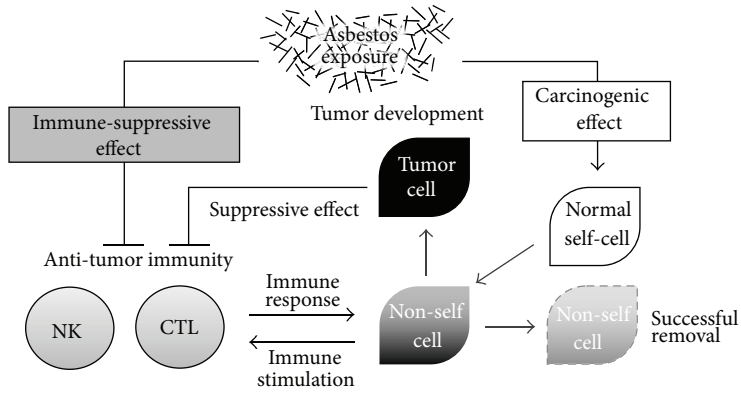
In fact, our study identified one appealing candidate for antitumor immunity in relation to asbestos exposure and malignant mesothelioma, namely, NKp46. NK cells in PBMCs showed decreased cell surface expression of NKp46 following exposure to asbestos, which was also shown by patients with malignant mesothelioma. Although our study using the cell line showed alteration in expression of activating receptors in a different manner, in which NKG2D and 2B4 decreased, these findings indicated that the decrease in activating receptors is attributed to low cytotoxicity through a decrease in signal transduction downstream of those receptors, and allowed us to understand that expression of activating receptors should be examined for primary cell cultures and specimens of malignant mesothelioma. It is interesting that the expression of activating receptors on both NK cells of asbestos-exposed PBMCs and patients with malignant mesothelioma is altered in a characteristic manner and is similar between these groups, in which there is a decrease of NKp46 but not NKG2D or 2B4, which suggests a relationship between the decrease in NKp46, asbestos exposure, and malignant mesothelioma. NK cells play a primary role in cytotoxicity against nonself targets in innate immunity before lymphocytes specific for those targets are clonally selected, proliferate, and acquire fully matured cytotoxicity. NKp46 might therefore be a useful tool for the evaluation of health conditions in asbestos-exposed individuals.

In addition, asbestos exposure suppressed development of CTL function during MLR, in which $CD8^+$ lymphocytes showed decreases in cytotoxicity and the percentage of intracellular granzyme B. These observations allowed us to speculate that $CD8^+$ lymphocytes in patients with malignant mesothelioma might show a decrease in granzymes or perforin similar to that shown by the MLR culture. However, there were no decreases in perforin or granzyme, but rather an increase in perforin in fresh $CD8^+$ lymphocytes from individuals with malignant mesothelioma, as well as those with pleural plaque, when compared with healthy individuals. These findings appear to be paradoxical, but careful discussion leads to clarification. As mentioned above, CTL function differs in induction and maturation from NK cell function. The former is induced by antigen stimulation, whereas the latter is ready to injure targets without stimulation. It is important to note in the results obtained from the analysis of blood specimens that $CD8^+$ lymphocytes showed an increase of perforin in plaque-positive individuals, which had been exposed to asbestos but did not develop any tumors. This means that they have some kind of "non-self" cells prior to tumors, probably caused by exposure to asbestos, which stimulate immune responses in the body. That explanation helped us to realize that both fresh $CD8^+$ lymphocytes of pleural plaque and malignant mesothelioma show such a similar character. On the other hand, healthy individuals have no stimulation with "non-self" cells, including no exposure to asbestos. Therefore, it is difficult to compare healthy and plaque-positive individuals as control and asbestos-exposed cultures in MLR, respectively, which could not have been anticipated before the study was performed. However, it is noteworthy that $CD8^+$ lymphocytes in plaque-positive individuals show an increase in perforin, suggesting that they are specifically fighting against "nonself."

Furthermore, it is interesting that the $CD8^+$ lymphocytes in patients with malignant mesothelioma showed characteristic impairment, in which the percentage of perforin$^+$ cells decreased after stimulation, even though it was as high as that of plaque-positive individuals before stimulation. These findings suggest that such impairment in CTL function might be related to the pathology or development of malignant mesothelioma. We can consider the two scenarios for impaired CTL function in malignant mesothelioma. The first may be caused just by the immune-suppressive effect of tumor cells after the onset of malignant mesothelioma. The second may be caused by the immune-suppressive effect of asbestos exposure before malignant mesothelioma, as suggested by results obtained from our experiment using the MLR culture. Although the study concerning $CD8^+$ cells did not find any impairment of function in individuals with pleural plaque, we have reported that $CD4^+$ T lymphocytes in individuals with pleural plaque showed a decrease in cell surface expression of CXCR3, a chemokine receptor dominantly expressed on Th1 cells [3], which supports the second aforementioned scenario. However, further studies are needed to conclude this matter. In either case, it is clear that CTL function is impaired in patients with malignant mesothelioma, which may be related to the pathology of this disease. Our study results and discussion are summarized in Figure 6.

11. Conclusion

Our overall findings highlight the following points. (1) Exposure to asbestos has the potential to suppress the function of NK cells and CTLs. (2) It is possible that analysis of immunological parameters, such as NKp46 expression, might



	Asbestos-exposed NK cell line or PBMC culture	Malignant mesothelioma	Pleural plaque
NK	• Cytotoxicity: ↓ • NKG2D and 2B4: ↓ (cell line) • NKp46: ↓ (PBMC)	• Cytotoxicity: ↓ • NKp46: ↓	
CTL	• Cytotoxicity induction: ↓ • Perf and GranB: ↓ • IFN-γ production: ↓	• Perf in fresh: ↑ • Perf after stimulation: ↓	• Perf in fresh: ↑ • Perf after stimulation: no change

Figure 6: Summary of our study results and discussions. It is illustrated that asbestos exposure may exert not only a carcinogenic effect, but also an immune-suppressive effect, and that there might be an interaction between antitumor immunity and "nonself" cells that results either in the successful removal of these cells or the development of tumor. Our study results are summarized below. Abbreviations: Perf, perforin; GranB, granzyme B.

contribute to the evaluation of health conditions in asbestos-exposed individuals. (3) $CD8^+$ lymphocytes in individuals with pleural plaque may be stimulated by some kind of "non-self" cells. (4) CTL function is impaired in patients with malignant mesothelioma in comparison to plaque-positive individuals. Following these studies, we have continued to examine the effect of asbestos exposure on immune function and analyze specimens from asbestos-exposed individuals and mesothelioma patients. We hope that our studies will contribute to a greater understanding of asbestos exposure-related health disorders, including malignant mesothelioma, in order to improve the cure rate of those diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Ms. Tamayo Hatayama and Shoko Yamamoto for their technical help. These studies were supported by Special Coordination Funds for Promoting Science and Technology (H18-1-3-3-1), JSPS KAKENHI Grants (19790431, 18390186, 20890270, 19659153, 19790411, 20390178, 21659161, and 23790679), The Takeda Science Foundation (Tokutei Kenkyu Josei I, 2008), Kawasaki Medical School Project Grants (19-407M, 19-603T, 19-205Y, 19-506, 21-107, 22-A29, 20-411I, 21-401, 23B-66, and 23P-3), The Kawasaki Foundation For Medical Science and Medical Welfare (KYOIKU KENKYU JOSEI, 2007, 2009), The Ryobi Teien Memory Foundation (KENKYU JOSEI, 2009), Okayama-Ken (Tokubetsu Dengen Syozai Ken Kagaku Gijyutsu Sinkou Jigyou KenkyuItaku, 2010–2012), Research Project Grant for Young Investigator (2010) in the Japanese Society of Hygiene, and Strategic Research Foundation Grant-aided Project for Private Universities from Ministry of Education, Culture, Sport, Science, and Technology, Japan.

References

[1] Y. Miura, Y. Nishimura, H. Katsuyama et al., "Involvement of IL-10 and Bcl-2 in resistance against an asbestos-induced apoptosis of T cells," *Apoptosis*, vol. 11, no. 10, pp. 1825–1835, 2006.

[2] M. Maeda, Y. Nishimura, H. Hayashi et al., "Reduction of CXC chemokine receptor 3 in an in vitro model of continuous exposure to asbestos in a human T-cell line, MT-2," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 45, no. 3, pp. 470–479, 2011.

[3] M. Maeda, Y. Nishimura, H. Hayashi et al., "Decreased CXCR3 expression in $CD4^+$ T cells exposed to asbestos or derived from asbestos-exposed patients," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 45, no. 4, pp. 795–803, 2011.

[4] M. Maeda, S. Yamamoto, Y. Chen et al., "Resistance to asbestos-induced apoptosis with continuous exposure to crocidolite on a human T cell," *Science of the Total Environment*, vol. 429, pp. 174–182, 2012.

[5] Y. Nishimura, Y. Miura, M. Maeda et al., "Impairment in cytotoxicity and expression of NK cell-activating receptors on human NK cells following exposure to asbestos fibers," *International Journal of Immunopathology and Pharmacology*, vol. 22, no. 3, pp. 579–590, 2009.

[6] Y. Nishimura, M. Maeda, N. Kumagai, H. Hayashi, Y. Miura, and T. Otsuki, "Decrease in phosphorylation of ERK following

decreased expression of NK cell-activating receptors in human NK cell line exposed to asbestos," *International Journal of Immunopathology and Pharmacology*, vol. 22, no. 4, pp. 879–888, 2009.

[7] N. Kumagai-Takei, Y. Nishimura, M. Maeda et al., "Effect of asbestos exposure on differentiation of cytotoxic t lymphocytes in mixed lymphocyte reaction of human peripheral blood mononuclear cells," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 49, no. 1, pp. 28–36, 2013.

[8] Y. Nishimura, M. Maeda, N. Kumagai-Takei et al., "Effect of asbestos on anti-tumor immunity and immunological alteration in patients with mesothelioma," in *Malignant Mesothelioma*, C. Belli, Ed., pp. 31–48, InTech d.o.o., Rijeka, Croatia, 2012.

[9] N. Kumagai-Takei, Y. Nishimura, M. Maeda et al., "Functional properties of $CD8^+$ lymphocytes in patients with pleural plaque and malignant mesothelioma," *Journal of Immunological Research*, vol. 2014, Article ID 670140, 10 pages, 2014.

[10] D. H. K. Lee and I. J. Selikoff, "Historical background to the asbestos problem," *Environmental Research*, vol. 18, no. 2, pp. 300–314, 1979.

[11] J. C. Wagner, C. A. Sleggs, and P. Marchand, "Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province," *British Journal of Industrial Medicine*, vol. 17, pp. 260–271, 1960.

[12] E. E. Keal, "Asbestosis and abdominal neoplasms," *The Lancet*, vol. 276, no. 7162, pp. 1211–1216, 1960.

[13] T. B. Mallory, B. Castleman, and E. E. Parris, "Case records of the Massachusetts general hospital, case 33441," *The New England Journal of Medicine*, vol. 237, pp. 673–676, 1947.

[14] S. R. Gloyne, "The morbid anatomy and histology of asbestosis," *Tubercle*, vol. 14, no. 10, pp. 445–451, 1933.

[15] P. Klempere and C. B. Robin, "Primary neoplasm of the pleura: a report of five cases," *Archives of Pathology*, vol. 11, pp. 385–412, 1931.

[16] H. E. Robertson, "'Endothelioma' of the Pleura," *The Journal of Cancer Research*, vol. 8, no. 3, pp. 317–375, 1924.

[17] T. A. Sporn and V. L. Roggli, "Mesothelioma," in *Pathology of Asbestos-Associated Diseases*, V. L. Roggli, T. D. Oury, and T. A. Sporn, Eds., pp. 104–168, Springer, New York, NY, USA, 2004.

[18] B. T. Mossman and A. Churg, "Mechanisms in the pathogenesis of asbestosis and silicosis," *The American Journal of Respiratory and Critical Care Medicine*, vol. 157, no. 5, pp. 1666–1680, 1998.

[19] B. T. Mossman, D. W. Kamp, and S. A. Weitzman, "Mechanisms of carcinogenesis and clinical features of asbestos-associated cancers," *Cancer Investigation*, vol. 14, no. 5, pp. 466–480, 1996.

[20] M. Dušinská, A. Collins, A. Kažimírová et al., "Genotoxic effects of asbestos in humans," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 553, no. 1-2, pp. 91–102, 2004.

[21] J. Topinka, P. Loli, P. Georgiadis et al., "Mutagenesis by asbestos in the lung of λ-lacI transgenic rats," *Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 553, no. 1-2, pp. 67–78, 2004.

[22] H. Bohlig and H. Otto, *Asbest und Mesotheliom: Fakten, Fragen, Umweltprobleme*, G. Thieme, Stuttgart, Germany, 1975.

[23] R. F. Dodson, J. Huang, and J. R. Bruce, "Asbestos content in the lymph nodes of nonoccupationally exposed individuals," *American Journal of Industrial Medicine*, vol. 37, no. 2, pp. 169–174, 2000.

[24] R. F. Dodson, M. G. Williams Jr., C. J. Corn, A. Brollo, and C. Bianchi, "A comparison of asbestos burden in lung parenchyma, lymph nodes, and plaques," *Annals of the New York Academy of Sciences*, vol. 643, pp. 53–60, 1991.

[25] L. Moretta and A. Moretta, "Unravelling natural killer cell function: Triggering and inhibitory human NK receptors," *EMBO Journal*, vol. 23, no. 2, pp. 255–259, 2004.

[26] W. M. Yokoyama and B. F. M. Plougastel, "Immune functions encoded by the natural killer gene complex," *Nature Reviews Immunology*, vol. 3, no. 4, pp. 304–316, 2003.

[27] X. Chen, P. P. Trivedi, B. Ge, K. Krzewski, and J. L. Strominger, "Many NK cell receptors activate ERK2 and JNK1 to trigger microtubule organizing center and granule polarization and cytotoxicity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 15, pp. 6329–6334, 2007.

[28] J. Banchereau and A. K. Palucka, "Dendritic cells as therapeutic vaccines against cancer," *Nature Reviews Immunology*, vol. 5, no. 4, pp. 296–306, 2005.

[29] J. T. Harty, A. R. Tvinnereim, and D. W. White, "Cd8+ T cell effector mechanisms in resistance to infection," *Annual Review of Immunology*, vol. 18, pp. 275–308, 2000.

[30] M. Catalfamo and P. A. Henkart, "Perforin and the granule exocytosis cytotoxicity pathway," *Current Opinion in Immunology*, vol. 15, no. 5, pp. 522–527, 2003.

[31] R. S. Allan, J. Waithman, S. Bedoui et al., "Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming," *Immunity*, vol. 25, no. 1, pp. 153–162, 2006.

[32] W. Weninger, N. Manjunath, and U. H. von Andrian, "Migration and differentiation of $CD8^+$ T cells," *Immunological Reviews*, vol. 186, pp. 221–233, 2002.

[33] G. Behrens, M. Li, C. M. Smith et al., "Helper T cells, dendritic cells and CTL Immunity," *Immunology and Cell Biology*, vol. 82, no. 1, pp. 84–90, 2004.

[34] G. Hillerdal and D. W. Henderson, "Asbestos, asbestosis, pleural plaques and lung cancer," *Scandinavian Journal of Work, Environment and Health*, vol. 23, no. 2, pp. 93–103, 1997.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 794968, 9 pages
http://dx.doi.org/10.1155/2015/794968

Review Article

Beyond the Immune Suppression: The Immunotherapy in Prostate Cancer

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Received 28 November 2014; Revised 6 February 2015; Accepted 6 February 2015

Academic Editor: Anshu Agrawal

Prostate cancer (PCa) is the second most common cancer in men. As well in many other human cancers, inflammation and immune suppression have an important role in their development. We briefly describe the host components that interact with the tumor to generate an immune suppressive environment involved in PCa promotion and progression. Different tools provide to overcome the mechanisms of immunosuppression including vaccines and immune checkpoint blockades. With regard to this, we report results of most recent clinical trials investigating immunotherapy in metastatic PCa (Sipuleucel-T, ipilimumab, tasquinimod, Prostvac-VF, and GVAX) and provide possible future perspectives combining the immunotherapy to the traditional therapies.

1. Introduction

Prostate cancer (PCa) is the second most common cancer in men. Although an estimated 15% of the cancers occurred in men in most developed countries, incidence rates are also relatively high in certain less developed regions. It represents the fifth leading cause of death from cancer in men [1]. Therefore screening and management of early prostate cancer are critical medical challenges. Even though the precise aetiology is not completely defined, both hereditary and environmental factors are important in the development of PCa. Human and animal studies suggest that the inflammation and the elusion of immune destruction can have an important role in PCa as well in the development of many other human cancers [2]. Immune evasion is now recognized as a hallmark feature of cancer [3].

Generally the inflammatory process restores the homeostasis but especially the chronic inflammation can produce a microenvironment that supports cancer initiation and progression [4, 5]. In addition to this extrinsic pathway, genetic alterations leading to cancer can also stimulate the inflammatory process, thus contributing to the establishment of a microenvironment favorable to tumor progression (Figure 1) [4]. Recent studies have demonstrated that the interaction between immune system inflammation and cancer is very complex and still far from fully understood, ranging from positive local effects, such as cytotoxicity mediated by T-cells to tumor progression until the destructive systemic effects such as cachexia [6].

In cancer patients immunity system is often altered with an excess of inhibitory functions induced by regulatory T cells (Treg) or myeloid-derived suppressor cells (MDSC) and by secretion of the immunosuppressive cytokines, tumor growth factor (TGF)-β, and interleukin (IL)-10. The manipulation of the immune system is also one of new promising therapies for cancer treatment, as detected in many different tumors (colon, breast, melanoma, and prostate) but until now only rarely established durable effects have been demonstrated [7]. Several ongoing trials have the purpose to identify new therapies that interfere with synergic activity of immunosuppressive environment and restore immune competence.

The aim of this review is to describe some of the agents that can activate different pathways involved in PCa promotion and progression, with particular interest to those

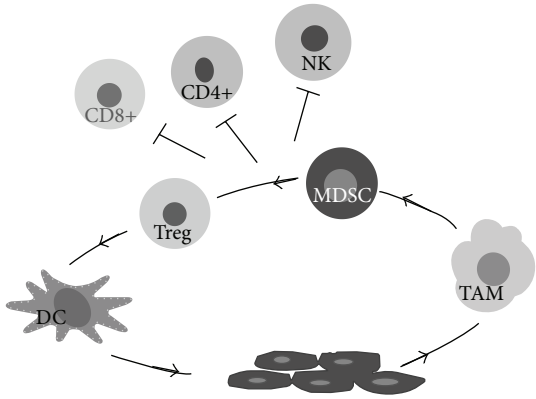

Figure 1: MDSCs produce high amount of IL-10 and drive polarized macrophage M2 (TAM) and active Tregs. MDSCs, TAMs, and Treg produce a cytokine subsets that interfere with DCs differentiation and enhance the suppressive phenotype of each cell type and inhibit $CD4^+$, $CD8^+$, and NK, and promove tumor progression. Tumor cells produce: PGE, COX, IL-6, VEGF, and other factors that recruit MDSCs, TAMS, and Tregs, induce defective DCs, and induce an immune suppressive microenvironment.

leukocytes that inhibit immunity response to cancer. We also specify some of the potential strategies aimed to alter cancer associated inflammation-immunity that are focused on the components of the tumor microenvironment.

2. Immunosystem in the Prostate Gland

An immune response in the prostate has been reported, and it is primarily cell-mediated [8]. The greatest concentration is in the stroma with a small but significant number of intraepithelial cells. The lymphocytes are chiefly T cells ($CD45RO^+$) in both stromal and intraepithelial compartments. Stromal T cells are mainly $CD4^+$ helper/inducer cells, whereas intraepithelial cells are $CD8^+$ cytotoxic/suppressor. The abundance of $CD8^+$ suggests that cytotoxic T cells are the first line of defense against luminal foreign agents. $CD4^+$ T cells can have different fates and are classified according to their cytokine profile: T helper (Th)-1 and T helper (Th)-2. Th1 express T-bet and produce interferon (IFN)-γ; Th2 express Gata-3 and produce IL-4 [9]. Tregs are $CD4^+$ lineage with essential immunosuppressive functions that often express transcription factor like Forkhead box P3 ($FoxP3^+$). Other T cells selectively produce IL-17 and the transcription factor RORγt (Th17), and finally newer T cells are identified and they are defined based on their cytokine production: Th9 and Th22 cells.

As helper cells can change their phenotype, it has become important to determine which T cells are more present in inflammatory lesions of prostate from BPH until carcinoma. In fact, an immune response is stimulated in PCa, as shown also by histological data revealing the presence of $CD4^+$ T cells, $CD8^+$ T cells, natural killer (NK) cells, dendritic cells (DC), and macrophages within tumors. Furthermore, it has been reported that a dense infiltration of lymphocytes is correlated with longer patient survival and that high grade prostatic adenocarcinomas have significantly less infiltration of T cells and macrophages as compared to benign nodular prostatic hyperplasia [10–13], suggesting that tumor progression may be associated with alterations in cell-mediated immune responses. On the contrary, [14] an increased inflammatory cell infiltrate within the tumor is associated with an increased risk of tumor recurrence. In some reports the presence of $CD4^+$ T cells infiltrate is related with poor cancer survival in patients with PCa, probably due to an increase of Tregs. So far it seems the presence of tumor associated macrophage (TAM) and Tregs correlate with a poorer prognosis [4, 12]. A crosstalk between these cells could promote synergy and amplify the immune suppressive effects of individual cell population [15].

2.1. Regulatory T Cells. $CD4^+CD25^+$ Tregs represent the major Treg population in the immune system [16] and are essential to maintain peripheral self-tolerance and avoid autoimmunity. They are also responsible of limiting tissue damage during ongoing and resolving immune responses [17, 18]. Expression of $Foxp3^+$ generally identifies natural, thymus derived Treg cells (nTregs) and may or may not be expressed in inducible Tregs (iTregs) [19–21]. $Foxp3^+$Tregs were detected in the peripheral blood and tumor tissue in many cancer patients suggesting their contribution to the reduction of the antitumor immune response [22]. The recruitment of Tregs (natural or induced) into tumors likely involves complex, multistep processes not yet completely defined [22, 23].

Tregs generally contribute to decreasing immunity during tumor development and progression, leading to poor outcomes in cancer patients [24, 25]. Likewise a relative enrichment of Tregs has been detected in prostate tissue and from peripheral blood of PCa patients compared to normal donors [26]. A significant association has been shown between the number of Tregs and poor prognosis in PCa [27, 28]. Moreover Tregs level decreases after androgen ablation and is elevated in the peripheral blood of patients with metastatic castration-resistant prostate cancer (mCRPC) [26, 28–30].

The mechanisms of suppression mediated by Tregs include cytotoxic T-lymphocytes-associated protein (CTLA)-4, programmed death-ligand (PD-L)-1, lymphocyte-activation gene (LAG)-3, neuropilin (Nrp)-1, and CD39/73 expression [31].

2.2. Myeloid-Derived Suppressor Cells. MDSCs are elevated at the tumor site, as well as in the peripheral blood of cancer patients and a correlation between tumor-MDSCs and patients survival has been described [32]. MDSCs are a heterogeneous cell population characterized by the ability to suppress T cells and NK cells functions. They consist of myeloid progenitor cells and immature myeloid cells (IMC). IMCs with a phenotype as MDSCs are continually generated in the bone marrow of healthy individuals and differentiate into mature myeloid cells without causing detectable immunosuppression [33]. Some pathological conditions, such as acute or chronic infections, trauma or sepsis, and cancer, prevent the differentiation and MDSCs exhibiting immunosuppressive functions derive [34, 35]. Until now two main MDSC populations have

been characterized primarily in mice: polymorphonuclear and monocytic MDSC. These cells share some characteristics but have also many different markers that complicate their studies and lead to controversial results. Nevertheless, there is a growing consensus to define human MDSCs as $CD11b^{+}CD33^{+}HLA\text{-}DR^{low/-}Lin^{-}$. Within this population, the $CD14^{+}CD15^{low/-}$ MDSCs share characteristics with murine M-MDSCs, while $CD14^{-}CD15^{+}$MDSCs resemble murine G-MDSCs [36]. Several different factors, including cyclooxygenase (COX)-2, prostaglandins (PGE), stem-cell factor (SCF), Macrophage-Colony Stimulating Factor (M-CSF), IL-6, granulocyte/macrophage CSF (GM-CSF), and vascular endothelial growth factor (VEGF), induce expansion and activation of MDSCs. These factors are also produced by tumor cells and promote the expansion of MDSCs through the stimulation of myelopoiesis and the inhibition of the differentiation in mature myeloid cells. In addition other signals are necessary to MDSCs activation, including IFN-γ, ligands for Toll-like receptors (TLRs), IL-4, IL-13, and TGF-β, produced mainly by activated T cells and tumor stromal cells after induction by different bacterial and viral products, or as a result of tumor-cell death [33].

The immunosuppressive activities of MDSCs are mediated by a variety of mechanisms. One mechanism consists in the depletion of essential nutrients, especially L-Arg, metabolized by arginase 1 and iNOS highly expressed in MDSCs. The depletion of Arg inhibits T-cell proliferation by decreasing their expression of CD3 ζ-chain and preventing upregulation of the expression of the cell cycle regulator, cyclin D3 [33, 37, 38]. Another mechanism is the generation of oxidative stress caused by the production of reactive oxygen species (ROS) and reactive nitrogen species by MDSCs, able to produce several molecular blocks in T cells, ranging from the loss of TCR ζ-chain expression and interference with IL-2 receptor signaling [33, 39]. MDSCs also disrupt T cell migration to lymph nodes by releasing ADAM 17 which down regulates L-selectin and prevents the homing receptor on T cells [40]. At last, MDSCs promote the recruitment and the expansion of Tregs by the production of IL-10, TGF-α, IFN-γ, and by CD40–CD40L interactions [41, 42]. In addition MDSCs enhance tumor growth by promoting angiogenesis [43], inducing tumor invasion and metastasis, and activating the protective pathways of tumor cells from chemotherapy-induced apoptosis [15, 33, 42].

Monocytic MDSCs have been detected elevated in the peripheral blood of patients with PCa and the level of MDSCs correlated with other negative prognostic factors for metastatic PCa, such as lactate dehydrogenase, alkaline phosphatase, PSA, and anemia [44]. It has also been described that $CD14^{+}HLA\text{-}DR^{low/-}$ monocytes isolated from PCa patients expressed high level of IL-10, inhibited autologous T cell proliferation more effectively than ($CD14^{+}HLA\text{-}DR^{+}$) monocytes from healthy individuals, and were defective in their ability to differentiate into phenotypically mature DCs [45].

2.3. Macrophages. Macrophages play a basic role that promote host survival by regulating adaptive immunity, inducing wound healing and eliminating infectious agents [46]. Their precursor cells, monocytes, after extravasation into target tissues differentiate to mature macrophages and polarize in response to microenvironment. Each polarized macrophage displays a differential expression profile of cytokines, enzymes, and cell-surface markers and they have been classified into two subsets. The classical M1, activated by IFN-γ and lipopolysaccharide (LPS), are characterized by their high expression of IL-12 and low expression of IL-10; the alternative M2, that are activated by IL-4, IL-13, IL-10, and glucocorticoid hormones, produce high levels of IL-10 and low levels of IL-12 [47, 48]. The role of macrophage in tumor development has been controversial. Even though macrophage surveillance mechanisms are essential for preventing the growth of transformed cells, activated macrophages contribute to early development of neoplasm through the free radicals production. Furthermore tumor microenvironment strongly polarizes macrophages towards a M2-like phenotype, the so-called TAMs, which facilitate tumor progression via both immunological and nonimmunological mechanisms. In fact in tumor microenvironment, molecules such as chemokines (CCL-2), cytokines (VEGF and M-CSF), and hypoxia promote monocytes recruitment as well as macrophages survival. TAMs expression correlates with tumor growth [49]. Often the same factors inhibit the differentiation of DCs. In turn, the recruited macrophages provide a transcriptional program, activated through Nuclear factor (NF-kB) and hypoxia-inducible transcription factors (HIF)-1, which support tumor progression and metastasis [4, 50, 51].

For many years a strict correlation between an increased number of macrophages and a poor prognosis has been described for many different tumors. TAMs are also a significant component of the inflammatory infiltrates in PCa. The detection of high density of M2 in both epithelial and stromal compartments was statistically associated to poorer prognosis [52, 53]. Moreover increased TAMs levels in biopsy are predictive of worse recurrence free survival in men treated with primary androgen deprivation therapy. An inverse correlation between total macrophage density and time to recurrence has also been reported from different analysis [54, 55].

2.4. Dendritic Cells. DCs are professional antigen-presenting cells (APC), which are critical to initiate innate and adaptive immune responses against pathogens and tumor cells, and because these cells orchestrate a large repertoire in T cell activation representing also a good tool for DCs-based cancer vaccination strategies [56].

DCs are terminal differentiated myeloid cells that are specialized in antigen processing and presentation. These cells differentiate in the bone marrow from various progenitors. In human, monocytes represent the major precursors of DCs. The differentiation leads to two major subsets of DCs, conventional DCs (cDCs), and plasmacytoid DCs (pDCs). They show different morphologies, markers, and functions. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) induce different pathway of differentiation in DCs [57]. The different pathways of differentiation define the fate of DCs and their

interaction with lymphocytes. In fact activated DCs produce a different setting of costimulatory molecules and cytokines inducing such contrasting states as immunity and tolerance. DCs after capturing and processing antigens present them to T cells through MHC and, by controlling Th1, mediate a resistance to intracellular microbes, by Th2 a defense to helminthes, by Th17 through IL-17 organize phagocytes at body surface to resist extracellular bacilli. Alternatively, DCs induce Tregs and cause tolerance. Maturing DCs also express more IL-15 and activate inflammation and NK [57].

DCs maturation is induced by tumor derived molecules, such as heat shock proteins (HPS) and high mobility-group box- (HMGB-) 1 protein, as well as proinflammatory cytokines produced by various tumor-infiltrating immune cells. Matured DCs have different tumoricidal activities often mediate by IFN production. DCs activate T cells and NK cells, both these cells have cytotoxic activity against tumors. DCs induce apoptosis and antiangiogenesis pathways via signaling through IFN [58]. Alternatively tumor may perturb this process by inducing the accumulation of immature DCs [33, 59]. The contact tumor-DCs or tumor-derived factors may affect DCs maturation and function. It has been demonstrated that tumor induces apoptosis or alters differentiation of DC as well as accumulation of immature cells with inhibitory function could impair immune responses [59, 60]. Defective DCs function has been found in many patients with a variety of cancers [61].

Some authors have detected in prostate carcinoma a significant correlation between low numbers of $CD1a^+$ cells (characterized DCs) and a high Gleason score, by contrast; DCs have been found elevated in low risk cancer [62]. Patients that suffer from metastatic PCa showed fewer circulating myeloid DCs than their age-matched controls [63]. These results indicate that in PCa patients monocytes do not develop into myeloid DCs as efficiently as they do in healthy individuals. This idea is also supported by observations that serum from PCa patients inhibited monocyte differentiation into DCs and that the degree of inhibition correlated with higher PSA levels [64].

3. Immunotherapy in Prostate Cancer: Clinical Data

Cancer immunotherapy has recently been introduced into the therapeutic field of metastatic PCa and mCPRC. The goal of immunotherapy is to harness the capabilities of immune system to effectively recognize and kill transformed cells whilst sparing healthy tissues [65, 66]. Over the past decade, strong evidences that PCa is immunogenic have emerged, which showed the rationale for using immune-based therapies for the treatment of metastatic PCa. This is confirmed by the support of the presence of several tumor associated antigens in the prostate; which include the PSA, prostatic acid phosphatase (PAP), prostate specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), mucin-1 (MUC-1), and the cancer testis antigen NY-ESO-1. As already mentioned, histological data revealed the presence of $CD4^+$, $CD8^+$ T cells, NK cells, DCs, and macrophages within tumors [7, 11]. Early studies reported that high grade PCa have significantly less infiltration of T cells, suggesting that tumor progression could be associated with defects in cell-mediated immune responses. A high prevalence of Tregs within tumors is associated with more lethal PCa, suggesting that therapeutic blockade of these cells may induce beneficial clinical response. Another recent observation consists in a reduced infiltration of $CD68^+$ macrophages that is associated with lymph node positivity and higher clinical stage. Increased NK infiltrate within tumors was also found to be associated with a lower risk of progression providing evidence that these innate immune cells may have a protective role against PCa. Four of the current immunologic therapeutic approaches with particular relevance to mCPRC are discussed in more detail in this section of the review (Table 1).

Sipuleucel-T is an autologous active cellular immunotherapy product that stimulates a T-cell immune response against cancer cells [65]. It is the first documented immunotherapy to prolong survival in mCRPC demonstrated in a phase III trial [67]. Autologous peripheral blood mononuclear cells (PBMCs) of patient are incubated *ex vivo* for 36/48 hours with a fusion protein (PA2024) of PAP and GM-CSF [68]. After about 40 hours, the fusion protein is washed out and the product is reinfused into the patient. This product contains at least 5×10^7 autologous activated $CD54^+$ DCs and a variable number of T cells, B cells, NK cells, and others [74]. Sipuleucel-T immunotherapy targets cells positive for PAP, a secreted glycoprotein enzyme that is expressed in 95% of prostate tissue and PCa [75]. Phase I/II clinical trials have shown that Sipuleucel-T is well-tolerated and the patients developed an appreciable antigen specific T-cell responses and antibodies against the fusion protein after the treatment [69, 70]. Actually three phase III clinical trials have been completed and showed promising findings of this DCs based vaccine. The two first studies compared patients with asymptomatic mCRPC assigned to placebo or Sipuleucel-T. There was no difference in time to progression but there was a significant increase of overall median survival (25.9 months versus 21.4 and 19.0 months versus 15.7) [76, 77]. A third phase III clinical trial known as Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT) trial showed a 4.1 months improvement in median OS and at 36-month interval the survival rate was 31.7% for treated patients compared to 23.0% for cases treated with placebo [67, 78].

Ipilimumab is a fully human IgG1 monoclonal antibody that Bind to and blocks the activity of CTLA-4. CTLA-4 has been shown to be potent negative T cells responses and is upregulated following T-cell stimulation to attenuate the response. CTLA-4 is also constitutively expressed on Tregs and mediates their immune suppressive effects [71]. Ipilimumab was approved by the FDA on 2011 for the treatment of advanced melanoma and is currently being trialled for the treatment of nonsmall cell lung cancer, metastatic renal cell cancer, and ovarian cancer. Regarding PCa, preclinical studies that combine ipilimumab with standard anticancer therapies are giving encouraging results. Synergic antitumor activity between radiotherapy and CTLA-4 blockade has emerged in a phase I, II study. In this study ipilimumab given alone in a dose escalation or in addition to a single fraction of radiation each day before starting the treatment, resulted in

Table 1: Current immunologic therapeutic approaches in PCa.

Therapy	Molecule	Mechanism of action	Clinical trials [Ref.]
Sipuleucel-T (Provenge)	Autologous cellular immune-therapy	Stimulates a T cell immune response against cancer cells (+ for PAP)	Phases I-II: [61] Phase II: [63–65]
Ipilimumab (Yervoy)	IgG1 Human monoclonal antibody	Blocks the activity of CTLA-4 and Treg expression	Phases I-II: [67] Phase III: [68]
Tasquinimod	Oral quinolone-3-carboxamide	Antitumor action through inhibition of angiogenesis and immunomodulation	Phase III: [69, 70]
Prostvac-VF	Vector based vaccing	A combination of two viral particles, vaccinia, and fowlpox that infect the APC cells promoting an immune response against PSA expressing cells	Phase II: [71]
GVAX	Granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transfected tumor cell vaccine	Evocation of a strong immunoreaction by antigens expressed on human prostate cell lines modified by GM-CSF	Phase III: [72, 73]

some very significant PSA declines [72]. There are currently phase III trials that are evaluating the effect of ipilimumab in patients with metastases who received or not docetaxel. A primary analysis showed no significant difference between the ipilimumab group and the placebo group in terms of overall survival. However, ipilimumab seems to be associated with better survival than placebo [73].

Tasquinimod, a quinoline-3-carboxamide analog, is in clinical development for treatment of prostate cancer and other solid tumors. In a placebo-controlled, phase II randomized trial, tasquinimod doubled the median progression-free survival (PFS) period and prolonged survival of patients with metastatic CRPC [79, 80]. A phase III clinical trial to test the effect of tasquinimod in the same patients population is ongoing (NCT01234311). Tasquinimod has been shown to inhibit prostate cancer growth and metastasis in animal models [81, 82]. Results from these studies have suggested that the antiangiogenic property of this molecule may be responsible for its antitumor activity, since tumor growth inhibition was associated with reduced microvasculature density, increased expression and secretion of the angiogenesis inhibitor thrombospondin-1 (TSP-1), and downregulation of VEGF and HIF-1α [83]. More recent data have suggested that tasquinimod may affect HIF by interfering with histone deacetylase 4 (HDAC 4) [84]. However, in an orthotopic, metastatic prostate cancer model, tasquinimod reduced the metastatic rate without affecting microvessel density in the primary tumor. Therefore, mechanisms other than impairing angiogenesis may play an important role in the antitumor and antimetastasis activities of tasquinimod. At this regard, S100A9 has been identified as a potential target of tasquinimod. S100A9 interacts with proinflammatory receptors: TLR4 and receptor of advanced glycation end products (RAGE). These receptors are expressed on MDSC, macrophages, DCs, and endothelial cells. S100A9 regulates the accumulation of MDSCs and inhibits DCs differentiation leading to immune response suppression [85].

Viral vectors are attractive for use in cancer immunotherapies as they can mimic natural infection and lead to the induction of immune response against the tumor antigen that they encode.

Prostvac-VF (viral-based vaccine) is a recombinant viral vaccine currently being trialled as an immunotherapy for PCa. Prostat-VF (TRICOM or PSA TRICOM) is based on a combination of two viral particles, vaccinia which is a potent immunologic priming agent, followed by fowlpox which is minimally or noncross reactive with vaccinia that is used as a boosting agent. Both recombinant viruses are engineered to encode the entire PSA gene with a modified agonist epitope and three costimulatory proteins B7-1 (facilitates T cell activation), lymphocyte function/associated antigen 3 (LFA-3; CD58), and intercellular adhesion molecule-1 (ICAM-1; CD54). The rationale behind this approach is that the virus will directly infect the APCs or somatic cells at the site of injection, leading to cell death and subsequent uptake of cellular debris containing PSA by the APCs [86]. The transduced APCs or antigen-loaded APCs upon interaction with $CD4^+$ and $CD8^+$T cells will effectively promote the T-cell mediated immune responses that destroy PSA expressing cells. Prostvac-VF/TRICOM was evaluated in a randomized phase II clinical trial in men with mCPRC. Comparing men who received Prostvac-VF and GM-CSF and men received empty vector plus placebo, this study showed positive results in median OS with a difference of eight months between treated groups. Medians OS in the control group was 16.6 versus 25.1 months in PROSTAVAC group [87]. There is also currently a global phase III trial that included 1200 men with mCRPC treated with PROSTAVAC or placebo that will determine the overall survival.

GVAX, granulocyte-macrophage colony-stimulating factor tumor cell vaccine, represents the whole-cell based immunotherapy. Whole autologous or allogeneic tumor cells as source of immunogens are genetically modified to express GM-CSF. GM-CSF enhances immune responses through the recruitment and activation of DCs at the injection site, necessary to process and present antigens, a critical step in the induction of an optimal immune response to any immunotherapy [88]. Because the small number of cells that can be obtained from surgically removed tumors limits autologous approach, GVAX for PCa is composed of two human prostate cell lines, LANCaP (androgen sensitive derived from a lymph node metastasis) and PC3 (androgen insensitive

derived from bone metastasis) as antigens source, transfected with GM-CSF, and then irradiated for safety [89, 90]. Phase I/II trials were performed: patients with hormone-refractory prostate cancer (HRPC), chemotherapy-naive, received an intradermal priming vaccination with GVAX-PCa (5×10^8 cells, half quantity of each cell line) followed by 12 weekly boost for 6 months [89] or ranged doses (1×10^8 cells to 5×10^8 cells) [90]. This immunotherapy resulted well tolerated and immunogenic for many of metastatic HRPC patients in terms of dose and time treatment with an encouraging OS rates. These data supported to initiation of two phase III trials to confirm the survival benefits. The first phase III study, Vaccine Immunotherapy with Allogeneic Prostate Cancer Cell Lines (VITAL)-1, was a phase III trial designed to compare GVAX to docetaxel plus prednisone in asymptomatic CRPC [91, 92]. VITAL-2 was conducted in symptomatic CRPC [91, 92]. The VITAL-2 study was terminated early due to increased deaths in the vaccine arm. Not long after, the VITAL-1 study was terminated based on a futility analysis of less than a 30% chance of meeting its end point [91].

The failure of GVAX immunotherapy to demonstrate clinical benefit in PCa has urged some considerations. The critical points are the lack of placebo arm and dose levels and the timing of chemotherapy was not conducted. Moreover the effects of immunotherapy may need a longer time than conventional therapy; other biological markers may be necessary to determine the effect of immunotherapy and finally the development of immunosuppression especially in metastatic disease that may require a different approach [91]. Emerging data suggest that this effect may be at least partially obviated by combining immunotherapy with immune checkpoint antagonist or immune agonist [91]. In this regard, a combined treatment with GVAX and ipilimumab in patients with metastatic CRPC was trialled. The tolerable dose and the safety profile resulted in a phase I study warrant further research [92]. In addition results from a trial including mCRPC patients treated with fixed initial doses of ipilimumab and PSA-Tricom vaccine have shown a raise of the median overall survival [93].

4. Conclusions

Immunotherapies have gained momentum in cancer therapeutics following the recent approvals of drugs for the treatment of prostate cancer and melanoma. Immunotherapy has the potential to mount an ongoing, dynamic immune response that can kill tumor cells for an extended time after the conventional therapy has been administered. Despite these clinical advances, further studies are still necessary to increase the understanding of the responses to these types of therapy and of the optimal management of different subset of patients. The most promising immune-based treatments are monoclonal antibodies that act as checkpoint inhibitors (e.g., ipilimumab and nivolumab), adoptive cell therapy (e.g., T cells expressing chimeric antigen receptors), and vaccines (e.g., Sipuleucel-T).

It is reasonable that a single immune therapeutic agent is unlikely to be clinically effective especially in metastatic patients and combing vaccines with immune check-point inhibitors can reorganize the immunological network to mount an immune response against the prostate cancer antigens.

Many trials are also ongoing to define the effects of immune therapy with established treatments: androgen deprivation therapy (ADT) and chemotherapy (CT) or radiotherapy (RT) [94]. As mentioned above, studies about possible combine immunotherapies are ongoing to better establish the safety and toxicity other than the efficacy of such treatments. In our opinion, identifying doses and timing and the sequences of combined treatments are crucial to gain a synergic effect.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] J. Ferlay, I. Soerjomataram, R. Dikshit et al., "Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012," *International Journal of Cancer*, vol. 136, no. 5, pp. E359–E386, 2015.

[2] A. M. De Marzo, E. A. Platz, S. Sutcliffe et al., "Inflammation in prostate carcinogenesis," *Nature Reviews Cancer*, vol. 7, no. 4, pp. 256–269, 2007.

[3] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *Cell*, vol. 144, no. 5, pp. 646–674, 2011.

[4] A. Mantovani, P. Allavena, A. Sica, and F. Balkwill, "Cancer-related inflammation," *Nature*, vol. 454, no. 7203, pp. 436–444, 2008.

[5] K. E. de Visser, A. Eichten, and L. M. Coussens, "Paradoxical roles of the immune system during cancer development," *Nature Reviews Cancer*, vol. 6, no. 1, pp. 24–37, 2006.

[6] C. I. Diakos, K. A. Charles, D. C. McMillan, and S. J. Clarke, "Cancer-related inflammation and treatment effectiveness," *The Lancet Oncology*, vol. 15, no. 11, pp. e493–e503, 2014.

[7] T. C. Bruno, J. D. French, K. R. Jordan et al., "Influence of human immune cells on cancer: studies at the University of Colorado," *Immunologic Research*, vol. 55, no. 1–3, pp. 22–33, 2013.

[8] D. G. Bostwick, G. De la Roza, P. Dundore, F. A. Corica, and K. A. Iczkowski, "Intraepithelial and stromal lymphocytes in the normal human prostate," *The Prostate*, vol. 55, no. 3, pp. 187–193, 2003.

[9] S. Nakayamada, H. Takahashi, Y. Kanno, and J. J. O'Shea, "Helper T cell diversity and plasticity," *Current Opinion in Immunology*, vol. 24, no. 3, pp. 297–302, 2012.

[10] M.-R. A. Hussein, M. AL-Assiri, and A. O. Musalam, "Phenotypic characterization of the infiltrating immune cells in normal prostate, benign nodular prostatic hyperplasia and prostatic adenocarcinoma," *Experimental and Molecular Pathology*, vol. 86, no. 2, pp. 108–113, 2009.

[11] K. S. Sfanos and A. M. de Marzo, "Prostate cancer and inflammation: the evidence," *Histopathology*, vol. 60, no. 1, pp. 199–215, 2012.

[12] P. A. McArdle, K. Canna, D. C. McMillan, A.-H. McNicol, R. Campbell, and M. A. Underwood, "The relationship between T-lymphocyte subset infiltration and survival in patients with prostate cancer," *British Journal of Cancer*, vol. 91, no. 3, pp. 541–543, 2004.

[13] S. Vesalainen, P. Lipponen, M. Talja, and K. Syrjanen, "Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma," *European Journal of Cancer Part A: General Topics*, vol. 30, no. 12, pp. 1797–1803, 1994.

[14] J. Irani, J. M. Goujon, E. Ragni et al., "High-grade inflammation in prostate cancer as a prognostic factor for biochemical recurrence after radical prostatectomy. Pathologist multi center study group," *Urology*, vol. 54, no. 3, pp. 467–472, 1999.

[15] S. Ostrand-Rosenberg, P. Sinha, D. W. Beury, and V. K. Clements, "Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression," *Seminars in Cancer Biology*, vol. 22, no. 4, pp. 275–281, 2012.

[16] S. Sakaguchi, N. Sakaguchi, M. Asano, M. Itoh, and M. Toda, "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases," *Journal of Immunology*, vol. 155, no. 3, pp. 1151–1164, 1995.

[17] S. Sakaguchi, N. Sakaguchi, J. Shimizu et al., "Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance," *Immunological Reviews*, vol. 182, no. 1, pp. 18–32, 2001.

[18] Z. Fehérvari and S. Sakaguchi, "Development and function of $CD25^+CD4^+$ regulatory T cells," *Current Opinion in Immunology*, vol. 16, no. 2, pp. 203–208, 2004.

[19] S. Z. Josefowicz, L.-F. Lu, and A. Y. Rudensky, "Regulatory T cells: mechanisms of differentiation and function," *Annual Review of Immunology*, vol. 30, pp. 531–564, 2012.

[20] X. Lin, M. Chen, Y. Liu et al., "Advances in distinguishing natural from induced Foxp3+ regulatory T cells," *International Journal of Clinical and Experimental Pathology*, vol. 6, no. 2, pp. 116–123, 2013.

[21] J. D. Fontenot, M. A. Gavin, and A. Y. Rudensky, "Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells," *Nature Immunology*, vol. 4, no. 4, pp. 330–336, 2003.

[22] M. Beyer and J. L. Schultze, "Regulatory T cells in cancer," *Blood*, vol. 108, no. 3, pp. 804–811, 2006.

[23] D. O. Adeegbe and H. Nishikawa, "Natural and induced T regulatory cells in cancer," *Frontiers in Immunology*, vol. 4, article 190, 2013.

[24] H. Nishikawa and S. Sakaguchi, "Regulatory T cells in tumor immunity," *International Journal of Cancer*, vol. 127, no. 4, pp. 759–767, 2010.

[25] C. M. Wilke, K. Wu, E. Zhao, G. Wang, and W. Zou, "Prognostic significance of regulatory T cells in tumor," *International Journal of Cancer*, vol. 127, no. 4, pp. 748–758, 2010.

[26] S. Tang, M. L. Moore, J. M. Grayson, and P. Dubey, "Increased $CD8^+$ T-cell function following castration and immunization is countered by parallel expansion of regulatory T cells," *Cancer Research*, vol. 72, no. 8, pp. 1975–1985, 2012.

[27] A. M. Miller, K. Lundberg, V. Özenci et al., "$CD4^+CD25^{high}$ T cells are enriched in the tumor and peripheral blood of prostate cancer patients," *The Journal of Immunology*, vol. 177, no. 10, pp. 7398–7405, 2006.

[28] A. Flammiger, L. Weisbach, H. Huland et al., "High tissue density of FOXP3+ T cells is associated with clinical outcome in prostate cancer," *European Journal of Cancer*, vol. 49, no. 6, pp. 1273–1279, 2013.

[29] V. Kärjä, S. Aaltomaa, P. Lipponen, T. Isotalo, M. Talja, and R. Mokka, "Tumour-infiltrating lymphocytes: a prognostic factor of psa-free survival in patients with local prostate carcinoma treated by radical prostatectomy," *Anticancer Research*, vol. 25, no. 6, pp. 4435–4438, 2005.

[30] N.-Y. Huen, A. L.-Y. Pang, J. A. Tucker et al., "Up-regulation of proliferative and migratory genes in regulatory T cells from patients with metastatic castration-resistant prostate cancer," *International Journal of Cancer*, vol. 133, no. 2, pp. 373–382, 2013.

[31] A. Facciabene, G. T. Motz, and G. Coukos, "T-Regulatory cells: key players in tumor immune escape and angiogenesis," *Cancer Research*, vol. 72, no. 9, pp. 2162–2171, 2012.

[32] D. I. Gabrilovich and S. Nagaraj, "Myeloid-derived suppressor cells as regulators of the immune system," *Nature Reviews Immunology*, vol. 9, no. 3, pp. 162–174, 2009.

[33] D. I. Gabrilovich, S. Ostrand-Rosenberg, and V. Bronte, "Coordinated regulation of myeloid cells by tumours," *Nature Reviews Immunology*, vol. 12, no. 4, pp. 253–268, 2012.

[34] J.-I. Youn and D. I. Gabrilovich, "The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity," *European Journal of Immunology*, vol. 40, no. 11, pp. 2969–2975, 2010.

[35] K. Movahedi, M. Guilliams, J. van den Bossche et al., "Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell suppressive activity," *Blood*, vol. 111, no. 8, pp. 4233–4244, 2008.

[36] J. Jiang, W. Guo, and X. Liang, "Phenotypes, accumulation, and functions of myeloid-derived suppressor cells and associated treatment strategies in cancer patients," *Human Immunology*, vol. 75, no. 11, pp. 1128–1137, 2014.

[37] P. C. Rodriguez, D. G. Quiceno, J. Zabaleta et al., "Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses," *Cancer Research*, vol. 64, no. 16, pp. 5839–5849, 2004.

[38] P. C. Rodríguez and A. C. Ochoa, "Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives," *Immunological Reviews*, vol. 222, no. 1, pp. 180–191, 2008.

[39] H. Sauer, M. Wartenberg, and J. Hescheler, "Reactive oxygen species as intracellular messengers during cell growth and differentiation," *Cellular Physiology and Biochemistry*, vol. 11, no. 4, pp. 173–186, 2001.

[40] E. M. Hanson, V. K. Clements, P. Sinha, D. Ilkovitch, and S. Ostrand-Rosenberg, "Myeloid-derived suppressor cells down-regulate L-selectin expression on $CD4^+$ and $CD8^+$ T cells," *Journal of Immunology*, vol. 183, no. 2, pp. 937–944, 2009.

[41] P.-Y. Pan, G. Ma, K. J. Weber et al., "Immune stimulatory receptor CD40 is required for T-cell suppression and T regulatory cell activation mediated by myeloid-derived suppressor cells in cancer," *Cancer Research*, vol. 70, no. 1, pp. 99–108, 2010.

[42] N. R. Monu and A. B. Frey, "Myeloid-derived suppressor cells and anti-tumor T cells: a complex relationship," *Immunological Investigations*, vol. 41, no. 6-7, pp. 595–613, 2012.

[43] L. Yang, L. M. DeBusk, K. Fukuda et al., "Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis," *Cancer Cell*, vol. 6, no. 4, pp. 409–421, 2004.

[44] M. Idorn, T. Køllgaard, P. Kongsted, L. Sengeløv, and P. thor Straten, "Correlation between frequencies of blood monocytic myeloid-derived suppressor cells, regulatory T cells and negative prognostic markers in patients with castration-resistant

metastatic prostate cancer," *Cancer Immunology, Immunotherapy*, vol. 63, no. 11, pp. 1177–1187, 2014.

[45] S. Vuk-Pavlović, P. A. Bulur, Y. Lin et al., "Immunosuppressive $CD14^{+}HLA\text{-}DR^{low/-}$ monocytes in prostate cancer," *The Prostate*, vol. 70, no. 4, pp. 443–455, 2010.

[46] D. M. Mosser and J. P. Edwards, "Exploring the full spectrum of macrophage activation," *Nature Reviews Immunology*, vol. 8, no. 12, pp. 958–969, 2008.

[47] A. Mantovani, B. Bottazzi, F. Colotta, S. Sozzani, and L. Ruco, "The origin and function of tumor-associated macrophages," *Immunology Today*, vol. 13, no. 7, pp. 265–270, 1992.

[48] M. Locati, A. Mantovani, and A. Sica, "Macrophage activation and polarization as an adaptive component of innate immunity," *Advances in Immunology*, vol. 120, pp. 163–184, 2013.

[49] J. W. Pollard, "Tumour-educated macrophages promote tumour progression and metastasis," *Nature Reviews Cancer*, vol. 4, no. 1, pp. 71–78, 2004.

[50] A. Sica and V. Bronte, "Altered macrophage differentiation and immune dysfunction in tumor development," *Journal of Clinical Investigation*, vol. 117, no. 5, pp. 1155–1166, 2007.

[51] T. Chanmee, P. Ontong, K. Konno, and N. Itano, "Tumor-associated macrophages as major players in the tumor microenvironment," *Cancers*, vol. 6, no. 3, pp. 1670–1690, 2014.

[52] M. Lanciotti, L. Masieri, M. R. Raspollini et al., "The role of M1 and M2 macrophages in prostate cancer in relation to extracapsular tumor extension and biochemical recurrence after radical prostatectomy," *BioMed Research International*, vol. 2014, Article ID 486798, 6 pages, 2014.

[53] I. F. Lissbrant, P. Stattin, P. Wikstrom, J. E. Damber, L. Egevad, and A. Bergh, "Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival," *International Journal of Oncology*, vol. 17, no. 3, pp. 445–451, 2000.

[54] N. Nonomura, H. Takayama, M. Nakayama et al., "Infiltration of tumour-associated macrophages in prostate biopsy specimens is predictive of disease progression after hormonal therapy for prostate cancer," *BJU International*, vol. 107, no. 12, pp. 1918–1922, 2011.

[55] S. Shimura, G. Yang, S. Ebara, T. M. Wheeler, A. Frolov, and T. C. Thompson, "Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression," *Cancer Research*, vol. 60, no. 20, pp. 5857–5861, 2000.

[56] J. Banchereau, F. Briere, C. Caux et al., "Immunobiology of dendritic cells," *Annual Review of Immunology*, vol. 18, pp. 767–811, 2000.

[57] R. M. Steinman and J. Banchereau, "Taking dendritic cells into medicine," *Nature*, vol. 449, no. 7161, pp. 419–426, 2007.

[58] J. Tel, S. Anguille, C. E. J. Waterborg, E. L. Smits, C. G. Figdor, and I. J. M. de Vries, "Tumoricidal activity of human dendritic cells," *Trends in Immunology*, vol. 35, no. 1, pp. 38–46, 2014.

[59] A. Pinzon-Charry, T. Maxwell, and J. A. López, "Dendritic cell dysfunction in cancer: a mechanism for immunosuppression," *Immunology and Cell Biology*, vol. 83, no. 5, pp. 451–461, 2005.

[60] B. Almand, J. R. Resser, B. Lindman et al., "Clinical significance of defective dendritic cell differentiation in cancer," *Clinical Cancer Research*, vol. 6, no. 5, pp. 1755–1766, 2000.

[61] A. Pinzon-Charry, C. S. K. Ho, R. Laherty et al., "A population of $HLA\text{-}DR^{+}$ immature cells accumulates in the blood dendritic cell compartment of patients with different types of cancer," *Neoplasia*, vol. 7, no. 12, pp. 1112–1122, 2005.

[62] Y. Liu, T. Sæter, L. Vlatkovic et al., "Dendritic and lymphocytic cell infiltration in prostate carcinoma," *Histology and Histopathology*, vol. 28, no. 12, pp. 1621–1628, 2013.

[63] A. Sciarra, M. Lichtner, A. A. Gomez et al., "Characterization of circulating blood dendritic cell subsets DC123+ (lymphoid) and DC11C+ (myeloid) in prostate adenocarcinoma patients," *The Prostate*, vol. 67, no. 1, pp. 1–7, 2007.

[64] M. Aalamian-Matheis, G. S. Chatta, M. R. Shurin, E. Huland, H. Huland, and G. V. Shurin, "Inhibition of dendritic cell generation and function by serum from prostate cancer patients: correlation with serum-free PSA," *Advances in Experimental Medicine and Biology*, vol. 601, pp. 173–182, 2007.

[65] P. Kantoff and C. S. Higano, "Integration of immunotherapy into the management of advanced prostate cancer," *Urologic Oncology: Seminars and Original Investigations*, vol. 30, supplement 5, pp. S41–S47, 2012.

[66] M. D. Vesely, M. H. Kershaw, R. D. Schreiber, and M. J. Smyth, "Natural innate and adaptive immunity to cancer," *Annual Review of Immunology*, vol. 29, pp. 235–271, 2011.

[67] P. W. Kantoff, C. S. Higano, N. D. Shore et al., "Sipuleucel-T immunotherapy for castration-resistant prostate cancer," *The New England Journal of Medicine*, vol. 363, no. 5, pp. 411–422, 2010.

[68] J. D. Wesley, J. Whitmore, J. Trager, and N. Sheikh, "An overview of sipuleucel-T: autologous cellular immunotherapy for prostate cancer," *Human Vaccines & Immunotherapeutics*, vol. 8, no. 4, pp. 520–527, 2012.

[69] E. Small, P. Fratesi, D. Reese et al., "Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells," *Journal of Clinical Oncology*, vol. 18, no. 23, pp. 3894–3903, 2000.

[70] P. A. Burch, J. K. Breen, J. C. Buckner et al., "Priming tissue-specific cellular immunity in a phase I trial of autologous dendritic cells for prostate cancer," *Clinical Cancer Research*, vol. 6, no. 6, pp. 2175–2182, 2000.

[71] K. Wing, Y. Onishi, P. Prieto-Martin et al., "CTLA-4 control over $Foxp3^{+}$ regulatory T cell function," *Science*, vol. 322, no. 5899, pp. 271–275, 2008.

[72] S. F. Slovin, C. S. Higano, O. Hamid et al., "Ipilimumab alone or in combination with radiotherapy in metastatic castration-resistant prostate cancer: results from an open-label, multicenter phase I/II study," *Annals of Oncology*, vol. 24, no. 7, Article ID mdt107, pp. 1813–1821, 2013.

[73] E. D. Kwon, C. G. Drake, H. I. Scher et al., "Ipilimumab versus placebo after radiotherapy in patients with metastatic castration-resistant prostate cancer that had progressed after docetaxel chemotherapy (CA184-043): a multicentre, randomised, double-blind, phase 3 trial," *The Lancet Oncology*, vol. 15, no. 7, pp. 700–712, 2014.

[74] C. J. Paller and E. S. Antonarakis, "Sipuleucel-T for the treatment of metastatic prostate cancer: promise and challenges," *Human Vaccines & Immunotherapeutics*, vol. 8, no. 4, pp. 509–519, 2012.

[75] B. W.-C. Tse, L. Jovanovic, C. C. Nelson, P. de Souza, C. A. Power, and P. J. Russell, "From bench to bedside: immunotherapy for prostate cancer," *BioMed Research International*, vol. 2014, Article ID 981434, 11 pages, 2014.

[76] E. J. Small, P. F. Schellhammer, C. S. Higano et al., "Placebo-controlled phase III trial of immunologic therapy with Sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer," *Journal of Clinical Oncology*, vol. 24, no. 19, pp. 3089–3094, 2006.

[77] C. S. Higano, P. F. Schellhammer, E. J. Small et al., "Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer," *Cancer*, vol. 115, no. 16, pp. 3670–3679, 2009.

[78] P. F. Schellhammer, G. Chodak, J. B. Whitmore, R. Sims, M. W. Frohlich, and P. W. Kantoff, "Lower baseline prostate-specific antigen is associated with a greater overall survival benefit from sipuleucel-T in the immunotherapy for prostate adenocarcinoma treatment (IMPACT) trial," *Urology*, vol. 81, no. 6, pp. 1297–1302, 2013.

[79] R. Pili, M. Häggman, W. M. Stadler et al., "Phase II randomized, double-blind, placebo-controlled study of tasquinimod in men with minimally symptomatic metastatic castrate-resistant prostate cancer," *Journal of Clinical Oncology*, vol. 29, no. 30, pp. 4022–4028, 2011.

[80] A. J. Armstrong, M. Häggman, W. M. Stadler et al., "Long-term survival and biomarker correlates of tasquinimod efficacy in a multicenter randomized study of men with minimally symptomatic metastatic castration-resistant prostate cancer," *Clinical Cancer Research*, vol. 19, no. 24, pp. 6891–6901, 2013.

[81] S. L. Dalrymple, R. E. Becker, and J. T. Isaacs, "The quinoline-3-carboxamide anti-angiogenic agent, tasquinimod, enhances the anti-prostate cancer efficacy of androgen ablation and taxotere without effecting serum PSA directly in human xenografts," *The Prostate*, vol. 67, no. 7, pp. 790–797, 2007.

[82] S. L. Dalrymple, R. E. Becker, H. Zhou, T. L. Deweese, and J. T. Isaacs, "Tasquinimod prevents the angiogenic rebound induced by fractionated radiation resulting in an enhanced therapeutic response of prostate cancer xenografts," *The Prostate*, vol. 72, no. 6, pp. 638–648, 2012.

[83] J. T. Isaacs, R. Pili, D. Z. Qian et al., "Identification of ABR-215050 as lead second generation quinoline-3- carboxamide anti-angiogenic agent for the treatment of prostate cancer," *Prostate*, vol. 66, no. 16, pp. 1768–1778, 2006.

[84] J. T. Isaacs, L. Antony, S. L. Dalrymple et al., "Tasquinimod is an allosteric modulator of HDAC4 survival signaling within the compromised cancer microenvironment," *Cancer Research*, vol. 73, no. 4, pp. 1386–1399, 2013.

[85] L. Shen, A. Sundstedt, M. Ciesielski et al., "Tasquinimod modulates suppressive myeloid cells and enhances cancer immunotherapies in murine models," *Cancer Immunology Research*, vol. 3, no. 2, pp. 136–148, 2015.

[86] C. G. Drake, "Prostate cancer as a model for tumour immunotherapy," *Nature Reviews Immunology*, vol. 10, no. 8, pp. 580–593, 2010.

[87] P. W. Kantoff, T. J. Schuetz, B. A. Blumenstein et al., "Overall survival analysis of a phase II randomized controlled trial of a poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer," *Journal of Clinical Oncology*, vol. 28, no. 7, pp. 1099–1105, 2010.

[88] D. T. Le, D. M. Pardoll, and E. M. Jaffee, "Cellular vaccine approaches," *Cancer Journal*, vol. 16, no. 4, pp. 304–310, 2010.

[89] E. J. Small, N. Sacks, J. Nemunaitis et al., "Granulocyte macrophage colony-stimulating factor-secreting allogeneic cellular immunotherapy for hormone-refractory prostate cancer," *Clinical Cancer Research*, vol. 13, no. 13, pp. 3883–3891, 2007.

[90] C. S. Higano, J. M. Corman, D. C. Smith et al., "Phase 1/2 dose-escalation study of a GM-CSF-secreting, allogeneic, cellular immunotherapy for metastatic hormone-refractory prostate cancer," *Cancer*, vol. 113, no. 5, pp. 975–984, 2008.

[91] C. G. Drake, "Immunotherapy for prostate cancer: walk, don't run," *Journal of Clinical Oncology*, vol. 27, no. 25, pp. 4035–4037, 2009.

[92] A. J. M. van den Eertwegh, J. Versluis, H. P. van den Berg et al., "Combined immunotherapy with granulocyte-macrophage colony-stimulating factor-transduced allogeneic prostate cancer cells and ipilimumab in patients with metastatic castration-resistant prostate cancer: a phase 1 dose-escalation trial," *The Lancet Oncology*, vol. 13, no. 5, pp. 509–517, 2012.

[93] R. A. Madan, M. Mohebtash, P. M. Arlen et al., "Ipilimumab and a poxviral vaccine targeting prostate-specific antigen in metastatic castration-resistant prostate cancer: a phase 1 dose-escalation trial," *The Lancet Oncology*, vol. 13, no. 5, pp. 501–508, 2012.

[94] C. G. Drake, P. Sharma, and W. Gerritsen, "Metastatic castration-resistant prostate cancer: new therapies, novel combination strategies and implications for immunotherapy," *Oncogene*, vol. 33, no. 43, pp. 5053–5064, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 178698, 9 pages
http://dx.doi.org/10.1155/2015/178698

Review Article

NKG2D and DNAM-1 Ligands: Molecular Targets for NK Cell-Mediated Immunotherapeutic Intervention in Multiple Myeloma

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Received 17 November 2014; Accepted 26 March 2015

Academic Editor: Swaleha Zubair

A pivotal strategy to improve NK cell-mediated antitumor activity involves the upregulation of activating ligands on tumor cells. Enhancement of NK cell-mediated recognition of multiple myeloma cells was reported by us and others showing increased surface expression of NKG2D and DNAM-1 ligands on tumor cells following treatment with a number of chemotherapeutic agents, such as genotoxic drugs or inhibitors of proteasome, histone deacetylases, GSK3, and HSP-90. These compounds have the capability to affect tumor survival but also to activate specific transduction pathways associated with the upregulation of different NK cell activating ligands on the tumor cells. Here, we will summarize and discuss the molecular pathways whereby these drugs can regulate the expression of NK cell activating ligands in multiple myeloma cells.

1. Introduction

Natural killer (NK) cells are important effectors in immune responses to tumors and viral infections whose effector function against target cells is generally related to their cytolytic activity. Moreover, by the secretion of different cytokines and chemokines, NK cells can also stimulate inflammatory responses and exert a control on adaptive immune responses [1, 2]. In this context, in the recent years, increased understanding of the mechanisms controlling NK cell activation has led to the development of therapeutic agents that can improve their responsiveness.

Multiple myeloma (MM) is a hematologic cancer characterized by clonal expansion of malignant plasma cells (PCs) that mainly reside in the bone marrow, able to interact with local microenvironment and bone marrow stromal cells (BMSCs) and these interactions are critical for survival and resistance to therapy [3]. Treatment strategies for MM have changed substantially in the past decade, and the use of autologous hematopoietic stem cell transplantation (HSCT) and the introduction of new drugs, such as bortezomib and immunomodulatory drugs (IMiDs), have significantly improved patients' survival [4–7]. Moreover, as an additional therapeutic strategy in young patients who experience early relapse or with very high risk features at diagnosis, allogeneic stem cell transplantation has been also considered, although often associated with significant transplantation-related morbidity or mortality [8]. However, despite advances in therapeutic strategies, MM remains an incurable disease (median survival around 4-5 years in adults) [9] and novel targeted therapies and synergistic combinations with appropriate antimyeloma agents are required.

Increasing evidences have shown that NK cells can elicit potent autologous and allogeneic responses to myeloma cells, strongly supporting their antitumor potential in response to immunomodulatory drugs or following stem cell transplantation [10–12]. Thus, an interesting strategy to treat this hematologic cancer could be to harness and boost NK cell

Table 1: Drug-induced pathways and molecular targets associated with the upregulation of NKG2D and DNAM-1L expression on MM cells.

Drug	Pathway/molecular target	Ligands	References
Genotoxic agents Doxorubicin Melphalan	ROS-dependent DDR	MICA/B, ULPB1-3 PVR, Nectin-2	Soriani et al., 2009 [19]; Soriani et al., 2014 [29]
GSK3 inhibitors LiCl BIO SB21	STAT3 inhibition	MICA	Fionda et al., 2013 [63]
Proteasome inhibitor Bortezomib	DDR	MICA, ULBP1-3, PVR, Nectin-2	Jinushi et al., 2008 [17] Soriani et al., 2009 [19]
Histone deacetylase inhibitor Valproic acid	ERK	MICA/B ULBP-2	Wu et al., 2012 [23]
Hsp90 inhibitors Radicicol, 17-AAG	HSR	MICA/B	Fionda et al., 2009 [22]

DDR: DNA damage response; ROS: reactive oxygen species.
HSR: heat shock response; ERK: extracellular signal-regulated kinase.

antitumor activity; in particular, since impaired recognition of tumor cells represents a critical mechanism of immune evasion, an intriguing approach could be to make myeloma cells more susceptible to receptor-mediated recognition and killing by NK cells. Indeed, anticancer immune responses may contribute to the control of tumor progression after conventional chemotherapy, and different observations have indicated that a number of chemotherapeutic agents, or radiotherapy, can induce immune responses that result in immunogenic cancer cell death and/or immunostimulatory effects [13, 14].

Several studies have shown that the engagement of different activating receptors, such as the NKG2D (natural killer group 2, member D) and DNAX accessory molecule-1 (DNAM-1), plays an important role in the NK cell-mediated recognition and killing of MM cells [15–17]. Indeed, MM cells can express the NKG2D ligands MICA/B [15, 18], different UL16-binding proteins, the DNAM-1 ligands poliovirus receptor (PVR/CD155), and Nectin-2 [19].

A cogent example of the functional connection between chemotherapy and therapeutical immunomodulation is the finding that several genotoxic agents or drugs, such as inhibitors of proteasome, histone deacetylases, or the HSP-90 molecular chaperone, can increase the expression of NKG2D or DNAM-1 ligands, thus facilitating the activation of NKG2D/DNAM-1-expressing lymphocytes (e.g., NK cells, NKT cells, and CTLs) against tumor cells, including MM [17, 19–23].

Combinatorial therapies, in which NK cells represent one important mediator, may become a pivotal instrument for the development of future immunochemotherapeutical strategies.

Here, we provide a description of the molecular pathways activated by different pharmacological treatments used in the therapy of MM, aimed at enhancing NK cell-mediated tumor killing (Table 1).

2. DNA Damage Response Pathways

The DDR is a complex network of signal transduction pathways that has the ability to sense DNA damage leading to the arrest of the cell cycle, either transiently or permanently, through the activation of cell cycle checkpoints, and of specific DNA repair pathways. However, if the DNA damage is irreparable, cells can undergo apoptosis in order to prevent any damaged DNA progressing to deleterious mutations that would be passed down to its progeny [24, 25].

Tumor cells often display a defect in the DDR, associated with mutated or nonfunctional proteins involved in these pathways. In particular, MM malignant plasma cells are characterized by marked genomic abnormalities during tumor progression and have aberrant DNA repair pathways [26]. In this regard, monoclonal gammopathy of undetermined significance (MGUS) or MM patients exhibit a deregulated expression of cyclin D genes leading to a defect in the cell cycle check points [27]. Other genetic alterations can involve p53, ARF, NF-κB, MYC, and KRAS genes, the gene products of which are critical in DNA repair pathways [27]. Several drugs used in MM therapy, such as melphalan, even at low doses, can induce DDR activation [28, 29]; in this context, we have recently contributed to delineating a link between the activation of DDR induced by chemotherapeutics and the transcriptional regulation of NKG2D and DNAM-1 ligands in MM. In particular, we observed the upregulation, at both protein and mRNA levels, of NKG2D and DNAM-1 ligand expression on MM cells (cell lines and primary malignant PCs) upon treatment with sublethal doses of commonly used genotoxic drugs such as melphalan and doxorubicin. In this context, the sublethal doses we used to treat the different MM cell lines corresponded to IC50 values 10 times lower, as previously described [19].

This effect was associated with the establishment of a chemotherapy-induced senescent phenotype characterized

by permanent cell cycle arrest at the G_2/M phase, flattened cell morphology, and positive senescence-associated β-galactosidase staining. Moreover, drug-induced ligand upregulation was dependent on the activity of the DDR protein kinases ATM/ATR and Chk-1/2 and on the E2F-1 transcription factor [19, 29]. Indeed, DDR activation leads to an ATM-dependent E2F1 accumulation, and a site for ATM/ATR phosphorylation in the amino terminus of E2F1 important for its stabilization has been identified [30]. Notably, at variance from our results showing that p53 is not involved in drug-induced ligand upregulation on malignant PCs, p53 involvement in ULBP1/2 upregulation on different human cancer cell lines was observed [31, 32], suggesting that p53 activity can exert opposite effects depending on the overall context of its activation.

We have also defined an important role for changes in the cellular redox state induced by sublethal doses of chemotherapy (melphalan, doxorubicin), in the control of DDR-dependent upregulation of ligand surface expression and gene transcriptional activity. Our observations, in accordance with much evidence indicating that DDR and oxidative stress are major determinants of cellular senescence, demonstrate that redox-dependent DDR activation plays a critical role for MM cell entry in premature senescence and is required for the preferential ligand upregulation on senescent cells [19, 29].

DDR is a tightly organized mechanism, governed by regulated protein-protein interactions and controlled also by a number of posttranslational modifications, including ubiquitination and sumoylation [33–36]. In this context, the disassembly, removal, and/or degradation of chromatin-associated DDR proteins represent an essential step in the double strand brakes (DSB) repair and postrepair processes and it is mostly coordinated by the ubiquitin-proteasome system (UPS) [37].

Bortezomib is a boronic acid 26S proteasome inhibitor which was approved by the Food and Drug Administration for the treatment of relapsed/refractory, relapsed, and newly diagnosed MM [38–40]. Interestingly, Jinushi and coworkers have demonstrated that bortezomib-mediated upregulation of MICA in myeloma cells required the activation of DDR, since shRNA silencing of ATM or Chk-2 blocked ligand induction [17]. Moreover, also DNAM-1 ligands expression is increased in response to bortezomib treatment both in primary malignant PCs and in MM cell lines [19].

Altogether, these data demonstrate a major role for the DDR pathways induced by genotoxic drugs or bortezomib, in the upregulation of NKG2D and DNAM-1 ligand on MM cells.

3. Hsp90 Inhibitors and Activation of HSF1

Hsp90 is a molecular chaperone able to directly bind, stabilize, and regulate the function of numerous client proteins, including many mediators of signal transduction and cell cycle progression [41]. Increased synthesis of Hsps is generally associated with stressful conditions which can cause protein denaturation/misfolding, but it is also a peculiarity of cancer cells whose proliferation depends on their capability to react to endogenous and exogenous stresses. In particular, Hsp90 is often overexpressed in different solid tumors and haematologic malignancies, such as MM, and can contribute to tumor cell survival by stabilizing many oncogenes and by interfering with apoptosis [42–44]. In MM, Hsp90 inhibition has been shown to affect multiple client proteins involved in pathways critical to tumor development and progression, angiogenesis, and osteoclastogenesis, such as IGF1 and IL-6 receptors, and PI3K/Akt, STAT3, and MAPK signaling pathways; moreover, upregulation of Hsp90 has been observed in MM cells interacting with BMSCs [45–47]. Accordingly, Hsp90 inhibitors have demonstrated potent antitumor activity in preclinical studies and several clinical trials of MM [46, 48, 49].

We found that treatment of MM cell lines with Hsp90 inhibitors [radicicol or 17-allylaminogeldanamycin (17AAG)] results in a significant upregulation of MICA and MICB expression, rendering these cells more efficient to activate NK cell degranulation [22]. To identify possible mechanisms underlying NKG2DL upregulation, we focused our attention on two different cellular responses induced by Hsp90 inhibitors: the "heat shock response" (HSR) and the "unfolded protein response" (UPR). In this regard, Hsp90 is considered a key factor in the regulation of HSF1, a transcription factor involved in the induction of the HSR. Under nonstress conditions, Hsp90 together with other components of the Hsp90 chaperone machinery interacts with HSF1 and represses its transcriptional activity [50]. Moreover, HSF1 is a known regulator of chaperone genes and its activation induces increased expression of Hsp90, thus providing an autoregulatory mechanism for its own inhibition. However, acute stress-induced HSF1 controls the expression of different target genes. In this regard, this transcription factor has been shown to mediate MICA and MICB promoter activation by heat shock [51]. Exposure of MM cells to Hsp90 inhibitors, able to block the HSF1/Hsp90 autoregulatory loop, induces the release, nuclear translocation, and binding of HSF1 to a heat shock response element (HSRE) on MICA/MICB promoters; moreover, knockdown of HSF1 using small hairpin RNA interference blocks these effects, indicating that HSF1 activation is essential for MICA and MICB upregulation by Radicicol and 17AAG [22]. The UPR consists in the accumulation of misfolded proteins and the induction of the ER stress, leading to the activation of complex signaling and transcriptional pathways [52, 53]. However, UPR activation, as revealed by XBP1 and CHOP presence, is weakly induced or inhibited by Hsp90 inhibitors in a time- and dose-dependent manner in MM cells [48]; moreover, treatment of MM cells with two classical ER stress inducers, such as tunicamycin or thapsigargin, failed to modulate MICA or MICB expression, suggesting that UPR activation, per se, is not sufficient to enhance levels of these ligands and that it is not involved in their regulation by drugs targeting Hsp90 [22].

4. GSK3 Inhibitors and STAT3

The serine/threonine kinase GSK3, for years considered only for its role in glycogen metabolism, now is a known component of diverse cellular signaling pathways involved in

the regulation of protein synthesis, cell motility, proliferation, and survival [54–57]. Moreover, GSK3 has been shown to have a positive role in cancer and its pharmacological inhibition holds promise for therapeutic intervention in several solid and hematologic tumors [58]. Interestingly, this protein kinase has emerged as a critical molecule in the pathogenesis of MM [59–61]. Studies on the expression and function of GSK3 in MM cells have reported an abundant expression of the two GSK3 subunits, α and β, and identified GSK3α as the prevailing active isoform. Indeed, GSK3 inhibitors can induce growth arrest or apoptosis in MM cell lines and can enhance the anti-MM cytotoxic effect of bortezomib, by modulating critical signaling pathways in these cells such as the forkhead transcription factors FHRL1 and FKHR, β-catenin, and extracellular signal-regulated kinase- (ERK-) 1/2 kinases. Moreover, GSK3-mediated phosphorylation can stimulate the activity of different transcription factors sustaining MM cell growth, such as NF-κB and Maf [62]. In this regard, administration of the GSK3 inhibitor [(2′Z,3′E)-6-bromoindirubin-3′-oxime] (BIO) in models of myeloma bone disease has been shown to ameliorate bone destruction associated with MM progression, enhancing the osteogenesis in mesenchymal stem cells and, in parallel, inducing regression of the tumor [61].

We found that different drugs targeting the GSK3 kinase [e.g., lithium chloride (LiCl), SB216763 (SB21), or BIO] can upregulate both MICA protein surface and mRNA expression in MM cells, with little or no effects on MICB and PVR expression [63]; moreover, exposure to GSK3 inhibitors renders myeloma cells more susceptible to NK cell-mediated cytotoxicity. Intriguingly, we also showed that STAT3 repression plays a critical role in the upregulation of MICA expression induced by GSK3 inhibitors [63]. Similarly, a previous study had demonstrated that STAT3 is a negative regulator of MICA transcription in different cancer cell lines [64]; moreover, GSK3 activity has been shown to positively influence the tyrosine^{705} (Tyr^{705}) phosphorylation and DNA-binding activity of STAT3 in response to different cytokines, and inhibition of this kinase could significantly modulate the expression of STAT3-regulated genes [65]. In this context, we showed that treatment of MM cells with the STAT3 inhibitor STA-21 or with the JAK2-specific inhibitor AG490 can increase MICA expression, thus confirming the repressive action of STAT3 on this gene also in this type of cancer cells. Indeed, our data showed that treatment of MM cells with drugs targeting GSK3 led to a marked reduction of the constitutive STAT3 phosphorylation in Tyr^{705} and its binding to the promoter fragment encompassing a repressive MICA/STAT3 response element. Moreover, overexpression of a constitutively active mutant form of STAT3 significantly inhibited MICA upregulation by GSK3 inhibitors, indicating that one of the mechanisms involved in GSK3-mediated regulation of *mica* gene expression could be related to the transcriptional activity of its promoter, where basal repression mediated by active STAT3 can be released by GSK3 inhibition. The mechanisms underlying MICA repression by STAT3 remain to be explored. STAT3 has been shown to inhibit certain tumor suppressor genes via epigenetic modifications, such as CpG island methylation [66, 67]. In this regard, NKG2D ligand expression by histone deacetylase (HDAC) or DNA methylation inhibitors was described in different cancer cells, suggesting that chromatin modifications can control the basal expression of these ligands on tumor cells [21, 68–71]. These findings suggest that epigenetic modifications likely can contribute to STAT3-dependent repression of *mica* promoter activity; however, additional experiments are needed to better analyze this hypothesis.

5. Histone Deacetylase Inhibitors (HDACi)

Histone deacetylase inhibitors (HDACi) are a novel class of anticancer agents undergoing evaluation in clinical trials for the potential treatment of patients with different cancers, including hematopoietic malignancies and MM. Indeed, HDACi are able to induce increased acetylation of DNA-associated histone proteins, leading to cell cycle arrest, differentiation, and/or apoptosis in a wide range of malignant cells [21, 72, 73]. A large body of evidence shows that treatment of different type of tumor cells with HDACi leads to the upregulation of NKG2D ligand surface expression resulting in a significant increase of NK cell-mediated lysis of tumor cells [21, 68–70]. In line with these results, a recent study performed on human MM cells demonstrates that valproic acid (VPA), a molecule originally described as an antiepileptic and then demonstrated to inhibit HDACs inducing antineoplastic activity), is able to enhance the expression of the NKG2D ligands MICA/B and ULBP-2 with a mechanism dependent on the activation of constitutively phosphorylated ERK [23]. Interestingly, treatment of MM cells with VPA increased the expression of pERK-1 and reduced pERK-2 levels; in this regard, although the reason for the preferential phosphorylation of ERK-1 in VPA-treated myeloma cells was not investigated, the underlying mechanism might be explained by loss of competition between ERKs for their binding/activation by mitogen-activated protein kinases [23].

6. Possible Cross Talk between Drug-Activated Pathways Inducing NKG2D and DNAM-1 Ligand Expression in MM Cells

Integration of different pathways regulating the expression of NKG2D and DNAM-1 ligand in MM cells could be beneficial to enhance NK cell recognition of tumor target cells. Moreover, treatment of MM implies the simultaneous administration of different pharmacologic agents, so it would be helpful to understand how and if multiple signaling events triggered by different drugs could affect surface levels of NK cell activating ligands; however, very few data are available about the potential effects of combined therapies on the expression of these molecules on MM cells.

Several studies have described synergistic antimyeloma effect of different pharmacologic agents including GSK3 or Hsp90 inhibitors with bortezomib [60, 74, 75] or with melphalan and doxorubicin [76, 77].

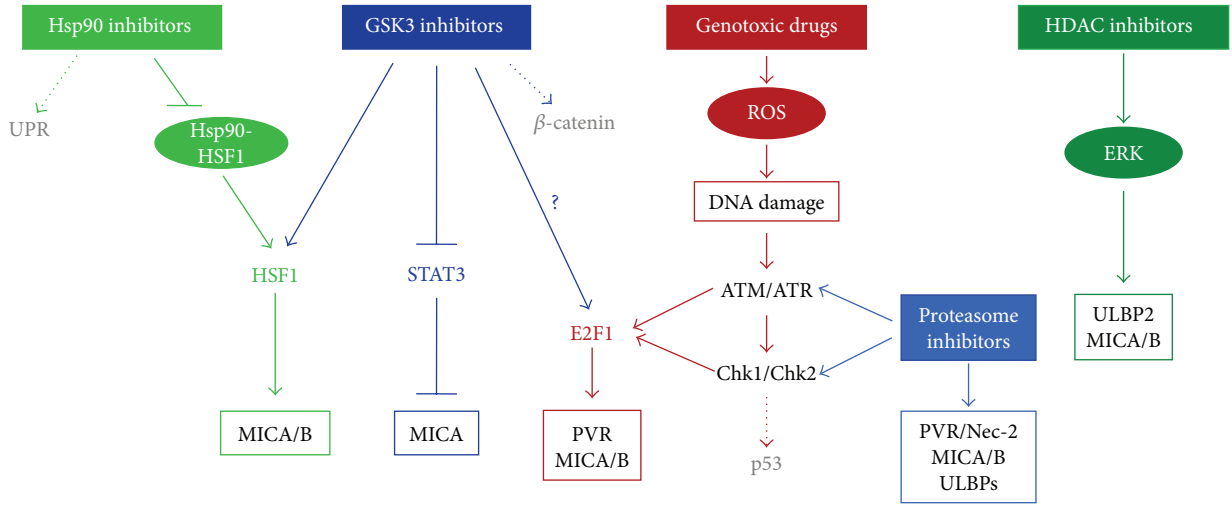

Figure 1: Drug-activated pathways regulating NK cell activating ligand expression. Hsp90 inhibitors regulate MICA/B expression via HSF1 activation. Drugs targeting GSK-3 repress STAT3 leading to MICA upregulation. Genotoxic drugs induce the expression of NKG2D or DNAM-1 ligands following the activation of DDR-dependent E2F1 transcription factor. Proteasome inhibitors induce MICA expression via ATM and Chk-2 activation. HDAC inhibitors increase MICA/B and ULBP2 levels with a mechanism dependent on ERK activation. Molecules or pathways not involved in the regulation of these ligands are indicated in grey color and with dotted raw.

In this regard, we have recently investigated the possibility of cross talk between pathways induced by chemotherapeutic agents. The cooperation between GSK3 inhibition and genotoxic agents in the induction of MICA expression has been investigated in MM cells. We observed that GSK3 inhibition can cooperate with drug-activated DDR to increase MICA expression, since treatment with melphalan increased the expression of MICA and this upregulation was further enhanced in the presence of LiCl. This cooperation may be due to different and independent cellular events triggered by two drugs; alternatively, treatment with GSK3 inhibitors may facilitate the action of melphalan and/or vice versa. Interestingly, STAT3 constitutive activation was shown to prevent the induction of MICA following genotoxic stress [64]. These observations suggest that constitutive activation of this transcription factor in MM cells may interfere with pathways triggered by DDR, increasing the threshold for optimal activation; it could be speculated that GSK3 inhibition may favor MICA upregulation after melphalan treatment by reducing the repressive activity of STAT3. In addition, GSK3 has been shown to regulate E2F1 activity by means of direct and indirect mechanisms. In particular, independently by its kinase activity, GSK3 has been found to physically interact with the transactivation domain of E2F1 and to inhibit its transcriptional activity [78, 79]. These observations can suggest E2F1 activation as a possible point of convergence between DDR and GSK/STAT3, resulting in further increase of activating ligand expression.

Despite the lack of data about the combined use of other drugs and NK cell ligand expression in MM cells, the fact that a growing number of studies described synergic antimyeloma effects of these pharmacologic agents strongly suggests that this aspect should be better investigated (Figure 1).

7. Chemotherapy and NKG2D Ligand Shedding: A Double Edge Sword?

The release of soluble NKG2D ligands has been suggested to be a major mechanism of tumor cell evasion from NKG2D-mediated immunosurveillance. As a matter of fact, soluble forms of NKG2D ligands are present in the serum of MM patients [17, 80] and other types of malignancies; in this regard, their levels correlate with tumor stage and metastasis and with reduced expression of NKG2D on NK cells and other cytotoxic lymphocytes [81, 82]. Soluble NKG2D ligands can be released through metalloproteinase-mediated cleavage, exosome secretion, or alternative splicing; however, little is known about the effect of different chemotherapeutic drugs on NKG2D ligands shedding. Although Kohga and coworkers have shown that epirubicin can reduce the shedding of MICA in epatocarcinoma cell lines [83], a large body of evidence denotes that conditions causing cellular stress, including chemotherapeutic agents and ROS, can lead to increased metalloproteinase-mediated release of cell surface molecules, including NKG2D ligands [84–87]. Interestingly, Huang and coworkers have shown that combination of valproate, known to upregulate cell surface MICA/B, and metalloproteinase inhibitors was found to significantly stabilize cell surface MICA/B on ovarian carcinoma cells and to enhance *in vivo* the efficacy of immune cell therapy [88]. Moreover, in osteosarcoma cells, valproate treatment can downregulate MMP9 expression and thereby upregulate cell surface MICA/B expression, inhibiting the release of soluble forms of these ligands [89].

Thus, metalloproteinases implicated in NKG2D ligands shedding could be targeted in novel therapeutic schemes to regulate the escape of malignant cells from stress-elicited

immune responses. In this regard, additional studies will be needed to better analyze NKG2D ligands shedding and the pathways involved in its regulation by chemotherapeutic agents in MM.

8. The Other Side of the Coin: How Pharmacologic Treatments Can Impact NK Cells

The setting of therapeutic approaches, based on chemotherapy-induced sensitization of tumor cells to NK cell-mediated cytotoxicity, should always consider the possible drug-induced effects when chemotherapy and the activity of NK cell-mediated actions are needed together. In this regard, standard and high dose chemotherapeutic regimens for malignancies can inhibit the activity of the immune system and also significantly decrease NK cell-mediated killing [90]. However, the effects of their immunomodulatory potential could be changed and improved by using different doses and schedules. In this context, we observed that treatment of NK cells, with sublethal concentrations of doxorubicin, does not affect the ability of NK cells to degranulate in response to MM cells, as well as the expression of NKG2D and DNAM-1 and ability to produce IFN-γ [29]. On the contrary, the activity of GSK3 kinase has been shown to modulate specific functions of NK cells; inhibition of its activity can increase cytokine secretion and cytotoxicity, possibly due to nuclear translocation of functional β-catenin [91]. Similarly, in different *in vivo* models of hematologic cancer, loss of STAT3 in NK cells enhances tumor surveillance by increasing their cytolytic activity [92]. Thus, the observations summarized above about the interplay GSK3/STAT3 and MICA expression in MM cells would be supported also by the additional information that inhibition of this kinase could directly enhance the activity of NK cells against the tumor.

Hsp90 is critical for regulation of phenotype and functional activity of NK cells. How, after Hsp90 inhibition, NK cells display decreased activating receptor expression which correlate with a downregulation of their cytolytic activity against tumor cells has been described [93]. Likewise, NK cell effector functions can be compromised following treatment with HDACi or bortezomib. Indeed, HDACi (e.g., vorinostat, trichostatin A, valproic acid, and apicidin) exert their suppressive effect on both resting and activated NK cells and at doses not affecting NK cell vitality, with reduced levels of the activating receptors NKG2D and NCRs [94, 95]. Moreover, these drugs can also downregulate ligands for NK cells-activating receptors, such as B7-H6 (a ligand for NKp30), and impair tumor cell recognition by NK cells [96].

Cytotoxic effects of bortezomib on immune-competent cells have also been observed. In this regard, bortezomib can trigger apoptosis and disrupt NKp46-dependent cytotoxicity in primary human NK cells [97]. Moreover, bortezomib can inhibit surface expression of TRAIL in activated human NK cells [98].

The development of combined cytoprotective strategies to prevent the adverse effects of bortezomib on NK cells, together with the use of adoptively transferred NK cells, will be needed to enable a more efficient use of this important class of drugs in MM patients.

9. Concluding Remarks

A number of experimental studies have shown that NK cells have the ability to eliminate cancer cells; in this context, the activity of NK cells can be exploited in therapeutic strategies against different cancers. As discussed above, a number of chemotherapy-induced molecular pathways can upregulate NKG2D and DNAM-1 activating ligands, able to increase activation and cytotoxic responses of NK cells toward MM. Future preclinical research and the standardization of combined therapeutic protocols using anticancer agents and NK cells should be encouraged to promote effective therapeutic immune responses to MM.

Abbreviations

DDR: DNA damage response
DNAM-1: DNAX accessory molecule-1
MM: Multiple myeloma
MMP: Matrix metalloprotease
NCR: Natural cytotoxicity receptors
NKG2D: NK group 2D
PVR: Poliovirus receptor-CD155
ROS: Reactive oxygen species.

Conflict of Interests

The authors declare they have no conflict of interests pending.

Acknowledgments

This work was supported by grants from the Italian Association for Cancer Research (AIRC Investigator Grant and AIRC 5x1000), the Sapienza University of Rome (Ateneo), and MIUR (PRIN/2010NECHBX_004/Marco Cippitelli).

References

[1] G. Trinchieri, "Biology of natural killer cells," *Advances in Immunology*, vol. 47, pp. 187–376, 1989.

[2] L. Moretta, E. Ciccone, M. C. Mingari, R. Biassoni, and A. Moretta, "Human natural killer cells: origin, clonality, specificity, and receptors," *Advances in Immunology*, vol. 55, pp. 341–380, 1994.

[3] M. H. Helfrich, E. Livingston, I. M. Franklin, and R. L. Soutar, "Expression of adhesion molecules in malignant plasma cells in multiple myeloma: comparison with normal plasma cells and functional significance," *Blood Reviews*, vol. 11, no. 1, pp. 28–38, 1997.

[4] R. A. Kyle and S. V. Rajkumar, "Multiple myeloma," *Blood*, vol. 111, no. 6, pp. 2962–2972, 2008.

[5] A. Mahindra, J. Laubach, N. Raje, N. Munshi, P. G. Richardson, and K. Anderson, "Latest advances and current challenges in the treatment of multiple myeloma," *Nature Reviews Clinical Oncology*, vol. 9, no. 3, pp. 135–143, 2012.

[6] B. Mohty, J. El-Cheikh, I. Yakoub-Agha, H. Avet-Loiseau, P. Moreau, and M. Mohty, "Treatment strategies in relapsed and refractory multiple myeloma: a focus on drug sequencing and retreatment approaches in the era of novel agents," *Leukemia*, vol. 26, no. 1, pp. 73–85, 2012.

[7] H. Ludwig, B. G. M. Durie, P. McCarthy et al., "IMWG consensus on maintenance therapy in multiple myeloma," *Blood*, vol. 119, no. 13, pp. 3003–3015, 2012.

[8] G. Koehne and S. Giralt, "Allogeneic hematopoietic stem cell transplantation for multiple myeloma: curative but not the standard of care," *Current Opinion in Oncology*, vol. 24, no. 6, pp. 720–726, 2012.

[9] S. K. Kumar, S. V. Rajkumar, A. Dispenzieri et al., "Improved survival in multiple myeloma and the impact of novel therapies," *Blood*, vol. 111, no. 5, pp. 2516–2520, 2008.

[10] C. Frohn, M. Höppner, P. Schlenke, H. Kirchner, P. Koritke, and J. Luhm, "Anti-myeloma activity of natural killer lymphocytes," *British Journal of Haematology*, vol. 119, no. 3, pp. 660–664, 2002.

[11] T. Hayashi, T. Hideshima, M. Akiyama et al., "Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application," *British Journal of Haematology*, vol. 128, no. 2, pp. 192–203, 2005.

[12] C. Y. Koh, A. Raziuddin, L. A. Welniak, B. R. Blazar, M. Bennett, and W. J. Murphy, "NK inhibitory-receptor blockade for purging of leukemia: effects on hematopoietic reconstitution," *Biology of Blood and Marrow Transplantation*, vol. 8, no. 1, pp. 17–25, 2002.

[13] E. Ullrich, M. Bonmort, G. Mignot, G. Kroemer, and L. Zitvogel, "Tumor stress, cell death and the ensuing immune response," *Cell Death and Differentiation*, vol. 15, no. 1, pp. 21–28, 2008.

[14] L. Zitvogel, L. Apetoh, F. Ghiringhelli, F. André, A. Tesniere, and G. Kroemer, "The anticancer immune response: indispensable for therapeutic success?" *The Journal of Clinical Investigation*, vol. 118, no. 6, pp. 1991–2001, 2008.

[15] E. Carbone, P. Neri, M. Mesuraca et al., "HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells," *Blood*, vol. 105, no. 1, pp. 251–258, 2005.

[16] Y. M. El-Sherbiny, J. L. Meade, T. D. Holmes et al., "The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells," *Cancer Research*, vol. 67, no. 18, pp. 8444–8449, 2007.

[17] M. Jinushi, M. Vanneman, N. C. Munshi et al., "MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 4, pp. 1285–1290, 2008.

[18] S. Girlanda, C. Fortis, D. Belloni et al., "MICA expressed by multiple myeloma and monoclonal gammopathy of undetermined significance plasma cells costimulates pamidronate-activated gammadelta lymphocytes," *Cancer Research*, vol. 65, no. 16, pp. 7502–7508, 2005.

[19] A. Soriani, A. Zingoni, C. Cerboni et al., "ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype," *Blood*, vol. 113, no. 15, pp. 3503–3511, 2009.

[20] S. Gasser, "DNA damage response and development of targeted cancer treatments," *Annals of Medicine*, vol. 39, no. 6, pp. 457–464, 2007.

[21] S. Armeanu, M. Bitzer, U. M. Lauer et al., "Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate," *Cancer Research*, vol. 65, no. 14, pp. 6321–6329, 2005.

[22] C. Fionda, A. Soriani, G. Malgarini, M. L. Iannitto, A. Santoni, and M. Cippitelli, "Heat shock protein-90 inhibitors increase MHC class I-related chain A and B ligand expression on multiple myeloma cells and their ability to trigger NK cell degranulation," *Journal of Immunology*, vol. 183, no. 7, pp. 4385–4394, 2009.

[23] X. Wu, Y. Tao, J. Hou, X. Meng, and J. Shi, "Valproic acid upregulates NKG2D ligand expression through an ERK-dependent mechanism and potentially enhances NK cell-mediated lysis of myeloma," *Neoplasia*, vol. 14, no. 12, pp. 1178–1189, 2012.

[24] Y. Shiloh, "The ATM-mediated DNA-damage response: taking shape," *Trends in Biochemical Sciences*, vol. 31, no. 7, pp. 402–410, 2006.

[25] I. B. Roninson, "Tumor cell senescence in cancer treatment," *Cancer Research*, vol. 63, no. 11, pp. 2705–2715, 2003.

[26] C. Gourzones-Dmitriev, A. Kassambara, S. Sahota et al., "DNA repair pathways in human multiple myeloma: role in oncogenesis and potential targets for treatment," *Cell Cycle*, vol. 12, no. 17, pp. 2760–2773, 2013.

[27] W. M. Kuehl and P. L. Bergsagel, "Molecular pathogenesis of multiple myeloma and its premalignant precursor," *Journal of Clinical Investigation*, vol. 122, no. 10, pp. 3456–3463, 2012.

[28] M. A. Dimopoulos, V. L. Souliotis, A. Anagnostopoulos et al., "Melphalan-induced DNA damage in vitro as a predictor for clinical outcome in multiple myeloma," *Haematologica*, vol. 92, no. 11, pp. 1505–1512, 2007.

[29] A. Soriani, M. L. Iannitto, B. Ricci et al., "Reactive oxygen species- and DNA damage response-dependent NK cell activating ligand upregulation occurs at transcriptional levels and requires the transcriptional factor E2F1," *The Journal of Immunology*, vol. 193, no. 2, pp. 950–960, 2014.

[30] W.-C. Lin, F.-T. Lin, and J. R. Nevins, "Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation," *Genes and Development*, vol. 15, no. 14, pp. 1833–1844, 2001.

[31] S. Textor, N. Fiegler, A. Arnold, A. Porgador, T. G. Hofmann, and A. Cerwenka, "Human NK cells are alerted to induction of p53 in cancer cells by upregulation of the NKG2D ligands ULBP1 and ULBP2," *Cancer Research*, vol. 71, no. 18, pp. 5998–6009, 2011.

[32] H. Li, T. Lakshmikanth, C. Garofalo et al., "Pharmacological activation of p53 triggers anticancer innate immune response through induction of ULBP2," *Cell Cycle*, vol. 10, no. 19, pp. 3346–3358, 2011.

[33] S. Panier and D. Durocher, "Regulatory ubiquitylation in response to DNA double-strand breaks," *DNA Repair*, vol. 8, no. 4, pp. 436–443, 2009.

[34] J. R. Morris, "SUMO in the mammalian response to DNA damage," *Biochemical Society Transactions*, vol. 38, no. 1, pp. 92–97, 2010.

[35] J. R. Morris, C. Boutell, M. Keppler et al., "The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress," *Nature*, vol. 462, no. 7275, pp. 886–890, 2009.

[36] Y. Galanty, R. Belotserkovskaya, J. Coates, S. Polo, K. M. Miller, and S. P. Jackson, "Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks," *Nature*, vol. 462, no. 7275, pp. 935–939, 2009.

[37] K. Ramadan and M. Meerang, "Degradation-linked ubiquitin signal and proteasome are integral components of DNA double strand break repair: new perspectives for anti-cancer therapy," *FEBS Letters*, vol. 585, no. 18, pp. 2868–2875, 2011.

[38] P. G. Richardson and K. C. Anderson, "Bortezomib: a novel therapy approved for multiple myeloma," *Clinical Advances in Hematology & Oncology*, vol. 1, no. 10, pp. 596–600, 2003.

[39] P. G. Richardson, R. Schlossman, C. Mitsiades, T. Hideshima, N. Munshi, and K. Anderson, "Emerging trends in the clinical use of bortezomib in multiple myeloma," *Clinical Lymphoma and Myeloma*, vol. 6, no. 2, pp. 84–88, 2005.

[40] J. F. San Miguel, R. Schlag, N. K. Khuageva et al., "Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma," *The New England Journal of Medicine*, vol. 359, no. 9, pp. 906–917, 2008.

[41] L. Neckers and S. P. Ivy, "Heat shock protein 90," *Current Opinion in Oncology*, vol. 15, no. 6, pp. 419–424, 2003.

[42] L. Whitesell and S. L. Lindquist, "HSP90 and the chaperoning of cancer," *Nature Reviews Cancer*, vol. 5, no. 10, pp. 761–772, 2005.

[43] P. Workman, F. Burrows, L. Neckers, and N. Rosen, "Drugging the cancer chaperone HSP90: combinatorial therapeutic exploitation of oncogene addiction and tumor stress," *Annals of the New York Academy of Sciences*, vol. 1113, pp. 202–216, 2007.

[44] J. Trepel, M. Mollapour, G. Giaccone, and L. Neckers, "Targeting the dynamic HSP90 complex in cancer," *Nature Reviews Cancer*, vol. 10, no. 8, pp. 537–549, 2010.

[45] M. Chatterjee, S. Jain, T. Stühmer et al., "STAT3 and MAPK signaling maintain overexpression of heat shock proteins 90α and β in multiple myeloma cells, which critically contribute to tumor-cell survival," *Blood*, vol. 109, no. 2, pp. 720–728, 2007.

[46] C. S. Mitsiades, N. S. Mitsiades, C. J. McMullan et al., "Antimyeloma activity of heat shock protein-90 inhibition," *Blood*, vol. 107, no. 3, pp. 1092–1100, 2006.

[47] J. Duus, H. I. Bahar, G. Venkataraman et al., "Analysis of expression of heat shock protein-90 (HSP90) and the effects of HSP90 inhibitor (17-AAG) in multiple myeloma," *Leukemia and Lymphoma*, vol. 47, no. 7, pp. 1369–1378, 2006.

[48] J. Patterson, V. J. Palombella, C. Fritz, and E. Normant, "IPI-504, a novel and soluble HSP-90 inhibitor, blocks the unfolded protein response in multiple myeloma cells," *Cancer Chemotherapy and Pharmacology*, vol. 61, no. 6, pp. 923–932, 2008.

[49] P. G. Richardson, C. S. Mitsiades, J. P. Laubach, S. Lonial, A. A. Chanan-Khan, and K. C. Anderson, "Inhibition of heat shock protein 90 (HSP90) as a therapeutic strategy for the treatment of myeloma and other cancers," *British Journal of Haematology*, vol. 152, no. 4, pp. 367–379, 2011.

[50] J. Zou, Y. Guo, T. Guettouche, D. F. Smith, and R. Voellmy, "Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1," *Cell*, vol. 94, no. 4, pp. 471–480, 1998.

[51] V. Groh, S. Bahram, S. Bauer, A. Herman, M. Beauchamp, and T. Spies, "Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 22, pp. 12445–12450, 1996.

[52] R. J. Kaufman, "Orchestrating the unfolded protein response in health and disease," *Journal of Clinical Investigation*, vol. 110, no. 10, pp. 1389–1398, 2002.

[53] J. W. Brewer and L. M. Hendershot, "Building an antibody factory: a job for the unfolded protein response," *Nature Immunology*, vol. 6, pp. 23–29, 2005.

[54] B. W. Doble and J. R. Woodgett, "GSK-3: tricks of the trade for a multi-tasking kinase," *Journal of Cell Science*, vol. 116, no. 7, pp. 1175–1186, 2003.

[55] L. Kockeritz, B. Doble, S. Patel, and J. R. Woodgett, "Glycogen synthase kinase-3—an overview of an over-achieving protein kinase," *Current Drug Targets*, vol. 7, no. 11, pp. 1377–1388, 2006.

[56] J. Abisheganaden, C. B. E. Chee, and Y. T. Wang, "Use of bilevel positive airway pressure ventilatory support for pathological flail chest complicating multiple myeloma," *European Respiratory Journal*, vol. 12, no. 1, pp. 238–239, 1998.

[57] H. Wang, J. Brown, and M. Martin, "Glycogen synthase kinase 3: a point of convergence for the host inflammatory response," *Cytokine*, vol. 53, no. 2, pp. 130–140, 2011.

[58] J. Luo, "Glycogen synthase kinase 3β (GSK3β) in tumorigenesis and cancer chemotherapy," *Cancer Letters*, vol. 273, no. 2, pp. 194–200, 2009.

[59] Y. Zhou, S. Uddin, T. Zimmerman, J.-A. Kang, J. Ulaszek, and A. Wickrema, "Growth control of multiple myeloma cells through inhibition of glycogen synthase kinase-3," *Leukemia & Lymphoma*, vol. 49, no. 10, pp. 1945–1953, 2008.

[60] F. Piazza, S. Manni, L. Q. Tubi et al., "Glycogen Synthase Kinase-3 regulates multiple myeloma cell growth and bortezomib-induced cell death," *BMC Cancer*, vol. 10, article 526, 2010.

[61] W. G. Gunn, U. Krause, N. Lee, and C. A. Gregory, "Pharmaceutical inhibition of glycogen synthetase kinase-3β reduces multiple myeloma-induced bone disease in a novel murine plasmacytoma xenograft model," *Blood*, vol. 117, no. 5, pp. 1641–1651, 2011.

[62] N. I. Herath, N. Rocques, A. Garancher, A. Eychène, and C. Pouponnot, "GSK3-mediated MAF phosphorylation in multiple myeloma as a potential therapeutic target," *Blood Cancer Journal*, vol. 4, no. 1, p. e175, 2014.

[63] C. Fionda, G. Malgarini, A. Soriani et al., "Inhibition of glycogen synthase kinase-3 increases NKG2D ligand MICA expression and sensitivity to NK cell-mediated cytotoxicity in multiple myeloma cells: role of STAT3," *The Journal of Immunology*, vol. 190, no. 12, pp. 6662–6672, 2013.

[64] R. Bedel, A. Thiery-Vuillemin, C. Grandclement et al., "Novel role for STAT3 in transcriptional regulation of NK immune cell targeting receptor MICA on cancer cells," *Cancer Research*, vol. 71, no. 5, pp. 1615–1626, 2011.

[65] E. Beurel and R. S. Jope, "Differential regulation of STAT family members by glycogen synthase kinase-3," *The Journal of Biological Chemistry*, vol. 283, no. 32, pp. 21934–21944, 2008.

[66] Q. Zhang, H. Y. Wang, M. Marzec, P. N. Raghunath, T. Nagasawa, and M. A. Wasik, "STAT3- and DNA methyltransferase 1-mediated epigenetic silencing of SHP-1 tyrosine phosphatase tumor suppressor gene in malignant T lymphocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 19, pp. 6948–6953, 2005.

[67] H. Lee, P. Zhang, A. Herrmann et al., "Acetylated STAT3 is crucial for methylation of tumor-suppressor gene promoters and inhibition by resveratrol results in demethylation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 20, pp. 7765–7769, 2012.

[68] N. Kato, J. Tanaka, J. Sugita et al., "Regulation of the expression of MHC class I-related chain A, B (MICA, MICB) via chromatin remodeling and its impact on the susceptibility of leukemic cells to the cytotoxicity of NKG2D-expressing cells," *Leukemia*, vol. 21, no. 10, pp. 2103–2108, 2007.

[69] A. Poggi, S. Catellani, A. Garuti, I. Pierri, M. Gobbi, and M. R. Zocchi, "Effective *in vivo* induction of NKG2D ligands in

acute myeloid leukaemias by all-*trans*-retinoic acid or sodium valproate," *Leukemia*, vol. 23, no. 4, pp. 641–648, 2009.

[70] S. Diermayr, H. Himmelreich, B. Durovic et al., "NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities," *Blood*, vol. 111, no. 3, pp. 1428–1436, 2008.

[71] K.-F. Tang, C.-X. He, G.-L. Zeng et al., "Induction of MHC class I-related chain B (MICB) by 5-aza-2′-deoxycytidine," *Biochemical and Biophysical Research Communications*, vol. 370, no. 4, pp. 578–583, 2008.

[72] O. Witt, H. E. Deubzer, T. Milde, and I. Oehme, "HDAC family: what are the cancer relevant targets?" *Cancer Letters*, vol. 277, no. 1, pp. 8–21, 2009.

[73] O. Khan and N. B. La Thangue, "HDAC inhibitors in cancer biology: emerging mechanisms and clinical applications," *Immunology and Cell Biology*, vol. 90, no. 1, pp. 85–94, 2012.

[74] T. Ishii, T. Seike, T. Nakashima et al., "Anti-tumor activity against multiple myeloma by combination of KW-2478, an Hsp90 inhibitor, with bortezomib," *Blood Cancer Journal*, vol. 2, no. 4, article e68, 2012.

[75] P. G. Richardson, A. A. Chanan-Khan, S. Lonial et al., "Tanespimycin and bortezomib combination treatment in patients with relapsed or relapsed and refractory multiple myeloma: results of a phase 1/2 study," *British Journal of Haematology*, vol. 153, no. 6, pp. 729–740, 2011.

[76] M. Kaiser, B. Lamottke, M. Mieth et al., "Synergistic action of the novel HSP90 inhibitor NVP-AUY922 with histone deacetylase inhibitors, melphalan, or doxorubicin in multiple myeloma," *European Journal of Haematology*, vol. 84, no. 4, pp. 337–344, 2010.

[77] B. Lamottke, M. Kaiser, M. Mieth et al., "The novel, orally bioavailable HSP90 inhibitor NVP-HSP990 induces cell cycle arrest and apoptosis in multiple myeloma cells and acts synergistically with melphalan by increased cleavage of caspases," *European Journal of Haematology*, vol. 88, no. 5, pp. 406–415, 2012.

[78] L. Espada, B. Udapudi, P. Podlesniy, I. Fabregat, C. Espinet, and A. Tauler, "Apoptotic action of E2F1 requires glycogen synthase kinase 3-β activity in PC12 cells," *Journal of Neurochemistry*, vol. 102, no. 6, pp. 2020–2028, 2007.

[79] G. García-Alvarez, V. Ventura, O. Ros, R. Aligué, J. Gil, and A. Tauler, "Glycogen synthase kinase-3beta binds to E2F1 and regulates its transcriptional activity," *Biochimica et Biophysica Acta*, vol. 1773, no. 3, pp. 375–382, 2007.

[80] V. Rebmann, P. Schütt, D. Brandhorst et al., "Soluble MICA as an independent prognostic factor for the overall survival and progression-free survival of multiple myeloma patients," *Clinical Immunology*, vol. 123, no. 1, pp. 114–120, 2007.

[81] V. Groh, J. Wu, C. Yee, and T. Spies, "Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation," *Nature*, vol. 419, no. 6908, pp. 734–738, 2002.

[82] E. S. Doubrovina, M. M. Doubrovin, E. Vider et al., "Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma," *The Journal of Immunology*, vol. 171, no. 12, pp. 6891–6899, 2003.

[83] K. Kohga, T. Takehara, T. Tatsumi et al., "Anticancer chemotherapy inhibits MHC class I-related chain a ectodomain shedding by downregulating ADAM10 expression in hepatocellular carcinoma," *Cancer Research*, vol. 69, no. 20, pp. 8050–8057, 2009.

[84] O. M. Fischer, S. Hart, A. Gschwind, N. Prenzel, and A. Ullrich, "Oxidative and osmotic stress signaling in tumor cells is mediated by ADAM proteases and heparin-binding epidermal growth factor," *Molecular and Cellular Biology*, vol. 24, no. 12, pp. 5172–5183, 2004.

[85] A. M. Vahdat, K. S. Reiners, V. L. Simhadri et al., "TNF-alpha-converting enzyme (TACE/ADAM17)-dependent loss of CD30 induced by proteasome inhibition through reactive oxygen species," *Leukemia*, vol. 24, no. 1, pp. 51–57, 2010.

[86] J. N. Kyula, S. Van Schaeybroeck, J. Doherty, C. S. Fenning, D. B. Longley, and P. G. Johnston, "Chemotherapy-induced activation of ADAM-17: a novel mechanism of drug resistance in colorectal cancer," *Clinical Cancer Research*, vol. 16, no. 13, pp. 3378–3389, 2010.

[87] X. Xu, G. S. Rao, V. Groh et al., "Major histocompatibility complex class I-related chain A/B (MICA/B) expression in tumor tissue and serum of pancreatic cancer: role of uric acid accumulation in gemcitabine-induced MICA/B expression," *BMC Cancer*, vol. 11, article 194, 2011.

[88] B. Huang, R. Sikorski, P. Sampath, and S. H. Thorne, "Modulation of NKG2D-ligand cell surface expression enhances immune cell therapy of cancer," *Journal of Immunotherapy*, vol. 34, no. 3, pp. 289–296, 2011.

[89] K. Yamanegi, J. Yamane, K. Kobayashi et al., "Downregulation of matrix metalloproteinase-9 mRNA by valproic acid plays a role in inhibiting the shedding of MHC class I-related molecules A and B on the surface of human osteosarcoma cells," *Oncology Reports*, vol. 28, no. 5, pp. 1585–1590, 2012.

[90] L. Markasz, G. Stuber, B. Vanherberghen et al., "Effect of frequently used chemotherapeutic drugs on the cytotoxic activity of human natural killer cells," *Molecular Cancer Therapeutics*, vol. 6, no. 2, pp. 644–654, 2007.

[91] A. Aoukaty and R. Tan, "Role for glycogen synthase kinase-3 in NK cell cytotoxicity and X-linked lymphoproliferative disease," *Journal of Immunology*, vol. 174, no. 8, pp. 4551–4558, 2005.

[92] D. Gotthardt, E. M. Putz, E. Straka et al., "Loss of STAT3 in murine NK cells enhances NK cell-dependent tumor surveillance," *Blood*, vol. 124, no. 15, pp. 2370–2379, 2014.

[93] J. Bae, A. Munshi, C. Li et al., "Heat shock protein 90 is critical for regulation of phenotype and functional activity of human t lymphocytes and nk cells," *Journal of Immunology*, vol. 190, no. 3, pp. 1360–1371, 2013.

[94] L. E. Rossi, D. E. Avila, R. G. Spallanzani et al., "Histone deacetylase inhibitors impair NK cell viability and effector functions through inhibition of activation and receptor expression," *Journal of Leukocyte Biology*, vol. 91, no. 2, pp. 321–331, 2012.

[95] H. Ogbomo, M. Michaelis, J. Kreuter, H. W. Doerr, and J. Cinatl Jr., "Histone deacetylase inhibitors suppress natural killer cell cytolytic activity," *FEBS Letters*, vol. 581, no. 7, pp. 1317–1322, 2007.

[96] N. Fiegler, S. Textor, A. Arnold et al., "Downregulation of the activating NKp30 ligand B7-H6 by HDAC inhibitors impairs tumor cell recognition by NK cells," *Blood*, vol. 122, no. 5, pp. 684–693, 2013.

[97] X. Wang, A. Ottosson, C. Ji et al., "Proteasome inhibition induces apoptosis in primary human natural killer cells and suppresses NKp46-mediated cytotoxicity," *Haematologica*, vol. 94, no. 4, pp. 470–478, 2009.

[98] X. Feng, J. Yan, Y. Wang et al., "The proteasome inhibitor bortezomib disrupts tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression and natural killer (NK) cell killing of TRAIL receptor-positive multiple myeloma cells," *Molecular Immunology*, vol. 47, no. 14, pp. 2388–2396, 2010.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 948501, 17 pages
http://dx.doi.org/10.1155/2015/948501

Review Article

Human Tumor Antigens and Cancer Immunotherapy

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Received 26 November 2014; Accepted 3 March 2015

Academic Editor: Yung-Fu Chang

With the recent developments of adoptive T cell therapies and the use of new monoclonal antibodies against the immune checkpoints, immunotherapy is at a turning point. Key players for the success of these therapies are the cytolytic T lymphocytes, which are a subset of T cells able to recognize and kill tumor cells. Here, I review the nature of the antigenic peptides recognized by these T cells and the processes involved in their presentation. I discuss the importance of understanding how each antigenic peptide is processed in the context of immunotherapy and vaccine delivery.

1. Introduction

Active cancer immunotherapy aims at activating the adaptive immune system of cancer patients to destroy tumors and prevent their recurrence. Key effector cells are the antitumor cytolytic T lymphocytes (CTL), which are poised to recognize and kill tumor cells. Strategies designed to activate antitumor CTL found in the blood and in the tumors of cancer patients are therefore promising for the development of strong and long-lasting antitumor responses. In the recent years, cancer immunotherapy made a significant breakthrough due to the development of adoptive T cell therapies and the use of monoclonal antibodies blocking the CTLA-4 and programmed cell death 1 (PD1) immune checkpoints [1–7]. However, in both cases, harmful autoimmune side effects have been observed, which likely result from the breakage of peripheral tolerance to antigens expressed by normal tissues.

The molecular nature of the antigens recognized by CTL on tumors was revealed in 1989, when Lurquin et al. showed that a mouse tumor-specific CTL recognized a peptide derived from a self-protein, mutated in cancer cells [8]. This observation demonstrated that MHC class I molecules continuously display on the cell surface peptides of 8 to 10 amino acids that are derived from a wide variety of, if not all, intracellular proteins [9]. In tumors, some of these peptides originate from altered or aberrantly expressed proteins, thereby marking the cells for CTL recognition [10].

Here, I review the nature of the peptides that are recognized by antitumor CTL together with the processes involved in their presentation to the immune system. I also discuss their use in the context of cancer immunotherapy.

2. Human Tumor Antigens

2.1. Identification of Tumor Antigens. Antitumor CTL clones have been isolated from the blood or tumors of cancer patients [11, 12]. One approach often employed to identify the peptides recognized by such CTL is expression cloning, which consists in isolating the peptide-encoding gene by transfecting a library of tumoral cDNA and testing the transfected cells for their ability to activate the CTL clone [13, 14]. Fragments of the identified gene can then be transfected to define the region encoding the antigenic peptide, and finally candidate peptides bearing adequate HLA-binding motifs are tested for their ability to sensitize target cells to lysis by the CTL. This approach was successfully used to identify a large number of antigenic peptides [13, 15–17].

Nowadays, tumor-associated antigenic peptides are often identified using the "reverse immunology" approach [18], which consists in selecting peptides with adequate HLA-binding motifs inside a protein of interest, such as proteins encoded by mutated oncogenes or genes that are either selectively expressed or overexpressed by tumors. Candidate peptides are synthesized and tested for HLA binding *in vitro*.

The most efficient binders are pulsed onto antigen-presenting cells, which are used to stimulate T lymphocytes *in vitro*, in order to derive CTL lines or clones that recognize peptide-pulsed target cells. A drawback of this approach is that the identified peptides might not be processed efficiently by tumors. It is therefore essential to verify that the CTL do recognize tumor cells that naturally express the peptide-encoding gene. Additionally, one should test transfectants that express normal levels of the gene [19] or cells where expression of the gene has been knocked down using si or shRNAs [20].

A third approach to antigen identification is based on the elution of antigenic peptides from MHC class I molecules immunopurified from the surface of tumor cells [21–24]. The direct identification by mass spectrometry of the sequence of the eluted peptides is technically demanding but proved useful to identify or to confirm the relevance of peptides that have undergone posttranslational modifications such as serine/threonine phosphorylation [25, 26], glycosylation-dependent asparagine deamidation [27], or peptide splicing [28].

A large number of antigenic peptides recognized by antitumor CTL have been identified using these various approaches. These antigens are conveniently classified according to the expression pattern of the parent gene [10, 29, 30] (Figure 1). A regularly updated database of those antigenic peptides effectively presented by tumor cells can be found on the http://www.cancerimmunity.org/ website [18].

2.2. Antigens with High Tumor Specificity. Antigens of three classes can induce tumor-specific T cell responses because they display a tumor-specific pattern of expression [30]: antigens derived from viral proteins, antigens derived from point mutations, and antigens encoded by cancer-germline genes (Figure 1).

2.2.1. Viral Antigens. Viruses are at the origin of several types of cancers including cervical carcinoma, nasopharyngeal carcinoma, hepatocarcinoma, and some leukemias [31]. Viral proteins are produced inside the tumor cells and therefore give rise to antigenic peptides that can be detected by T cells (Figure 1). Vaccines containing long HPV peptides recently emerged as a promising therapeutic modality for HPV-related cancers, as these long peptides proved capable of increasing the number and activity of HPV-16-specific CD4 and CD8 T cells [32, 33].

2.2.2. Antigens Encoded by Mutated Genes. Many CTL isolated from the blood or tumors of cancer patients were found to recognize antigens that arise from point mutations in ubiquitously expressed genes [34–36]. In most cases, the mutation changes one amino acid in the peptide sequence, either enabling the peptide to bind to the MHC class I molecule or creating a new antigenic determinant that is recognized by the CTL (Figure 1). In some cases, the mutation causes a frameshift leading to the production of a new antigenic peptide [37, 38]. Some mutated antigenic peptides result from oncogenic mutations. A mutation in gene *CDK4* was shown to affect the binding of CDK4 to its inhibitor p16/INK4a, thereby favoring uncontrolled cell division [39]. A mutation in gene *CTNNB1* produces an antigenic peptide [40], stabilizes β-catenin, and increases its association with the transcription factor Lef-Tcf. This may result in the persistent transactivation of genes involved in melanoma progression [41]. A mutation in gene *CASP8*, recognized by a CTL, was shown to decrease cell sensitivity to apoptosis [42]. In most cases however, these mutations are passenger mutations and the corresponding antigenic peptides are unique to the tumors in which they were identified. Tumors with a high mutation rate, such as melanoma, lung carcinoma, or microsatellite instability $(MSI)^+$ colorectal carcinoma are expected to bear more mutated antigens and are therefore more immunogenic. Noteworthy in some patients, the antitumor CTL response is directed mostly against mutated epitopes [43].

Surprisingly, only a few CD8 T cells recognizing peptides derived from genes that are often mutated in cancer, such as *P53*, *KRAS*, or *NRAS*, were isolated [44–46]. Most of these CTL were found using the reverse immunology approach and not the mixed-lymphocyte tumor culture, suggesting that the corresponding peptides might be poorly immunogenic. Peptides derived from chromosomal translocations such as BCR-ABL or ETV6-AML1 were also identified [47, 48].

2.2.3. Cancer-Germline Genes. Another important source of tumor specific antigens is the cancer-germline genes (Figure 1). They include the melanoma-antigen encoding (MAGE) genes, comprising 25 functional genes clustered in three regions of the X chromosome: *MAGEA*, *MAGEB*, and *MAGEC* [49–51]. Other cancer-germline gene families on the X chromosome include the *BAGE* [52], *GAGE* [53, 54], *LAGE/NY-ESO1* [55, 56], and *SSX* genes [57, 58]. Cancer-germline genes are expressed in a wide variety of cancer types and not in normal tissues except germline and trophoblastic cells [13, 59]. Their tumor-specific pattern of expression results from the demethylation of their promoter sequence, as part of a genome-wide demethylation that takes place in male germ cells and in some advanced cancers [60–65]. Because male germline cells and trophoblastic cells do not display MHC class I molecules on their surface [66], they cannot display antigens to T cells. The antigenic peptides derived from cancer-germline genes, also called MAGE-type antigens, therefore appear to be strictly tumor-specific and their use as immunotherapeutic targets should not be deleterious to the patient. It is important to note, however, that a low level of expression of MAGE-A12 was recently reported in brain cells [67]. Besides cancer-germline genes, a few examples of aberrant transcripts expressed in tumors but silent or expressed at very low levels in normal tissues have been shown to encode antigenic peptides [68–70]. Recently, peptides were identified that derive from cyclin-A1, a protein with pro-proliferative and anti-apoptotic properties, which is expressed in testis and acute myeloid leukemia [71].

2.3. Antigens with Low Tumor Specificity. This category of antigens comprises differentiation antigens and antigens derived from genes that are overexpressed in tumors [30] (Figure 1).

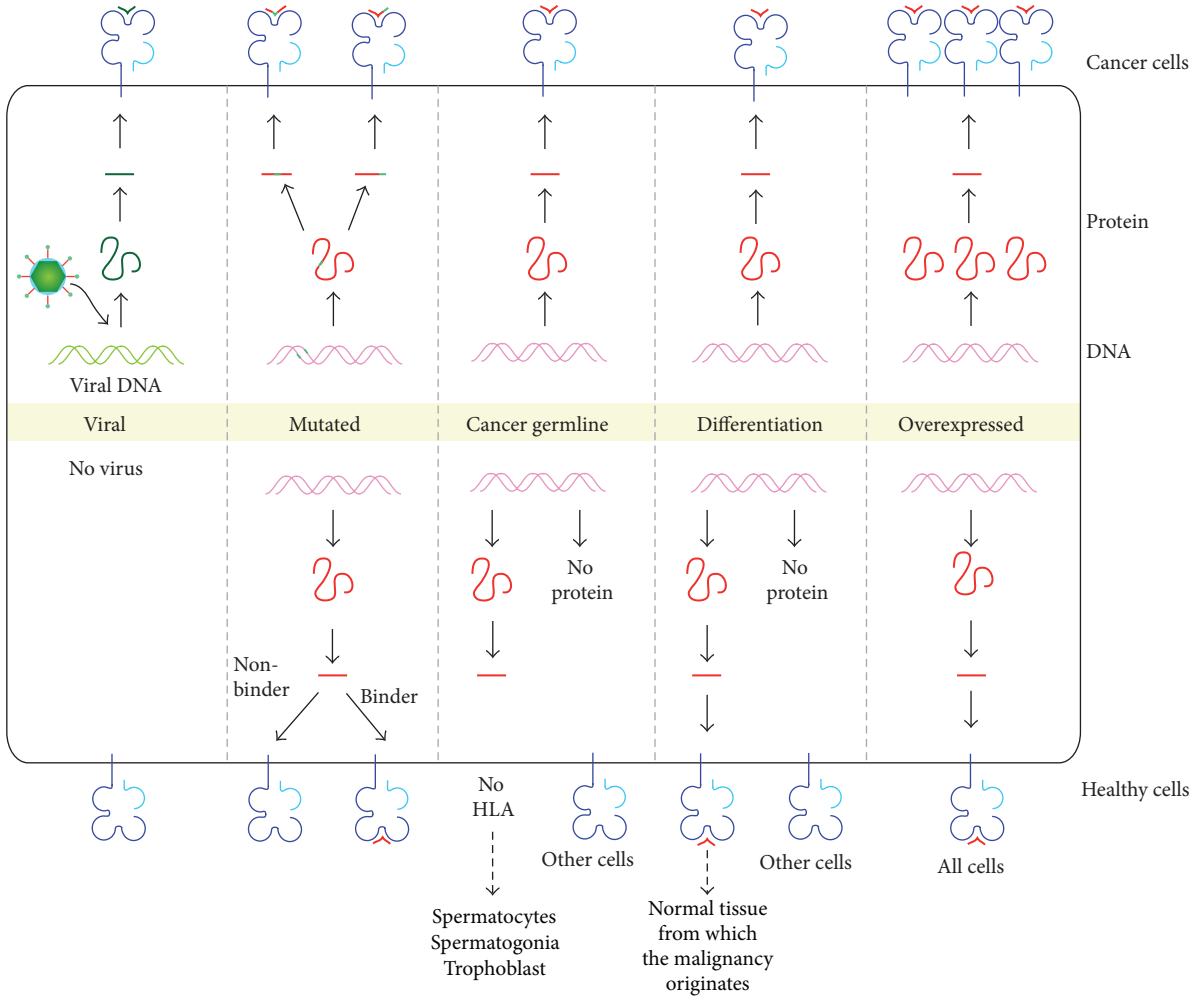


Figure 1: Tumor antigens recognized by cytolytic T lymphocytes. Tumor antigens are classified according to the pattern of expression of the parental gene. The production of antigenic peptides by cancer cells (upper panel) and healthy cells (lower panel) is depicted. Viral antigens are only expressed in virally infected cells. Mutated genes can give rise to a modified peptide that is able to bind the HLA class I molecules while the wild-type peptide cannot (left). The mutation can also alter a peptide, which is able to bind the HLA class I molecule, so that this modified peptide is now recognized as nonself by circulating CTL. Cancer-germline genes are expressed in tumors or germline cells as a result of whole genome demethylation. MAGE-type antigens encoded by cancer-germline genes are not expressed at the surface of healthy cells nor on germline cells since the latter do not express HLA class I molecules. Differentiation antigens are encoded by genes with a tissue-specific expression. They are therefore expressed by some types of tumors and the corresponding healthy tissue. Some genes are overexpressed in tumors as a result of increased transcription or gene amplification. The resulting peptides are highly expressed on these tumors but also show a low level of expression in some or all healthy tissues.

2.3.1. Differentiation Antigens. Differentiation antigens are derived from proteins that are expressed in a given type of tumor and the corresponding healthy tissue. Most identified differentiation antigens are present on melanoma cells, in which the corresponding protein is often involved in melanin biosynthesis or melanosome biogenesis. The expression of the corresponding genes depends on transcription factor MITF (microphthalmia associated transcription factor) [72]. Interestingly, spontaneous responses to peptides derived from proteins such as tyrosinase [15, 73], gp100/pmel17 [19, 74, 75], Melan-A/MART-1 [76, 77], gp75/TRP1 [78], or TRP2 [79] are frequent in melanoma patients and healthy donors [80, 81], suggesting that central tolerance to these antigens is not complete. T cell responses to differentiation antigens can lead to vitiligo, a partial skin depigmentation often observed in melanoma patients and generally associated with a good prognosis [82–84]. Peptides were also identified from the prostate specific antigen and the prostatic acidic phosphatase, two proteins expressed in normal prostate and tumoral prostate tissues [85, 86]. Finally, the carcinoembryonic antigen (CEA) is often highly expressed in colorectal cancer and other epithelial tumors but is also present at lower level in a variety of normal epithelial cells of the intestinal tract [87].

2.3.2. Overexpressed Antigens. Overexpressed antigens also provide candidates for the development of immunotherapeutic vaccines. The difficulty with these antigens is the reliability of the quantification of their amounts on the surface of

tumoral versus normal cells, on the basis of which one predicts that there might be a threshold of expression below which the CTL will not recognize the antigen. A number of antigenic peptides have been reported to be "overexpressed," most of which identified using the reverse immunology approach [18]. An interesting example of overexpressed antigen is the peptide recognized by a CTL on a renal cell carcinoma and encoded by gene *MOK* (*RAGE-1*) [88]. *RAGE-1* is expressed in tumors of different histological types but is silent in normal tissues except retina, where low expression is observed. Because the eye is an immunologically privileged site [89, 90] and because retina cells do not seem to express MHC class I molecules [91], immunization against RAGE-1 could be considered against renal cell carcinoma, which express no cancer-germline genes. At least five peptides recognized by CTL in melanoma patients were shown to derive from *PRAME*, a gene overexpressed in a number of tumor types, but expressed at low levels in various normal tissues [92, 93]. Other examples of peptides derived from overexpressed genes include those derived from the inhibitor of apoptosis protein survivin [94, 95], the wild-type p53 protein [96, 97], or the oncogene and growth factor ERBB2 (HER2/NEU) which is overexpressed in many epithelial tumors such as ovarian and breast carcinoma due to increased gene transcription and gene amplification [98–100]. Peptides were also identified that derive from the protein Wilms tumor 1 (WT1), a transcription factor expressed at 10- to 1000-fold higher levels in leukemic versus normal cells [101–103]. A decrease in the number of leukemic cells was observed in leukemia patients following allogeneic stem cell transplantation and injection of donor-derived CTL recognizing the HLA-A2-restricted peptide $WT1_{126–134}$, without evidence of autoimmune toxicity [104]. As overexpressed antigens are shared by numerous tumors, they represent attractive targets for the development of immunotherapy; however their use is not devoid of the risk of developing autoimmune reactions due to the low but still detectable expression of the corresponding genes in healthy tissues.

2.4. The Importance of Tumor Specificity in Cancer Immunotherapy. Although antibodies targeting the immune checkpoints CTLA-4 and PD1 proved efficient at inducing sustained clinical responses in cancer patients, harmful autoimmune side effects have been observed in a large number of patients. This damage to healthy tissues likely results from the fact that checkpoints inhibitors boost overall T cell immunity, thereby unleashing peripheral tolerance to antigens expressed by these tissues. The T cells responsible for these toxicities were not yet characterized but they likely correspond to autoreactive T cells that are not specific for the tumor.

To be safe and efficient, immunotherapy strategies should therefore elicit efficient T cell responses against antigenic peptides that are present on tumor cells but not on healthy cells in order to avoid such autoimmune side effects. In that regard, antigens encoded by mutated genes are among the safest. So far, their identification relied mostly on the screening of autologous cDNA libraries [18]. In melanoma patients, about one half of the currently defined tumor-specific antigens recognized by CTL are mutated antigens, while the other half correspond to MAGE-type antigens [30]. Using whole genome sequencing of tumor samples, Robbins et al. have speeded up the identification procedure of antigens derived from passenger mutations recognized by tumor infiltrating lymphocytes (TIL) [105]. Adoptive transfer of these TIL was followed by objective tumor regressions [105, 106]. Because of the therapeutic potential of mutated antigens, identification approaches based on exome sequencing or mass spectrometry might lead to patient-tailored procedures.

For the nonmutated antigens, strict tumor specificity is often hard to certify. Even if an antigen shows a tumor-specific pattern of expression, one can never exclude that it could have a substantial degree of expression in a small subset of cells of the body. No toxicity was observed in cancer vaccine trials based on the activation of patient T cells against such antigens; however, vaccination approaches generally induce only low amounts of CTL [107]. The risk is much higher, for example, with adoptive T cell therapy, which is based on the infusion of a large number of high avidity T cells. About 30% of melanoma patients infused with T cells transduced with a high affinity receptor recognizing melanoma differentiation antigens Melan-A/MART1 or gp100 showed objective cancer regression, while 50% of the patients developed vitiligo and sometimes destruction of melanocytes in the eye and inner ear [108]. The transfer of T cells engineered to express a single-chain murine antibody-type receptor recognizing carbonic anhydrase IX, a protein present on renal cell carcinoma but also on bile epithelial cells, was found to encounter liver toxicity [109]. Recent clinical trial based on the adoptive transfer of T cells engineered to express a murine TCR recognizing the carcinoembryonic antigen led to objective tumor regression in one patient out of four; however, all patients treated developed severe colitis [110]. Finally, transferring T cells carrying a chimeric receptor against ERBB2 was shown to be lethal, due to the rapid gathering of the infused T cells at the lung epithelium, where ERBB2 is expressed at low level [111]. One should therefore ensure that the antigenic peptide targeted by such therapies is strictly specific to the tumor. This is particularly the case when using TCR engineered to increase their affinity, because they bypass the mechanisms responsible for the establishment of natural immune tolerance. Moreover, problems of cross-reaction with unrelated peptides expressed on healthy tissues can also arise. In a recent trial, administration to patients of T cells engineered to express an affinity-enhanced TCR recognizing the peptide EVDPIGHLY derived from MAGE-A3 resulted in a serious adverse event and fatal toxicity against cardiac tissue, probably caused by a cross-recognition of the peptide ESDPIVAQY derived from the muscle protein TITIN [112, 113]. In another trial, adoptive transfer of T cells engineered to recognize the MAGE-A3 peptide KVAELVHFL shared by MAGE-A9 and closely related to epitopes from MAGE-A2, MAGE-A6, and MAGE-A12, three patients experienced mental status changes, and two patients lapsed into comas and subsequently died [67]. This was most likely related to a previously unrecognized expression of MAGE-A12 in the brain, which resulted in the destruction of the

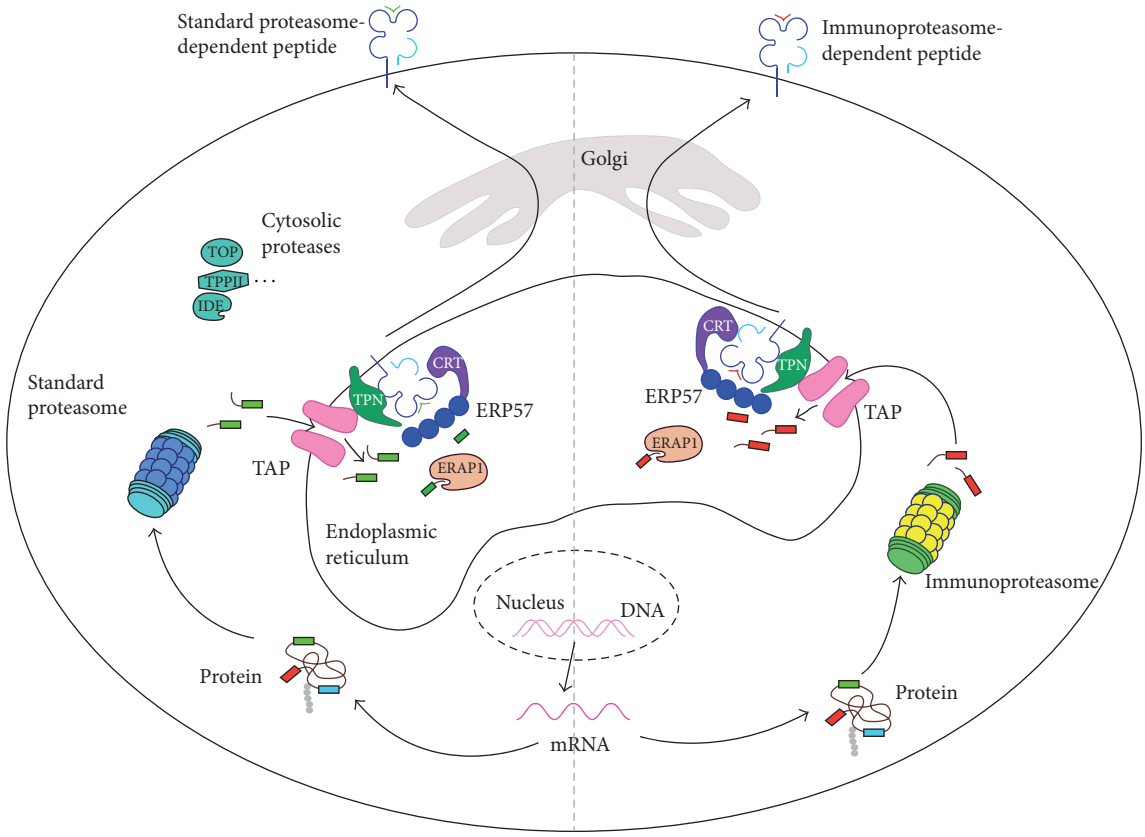


Figure 2: Processing of tumor antigens recognized by $CD8^+$ T cells. CTL recognize peptides that are produced by the degradation of cellular proteins by the proteasome. Four types of proteasome exist, two of which are represented here (standard proteasome and immunoproteasome). Peptides resulting from proteasome degradation are then transported in the lumen of the ER by the TAP transporter. Peptides bearing an extended N-terminus can be further trimmed by additional proteases such as ERAP1 before being loaded on HLA class I molecules with the help of the peptide loading complex, which is composed of TAP, tapasin (Tpn), the oxidoreductase ERp57, and the chaperone calreticulin (CRT). Peptide/HLA complexes are then transferred to the cell surface through the secretory pathway.

neuronal tissue by engineered T cells. In this case, the TCR used to engineer the transferred T cells was obtained from an HLA-A2 transgenic mouse immunized using MAGE-A3 and the TCR was further modified to improve peptide reactivity. Moreover, the toxicity of the transfused T cells appeared to be related to the amount of cells transfused. Altogether, these results confirm the antitumor activity of T cells engineered to express TCR and illustrate the risk of this approach when dealing with antigens that are not truly tumor-specific or with manipulated TCR that have bypassed the mechanisms of natural tolerance.

3. Processing of Human Tumor Antigens

3.1. The MHC Class I Presentation Pathway. Antigenic peptides recognized by CTL usually originate from the degradation of cellular proteins by a large cytosolic complex called the proteasome (Figure 2). Peptides released from proteasomal degradation are transferred into the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) (Figure 2). Once in the ER, peptides can be further trimmed by aminopeptidases such as ERAP1 and ERAP2. Peptides with suitable size and sequence will bind to MHC class I molecules, with the help of the peptide loading complex (PLC) composed of TAP, tapasin, ERp57, and calreticulin (CRT). Stable peptide/MHC class I complexes will leave the ER and migrate to the cell surface (Figure 2). Most peptide/MHC complexes displayed at the surface of healthy cells are not recognized by T cells as a result of self-tolerance. However, in tumor cells or cells infected by a virus, a new repertoire of peptides is produced, which is derived from viral or tumor-associated proteins and against which an immune response can be mounted. Since CTL can recognize cells bearing as few as 10 peptide/MHC complexes [114], small changes in the cellular protein content can be detected by the immune system.

3.2. The Proteasome. The 20S proteasome is a large barrel-shape structure made of four stacked heptameric rings that delimit a catalytic chamber inside which proteins are degraded. The two outer rings of 20S proteasome are made of α-subunits (α1–7), while the two inner rings contain β-type subunits (β1–7), three of which (β1, β2, and β5) are catalytic in the vertebrate proteasome. The 20S proteasome associates with the 19S regulatory complex to form the 26S proteasome, a complex responsible for the breakdown

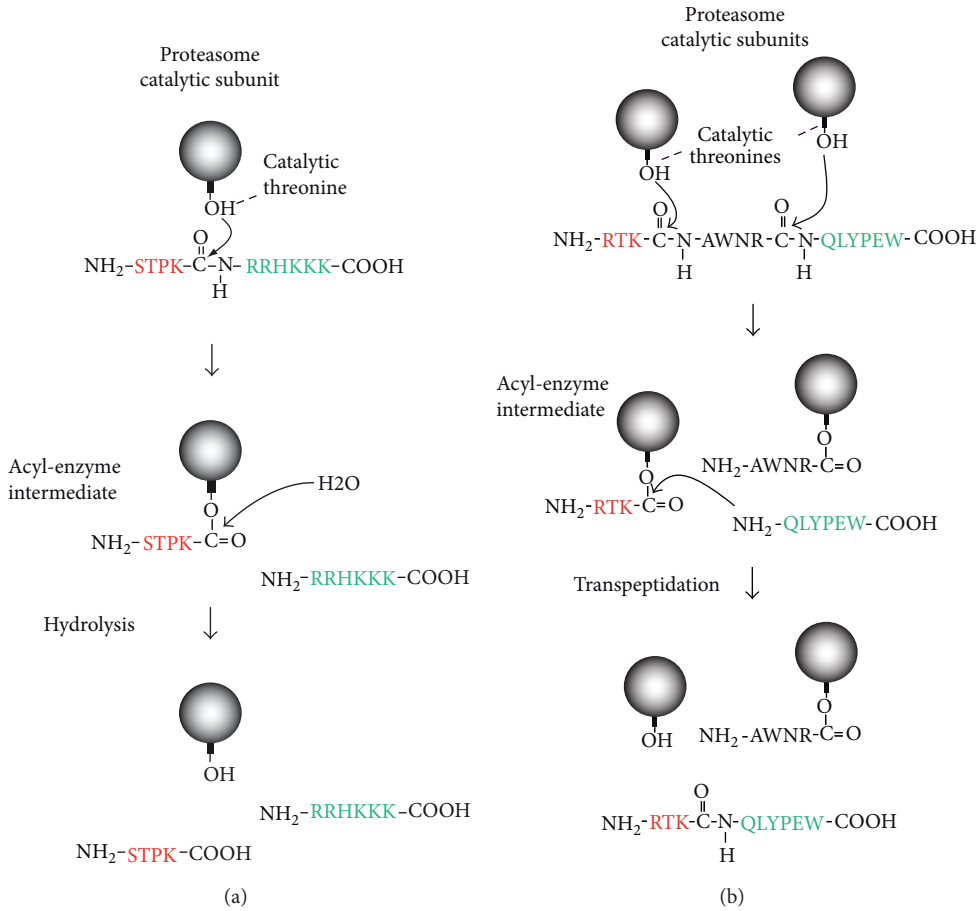


Figure 3: Proteasome activities. (a) Peptide-bond hydrolysis. In the course of peptide-bond hydrolysis, the hydroxyl group of the N-terminal threonine produces a nucleophile attack on the carbonyl of the peptide bond. This leads to the formation of an acyl-enzyme intermediate in which a peptide fragment remains attached to the proteasome through an ester link. Finally hydrolysis of the acyl-enzyme intermediate by a water molecule present in the proteasome chamber will restore the hydroxyl group of the catalytic threonine and release the peptide. (b) Peptide splicing by the proteasome. Splicing of the antigenic peptide RTK_QLYPEW derived from the differentiation antigen gp100. After formation of the acyl-enzyme intermediate involving the fragment RTK, the free N-terminal amino-group of peptide QLYPEW present in the proteasome chamber performs a nucleophilic attack on the acyl-enzyme intermediate. This leads to the creation of a new peptide bond, which assembles both fragments of the spliced peptide. Balls represent the catalytic β subunits of the proteasome. The hydroxyl group of the N-terminal threonine is indicated.

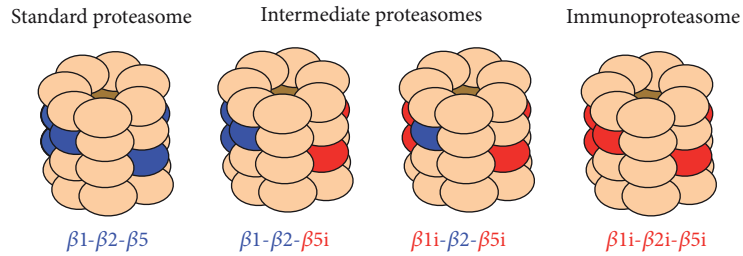
of polyubiquitinylated proteins [115]. One of the earliest hints that production of antigenic peptides was dependent on the proteasome was the observation that ubiquitination was necessary for the presentation of the model antigen ovalbumin [116]. Later on, the use of specific cell-permeable proteasome inhibitors, which reversibly or irreversibly bind to the hydroxyl group of the proteasome catalytic subunits, confirmed the proteasome involvement in the production of antigenic peptides and in the overall peptide supply to MHC class I molecules [117–120]. Mutagenesis studies as well as the analysis of crystal structures of inhibitor-bound proteasomes have shown that the hydroxyl group of the N-terminal threonine residue of each catalytic subunit was responsible for the nucleophilic attack that initiates hydrolysis of the peptide bond [121–123]. This nucleophilic attack on the peptide bond leads to the formation of an acyl-enzyme intermediate in which a peptide fragment remains attached to the proteasome by an ester link. This acyl-enzyme is then rapidly hydrolyzed by water molecules found in the catalytic chamber and the released peptide is then transferred back into the cytosol (Figure 3(a)) [124].

Three major types of catalytic activities are associated with proteasome function: the PGPH (peptidyl-glutamyl peptide bond hydrolysing) or caspase-like activity, the trypsin-like activity, and the chymotrypsin-like activity, which cleave after acidic, basic, and hydrophobic residues, respectively. Based on the study of yeast proteasome mutants, the caspase-like activity was linked to the β1 subunit, while the trypsin-like and the chymotrypsin-like activities were associated to the β2 and β5 subunits, respectively [125–130]. However, other parameters are also involved as some proteasome subunits show overlapping specificities [128] and the sequences surrounding the cleavage site also influence cleavage strength [131, 132]. The quality of proteasome degradation

will influence the nature of the peptides displayed at the cell surface by MHC class I molecules. A few years ago, my colleagues and I discovered that the proteasome was able to produce antigenic peptides by assembling peptide fragments that are distant in the parental protein. This process, called peptide splicing, takes place inside the catalytic chamber of the proteasome as a consequence of protein degradation (Figure 3(b)). We showed that peptide splicing takes place through a transpeptidation that involves the nucleophilic attack of the acyl-enzyme intermediate by the N-terminus of a peptide fragment present in the chamber [133]. This leads to the creation of a new peptide bond between the C-terminus of the peptide released from the acyl-enzyme intermediate and the N-terminus of the nucleophilic peptide (Figure 3(b)). So far, five spliced antigenic peptides were identified [28, 133–137], three of which were composed of fragments that were assembled in the reverse order to that in which they occur in the parental protein [28, 135, 137]. Studying a spliced peptide derived from gp100 and composed of fragment RSYVPLAH linked to a single arginine (R) at its C-terminus (RSYVPLAH_R), we showed that the attacking nucleophilic peptide required a minimal size of three amino acids in order to perform the splicing reaction. In the case of peptide RSYVPLAH_R, the spliced peptide bears an extended C-terminus that needs to be further trimmed by the proteasome to produce the final antigenic peptide [137]. Mishto et al. recently suggested that, in the catalytic chamber, the nucleophilic peptide would be accommodated in a dedicated pocket, different from the primed substrate binding site. The latter, during proteolysis, accommodates the part of the peptide substrate that is located C-terminally from the cleavage site [138]. The accommodation of the nucleophilic peptide inside a dedicated pocket might facilitate and speed up the peptide splicing process. The existence of this dedicated pocket would explain why, in the study from Mishto et al., the fragments that are the most frequently involved in peptide splicing did not always correspond to the most abundant fragments observed after cleavage of the parental protein, suggesting that affinity of the spliced reactant for this pocket might also rule the peptide splicing reaction [138].

3.3. Standard and Immunoproteasome. In immune cells and in the presence of inflammatory cytokines, three additional catalytic subunits (LMP-2(β1i), MECL-1(β2i), and LMP-7(β5i)) are expressed, which incorporate into proteasomes instead of subunits β1, β2, and β5, resulting in the formation of immunoproteasomes [139] (Figure 4). The immunoproteasome has an increased propensity to cleave after basic and hydrophobic residues, thereby improving the cell ability to produce peptides for loading on MHC class I molecules, which often require basic and hydrophobic residues at their C-terminus [140–142]. This idea was corroborated by the study of mice knockout for 2 (β1i or β5i) or 3 immunosubunits, which fail to present a number of MHC class I epitopes [143–145]. Consequently, the peptide repertoire displayed by cells expressing immunoproteasomes is different from that of cells expressing standard proteasomes, and this might have important consequences for the development of cancer immunotherapy strategies [132, 140, 141, 145–147]. Several human tumor antigens were found to be more efficiently produced by the immunoproteasome, such as the HLA-B40-restricted peptide MAGE-A3$_{114-122}$, HLA-A2-restricted peptide MAGE-C2$_{336-344}$, and HLA-B57-restricted peptide MAGE-C2$_{42-50}$ [17, 142, 148–150]. On the other hand, some tumor-associated peptides are better processed by the standard proteasome. The first example, a peptide derived from the ubiquitous protein RU1, was presented to CTL by a renal cell carcinoma but not by the autologous EBV-transformed B cells [118]. This was explained by the fact that, contrary to the tumor cells, EBV-transformed B cells contain a large amount of immunoproteasome, which is not able to produce the antigenic peptide. Since then, several other peptides were found to be processed by the standard proteasome but not the immunoproteasome, among which the HLA-A2-restricted peptides Melan-A/MART-1$_{26-35}$ [118], gp100$_{209-217}$ [149], and tyrosinase$_{369-377}$ [149]. As shown by *in vitro* digestion of long peptide precursors, the lack of processing of antigenic peptides by one proteasome type is generally caused by an internal cleavage that destroys the peptide [142, 149, 150]. Interestingly, studying the differential processing of the spliced peptides by the proteasome, Dalet et al. observed that three spliced peptides were better produced by the standard proteasome, while one peptide was better produced by the immunoproteasome [151]. The efficiency of the peptide splicing reaction depended on the ability of each proteasome type to liberate the peptide fragments involved in the splicing [151].

3.4. Intermediate Proteasomes. The existence of intermediate proteasomes, composed of a mixture of standard and immunosubunits, was suggested by the observation that some tissues contain some but not all proteasome immunosubunits [152] (Figure 4). Using a unique set of antibodies directed against proteasome catalytic subunits and recognizing proteasomes in their native configuration, Guillaume et al. demonstrated the existence of intermediate proteasomes containing one (β5i) or two (β1i β5i) immunosubunits [142]. The absence of other types of intermediate proteasomes is in agreement with the rules of proteasome assembly, which depends on the nature of the subunit propeptide [153, 154]. Indeed, the fact that the incorporation of β2i depends on the previous incorporation of β1i explains why Guillaume et al. did not detect intermediate proteasomes containing β2i only [142]. Moreover, the presence of the immunosubunit β5i is required for the maturation of proteasomes containing β1i and β2i [139], explaining why intermediate proteasomes lacking β5i were never detected. Interestingly, intermediate proteasomes β1-β2-β5i and β1i-β2-β5i represent 10 to 20% of the total proteasomes found in tumors and 30 to 50% of those found in liver, kidney, small bowel, colon, and dendritic cells [142]. Intermediate proteasomes β1-β2-β5i and β1i-β2-β5i were shown to display chymotrypsin-like and trypsin-like activities that are intermediate between standard and immunoproteasomes [142]. The β1 β2 β5i intermediate proteasome displays a caspase-like (cleavage after acid amino acids) activity similar to that of the standard proteasome, while this activity is low in β1i-β2-β5i proteasome. This originates from the fact that the caspase-like



Differentiation						
$gp100_{209–217}$	HLA-A2	ITDQVPFSV	+ +	+/−	+/−	+/−
$Tyrosinase_{369–377}$	HLA-A2	YMDGTMSQV	+ +	−	+/−	+/−
$Melan\text{-}A_{26–35}$	HLA-A2	EAAGIGILTV	+ +	+/−	+/−	−
Cancer germline						
$MAGE\text{-}A3_{114–122}$	HLA-B40	AELVHFLLL	−	+ +	+ +	+ +
$MAGE\text{-}A3_{271–279}$	HLA-A2	FLWGPRALV	−	+ +	−	−
$MAGE\text{-}A10_{254–262}$	HLA-A2	GLYDGMEHL	−	−	+ +	−
$MAGE\text{-}C2_{336–344}$	HLA-A2	ALKDVEERV	−	−	+ +	+ +
$MAGE\text{-}C2_{42–50}$	HLA-B57	ASSTLYLVF	−	+ +	+ +	+ +
$MAGE\text{-}C2_{191–200}$	HLA-A2	LLFGLALIEV	−	−	+ +	−

Figure 4: Proteasome subtypes. Mammalian 20S proteasomes are composed four stacked rings of seven subunits each. The two outer rings are made of α-subunits and delimit the entrance of the catalytic chamber. The two inner rings are made of β subunits, three of which (β1, β2, and β5) are catalytically active. In immune cells or upon induction with IFNγ, catalytic subunits β1, β2, and β5 are replaced with their inducible counterparts β1i, β2i, and β5i to form immunoproteasomes. Besides standard and immunoproteasomes, two additional forms of proteasome exist, which contain a mixture of standard and immune catalytic subunits, as indicated. The processing ability of these four types of proteasomes was studied for the indicated peptides (lower part of the figure). ++: efficiently produced, +/−: slightly produced, and −: not produced.

activity is generally assigned to the β1 subunit, which is present in standard and intermediate proteasome β1-β2-β5i and absent in immunoproteasome and intermediate proteasome β1i-β2-β5i. Because of their particular cleavage properties, intermediate proteasomes were shown to produce a unique repertoire of peptides. Indeed, some antigenic peptides such as the HLA-A2-restricted peptides $MAGE\text{-}A10_{254–262}$ and $MAGE\text{-}C2_{191–200}$ are exclusively produced by the intermediate proteasome β1i-β2-β5i while the HLA-A2-restricted peptide $MAGE\text{-}A3_{271–279}$ is only produced by intermediate proteasome β1-β2-β5i [142, 150]. Trying to induce T cell responses against antigenic peptides that are produced by both immunoproteasome and intermediate proteasomes should therefore enable the recognition by the immune system of tumors in any circumstances.

3.5. Other Proteases Producing Antigenic Peptides. Although most antigenic peptides appear to be produced by the proteasome, others are processed independently from the proteasome through the action of cytosolic proteases such as TPPII or insulin-degrading enzyme [120, 155, 156]. Recently, the production of a PRAME peptide was shown to involve the proteasome for cleavage at the N-terminus while the C-terminus required sequential cleavages by nardilysin and thimet oligopeptidase [157]. Angiotensin-converting enzyme (ACE) was also suggested to play a role in the edition of the carboxyl-terminus of proteasome produced MHC class I peptides [158]. Antigenic peptides produced through proteasome-independent pathways might represent interesting targets in the context of immunotherapy, as some of those antigens might keep being expressed by tumors without being influenced by the surrounding environment.

3.6. Central Tolerance to Human Tumor Antigens. Central tolerance to self-antigens is established in the thymus during T cell development, through the sequential steps of positive selection, which occurs in the thymic cortex and retains only T cells whose T cell receptor (TCR) can interact with self-MHC, and negative selection, which eliminates T cells that recognize self-peptides presented in the thymic medulla. Thus, the antitumor T cell repertoire present in the periphery is shaped by the ability of the thymic medulla to process and present tumor-associated antigenic peptides. T cells directed against mutated (and viral) antigens, which are not expressed by the thymic medulla, are not eliminated in the thymus and high affinity T cells to these antigens are therefore present in the periphery, probably explaining the prevalence of the spontaneous antitumor responses to mutated antigens [30, 43]. The presence in the blood of cancer patients of CTL directed against antigens encoded by cancer-germline genes or differentiation antigens indicates that natural immune tolerance against these antigens is either incomplete or absent. Interestingly, antigenic peptides encoded by *MAGE* genes are usually better produced by the immuno- or the

intermediate proteasomes than by the standard proteasome (Figure 4) [10, 159]. This contrasts with differentiation antigens, usually produced by the standard proteasome but not by the immuno- or the intermediate proteasomes (Figure 4). The exact proteasome content of thymic epithelial cells is not yet known; however, it was suggested to contain mostly immunoproteasome [160]. For differentiation antigens, the absence in the blood or tumor of cancer patients of T cells recognizing immunoproteasome-dependent peptides probably reflects the efficient central tolerance toward these antigens: T cells directed against immunoproteasome-dependent differentiation peptides do not survive thymic selection. The situation is more complex for antigens encoded by cancer-germline genes. As stated above, these antigens are usually processed by the immunoproteasome or the intermediate proteasomes, suggesting a lack of central tolerance to these antigens. It was initially considered that this lack of central tolerance resulted from the lack of expression of these genes in the thymus. However, Gotter et al. showed that a number of cancer-germline genes were expressed at low levels in medullary thymic epithelial cells (mTEC), which are the cells that present antigens for negative selection [161]. It was then considered that the low expression level of these genes in mTEC might result in partial tolerance, leading to the negative selection of high avidity T cells only. The antitumor T lymphocytes present in the blood of cancer patients would then derive from low avidity T cells that have escaped negative selection in the thymus. To confirm this concept, Huijbers et al. produced knockout mice for the murine cancer-germline gene *P1A* [162]. The expectation was that these mice should contain an unselected anti-P1A T cell repertoire containing high affinity T cells. Surprisingly, while the number of anti-P1A T cells was slightly higher in the P1A-KO mice, their repertoire was barely different from that of wild-type mice, indicating that there is only minimal tolerance against this antigen in WT mice, despite the detectable expression of P1A in mTEC [162]. The apparent lack of central tolerance to the P1A antigen could result from the inability of mTEC to process the P1A peptide. Identifying exactly the type of proteasome present in thymic epithelial cells should therefore shed a better light on the lack of central tolerance to cancer-germline genes and help identifying those antigens for which central tolerance is limited and against which T cells with a relatively high affinity should be found in the periphery.

4. Future Challenges for Cancer Immunotherapy

4.1. The Potential of Cancer Vaccines. Current developments based on the use of T cell stimulating antibodies or on the adoptive transfer of antitumor T cells have highlighted the power of cancer immunotherapy strategies [1–5, 163]. A significant proportion of the patients treated using these therapies shows remarkable clinical responses and some of these patients even appear disease-free several years after initiation of the treatment. Nevertheless, as discussed above, both therapies often lead to the development of strong autoimmune side effects that need to be controlled. Additionally, although the response to adoptive transfer and stimulating antibodies therapies shows great promises, a large number of patients still remain refractory to these treatments. Therapeutic cancer vaccines aim at specifically activating antitumor CTL already present in the blood or the tumor of cancer patients. They appear safer and better controlled, first because vaccination focuses specifically on the activation of anti-vaccine T cells and second because the T cells targeted by the vaccine have undergone thymic selection, a process that should minimize the occurrence of undesirable immune responses against self-antigens expressed by normal tissues. More specifically, antigenic peptides identified from mixed-tumor lymphocytes cultures, that is, by the *in vitro* stimulation with autologous tumor cells of T cells originating from cancer patients, should be the safest, as they generally target antigens against which a previous spontaneous response has been mounted, usually without any noticeable side effects [30]. On the other hand, the safety of antigens identified using the reverse immunology approach, that is, the *in vitro* priming of healthy donor or cancer patient T cells with pulsed dendritic cells, should be appropriately evaluated, as it is not always known whether these antigens can be safely targeted by the immune system.

No adverse events were observed in the cohorts of patients who were previously vaccinated using peptide, full-length protein or virus containing MAGE-1 or MAGE-3, even in the few responding patients [164–166]. Interestingly, following vaccination, patients who responded to the vaccine were shown to display a considerable enrichment of antitumor T cells in their metastases, when compared to anti-vaccine T cells, suggesting that activation of only a few anti-vaccine CTL can lead to the priming or reactivation of T cells recognizing tumor antigens unrelated to the vaccine [167, 168].

4.2. Challenges of Immunotherapy: The Tumor Escape and Immunosuppression. It seems now evident that most melanoma patients develop a spontaneous T cell response to their tumor [12, 168]. Although the presence of TIL at the tumor site is often associated with a good prognosis [169], in many cases, these TIL are overwhelmed by tumor development and they remain inactive and anergized at the tumor site probably explaining why, so far, vaccination of cancer-bearing patients only brought about 5 to 10% clinical responses [29]. The inability of antitumor lymphocytes to eliminate the tumor can be caused by a multiplicity of factors. One of these factors is the loss of those antigens that were initially recognized by T lymphocytes on tumors. This loss of antigen can result from genetic defects affecting either the antigenic peptide, the HLA molecule, or any of the proteins involved in the MHC class I processing machinery. A drastic example is the downregulation of the TAP transporter or tapasin, which is observed in a number of cancer types and affects the processing and presentation of a large number of antigenic peptides [170–174]. Cytokines such as IFNγ, which is produced by tumor-infiltrating lymphocytes or NK cells, can also modify the peptide repertoire expressed by tumors. Indeed, although IFNγ increases the presentation of a number of antigenic peptides by upregulating the expression of HLA class I heavy chains, β2m, TAP1, TAP2, or Tapasin, it also decreases the presentation of those peptides that are destroyed or

poorly produced by the standard proteasome [142, 149, 150]. This is true, for example, for peptides derived from melanoma differentiation antigens, which are processed by the standard proteasome but not by the immunoproteasome. Consequently, the following sequence of events might occur: during the initial phase of tumor infiltration by T cells, antitumor lymphocytes attack the tumor cells. The antigens recognized by these lymphocytes are probably mostly produced by standard or intermediate proteasomes, which are expressed by tumors at steady state. These T cells produce INFγ, which modifies the proteasome content of the surrounding tumor cells. As a result, tumor cells no longer express the standard-proteasome dependent antigens against which the immune response was initially mounted, and they start expressing immunoproteasome-dependent antigens potentially recognized by other antitumor T cells. During this whole process, under the selective pressure of antitumor CTL, the tumor can sometimes escape the immune response by losing the expression of genes that are necessary for the presentation of peptides to antitumor T cells. Additionally, the IFNγ produced by activated lymphocytes at the tumor site induces the expression of immunomodulatory molecules that dampen the immune response and inhibit the function of antitumor CTL. One of these immunomodulatory molecules is the enzyme indoleamine 2,3-dioxygenase (IDO), which creates an immunosuppressive environment by depleting tryptophan and releasing metabolites that inhibit T cell proliferation and function while promoting tumor survival and motility [175]. Another example is the programmed death ligand-1 (PD-L1), whose expression on tumor cells is also stimulated by IFNγ and whose engagement with its T cell receptor PD-1 leads to a decrease in T cell proliferation, cytokine production, and T cell adhesion [176]. In that context, reverting local immunosuppression by applying anti-PD1 or anti-PDL-1 neutralizing antibodies concomitantly to cancer vaccines might help maintaining the antitumor T cell responses effective. In preclinical models of melanoma and colon carcinoma, PD-1 blockade enhanced the effectiveness of granulocyte macrophage colony stimulating factor (GM-CSF)-secreting tumor cells immunotherapy by increasing survival of tumor-bearing mice and by enhancing antigen-specific immune responses [177]. In another model of murine melanoma, PD1 blockade combined to 4-1BB stimulation greatly improved vaccination with a recombinant human adenovirus expressing the dopachrome tautomerase antigen and resulted in a complete tumor regression [178]. Finally, another study showed that combination of both CTLA-4 and PD1 blocking antibodies with GM-CSF-expressing tumor cell vaccine also efficiently promoted tumor rejection in mouse models of colon carcinoma and ovarian cancer [179, 180]. This was associated with an increased proliferation, function, and tumor infiltration of tumor-specific CTL. On the other hand, suppressive Tregs were inhibited and found in lower number at the tumor site. These results highlight the potential of combination therapies to enhance the effectiveness of cancer vaccines.

4.3. Perspectives for Tumor Vaccines. Trying to overcome issues related to tumor escape and immunosuppression is the current challenge for the development of more efficient immunotherapeutic vaccines. To limit the possibilities of developing antigen-loss variants, cancer vaccines should be designed to activate T cells against a great variety of antigens. One should also favor vaccines targeting antigens that have a high tumor specificity and can be presented by tumors in any circumstance. In that regard, tumor antigens processed by both intermediate proteasomes and immunoproteasomes appear as the best choice, since intermediate proteasomes are found in most tumors at steady state while the immunoproteasome is prominent in tumors exposed to an inflammatory environment. Antigens that can still be expressed when components of the antigen processing machinery (such as TAP, tapasin, or ERAP) are affected should also be considered as relevant targets. TAP-independent antigenic peptides mostly include peptides derived from the signal sequences of ER-targeted proteins and can be released in the ER following cleavage by signal peptidase and signal peptide peptidase [181, 182]. Antigens that are specifically expressed in cells lacking components of the MHC class I processing machinery belong to a special category of antigens called T cell epitopes associated with impaired peptide processing (TEIPP) [183]. The absence of TEIPP antigens at the surface of TAP-positive cells results from the fact that these peptides are underrepresented in the ER lumen, when compared to peptides that are pumped in by TAP [184]. Interestingly and contrarily to what is observed in TAP-knockout mice, thymic epithelial cells from wild-type mice could not process the TEIPP peptides despite efficient expression of the parent protein in thymic cells [184]. As a consequence, negative selection of T cells recognizing such TEIPP antigens should not be achieved in wild-type animals, and TEIPP T cells are expected to display a higher avidity when compared to other antitumor T cells, which underwent negative selection [185]. Identification of novel tumor antigens presented in the absence of TAP or other components of the MHC class I machinery, therefore, represents a new avenue of exploration to overcome tumor escape strategies.

Additionally, the success of cancer vaccines will largely depend on their mode of delivery. Optimally, vaccines should elicit strong CTL responses. In that regard peptide-based or protein-based vaccines are not optimal. On the contrary, viral vector-based vaccines designed to express antigens of interest are very immunogenic as they were shown to activate both the innate and adaptive immune system. More specifically, viral based vaccines efficiently infect DC, which are required for the priming of antitumor CTL. Recombinant viral vectors were found to induce very strong CTL responses associated with antitumoral effects [186]. However, they usually require heterologous prime-boost protocols, where a different vector is used for the priming and the boost, to circumvent the negative effect of vector-specific neutralizing antibodies induced by some vectors after a single injection [186–190].

Finally, as discussed above, reverting local immunosuppression will be a key parameter for the progression of cancer immunotherapy. Indeed, patients responding to the antitumor vaccine might be those whose tumor displays the lowest degree of immunosuppression. The development of efficient inhibitors of immunomodulatory enzymes such as

IDO, tryptophan 2,3-dioxygenase (TDO), or arginase has become a major topic of research. Other immunosuppressive factors that could also be targeted are numerous. They include soluble factors such as transforming growth factor-β (TGF-β), interleukin 10, and galectins or suppressive cells found at the tumor site such as regulatory T cells, tolerogenic dendritic cells, or myeloid-derived suppressor cells. The concomitant delivery of new drugs blocking these immunosuppressive factors together with cancer vaccines, checkpoint blockade inhibitors, or adoptive T cell transfer might show great promises for future therapies.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The author is grateful to Professor Pierre Coulie and Professor Benoît Van den Eynde for critical reading and to Mrs. Julie Klein for her precious help in the preparation of this paper. She is supported by a grant from the Belgium Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming.

References

[1] F. S. Hodi, S. J. O'Day, D. F. McDermott et al., "Improved survival with ipilimumab in patients with metastatic melanoma," *The New England Journal of Medicine*, vol. 363, no. 8, pp. 711–723, 2010.

[2] C. Robert, L. Thomas, I. Bondarenko et al., "Ipilimumab plus dacarbazine for previously untreated metastatic melanoma," *The New England Journal of Medicine*, vol. 364, no. 26, pp. 2517–2526, 2011.

[3] S. L. Topalian, F. S. Hodi, J. R. Brahmer et al., "Safety, activity, and immune correlates of anti-PD-1 antibody in cancer," *The New England Journal of Medicine*, vol. 366, no. 26, pp. 2443–2454, 2012.

[4] S. L. Topalian, C. G. Drake, and D. M. Pardoll, "Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity," *Current Opinion in Immunology*, vol. 24, no. 2, pp. 207–212, 2012.

[5] J. R. Brahmer, S. S. Tykodi, L. Q. M. Chow et al., "Safety and activity of anti-PD-L1 antibody in patients with advanced cancer," *The New England Journal of Medicine*, vol. 366, no. 26, pp. 2455–2465, 2012.

[6] S. L. Topalian, M. Sznol, D. F. McDermott et al., "Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab," *Journal of Clinical Oncology*, vol. 32, no. 10, pp. 1020–1030, 2014.

[7] C. Lebbe, J. S. Weber, M. Maio et al., "Survival follow-up and ipilimumab retreatment of patients with advanced melanoma who received ipilimumab in prior phase II studies," *Annals of Oncology*, vol. 25, no. 11, pp. 2277–2284, 2014.

[8] C. Lurquin, A. van Pel, B. Mariamé et al., "Structure of the gene of tum- transplantation antigen P91A: the mutated exon encodes a peptide recognized with Ld by cytolytic T cells," *Cell*, vol. 58, no. 2, pp. 293–303, 1989.

[9] H.-G. Rammensee, K. Falk, and O. Rötzschke, "Peptides naturally presented by MHC class I molecules," *Annual Review of Immunology*, vol. 11, pp. 213–244, 1993.

[10] N. Vigneron and B. J. van den Eynde, "Insights into the processing of MHC class I ligands gained from the study of human tumor epitopes," *Cellular and Molecular Life Sciences*, vol. 68, no. 9, pp. 1503–1520, 2011.

[11] Y. Kawakami, P. F. Robbins, X. Wang et al., "Identification of new melanoma epitopes on melanosomal proteins recognized by tumor infiltrating T lymphocytes restricted by HLA-A1, -A2, and -A3 alleles," *Journal of Immunology*, vol. 161, no. 12, pp. 6985–6992, 1998.

[12] C. Germeau, W. Ma, F. Schiavetti et al., "High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens," *The Journal of Experimental Medicine*, vol. 201, no. 2, pp. 241–248, 2005.

[13] P. van der Bruggen, C. Traversari, P. Chomez et al., "A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma," *Science*, vol. 254, no. 5038, pp. 1643–1647, 1991.

[14] N. Vigneron, W. Ma, A. Michaux, and B. J. van den Eynde, "Identifying source proteins for mhc class I-presented peptides," *Methods in Molecular Biology*, vol. 960, pp. 187–207, 2013.

[15] V. Brichard, A. van Pel, T. Wolfel et al., "The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas," *The Journal of Experimental Medicine*, vol. 178, no. 2, pp. 489–495, 1993.

[16] W. Ma, C. Germeau, N. Vigneron et al., "Two new tumor-specific antigenic peptides encoded by gene MAGE-C2 and presented to cytolytic T lymphocytes by HLA-A2," *International Journal of Cancer*, vol. 109, no. 5, pp. 698–702, 2004.

[17] W. Ma, N. Vigneron, J. Chapiro et al., "A MAGE-C2 antigenic peptide processed by the immunoproteasome is recognized by cytolytic T cells isolated from a melanoma patient after successful immunotherapy," *International Journal of Cancer*, vol. 129, no. 10, pp. 2427–2434, 2011.

[18] N. Vigneron, V. Stroobant, B. J. van den Eynde, and P. van der Bruggen, "Database of T cell-defined human tumor antigens: the 2013 update," *Cancer immunity*, vol. 13, p. 15, 2013.

[19] N. Vigneron, A. Ooms, S. Morel, W. Ma, G. Degiovanni, and B. J. van den Eynde, "A peptide derived from melanocytic protein gp100 and presented by HLA-B35 is recognized by autologous cytolytic T lymphocytes on melanoma cells," *Tissue Antigens*, vol. 65, no. 2, pp. 156–162, 2005.

[20] Y. Tomita, K. Imai, S. Senju et al., "A novel tumor-associated antigen, cell division cycle 45-like can induce cytotoxic T-lymphocytes reactive to tumor cells," *Cancer Science*, vol. 102, no. 4, pp. 697–705, 2011.

[21] D. F. Hunt, R. A. Henderson, J. Shabanowitz et al., "characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry," *Science*, vol. 255, pp. 1261–1263, 1992.

[22] R. A. Henderson, H. Michel, K. Sakaguchi et al., "HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation," *Science*, vol. 255, no. 5049, pp. 1264–1266, 1992.

[23] A. L. Cox, J. Skipper, Y. Chen et al., "Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines," *Science*, vol. 264, no. 5159, pp. 716–719, 1994.

[24] M. Schirle, W. Keilholz, B. Weber et al., "Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach," *European Journal of Immunology*, vol. 30, no. 8, pp. 2216–2225, 2000.

[25] A. L. Zarling, J. M. Polefrone, A. M. Evans et al., "Identification of class I MHC-associated phosphopeptides as targets for cancer immunotherapy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 40, pp. 14889–14894, 2006.

[26] M. Cobbold, H. de la Peña, A. Norris et al., "MHC class I-associated phosphopeptides are the targets of memory-like immunity in leukemia," *Science Translational Medicine*, vol. 5, no. 203, Article ID 203ra125, 2013.

[27] J. C. A. Skipper, R. C. Hendrickson, P. H. Gulden et al., "An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins," *Journal of Experimental Medicine*, vol. 183, no. 2, pp. 527–534, 1996.

[28] A. Dalet, P. F. Robbins, V. Stroobant et al., "An antigenic peptide produced by reverse splicing and double asparagine deamidation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 29, pp. E323–E331, 2011.

[29] T. Boon, P. G. Coulie, B. J. van den Eynde, and P. van der Bruggen, "Human T cell responses against melanoma," *Annual Review of Immunology*, vol. 24, pp. 175–208, 2006.

[30] P. G. Coulie, B. J. van den Eynde, P. van der Bruggen, and T. Boon, "Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy," *Nature Reviews Cancer*, vol. 14, no. 2, pp. 135–146, 2014.

[31] D. Iacovides, S. Michael, C. Achilleos, and K. Strati, "Shared mechanisms in stemness and carcinogenesis: lessons from oncogenic viruses," *Frontiers in Cellular and Infection Microbiology*, vol. 3, article 66, 2013.

[32] M. J. P. Welters, G. G. Kenter, S. J. Piersma et al., "Induction of tumor-specific $CD4^+$ and $CD8^+$ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine," *Clinical Cancer Research*, vol. 14, no. 1, pp. 178–187, 2008.

[33] G. G. Kenter, M. J. P. Welters, A. R. P. M. Valentijn et al., "Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia," *The New England Journal of Medicine*, vol. 361, no. 19, pp. 1838–1847, 2009.

[34] P. G. Coulie, F. Lehmann, B. Lethé et al., "A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 17, pp. 7976–7980, 1995.

[35] Y. Kawakami, X. Wang, T. Shofuda et al., "Isolation of a new melanoma antigen, MART-2, containing a mutated epitope recognized by autologous tumor-infiltrating T lymphocytes," *Journal of Immunology*, vol. 166, no. 4, pp. 2871–2877, 2001.

[36] N. Vigneron, A. Ooms, S. Morel, G. Degiovanni, and B. J. Van Den Eynde, "Identification of a new peptide recognized by autologous cytolytic T lymphocytes on a human melanoma," *Cancer Immunity*, vol. 2, pp. 9–18, 2002.

[37] J. Huang, M. El-Gamil, M. E. Dudley, Y. F. Li, S. A. Rosenberg, and P. F. Robbins, "T cells associated with tumor regression recognize frameshifted products of the CDKN2A tumor suppressor gene locus and a mutated HLA class I gene product," *Journal of Immunology*, vol. 172, no. 10, pp. 6057–6064, 2004.

[38] M. Linnebacher, J. Gebert, W. Rudy et al., "Frameshift peptide-derived T-cell epitopes: a source of novel tumor-specific antigens," *International Journal of Cancer*, vol. 93, no. 1, pp. 6–11, 2001.

[39] T. Wölfel, M. Hauer, J. Schneider et al., "A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma," *Science*, vol. 269, no. 5228, pp. 1281–1284, 1995.

[40] P. F. Robbins, M. El-Gamil, Y. F. Li et al., "A mutated β-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes," *The Journal of Experimental Medicine*, vol. 183, no. 3, pp. 1185–1192, 1996.

[41] B. Rubinfeld, P. Robbins, M. El-Gamil, I. Albert, E. Porfiri, and P. Polakis, "Stabilization of β-catenin by genetic defects in melanoma cell lines," *Science*, vol. 275, no. 5307, pp. 1790–1792, 1997.

[42] S. Mandruzzato, F. Brasseur, G. Andry, T. Boon, and P. van der Bruggen, "A CASP-8 mutation recognized by cytolytic T lymphocytes on a human head and neck carcinoma," *The Journal of Experimental Medicine*, vol. 186, no. 5, pp. 785–793, 1997.

[43] V. Lennerz, M. Fatho, C. Gentilini et al., "The response of autologous T cells to a human melanoma is dominated by mutated neoantigens," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 44, pp. 16013–16018, 2005.

[44] M. K. Gjertsen, J. Bjørheim, I. Saeterdal, J. Myklebust, and G. Gaudernack, "Cytotoxic $CD4^+$ and $CD8^+$ T lymphocytes, generated by mutant p21-ras (12Val) peptide vaccination of a patient, recognize 12Val-dependent nested epitopes present within the vaccine peptide and kill autologous tumour cells carrying this mutation," *International Journal of Cancer*, vol. 72, no. 5, pp. 784–790, 1997.

[45] B. Linard, S. Bézieau, H. Benlalam et al., "A *ras*-mutated peptide targeted by CTL infiltrating a human melanoma lesion," *The Journal of Immunology*, vol. 168, no. 9, pp. 4802–4808, 2002.

[46] D. Ito, C. Visus, T. K. Hoffmann et al., "Immunological characterization of missense mutations occurring within cytotoxic T cell-defined p53 epitopes in HLA-A^*0201^+ squamous cell carcinomas of the head and neck," *International Journal of Cancer*, vol. 120, no. 12, pp. 2618–2624, 2007.

[47] P. Yotnda, H. Firat, F. Garcia-Pons et al., "Cytotoxic T cell response against the chimeric p210 BCR-ABL protein in patients with chronic myelogenous leukemia," *The Journal of Clinical Investigation*, vol. 101, no. 10, pp. 2290–2296, 1998.

[48] P. Yotnda, F. Garcia, M. Peuchmaur et al., "Cytotoxic T cell response against the chimeric ETV6-AML1 protein in childhood acute lymphoblastic leukemia," *Journal of Clinical Investigation*, vol. 102, no. 2, pp. 455–462, 1998.

[49] C. Lurquin, C. de Smet, F. Brasseur et al., "Two members of the human *MAGEB* gene family located in Xp21.3 are expressed in tumors of various histological origins," *Genomics*, vol. 46, no. 3, pp. 397–408, 1997.

[50] S. Lucas, C. De Smet, K. C. Arden et al., "Identification of a new MAGE gene with tumor-specific expression by representational difference analysis," *Cancer Research*, vol. 58, no. 4, pp. 743–752, 1998.

[51] P. Chomez, O. De Backer, M. Bertrand, E. De Plaen, T. Boon, and S. Lucas, "An overview of the MAGE gene family with the identification of all human members of the family," *Cancer Research*, vol. 61, no. 14, pp. 5544–5551, 2001.

[52] P. Boel, C. Wildmann, M. L. Sensi et al., "BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes," *Immunity*, vol. 2, no. 2, pp. 167–175, 1995.

[53] B. van den Eynde, B. van den Eynde, O. Peeters et al., "A new family of genes coding for an antigen recognized by autologous

cytolytic T lymphocytes on a human melanoma," *The Journal of Experimental Medicine*, vol. 182, no. 3, pp. 689–698, 1995.

[54] O. de Backer, K. C. Arden, M. Boretti et al., "Characterization of the GAGE genes that are expressed in various human cancers and in normal testis," *Cancer Research*, vol. 59, no. 13, pp. 3157–3165, 1999.

[55] Y.-T. Chen, M. J. Scanlan, U. Sahin et al., "A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 5, pp. 1914–1918, 1997.

[56] B. Lethé, S. Lucas, L. Michaux et al., "LAGE-1, a new gene with tumor specificity," *International Journal of Cancer*, vol. 76, no. 6, pp. 903–908, 1998.

[57] Ö. Türeci, U. Sahin, I. Schobert et al., "The SSX-2 gene, which is involved in the t(X;18) translocation of synovial sarcomas, codes for the human tumor antigen HOM-MEL-40," *Cancer Research*, vol. 56, no. 20, pp. 4766–4772, 1996.

[58] A. O. Gure, Ö. Türeci, U. Sahin et al., "SSX: a multigene family with several members transcribed in normal testis and human cancer," *International Journal of Cancer*, vol. 72, no. 6, pp. 965–971, 1997.

[59] E. de Plaen, K. Arden, C. Traversari et al., "Structure, chromosomal localization, and expression of 12 genes of the MAGE family," *Immunogenetics*, vol. 40, no. 5, pp. 360–369, 1994.

[60] M. A. Gama-sosa, V. A. Slagel, R. W. Trewyn et al., "The 5-methylcytosine content of DNA from human tumors," *Nucleic Acids Research*, vol. 11, no. 19, pp. 6883–6894, 1983.

[61] M. Ehrlich, M. A. Gama-Sosa, L.-H. Huang et al., "Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells," *Nucleic Acids Research*, vol. 10, no. 8, pp. 2709–2721, 1982.

[62] E. S. Diala, M. S. C. Cheah, D. Rowitch, and R. M. Hoffman, "Extent of DNA methylation in human tumor cells," *Journal of the National Cancer Institute*, vol. 71, no. 4, pp. 755–764, 1983.

[63] J. Weber, M. Salgaller, D. Samid et al., "Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-Aza-2′-deoxycytidine," *Cancer Research*, vol. 54, no. 7, pp. 1766–1771, 1994.

[64] C. de Smet, C. Lurquin, B. Lethé, V. Martelange, and T. Boon, "DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter," *Molecular and Cellular Biology*, vol. 19, no. 11, pp. 7327–7335, 1999.

[65] L. Sigalotti, E. Fratta, S. Coral et al., "Intratumor heterogeneity of cancer/testis antigens expression in human cutaneous melanoma is methylation-regulated and functionally reverted by 5-Aza-2′-deoxycytidine," *Cancer Research*, vol. 64, no. 24, pp. 9167–9171, 2004.

[66] G. G. Haas Jr., O. J. D'Cruz, and L. E. De Bault, "Distribution of human leukocyte antigen-ABC and -D/DR antigens in the unfixed human testis," *American Journal of Reproductive Immunology and Microbiology*, vol. 18, no. 2, pp. 47–51, 1988.

[67] R. A. Morgan, N. Chinnasamy, D. Abate-Daga et al., "Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy," *Journal of Immunotherapy*, vol. 36, no. 2, pp. 133–151, 2013.

[68] Y. Guilloux, S. Lucas, V. G. Brichard et al., "A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanomas is encoded by an intron sequence of the N-acetylglucosaminyltransferase V gene," *The Journal of Experimental Medicine*, vol. 183, no. 3, pp. 1173–1183, 1996.

[69] B. J. Van Den Eynde, B. Gaugler, M. Probst-Kepper et al., "A new antigen recognized by cytolytic T lymphocytes on a human kidney tumor results from reverse strand transcription," *Journal of Experimental Medicine*, vol. 190, no. 12, pp. 1793–1799, 1999.

[70] A. Moreau-Aubry, S. le Guiner, N. Labarrière, M.-C. Gesnel, F. Jotereau, and R. Breathnach, "A processed pseudogene codes for a new antigen recognized by a $CD8^+$ T cell clone on melanoma," *The Journal of Experimental Medicine*, vol. 191, no. 9, pp. 1617–1624, 2000.

[71] S. Ochsenreither, R. Majeti, T. Schmitt et al., "Cyclin-A1 represents a new immunogenic targetable antigen expressed in acute myeloid leukemia stem cells with characteristics of a cancer-testis antigen," *Blood*, vol. 119, no. 23, pp. 5492–5501, 2012.

[72] K. S. Hoek, N. C. Schlegel, O. M. Eichhoff et al., "Novel MITF targets identified using a two-step DNA microarray strategy," *Pigment Cell and Melanoma Research*, vol. 21, no. 6, pp. 665–676, 2008.

[73] T. Wölfel, A. van Pel, V. Brichard et al., "Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes," *European Journal of Immunology*, vol. 24, no. 3, pp. 759–764, 1994.

[74] Y. Kawakami, S. Eliyahu, C. Jennings et al., "Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with *in vivo* tumor regression," *Journal of Immunology*, vol. 154, no. 8, pp. 3961–3968, 1995.

[75] H. Zarour, C. De Smet, F. Lehmann et al., "The majority of autologous cytolytic T-Lymphocyte clones derived from peripheral blood lymphocytes of a melanoma patient recognize an antigenic peptide derived from gene pme117/gp100," *Journal of Investigative Dermatology*, vol. 107, no. 1, pp. 63–67, 1996.

[76] P. G. Coulie, V. Brichard, A. van Pel et al., "A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas," *The Journal of Experimental Medicine*, vol. 180, no. 1, pp. 35–42, 1994.

[77] Y. Kawakami, S. Eliyahu, K. Sakaguchi et al., "Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes," *Journal of Experimental Medicine*, vol. 180, no. 1, pp. 347–352, 1994.

[78] R.-F. Wang, M. R. Parkhurst, Y. Kawakami, P. F. Robbins, and S. A. Rosenberg, "Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen," *Journal of Experimental Medicine*, vol. 183, no. 3, pp. 1137–1140, 1996.

[79] R.-F. Wang, E. Appella, Y. Kawakami, X. Kang, and S. A. Rosenberg, "Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes," *The Journal of Experimental Medicine*, vol. 184, no. 6, pp. 2207–2216, 1996.

[80] M. J. W. Visseren, A. van Elsas, E. I. H. van der Voort et al., "CTL specific for the tyrosinase autoantigen can be induced from healthy donor blood to lyse melanoma cells," *Journal of Immunology*, vol. 154, no. 8, pp. 3991–3998, 1995.

[81] M. J. Pittet, D. Valmori, P. R. Dunbar et al., "High frequencies of naive Melan-A/MART-1-specific $CD8^+$ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals," *Journal of Experimental Medicine*, vol. 190, no. 5, pp. 705–715, 1999.

[82] J. J. Nordlund, J. M. Kirkwood, B. M. Forget, G. Milton, D. M. Albert, and A. B. Lerner, "Vitiligo in patients with metastatic melanoma: a good prognostic sign," *Journal of the American Academy of Dermatology*, vol. 9, no. 5, pp. 689–696, 1983.

[83] S. A. Rosenberg and D. E. White, "Vitiligo in patients with melanoma: normal tissue antigens can be targets for cancer immunotherapy," *Journal of Immunotherapy*, vol. 19, no. 1, pp. 81–84, 1996.

[84] C. Yee, J. A. Thompson, P. Roche et al., "Melanocyte destruction after antigen-specific immunotherapy of melanoma: direct evidence of T cell-mediated vitiligo," *Journal of Experimental Medicine*, vol. 192, no. 11, pp. 1637–1643, 2000.

[85] B. M. Olson, T. P. Frye, L. E. Johnson et al., "HLA-A2-restricted T-cell epitopes specific for prostatic acid phosphatase," *Cancer Immunology, Immunotherapy*, vol. 59, no. 6, pp. 943–953, 2010.

[86] P. Correale, K. Walmsley, C. Nieroda et al., "*In vitro* generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen," *Journal of the National Cancer Institute*, vol. 89, no. 4, pp. 293–300, 1997.

[87] S. Hammarström, "The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues," *Seminars in Cancer Biology*, vol. 9, no. 2, pp. 67–81, 1999.

[88] B. Gaugler, N. Brouwenstijn, V. Vantomme et al., "A new gene coding for an antigen recognized by autologous cytolytic T lymphocytes on a human renal carcinoma," *Immunogenetics*, vol. 44, no. 5, pp. 323–330, 1996.

[89] C. F. Barker and R. E. Billingham, "Immunologically privileged sites," *Advances in Immunology*, vol. 25, pp. 1–54, 1978.

[90] T. S. Griffith, T. Brunner, S. M. Fletcher, D. R. Green, and T. A. Ferguson, "Fas ligand-induced apoptosis as a mechanism of immune privilege," *Science*, vol. 270, no. 5239, pp. 1189–1192, 1995.

[91] D. Abi-Hanna, D. Wakefield, and S. Watkins, "HLA antigens in ocular tissues. I. *In vivo* expression in human eyes," *Transplantation*, vol. 45, no. 3, pp. 610–613, 1988.

[92] H. Ikeda, B. Lethé, F. Lehmann et al., "Characterization of an antigen that is recognized on a melanoma showing partial HLA loss by CTL expressing an NK inhibitory receptor," *Immunity*, vol. 6, no. 2, pp. 199–208, 1997.

[93] J. H. Kessler, N. J. Beekman, S. A. Bres-Vloemans et al., "Efficient identification of novel HLA-A∗0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis," *The Journal of Experimental Medicine*, vol. 193, no. 1, pp. 73–88, 2001.

[94] M. Schmitz, P. Diestelkoetter, B. Weigle et al., "Generation of survivin-specific $CD8^+$ T effector cells by dendritic cells pulsed with protein or selected peptides," *Cancer Research*, vol. 60, no. 17, pp. 4845–4849, 2000.

[95] S. M. Schmidt, K. Schag, M. R. Müller et al., "Survivin is a shared tumor-associated antigen expressed in a broad variety of malignancies and recognized by specific cytotoxic T cells," *Blood*, vol. 102, no. 2, pp. 571–576, 2003.

[96] M. Röpke, J. Hald, P. Guldberg et al., "Spontaneous human squamous cell carcinomas are killed by a human cytotoxic T lymphocyte clone recognizing a wild-type p53-derived peptide," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 25, pp. 14704–14707, 1996.

[97] A. M. Barfoed, T. R. Petersen, A. F. Kirkin, P. Thor Straten, M. H. Claesson, and J. Zeuthen, "Cytotoxic T-lymphocyte clones, established by stimulation with the HLA- A2 binding p5365-73 wild type peptide loaded on dendritic cells *in vitro*, specifically recognize and lyse HLA-A2 tumour cells overexpressing the p53 protein," *Scandinavian Journal of Immunology*, vol. 51, no. 2, pp. 128–133, 2000.

[98] M. H. Kraus, N. C. Popescu, S. C. Amsbaugh, and C. R. King, "Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms," *The EMBO Journal*, vol. 6, no. 3, pp. 605–610, 1987.

[99] D. J. Slamon, G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire, "Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene," *Science*, vol. 235, no. 4785, pp. 177–182, 1987.

[100] B. Fisk, T. L. Blevins, J. T. Wharton, and C. G. Ioannides, "Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines," *The Journal of Experimental Medicine*, vol. 181, no. 6, pp. 2109–2117, 1995.

[101] K. Inoue, H. Ogawa, Y. Sonoda et al., "Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia," *Blood*, vol. 89, no. 4, pp. 1405–1412, 1997.

[102] H. Ohminami, M. Yasukawa, and S. Fujita, "HLA class I-restricted lysis of leukemia cells by a $CD8^+$ cytotoxic T-lymphocyte clone specific for WT1 peptide," *Blood*, vol. 95, no. 1, pp. 286–293, 2000.

[103] A. M. Asemissen, U. Keilholz, S. Tenzer et al., "Identification of a highly immunogenic HLA-A01-binding T cell epitope of WT1," *Clinical Cancer Research*, vol. 12, no. 24, pp. 7476–7482, 2006.

[104] A. G. Chapuis, G. B. Ragnarsson, H. N. Nguyen et al., "Transferred WT1-reactive $CD8^+$ T cells can mediate antileukemic activity and persist in post-transplant patients," *Science Translational Medicine*, vol. 5, no. 174, Article ID 174ra27, 2013.

[105] P. F. Robbins, Y.-C. Lu, M. El-Gamil et al., "Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells," *Nature Medicine*, vol. 19, no. 6, pp. 747–752, 2013.

[106] Y.-C. Lu, X. Yao, Y. F. Li et al., "Mutated PPP1R3B is recognized by T cells used to treat a melanoma patient who experienced a durable complete tumor regression," *Journal of Immunology*, vol. 190, no. 12, pp. 6034–6042, 2013.

[107] N. van Baren, J. F. Baurain, F. Brasseur, and P. G. Coulie, "Melanoma antigens recognized by T lymphocytes," in *Targeted Therapeutics in Melanoma*, T. F. Gajewski and F. S. Hodi, Eds., Current Clinical Oncology, pp. 187–205, Springer Science+Business Media, LLC, 2012.

[108] L. A. Johnson, R. A. Morgan, M. E. Dudley et al., "Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen," *Blood*, vol. 114, no. 3, pp. 535–546, 2009.

[109] C. H. J. Lamers, S. Sleijfer, A. G. Vulto et al., "Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience," *Journal of Clinical Oncology*, vol. 24, no. 13, pp. e20–e22, 2006.

[110] M. R. Parkhurst, J. C. Yang, R. C. Langan et al., "T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis," *Molecular Therapy*, vol. 19, no. 3, pp. 620–626, 2011.

[111] R. A. Morgan, J. C. Yang, M. Kitano, M. E. Dudley, C. M. Laurencot, and S. A. Rosenberg, "Case report of a serious adverse event following the administration of t cells transduced with a chimeric antigen receptor recognizing ERBB2," *Molecular Therapy*, vol. 18, no. 4, pp. 843–851, 2010.

[112] B. J. Cameron, A. B. Gerry, J. Dukes et al., "Identification of a titin-derived HLA-A1-presented peptide as a cross-reactive

target for engineered MAGE A3-directed T cells," *Science Translational Medicine*, vol. 5, no. 197, Article ID 197ra103, 2013.

[113] G. P. Linette, E. A. Stadtmauer, M. V. Maus et al., "Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma," *Blood*, vol. 122, no. 6, pp. 863–871, 2013.

[114] S. Kageyama, T. J. Tsomides, Y. Sykulev, and H. N. Eisen, "Variations in the number of peptide-MHC class I complexes required to activate cytotoxic T cell responses," *Journal of Immunology*, vol. 154, no. 2, pp. 567–576, 1995.

[115] N. Vigneron and B. van den Eynde, "Proteasome subtypes and regulators in the processing of antigenic peptides presented by class I molecules of the major histocompatibility complex," *Biomolecules*, vol. 4, no. 4, pp. 994–1025, 2014.

[116] M. T. Michalek, E. P. Grant, C. Gramm, A. L. Goldberg, and K. L. Rock, "A role for the ubiquitin-dependent proteolytic pathway in MHC class I- restricted antigen presentation," *Nature*, vol. 363, no. 6429, pp. 552–554, 1993.

[117] K. L. Rock, C. Gramm, L. Rothstein et al., "Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules," *Cell*, vol. 78, no. 5, pp. 761–771, 1994.

[118] S. Morel, F. Lévy, O. Burlet-Schiltz et al., "Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells," *Immunity*, vol. 12, no. 1, pp. 107–117, 2000.

[119] S. Ottaviani, Y. Zhang, T. Boon, and P. van der Bruggen, "A MAGE-1 antigenic peptide recognized by human cytolytic T lymphocytes on HLA-A2 tumor cells," *Cancer Immunology, Immunotherapy*, vol. 54, no. 12, pp. 1214–1220, 2005.

[120] N. Parmentier, V. Stroobant, D. Colau et al., "Production of an antigenic peptide by insulin-degrading enzyme," *Nature Immunology*, vol. 11, no. 5, pp. 449–454, 2010.

[121] J. Löwe, D. Stock, B. Jap, P. Zwickl, W. Baumeister, and R. Huber, "Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution," *Science*, vol. 268, no. 5210, pp. 533–539, 1995.

[122] M. Groll, L. Ditzel, J. Löwe et al., "Structure of 20S proteasome from yeast at 2.4 Å resolution," *Nature*, vol. 386, no. 6624, pp. 463–471, 1997.

[123] E. Seemuller, A. Lupas, D. Stock, J. Lowe, R. Huber, and W. Baumeister, "Proteasome from *Thermoplasma acidophilum*: a threonine protease," *Science*, vol. 268, no. 5210, pp. 579–582, 1995.

[124] M. Groll and R. Huber, "Substrate access and processing by the 20S proteasome core particle," *The International Journal of Biochemistry & Cell Biology*, vol. 35, no. 5, pp. 606–616, 2003.

[125] W. Heinemeyer, A. Cruhler, V. Mohrle, Y. Mahe, and D. H. Wolf, "PRE2, highly homologous to the human major histocompatibility complex- linked Ring10 gene, codes for a yeast proteasome subunit necessary for chymotryptic activity and degradation of ubiquitinated proteins," *The Journal of Biological Chemistry*, vol. 268, no. 7, pp. 5115–5120, 1993.

[126] C. Enenkel, "PRE3, highly homologous to the human major histocompatibility complex-linked LMP2 (RING12) gene, codes for a yeast proteasome subunit necessary for the peptidylglutamyl-peptide hydrolyzing activity," *FEBS Letters*, vol. 341, no. 2-3, pp. 193–196, 1994.

[127] W. Heinemeyer, M. Fischer, T. Krimmer, U. Stachon, and D. H. Wolf, "The active sites of the eukaryotic 20 S proteasome and their involvement in subunit precursor processing," *Journal of Biological Chemistry*, vol. 272, no. 40, pp. 25200–25209, 1997.

[128] T. P. Dick, A. K. Nussbaum, M. Deeg et al., "Contribution of proteasomal β-subunits to the cleavage of peptide substrates analyzed with yeast mutants," *The Journal of Biological Chemistry*, vol. 273, no. 40, pp. 25637–25646, 1998.

[129] R. Gueckel, C. Enenkel, D. H. Wolf, and W. Hilt, "Mutations in the yeast proteasome β-type subunit Pre3 uncover position-dependent effects on proteasomal peptidase activity and in vivo function," *Journal of Biological Chemistry*, vol. 273, no. 31, pp. 19443–19452, 1998.

[130] U. Salzmann, S. Kral, B. Braun et al., "Mutational analysis of subunit iβ2 (MECL-1) demonstrates conservation of cleavage specificity between yeast and mammalian proteasomes," *FEBS Letters*, vol. 454, no. 1-2, pp. 11–15, 1999.

[131] A. K. Nussbaum, T. P. Dick, W. Keilholz et al., "Cleavage motifs of the yeast 20S proteasome β subunits deduced from digests of enolase 1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 21, pp. 12504–12509, 1998.

[132] R. E. M. Toes, A. K. Nussbaum, S. Degermann et al., "Discrete cleavage motifs of constitutive and immunoproteasomes revealed by quantitative analysis of cleavage products," *The Journal of Experimental Medicine*, vol. 194, no. 1, pp. 1–12, 2001.

[133] N. Vigneron, V. Stroobant, J. Chapiro et al., "An antigenic peptide produced by peptide splicing in the proteasome," *Science*, vol. 304, no. 5670, pp. 587–590, 2004.

[134] K.-I. Hanada, J. W. Yewdell, and J. C. Yang, "Immune recognition of a human renal cancer antigen through post-translational protein splicing," *Nature*, vol. 427, no. 6971, pp. 252–256, 2004.

[135] E. H. Warren, N. J. Vigneron, M. A. Gavin et al., "An antigen produced by splicing of noncontiguous peptides in the reverse order," *Science*, vol. 313, no. 5792, pp. 1444–1447, 2006.

[136] A. Dalet, N. Vigneron, V. Stroobant, K.-I. Hanada, and B. J. van den Eynde, "Splicing of distant peptide fragments occurs in the proteasome by transpeptidation and produces the spliced antigenic peptide derived from fibroblast growth factor-5," *Journal of Immunology*, vol. 184, no. 6, pp. 3016–3024, 2010.

[137] A. Michaux, P. Larrieu, V. Stroobant et al., "A spliced antigenic peptide comprising a single spliced amino acid is produced in the proteasome by reverse splicing of a longer peptide fragment followed by trimming," *The Journal of Immunology*, vol. 192, no. 4, pp. 1962–1971, 2014.

[138] M. Mishto, A. Goede, K. T. Taube et al., "Driving forces of proteasome-catalyzed peptide splicing in yeast and humans," *Molecular and Cellular Proteomics*, vol. 11, no. 10, pp. 1008–1023, 2012.

[139] T. A. Griffin, D. Nandi, M. Cruz et al., "Immunoproteasome assembly: cooperative incorporation of interferon γ (IFN-γ)-inducible subunits," *Journal of Experimental Medicine*, vol. 187, no. 1, pp. 97–104, 1998.

[140] J. Driscoll, M. G. Brown, D. Finley, and J. J. Monaco, "MHC-linked LMP gene products specifically alter peptidase activities of the proteasome," *Nature*, vol. 365, no. 6443, pp. 262–264, 1993.

[141] M. Gaczynska, K. L. Rock, and A. L. Goldberg, "γ-Interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes," *Nature*, vol. 365, no. 6443, pp. 264–267, 1993.

[142] B. Guillaume, J. Chapiro, V. Stroobant et al., "Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 43, pp. 18599–18604, 2010.

[143] H. J. Fehling, W. Swat, C. Laplace et al., "MHC class I expression in mice lacking the proteasome subunit LMP-7," *Science*, vol. 265, no. 5176, pp. 1234–1237, 1994.

[144] L. van Kaer, P. G. Ashton-Rickardt, M. Eichelberger et al., "Altered peptidase and viral-specific T cell response in LMP2 mutant mice," *Immunity*, vol. 1, no. 7, pp. 533–541, 1994.

[145] E. Z. Kincaid, J. W. Che, I. York et al., "Mice completely lacking immunoproteasomes show major changes in antigen presentation," *Nature Immunology*, vol. 13, no. 2, pp. 129–135, 2012.

[146] B. Boes, H. Hengel, T. Ruppert, G. Multhaup, U. H. Koszinowski, and P.-M. Kloetzel, "Interferon γ stimulation modulates the proteolytic activity and cleavage site preference of 20S mouse proteasomes," *The Journal of Experimental Medicine*, vol. 179, no. 3, pp. 901–909, 1994.

[147] D. de Verteuil, T. L. Muratore-Schroeder, D. P. Granados et al., "Deletion of immunoproteasome subunits imprints on the transcriptome and has a broad impact on peptides presented by major histocompatibility complex I molecules," *Molecular and Cellular Proteomics*, vol. 9, no. 9, pp. 2034–2047, 2010.

[148] E. S. Schultz, J. Chapiro, C. Lurquin et al., "The production of a new MAGE-3 peptide presented to cytolytic T lymphocytes by HLA-B40 requires the immunoproteasome," *The Journal of Experimental Medicine*, vol. 195, no. 4, pp. 391–399, 2002.

[149] J. Chapiro, S. Claverol, F. Piette et al., "Destructive cleavage of antigenic peptides either by the immunoproteasome or by the standard proteasome results in differential antigen presentation," *The Journal of Immunology*, vol. 176, no. 2, pp. 1053–1061, 2006.

[150] B. Guillaume, V. Stroobant, M.-P. Bousquet-Dubouch et al., "Analysis of the processing of seven human tumor antigens by intermediate proteasomes," *The Journal of Immunology*, vol. 189, no. 7, pp. 3538–3547, 2012.

[151] A. Dalet, V. Stroobant, N. Vigneron, and B. J. Van Den Eynde, "Differences in the production of spliced antigenic peptides by the standard proteasome and the immunoproteasome," *European Journal of Immunology*, vol. 41, no. 1, pp. 39–46, 2011.

[152] B. Dahlmann, T. Ruppert, L. Kuehn, S. Merforth, and P.-M. Kloetzel, "Different proteasome subtypes in a single tissue exhibit different enzymatic properties," *Journal of Molecular Biology*, vol. 303, no. 5, pp. 643–653, 2000.

[153] D. J. Kingsbury, T. A. Griffin, and R. A. Colbert, "Novel propeptide function in 20 S proteasome assembly influences β subunit composition," *Journal of Biological Chemistry*, vol. 275, no. 31, pp. 24156–24162, 2000.

[154] M. De, K. Jayarapu, L. Elenich, J. J. Monaco, R. A. Colbert, and T. A. Griffin, "β2 subunit propeptides influence cooperative proteasome assembly," *The Journal of Biological Chemistry*, vol. 278, no. 8, pp. 6153–6159, 2003.

[155] U. Seifert, C. Marañón, A. Shmueli et al., "An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope," *Nature Immunology*, vol. 4, no. 4, pp. 375–379, 2003.

[156] S. Guil, M. Rodríguez-Castro, F. Aguilar, E. M. Villasevil, L. C. Antón, and M. Del Val, "Need for tripeptidyl-peptidase II in major histocompatibility complex class I viral antigen processing when proteasomes are detrimental," *Journal of Biological Chemistry*, vol. 281, no. 52, pp. 39925–39934, 2006.

[157] J. H. Kessler, S. Khan, U. Seifert et al., "Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes," *Nature Immunology*, vol. 12, no. 1, pp. 45–53, 2011.

[158] X. Z. Shen, S. Billet, C. Lin et al., "The carboxypeptidase ACE shapes the MHC class I peptide repertoire," *Nature Immunology*, vol. 12, no. 11, pp. 1078–1085, 2011.

[159] N. Vigneron and B. J. van den Eynde, "Proteasome subtypes and the processing of tumor antigens: increasing antigenic diversity," *Current Opinion in Immunology*, vol. 24, no. 1, pp. 84–91, 2012.

[160] A. Nil, E. Firat, V. Sobek, K. Eichmann, and G. Niedermann, "Expression of housekeeping and immunoproteasome subunit genes is differentially regulated in positively and negatively selecting thymic stroma subsets," *European Journal of Immunology*, vol. 34, no. 10, pp. 2681–2689, 2004.

[161] J. Gotter, B. Brors, M. Hergenhahn, and B. Kyewski, "Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters," *The Journal of Experimental Medicine*, vol. 199, no. 2, pp. 155–166, 2004.

[162] I. J. Huijbers, S. M. Soudja, C. Uyttenhove et al., "Minimal tolerance to a tumor antigen encoded by a cancer-germline gene," *Journal of Immunology*, vol. 188, no. 1, pp. 111–121, 2012.

[163] J. D. Wolchok, B. Neyns, G. Linette et al., "Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study," *The Lancet Oncology*, vol. 11, no. 2, pp. 155–164, 2010.

[164] W. H. Kruit, N. van Baren, Y. Humblet et al., "Phase I/II study of vaccination with MAGE-3 protein plus immunological adjuvant SB AS-2 in HLA Class I selected patients with MAGE-3 positive tumors," *Proceedings of the American Society of Clinical Oncology*, vol. 19, abstract 1882, p. 479a, 2000.

[165] W. H. J. Kruit, H. H. van Ojik, V. G. Brichard et al., "Phase 1/2 study of subcutaneous and intradermal immunization with a recombinant MAGE-3 protein in patients with detectable metastatic melanoma," *International Journal of Cancer*, vol. 117, no. 4, pp. 596–604, 2005.

[166] N. van Baren, M.-C. Bonnet, B. Dréno et al., "Tumoral and immunologic response after vaccination of melanoma patients with an ALVAC virus encoding MAGE antigens recognized by T cells," *Journal of Clinical Oncology*, vol. 23, no. 35, pp. 9008–9021, 2005.

[167] J. Carrasco, A. van Pel, B. Neyns et al., "Vaccination of a melanoma patient with mature dendritic cells pulsed with MAGE-3 peptides triggers the activity of nonvaccine anti-tumor cells," *Journal of Immunology*, vol. 180, no. 5, pp. 3585–3593, 2008.

[168] C. Lurquin, B. Lethé, E. de Plaen et al., "Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen," *The Journal of Experimental Medicine*, vol. 201, no. 2, pp. 249–257, 2005.

[169] I. S. van Houdt, B. J. R. Sluijter, L. M. Moesbergen et al., "Favorable outcome in clinically stage II melanoma patients is associated with the presence of activated tumor infiltrating T-lymphocytes and preserved MHC class I antigen expression," *International Journal of Cancer*, vol. 123, no. 3, pp. 609–615, 2008.

[170] M. J. Maeurer, S. M. Gollin, D. Martin et al., "Tumor escape from immune recognition: lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen," *The Journal of Clinical Investigation*, vol. 98, no. 7, pp. 1633–1641, 1996.

[171] J. Kamarashev, S. Ferrone, B. Seifert et al., "TAP1 down-regulation in primary melanoma lesions: an independent marker of poor prognosis," *International Journal of Cancer*, vol. 95, no. 1, pp. 23–28, 2001.

[172] M. Vitale, R. Rezzani, L. Rodella et al., "HLA class I antigen and transporter associated with antigen processing (TAP1 and

TAP2) down-regulation in high-grade primary breast carcinoma lesions," *Cancer Research*, vol. 58, no. 4, pp. 737–742, 1998.

[173] T. Ogino, H. Shigyo, H. Ishii et al., "HLA class I antigen down-regulation in primary laryngeal squamous cell carcinoma lesions as a poor prognostic marker," *Cancer Research*, vol. 66, no. 18, pp. 9281–9289, 2006.

[174] N. Vigneron, D. R. Peaper, R. M. Leonhardt, and P. Cresswell, "Functional significance of tapasin membrane association and disulfide linkage to ERp57 in MHC class I presentation," *European Journal of Immunology*, vol. 39, no. 9, pp. 2371–2376, 2009.

[175] M. Platten, W. Wick, and B. J. van den Eynde, "Tryptophan catabolism in cancer: beyond IDO and tryptophan depletion," *Cancer Research*, vol. 72, no. 21, pp. 5435–5440, 2012.

[176] A. Pedoeem, I. Azoulay-Alfaguter, M. Strazza, G. J. Silverman, and A. Mor, "Programmed death-1 pathway in cancer and autoimmunity," *Clinical Immunology*, vol. 153, no. 1, pp. 145–152, 2014.

[177] B. Li, M. Vanroey, C. Wang, T.-H. T. Chen, A. Korman, and K. Jooss, "Anti-programmed death-1 synergizes with granulocyte macrophage colony-stimulating factor-secreting tumor cell immunotherapy providing therapeutic benefit to mice with established tumors," *Clinical Cancer Research*, vol. 15, no. 5, pp. 1623–1634, 2009.

[178] A. J. R. McGray, D. Bernard, R. Hallett et al., "Combined vaccination and immunostimulatory antibodies provides durable cure of murine melanoma and induces transcriptional changes associated with positive outcome in human melanoma patients," *OncoImmunology*, vol. 1, no. 4, pp. 419–431, 2012.

[179] J. Duraiswamy, G. J. Freeman, and G. Coukos, "Therapeutic PD-1 pathway blockade augments with other modalities of immunotherapy T-cell function to prevent immune decline in ovarian cancer," *Cancer Research*, vol. 73, no. 23, pp. 6900–6912, 2013.

[180] J. Duraiswamy, K. M. Kaluza, G. J. Freeman, and G. Coukos, "Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors," *Cancer Research*, vol. 73, no. 12, pp. 3591–3603, 2013.

[181] C. Wölfel, I. Drexler, A. Van Pel et al., "Transporter (TAP)- and proteasome-independent presentation of a melanoma-associated tyrosinase epitope," *International Journal of Cancer*, vol. 88, no. 3, pp. 432–438, 2000.

[182] F. El Hage, V. Stroobant, I. Vergnon et al., "Preprocalcitonin signal peptide generates a cytotoxic T lymphocyte-defined tumor epitope processed by a proteasome-independent pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 29, pp. 10119–10124, 2008.

[183] C. C. Oliveira and T. van Hall, "Importance of TAP-independent processing pathways," *Molecular Immunology*, vol. 55, no. 2, pp. 113–116, 2013.

[184] C. C. Oliveira, B. Querido, M. Sluijter, J. Derbinski, S. H. van der Burg, and T. van Hall, "Peptide transporter TAP mediates between competing antigen sources generating distinct surface MHC class I peptide repertoires," *European Journal of Immunology*, vol. 41, no. 11, pp. 3114–3124, 2011.

[185] U. J. E. Seidel, C. C. Oliveira, M. H. Lampen, and T. Van Hall, "A novel category of antigens enabling CTL immunity to tumor escape variants: Cinderella antigens," *Cancer Immunology, Immunotherapy*, vol. 61, no. 1, pp. 119–125, 2012.

[186] T. I. Näslund, C. Uyttenhove, E. K. L. Nordström et al., "Comparative prime-boost vaccinations using semliki forest virus, adenovirus, and ALVAC vectors demonstrate differences in the generation of a protective central memory CTL response against the P815 tumor," *Journal of Immunology*, vol. 178, no. 11, pp. 6761–6769, 2007.

[187] S. J. Dunachie and A. V. S. Hill, "Prime-boost strategies for malaria vaccine development," *The Journal of Experimental Biology*, vol. 206, part 21, pp. 3771–3779, 2003.

[188] A. C. Moore and A. V. S. Hill, "Progress in DNA-based heterologous prime-boost immunization strategies for malaria," *Immunological Reviews*, vol. 199, pp. 126–143, 2004.

[189] A. J. McMichael, "HIV vaccines," *Annual Review of Immunology*, vol. 24, no. 1, pp. 227–255, 2006.

[190] C. Larocca and J. Schlom, "Viral vector-based therapeutic cancer vaccines," *Cancer Journal*, vol. 17, no. 5, pp. 359–371, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 830847, 11 pages
http://dx.doi.org/10.1155/2014/830847

Research Article

Increased Sialylation of Anti-Thomsen-Friedenreich Antigen (CD176) Antibodies in Patients with Gastric Cancer: A Diagnostic and Prognostic Potential

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Received 21 July 2014; Revised 18 August 2014; Accepted 18 August 2014; Published 4 September 2014

Academic Editor: Anshu Agrawal

Aim. To study whether alterations in the sialylation of antibodies (Ab) specific to the Thomsen-Friedenreich (TF) glycotope have a diagnostic and prognostic potential in gastric cancer. *Methods*. Serum samples were taken from patients with gastric carcinoma ($n = 142$) and controls ($n = 61$). The level of TF-specific antibodies and their sialylation was detected using ELISA with synthetic TF-polyacrylamide conjugate as antigen and sialic acid-specific *Sambucus nigra agglutinin* (SNA). *Results*. The level of TF-specific IgM was significantly decreased in cancer compared with controls ($P \leq 0.001$). Cancer patients showed a higher level of SNA binding to anti-TF IgM and IgA ($P \leq 0.001$) irrespective of disease stage, tumor morphology, and gender. Changes in the SNA/Ab index demonstrated moderate sensitivity (66–71%) and specificity (60–73%) for stomach cancer. The best diagnostic accuracy (100%) was achieved in 29% patients with high SNA binding and low anti-TF IgM level. This subset of patients demonstrated the poorest survival. *Conclusion*. Our findings are the first evidence that the increased sialylation of TF-specific Abs combined with a low level of anti-TF IgM is strongly linked to gastric cancer and patients survival, which can be used as a novel biomarker for cancer detection and prognosis.

1. Introduction

Early detection is vital for an effective treatment of cancer. The discovery and characterization of new easily applied sensitive and specific cancer biomarkers are promising ways for further success in early cancer diagnostics, patient monitoring, and prognostics. The altered glycosylation observed in cancer cells leads to the expression of modified tumor-associated glycans (TAG) such as Thomsen-Friedenreich antigen (Galβ1-3GalNAcα/β-O-Ser/Thr; TF, CD176) that may be autoimmunogenic and may be recognized by autoantibodies [1–6]. TAG are considered as promising targets for cancer immunotherapy [6–8]. The TF glycotope overexpression observed in the majority of adenocarcinomas and the reduced level of anti-TF antibodies are associated with more aggressive tumors and the induction of invasion, cancer surveillance mechanisms, and patients survival rate [3, 7, 9–13]. The TF antigen seems to play a crucial role in the adhesion of cancer cells to the endothelium through the interaction with galectin-3, thereby promoting metastases [14, 15]. In cancer patients, an abnormal glycosylation pattern has been demonstrated for many circulating glycoconjugates [2, 4, 16–18], including immunoglobulins which have a set of glycoforms differing in number, type, and site of oligosaccharide attachment [19]. It is now clear that the N-glycans of the Fc-fragment strongly influence IgG-Fcγ receptor interactions and thus the Fc-mediated effector mechanisms [20–22].

Appreciable amounts of TF-specific antibodies of different isotypes are present in normal human serum. Their level is decreased in patients with cancer although there are large interindividual variations [1, 3]. Little attention has been paid so far to the glycosylation of naturally-occurring TF-specific antibodies. Recently, we reported that the sialic acid specific SNA lectin reactivity of anti-TF IgG determined in the total IgG purified from the serum of patients with stomach cancer was significantly decreased compared to that of healthy blood donors and patients with nonmalignant gastric diseases [23]. As detected by LC-ESI-MS, the sialylation of the total_IgG

Table 1: Characteristics of the subjects tested.

Group	*n*	Males	Females	M/F	Median age (range), yr
Donors	**31**	13	18	0.72	53.6 (31–70)
Benign diseases group[1]	**30**	16	14	1.14	65.0 (44–76)
Noncancer[2]	**61**	29	32	0.91	59.5 (31–76)
Cancer patients Stages 1–4	**142**	82	60	1.36	69.2 (25–84)
Stage 1	**39**	19	20	0.95	66.0 (28–84)
Stage 2	**32**	18	14	1.28	66.5 (46–80)
Stage 3	**51**	30	21	1.42	67.0 (37–76)
Stage 4	**20**	15	5	3.0	65.0 (49–81)

[1]A group of patients with chronic gastric diseases: peptic ulcer disease, $n = 9$; chronic gastritis, $n = 11$; and atrophic gastritis, $n = 10$. [2]A combined group of donors and patients with nonmalignant stomach diseases. The number of subjects examined using a particular method is given in the corresponding section of the results.

Fc glycan was also found to be much less pronounced in cancer patients [24]. These findings prompted us to further investigate whether the sialylation of anti-TF Abs of various isotypes reveals cancer-associated changes that could be used as a biomarker of gastric cancer. The *Sambucus nigra* agglutinin (SNA) directed against glycans with the terminal α2,6-linked sialic acid has been shown to bind mostly to Fab glycans that, in contrast to Fc glycans, are fully sialylated and strongly SNA reactive [25–28].

In the present study, we show that, in contrast to the anti-TF IgG or Fc glycans detected in the purified total IgG samples, the sialylation of TF-specific IgM and IgA antibodies is significantly increased in patients with gastric cancer already in the early stages of the disease. The combined analysis of the anti-TF IgM antibody level and SNA reactivity revealed its promising diagnostic (ACC = 69%) and prognostic potential. Moreover, using the further stratification of patients by these two parameters, we were able to diagnose gastric cancer in 29% of patients with 100% accuracy, irrespective of cancer stage, tumor morphology, or gender. In addition, the poor survival of cancer patients with a low level of TF-IgM and high SNA reactivity of TF-specific antibodies was demonstrated.

2. Material and Methods

2.1. Subjects. Serum samples were taken from healthy blood donors, patients with benign stomach diseases, and individuals with histologically verified gastric carcinoma (Table 1). The investigation was carried out in accordance with the ICH GCP Standards and approved by the Tallinn Medical Research Ethics Committee, Estonia. A written informed consent was obtained from each subject. Tumor staging and morphology were based on the histopathological (pTNM) classification of malignant tumors and evaluated according to the system of Lauren 1965 [29] as an intestinal or diffuse type of tumor growth. The serum samples were stored in aliquots at −20°C until use.

2.2. The TF-Specific Antibody Assay. The level of anti-TF IgG, IgM, and IgA was determined by the enzyme-linked immunosorbent assay (ELISA) as described elsewhere [12], with minor modifications. Briefly, the plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with a synthetic TF-polyacrylamide conjugate (TF-PAA, Lectinity, Russia, 10 mol% of carbohydrate) in the carbonate buffer, pH 9.6. After the overnight incubation, triple washing and blocking with a Superblock solution (Pierce, USA) for 15 min at 25°C the serum samples diluted 1 : 50 in PBS-0.05% Tween (Tw) were applied for 1.5 h at 25°C. After the subsequent washing with PBS-Tw, the level of bound anti-TF Ab was determined using the alkaline phosphatase conjugated goat anti-human IgG, IgM (Sigma, USA), or IgA (Dako, USA) and p-nitrophenylphosphate disodium hexahydrate (Sigma, USA). The absorbance values were read at 492 nm (Tecan Reader, Austria). The optical density value (O.D.) of control wells (blank: the Superblock solution instead of TF-PAA) was subtracted from that of Ab-coated wells and each sample was analysed in duplicate.

2.3. The SNA Lectin Reactivity of TF-Specific Antibodies. The lectin reactivity of TF glycotope specific antibodies was measured in a similar way, except that the binding of the neuraminic acid (sialic acid) specific *Sambucus nigra* agglutinin (SNA) to the absorbed anti-TF antibodies was determined as described by Kodar et al. [23]. The biotinylated SNA (Vector Laboratories Inc., USA) in 10 mmol/L Hepes, 0.15 mol/L NaCl, 0.1 mmol/L $CaCl_2$, and pH 7.5 was applied at a concentration of 5 μg/mL for 1.5 h at 25°C. The bound lectin was detected with a streptavidin-alkaline phosphatase conjugate (Dako, USA) and p-nitrophenylphosphate (Sigma, USA). The optical density value (O.D.) of control wells (no sample) was subtracted from that of Ab-coated wells to determine the lectin binding. Each sample was analysed in duplicate. The value of the SNA binding to all TF-specific Abs and the ratio of SNA binding to TF-specific IgG, IgM, and IgA level (SNA/Ig index) were determined.

2.4. Statistical Analysis. Comparisons between the groups were made using the nonparametric Mann-Whitney U test for unpaired data (or Student's t-test, where appropriate), the discriminant analysis, and the Pearson two-tailed correlation. The survival of cancer patients with weak and strong response was analysed by the Kaplan-Meier method,

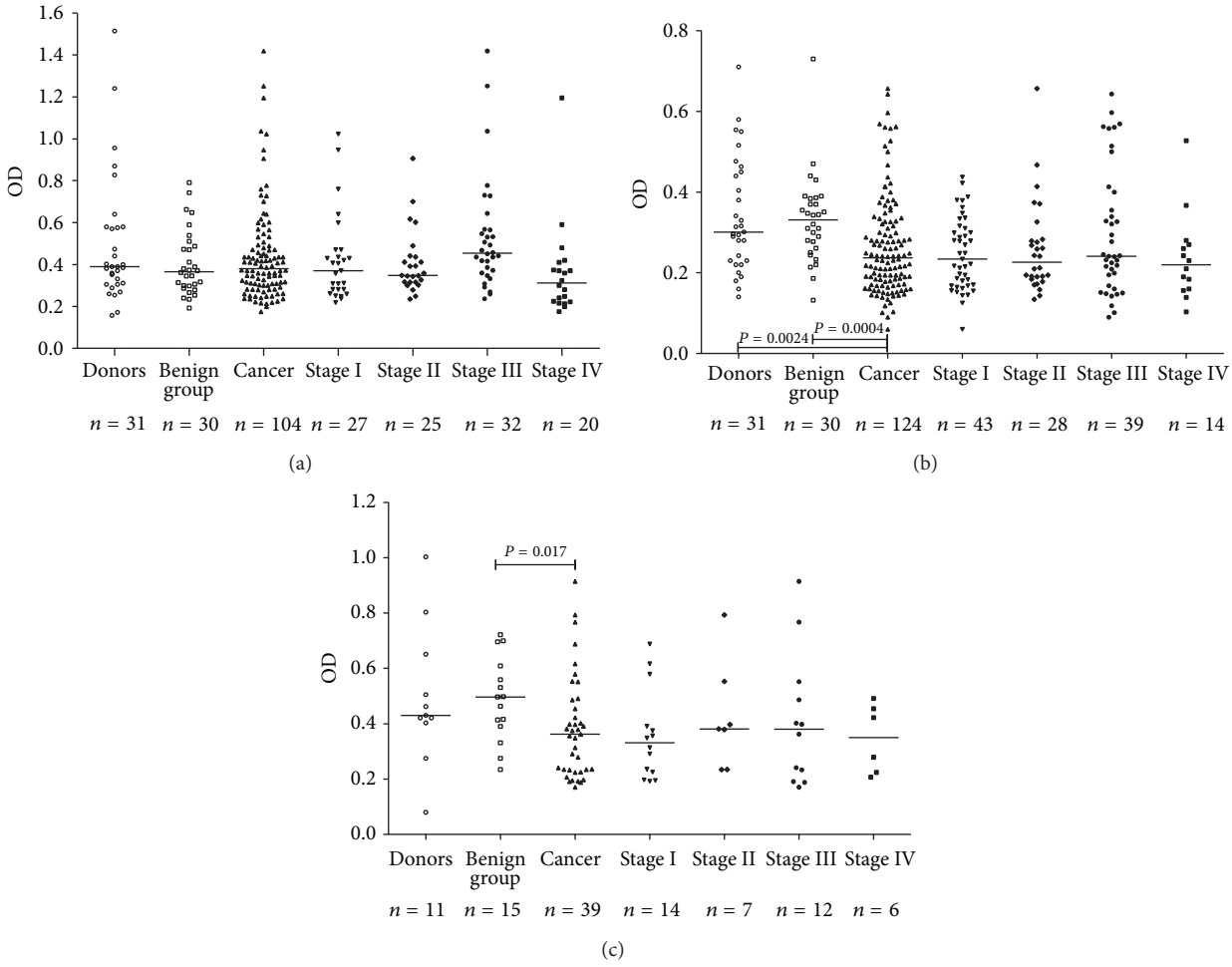

Figure 1: The TF-specific antibody level in patients with stomach cancer and controls. Anti-TF antibody level pattern in controls and cancer patients by stage of cancer; each dot represents one individual and group median is indicated by horizontal lines: (a) anti-TF IgG; (b) anti-TF IgM; (c) anti-TF IgA. *P* values were calculated by the Mann-Whitney *U*test and are shown for significant differences.

using the Estonian Cancer Registry database. The median of anti-TF antibody or SNA binding levels was used as cut-off. Patients whose median is equal to or greater than the corresponding median O.D. value were classified as strong responders and those with levels below the median as weak responders. The receiver operator characteristic (ROC) curve analysis was used to evaluate the sensitivity and specificity of changes found for stomach cancer. The area under the ROC curve and the *P* value of the ROC curve were calculated. The difference between the groups was considered to be significant when $P \leq 0.05$. All calculations were performed using the GraphPad Prism 5 and SPSS 15.0 software.

3. Results

3.1. The Level of TF-Specific Antibodies in the Serum of Cancer Patients and Controls. There was no significant difference in anti-TF IgG antibody level between cancer patients and both of the control groups (Figure 1(a)). A trend to a lower IgG Ab level was observed only in stage 4 patients: *P* was 0.033 and 0.09 compared to donors and the benign gastric diseases group, respectively. The anti-TF-IgM serum level was significantly lower in cancer patients than in blood donors ($P = 0.0024$) and the benign diseases group ($P = 0.0004$) and for the combined group of controls ($P = 0.0001$), with no relation to the stage of cancer (Figure 1(b)). This decrease was mostly observed in patients with an intestinal type of cancer ($P = 0.012$), unlike those with a diffuse type of tumor growth, especially in females ($P = 0.007$) (Figure 2(b)). Similar anti-TF IgM Ab levels were observed in blood donors and the benign diseases group ($P = 0.88$). The TF-specific IgA antibody level was also lower in cancer patients than in donors ($P = 0.06$) and the benign diseases group ($P = 0.017$) (Figure 1(c)). Like anti-TF IgM, a lower anti-TF IgA Ab levels were found in patients with intestinal type tumors (Figure 2(c)). For all the groups under study, there were rather big interindividual variations in any Ig isotype. No significant correlations between the levels of anti-TF antibodies of different Ig isotypes were observed in both

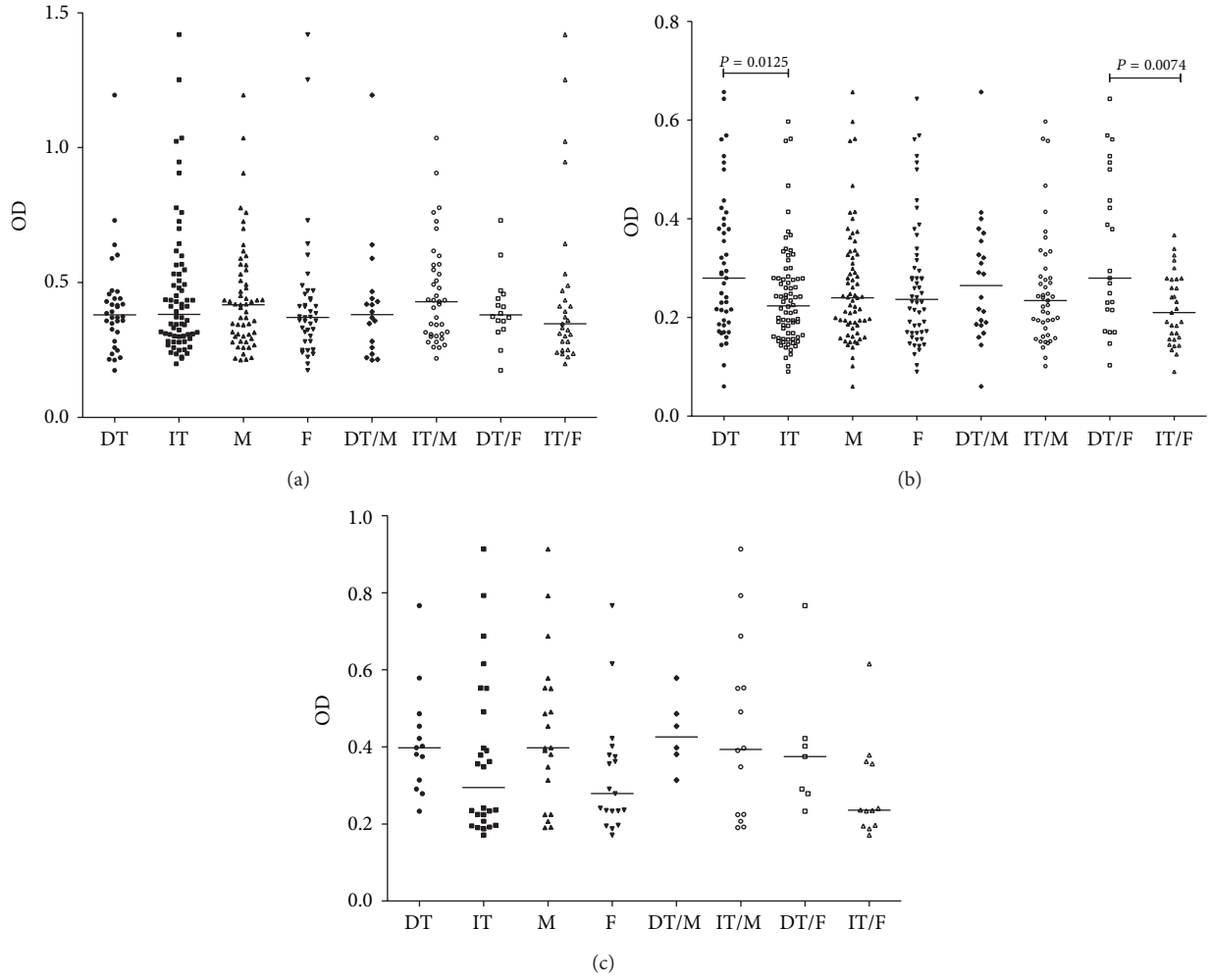

Figure 2: The TF-specific antibody level in cancer patients by gender and tumor morphology. Each dot represents one individual and group median is indicated by horizontal lines: (a) anti-TF IgG; (b) anti-TF IgM; (c) anti-TF IgA. Tumor morphology was evaluated by the Lauren classification as an intestinal (IT) or diffuse (DT) type of tumor growth. M: males; F: females. *P*values are shown for significant differences.

patients and controls: IgG versus IgM, $r = -0.1$ and IgG or IgM versus IgA, $r = 0.23$–0.31 ($P > 0.05$).

Thus, the TF-specific IgM and IgA antibody levels were decreased in gastric cancer patients irrespective of the stage of cancer with some dependency on tumor morphology, while the anti-TFIgG level was slightly decreased in patients with advanced cancer only.

3.2. Interaction of TF-Specific Antibodies with Sambucus nigra Agglutinin (SNA). The binding of SNA to anti-TF Abs (pool of all Ig isotypes) was significantly higher in cancer patients compared with that of blood donors and patients with nonmalignant gastric diseases or the combined group of controls: P was 0.0003, 0.005, and <0.0001, respectively, (Figure 3). The increase in the SNA lectin reactivity was not dependent on the stage of cancer except the slightly higher values in stage 2 versus stages 3 or 4 patients ($P = 0.15$) and tumor morphology (DT/IT); it was similar in males and females and in patients under and over 50 (data not shown).

The SNA binding assay results were further correlated with the level of anti-TF IgG, IgM, and IgA, and the SNA binding/Ig level ratio, or the SNA index, was calculated for each Ig isotype. A significant increase of the SNA/anti-TF IgM index was found in patients with cancer unlike both blood donors and the benign diseases group or the combined group of controls: P was 0.0001, 0.0003, and <0.0001, respectively (Figure 4). The same was true for anti-TF IgA: P was 0.0013 and 0.0007 for donors and the benign diseases group, respectively. An increase of the anti-TF IgG SNA index in cancer was a bit less expressed: P was 0.0089 and 0.033 for blood donors and the benign diseases group, respectively.

The discriminant analysis of three variables (level of anti-TF IgG, anti-TF IgM, and SNA binding value as a dependent variable ($n = 104$)) showed that the anti-TF IgM antibodies played the main role in the SNA binding ($P = 0.0001$), while the role of TF IgG was negligible ($P = 0.82$). The anti-TF IgA level was not included in the analysis due to the small number of patients tested.

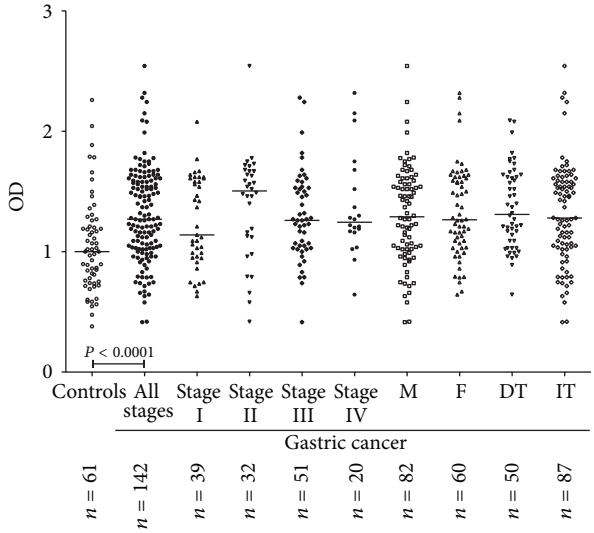

Figure 3: The binding of *Sambucus nigra* agglutinin to TF-specific antibodies in controls and gastric cancer patients by stage of disease, tumor morphology, and gender. DT: Diffuse type of tumor growth; IT: intestinal type of tumor growth. M: males; F: females. *P* values are shown for significant differences.

Thus, the significantly higher SNA reactivity of TF-specific Abs, which reflects the interaction with the terminal alpha 2,6-linked sialic acid of Ab glycans, was revealed in patients with gastric cancer irrespective of disease stage, tumor morphology, or gender. It appears that anti-TF IgM is the main target of the changes observed.

3.3. Changes of the Anti-TF Abs Sialylation as Biomarker for Gastric Cancer Diagnostics: Receiver Operator Curve (ROC) Analysis. The sensitivity and specificity of changes of the anti-TF Abs level and SNA reactivity for gastric cancer were assessed using the ROC curves analysis (Table 2).

The level of anti-TF IgG showed very low sensitivity, specificity, and accuracy (ACC = 0.56) for gastric cancer. The level of anti-TF IgM demonstrated a bit higher predicted group membership for cancer (ACC = 0.67). Compared to the SNA binding assay alone, a higher diagnostic accuracy was demonstrated for the SNA/IgM and SNA/IgA indexes (ACC = 0.69 and 0.72). Considering that both the anti-TF IgM level and SNA binding values were significantly changed in cancer patients and the respective changes showed quite an opposite direction, the same value of the SNA/anti-IgM index may be obtained if both variables are similarly either low or high. Therefore, cancer patients were further stratified into four subgroups by using the median of SNA binding and anti-TF IgM level values for the combined group of cancer patients as follows: (1) patients with an SNA binding value that is equal to or more than median and anti-TF IgM level that is equal to or more than median; that is, SNA ≥ IgM ≥, (2) SNA < IgM ≥, (3) SNA ≥ IgM <, and (4) SNA < IgM < subgroup (see the table in Figure 5). The controls (the combined group of donors and the benign diseases group) were stratified in a similar way by using the corresponding medians of the cancer group. All subgroups of cancer patients were subjected to the ROC curve analysis for sensitivity, specificity, and accuracy of cancer diagnostics and the results were compared with those of the corresponding subgroup of controls (Figure 5).

It is obvious from Figure 5 that the 3rd subgroup, that is, subjects with a high SNA/anti-IgM ratio due to the high level of SNA binding and low level of TF IgM, exclusively belongs to the cancer patients group. Using the ROC analysis the cut-off level of the SNA/anti-IgM index that allows the best discrimination of cancer patients with high SNA binding and low anti-TF IgM level from controls was determined to be equal to 6.1 (cut-off 1). Only 2 out of 61 control subjects had the SNA/IgM ratio value above this cut-off: one from (≥≥) subgroup and one from (≪) subgroup. No controls belonged to the SNA ≥ IgM < subgroup. It is notable that no appreciable differences between the subgroups in the distribution of patients by stage of disease, tumor morphology, and gender were found (Table 3). Thus, using this approach subgroup 3 (SNA ≥ IgM <) may be selected (36 patients out of 124 (29.03%)) whose specificity, sensitivity, and ACC × 100 of gastric cancer diagnostics have reached 100% (Figure 5). The analogous analysis of patients and controls by SNA binding and anti-TF IgA level, as has been done for SNA and IgM, showed that subgroup 3 (SNA ≥ IgA <) demonstrated the ACC value equal to 52%; that is, there was no discrimination between patients and controls.

3.4. Survival Analysis. The relation of cancer-associated changes in the level of anti-TF antibodies and their SNA reactivity to survival is illustrated in Figures 6 and 7. High level of anti-TF IgG was associated with a better survival in patients of stages 3-4, compared with low responders ($P = 0.005$) (Figure 6(a)). A similar trend was observed for anti-TF IgM level ($P = 0.064$) especially in stage 3 patients ($P = 0.01$) (Figure 6(b)) irrespective of tumor morphology and gender (data not shown). The SNA reactivity of TF-specific Abs (pool of all isotypes) showed no relation to survival (HR = 0.88 (0.53–1.46), $P = 0.63$, $n = 136$). The high SNA binding/anti-TF IgG index did not show any association with survival either (HR = 0.97). In contrast, the high SNA/anti-TF IgM index was associated with poor prognosis ($n = 112$, HR = 0.44 (0.25–0.77), $P = 0.0038$) (Figure 6(c)), especially in patients with intestinal tumors (Figure 6(d)) exhibiting a more pronounced association ($P = 0.005$; HR = −0.34 (0.16–0.72); $n = 69$) in both males and females: P was 0.07 and 0.026, respectively. Patients with low differentiated (diffuse type) tumors showed a weaker association (HR = 0.69 (0.29–1.61), $P = 0.39$).

However, in subgroups of patients stratified by SNA reactivity and anti-TF IgM level (as shown in Figure 5 and Table 3) association with survival was different (Figure 7). For instance, group 3 with the highest SNA/anti-IgM index values showed a significantly poorer survival: HR = 0.50 (0.26–0.99), $P = 0.047$ compared with group 2 [<≥] in spite of the highly similar distribution by stage of disease, tumor morphology type, and gender in both groups (Table 3).

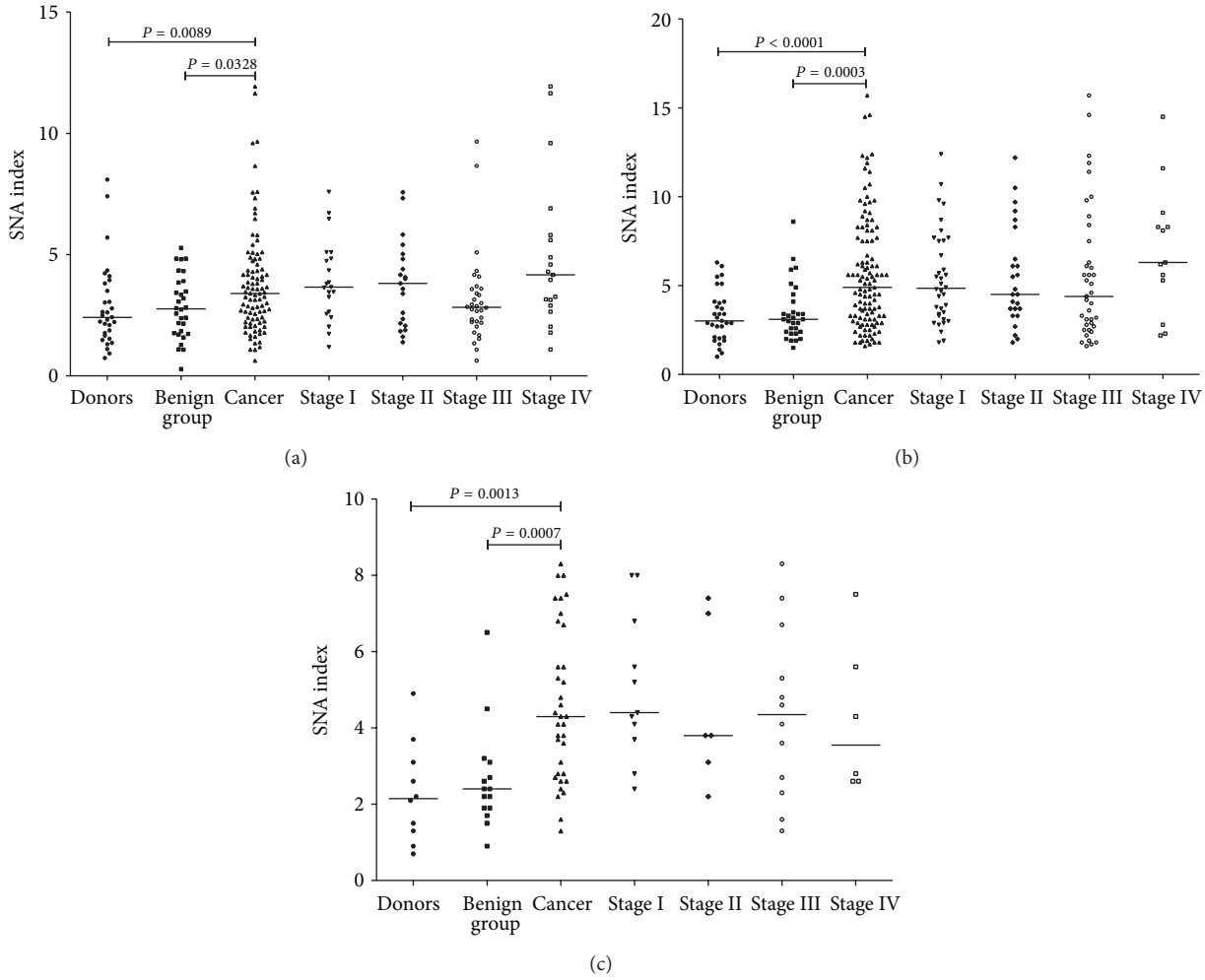

Figure 4: The binding of SNA to anti-TF antibodies depending on the level of TF-specific IgG, IgM, and IgA (SNA/Ig index). (a) SNA/anti-TF IgG, (b) SNA/anti-TF IgM, and (c) SNA/anti-TF IgA index.*P* values are shown for significant differences.

Table 2: The sensitivity, specificity, and accuracy of diagnostics (ACC) for the main parameters under study.

	Sensitivity (95% CI)	Specificity (95% CI)	ROC curve area (95% CI)	*P* Value	ACC
Anti-TF IgA	0.68 (0.51–0.82)	0.77 (0.56–0.91)	0.70 (0.57–0.83)	0.007	0.719
Anti-TF IgG	0.55 (0.44–0.67)	0.58 (0.41–0.74)	0.53 (80.42–0.65)	0.543	0.563
Anti-TF IgM	0.66 (0.57–0.75)	0.69 (0.56–0.81)	0.69 (0.61–0.77)	<0.0001	0.670
SNA	0.60 (0.51–0.69)	0.62 (0.49–0.74)	0.66 (80.58–0.7)	0.000	0.611
SNA/anti-TF IgA	0.71 (0.54–0.85)	0.73 (0.52–0.88)	0.79 (0.67–0.91)	<0.0001	0.719
SNA/anti-TF IgG	0.66 (0.54–0.77)	0.60 (0.43–0.76)	0.64 (0.53–0.75)	0.017	0.643
SNA/anti-TF IgM	0.70 (0.61–0.78)	0.67 (0.54–0.79)	0.73 (0.66–0.80)	<0.0001	0.692

95% CI: 95% confidence interval (in brackets).
ACC: accuracy of diagnostics for each subgroup calculated by the ROC analysis as compared with that of the corresponding subgroup of controls (31 blood donors and 30 patients with nonmalignant gastric diseases). ACC = (TP + TN)/(TP + TN + FP + FN) where TP: true positive cases, TN: true negative cases, FP: false positive cases, and FN: false negative cases.

4. Discussion

The majority of natural tumor-specific Abs belong to germline coded IgM antibodies directed mostly against carbohydrate epitopes and may be responsible for Abs-mediated tumour defence [4, 30, 31]. The origin of these Abs is still a matter of debate, but it appears that some of them (anti-TF and anti-alpha Gal Abs of different isotypes) are directed against microbial glycans or antigens cross-reactive with them [32–34]. Since the 1980s [1] it has been established that the level of anti-TF IgM is lower in cancer patients and is related to higher breast cancer risk. It is notable

Table 3: Distribution of cancer patients by stage of disease, tumor morphology, and gender in subgroups stratified by TF-specific antibody reactivity to SNA and anti-TF IgM level.

Group of patients	n	SNA/IgM subgroup			
		1	2	3	4
		SNA≥IgM≥	SNA<IgM≥	SNA≥IgM<	SNA<IgM<
	124*	25	37	36	26
Stage 1	43	8 (18.6)	14 (32.2)	13 (30.2)	8 (18.6)
Stage 2	28	7 (25.0)	7 (25.0)	8 (28.6)	6 (21.4)
Stage 3	39	7 (17.9)	13 (33.3)	12 (30.7)	7 (17.9)
Stage 4	14	3 (21.4)	3 (21.4)	3 (21.4)	5 (35.7)
DT	49	11 (22.4)	16 (32.7)	14 (28.6)	8 (16.3)
IT	73	14 (19.2)	19 (26.0)	22 (30.1)	18 (24.6)
males	70	15 (21.7)	18 (25.7)	20 (28.6)	17 (24.3)
females	54	10 (18.5)	19 (35.2)	16 (29.6)	9 (16.7)

*Two patients had morphologically mixed (unclassified) tumors.
In brackets: the percentage in the corresponding group.

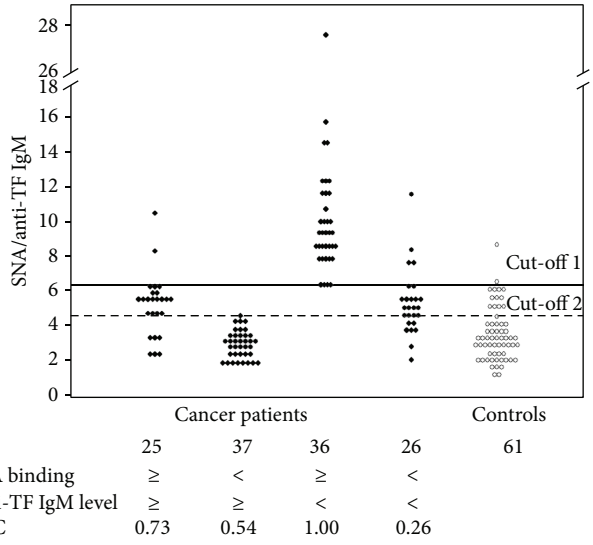

Figure 5: The accuracy of gastric cancer diagnostics (ACC) after the stratification of gastric cancer patients by SNA binding and anti-TF IgM level. The median of the SNA binding and anti-TF IgM level was used to distinguish four subgroups as shown in Figure 5. A solid horizontal line shows the SNA/anti-TF IgM index cut-off 1 (6.1) that allows the best discrimination between subgroup 3 (SNA ≥ IgM <) and controls as determined by the ROC curves analysis. Cut-off 2 (a dotted line) indicates the best discrimination between the combined group of cancer patients and controls. ACC: accuracy of diagnostics as calculated by the ROC curve analysis.

that the level of Abs to many TAGs is often decreased in cancer patients [3, 4, 12]. Circulating autoantibodies to tumor-associated antigens, including TAGs, are considered as promising biomarkers for an early detection of cancer [6, 35] although up to now these antibodies have not shown any sufficient diagnostic accuracy or clinical applicability.

In the present study, we found that all isotypes of TF-specific Abs demonstrated a common trend to a lower level in patients with gastric cancer though IgM Abs revealed the most pronounced decrease. To our knowledge, the level of anti-TF IgA Abs in patients with cancer has not been studied before. The level of anti-TF IgA was similar to that of IgG, but no correlation between anti-TF IgA and the two other isotypes was observed. Rather big interindividual variations in TF-specific Ab level were observed in patients and controls suggesting that some ohter reasons but cancer may be involved, for instance, the profile of individual microbiota.

While many serum glycoproteins exhibit carbohydrate changes in malignancy [5, 17, 18, 36], comparably little is known about the glycosylation of immunoglobulins in patients with cancer, especially in regard to antibodies directed against tumor-associated antigens. Gerçel-Taylor et al. [37] reported that the tumor-derived IgG exhibited more pronounced changes in the glycosylation than serum IgG in patients with ovarian cancer. These authors have supposed that the aberrantly glycosylated serum IgG either may be of tumor origin or may accumulate in tumor tissue. Oaks et al. [38] demonstrated that IgG antibodies against melanoma-associated antigens were much more sialylated compared to the total serum IgG or anti-infectious Abs obtained in melanoma patients, as measured by the SNA lectin binding assay. In some autoimmune disorders, the variable region glycosylation of antigen-specific autoantibodies was also different from that of total IgG, [28]. Thus the determination of the total serum Abs glycosylation does not reflect the glycosylation profile of antigen-specific Abs. This implies that the glycosylation pattern of Abs against the target antigens involved in the pathogenesis of a specific disease may be more informative.

The lectin-based analysis of TF-specific IgG revealed significant alterations in sialo- and fucose-specific lectin reactivity in cancer patients [23]. Interestingly, in patients with gastric cancer the TF-specific IgG antibodies in the total purified IgG were, on the contrary, significantly less SNA-reactive [23] and similar decrease in the purified IgG Fc glycan sialylation was demonstrated by mass spectrometry [24]. Recently, we established a much higher level of anti-TF IgG in purified IgG than in serum samples (unpublished).

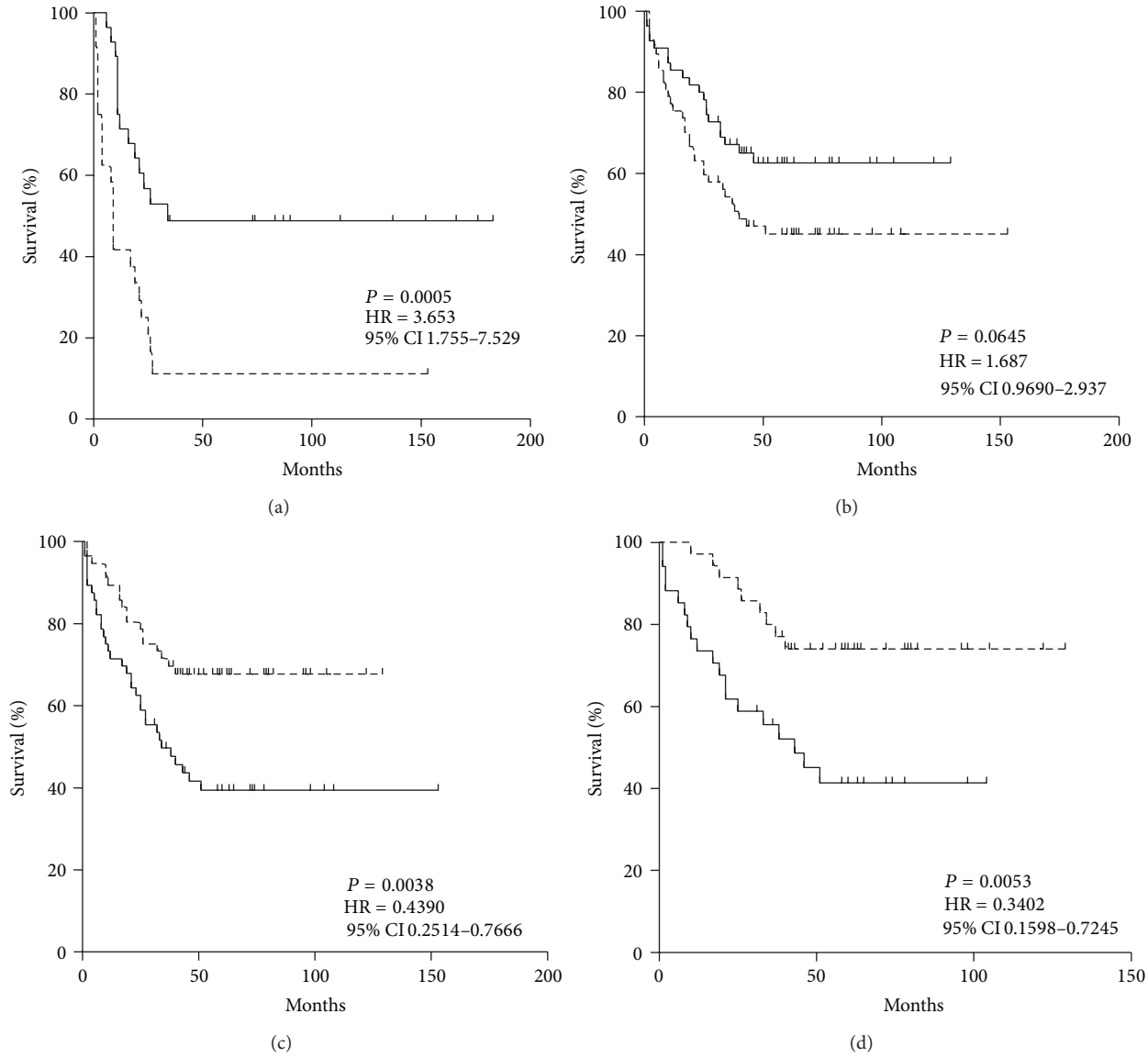

Figure 6: The probability of survival of stomach cancer patients in relation to the level of TF-specific antibodies and their SNA reactivity. Patients with either lower, equal (a dashed line), or higher values (level of Abs or SNA binding) than median (a solid line) are compared using the Kaplan-Meier method. HR: hazard ratio with 95% confidence interval and P values are shown. (a) Anti-TF IgG level and survival of patients in stages 3-4 of the disease. (b) Anti-TF IgM level and survival of cancer patients (stages 1–4). (c) SNA/anti-TF IgM index (all patients). (d) SNA/anti-TF IgM index (patients with intestinal tumors).

This suggests that some additional, the so-called "hidden," anti-TF IgG have been detected in the purified IgG, which may be due to several reasons such as: (i) presence of IgG Abs in a bound form in serum (in complexes with TF-positive ligands); (ii) modification of IgG during the purification (acid elution, dissociation of IC); and (ii) appearance of IgG polyreactivity in the absence of some serum factors that block or inhibit the TF-specific IgG reactivity. Those hidden Abs cannot be detected by conventional methods and may be aberrantly glycosylated (hypersialylated). We suggest that unmasking such hidden Abs and analysis of the whole spectrum (free and hidden Abs) of anti-TF antibodies may lead to the discovery of new biomarkers in tumor immunity and autoimmunity.

To our knowledge, no special studies of TF-specific IgM and IgA antibody glycosylation have been performed before. We established a significant increase of the SNA binding (to a pool of anti-TF Abs) and anti-TF IgM SNA index values in patients with gastric cancer, unlike both control groups. In our lectin-ELISA format, the anti-TF Abs bind the TF-PAA conjugate immobilized on plastic. Thus it might be expected that some SNA reactive glycans, especially in the Fab fragment, would be inaccessible or more accessible to lectin after interaction of Abs with TF, as has been shown for

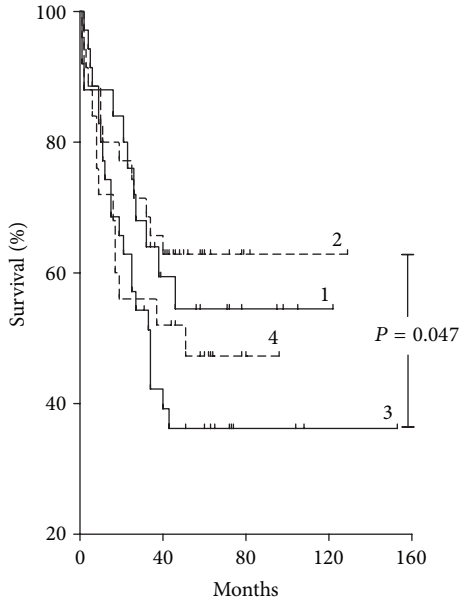

Figure 7: The probability of survival of gastric cancer patients in subgroups stratified by TF-specific antibody SNA reactivity and TF IgM level. The stratification of cancer patients into four subgroups was performed as shown in Figure 5 by using the median of SNA binding and anti-TF IgM level value as cut-off: (1) patients with an SNA binding value that is equal to or more than median and anti-TF IgM level that is equal to or more than median; that is, SNA ≥ IgM ≥, (2) SNA < IgM ≥, (3) SNA ≥ IgM<, and (4) SNA < IgM < subgroup.

the interaction of mannan binding lectin (MBL) after IgM-antigen interaction [39].

This study showed that the level of anti-TF IgG in serum samples did not change much in the cancer group (Figure 1(a)), but the SNA/anti-TF IgG index was significantly higher in cancer patients (Figure 4(b)) suggesting that IgG Fab sialylation may be also related to the increased SNA binding observed in the cancer group. However, the serum IgM is much more glycosylated compared to IgG and more than 80% of IgM complex glycans are terminated in sialic acid [39]. Therefore, we suggested that, despite its lower level in cancer group, the anti-TF IgM is obviously the main component responsible for the increased SNA lectin binding to anti-TF Abs in our model. In fact, the discriminant analysis of SNA binding has indicated that it is anti-TF IgM but not IgG level that was significantly associated with changes in SNA reactivity of TF-specific Abs in cancer patients.

Several reasons might be considered to explain the higher SNA reactivity of anti-TF Abs in cancer patients: (i) in cancer anti-TF IgM is actually more sialylated due to the altered activity of glycosyltransferases in tumor cells and/or in tumor-bearing host; (ii) the Fab glycans of TF-specific Abs are more accessible to SNA due to IgM conformational modifications after interaction with some ligands, such as MBL or other endogeneous lectins; (iii) the anti-TF IgM sialic acids of controls are masked by some TF-positive ligands that are absent in cancer patients but present in healthy state.

Both the level of anti-TF Abs and SNA binding alone had a relatively moderate diagnostic value in gastric cancer with maximal diagnostic accuracy for anti-TF IgM and IgA level (ACC was 0.67 and 0.72, resp., Table 2). The same was true for the SNA/IgM and SNA/IgG indexes (ACC was 0.69 and 0.64, resp.). However, after the stratification of patients by the level of anti-TF IgM and SNA binding we were able to select a group of cancer patients (29%) where the diagnostic accuracy reached 100%. It is important that the high accuracy of diagnostics in patients with a high SNA binding and low TF-IgM level was not appreciably dependent on the stage of disease or tumor morphology, indicating that this biomarker is highly suitable for early gastric cancer diagnostics. Our preliminary data in breast cancer show similar anti-TF Ab sialylation changes (unpublished). Notably, the similar stratification of patients and controls by SNA binding and anti-TF IgA level as has been done for SNA and IgM, showed that patients with high SNA binding and low IgA level demonstrated the ACC value equal to 52%, which allowed no cancer-noncancer group discrimination, thus supporting the idea that IgM is the main target for changes in the increased anti-TF antibody sialylation in cancer. However, the group is rather small. Therefore we consider these data as preliminary ones and a further pertinent study will be required to draw to any final conclusions.

The better survival of patients with a high level of anti-TF IgG antibodies supports our previous findings [12], and a similar association was also found for anti-TF IgM antibodies. No relation to survival was found for the SNA reactivity of TF-specific antibodies. However, a significant (negative) association of the SNA/anti-TF IgM index with survival was demonstrated that is a benefit in survival of patients with a low level of SNA/IgM index (Figure 6(c)) especially for patients with intestinal type tumors (Figure 6(d)). In contrast, the SNA/IgG and SNA/IgA indexes did not show such association.

Since the sialylated Abs display immunosuppressive or tolerogenic effects [40, 41], the higher sialylation of TF antigen-specific IgM Abs may have a negative effect on tumor immunity possibly by interfering with binding of more active anti-TF IgG to tumor cells. Alternatively, highly sialylated (anti-inflammatory) Abs may eliminate undesirable Ab-induced inflammatory reactions in tumor tissue that may promote tumor growth [42–44]. It is logical to assume that different immune mechanisms may be involved in Ab-mediated reactions with circulating tumor cells or tumor cells in tumor tissue, depending on the beneficial or detrimental microenvironment *in situ*. The functional activities of aberrantly glycosylated TF-specific antibodies remain to be determined. Our research currently aims to define the function of differently sialylated TF-specific Ab subsets and their further characterization.

Our findings support the idea that it is hardly possible to have a universal diagnostic biomarker applicable for all cancer patients even within a specific cancer site. This implies that, like cancer therapy, diagnostics also badly needs personalization and the success may be anticipated only if a proper test in a suitable subgroup of patients is applied. Thus the challenge is which criteria should be used to stratify correctly

both patients and markers. Being present in each individual, naturally occurring anti-TF autoantibodies represent a good target for Abs glycoprofiling investigation in cancer detection, screening programs or risk factor studies, in contrast to many other tumor-related Abs that may be revealed in a minority of patients.

In conclusion, our findings are the first evidence that the sialylation of naturally occurring autoantibodies to the tumor-associated Thomsen-Friedenreich antigen is significantly increased in patients with gastric cancer. This increase is mainly related to the anti-TF IgM and IgA isotypes observed already at the early stages of cancer and is independent of tumor morphology or gender. Coming of the opposite character of changes in anti-TF IgM level and the sialylation degree of TF-targeting antibodies in cancer, a combination of these two parameters may be recommended as a novel biomarker for an early diagnosis of gastric cancer and disease prognosis. Such noninvasive approach may be a good prerequisite for the improvement of the clinical utility of antibody-based biomarkers. This information can be exploited for the structural-based functional study of antibodies to tumor-related glycans to further evaluate the clinical relevance of tumor-specific antibody glycovariants.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by the Estonian Research Council grant PUT371.

References

[1] G. F. Springer, "T and Tn, general carcinoma autoantigens," *Science*, vol. 224, no. 4654, pp. 1198–1206, 1984.

[2] S. Hakomori, "Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens," *Advances in Cancer Research*, vol. 52, pp. 257–331, 1989.

[3] O. Kurtenkov, L. Miljukhina, J. Smorodin et al., "Natural IgM and IgG antibodies to Thomsen-Friedenreich (T) antigen in serum of patients with gastric cancer and blood donors—relation to Lewis (a,b) histo-blood group phenotype," *Acta Oncologica*, vol. 38, no. 7, pp. 939–943, 1999.

[4] H. P. Vollmers and S. Brändlein, "Natural antibodies and cancer," *Journal of Autoimmunity*, vol. 29, no. 4, pp. 295–302, 2007.

[5] S. A. Brooks, T. M. Carter, L. Royle et al., "Altered glycosylation of proteins in cancer: what is the potential for new anti-tumour strategies," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 8, no. 1, pp. 2–21, 2008.

[6] H. H. Wandall, O. Blixt, M. A. Tarp et al., "Cancer biomarkers defined by autoantibody signatures to aberrant O-glycopeptide epitopes," *Cancer Research*, vol. 70, no. 4, pp. 1306–1313, 2010.

[7] G. F. Springer, "Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy," *Journal of Molecular Medicine*, vol. 75, no. 8, pp. 594–602, 1997.

[8] A. Almogren, J. Abdullah, K. Ghapure, K. Ferguson, V. V. Glinsky, and K. Rittenhouse-Olson, "Anti-Thomsen-Friedenreich-Ag (anti-TF-Ag) potential for cancer therapy," *Frontiers in Bioscience*, vol. 4, pp. 840–863, 2012.

[9] S. E. Baldus, T. K. Zirbes, F.-G. Hanisch et al., "Thomsen-Friedenreich antigen presents as a prognostic factor in colorectal carcinoma: a clinicopathologic study of 264 patients," *Cancer*, vol. 88, no. 7, pp. 1536–1543, 2000.

[10] P. R. Desai, "Immunoreactive T and Tn antigens in malignancy: role in carcinoma diagnosis, prognosis, and immunotherapy," *Transfusion Medicine Reviews*, vol. 14, no. 4, pp. 312–325, 2000.

[11] C. Schindlbeck, U. Jeschke, S. Schulze et al., "Prognostic impact of Thomsen-Friedenreich tumor antigen and disseminated tumor cells in the bone marrow of breast cancer patients," *Breast Cancer Research and Treatment*, vol. 101, no. 1, pp. 17–25, 2007.

[12] O. Kurtenkov, K. Klaamas, S. Mensdorff-Pouilly, L. Miljukhina, L. Shljapnikova, and V. Chužmarov, "Humoral immune response to MUC1 and to the Thomsen-Friedenreich (TF) glycotope in patients with gastric cancer: relation to survival," *Acta Oncologica*, vol. 46, no. 3, pp. 316–323, 2007.

[13] L.-G. Yu, "The oncofetal Thomsen-Friedenreich carbohydrate antigen in cancer progression," *Glycoconjugate Journal*, vol. 24, no. 8, pp. 411–420, 2007.

[14] V. V. Glinsky, G. V. Glinsky, K. Rittenhouse-Olson et al., "The role of Thomsen-Friedenreich antigen in adhesion of human breast and prostate cancer cells to the endothelium," *Cancer Research*, vol. 61, no. 12, pp. 4851–4857, 2001.

[15] J. Heimburg, J. Yan, S. Morey et al., "Inhibition of spontaneous breast cancer metastasis by anti-Thomsen-Friedenreich antigen monoclonal antibody JAA-F11," *Neoplasia*, vol. 8, no. 11, pp. 939–948, 2006.

[16] J. Bones, J. C. Byrne, N. Odonoghue et al., "Glycomic and glycoproteomic analysis of serum from patients with stomach cancer reveals potential markers arising from host defense response mechanisms," *Journal of Proteome Research*, vol. 10, no. 3, pp. 1246–1265, 2011.

[17] J. N. Arnold, R. Saldova, M. C. Galligan et al., "Novel glycan biomarkers for the detection of lung cancer," *Journal of Proteome Research*, vol. 10, no. 4, pp. 1755–1764, 2011.

[18] W. R. Alley Jr., J. A. Vasseur, J. A. Goetz et al., "N-linked glycan structures and their expressions change in the blood sera of ovarian cancer patients," *Journal of Proteome Research*, vol. 11, no. 4, pp. 2282–2300, 2012.

[19] J. N. Arnold, M. R. Wormald, R. B. Sim, P. M. Rudd, and R. A. Dwek, "The impact of glycosylation on the biological function and structure of human immunoglobulins," *Annual Review of Immunology*, vol. 25, pp. 21–50, 2007.

[20] R. A. Clynes, T. L. Towers, L. G. Presta, and J. V. Ravetch, "Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets," *Nature Medicine*, vol. 6, no. 4, pp. 443–446, 2000.

[21] F. Nimmerjahn and J. V. Ravetch, "Antibodies, Fc receptors and cancer," *Current Opinion in Immunology*, vol. 19, no. 2, pp. 239–245, 2007.

[22] S. Iida, R. Kuni-Kamochi, K. Mori et al., "Two mechanisms of the enhanced antibody-dependent cellular cytotoxicity (ADCC) efficacy of non-fucosylated therapeutic antibodies in human blood," *BMC Cancer*, vol. 9, article 58, 2009.

[23] K. Kodar, J. Izotova, K. Klaamas, B. Sergeyev, L. Järvekülg, and O. Kurtenkov, "Aberrant glycosylation of the anti-Thomsen-Friedenreich glycotope immunoglobulin G in gastric cancer patients," *World Journal of Gastroenterology*, vol. 19, no. 23, pp. 3573–3582, 2013.

[24] K. Kodar, J. Stadlmann, K. Klaamas, B. Sergeyev, and O. Kurtenkov, "Immunoglobulin G Fc N-glycan profiling in patients with gastric cancer by LC-ESI-MS: relation to tumor progression and survival," *Glycoconjugate Journal*, vol. 29, no. 1, pp. 57–66, 2012.

[25] J. Stadlmann, M. Pabst, and F. Altmann, "Analytical and functional aspects of antibody sialylation," *Journal of Clinical Immunology*, vol. 30, supplement 1, pp. S15–S19, 2010.

[26] T. Guhr, J. Bloem, N. I. L. Derksen et al., "Enrichment of sialylated IgG by lectin fractionation does not enhance the efficacy of immunoglobulin G in a murine model of immune thrombocytopenia," *PLoS ONE*, vol. 6, no. 6, Article ID e21246, 2011.

[27] F. Käsermann, D. J. Boerema, M. Rüegsegger et al., "Analysis and functional consequences of increased Fab-sialylation of intravenous immunoglobulin (IVIG) after lectin fractionation," *PloS ONE*, vol. 7, no. 6, Article ID e37243, 2012.

[28] P.-C. Xu, S.-J. Gou, X.-W. Yang et al., "Influence of variable domain glycosylation on anti-neutrophil cytoplasmic autoantibodies and anti-glomerular basement membrane autoantibodies," *BMC Immunology*, vol. 13, article 10, 2012.

[29] P. Lauren, "The two histological main types of gastric carcinoma: an attempt at a histo-clinical classification," *Acta Pathologica et Microbiologica Scandinavica*, vol. 64, pp. 31–49, 1965.

[30] R. Schwartz-Albiez, S. Laban, S. Eichmüller, and M. Kirschfink, "Cytotoxic natural antibodies against human tumours: an option for anti-cancer immunotherapy?" *Autoimmunity Reviews*, vol. 7, no. 6, pp. 491–495, 2008.

[31] A. Pashov, B. Monzavi-Karbassi, G. P. S. Raghava, and T. Kieber-Emmons, "Bridging innate and adaptive antitumor immunity targeting glycans," *Journal of Biomedicine and Biotechnology*, vol. 2010, Article ID 354068, 19 pages, 2010.

[32] G. F. Springer and H. Tegtmeyer, "Origin of anti-Thomsen-Friedenreich (T) and Tn agglutinins in man and in white leghorn chicks," *British Journal of Haematology*, vol. 47, no. 3, pp. 453–460, 1981.

[33] U. Galili, R. E. Mandrell, R. M. Hamadeh, S. B. Shohet, and J. M. Griffiss, "Interaction between human natural anti-α-galactosyl immunoglobulin G and bacteria of the human flora," *Infection and Immunity*, vol. 56, no. 7, pp. 1730–1737, 1988.

[34] K. Klaamas, O. Kurtenkov, K. Rittenhouse-Olson et al., "Expression of tumor-associated Thomsen-Friedenreich antigen (T Ag) in *Helicobacter pylori* and modulation of T Ag specific immune response in infected individuals," *Immunological Investigations*, vol. 31, no. 3-4, pp. 191–204, 2002.

[35] J. W. Pedersen and H. H. Wandall, "Autoantibodies as biomarkers in cancer," *LabMedicine*, vol. 42, no. 10, pp. 623–628, 2011.

[36] S. Hakomori, "Glycosylation defining cancer malignancy: new wine in an old bottle," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 16, pp. 10231–10233, 2002.

[37] Ç. Gerçel-Taylor, L. B. Bazzett, and D. D. Taylor, "Presence of aberrant tumor-reactive immunoglobulins in the circulation of patients with ovarian cancer," *Gynecologic Oncology*, vol. 81, no. 1, pp. 71–76, 2001.

[38] M. Oaks, S. Taylor, and J. Shaffer, "Autoantibodies targeting tumor-assdociated antigens in metastatic cancer. Sialated IgGs as candidate anti-inflammatory antibodies," *Oncoimmunology*, vol. 2, no. 6, Article ID e24841, 2013.

[39] J. N. Arnold, M. R. Wormald, D. M. Suter et al., "Human serum IgM glycosylation: identification of glycoforms that can bind to Mannan-binding lectin," *Journal of Biological Chemistry*, vol. 280, no. 32, pp. 29080–29087, 2005.

[40] S. Böhm, I. Schwab, A. Lux, and F. Nimmerjahn, "The role of sialic acid as a modulator of the anti-inflammatory activity of IgG," *Seminars in Immunopathology*, vol. 34, no. 3, pp. 443–453, 2012.

[41] M. Collin and M. Ehlers, "The carbohydrate switch between pathogenic and immunosuppressive antigen-specific antibodies," *Experimental Dermatology*, vol. 22, no. 8, pp. 511–514, 2013.

[42] Y. Kaneko, F. Nimmerjahn, and J. V. Ravetch, "Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation," *Science*, vol. 313, no. 5787, pp. 670–673, 2006.

[43] T.-T. Tan and L. M. Coussens, "Humoral immunity, inflammation and cancer," *Current Opinion in Immunology*, vol. 19, no. 2, pp. 209–216, 2007.

[44] D. Raman, P. J. Baugher, Y. M. Thu, and A. Richmond, "Role of chemokines in tumor growth," *Cancer Letters*, vol. 256, no. 2, pp. 137–165, 2007.