Journal of Immunology Research

Cancer Immunotherapy: Theory and Application

Lead Guest Editor: Guobing Chen
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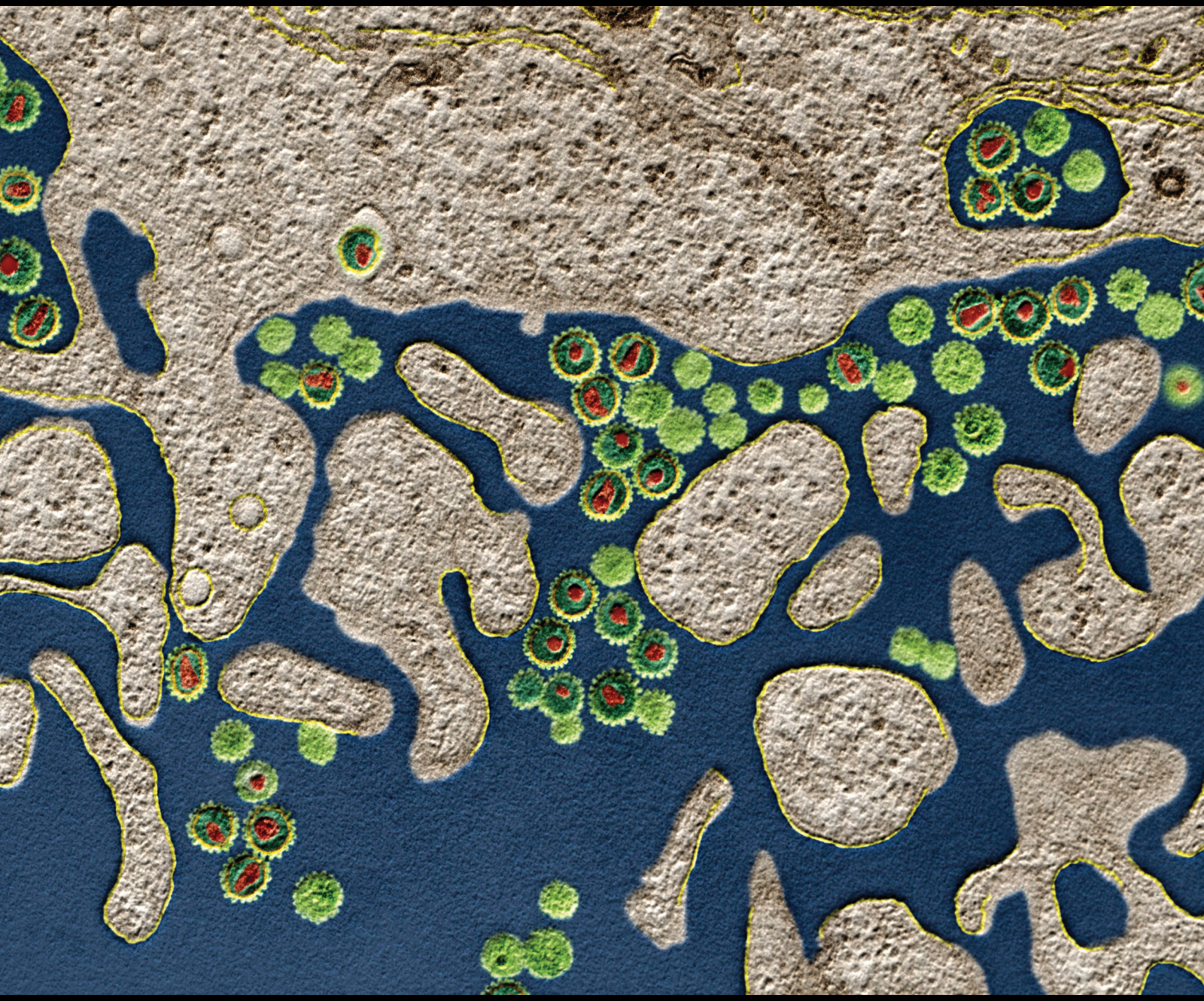

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Editorial Board

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Editorial

Cancer Immunotherapy: Theory and Application

Guobing Chen[1], **Monica Bodogai**[2], **Norimasa Tamehiro**[3], **Chuanlai Shen**[4] and **Jun Dou**[4]

[1]*Medical School, Jinan University, Guangzhou, Guangdong Province, China*
[2]*National Institute on Aging, NIH, Baltimore, MD, USA*
[3]*National Institute of Health Sciences, Tokyo, Japan*
[4]*Southeast University Medical School, Nanjing, Jiangsu Province, China*

Correspondence should be addressed to Guobing Chen; guobingchen@jnu.edu.cn

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In the last few decades, immunotherapy has become an important part of treating some types of cancer. Through either strengthening the host immune responses against tumors, supplying modified immune system components, or counteracting signals produced by cancer cells that suppress immune responses, immunotherapy has become an effective regimen alone or combined with other treatments, such as surgery, chemotherapy, and radiation therapy for cancer patients. With the rapid increase in our understanding of the immune system, more and more small molecules, peptides, recombinant antibodies, vaccines, and cellular therapeutic modalities are being applied to manipulate the immune response for cancer treatment. These immunotherapies have provided significant benefits against cancer, especially the application of immune checkpoint inhibitors [1] and cell therapies [2]. To reflect the advancement and diversity of this field, we invited prospective authors to contribute original manuscripts, case reports, clinical studies, and reviews that focused on antitumor immunotherapy.

In this issue, K. Łukasiewicz and M. Fol summarize the advantages and limitations of microorganisms for cancer treatment. Microorganisms, or a part of them, could stimulate the immune system generally or specifically to eliminate cancer cells. The microorganisms could also be developed as delivery vehicles with exceptional properties. However, the consideration of accompanied infection and limited types of the cancer candidates restricts the wide application of microorganisms, which need more attention and effort in the future.

R. Arai et al. observed decreased peripheral blood dendritic cell (DC) number and function with the lipid accumulation in lung cancer patients. DCs are critical antigen-presenting cells (APC) which present antigen peptides to T cells to initiate specific antitumor immune response. The accumulation of abnormal triglycerides in DC caused the decline of both APC number and function in cancer progression and metastasis. This offers a new potential antitumor target for research and development.

M. R. Rollins and R. M. Gibbons Johnson focused on PD-L1 in antitumor immunity. Checkpoint-associated antitumor therapy has recently had great successes in many types of cancers and advanced a new field that may have the potential to conquer some types of cancer. In this issue, Dr. Johnson's group demonstrated that activated CD8+ T cells could survive better without CD80 expression, which is one of the PD-L1 ligands. It raises the importance of CD80 in the design and implementation of checkpoint blockage for antitumor therapies.

Cell therapy is another excellent implementation with a rapid development in the last few years. Despite advanced manipulation, such as CAR-T and TCR-T therapies [3], the original tumor-infiltrating lymphocyte (TIL) therapy still demonstrated promising outcomes because of safety and longtime development. In a clinical study carried by W. Li et al., TIL combined with IFN-alpha therapy had significant, long disease-free survival and overall survival rates compared to that of no cell therapy in malignant melanoma patients.

Cereblon is a key protein in autosomal recessive nonsyndromic mental retardation and metabolic diseases because of the important regulation roles on the genes involved in cell proliferation and metabolism [4]. It also has different roles in immunomodulatory drug treatment of cancer patients. In this issue, Q. Shi and L. Chen summarize the function of cereblon in cell metabolism and generation of related diseases, as well as the multiple functions and mechanisms in the implementation of immunomodulatory drugs, which could greatly benefit any immunomodulatory drug.

Undoubtedly, there remain many more topics to be discussed, but this special issue includes a number of original research articles, clinical studies, and systematic reviews of cancer immunotherapy from different angles. We hope that this special issue can provide valuable information to researchers as well as clinicians and not only lead to enhancement of knowledge but also serve for better immunotherapy implementation for cancer patients.

Acknowledgments

We would like to thank all the authors for the excellent research stories and stimulating ideas. We would also like to express our great appreciation to all the special issue reviewers and editors, whose efforts substantially contributed to the improvement of the overall quality of this issue.

Guobing Chen
Monica Bodogai
Norimasa Tamehiro
Chuanlai Shen
Jun Dou

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Research Article

Lipid Accumulation in Peripheral Blood Dendritic Cells and Anticancer Immunity in Patients with Lung Cancer

Ryo Arai, Sayo Soda, Tomoko Okutomi, Hiroko Morita, Fumito Ohmi, Tomoe Funakoshi, Akihiro Takemasa, and Yoshiki Ishii

Department of Pulmonary Medicine and Clinical Immunology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu, Tochigi 321-0293, Japan

Correspondence should be addressed to Yoshiki Ishii; ishiiysk@dokkyomed.ac.jp

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We studied the subsets of peripheral blood dendritic cells (DCs) and lipid accumulation in DCs to investigate the involvement of DCs in the decreased anticancer immunity of advanced lung cancer patients. We analyzed the population of DC subsets in peripheral blood using flow cytometry. We then determined lipid accumulation in the DCs using BODIPY 650/665, a fluorophore with an affinity for lipids. Compared with healthy controls, the number of DCs in the peripheral blood of treatment-naive cancer patients was significantly reduced. In patients with stage III + IV disease, the numbers of myeloid DCs (mDCs) and plasmacytoid DCs were also significantly reduced. Lipid accumulation in DCs evaluated based on the fluorescence intensity of BODIPY 650/665 was significantly higher in stage III + IV lung cancer patients than in the controls. In the subset analysis, the fluorescence was highest for mDCs. The intracellularly accumulated lipids were identified as triglycerides. A decreased mixed leukocyte reaction was observed in the mDCs from lung cancer patients compared with those from controls. Taken together, the results show that lung cancer patients have a notably decreased number of peripheral blood DCs and their function as antigen-presenting cells is decreased due to their high intracellular lipid accumulation. Thereby, anticancer immunity is suppressed.

1. Introduction

Lung cancer has a poorer prognosis compared to other cancers. The pathologic analysis of tumor immunity in patients with lung cancer is important for the advancement of immunotherapy. Although treatments based on a dendritic cell (DC) vaccine for immunogenic malignant tumors, such as melanoma and renal cell carcinoma, are considered promising [1, 2], clinical trials involving lung cancer patients have also shown good outcomes [3–6]. DCs play an important role in the immune response as they are antigen-presenting cells that infiltrate cancer tissues, consistently activate cancer-specific T helper cells and cytotoxic T lymphocytes (CTL), and mediate the early stage of the antitumor response.

Human-derived DCs are typically classified into two types, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [7, 8]. mDCs are derived from monocytes in the peripheral blood and are differentiated by the influence of granulocyte/macrophage colony-stimulating factor and interleukin- (IL-) 4, and they preferentially induce mature T helper 1 (Th1) cells, from naive T cells [9–12]. In vivo, mDCs possess strong phagocytotic and antigen-presenting abilities and are involved in the acquisition of cell-mediated immunity. Meanwhile, pDCs are induced by culture with IL-3 and mediate immunotolerance [13, 14].

DCs exist in the peripheral blood in an immature state and capture and recognize specific tumor antigens. They respond to inflammatory mediators such as interferon-alpha and toll-like receptor (TLR) agonists, and when they mature, they present antigens to T cells and acquire the ability to activate a specific antitumor T cell response and migrate to other tissues [15, 16]. DCs produce biologically active IL-12 p70 inducing a remarkable anticancer immunity by potentiating the activity of natural killer cells

and inducing a Th1 response and tumor-specific CD8(+) cells [17, 18].

It is known that anticancer immunity is reduced in patients with cancer. Several tumors produce cytokines and other factors that suppress the maturation or differentiation of DCs in order to avoid the immune response [15]. CTL, which are activated by Th1-derived cytokines, show cytotoxic activity and induce apoptosis in cancer cells, but T cell activation is reduced in cancer patients, and one possible mechanism by which this occurs is through transforming growth factor-β (TGF-β), which is released by tumor cells and suppresses the activation of Th1 cells and CTL [19, 20].

Pathological impairment of DC function is considered to be a cause of decreased tumor immunity in cancer patients. All the causes of DC dysfunction have not been sufficiently elucidated, but one of the known causes is lipid accumulation in DCs in cancer patients, which may suppress DC function [21]. As we expect that elucidating the mechanism of lipid accumulation in DCs will lead to the development of new immunotherapy, we investigated the DC subsets and lipid accumulation in the peripheral blood of lung cancer patients to elucidate the changes in tumor immunity in lung cancer.

2. Methods

2.1. Patients. Subjects were treatment-naive lung cancer patients diagnosed histopathologically. Lung cancer disease staging was performed according to the 7th edition of the TNM Classification of Malignant Tumours [22]. The controls consisted of healthy individuals with no allergic disease, infection, or autoimmune disease and no history of malignant tumor. Informed consent was obtained from all subjects. The study was approved by the institutional ethics committee and was conducted in accordance with the ethical principles embodied in the Declaration of Helsinki.

2.2. Flow Cytometry. Twenty ml of heparinized peripheral blood was obtained from the controls and lung cancer patients. The following monoclonal antibodies were added to the fresh blood, which was cultured at room temperature for 2 h: Lineage-1, fluorescein isothiocyanate (FITC; BD Biosciences, San Jose, CA, USA); HLA-DR, Per-CP (BD Biosciences); CD11c, FITC (BD Biosciences); CD11c, PE (BD Biosciences); and CD123, PE (BD Biosciences). Thereafter, erythrocytes were hemolyzed by cell lysing solution (BD Biosciences). The DC subtype and percentage of each subtype were analyzed by flow cytometry (FACSCalibur, BD Biosciences). Data were analyzed using CellQuest Pro (BD Biosciences). After gating mononuclear cells based on side scatter and forward scatter, the blood DC population was identified as the lin^-/HLA-DR^+ fraction. DCs were divided into a $CD11c^+$DC subset (mDCs) and a $CD123^+$DC subset (pDCs). The number of total events was 200,000, and data are expressed as DC counts per 200,000 leukocytes.

2.3. Lipid Accumulation Analysis. Peripheral blood was collected from controls and lung cancer patients, and the Lineage(−) and HLA-DR(+) DC fractions were sorted using a FACSAria cell sorter (BD Biosciences) and stained with BODIPY 650/665 and DAPI (Polysciences, Warrington, PA, USA), a chromatin dye. The cells were analyzed by fluorescence microscopy for DC lipid accumulation. For the flow cytometry analysis, sorted DCs were immobilized and stained with BODIPY 650/665 at room temperature for 15 min.

2.4. Oil Red O Staining. Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) is a type of azo dye, and because it is nonpolar and lipophilic, it is incorporated into intracellular lipids (i.e., triglycerides) and provides a means of specific triglyceride staining [23]. mDCs and pDCs were isolated from the peripheral blood of treatment-naive lung cancer patients and controls using the FACSAria, and slide specimens were made using a Cytospin. After fixation with 10% formalin, specimens were stained using Oil Red O, thus staining the intracellular triglycerides.

2.5. Quantification of DC Lipids. Intracellular triglycerides content in cell homogenate of DCs were measured using the Adipogenesis Assay Kit (BioVision, Milpitas, CA, USA), a high-sensitivity quantitation kit for intracellular triglycerides.

2.6. Mixed Lymphocyte Culture Reaction. T cell proliferation ability of DCs was evaluated by mixed leukocyte reaction. Peripheral blood was collected from lung cancer patients and controls. The Lineage(−), HLA-DR(+), CD11c(+), and CD123(−) mDC fractions were sorted using the FACSAria cell sorter and cocultured in 96-well plates with allogenic naive T cells at a ratio of 1 : 6. naive T cells were purified from PBMC by a negative selection method using naive CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the supplier's instructions. The purity of $CD4^+$, $CD45RA^+$, and $CD45RO^-$ T cells was evaluated to be over 98% using flow cytometry. After 6 days of culture, BrdU (10 μM 5-bromo-2′-deoxyuridine phosphate-buffered saline, pH 7.4; Roche Applied Science, Basel, Switzerland) was added, and the intracellular uptake of BrdU was conducted using a BrdU Cell Proliferation ELISA kit (Roche Applied Science) and measured using a microplate reader (Molecular Devices, Tokyo, Japan).

2.7. Statistics. Because the data obtained in this study were not normally distributed, we used nonparametric Wilcoxon test for comparisons between two groups and Kruskal–Wallis test for comparisons among multiple groups. Differences were considered significant for values of $p < 0.05$. Data are presented as the means ± SD. JMP Pro Cary (SAS Institute Inc., Cary, NC) was used for statistical analysis.

3. Results

3.1. Patient Background. Patient background is shown in Table 1. The mean age of the 29 lung cancer patients (21 men and 8 women) was 71.6 years (range: 53–84 years) and that of the 25 controls (10 men and 15 women) was 55.7 years (range: 28–67 years). Histological types of lung cancer were adenocarcinoma in 18 subjects, squamous cell carcinoma in 7 subjects, small-cell carcinoma in 3 subjects, and non-small-cell carcinoma in 1 subject. Clinical stage was I

Table 1: Patient characteristics.

	Healthy volunteers	Lung cancer patients
Number	25	29
Age (range)	55.7 (28–67)	71.6 (53–84)
Gender (male/female)	10/15	21/8
Histology		
Adenocarcinoma		18
Squamous cell carcinoma		7
Small-cell carcinoma		3
NSCLC		1
Stage (I/II/III/IV)		10/1/6/12

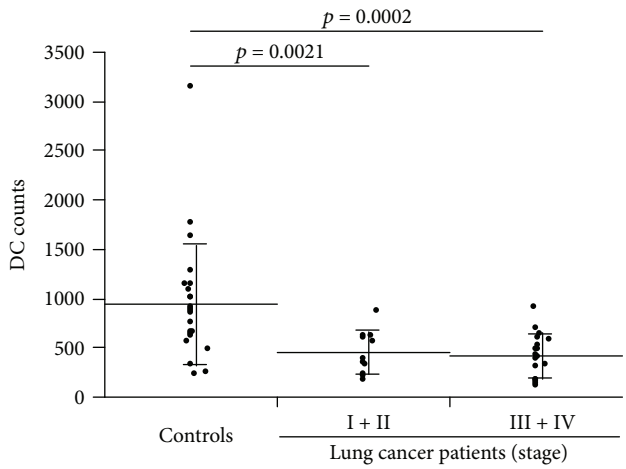


Figure 1: Peripheral blood dendritic cell (DC) count. After adding monoclonal antibody, peripheral blood samples were hemolyzed, and cell immobilization was conducted. HLA-DR(+) and Lineage-1(−) DC fractions were identified using a FACSCalibur, and the number of cells per 200,000 leukocytes was analyzed. Significant differences were observed between the stage I + II lung cancer patients and the controls ($p = 0.0021$) and between the stage III + IV lung cancer patients and the controls ($p = 0.0002$). Horizontal lines represent the means, and vertical lines represent the standard deviation.

in 10 patients, II in 1 patient, III in 6 patients, and IV in 12 patients.

3.2. Number of Peripheral Blood DCs. Lung cancer patients had significantly fewer DCs (groups I + II: 452.6 ± 221.2/200,000 leukocytes, $p = 0.0021$; groups III + IV: 418.0 ± 219.9/200,000 leukocytes, $p = 0.0002$) than the controls (954.9 ± 629.2/200,000 leukocytes) (Figure 1). Patients with stage III + IV lung cancer had a significantly reduced number of mDCs (207.7 ± 201.3/200,000 leukocytes, $p = 0.0007$) than the controls (516.3 ± 386.8/200,000 leukocytes) (Figure 2). The number of pDCs in patients with stage I + II lung cancer (35.0 ± 14.9/200,000 leukocytes, $p = 0.0009$) and stage III + IV lung cancer (32.9 ± 38.8/200,000 leukocytes, $p = 0.0004$) was significantly lower than that in the controls (84.0 ± 45.8/200,000 leukocytes).

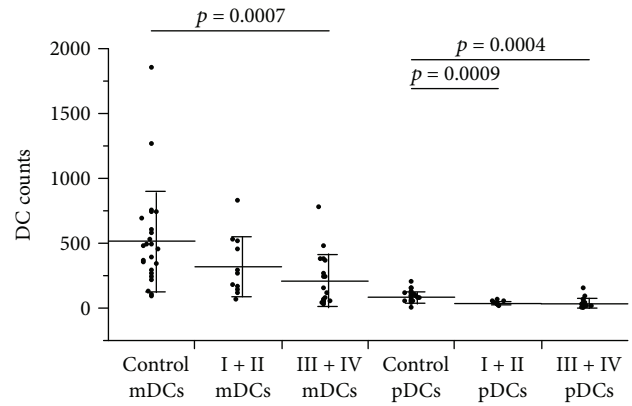


Figure 2: DC count by subtype. Lineage(−), HLA-DR(+), and CD11c(+) myeloid DC (mDC) and Lineage(−), HLA-DR(+), and CD123(−) plasmacytoid DC (pDC) fractions were counted and compared between the stage I + II and stage III + IV cancer patients. Significantly fewer mDCs were observed in stage III + IV cancer patients compared with the controls, and significantly fewer pDCs were observed in stage I + II and stage III + IV lung cancer patients compared with the controls.

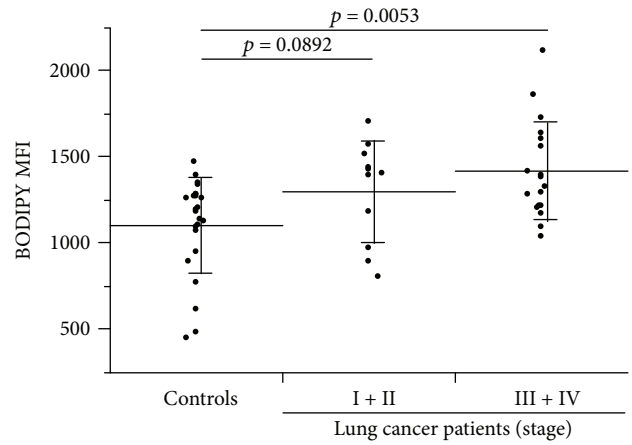
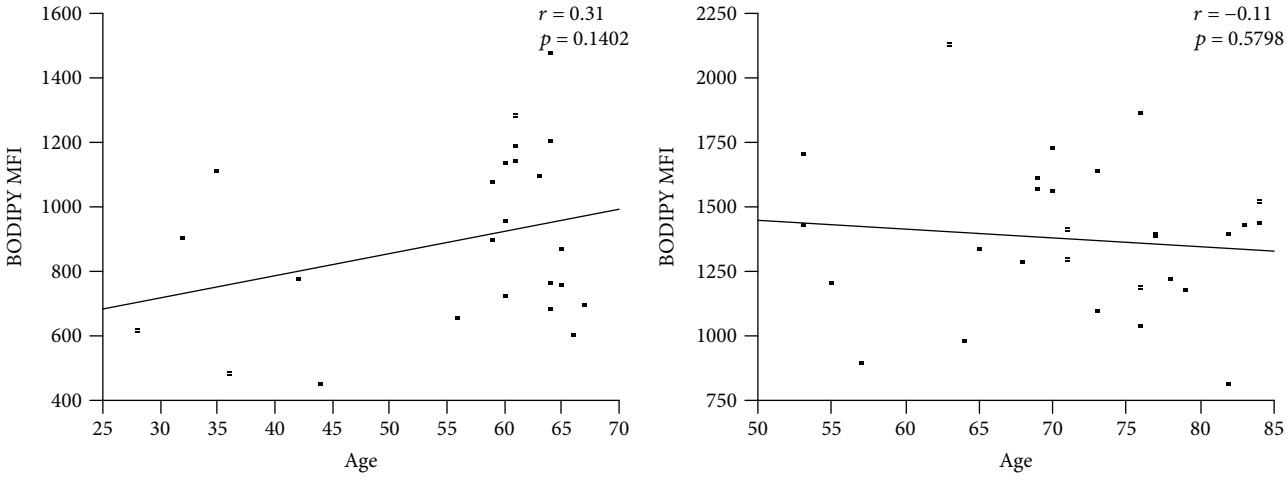


Figure 3: BODIPY 650/665 fluorescence intensity in peripheral blood DCs. The BODIPY 650/665 mean fluorescence intensity of HLA-DR(+) and Lineage-1(−) DC fractions was assessed in lung cancer patients according to clinical stages. A significant difference was observed between the stage III + IV lung cancer patients and the controls ($p = 0.0053$).

3.3. Lipid Accumulation in Peripheral Blood DC. The mean fluorescence intensity (MFI) of peripheral blood DCs stained with BODIPY 650/665 was greater in patients with more advanced cancer stages than in the controls (1148.6 ± 237.1). The lipid accumulation in peripheral blood DCs was not significant in stage I + II patients (MFI, 1299.9 ± 293.8, $p = 0.0892$), but was significant in stage III + IV patients (MFI, 1419.7 ± 283.7, $p = 0.0053$) (Figure 3). There was no correlation between age and DC BODIPY 650/665 MFI in lung cancer patients or controls (Figure 4). The mDCs in stage I + II patients (0.185 ± 0.183%, $p = 0.0003$) and stage III + IV patients (0.177 ± 0.212%, $p = 0.0001$) were significantly elevated compared with the controls (0.036 ±



(a) Controls (b) Lung cancer patients

Figure 4: Correlation between age and lipid accumulation in DCs in the control group or lung cancer patient group. No significant correlation was observed between age and BODIPY MFI in DCs. The r value was 0.31 ($p = 0.1402$) in the control group (a) and −0.11 ($p = 0.5798$) in the lung cancer patient group (b).

0.063%) (Figure 5). In contrast to mDCs, increased lipid accumulation was not seen in pDCs (Figure 5).

3.4. Fluorescence Microscopy for Intracellular Lipid Accumulation. Fluorescence microscopy photographs of DCs stained with BODIPY 650/665 from representative cases were shown in Figure 6. Control DCs showed only slight fluorescence. On the contrary, DCs from stage IV lung cancer patients showed strong and intense fluorescence indicating intracellular lipid accumulation.

3.5. Oil Red O Staining. More cells from stage IV lung cancer patients were stained with Oil Red O, suggesting that the intracellular lipids observed in BODIPY staining were triglycerides. DCs from representative cases were shown in Figure 7.

3.6. Quantitation of Intracellular Triglycerides in DCs. In some cases, intracellular triglycerides content were measured in homogenized DCs. The mDCs in stage IV lung cancer patients ($n = 3$) had significantly higher levels of triglycerides (17.73 ± 3.89 nmol, $p = 0.0339$) than those in the controls ($n = 4$, 4.23 ± 2.52 nmol). However, pDC triglyceride levels were low in both the lung cancer patients and the controls (Figure 8).

3.7. Mixed Lymphocyte Reaction (MLR). T cell proliferation ability of DCs which was evaluated by MLR was significantly lower in the mDCs from stage IV lung cancer patients (0.32 ± 0.26 rlu/s $\times 10^3$, $p = 0.0195$) than in those from the controls (1.21 ± 0.19 rlu/s $\times 10^3$) (Figure 9). In the case of pDCs, MLR was low in both the stage IV lung cancer patients and the controls.

4. Discussion

We confirmed increased lipid accumulation of triglycerides in the mDCs of lung cancer patients when assessing the

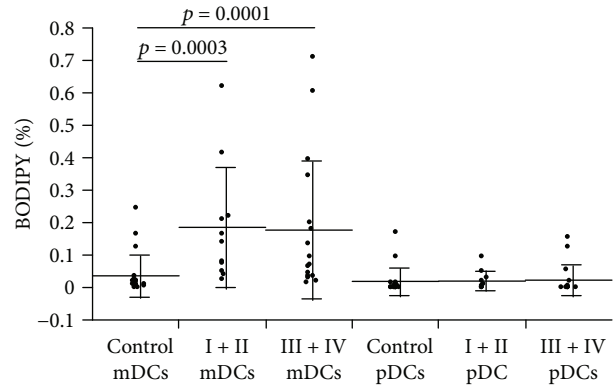
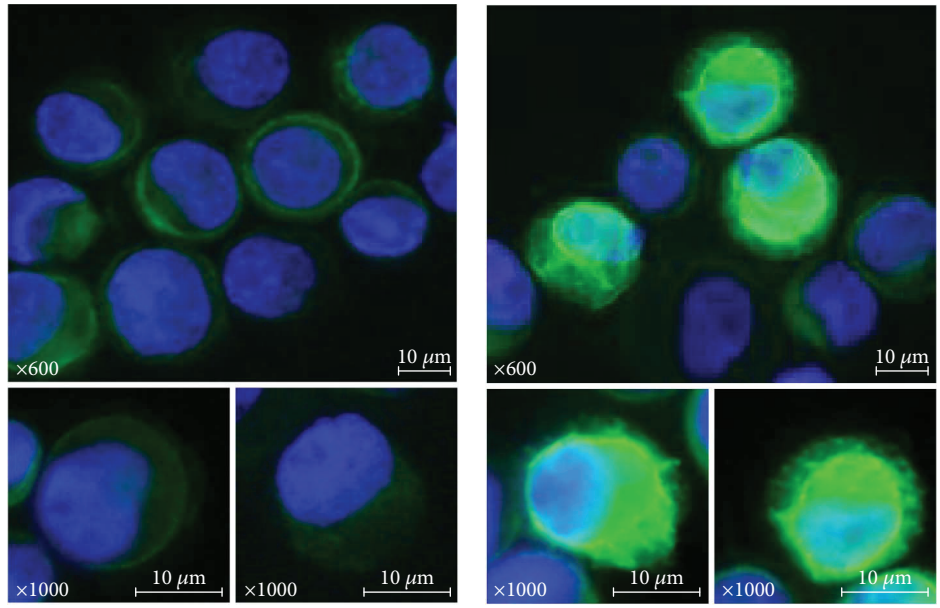


Figure 5: BODIPY 650/665 fluorescence intensity in mDC and pDC. This figure shows the percentages of HLA-DR(+), Lineage(−) DCs, and BODIPY-positive mDCs and pDCs. Significantly, more mDCs were observed in stage I + II and stage III + IV lung cancer patients than in the controls.

DCs obtained from peripheral blood. Furthermore, this study showed for the first time an increase in DC lipid accumulation in line with cancer progression and metastasis. DCs with accumulated lipids are known to reduce lymphoproliferative ability, which in turn reduces anticancer immunity.

Lipids exist in various states, such as fatty acids, phospholipids, sphingolipids, sterols, and lipoproteins, and their metabolism and oxidation are known to influence immune cells. Recently, lipids have been reported to affect macrophage function, but few studies have reported the influence of lipids on DCs. Loscher et al. reported that conjugated linoleic acid suppresses the activity of nuclear factor-κB (NF-κB) and the production of IL-12, while inducing IL-10 via extracellular signal-regulated kinase in bone marrow-derived murine DCs [24]. In addition, Zapata-Gonzalez, et al. reported that fatty acids regulate the activity of human-derived DCs mainly via peroxisome proliferator-activated



(a) Control DCs (b) Lung cancer DCs

Figure 6: DC lipid accumulation. DCs isolated from the peripheral blood were stained using BODIPY 650/665 and confirmed under a light microscope. (a) Control DCs showing only slight fluorescence. (b) Stage IV lung cancer DCs showing strong fluorescence. Fluorescence microscopy photographs of DCs from representative cases were indicated.

receptor-γ (PPAR-γ) [25]. Fatty acids are related to the maturity and function of DCs, and signal transmission may occur via membrane receptors (e.g., TLR), PPAR-γ, and NF-κB.

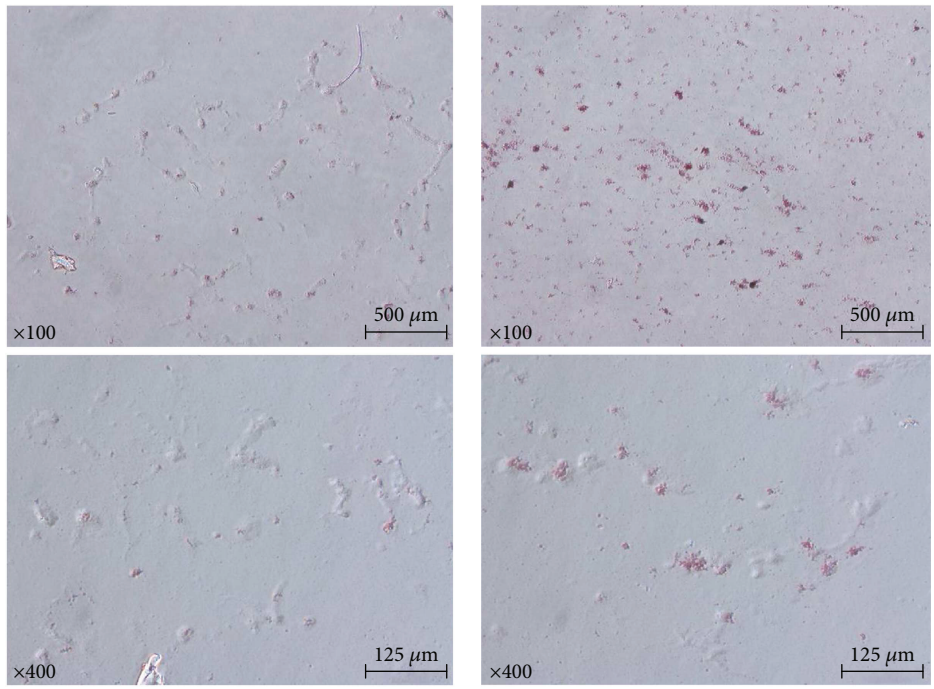
In terms of the influence of lipid accumulation on DC function, the accumulation of intracellular lipids, particularly triglycerides, has been reported in murine DCs [21]. Furthermore, lipid accumulation has also been observed in DCs in non-small-cell lung cancer (NSCLC) and renal cell carcinoma. However, as these studies involved a small population of only 6 patients, further investigation was warranted. Therefore, we conducted the present study. By means of highly sensitive intracellular triglyceride quantitation and Oil Red O staining, we showed lipid accumulation of triglycerides in peripheral blood DCs in lung cancer patients, which supports the findings of Herber et al. [21]. In addition, our study made some new discoveries, such as that the number of DCs decreases and lipid accumulation by DCs is potentiated in line with cancer progression, and that lipid accumulation occurs in mDCs.

Because it was confirmed that accumulated lipids in the DCs obtained from lung cancer patients are mainly triglycerides from the results of Oil Red O staining and measurement of triglyceride content, dysfunction of DCs is thought to be attributed to the triglycerides. However, we cannot deny the participation of other lipids except triglycerides because we did not analyze all lipids.

Factors influencing lipid accumulation may include advanced age and serum triglyceride levels, as well as cancer stage. Although the mean age of the controls (55 years) was lower than that of the lung cancer patients, no correlation was observed between age and DC BODIPY 650/665 MFI in lung cancer patients, or controls, suggesting that age may not play a role. Moreover, no correlation was observed between serum triglyceride levels and DC BODIPY 650/665 MFI, suggesting that serum triglycerides may also not play a role (data not shown).

The receptors mediating DC lipid accumulation are scavenger receptors, and the expression of macrophage scavenger receptor 1 (Msr-1) is reported to increase in DCs with high lipid accumulation [21]. In our study, however, no increased expression of Msr-1 as a protein on the surface of peripheral blood DCs was observed in lung cancer patients (data not shown). Although expression of scavenger receptor B is potentiated during lipid accumulation in mouse bone marrow and spleen-derived DCs [26], the receptors mediating DC lipid accumulation may differ between mice and humans. Going forward, we need to elucidate the lipid accumulation mechanisms, including lipid synthesis, metabolism, and expressed receptors, in the DCs of lung cancer patients.

In the process of tumor immunity, DCs ingest cancer cell antigens and present them to T cells, which recognize the cancer cell antigens presented via the major histocompatibility complex (MHC) and induce apoptosis. Since we suspected a dysfunction of DCs in lung cancer patients during this process, we studied the mixed leukocyte reaction (MLR) of DCs in cancer patients. As the MLR response was reported to be high when the ratio of DCs to allogenic naive T cells was 1 : 6 [27], we used the same ratio in our study. The MLR using DCs with high levels of lipid accumulation in cancer patients was significantly lower than that in the controls, and DC lipid accumulation decreased antigen presentation to naive T cells, which was believed to suppress T cell-induced apoptosis. In addition, the decreased number of



(a) Control DCs (b) Lung cancer DCs

Figure 7: Oil Red O staining. Oil Red O staining is (a) low in the controls and (b) high in the stage IV lung cancer patients (representative cases).

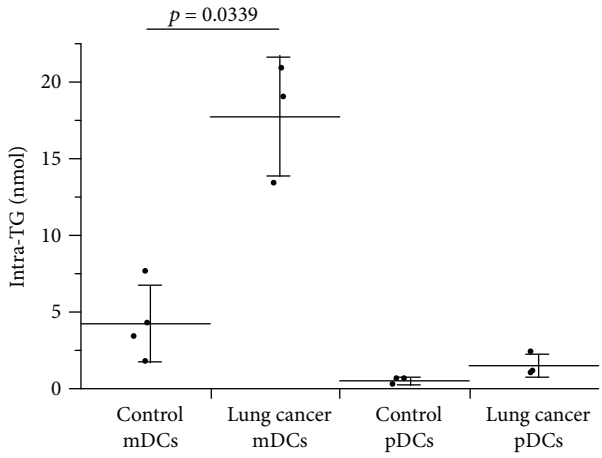


Figure 8: Quantitation of intracellular triglycerides in DCs. Lineage(−), HLA-DR(+), CD11c(+), and CD123(−) mDC fractions and Lineage(−), HLA-DR(+), and CD123(−) pDC fractions were sorted in the peripheral blood from the stage IV lung cancer patients and controls, and the cells were lysed to quantify the intracellular triglycerides. The intracellular triglyceride levels in mDCs were significantly higher in the lung cancer patients ($n = 3$) than in the controls ($n = 4$).

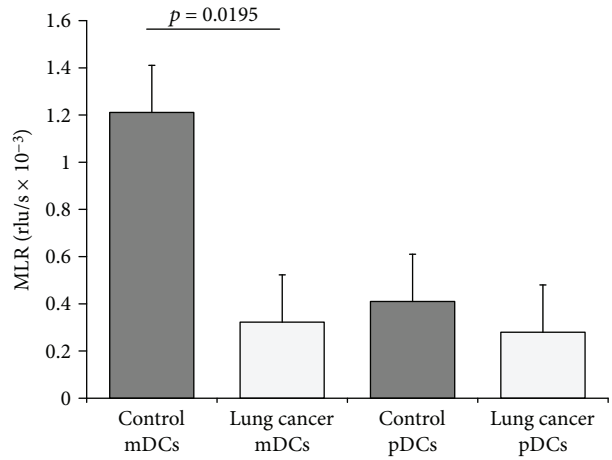


Figure 9: Mixed lymphocyte reaction (MLR). Peripheral blood DCs were cocultured with allogenic native T cells at a ratio of 1 : 6, and the rate of T cell proliferation was measured using BrdU. A lower MLR was observed in the stage IV lung cancer patient mDCs ($n = 4$) than in control mDCs ($n = 4$). These data represent means ± SEM of four independent experiments.

CD8(+) T cells observed in the peripheral blood of cancer patients (data not shown), and the possibly decreased induction and suppressed activation of CTL, may be influenced by factors such as TGF-β and IL-10 from cancer cells, as well as by DC lipid accumulation.

Various factors are reported to participate in decreased tumor immunity. For example, during long-term exposure to cancer antigens, expression of programmed death receptor-1 (PD-1), a costimulatory receptor, is observed in T cells, and the PD-1 ligand is expressed on cancer cells. Binding of these two components suppresses T cell activation [28, 29]. Development of anti-PD-1 antibodies results in

competition for the PD-1 ligand binding site and may be a treatment strategy for overcoming T cell suppression and could therefore have clinical applications. The PD-1 antibodies have been already clinically used and placed as an important option of the lung cancer therapeutic drug [30–33]. The involvement of inhibitory and costimulatory markers other than PD-1, such as TIM-3, BTLA, and LAG-3, has also been reported [34–36].

Taken together, various mechanisms involved in tumor immunity cause immunosuppression and immunotolerance, thereby enabling cancer cells to evade tumor immunity. We focused on lipid accumulation in DCs and found a significant increase in lipid accumulation in mDCs associated with cancer progression. Reduced MLR levels were observed in DCs from cancer patients, thereby decreasing anticancer immune response. Going forward, it is necessary to elucidate the mechanism of lipid uptake by DCs to develop drugs that suppress lipid uptake. If this is achieved, a new form of immunotherapy to increase the anticancer immunity of cancer patients may become a reality.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Review Article

Microorganisms in the Treatment of Cancer: Advantages and Limitations

Klaudia Łukasiewicz and Marek Fol

Division of Cellular Immunology, Department of Immunology and Infectious Biology, Faculty of Biology and Environmental Protection, University of Lodz, Łódź, Poland

Correspondence should be addressed to Marek Fol; marekfol@poczta.onet.pl

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Cancer remains one of the major challenges of the 21st century. The increasing numbers of cases are not accompanied by adequate progress in therapy. The standard methods of treatment often do not lead to the expected effects. Therefore, it is extremely important to find new, more effective treatments. One of the most promising research directions is immunotherapy, including the use of specific types of microorganisms. This type of treatment is expected to stimulate the immune system for the selective elimination of cancer cells. The research results seem to be promising and show the intensive activation of the immune response as a result of bacterial stimulation. In addition, it is possible to use microorganisms in many different ways, based on their specific properties, that is, toxin production, anaerobic lifestyle, or binding substances that can be delivered to a specific location (vectors). This paper provides an overview of selected microorganisms which are already in use or that are in the experimental phase. Just like any other therapy, the use of microbes for cancer treatment also has some disadvantages. Nevertheless, this kind of treatment can supplement conventional anticancer therapy, giving cancer patients a chance and hope of recovery.

1. Introduction

According to the report of Ferlay et al. [1], it is estimated that in 2012 in Europe, approximately 3.45 million people suffered from different types of cancer and 1.75 million died. Cancers of breast, rectum or colon, lung, and prostate are responsible for half of all cancer cases in Europe; furthermore, the first three of them and additionally the stomach cancer are the most common causes of death from cancer in the European Union. Cancer is the second major cause of death in the USA. It is prognosticated that during the year 2017, more than 1.6 million cases will be registered, which means that more than 4600 cancer cases will be reported every day [2]. It should be stressed that the statistics may be underestimated as many cancer lesions develop over the years and are only diagnosed at a high stage of the disease. There are many factors that influence the development of cancer. One of the best recognized risk factors is tobacco smoking, which can cause cancers in lungs, head, and neck [3]. Other examples are chemicals, including those being in use in the research laboratories, such as ethidium bromide, which is a highly mutagenic agent [4]. Mutations in the genetic material may also be the result of irradiation, such as UV or X rays [5], or the effect of infection with a pathogen such as HPV (cervical cancer) [6] or HCV and HBV (liver cancer) [7, 8]. Neoplasms can also be inherited as a polygenic disorder. This is due to the overlap of hereditary changes in the carriers of the defective gene and the DNA damage at sites that are important for the process of cancerogenesis that occurred during human development. The flagship examples are inherited damaged BRCA 1, which is responsible for the development of breast and ovarian cancer, and RB1, which is responsible for the development of retinoblastoma [9]. There are some genetic predispositions, so-called "genetic background," including for instance single mutations in the genetic material or epigenetic changes that may increase the risk of cancer development [10].

The main priorities in cancer research are prevention, early detection, and the development of new therapies, including personalized therapies, which are intended to

include the molecular biology of a particular tumor and the predisposition of the patient's immune system. Among the known and practiced anticancer therapies, the use of microbes appears to be one of the most original strategies. Although now somewhat forgotten, it has a large potential to play a significant role in the treatment of cancer. This paper reviews the perspectives for the use of microorganisms in anticancer therapy. It presents microorganisms that have already been commonly used and those going through phase II and phase III clinical trials.

2. Anticancer Therapy: General Characteristics

Treatment methods can be divided into local/regional treatment and systemic treatment. The combination of both methods is combination therapy. Local treatment includes oncological surgery and radiotherapy. Surgical treatment plays the most important role in cancer treatment, because it often gives a chance of a complete cure (radical treatment). In addition, it is used in palliative treatment that does not give any chance of a cure, but allows incurable patients to alleviate the symptoms of the disease and ensure optimal functioning in the last months of life. Surgery also allows reducing the tumor mass, which significantly improves the effects of systemic treatment. Another method of regional treatment is radiotherapy, which involves irradiation of the tumor, leading to impaired cell division capacity and metabolic functions. Radiotherapy can be applied in two ways: using an external source and by introducing a source into or near a tumor [11]. Systemic treatment has a smaller or greater impact on the entire body of the patient. We can distinguish between chemotherapy, hormone therapy, and biological therapy. Chemotherapy uses drugs that block cell division. All quickly divided cells are destroyed—both cancerous and normal cells of the body. Chemotherapy is accompanied by a number of side effects and general worsening of the patient's condition. Therefore, new, more precise treatments are being sought [11]. Hormone therapy is used in tumors that express receptors for appropriate hormones, such as breast, prostate, or ovary. This method is based on hormonal imbalance, but it is important to evaluate the expression of receptors before treatment, as they may change with the progression of the tumor. Hormone therapy is primarily used for tumor recurrences [12]. In terms of biological treatment, therapy with monoclonal antibodies plays a dominant role. They are directed against specific antigens of tumor cells. In addition, biological substances are used that block the pathways of cancer cell metabolism. Anticancer therapy also involves vaccination with the use of precisely prepared dendritic cells or cancer cells. Interestingly, as early as decades ago, the immunization with the use of microorganisms was already applied as anticancer therapy to stimulate the patient's immune system to fight the disease; however, this kind of treatment is currently poorly explored [13, 14].

2.1. Microorganisms as an Element of Immunotherapy. Intrusion of microorganisms into the body leads to the activation of immune mechanisms, which manifests itself in increasing the number and recruitment of congenital immune cells (especially neutrophils, monocytes/macrophages, and NK cells), activation of acquired immunity cells, that is, T and B lymphocytes, and intensification of proinflammatory cytokine production. It is assumed that the "mobilized" immune system, by intentionally introducing microorganisms into the oncological patient, is able to at least limit the development of cancer. This is a method in which microbes indirectly lead to cancer regression—especially in those in whom other commonly used treatments have failed [15]. The safety of the used microorganisms is extremely important, because the aim of the therapy is to fight cancer, not to harm the patient's organism by infecting it with a pathogen. Various methods are used to ensure the safety of the formulations [16]. First and foremost, microbes are deprived of their pathogenicity (attenuation), for example, by culturing under appropriate environmental conditions or by the treatment of certain substances, resulting in mutation and weakening/loss of pathogenic properties [17].

Bacteria can be applied in various forms for therapeutic purposes. Apart from whole, living attenuated cells, we can use genetically engineered bacteria expressing particularly desirable factors [18]. Microorganisms are also applied as vectors, which are carriers of specific antineoplastic agents (e.g., chemotherapeutics) or enzymes useful in cancer cell destruction. The use of bacteria as a vector to transfer a chemotherapeutic agent directly into the tumor allows a significant reduction of the side effects of treatment that usually accompany traditional chemotherapy [18, 19]. In addition, there is a therapeutic potential in using bacterial secretion products, for example, toxins. Their presence in the tumor environment could have destructive effect on cancer cells [18, 20–22]. The use of sporangial bacteria, which can survive under unfavorable environmental conditions, represents another approach, which has been applied in the experiments with *Clostridium novyi*. This microorganism prefers anaerobic conditions, which are found in the tumor. Instead of spreading over the entire organism, the bacteria are directed to the tumor site only, where they have the optimal conditions for growth. This bacterial property allows the patient to be protected against the development of serious infections [16].

3. Back to Sources

The beginnings of the use of microbes in cancer therapy date back to the nineteenth century. Dr. William Coley (1862–1936) developed a mixture of bacterial microbes and, for the first time in modern medicine, he successfully treated certain types of cancer, thus becoming the father of immunotherapy [23]. Dr. William Coley was employed at the New York Cancer Hospital and then at the Hospital for Special Surgery in New York, as a surgeon specializing in sarcoma, especially bone cancer. He was deeply shocked when one of his first oncological patients died, and that was a reason he began seeking more effective forms of cancer treatment. Coley studied in-depth the case report forms of his contemporary and much earlier oncological patients. He came across information on spontaneous regression of sarcoma in

patients with severe bacterial infection. This prompted him to undertake experimental therapy, which involved the administration of *Streptococcus pyogenes* to a patient with nonoperative bone sarcoma. The results were extremely promising because remarkable tumor regression was observed [24]. Coley worked on new treatment method for the next forty years, preparing other variants of microbial mixture. This preparation could be called a vaccine because it stimulated/activated the immune system by the introduction of antigens (bacterial components). Twenty different versions of the vaccine (called the Coley's toxin) were devised at that time, and each of them had a different effectiveness [25]. It is also very important that the microbes were administered in different ways: intramuscularly, intravenously, or directly into the tumor. Coley's toxin was given to hundreds of patients, and more than a quarter of them were cured. After Coley's death, due to the lack of systematic and precise documentation on the research methodology and preparation of the vaccines, such spectacular results were not replicated [24]. Nevertheless, many attempts were undertaken to reconstitute Coley's toxin [23], for example, by the company MBVax. It has been decided to reproduce the version of a vaccine that was created by the bacteriologist Martha Tracy, who co-operated with William Coley at that time. This vaccine was supposed to have the greatest efficacy. The formulation was based on two types of microorganisms: beta-hemolytic *Streptococcus pyogenes*—as the main factor activating the immune system, and *Serratia marcescens*, producing a red colorant, prodigiosin, which is an apoptosis factor of tumor cells [25, 26]. After administration of the bacteria to the patient, there was a significant increase in the level of cytokines as well as the number of neutrophils, macrophages, T and B lymphocytes, and NK cells. The antigen-presenting cells (APC) initiated the immune response by presenting bacterial antigens to naive $CD4^+$ T cells and $CD8^+$ cells, leading to the production of proinflammatory cytokines, such as interleukins (IL) 1, IL-2, and IL-12, and tumor necrosis factor alpha (TNF), but a total regression of the tumor occurred only in one case [25, 27].

Currently, Coley's toxin is not used in the treatment of bone sarcoma, but the so-called antineoplastic vaccines are commonly used in the treatment of other cancers. The method based on stimulating the immune system is constantly being developed, and more and more studies on immunotherapy appear. Attention is particularly focused on finding new ways to induce the production of proinflammatory cytokines, including TNF or interferon (IFN), which have the capacity to destroy tumor cells. That is why it can be claimed that Dr. William Coley was ahead of his time [24].

4. Bacteria Used as Anticancer Agents

The antitumor efficacy of microorganisms is extremely diverse. Results of clinical trials allow determining whether a particular product can be intended for general use. Currently used anticancer bacterial microbial preparations have the status of a therapy complementary to standard treatment, increasing the patient's chances of complete recovery. The chapter reviews the microorganisms going through phase II and phase III clinical trials and presents those that have already been commonly used in cancer therapy.

4.1. Mycobacterium bovis BCG. Bacillus Calmette-Guérin (BCG) is a strain of *Mycobacterium bovis* developed by Albert Calmett and Camille Guérin as a tuberculosis vaccine and has been used since 1921. In many countries, this vaccine has been induced in the mandatory vaccination schedule and is administered to children within 24 hours after birth, in a single dose, intradermally.

Mycobacterium bovis is an etiological agent of bovine tuberculosis. However, in certain circumstances (e.g., after ingestion of untreated milk from an infected animal), it can cause tuberculosis symptoms in humans as well. That is why it was necessary to attenuate this microorganism. Calmett and Guérin have passaged *M. bovis* (231 passages in total) for 13 years on a medium consisting mainly of cooked potato slices soaked in ox bile and glycerin. Only then did it become safe for human use, as an avirulent but immunogenic strain [28].

At the beginning of the twentieth century there were some links between the occurrence of tuberculosis and cancer regression [28]. However, only after Morales and his colleagues demonstrated in 1976 that the use of BCG was accompanied with the cancer regression, the vaccine was approved as the complementary treatment of bladder cancer [29]. Treatment of this type of cancer with the *M. bovis* BCG strain requires the intravesical infusion of the microbial suspension using urethral catheters. This therapy is most often used after resection to eliminate accurately the cancer cells and to prevent recurrence [29]. The dose and duration of treatment are strictly dependent on the stage of cancer. Clinical observations show that recurrence is much less likely to occur after tumor resection or resection and chemotherapy when BCG is administered intravesically [30].

BCG's mechanism of action is based on stimulating the patient's immune system. It appears that IFN-γ and effector cells, that is, $CD4^+$ and $CD8^+$ lymphocytes, play an extremely important role in the recognition of tumor antigens. In addition, the pool of proinflammatory cytokines is increasing, which enhances the immune response of the body by activating the phagocytosis of cancer cells. Providing the selected vitamins during therapy may increase the survival of *M. bovis* BCG cells, which improves the quality of therapy [16, 29, 31, 32].

4.2. Streptococcus pyogenes OK-432. Streptococcus pyogenes was originally used in the treatment of bone sarcoma by Dr. William Coley. However, the emergence and development of other treatments for cancer, especially chemotherapy and radiotherapy, caused that for many years, the concept of using this microorganism was forgotten. Fortunately, the concept of anticancer therapy with the use of *S. pyogenes* has endured and the bacteria are currently applied in the treatment of lymphangiomas in children. Presently, the *S. pyogenes* OK-432 strain has been used in that way in many countries around the world [25, 33].

Lymphangiomas are tumors formed by excessive division of lymphatic vessels' endothelial cells. They are most often

found in the head and neck area of children under the age of two. The pathological development of lymphatic vessels is primarily associated with impaired lymph flow, which in turn manifests itself in the formation of cysts. Changes in children resemble goiter, similar to that one, which is associated with an enlarged thyroid gland. Treatment primarily involves surgical removal of the cyst, but this is not an easy task, and is often burdened with numerous adverse effects, including death [34, 35].

An alternative and safer method of treatment is sclerotherapy. *Streptococcus pyogenes* OK-432 is injected into pathologically changed lymphatic vessels. In Japan, this microorganism has been successfully used in the treatment of lymphangiomas in children since 1987. Studies show that the strain is safe and results in at least 50% reduction of cyst volume [33, 35].

The mechanism of action of the microorganism is also based on the sensitization of the immune system. Activated cells destroy the neoplasm, further growth is inhibited, and the lymphangioma is reduced. Studies using flow cytometry have shown that the first day after suspension administration, the numbers of neutrophils and macrophages, as well as lymphocytes, rapidly increase. NK $CD56^+$ cells, TNFα, IL-6, IL-8, IFNγ, and VEGF (vascular endothelial growth factor) levels also increase. Due to the appearance of inflammation immediately after the procedure, the lesion may be swollen, but therapeutic effects are noticeable after a few months [33, 35–37]. Moreover, studies conducted in the years 2005–2015 showed the great effectiveness of this strain also in the treatment of intraoral ranula. Complete regression occurred in 78.2% of patients [38].

4.3. Clostridium novyi and Salmonella enterica Serovar Typhimurium. Obligate anaerobes and facultative anaerobes have potential to be used in anticancer therapies because they grow best under conditions of significant oxygen unavailability (hypoxia). Oxygen is delivered to the cells through blood vessels which penetrate mainly the tumor surface area. That results in impaired diffusion of oxygen into the tumor and hypoxia. The anaerobic environment creates favourable conditions for the development of anaerobic bacteria, for example, *Clostridium* spp., *Salmonella spp*., *Bifidobacterium* spp., or *Listeria spp*. [16, 39]. The greatest advantage of using these microorganisms is that they locate directly inside the tumor, in contrast to chemotherapeutics, which spread throughout the body with blood, also destroying normal, healthy cells [39–41].

In the context of hypoxia and the antineoplastic therapy, the most common type of bacteria being in use is *Clostridium*, due to the anaerobic nature of the rods. Bacteria develop in the tumor's necrotic areas and can directly damage tumor cells [39–41]. The history of the use of *Clostridium* in the fight against cancer dates back to 1935, when Connell published an article describing the regression of advanced cancer under the influence of enzymes produced by *Clostridium histolyticum* [42]. Since then, more research has been done on the use of *Clostridium*. The attenuated strain of *Clostridium novyi*-NT has positively undergone phase I and phase II clinical trials, giving extremely promising results for the treatment of leiomyoma [39–41]. The mechanism of the anticancer activity of *Clostridium* spp. is unknown yet, but it is common knowledge that bacterium is capable of producing specific enzymes and toxins that destroy cancer cells. In addition, it produces specific proteins that can be conjugated to specific chemotherapeutics. This allows the drug to enter the tumor. In traditional chemotherapy, drugs are not able to penetrate into the tumor precisely due to its external vascularization and internal hypoxia [39–41].

Salmonella enterica serovar Typhimurium, an etiological agent of typhoid fever, shows similar features as *Clostridium*. It is a relatively anaerobic rod that can also be located in the necrotic tumor regions. In the treatment of cancer, the attenuated strain *Salmonella typhimurium* VNP20009 is used for safety reasons [43]. Clinical trials on the use of this microorganism for melanoma treatment began in 2002 [16]. In addition, the VXM01 antitumor vaccine, which is based on the attenuated strain of *Salmonella typhi*, has successfully passed phase I clinical trials. This bacterium has a plasmid-encoding expression of VEGFR2 (vascular endothelial growth factor receptor-2). The vaccine blocks the angiogenesis process. The formulation was tested in individuals with pancreatic cancer [44].

5. Perspectives for the Use of New Species of Microorganisms

Man has always looked for a mythical panacea, the cure for every illness. Alchemists sought it out in the Middle Ages. Such a legendary substance does not exist, but ideal drugs are still sought by biologists, chemists, physicians, and the other researchers. Ideal means as much as possible safe and effective. This concept is also rooted in research into cancer therapies, which can be evidenced by ever more courageous and original ideas, including the use of microbes; today too daring, in the future they could set standards [45].

5.1. Magnetococcus marinus. The most recent anticancer strategies use the achievements of various scientific disciplines, for instance, nanobiotechnology. Nanoparticles (nanocapsules), lipid vesicles with a chemotherapeutic drug inside, are the object of growing interest. Nanoliposomes are able to deliver the drug inside the tumor [46]. However, they are not a perfect solution because many of the particles do not reach the target. As mentioned earlier, the tumor is only vascularized from the outside, which makes it impossible for chemotherapeutics to reach the inside of the lesion. Hence, the idea of delivering drugs directly to the tumor with vectors/carriers would allow for more precise targeting of the cancer site. Limiting the spread of the drug only to the tumor area would significantly reduce the adverse effects of chemotherapy [40]. For the mentioned reasons, it was decided to take a closer look at very original bacteria named *Magnetococcus marinus* MC1 [19].

Magnetococcus marinus MC1 is a Gram-negative coccus found in the Atlantic Ocean near Rhode Island, USA. This microorganism has cilia, arranged in two bundles located at one pole, which enable the bacteria to move. The unique feature of this bacterium structure is the presence of

magnetosomes—special elements which are magnetite particles (Fe_3O_4) surrounded by membranes, forming chains in the cytosol [47]. The presence of magnetite orients the bacteria with the Earth's magnetic field. In addition, this microorganism shows negative aerotaxis capacity, that is, prefers an environment that is poor in oxygen [48]. These properties make the *Magnetococcus marinus* a useful tool to destroy cancer cells. Using a powerful magnetic field, the same as in the MRI technique (magnetic resonance imaging), it would be possible to direct bacteria containing magnetosomes to the site of the tumor. The bacteria will be located precisely in the areas of hypoxia, in that case inside the tumor, where they would deliver a chemotherapeutic encapsulated in nanoliposomes attached to the bacteria surface. Animal studies have shown that approximately 55% of nanoliposomal transmission cells reach tumors [19, 48].

5.2. Toxoplasma gondii. *Toxoplasma gondii* is an obligatory intracellular protozoan. It can be life-threatening to people with impaired immunity or pregnant women, who can suffer abortion or foetal malformation. The primary hosts are *Felidae* (e.g. cats), in which the sexual phase of pathogen development occurs. Feces containing parasite's oocytes are the source of infection for birds and mammals, including rodents and people, who are the intermediate hosts. In the intermediate hosts' organisms, the parasite divides and cysts are formed in the muscles and brain. In healthy individuals, the immune system inhibits further development of the protozoa [49, 50].

It turns out that the protozoan and its lysate, called TLA (Toxoplasma lysate antigen), containing antigens of the microorganism, can be used to treat not only neurodegenerative diseases [49] but also cancer [49, 51, 52]. In particular, the research focuses on the use of the uracil auxotrophic carbamoyl phosphate synthase mutant *Toxoplasma gondii* (CPS) in the treatment of the most aggressive types of cancer: melanoma, pancreatic cancer [53], lung cancer [49, 52], and ovarian cancer [54]. As a result of the administration of this strain, an increase in the level of IL-12, a cytokine which mediates the inflammation, and the activation of other immune cells were observed. In addition, IL-12 is responsible for inhibition of angiogenesis, leading to hypoxia and tumor growth slackening [54]. Moreover, the expression of the CD31 molecule (angiogenesis marker) is reduced, and the Th1 lymphocytes appear, which also causes a significant inhibition of the formation of blood vessels [49]. In addition, $CD4^+$, $CD8^+$, and IFNγ levels are significantly increased [53]. Recent studies in the mouse model indicate that the use of *T. gondii* CPS therapy provides long-term protection from recurrence, which is connected with the development of immune memory and the high titre of IgG recognizing the specific tumor's antigens [53].

5.3. Plasmodium falciparum. Malaria, caused by protozoa of the genus *Plasmodium*, is one of the most common parasitic diseases in the world. The parasite is transmitted from a healthy person through an *Anopheles* mosquito. The life cycle includes two hosts, an intermediate host—a human being, and a primary one—a mosquito. When the mosquito bites, sporozoites enter the body through blood vessels and then move to the liver where they enter hepatocytes very rapidly, thanks to the apical complex, and in that way, they avoid contact with the host immune system. Here, the sporozoites form schizonts, within which there are numerous divisions, and merozoites are formed. Merozoites are released into the bloodstream about 30 days after the infection. From this point, an erythrocytic cycle starts, and it is responsible for the clinical symptoms of malaria. Merozoites penetrate erythrocytes and turn into trophozoites and then again into schizonts with merozoites inside. Every 48 hours, new merozoites are released and the cycle repeats, destroying more and more red blood cells. After several cycles, some of the merozoites create gametocytes that can be sucked out with blood by a mosquito. There is sporogenesis (a sexual development phase) inside the mosquito's digestive system. Gametes in the body of the mosquito combine to create a zygote and then an ookinete that penetrates the intestinal epithelium of the mosquito, forming an oocyte [55, 56].

Plasmodium falciparum is considered to be the most malignant causative agent of malaria because it aggregates erythrocytes and thrombocytes that adhere to the vascular endothelium, which can lead to the closure of vascular light and thus damage to vascular walls and even necrosis [57]. However, despite all the negative features of the parasite, it can be used to treat cancer. Salanti et al. [22] demonstrated that *Plasmodium falciparum*, after penetrating into erythrocytes, expresses malarial protein VAR2CSA, which is responsible for binding to mucopolysaccharide-chondroitin sulphate A (CSA), present in physiological conditions on the surface of placenta cells [22, 56, 58]. The placenta is a specialized organ whose main function is acting as a mediator between the mother and the baby. It develops extremely fast—from the time of implantation of the embryo into the uterus until the fetus' heart is a fully functional organ. Cells proliferate, and proangiogenic factors cause vascularization of the placenta, which develops and grows throughout the pregnancy, forming a cellular syncytium [58]. It turns out that the placenta and tumors have more in common than just the cell proliferation rate. Chondroitin sulphate is also present on the surface of many tumor cells. Thus, the rVAR2 protein, which is a recombinant version of the VAR2CSA malarial protein, was developed and after being conjugated to the appropriate part of the diphtheria toxoid it was tested for suitability in the destruction of tumor cells. Both *in vitro* studies on tumor cell lines and *in vivo* studies on the mouse model showed the high effectiveness of the strategy used, with the best effects observed for certain types of melanoma with high expression of chondroitin sulfate [22, 58].

6. Summary and Conclusions

Anticancer therapy with the use of microorganisms is often marginalized and neglected. A very narrow group of researchers strive to investigate and develop cancer treatment methods using microorganisms, either as vaccines that activate the immune system to fight disease or as vectors for the transmission of antitumor therapeutics.

Table 1: A representative list of microorganisms used/planned to be used in anticancer therapy.

Microorganism	Strain/antigen	Cancer	Type of treatment	Deployment
Mycobacterium bovis	Atenuated strain Calmette-Guérin	Superficial bladder cancer	Complementary therapy	Commonly used
Streptococcus pyogenes	OK-432	Lymphangioma	Alternative therapy for surgical treatment	Commonly used
Clostridium novyi	Strain NT	Solid tumors	No data	Clinical trials
Salmonella enterica serovar Typhimurium	Strain VNP20009	Melanoma	No data	Clinical trials
Magnetococcus marinus	MC1	Solid tumors and some metabolic tumors	Additional therapy supporting chemotherapy	Experimental research (animal studies)
Toxoplasma gondii	*CPS*/TLA	Pancreas, lung and ovarian cancer, and melanoma	No data	Experimental research
Plasmodium falciparum	rVAR2-DT	Melanoma expressing CS	No data	Experimental research

Very often, these studies go unnoticed, despite significant achievements in the field of immunotherapy. With this method of treatment, people who have been failed by conventional treatment are more likely to recover, what is more important, this type of therapy is more selective and therefore less burdensome for the entire organism of the patient [18–22]. Of course, like every treatment, this one also has certain disadvantages. There is primarily a risk of developing infection and related consequences, including death. In experimental studies, laboratory animals have been used to show that the most effective strain actually destroyed cancer, but animals died because of infection by pathogens. It is therefore very important to ensure the safety of the patients, especially by using only adequately attenuated microorganisms. Only a perfect balance between the attenuation of a microorganism and its immune stimulatory ability can guarantee the proper effect. In addition, the costs associated with clinical trials and the introduction of a new product to the market are extremely high. Legal regulations are also very complicated, due to the not fully known impact of microbes on cancer.

Another issue is the need to take into account the patient's condition. An accurate diagnosis and carrying out proper tests are absolutely necessary. The research on *Plasmodium falciparum* is a good example of how difficult it is to move from the experimental phase to the implementation stage. Another concern at the moment is the limited use of microbial preparations. As mentioned above, there are over two hundred different cancer diseases and only for a few of them where the bacterial preparations have been developed or introduced. So far, there is no general-purpose (universal) bacterial preparation, each type of cancer requires a specially selected (optimized) strain (Table 1), and it is difficult to believe that this kind of universal microbe-based treatment could be ever compiled. However, microbial therapy and research on other bacterial preparations should not be stopped. Relatively recently, a number of reports have been published regarding the use of a padeliporfin derivative (palladium bacteriopheophorbide monolysine taurine, WST-11) in the treatment of prostate cancer [59–62]. This is a vascular-targeted photodynamic therapy (VTP) with the use of the water-soluble WST-11 complex directly administrated into the tumor and subsequently a 753 nm wavelength laser beam aiming the cancer cells to activate the compound. WST-11, in contact with infrared light, induces the synthesis of reactive oxygen species and inhibits angiogenesis, which leads to tumor necrosis. The compound that was the starting point for WST-11 was isolated from ocean-bottom bacteria. The bacteria have developed photosynthetic pigment (bacteriochlorophyll) to adapt to near-total darkness. They use the smallest light source as energy. The success of this therapy undoubtedly proofs of the need for further research into the use of microbes and their compounds/products in the treatment of cancer.

Conflicts of Interest

There is no conflict of interest related to this work.

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Research Article

CD80 Expressed by CD8$^+$ T Cells Contributes to PD-L1-Induced Apoptosis of Activated CD8$^+$ T Cells

Meagan R. Rollins[1,2] and Rachel M. Gibbons Johnson[1]

[1]*Biology Discipline, University of Minnesota, Morris, MN, USA*
[2]*Department of Immunology, Mayo Clinic, Rochester, MN, USA*

Correspondence should be addressed to Rachel M. Gibbons Johnson; rmjohns@morris.umn.edu

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Tumor cells are capable of limiting antitumor CD8$^+$ T cell responses through their cell surface expression of PD-L1. In addition to PD-1 expressed by CD8$^+$ T cells, PD-L1 also binds to CD80 expressed by CD8$^+$ T cells. The influence of the PD-L1/CD80 interaction on CD8$^+$ T cell function has not been fully characterized, so we sought to investigate the impact of the PD-L1/CD80 interaction on PD-L1-induced apoptosis of activated CD8$^+$ T cells. We found that CD8$^+$ T cells that lacked CD80 expression got activated to the same extent as wild-type CD8$^+$ T cells, but when cultured with anti-CD3 and PD-L1/Fc protein, activated CD8$^+$ T cells that lacked CD80 expression survived better than activated wild-type CD8$^+$ T cells. These findings indicate that PD-L1 induces apoptosis in activated CD8$^+$ T cells in part by signaling through CD80. Thus, in the design and implementation of checkpoint blockade therapies that target PD-L1, it is essential that both binding partners for PD-L1, PD-1, and CD80 are considered.

1. Introduction

Cell surface expression of the checkpoint protein programmed death ligand 1 (PD-L1, also named B7-H1 and CD274) is a potent mechanism of immune evasion employed by a wide variety of tumor types and is the target of several checkpoint blockade immunotherapies for cancer [1]. PD-L1 limits an antitumor immune response by signaling through its receptors, PD-1 and CD80 (also named B7-1), expressed on the surface of activated CD8$^+$ T cells. The influence of the PD-L1/PD-1 interaction on CD8$^+$ T cell function has been extensively characterized and is known to limit CD8$^+$ T cell responses by inhibiting TCR signaling, thus restricting CD8$^+$ T cell survival, proliferation, and cytokine production [2, 3]. The PD-L1/PD-1 interaction is the target of the checkpoint blockade therapies pembrolizumab and nivolumab. Both of these drugs are humanized antibodies that bind to PD-1 and prevent PD-L1 from binding to PD-1, thus eliminating the negative signaling delivered to CD8$^+$ T cells by PD-L1 [4, 5]. To date, pembrolizumab is approved for use in metastatic melanoma, both squamous and nonsquamous non-small-cell lung cancer (NSCLC), head and neck squamous cell carcinoma, and Hodgkin's lymphoma. Nivolumab is approved for the treatment of metastatic melanoma, both squamous and nonsquamous NSCLC, and renal cell carcinoma. In clinical trials for both drugs, significant portions of enrolled patients exhibited durable responses or complete tumor elimination [6–14]. There are additional checkpoint blockade therapies, durvalumab, atezolimuab, and avelumab, which bind to PD-L1, blocking the interaction of PD-L1 with both PD-1 and CD80. Currently, durvalumab is approved for the treatment of urothelial carcinoma, atezolimuab is approved for the treatment of NSCLC and urothelial carcinoma, and avelumab is approved for the treatment of Merkel cell carcinoma [15–18]. As durvalumab, atezolimuab, avelumab, and other drugs that target the PD-L1/CD80 interaction in addition to the PD-L1/PD-1 interaction are being designed and implemented, it is necessary to gain a better understanding of how the PD-L1/CD80 interaction is involved in limiting antitumor CD8$^+$ T cell responses.

The interaction between PD-L1 and CD80 was first characterized in 2007 [19] but has not been extensively studied since then. The interaction occurs in both mice and humans and has an affinity that is threefold weaker than that of the PD-L1/PD-1 interaction and threefold stronger than that of the CD28/CD80 interaction [19, 20]. When mouse $CD4^+$ T cells were cultured with plate-bound PD-L1 and anti-CD3, proliferation and production of proinflammatory cytokines were inhibited, even if the $CD4^+$ T cells lacked expression of PD-1 [19]. These results were the first indication that the PD-L1/CD80 interaction functions to limit T cell responses. In related studies, when the PD-L1/CD80 interaction was blocked by an antibody and the PD-L1/PD-1 interaction was left intact, $CD8^+$ T cells exhibited an extended period of expansion and decreased induction of anergy in an *in vivo* peptide immunization model [21]. In a cardiac allograft model in mice, specifically blocking the PD-L1/CD80 interaction accelerated graft rejection and led to an increased production of proinflammatory cytokines [22]. Similarly, using the nonobese diabetic mouse model, the blockade of the PD-L1/CD80 interaction accelerated diabetes in older mice [23]. All together, these findings demonstrate that CD80 expressed by T cells can deliver "reverse signaling" into the T cell upon interaction with PD-L1 that is anti-inflammatory and protolerogeneic. Accordingly, tumor cells are likely capable of inhibiting antitumor $CD8^+$ T cell responses by signaling through both PD-1 and CD80.

In this study, we specifically investigated the role of PD-L1/CD80 signaling in limiting the survival of activated $CD8^+$ T cells. During an immune response, activated $CD8^+$ T cells go through a period of expansion; then, after antigen clearance, there is a contraction phase during which a majority of the activated $CD8^+$ T cells die by apoptosis. The contraction phase is largely mediated by the mitochondrial pathway of apoptosis [24–26], and we previously demonstrated that PD-L1 signaling is involved in the induction of apoptosis of activated $CD8^+$ T cells during the contraction phase. We found that when either the PD-L1/PD-1 interaction or the PD-L1/CD80 interaction was blocked, activated $CD8^+$ T cells expressed decreased levels of the proapoptotic protein Bim [27], indicating a novel role for PD-L1/CD80 signaling in limiting the survival of activated $CD8^+$ T cells. In this study, CD80-deficient mice were used to demonstrate that the PD-L1/CD80 interaction contributes to the induction of PD-L1-induced apoptosis in activated $CD8^+$ T cells. This new information is important to consider in the design and implementation of checkpoint blockade therapies that target PD-L1, as therapies that targetly block the PD-L1 interaction with both PD-1 and CD80 may be more effective than those that only block the PD-L1/PD-1 interaction.

2. Materials and Methods

2.1. Mice. C57BL/6J wild-type (WT) and CD80-knockout (KO) mice (B6.129S4-Cd80tm1Shr/J) were purchased from Jackson Laboratories. Homozygous CD80-KO mice were bred from heterozygous CD80-KO mice. Mice were used at 6–12 weeks of age. Studies were conducted in accordance with the National Institutes of Health guidelines for the proper use of animals in research and with local Institutional Animal Care and Use Committee approval.

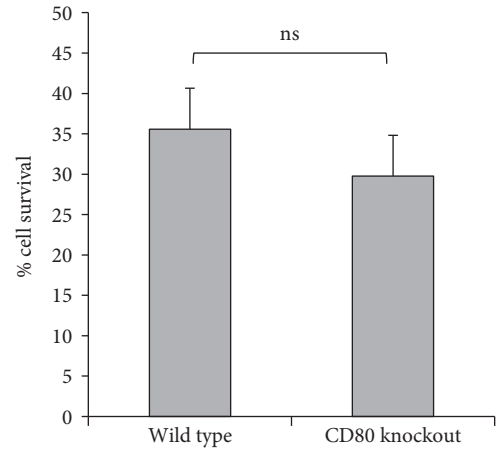

Figure 1: CD80-KO and WT $CD8^+$ T cells survive at equal levels after ConA activation. WT and CD80-KO cells were activated with ConA, then harvested for analysis. Live cells were counted by Trypan blue exclusion ($n = 3$, ±SD, $p = 0.24$, ns: not significant).

2.2. In Vitro $CD8^+$ T Cell Activation and Culturing with Fusion Proteins. The spleen and lymph nodes of WT and CD80-KO mice were harvested at 6–12 weeks of age. The cells were activated with concanavalin A (ConA, 5 μg/mL, L7647, Sigma-Aldrich) for 48 hours. Following activation, $CD8^+$ T cells were purified from the whole cell population (EasySep $CD8^+$ T cell negative selection kit, Stem Cell Technologies) and were incubated with plate-bound PD-L1/Fc or recombinant human IgG1/Fc (control/Fc) fusion proteins (R&D Systems) for 48 hours in the presence of anti-CD3 (clone 2C11, BD Biosciences) in ConA-conditioned media (RPMI 1640 medium with L-glutamine and 25 mM HEPES (Lonza) with 10% FBS (Gibco), 1 U/mL penicillin (Gibco), and 1 μg/mL streptomycin (Gibco)). Live cells were counted by Trypan blue (Millipore) exclusion using a hemocytometer.

2.3. Western Blotting. Cells were lysed on ice with lysis buffer containing 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and protease inhibitors (Millipore). 0.5×10^6 cells were lysed for each condition and run on SDS-PAGE gels, transferred to nitrocellulose (Bio-Rad), and blotted using standard procedures. Rat anti-mouse Bim mAb (3C5) was purchased from Enzo Life Sciences. Goat anti-rat HRP was purchased from BioLegend. Rabbit anti-mouse actin mAb (D18C11) was purchased from Cell Signaling. Goat anti-rabbit HRP was purchased from Bio-Rad.

2.4. Flow Cytometry Analysis. Samples were run on a BD Accuri™ C6 Flow Cytometer and analyzed by BD Accuri C6 Software. For analysis, gates were drawn from live $CD8^+$ cells. Fluorochrome-conjugated antibodies against CD8, CD86, and PD-1 were purchased from BioLegend or eBiosciences.

2.5. Statistical Analysis. A two-sided paired Student's t-test was used to assess statistical differences in experimental groups. A p value < 0.05 was considered statistically significant.

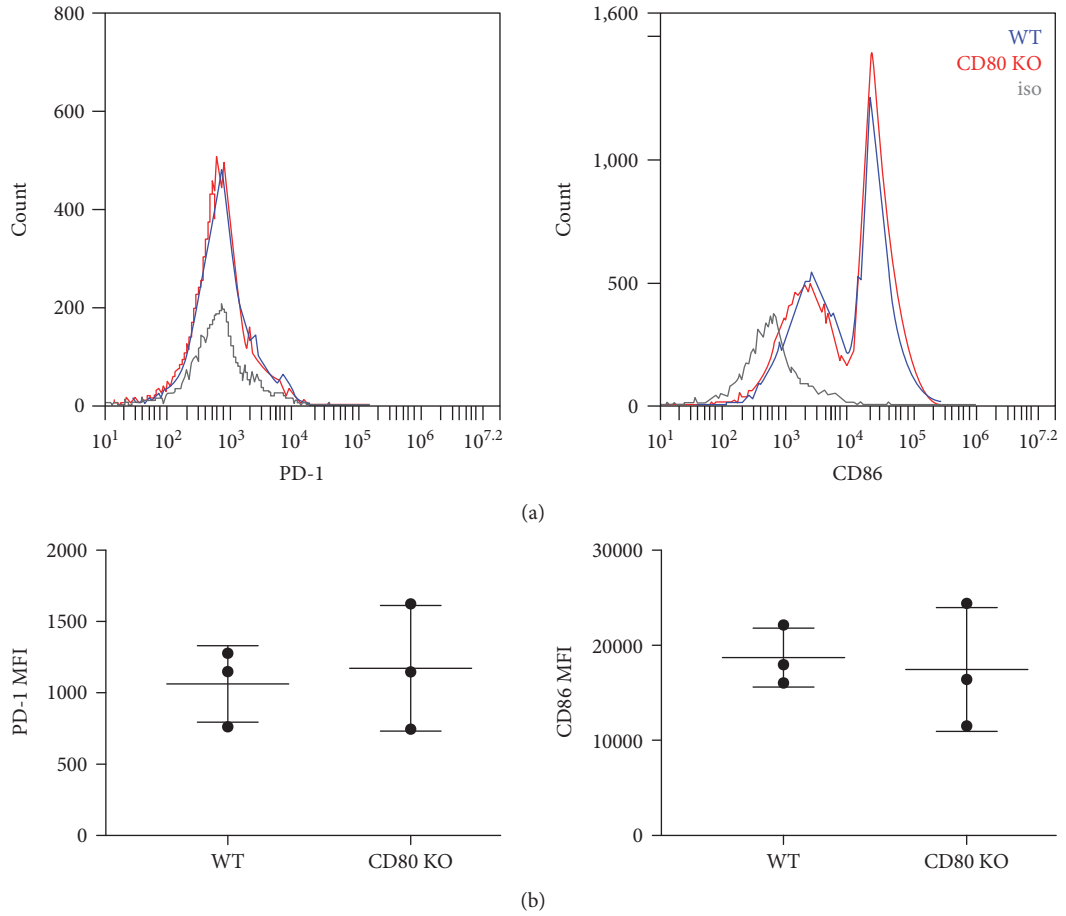

Figure 2: CD80-KO and WT $CD8^+$ T cells express equivalent levels of cell surface markers of activation after ConA activation. WT and CD80-KO cells were activated with ConA, then harvested for analysis. Cells were then analyzed by flow cytometry. (a) Histograms are of live $CD8^+$ cells and representative of 3 separate experiments. (b) Mean fluorescent intensity (MFI) for PD-1 and CD86 ($n = 3$, ±SD, not significant).

3. Results

3.1. Characterization of $CD8^+$ T Cells Activated in the Absence of PD-L1/CD80 Signaling. In order to investigate the influence of PD-L1/CD80 signaling on activated $CD8^+$ T cell survival using CD80-KO $CD8^+$ T cells, we first needed to determine whether or not CD80-KO and WT $CD8^+$ T cells were activated equivalently. We used an *in vitro* culture system in which splenocytes were harvested from naïve WT and CD80-KO mice and activated for 48 hours with ConA. Cells were then harvested for analysis. CD80-KO and WT $CD8^+$ T cells survived at equivalent levels after ConA activation as shown in Figure 1. We also assessed the expression of cell surface markers of activation, including CD86 and PD-1, and found that CD80-KO and WT $CD8^+$ T cells expressed equivalent levels of these markers after *in vitro* activation (Figure 2). Since PD-1 expression was equivalent between CD80-KO and WT $CD8^+$ T cells, it appears that the defect in CD80 expression in the CD80-KO $CD8^+$ T cells does not affect the expression of PD-1 by these cells. Based on these findings, we concluded that $CD8^+$ T cells get activated in our *in vitro* culture system equivalently in the absence of PD-L1/CD80 signaling.

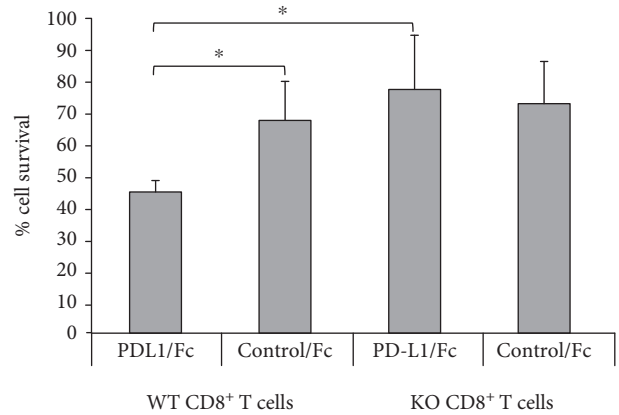

Figure 3: CD80-KO $CD8^+$ T cells survive better than WT $CD8^+$ T cells when cultured with PD-L1/Fc. ConA-activated WT and CD80-KO CD8+ cells were cultured with anti-CD3 and either recombinant mouse PD-L1/Fc or control/Fc for 48 hours, then harvested for analysis. Live cells were counted by Trypan blue exclusion ($n = 3$, ±SD, $^*p \leq 0.05$).

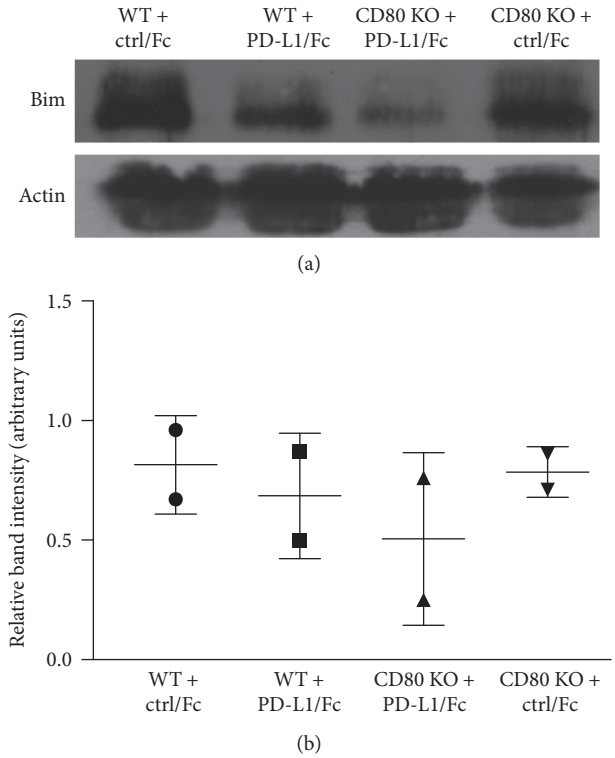

Figure 4: Decreased Bim protein levels in activated CD80-KO $CD8^+$ T cells cultured with PD-L1 as compared to WT cells. (a) ConA-activated WT and CD80-KO $CD8^+$ cells were cultured with anti-CD3 (clone 2C11) and either recombinant mouse PD-L1/Fc or control/Fc (ctrl/Fc) for 48 hours, then harvested for analysis. Cells were lysed and analyzed by Western blotting for Bim and actin protein levels. Representative of two separate experiments. (b) Bim signals were quantified using ImageJ and normalized to actin signals ($n = 2$, ±SD, not significant).

3.2. Activated $CD8^+$ T Cells Survive Better in the Absence of PD-L1/CD80 Signaling. We next went on to investigate the influence of PD-L1/CD80 signaling on the survival of activated $CD8^+$ T cells. We used the same *in vitro* activation with ConA as above; then, after harvesting the activated cells, we isolated the activated $CD8^+$ T cells and cultured them for an additional 48 hours on plates coated with anti-CD3 and either PD-L1/Fc or control/Fc protein. Cells were then harvested for analysis. Activated CD80-KO and WT $CD8^+$ T cells cultured with anti-CD3 and control/Fc protein were recovered at equal levels after the culture period, but more activated CD80-KO $CD8^+$ T cells cultured with anti-CD3 and PD-L1/Fc protein were recovered than activated WT $CD8^+$ T cells cultured with anti-CD3 and PD-L1/Fc (Figure 3). These data indicate that PD-L1/CD80 signaling limited the survival of activated $CD8^+$ T cells.

3.3. Bim Expression Is Decreased in Activated $CD8^+$ T Cells in the Absence of PD-L1/CD80 Signaling. We went on to investigate the mechanism by which PD-L1/CD80 signaling limited the survival of activated $CD8^+$ T cells. CD80-KO and WT $CD8^+$ T cells were activated and cultured with anti-CD3 and PD-L1/Fc protein as described above; then, the expression levels of the proapoptotic protein Bim were analyzed by Western blotting. As shown in Figure 4(a), activated CD80-KO $CD8^+$ T cells cultured with anti-CD3 and PD-L1/Fc protein expressed decreased levels of Bim as compared to WT cells. This finding was supported by two separate experiments. The Bim signals from the Western blots were quantified using ImageJ and normalized to actin signals as shown in Figure 4(b). This finding demonstrates that PD-L1/CD80 signaling contributes to the induction of apoptosis in activated $CD8^+$ T cells by inducing increased expression of Bim.

4. Discussion

Activated $CD8^+$ T cells are potent killer cells but are themselves very sensitive to being killed by apoptosis. The PD-L1/PD-1 signaling pathway is well known to induce apoptosis of activated $CD8^+$ T cells, but the contribution of the PD-L1/CD80 signaling pathway to apoptosis of activated $CD8^+$ T cells has not been extensively investigated. In this study, we demonstrate that PD-L1/CD80 signaling contributes to the induction of apoptosis of activated $CD8^+$ T cells by inducing increased expression of Bim. We used an *in vitro* ConA activation system for our studies and first confirmed that CD80-KO and WT $CD8^+$ T cells get activated to the same extent (Figures 1 and 2). We found that there are no intrinsic differences between CD80-KO and WT $CD8^+$ T cells upon activation. We then went on to culture the ConA-activated $CD8^+$ T cells with PD-L1 and found that the CD80-KO $CD8^+$ T cells survived better than the WT $CD8^+$ T cells (Figure 3). The increased survival of the $CD8^+$

T cells that lacked PD-L1/CD80 signaling was due, at least in part, to decreased levels of Bim expression (Figure 4).

The goal of checkpoint blockade therapies that target PD-L1 expressed by tumor cells is to reactivate an antitumor $CD8^+$ T cell response; thus, it is crucial that we fully understand the mechanisms by which PD-L1 signaling limits antitumor $CD8^+$ T cell responses. Based on our findings reported here, if a checkpoint blockade therapy only inhibits the PD-L1/PD-1 signaling pathway and leaves the PD-L1/CD80 signaling pathway intact, then PD-L1 expressed by tumor cells will still be able to induce apoptosis of tumor-infiltrating $CD8^+$ T cells by signaling through CD80. It has also been reported that PD-L1 limits $CD8^+$ T cell responses in part by inhibiting glycolysis downstream of PD-1 signaling [28, 29]. It was reported that PD-L1/PD-1 blockade led to a metabolic reprogramming in activated $CD8^+$ T cells that resulted in increased rates of glycolysis. This metabolic switch induced by the PD-L1/PD-1 blockade in $CD8^+$ T cells was due to increased Akt activation in the absence of PD-1 signaling. Bim expression levels are also regulated by Akt signaling in $CD8^+$ T cells [30], so it is possible that PD-L1/CD80 signaling, in addition to influencing Bim expression levels, may also influence the metabolism of $CD8^+$ T cells. Continued studies into the influence of the PD-L1/CD80 pathway on $CD8^+$ T cell functions are necessary.

Abbreviations

ConA: Concanavalin A
NSCLC: Non-small-cell lung cancer
PD-L1: Programmed death ligand 1
KO: Knockout
WT: Wild type.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Rachel M. Gibbons Johnson conceived the project and was responsible for the research design and data analysis. Meagan R. Rollins performed the experiments and contributed to the research design and data analysis. Both authors drafted the manuscript.

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Clinical Study

Efficacy of Tumor-Infiltrating Lymphocytes Combined with IFN-α in Chinese Resected Stage III Malignant Melanoma

Wei Li,[1,2] Linping Xu,[3] Yaomei Wang,[2] Lingdi Zhao,[1] Daniel B. Kellner,[4] and Quanli Gao[1]

[1]*Department of Immunotherapy, The Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, Henan 450008, China*
[2]*School of Life Sciences, Zhengzhou University, Zhengzhou, Henan 450001, China*
[3]*Department of Research and Foreign Affairs, The Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, Henan 450008, China*
[4]*Department of Medicine, Weill Cornell Medical College, New York, NY 10065, USA*

Correspondence should be addressed to Wei Li; 15903996841@163.com and Quanli Gao; gaoquanli1@aliyun.com

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Background. This study aims to explore the efficacy of tumor-infiltrating lymphocytes (TIL) along with interferon-α (IFN-α) to treat stage III malignant melanoma (MM) patients in China. *Methods*. Between May 2010 and October 2014, 77 patients of stage III MM who underwent surgery were collected in this study. These patients were divided into two groups: patients who received TIL + IFN-α ± RetroNectin-activated cytokine-induced killer cells (R-CIK) in Arm 1 ($n = 27$) and IFN-α ± R-CIK in Arm 2 ($n = 50$) as adjuvant therapy. The primary endpoints were disease-free survival (DFS) time and DFS rates measured at time points of 1, 2, and 3 years. The secondary endpoints were overall survival (OS) rates measured at time points of 1, 2, 3, and 5 years as well as OS as evaluated by Kaplan-Meier. *Results*. Our results indicated that the median DFS and OS in Arm 1 were significantly better than those in Arm 2. The data also demonstrated that DFS rate and OS rates in Arm 1 were significantly better than those in Arm 2 at all measured time points. *Conclusion*. Patients who undergo surgical excision of stage III MM appear to enjoy prolonged DFS and OS when treated with TIL + IFN-α compared to IFN-α alone.

1. Introduction

The epidemiology data of the United States in 2014 indicated that an estimated 76,100 patients were diagnosed with melanoma and 9710 patients died from the disease [1]. Worse yet, incidence of this disease appeared to be rising rapidly. From 2002 to 2006, the incidence of melanoma increased by 33% among men and 23% among women [2]. Currently, definitive surgical excision is still the primary treatment for candidate malignant melanoma patients. However, the rate of relapse for stage III malignant melanoma patients remains very high even with the administration of adjuvant high-dose interferon-α (IFN-α) [3]. In numerous clinical trials, this IFN-α adjuvant therapy has been shown to improve DFS but not OS [4–6]. In cases of metastatic disease, prognosis is exceptionally poor with mOS of 6 to 8 months and a 5-year OS rate of approximately 6% [7, 8]. Recently, numbers of novel immunotherapies such as anticytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4) and programmed death 1 (anti-PD-1) antibodies have gained FDA approval. While, anti-PD-1 antibody, which has been associated with a 38% objective response rate (ORR), is only approved for advanced malignant melanoma [9]. Therefore, the identification of new postoperative therapies for malignant melanoma patients is of urgent importance.

For a long time, TIL therapy had already shown promise for advanced melanoma patients, with 51% to 72% ORR by

Rosenberg et al. [10–14]. Then, more and more clinical trials applied TIL to treat advanced malignant melanoma patients; however, the data of applying TIL to treat postoperative malignant melanoma patients is still few. In 2002, Labarriere et al. reported that TIL treatment combined with interleukin-2 (IL-2) can prolong the DFS of stage III malignant melanoma patients, who emerged only one metastatic lymph node [15]. Unfortunately, in 2007, the 7 years' follow-up data from that same trial failed to show that TIL treatment combined with IL-2 prolonged RFS or OS overall. Intriguingly, however, in patients with only one positive lymph node, the estimated DFS and OS were significantly prolonged from the TIL + IL-2 therapy compared with IL-2 alone therapy [16]. In 2014, this same team of researchers updated their data and reported that TIL therapy can enhance the curative efficacy of patients with low tumor burden [17]. These data suggest that TIL treatment can be effective against malignant melanoma when applied in the right patient population. However, the efficacy of combining TIL therapy with administration of IFN-α to treat stage III malignant melanoma is unclear. The aim of our current study is to evaluate the efficacy of adjuvant TIL therapy with IFN-α for patients undergoing resection of stage III malignant melanoma.

2. Methods

2.1. Patients. From May 2010 to October 2014, 77 patients undergoing surgical resection of stage III malignant melanoma were collected in this study. Then, TIL + IFN-α ± R-CIK treatment was provided to 27 patients of Arm 1 and IFN-α ± R-CIK treatment was provided to 50 patients of Arm 2. This study was approved by the ethics committee at The Affiliated Cancer Hospital of Zhengzhou University, and an approved consent form was signed by all patients. The procedures were in accordance with the Helsinki Declaration of 1975 and Good Clinical Practice guidelines. Although the two groups have different sample sizes, the baselines of the two Arms were relatively well balanced. The detailed baseline of the 77 patients is listed in Table 1.

2.2. Retrospective Analysis and Follow-Up. The primary endpoint was DFS, with DFS rates measured at time points of 1, 2, and 3 years. The secondary endpoints were 1-, 2-, 3-, and 5-year OS rates as well as OS as evaluated by Kaplan-Meier analysis, and potential prognostic factors were also analyzed by univariate analysis and multivariate analysis. Following surgery, patients were seen for follow-up every 3 months for a two-year period. During postoperative years 2 to 5, patients were reevaluated every 6 months. Beyond the 5-year mark, follow-up evaluation occurred annually. The follow-up deadline was December 8, 2016. When follow-up evaluation revealed metastatic disease, other therapies were employed, including surgery, immunotherapy, chemotherapy, and radiotherapy (Table 1).

2.3. Preparation of TIL. Following surgery, fresh excised tumor tissues were used to culture TILs. Firstly, the excised tumor tissues were sliced into pieces of approximately 2 to 3 mm^3 in size using a scalpel. Secondly, collagenase, DNase I type IV, and hyaluronidase type V (Sigma-Aldrich, St. Louis, MO, United States) were used to perform enzymatic digestion of these tissues for 2 to 3 hours at room temperature to obtain single-cell suspension. Thirdly, the single-cell suspension was filtered, washed twice with phosphate-buffered saline (PBS), and then incubated in a 12-well plate at a concentration of 1.0×10^6 TIL/ml in X-VIVO medium (Muenchensteinerstrasse 38 CH-4002 Basel, Switzerland) with 7000 IU/ml recombinant human interleukin-2 (rhIL-2). The next day, the cell suspension was removed and further purified via Ficoll gradient. The purified bulk TIL culture was maintained at a concentration of $1\text{-}2 \times 10^6$ cells/ml in X-VIVO medium with 7000 IU/ml rhIL-2 until all melanoma cells were eliminated and a cell number of at least 5×10^7 TIL cells were achieved. This culture process required approximately 10 to 14 d. Finally, the cultured TIL cells were immediately used with anti-CD3 antibody (GE Healthcare Biosciences, Pittsburgh, PA, USA; 5 μg/ml) and 1000 IU/ml rhIL-2 for large-scale expansion. By this

Table 1: Clinical characteristics of 77 patients in this study.

	Arm 1 ($n = 27$)	Arm 2 ($n = 50$)	P value
Sex			
Male	12	29	0.255
Female	15	21	
Age (year)			
>60	11	21	0.915
≤60	16	29	
KPS			
≥80	22	45	0.289
<80	5	5	
Primary tumor site			
Mucosa type*	4	15	0.140
No-mucosa type*	23	35	
R-CIK			
Yes	22	45	0.289
No	5	5	
Treatment after metastasis			
Surgical	8	10	—
Immunotherapy	27	45	—
Chemotherapy	5	10	—
Radiotherapy	3	6	—
Any 2 or more	16	35	—
Any 3 or more	3	8	0.912

*In Arm 1, mucosa type patients include 3 patients with nasal cavity mucosa melanoma and one patient with mouth cavity melanoma. No-mucosa type patients include 12 patients with acral lentiginous melanoma, 7 patients with nodular melanoma, and 4 patients with superficial spreading melanoma. In Arm 2, mucosa type patients include 5 patients with rectal mucosa melanoma, 6 patients with nasal cavity mucosa melanoma, 2 patients with mouth cavity melanoma, one patient with penis mucosa melanoma, and one patient with vaginal mucosa melanoma. No-mucosa type patients include 14 patients with acral lentiginous melanoma, 11 patients with nodular melanoma, and 10 patients with superficial spreading melanoma.

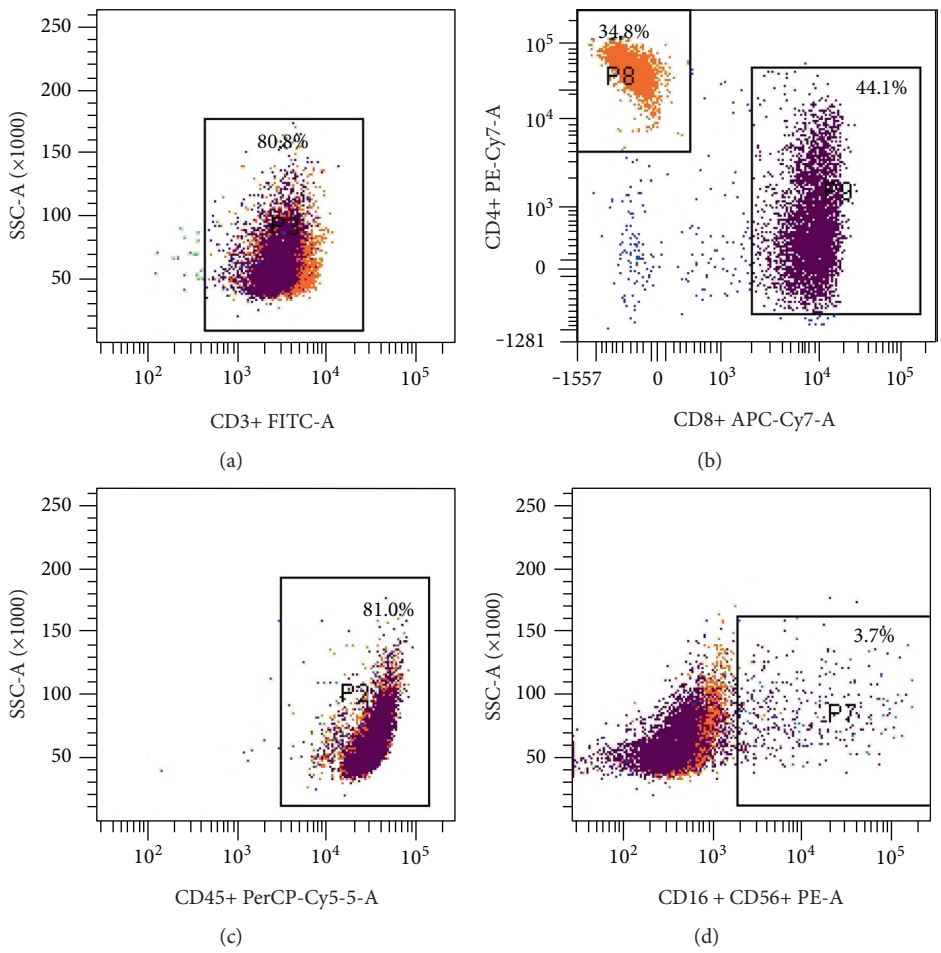

Figure 1: (a) The proportion of CD3+ T cells among TIL cells. (b) The proportion of CD3+CD4+ and CD3+CD8+ T cells among TIL cells. (c) The proportion of CD45+ T cells among TIL cells. (d) The proportion of CD3−CD16+CD56+ T cells among TIL cells.

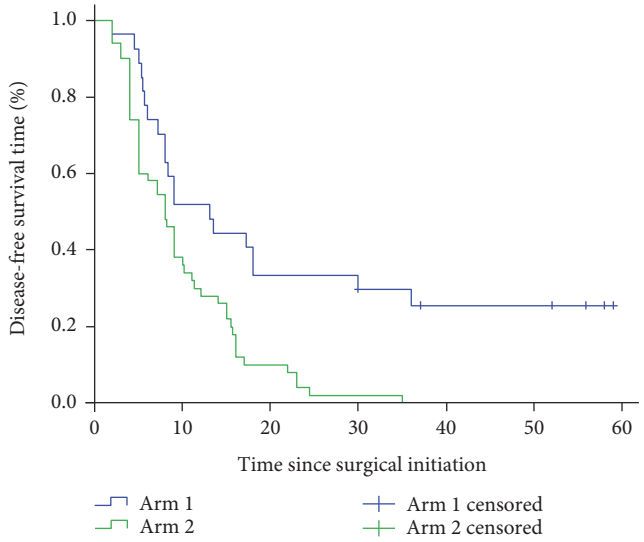

Figure 2: Disease-free survival time of Arm 1 versus Arm 2 was calculated in 27 patients in Arm 1 compared with 50 patients in Arm 2.

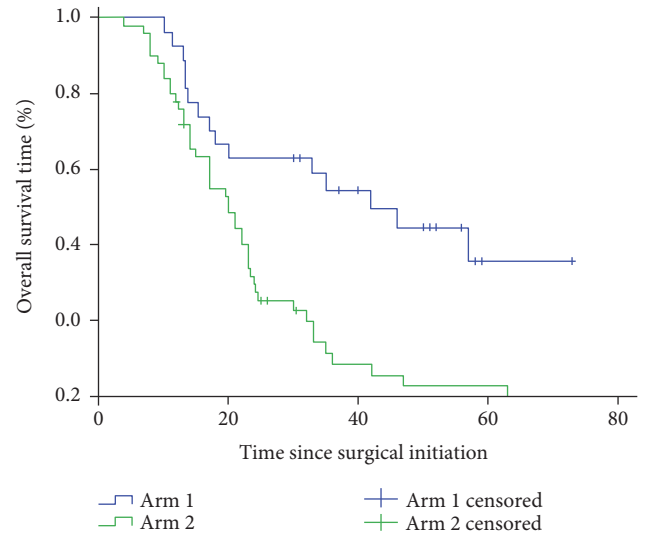

Figure 3: Overall survival time of Arm 1 versus Arm 2 was calculated using 27 patients in Arm 1 compared with 50 patients in Arm 2.

Table 2: Treatment group outcomes.

Treatment group	DFS			OS		
	Arm 1	Arm 2	*P*	Arm 1	Arm 2	*P*
1-year DFS or OS rates	48.21% (95% CI 29.76–64.46%)	28.00% (95% CI 16.45–40.75%)	*0.04*	92.59% (95% CI 73.50–98.09%)	78.00% (95% CI 63.81–87.16%)	*0.03*
2-year DFS or OS rates	33.38% (95% CI 17.80–49.78%)	2.00% (95% CI 0.16–9.23%)	0.00	62.96% (95% CI 42.12–78.07%)	32.00% (95% CI 19.70–44.97%)	0.00
3-year DFS or OS rates	25.96% (95% CI 12.47–41.75%)	0% (95% CI 0-0%)	0.00	55.56% (95% CI 35.22–71.81%)	16.00% (95% CI 7.50–27.37%)	0.00
5-year DFS or OS rates	—	—	—	48.14% (95% CI 28.69–65.19%)	12.00% (95% CI 4.88–22.60%)	0.00

process, cultures were expanded to 5×10^9 TIL cells and were harvested. Finally, these cells were infused back into patients.

2.4. Preparation of R-CIK. Peripheral blood mononuclear cells (PBMCs) of the patients were used to culture R-CIK. The detailed process of R-CIK preparation is the same to our published data [18]. Then, at the transfusion day, the dose of R-CIKs is about 5×10^9 cells.

2.5. Phenotype Detection. In order to analyze the cell population of TILs before transfusion, they were stained with antibodies against CD3-FITC, CD4-PE-Cy7-A, CD8-APC-Cy7-A, and CD16/CD56-PE (BD Bioscience, San Jose, CA, USA) and flow cytometry was performed using a BD FACS-Canto cell sorter (BD Bioscience, San Jose, CA, USA). Finally, the proportion of CD3+CD4+ and CD3+CD8+ cells of TILs was analyzed by gating the CD3+ population, and the percentage of CD3-CD16+ CD56+ cells of TILs was analyzed by CD45+ gating.

2.6. Statistical Analysis. Spss17.0 software was used to perform the statistical analysis. The Kaplan-Meier method was used to analyze the DFS and OS. Univariate and multivariable analyses also were used to analyze the prognostic factors. $P < 0.05$ was considered to demonstrate a statistically significant difference.

3. Results

3.1. Phenotype Analysis. Before transfusion of TIL cells to patients, we used flow cytometry to detect the proportion of CD3+, CD3+CD4+, CD3+CD8+, and CD3-CD16+CD56+ cells (Figures 1(a), 1(b), 1(c), and 1(d)). When the proportion of CD3+, CD3+CD4+, CD3+CD8+, and CD3-CD16+CD56+ cells reached appropriate levels, then we transfused the TIL back to patients. At the time of delivery of cultured TIL back to patients, the composition of the transfused cells was as follows: CD3+ $80.8\% \pm 3.23\%$, CD3+CD4+ $34.8\% \pm 2.14\%$, CD3+CD8+ $44.1\% \pm 2.56\%$, and CD3-CD16+CD56+ $3.7\% \pm 0.34\%$.

3.2. Treatment Outcomes. Our data demonstrated that the mDFS and mOS of Arm 1 versus Arm 2 were 23.66 months versus 9.78 months ($\chi^2 = 11.559$, $P \leq 0.001$, Figure 2) and 43.75 months versus 21.86 months ($\chi^2 = 15.03$, $P \leq 0.001$, Figure 3), respectively. Then, we also analyzed the 1-year DFS rates and OS rates, 2-year DFS rates and OS rates, 3-year DFS rates and OS rates, and 5-year OS rates. The data indicated that DFS rate and OS rates in Arm 1 were significantly better than those in Arm 2 at all measured time points. The detailed data was listed in Table 2. Thus, it appears that stage III malignant melanoma patients can benefit from TIL + IFN-α treatment.

3.3. Prognostic Factors of TIL + IFN-$\alpha \pm$ R-CIK Treatment in Arm 1. The DFS and OS of Arm 1 patients achieved greater improvement compared with those of Arm 2 patients. In order to observe potential prognostic factors in the Arm 1 treatment group, then we analyzed many factors such as sex, age, KPS scores, cell numbers for transfusion, number of culture days, and use of R-CIK therapy. Although univariate analyses indicated that KPS scores, transfused cell numbers, and increased duration of culture were potential predictive factors (Table 3), there were no significant differences by multivariate analysis based on these predictive factors (Tables 4 and 5).

Table 3: Univariate analysis.

	DFS (months)	P	mOS (months)	P
Age (years)				
>60	25.69	0.637	42.79	0.814
≤60	21.27		38.82	
Sex				
Male	20.47	0.560	37.33	0.861
Female	28.17		44.10	
KPS scores				
≥80	26.69	0.032	49.33	0.020
<80	13.27		21.50	
Cell numbers for transfusion				
$<8 \times 10^9$	6.91	0.000	16.94	0.000
$\geq 8 \times 10^9$	30.97		53.46	
Culture days				
<30	30.77	0.014	55.78	0.001
≥30	13.79		24.43	
R-CIK				
Yes	29.22	0.578	44.29	0.791
No	22.35		35.28	

3.4. Prognostic Factors of IFN-$\alpha \pm$ R-CIK Treatment in Arm 2. In Arm 1, our data indicated that adding R-CIK might not improve the DFS and OS of stage III malignant melanoma patients. To investigate whether adding R-CIK can improve the DFS or/and OS of the patients of Arm 2, we also used univariate analysis. Unfortunately, our data demonstrated that there were no significant differences whether with R-CIK therapy or not by univariate analysis (DFS: 9.94 months versus 8.40 months, $P = 0.707$; OS: 21.66 months versus 23.83 months, $P = 0.770$). Thus, it appears that stage III malignant melanoma patients cannot benefit from R-CIK based on IFN-α therapy.

3.5. Adverse Events. In this retrospective analysis, all patients completed our immunotherapy. There were no severe adverse effects (grade 3 or grade 4) associated with TIL, IFN-α, or R-CIK therapy. The primary side effects of immunotherapy (grade 1 or grade 2) were fever, arthralgia, nausea, leucopenia, liver dysfunction, anemia, and vitiligo (Table 6).

4. Discussion

Clearly, surgery is still the appropriate primary treatment for candidate malignant melanoma. However, in high-dose IFN-α as a current adjuvant therapy, the rate of relapse for stage III malignant melanoma patients remains very high [3].

Table 4: Multivariate analysis (DFS).

Parameters	Hazard ratio	95% confidence interval	P value
KPS (≥80 scores versus <80 scores)	0.948	(0.889–1.011)	0.104
Cell numbers for transfusion ($\geq 8 \times 10^9$ versus $< 8 \times 10^9$)	0.912	(0.782–1.064)	0.276
Culture days (<30 days versus ≥30 days)	1.038	(0.976–1.105)	0.268

Table 5: Multivariate analysis (OS).

Parameters	Hazard ratio	95% confidence interval	P value
KPS (≥80 scores versus <80 scores)	0.944	(0.878–1.015)	0.119
Cell numbers of transfusion ($\geq 8 \times 10^9$ versus $< 8 \times 10^9$)	0.794	(0.586–1.075)	0.136
Culture days (<30 days versus ≥30 days)	1.064	(0.981–1.153)	0.134

Table 6: Distribution of adverse events.

Side effects	Arm 1		Arm 2	
	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4
Fever	13	0	20	0
Arthralgia	5	0	13	0
Nausea	3	0	12	0
Leukopenia	4	0	8	0
Liver dysfunction	2	0	9	0
Anemia	3	0	10	0
Vitiligo	2	0	1	0

The treatment of stage III malignant melanoma patients with TIL in combination with IL-2 has previously demonstrated promising results [15–17]. Unfortunately, this combination appears to prolong DFS and OS only among those patients with a single-positive lymph node.

Based upon these encouraging results, efforts to improve the treatment method will be an urgent significance. In our study, administration of cultured autologous TIL combined with IFN-α therapy was employed as an adjuvant treatment strategy for stage III malignant melanoma patients. This was compared to the use of IFN-α alone. Our data indicates that TIL combined with IFN-α therapy can improve the DFS and OS of stage III malignant melanoma patients. From 1991 to present, CIK cell therapy has been applied as an immunotherapy for cancer patients in many clinical trials, including in patients with hepatocellular carcinoma (HCC), non-small-cell lung cancer (NSCLC), and renal cell carcinoma (RCC) [19–26]. And in our Immunotherapy Center of The Affiliated Cancer Hospital of Zhengzhou University, we also did some work to demonstrate that R-CIK (or CIK) combined with chemotherapy or not can prolong the mOS of HCC, RCC, pancreatic cancer, and so on [18, 27–31]. Therefore, R-CIK therapy was often employed in both Arm 1 and Arm 2 patients in order to increase the treatment efficacy. However, in our analysis of Arm 1 and Arm 2 data, there was no difference in DFS and OS with or without R-CIK. Up to now, many experiments indicate that CIK or R-CIK is considered to a nonspecific immunotherapy, which has major histocompatibility- (MHC-) unrestricted cytotoxic effect [26, 32]. Therefore, R-CIK and IL-2 are all nonspecific immunotherapy methods. It appears that combined two nonspecific immunotherapies (R-CIK and IL-2) may not improve the prognosis of stage III malignant melanoma patients.

As we all have known, Rosenberg et al. had done many experiments for metastatic melanoma by applying TIL combined with nonmyeloablative chemotherapy with or without 1200 cGy total body irradiation. The objective response rate can achieve more than 50% [33–35]. In our study, we applied TIL combined with IFN-α to treat stage III malignant melanoma as an adjuvant therapy, which indicated that this therapy can prolong the DFS and OS of these patients. Therefore, we conclude that patients diagnosed with stage III malignant melanoma can benefit from TIL + IFN-α treatment after surgery.

To explore the prognostic factors governing the efficacy of TIL + IFN-α treatment in Arm 1, we analyzed individual results in Arm 1 and correlated them to sex, age, KPS scores, cell numbers at time of transfusion, duration of culture, and presence or absence of R-CIK therapy. Although univariate analyses identified KPS scores, cell numbers for transfusion, and number of culture days as potential predictive factors, there were no significant differences based on these potential predictive factors by multivariate analysis. This leads us to conclude that adjuvant TIL therapy combined with administration of IFN-α can prolong DFS and OS in stage III malignant melanoma patients generally. However, adding R-CIK cannot improve the DFS and OS of stage III malignant melanoma patients further. In addition, in our study of all the patients, there were no treatment-related mortalities, and the toxic effects were comparable with previous TIL studies and IFN-α studies. While TIL cultures and transfusions require high laboratory expertise, the quality of cultured TIL is the key problem in clinical use. Most of all, our study demonstrated that TIL combined with IFN-α might be a good method for stage III malignant melanoma patients. In the future, a multicenter randomized phage study will become a better way to reveal the true clinical contribution of TIL combined with IFN-α for the treatment of stage III malignant melanoma.

5. Conclusions

In summary, adjuvant adoptive TIL therapy combined with IFN-α therapy can prolong the DFS and OS of stage III malignant melanoma patients who undergo surgical excision. Toxicity and side effects were quite manageable. In the future, more studies should be performed to provide additional data regarding the efficacy of adjuvant TIL combined with IFN-α therapy in the management of stage III malignant melanoma.

Abbreviations

IFN-α: Interferon-α
DFS: Disease-free survival time
OS: Overall survival time
TIL: Tumor-infiltrating lymphocytes
R-CIK: RetroNectin-activated cytokine-induced killer cells
ORR: Overall response rate
ACT: Adoptive cell transfer
PBMC: Peripheral blood mononuclear cells.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Wei Li, Quanli Gao, and Linping Xu designed the study. Wei Li and Yaomei Wang analyzed the data and wrote the paper. Lingdi Zhao and Daniel B. Kellner read and edited the manuscript. All authors read and provided comments on the report. All authors read and approved the final manuscript. Wei Li, Linping Xu, and Yaomei Wang share co-first authorship.

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Review Article

Cereblon: A Protein Crucial to the Multiple Functions of Immunomodulatory Drugs as well as Cell Metabolism and Disease Generation

Qinglin Shi and Lijuan Chen

Department of Hematology, The First Affiliated Hospital of Nanjing Medical University/Jiangsu Province Hospital, 300 Guangzhou Road, Nanjing 210029, China

Correspondence should be addressed to Lijuan Chen; chenljb@126.com

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It is well known that cereblon is a key protein in autosomal recessive nonsyndromic mental retardation. Studies have reported that it has an intermediary role in helping immunomodulatory drugs perform their immunomodulatory and tumoricidal effects. In addition, cereblon also regulates the expression, assembly, and activities of other special proteins related to cell proliferation and metabolism, resulting in the occurrence and development of metabolic diseases. This review details the multiple functions of cereblon and the underlying mechanisms. We also put forward some unsolved problems, including the intrinsic mechanism of cereblon function and the possible regulatory mechanisms of its expression.

1. Introduction

The gene that encodes cereblon, *CRBN*, was first identified by Higgins et al. while studying genes that were related to memory and learning. Their team found a nonsense mutation (R419X) in a newly discovered gene located on 3p26.2 in an ethnic group with a mild type of nonsyndromic mental retardation. The gene was assigned the name *CRBN* (cereblon, NM_016302) based on its supposed role in the development of cerebral tissues and because its expression in the hippocampus among other areas is associated with memory and learning processes [1]. Although there are numerous causes of mental retardation, the stop codon of the CRBN gene is thought to be the major factor [2].

CRBN interacts with the DNA damage-binding protein-1 (DDB1), Cullin 4 (Cul4A or Cul4B), and regulator of Cullins 1 (RoC1) to form the functional E3 ubiquitin ligase complex. In this complex, CRBN functions as a substrate receptor of E3 ubiquitin ligase complex and targets proteins for proteolysis through a ubiquitin-proteasome pathway [2–4]. E3 ubiquitin ligase complex can achieve different effects by targeting different substrates.

In recent years, CRBN has been extensively studied because it is involved in many biological processes and is responsible for the multiple effects of immunomodulatory drugs (IMiDs). CRBN performs these functions generally under two circumstances: with and without IMiDs. CRBN expression in the cells can affect cell metabolism and cause disease in the absence of IMiDs. In addition, CRBN is also the target protein of IMiDs and enhances their effects when present. In this review, we will describe the various functions of CRBN and the underlying mechanisms involved.

2. CRBN Expression Affects Cell Metabolism and Disease Generation

CRBN, a 442-amino acid protein with multifunction, locates in the cytoplasm, nucleus, and peripheral membrane of the human brain and other tissues [5]. The diverse roles of

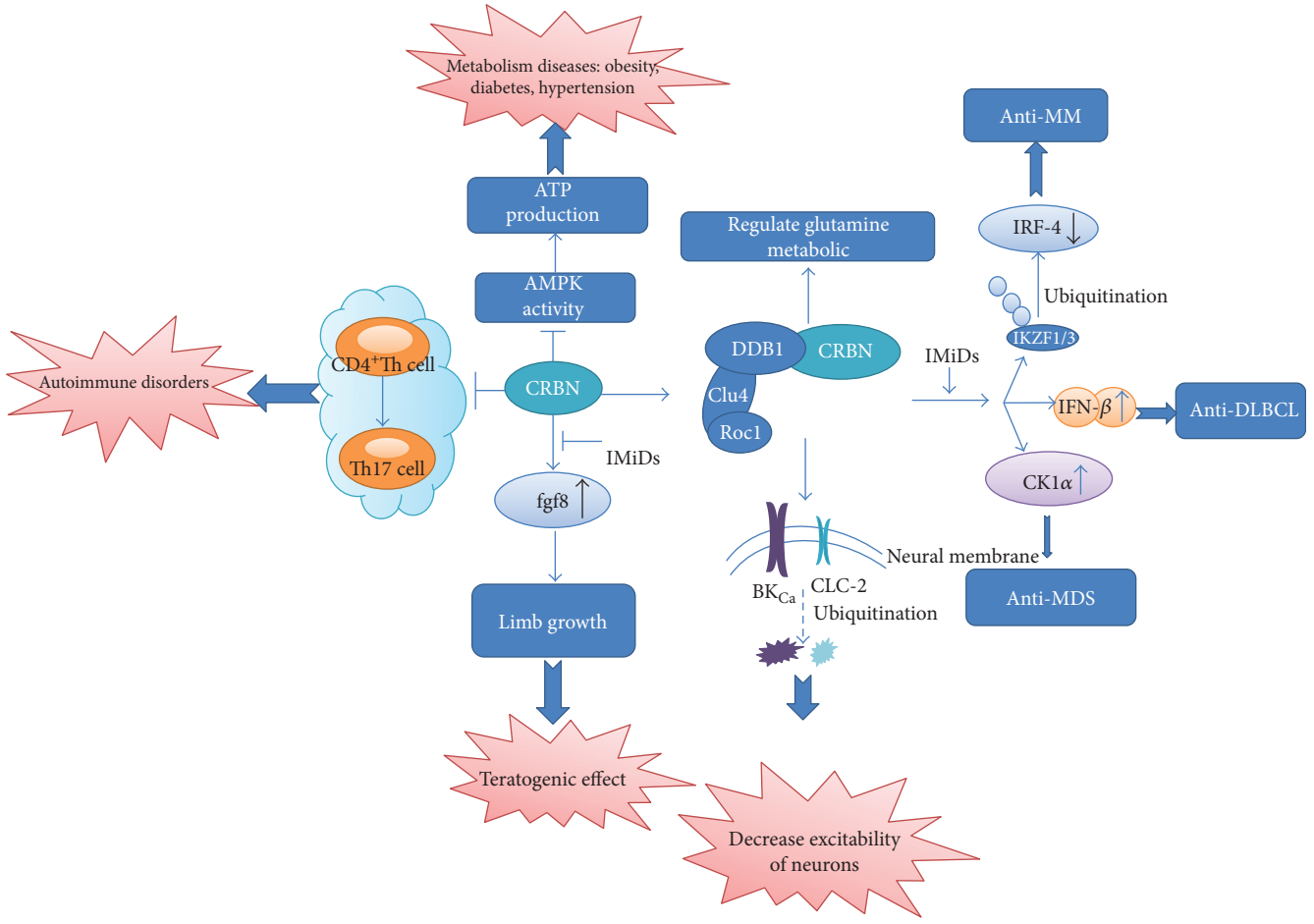

FIGURE 1: Schematic representation of CRBN-mediated function regulation. In the absence of immunomodulatory drugs (IMiDs), CRBN has an important role in the regulation of ion transport, modulation of AMP-activated protein kinase (AMPK) signaling, and metabolism in cell and whole tissues or organs. In the presence of IMiDs, CRBN is the target protein of IMiDs and helps exert their multiple functions.

CRBN on cell metabolism and disease genesis have been extensively studied.

2.1. CRBN and Cell Metabolism. CRBN has an important role in the regulation of ion transport, modulation of AMP-activated protein kinase (AMPK) signaling pathway, and metabolism in cell and whole tissues or organs (Figure 1) [1, 6, 7]. Intriguingly, CRBN also influences cell proliferation and apoptosis.

Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are ubiquitous in many tissues and are activated by membrane depolarization and high levels of intracellular calcium [8]. BK_{Ca} channels have an essential role in neuronal excitability. Evidence showed that CRBN might regulate the activity of BK_{Ca} channels by affecting their expression and assembly in the cell membrane [9]. BK_{Ca} channels are ubiquitinated in the endoplasmic reticulum by the E3 ubiquitin ligase complex, through direct interaction with CRBN. This process decreases the expression of BK_{Ca} channels in the neuronal membrane surface and retains them in the endoplasmic reticulum [4, 10]. When this control process is inhibited, BK_{Ca} channels in the cell membrane are enriched and the excitability of neurons is increased. These effects increase excitability of nerve cells and eventually increase the risk of epilepsy [8, 10]. In addition to BK_{Ca} channels, experiments showed that CRBN also interacts with the voltage-gated chloride channel-2 (ClC-2), which is ubiquitous in cell membranes and functions by regulating cell excitability in neurons [11, 12]. CRBN helps the E3 ubiquitin ligase complex target ion channels for ubiquitination and therefore maintains the ion balance and reduces the incidence of ion channel disease.

CRBN is a metabolic regulator that directly binds to AMPK and inhibits its function of increasing ATP production and decreasing ATP consumption. Its regulation of energy metabolism on a cellular level helps to control appetite, intake of nutrients, and the endocrine system, which are

related to many physiological processes [13, 14]. AMPK regulates the metabolism of carbohydrates, liquid, proteins, and the energy balance of the whole body. The dysfunction of AMPK causes a wide spectrum of metabolic diseases such as obesity, diabetes, hypertension, and even cancer [13].

The CRBN protein also participates in the regulation of glutamine whose dysregulation is responsible for many diseases including cancer and other metabolic abnormalities [15, 16]. Glutamine synthetase (GS) is essential in the signaling pathway that regulates glutamine levels. Nguyen et al. reported that GS binds directly to CRBN, leading to GS ubiquitination by E3 ubiquitin ligase complex and the eventual decrease of glutamine. This process functions as a negative feedback mechanism under high glutamine concentrations [17, 18]. Likewise, the ubiquitination of GS will be reduced under low glutamine concentrations.

In addition to the regulation of key proteins and signal pathways related to cell metabolism, CRBN has effects on cell proliferation and apoptosis. CRBN knockdown in multiple myeloma cells showed significantly reduced CRBN expression and decreased cell viability [19]. In contrast, CRBN overexpression promotes cell proliferation.

2.2. CRBN and Disease Genesis. Many diseases, such as cardiovascular disease, obesity, and fatty liver, have been linked to the CRBN-mediated inactivation of AMPK. Several studies demonstrated that activated AMPK protected myocardial tissues, thus decreasing ischemia-reperfusion injury. The activity inhibition of AMPK by CRBN may cause cardiac diseases [20]. Furthermore, increased AMPK activity might protect cells from injury caused by high-fat induced disorders of lipometabolism and alcohol-induced accumulation of liquid in liver cells [13, 21, 22]. It was reported that CRBN-deficient mice were not susceptible to metabolic-related diseases (Figure 1) [23].

Recent studies also found that CRBN has a negative regulation role of CD4+ T cell activation. CRBN deficiency increased the activation of CD4+ T cells and enhanced IL-2 secretion, helping CD4+ T cells differentiate into Th17 cells [24]. Enhanced T cell activation can sometimes cause T-cell-mediated autoimmune disorders such as experimental autoimmune encephalomyelitis and delayed-type hypersensitivity reaction [25, 26]. These experiments reflect the regulatory role of CRBN in disease genesis.

Sawamura et al. reported, in 2015, that CRBN was recruited to aggresome and had a protective effect against extracellular stresses, such as ubiquitin-proteasome system (UPS) impairment and oxidative stress. Aggresomes are thought to be cytoprotective because they sequester toxic, aggregate proteins and eliminate them by autophagy. Actually, aggresomes which contain misfolded proteins can also be observed in many neuropsychiatric diseases such as Parkinson's disease and Schizophrenia. The team showed that CRBN plays a vital role in aggresome formation and cytoprotection against UPS impairment. The normal CRBN function is cytoprotective against UPS dysfunction-induced cell death and the defect may be of great importance to intellectual disability (ID) pathogenesis [27].

3. CRBN Is the Target Protein of IMiDs and Is Responsible for Their Multiple Functions

IMiDs, such as thalidomide, lenalidomide, and pomalidomide, are oral medications used for treating multiple myeloma (MM), deletion (5q) myelodysplastic syndrome (del(5q) MDS), chronic lymphocytic lymphoma (CLL), and activated B-cell-like subtype diffuse large B-cell lymphoma (ABC-DLBCL) (Figure 1). Thalidomide was first introduced in the late 1950s as a sedative for pregnant women to prevent morning sickness [28]. However, treatment with this drug caused serious side effects including limb deformity [28]. Because of this serious teratogenic effect, thalidomide was withdrawn from the market in 1960s [29]. Accumulating evidence indicated that CRBN was responsible for the teratogenic activities of thalidomide, until 2010, when a group of scientists proved that CRBN was the bona fide target of thalidomide [4]. Surprisingly, it was subsequently discovered that thalidomide possessed other activities including antiangiogenesis and anti-inflammatory effects. Based on the reports of thalidomide's effects against multiple myeloma, the Food and Drug Administration (FDA) approved the use of thalidomide for the treatment of the newly diagnosed MM patients in 1999 [29, 30]. Lenadilomide and pomalidomide were approved by FDA for MM treatment in 2006 and 2013, respectively. Those findings triggered increased researches in the cancer treatment field of IMiDs. The molecular mechanism of the antimyeloma effect of IMiDs has been studied over the last 20 years [31]. It was gradually discovered that the antitumor effect of IMiDs on multiple myeloma and other hematologic diseases is mediated by CRBN either through a ubiquitin-dependent or a ubiquitin-independent pathway. Furthermore, the response to IMiD therapy is also related to the expression of CRBN.

3.1. CRBN and the Teratogenic Effect of IMiDs. When it was discovered that CRBN directly interacted with thalidomide, researchers then investigated the role of CRBN *in vivo* [4]. Experiments on a zebrafish model finally verified the assumption that CRBN was responsible for the thalidomide teratogenic effect. In their experiments, thalidomide decreased the protein level of fibroblast growth factor 8 (fgf8), which is essential for limb growth [32]. Knockdown of zebrafish CRBN had the same effect as thalidomide treatment. Furthermore, the teratogenic effect induced by thalidomide was reversed by overexpression of wild-type CRBN. They also found that the overexpression of Y374A/W376A-mutated zebrafish CRBN, which cannot bind to thalidomide, had no obvious malformation of limb development. Therefore, CRBN was considered the direct target protein of thalidomide. Inhibition of CRBN activity by thalidomide downregulated fgf8. This is one of the mechanisms that caused the thalidomide teratogenic effect [4].

fgf8 can be upregulated by the CD147-MCT1 complex whose activity is promoted by CRBN through their combination. In the presence of thalidomide, the combination of CRBN and the CD147-MCT1 complex is weakened. Consequently, fgf8 expression is downregulated. This may be another mechanism of the teratogenic effect of IMiDs [33].

3.2. CRBN and the Antimyeloma Effect of IMiDs. The mechanism of the antimyeloma effect of thalidomide and other IMiDs remained unclear until researchers discovered the mechanism of the teratogenic effect of thalidomide and its direct combination with CRBN (Figure 1). Several researchers reported that Ikaros (IKZF1) and Aiolos (IKZF3) were direct substrates of E3 ubiquitin ligase complex. IKZF1 and IKZF3 are specific members of the B-cell transcription factors family and are critical for plasma cell development and proliferation [34]. After binding of IMiDs, E3 ubiquitin ligase complex changed its specificity and ubiquitinates and marks for degradation these two factors [34–36]. Interestingly, it was reported that a single amino acid substitution of IKZF3 decreased the inhibition of cell proliferation and conferred resistance to degradation induced by IMiDs [37]. These results suggested that the repression and degradation of IKZF1 and IKZF3 is potentially involved in the mechanism of IMiDs against multiple myeloma.

The degradation of IKZF1 and IKZF3 induces cytotoxicity in myeloma cells because they are critical factors for B-cell differentiation. Nevertheless, some investigators further studied the targets of these two factors to identify the most direct factors leading to this antitumor effect. Their results showed that the knockdown of IKZF1 and IKZF3 by shRNA contributed to the suppression of IRF4 levels and the increase of interleukin-2 (IL-2) levels, which affected the survival of MM cells. Similar results were observed under lenalidomide treatment [34].

The transcription factor IRF4, identified as an essential factor for myeloma cell survival, is involved in the activity of lenalidomide to treat multiple myeloma [38, 39]. The inhibition of IRF4 was toxic to myeloma cell lines. The direct targets of IRF4 include several key regulators that have a great impact on cell proliferation and survival, such as Myc, CDK6, and STAG2 [30, 38]. IRF4 regulates cell metabolism, cell cycle progression, cell death, and plasma cell differentiation via these direct targets [38].

It was reported previously that IL-2 inhibits tumor formation suggesting that the IL-2-mediated suppression of tumors might be a new approach for treating myeloma or other malignancies. IKZF1 and IKZF3 are transcriptional suppressors of the *IL-2* gene whose expression product can regulate T cell function [40, 41]. Therefore, lenalidomide-induced IL-2 production in T cells is caused by rescuing inhibition of IL-2 whose expression is repressed by IKZF1 and IKZF3 [40, 42].

In addition to the Ikaros family members, many other interesting proteins participating in important physiological processes also bind to CRBN and are regulated by IMiDs in a ubiquitin-dependent mechanism [37, 43].

In addition to the ubiquitin-dependent pathway mentioned above, a recent study reported a ubiquitin-independent pathway through which CRBN mediates the antimyeloma effect of IMiDs. CRBN promotes the activation of the CD147-MCT1 complex, which is upregulated in MM cells. CD147 enhances angiogenesis, cell proliferation, cell survival, and tumor aggressiveness, which can be observed in many kinds of malignances [44]. CD147 and MCT1 form a complex that functions in the cellular metabolism of tumor cells [45]. IMiDs compete with CRBN to combine with the CD147-MCT1 complex to weaken the CRBN and CD147-MCT1 complex, thus inhibiting tumor growth [33]. The suppression of CD147-MCT1 complex activity also decreases the aggressiveness of B cell neoplasms.

Recently, Xu et al. identified that argonaute 2 (AGO2), as a CRBN binding partner, plays an important role in regulating angiogenesis and MM cell survival. Treating the IMiD-sensitive MM cells with lenalidomide induced the steady-state levels of CRBN which were significantly increased whereas the levels of AGO2 were significantly decreased. It has been reported that AGO2 plays a pivotal role in microRNA (miRNA) maturation, stability, and function. Under the treatment of IMiDs, the steady-state levels of AGO2 and miRNA were significantly downregulated and ultimately inhibited angiogenesis and cell growth [46].

3.3. CRBN and Its Downstream Protein Expression Level Affect the Response Rate of IMiDs. Researchers started to explore whether CRBN protein level could be used to guide the rational clinical use of IMiDs. A study enrolled 107 MM patients to assess the expression level of CRBN protein by immunohistochemical staining. Among these patients, 60 were relapsed and/or refractory MM patients who had received lenalidomide and dexamethasone (LD) as their salvage treatment, 45 were newly diagnosed MM patients who had received thalidomide and dexamethasone (TD) as their induction therapy, and 22 were newly diagnosed patients who had melphalan, bortezomib, and prednisolone (MVP) as their induction therapy. Results suggested that higher CRBN protein level was associated with superior treatment response to IMiD-based therapy instead of the regimen without IMiDs [36]. Another study compared the changes of CRBN expression level pre- and post-lenalidomide therapy in nine lenalidomide-resistant patients. They were surprised to find that the CRBN expression level was significantly reduced at the time of drug resistance [19]. Similar studies have shown that CRBN is a unique biomarker for IMiD sensitivity and that high CRBN expression is an independent factor related to a better prognosis in MM patients treated with IMiDs [36, 47–49]. Some studies showed that the downstream of CRBN, such as IKZF1/3, may also affect the prognosis and the overall survival and progression-free survival of MM patients [36, 50, 51]. However, there is still much controversy in the relationship between CRBN-IKZF1/3-IRF4 expression level and prognosis in MM patients.

In line with the analysis of clinical data, the sensitivity to IMiDs is strongly related to CRBN protein level on a cellular level. Zhu et al. reported that the therapeutic effect of IMiDs was almost completely cancelled and IMiD-resistant cells formed if CRBN was knocked down [19]. Zhu et al. reported that *CRBN* knockdown caused MM cell lines to acquired resistance to lenalidomide compared with their counterparts. Subsequently, they did more tests with other antimyeloma drugs, such as pomalidomide, melphalan, dexamethasone, and bortezomib, and found that CRBN knockdown cells acquire resistance to pomalidomide but retained sensitivity to other drugs [19].

The same group compared the CRBN expression level of IMiDs primary resistance MM cell lines such as OCI-My5 and OPM1 with relatively sensitive cell lines such as MM1S and OPM2. Results showed that the sensitive cell lines expressed higher levels of CRBN protein and transcriptional levels than resistance cell lines [30]. Furthermore, the sensitivity of lenalidomide was increased by the upregulation of CRBN levels in the primary LEN-resistance cell lines [30]. Many other experiments also confirmed that acquired resistance to lenalidomide was accompanied by decreased CRBN. This evidence further demonstrated that CRBN is an independent factor that is predictive for the prognosis of MM patients using IMiD-based therapy [47, 52].

3.4. CRBN and the Therapeutic Effect of IMiDs in Other Hematologic Diseases. CRBN-associated substrates and downstream signaling vary in different cell types, which accounts for the multiple effects of IMiDs [30]. In addition to the antimyeloma effects, IMiDs are effective for the treatment of ABC-DLBCL, CLL, and deletion (5q) myelodysplastic syndrome (MDS). Although these therapeutic effects depend on CRBN, the pathways involved in cell proliferation and survival are different from those involved in the antimyeloma effect (Figure 1) [39, 53].

Lenalidomide-induced tumoricidal effects on ABC-DLBCL cells have gained much attention in recent years. CRBN is required for the toxic effect of lenalidomide in ABC-DLBCL. Knockdown of CRBN reduces the toxic effect of lenalidomide and affects its therapeutic ability on ABC-DLBCL. However, these effects can be reversed by the ectopic expression of CRBN [39]. Indeed, these therapeutic effects are achieved by enhancing interferon beta (IFN-β) production and inhibiting the activity of nuclear factor-κB (NF-κB) through the downregulation of IRF4 and SPi-B (an Ets family transcription factor) in a cereblon-dependent manner [54]. CRBN has an important role in maintaining the levels of SPi-B and IRF4 in ABC-DLBCL [39]. Following CRBN downregulation, the mRNA and protein levels of SPi-B and IRF4 were reduced [39]. SPi-B is required for ABC-DLBCL cells survival and its knockdown may have toxic effects on ABC-DLBCL cells [54]. Similarly, IRF4 expression is regarded as a hallmark of ABC DLBCL and its overexpression confers ABC-DLBCL cells resistant to lenalidomide [29, 39]. Spi-B together with IRF4 reduces the expression of INF-β and influences the survival and proliferation of cells in ABC-DLBCL [55]. There may be a positive interaction mechanism between IRF4 and NF-κB, because NF-κB enhances IRF4 transcription and IRF4 enhance NF-κB activity. The downregulation of IRF4 inhibits NF-κB activity, and conversely, the overexpression of IRF4 enhances NF-κB activity and results in lenalidomide resistance in ABC-DLBCL cells [53].

Research indicates that treatment with lenalidomide significantly inhibited CLL cells proliferation, which is associated with the p53-independent upregulation of a cyclin-dependent kinase inhibitor p21. Silencing of CRBN impaired the effect of lenalidomide to induce p21 expression as well as CLL cell proliferation. These results indicate that lenalidomide directly inhibits the CLL cell proliferation in a CRBN/p21-dependent manner [56].

The CSNK1A1 gene, whose expression product is CK1α, located in the common deleted region for del (5q) MDS [57]. CK1α has tumor-suppressing capabilities and is closely related to the biological and therapeutic effect of del (5q) MDS [57, 58]. Lenalidomide application reduces its expression [59]. However, no similar phenomenon was observed when cells treated with the proteasome inhibitor or other nonimmunomodulatory drugs. The lenalidomide-dependent decrease in CK1α protein level was induced by the ubiquitin action of the CRBN-related E3 ubiquitin ligase complex. It was shown that the lenalidomide-induced ubiquitination of endogenous CK1α only occurred in the presence of CRBN [60]. Last year, Fang et al. identified a candidate gene GPR68, whose expression products has been implicated in calcium metabolism, for modulating the sensitivity to LEN in MDS cells. They found lenalidomide-induced GPR68 expression via IKZF1, resulting in the increasing level of cytosolic calcium and activating a calcium-dependent calpain CAPN1, which plays an important role in the induction of apoptosis of MDS cells. Depletion of calpastatin, an endogenous CAPN1 inhibitor which is encoded by a gene deleted in del(5q) MDS, increased the expression of CAPN1 and enhanced the sensitivity of del(5q) MDS to lenalidomide [61]. Taken together, these studies indicate the essential role of CRBN in the treatment of del(5q) MDS and provide an explanation for the superior responses of patients with del(5q) MDS to lenalidomide treatment.

3.5. Novel Agents and Newly Discovered Signal Pathways of CRBN. Many researchers have sought to identify new CRBN agents and new signal pathways of CRBN, which may allow a wider range of application in treating more diseases. Matyskiela et al. reported a novel CRBN agent CC-885 with potent ability to inhibit cancer cell line growth as well as patient-derived acute myeloid leukemia cells. CC-885 promoted ubiquitination and degradation of translation termination factor GSPT1 (eRF3a) [62]. GSPT1, cooperatively with eRF1, induces effective stop codon recognition [63]. The degradation of GSPT1 leads to cell cycle arrest thereby ensuring the antitumor effect of CC-885 [62].

CRBN also negatively regulates the Toll-like receptor 4-(TLR4-) mediated signal pathway, thereby downregulating NF-κB expression and proinflammatory cytokine production [64]. CRBN binds to the zinc finger domain of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which contains an autoubiquitination site [65]. The CRBN-TRAF6 interaction attenuates the ubiquitination of TRAF6 and TAB2, thereby inhibiting the activity of NF-κB and the production of proinflammatory cytokines [65]. *In vivo* experiments showed that CRBN knockdown mice were more vulnerable to lipopolysaccharide (LPS) challenge and that this effect might be associated with enhanced NF-κB activity and increased proinflammatory cytokines [64].

4. Conclusions

CRBN is closely associated with the proliferation and metabolism of normal cells as well as tumor cells. Its existence ensures normal metabolic function and normal physiological

function of ion channels, thereby maintaining cell growth and proliferation. CRBN is also involved in the occurrence of many diseases. In addition, with the identification of CRBN and its multiple functions, the antitumor action and the relative side effects of IMiDs have become more understandable. Evidence indicates that CRBN is an indispensable protein for IMiDs function. Furthermore, CRBN is a direct target of IMiDs and is related to the sensitivity and response to IMiDs, providing a theoretical foundation for individualized clinical therapy.

However, the mechanisms involved in CRBN functions are still poorly understood. What is the underlying mechanism through which cellular contents of CRBN can be decreased after long-term treatment with IMiDs in MM patients or MM cells? What is the intrinsic mechanism of the different CRBN protein levels among different cell lines sensitive or resistant to IMiDs? If eliminating CRBN expression can suppress cell proliferation and induce apoptosis, then why is the viability of cells that express low levels of CRBN protein similar to cells that highly express CRBN protein? Many animal experiments have shown that the inhibition of CRBN reduces the genesis of cardiovascular disease and obesity: could this be the same in humans? The development of drugs that combine with IMiDs to overcome IMiDs resistance resulting from the IMiD-mediated downregulation of CRBN might provide better combined clinical treatment.

Conflicts of Interest

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

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