Journal of Oncology

Immunotherapy: A New Hope for Cancer Patients

Lead Guest Editor: Shalini Gupta
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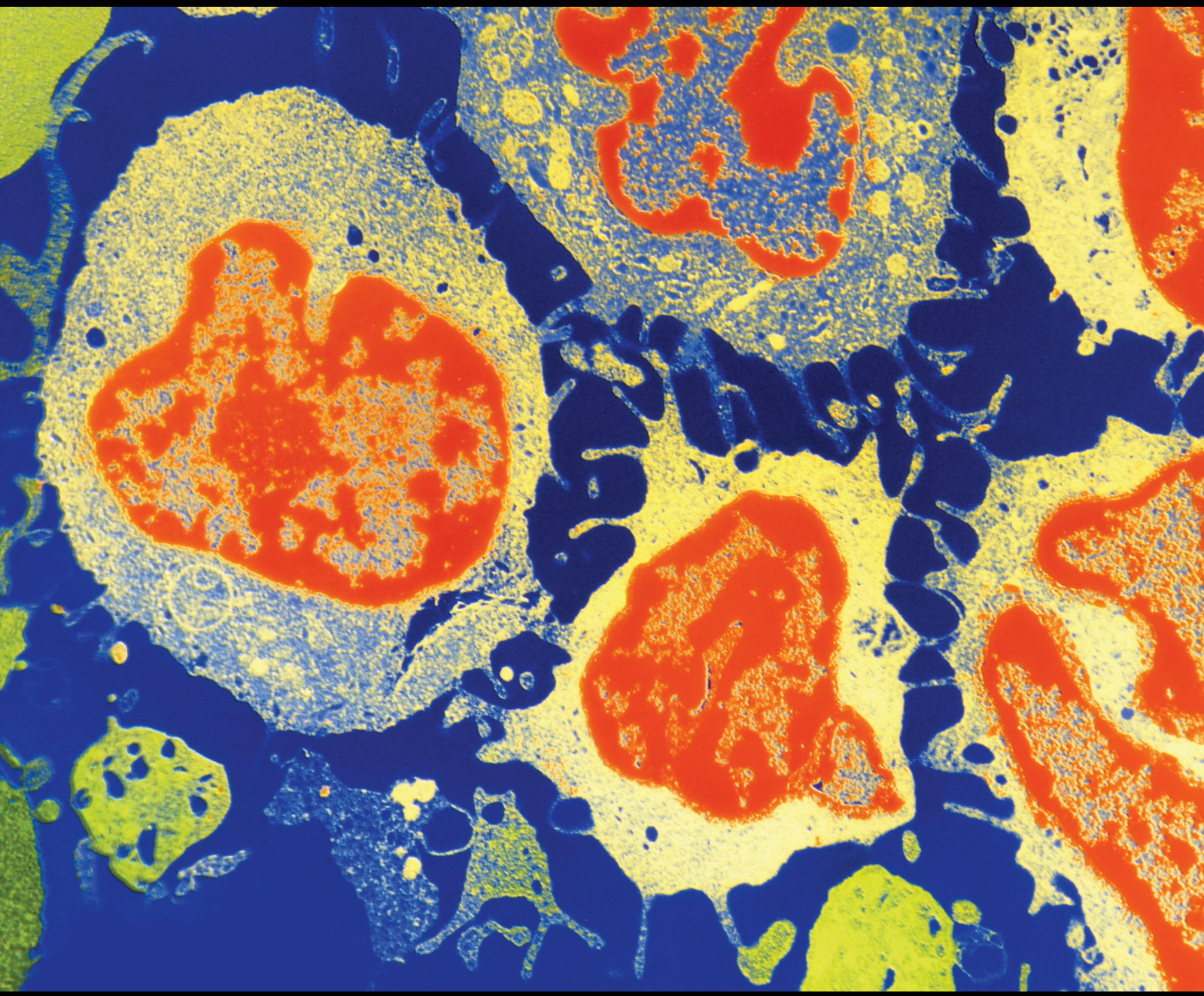

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Editorial

Immunotherapy: A New Hope for Cancer Patients

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Every year, a large number of people fall prey to cancer worldwide and more than half lose their battle to cancer. Among the multimodalities of cancer management, including surgery, hormonal therapy, radiotherapy, and chemotherapy, immunotherapy has revolutionized the treatment of cancer. Immune-oncology is a rapidly developing and exciting field of cancer treatment with the prospective of impacting the management of a wide array of malignancies which have recently witnessed a steep rise.

The major goal of cancer immunotherapy is to alleviate tumor-associated suppression of anticancer immune responses. The basic notion of utilizing a patient's immune system against cancer cells dates back to 1997, since the immune system response versus virulent cells during initial transformation in the immune surveillance process was discovered. Cancer immunotherapy, which is occasionally referred to as immune-oncology, induces the patient's own immune system and attempts to exploit the exquisite power and specificity of the immune system for cancer patient treatment. Immunotherapy against cancers encompasses a diverse continuum of approaches, ranging from stimulating effector mechanisms to counteracting inhibitory and suppressive mechanisms. Owing to the swift knowledge amassed by the scientific community about the immune system, small molecules, peptides, recombinant antibodies, vaccines, and cellular therapeutic modalities are being applied to manipulate the immune response to treat cancer.

With recent success, immunotherapy has emerged as a pragmatic strategy and a reputable pillar of cancer treatment improving the prognosis of many patients suffering from different malignancies. The two main drivers behind this success are immune checkpoint inhibitors (CPIs) and chimeric antigen receptor- (CAR-) T cells. For checkpoint blockade, current studies emphasize combinational approaches, perioperative use, response prediction, new tumor entities, toxicity management, and use in special patient populations. Regarding cellular immunotherapy, recent studies confirmed safety and efficacy of CAR-T cells in larger cohorts of patients with acute lymphoblastic leukemia or diffuse large B-cell lymphoma. Different strategies to translate the conspicuous triumph of CAR-T cells in B-cell malignancies to other hematological and solid cancer types are currently in clinical investigation phase. Since cancer is still one of the deadly challenges that are faced by humans, treatment regimes with single-drug therapies are not yielding very effective results in terms of improving the patient survival and treatment, and therefore, a combination of two or more therapies targeting different mechanisms could be more efficacious.

The purpose of this special issue is to present the recent progress in this exciting field. A brief summary of all accepted papers is provided as follows.

In the review article by M. Sambi et al., the authors have described the options to improve patient response rate to immunotherapy with an emphasis on adopting a multimodal approach with an emphasis on the novel role that the gut microbiota may play in modulating the efficacy of cancer immunotherapy.

The paper by D. D. Goycoechea et al. provides an extensive and critical review of the literature regarding

immune checkpoint inhibition in classical Hodgkin lymphoma. The paper discusses the early achievements and provides new perspectives on immune checkpoint inhibition in a disease that constitutes a model of sensitivity to this treatment.

The paper by A. Rogiers et al. presents an updated and comprehensive review of the results of the main clinical trials in the field of immunotherapy in melanoma and highlights potential consequences on the psychosocial wellbeing, neurocognitive functions, and life quality of patients.

In the research article by P. Darvin et al., the authors investigated the regulation of PD-L1 expression in breast cancer tumor spheres compared to cell lines. They show that PD-L1 is likely regulated by histone modifications rather than DNA methylation.

In the paper by D. Wu et al., the authors reached the conclusion that CTP is currently the most effective neoadjuvant regimen for the chance of achieving pathological complete response (pCR), with little additional toxicity compared, and MP has the best tolerability and acceptable efficacy.

In the research article by A. Rossi et al., the authors point out that ALDH levels are elevated in the serum of NSCLC patients with advanced stage as well as in early stage disease and therefore can be evaluated as part of the panel of markers for noninvasive detection of early NSCLC in a larger cohort of patients at risk.

In the paper by A. C. Martínez-Torres et al., the authors have discovered that PKHB1, a TSP1-derived CD47 agonist peptide, can induce immunogenic cell death in tumoral T lymphoblasts. Tumor cell lysate (TCL) from PKHB1-treated lymphoblasts can induce DC maturation and T-cell activation ex vivo. Also, TCL can be used as tumor vaccine, when administrated in either prophylactic or therapeutic settings.

In the review article by C. Fiorentini et al., the authors have reviewed clinical results of immunotherapy in ACC and highlighted molecular mechanisms that lead to immunotherapy failure in ACC. They have also suggested possible approaches to prevail over resistance.

In the review article by R. E. Cooke et al., the authors have discussed the concept of immune profiling to target patients of multiple myeloma who might benefit the most from immunotherapeutics. The review also covers the advances and use of humanized mice as well as 3D culture systems for personalized medicine.

In the paper by M. Kreidieh et al., the authors have reviewed in detail about treatment strategy in liver cancer including radiation therapy and immunotherapy alone and in combination. The study also explores the evidence surrounding the use of SBRT and immunotherapy for the treatment of HCC and CCA.

In the paper by F. Toia et al., the authors have presented a systematic review of preclinical and clinical studies on $\gamma\delta$ T cell-based immunotherapy and melanoma, in which the discussion is mainly focused on research state of the art and future perspectives.

In the review article by F. Ghali et al., the authors have reviewed the current status of adjuvant and neoadjuvant immunotherapy in localized and locally advanced renal cell carcinoma, combining discussion of recently published, ongoing clinical trials and future concepts in this fast moving area of investigation.

In the paper by M. A. I. Al-Hatamleh et al., the authors have reviewed the implications of tumour necrosis factor and its receptor 2 (TNFR2) expressions in breast cancer, the oncogenic consequences, and their role in the suppressive immune responses by TRegs. They have described the use of nanoparticles as a targeted drug delivery agent in immunotherapy, based on their advantageous properties. The review also discusses the manipulation of NPs with TNF-antagonist to modulate TNF-TNFR2 interaction that inhibits breast cancer progression.

In the research article by E.-G. Chavez-Cortez et al., the authors show a novel, affordable, and effective tumor, glioblastoma. The immunotoxin produced and tested in this investigation, i.e., IgY-abrin immunotoxin, had cytotoxic activity against CD133+ malignant glioma stem cells and provided a novel approach for the immunotherapy of glioblastoma.

The research article by U. Swami et al. suggests that advanced melanoma patients discontinuing anti-PD-1 therapy due to irAE usually experience durable clinical benefit. However, caution is needed with these agents in patients with underlying autoimmune diseases. The study explored durable clinical benefit in this subset of patients. The manuscript goes in detail of each immune-toxicity and its management.

The review by S. P. Singh et al. provides an interesting overview of the role of cancer stem cells and molecular pathways that are aberrantly expressed in these cells. This review article also provides an updated description of markers expressed by cancer stem cells and used for their isolation. It also covers the options of treatment of cancer stem cells with immunotherapy. Therefore, examples of molecules/pathways expressed by cancer stem cells that could represent target for immunotherapy approaches are also included in the review.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this Special Issue.

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Review Article

Immunotherapy: Newer Therapeutic Armamentarium against Cancer Stem Cells

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Mounting evidence from the literature suggests the existence of a subpopulation of cancer stem cells (CSCs) in almost all types of human cancers. These CSCs possessing a self-renewal capacity inhabit primary tumors and are more defiant to standard antimitotic and molecularly targeted therapies which are used for eliminating actively proliferating and differentiated cancer cells. Clinical relevance of CSCs emerges from the fact that they are the root cause of therapy resistance, relapse, and metastasis. Earlier, surgery, chemotherapy, and radiotherapy were established as cancer treatment modalities, but recently, immunotherapy is also gaining importance in the management of various cancer patients, mostly those of the advanced stage. This review abridges potential off-target effects of inhibiting CSC self-renewal pathways on immune cells and some recent immunological studies specifically targeting CSCs on the basis of their antigen expression profile, even though molecular markers or antigens that have been described till date as expressed by cancer stem cells are not specifically expressed by these cells which is a major limitation to target CSCs. We propose that owing to CSC stemness property to mediate immunotherapy response, we can apply a combination therapy approach by targeting CSCs and tumor microenvironment (TME) along with conventional treatment strategies as an effective means to eradicate cancer cells.

1. Introduction

Cancer is a diverse heterogeneous disease which is characterized by phenotypically and functionally discrete subsets of cells. Data amassed from the literature suggests the presence of a small population of cancer cells with stemlike properties in a wide continuum of human cancers. Characterized by self-renewal and differentiation, these cells have been termed as cancer stem cells or tumor-initiating cells, and we have used CSCs to denote these cells throughout the review. CSCs are biologically similar to normal stem cells (SCs) [1, 2]. CSCs are characterized functionally by the intrinsic ability to initiate and long-term repopulate tumors with a recapitulation of the lineage/cellular heterogeneity seen in parental tumors [3]. Accumulated evidences advocate that from the time when CSCs were initially identified in human acute myeloid leukemia (AML), they have been isolated from many divergent malignancies, including cancers of the breast, prostate, colon, brain, pancreas, lung, liver, bladder, and ovary [4–8].

CSCs also appear to have resistance to anticancer therapies leading to relapse. This deleterious feature of CSC causes a dire impact on cancer management and hence makes CSCs promising targets for elimination. There are a few ongoing trials that involve immunotherapy strategies against CSCs [9]. However, in order to design newer therapeutic approaches, we need a clearer understanding of the biology of these cells. The present review aims to

determine the feasibility of immune targeting CSCs in solid tumors and also highlights that some of the biological targetings of CSCs may be ambivalent by also affecting immune responses.

2. Role of Developmental Signalling Pathways in the Regulation of CSCs

Tumorigenesis bears resemblance to abnormal organogenesis. CSCs exhibit three cell-intrinsic fundamental properties: self-renewal, quiescence, and differentiation. Therefore, any genetic or epigenetic program that can regulate one or more of these three properties could theoretically have an impact on CSC biology [3]. CSC phenotypes change due to altered genetics via various mechanisms. CSC biology is majorly governed by developmental pathways, stem cell factors, cell cycle regulation and apoptosis, epithelial-mesenchymal transition (EMT), and epigenetics apart from physiological metabolism. Owing to complex interactions and overlap between mechanistic programs driving CSC regulation, defining five mechanisms is rather arbitrary, as they could all ultimately converge on transcriptional regulation driven by myriad transcription factors [1].

An array of signalling pathways, namely, Myc, Notch, Hedgehog (Hh), Wnt, FGF/FGFR, EGF/EGFR, NF-κB, MAPK, PTEN/PI3K, HER2, and JAK/STAT operative in normal SCs during development and homeostasis are frequently found to be deregulated in CSCs [10–14]. Developmental pathways such as Notch, Wnt/β-catenin, and Hedgehog participate instrumentally in cancers and are frequently altered and are implicated in CSCs regulation [15–18].

Notch ligands, residing in plasma membrane of neighbouring cells, are transactivated, eliciting the transcription of Notch target genes, such as c-myc, p21 and p27, PI3K, the hairy and enhancer of split- (HES-) related family, protein kinase B (AKT), peroxisome proliferator-activated receptor (PPAR), NF-κB, and cyclin D1. Depending on the particular signalling milieu, these downstream targets get activated and in turn regulate cell fate leading to differentiation, cell-cycle progression, and survival. Notch may decelerate differentiation and endorse cell survival in stem-like cells [19, 20]. There are numerous ongoing Phase I and II clinical trials in cancer with a range of targets and mechanisms investigating the efficacy of Notch targeting, singly or in amalgamation with other therapeutic strategies [19]. Notch signalling has, nevertheless, also been correlated to peripheral T-cell maturation into effector cells, such as developing cytotoxic T-cell function, or cytokine production [21]. Similarly, T-cell activity has been weakened by Notch-inhibition using γ-secretase inhibitor [22].

Two signalling pathways have been recognized which are the β-catenin-dependent pathway that is involved in cell fate determination (canonical), and the β-catenin independent pathway that is involved in cell polarity and movement (noncanonical). Commencement of Wnt signalling is fostered by the release of soluble ligands by the cells in the near vicinity. The gain of function, regulation by methylation, and histone modification have all been implicated in carcinogenesis due to this pathway [17, 23, 24]. The focus of treatment research in cancer is to inhibit the adverse pathway in CSCs [25]. However, the Wnt/β-catenin pathway is a double-edged sword as it functions as a major player in the regulation of T-cell development and activation, Wnt/β-catenin signalling is crucial for $CD8^+$ memory T-cell development, and on the other hand, the efficacy of immunotherapy responses is improved by agonists [26, 27].

The Hedgehog (Hh) signalling pathway plays a key role in tissue wear and tear and homeostasis and epithelial to mesenchymal transition (EMT) in normal tissues. Belligerent manifestations like cancer may be prompted by tumorigenesis via aberrant Hh signalling defined by the overexpression or loss of function of its ligands or receptor and dysregulation of transcription factors. Hh signalling can be triggered by an array of factors in the tumor microenvironment, such as transforming growth factor- (TGF-) β, tumor necrosis factor- (TNF-) α, and interleukin (IL)-6. Attenuation of Hh signalling is also attracting focus of researchers worldwide and undergoing intense exploration for cancer treatment [28]. Hh signalling is relevant in immune cell development and function [29–32]. Also, Hh inhibitors may deliver additional benefits as they are also involved in myeloid-derived suppressor cell (MDSC) function. Singly targeting any pathway may not yield a physiologically relevant level of inhibition as there is a considerable overlap between these pathways. Since it is responsible for normal tissue homeostasis and development, including immune cell behaviour and peripheral effector function, targeting them is a challenging job [33].

3. Identification and Isolation of CSCs

3.1. Identification Based on Immunophenotypic Marker. Coherent immune targeting of CSCs depends on the categorization of (i) stem cell-like CSC markers used for its isolation and (ii) Antigens expressed on CSCs which are not preferably express on non-CSC/normal cells. The SC biology of the tissues from which tumor originates forms the basis of identification of CSC markers since the lineage relationship between CSCs and tissue SCs remains vague in most tumor systems. For detection and segregation of alleged CSC populations, not only are functional assays like ALDEFLUOR, mammosphere, and neurosphere employed, but also side population (SP) and cell surface marker coupled with a fluorescence-activated cell sorter (FACS) are put to use. The immunophenotypic markers for potential CSCs have been reported in many human malignancies [1, 3, 6–8] (Table 1). A range of CSC markers (CD44, CD133, and HER2) represent promising targets for CSC immunotherapy, such as antibody therapy. However, probable safety issues should be addressed when utilizing these markers as CSCs since normal SCs share similar phenotypic marker profiles. Also, a limited number of CSC markers in solid malignancies are currently accessible and their biological function is less categorized.

It is a well-established notion that CSCs in different tumor types demonstrate distinctive transcriptomic profiles

Table 1: CSC Markers in different cancers.

Cancer type	CSC markers reviewed in [1, 3, 6–8]	
	Positive markers	Negative markers
Leukemia	CD34, CD96, ALDH, CD47, CD44, CD123, TIM-3, CD32, CD25, CLL-1	CD38
Prostate	ABCG2, ALDH, CD44, $\alpha 2\beta 1$, CXCR4, CD133, NANOG, TRA-1-60, CD151, CD 166	PSA, CK18, CK20, EMA, CD66c, CD24
Bladder	CD44, CK5, CD44v6, 67LR, CK17, ALDH, SOX2	
Breast	CD44, PKH26, CD49f, ALDH, CD133, CD90	
Lung	CD133, ALDH, CD117/c-kit, OCT4, NANOG, CD44, TPBG/5 T4, CD166, CD44, EpCAM	
Pancreatic	CD44v6, $\alpha 6\beta 4$, TSPAN8, CXCR4, CD44, CD24, ESA, c-Met, ALDH, CD133,	
Glioblastoma	CD133, ABCG2, SSEA-1, SOX2, BMI1, MUSASHI1, NESTIN, OLIG2, CD49f, A2B5, L1CAM, EGFR, CD44, ID1, MYC, ALDH	
Ovarian	ALDH, CD44, CD117/c-kit, CD44, MyD88, CD24, CD133, CXCR4	
Colon	CD133, EpCAM, CD44, CD166, ALDH, LGR5, ABCB5	
Liver	CD133, ALDH, EpCAM, CD90, CD44, CD13, SALL4	CD45

and thereby express discrete antigens. They express a continuum of tumor-associated antigens (TAAs) that have the capacity to be identified by the immune system of the host [3]. TAAs may be categorically illustrated in CSCs as four subgroups [3, 8, 31], namely, (i) nominal expression by normal tissues but constitutively highly expressed by tumors as part of being their malignant phenotype like EGFR and surviving, (ii) differentiation antigens that are tissue-specific and expressed by both benign and cancer cells, (iii) CT cancer/testis antigens inadvertently activated in tumors like MAGE-A3 and MAGE-A4 that are normally confined to placenta and testicular germ cells, and (iv) neoantigens in cancer genomes derived from mutations leading to new epitopes identifiable by immune system like MUM-1 and CDK4 in melanoma. Innumerable lineage-specific markers are categorised as differentiation antigens, namely, PSA in PCa and MART-1 in melanoma. CSC markers can henceforth be thought to act as overexpressed antigens although not all are expressed minimally in normal cells. CT antigens and neoantigens have been considered best for CSC-targeting immunotherapy although lineage differentiation antigens are expressed in low amounts in CSCs [3, 8, 31].

3.2. Identification Based on Metabolism. There is a metabolism-based identification which is based on the activity of certain enzymes such as aldehyde dehydrogenase (ALDH) and mitochondrial glycolytic activity.

The ALDH family is encompassed by a gamut of 19 genes (in humans) that articulate enzymes for catalysis of aldehyde oxidation. Metabolism of vitamins, amino acids, and lipids requires aldehyde oxidation [34]. Elevated ALDH activity is linked with both normal SCs and CSCs. These enzymes also exert a shielding detoxifying effect by catabolizing aldehydes derived from pharmacological substrates. Identification of CSCs in the liver, breast, colon, and head and neck is done measuring ALDH activity employing flow cytometry-based ALDEFLUOR assay. This method is more instantaneous and is the direct readout of a fluorescent signal, based on enzyme activity as compared to other phenotypical detection methods. This method is less prone to discrepancies encountered by antibody-based staining, namely, epitope downregulation or masking, or expression of splice variants. The ALDEFLUOR assay uses N,N-diethylaminobenzaldehyde (DEAB) to measure the activity of the ALDH1 family for which it acts as a specific inhibitor [35]. Nevertheless, it can be inferred that the occurrence of $ALDH^{high}$ cells is underrated in tissues in which the chief ALDH isozyme is not a member of the ALDH1 family.

Studies on a variety of cancers such as prostate cancer, ovarian cancer, breast cancer, lung cancer-derived primary culture, and nonmetastatic/metastatic cell lines of these cancers suggest variation in ALDH expression [36–47]. This inconsistency could refute potentially applicable CSC markers and also brings into limelight the significance of testing in multiple cell lines/primary tissues. The clinical outcome in a variety of cancers has been attributed to ALDH expression [48–50]. ALDH has been implicated in resistance mechanisms to radiotherapy and chemotherapy; thus, a more specific, diverse therapeutic approach must be embarked upon. Some research groups have been attracted towards investigating inhibitors and involved in developing more specific inhibitors through drug discovery approaches, as the common expression of ALDH in healthy stem cells which can cause an off-target activity [35]. It is possible that functional redundancy within the isozyme family could recompense the inhibition for one ALDH. $ALDH^{high}$ CSC-loaded dendritic cells (DCs) have been used fruitfully in two in vivo melanoma models in the immunotherapy setting [51, 52]. In transplantation setting, ALDH activity has been demonstrated in the T regulatory cell (T_{reg}) immune subset; thus, targeting of ALDH may also show an antitumor impact through modulation of T_{reg} subset [53].

CSCs possess a lesser number of mitochondria and consequently show more glycolytic activity than other tumor cells, as evident in liver, breast, melanoma, and lung cancer [54–57]. It can be perceived by attenuated mitochondrial activity, diminished intracellular concentrations of reactive oxygen species (ROS) and ATP, perinuclear mitochondrial distribution, and a smaller concentration of mitochondrial DNA in CSCs [58]. CSC inhibitors have promising off-target effects on other cell types focussing on either self-renewal pathways, surface markers, or enzymes. Table 2 illustrates the potential off-target activities of some of these inhibitors on immune cells [59–61].

Table 2: Off-target effect of CSC-targeting inhibitors on immune cells.

Inhibition of CSC pathways, function and markers	Off targeting effect on immune cells	Reference
β-catenin	Impairs polarization, differentiation, and maturation of T cells	[23, 24]
Notch receptor	Blocks cytotoxic T cells' function and cytotoxin production	[19, 20]
WnT receptor	Inhibits CD8 positive T cell's development	[23, 24]
Hedgehog receptor	Inhibits myeloid-derived suppressor cell function	[30]
ABCB complex	Inhibits transporter associated with antigen processing (TAP) in antigen-presenting cells	[69]
ALDH	Inhibits regulatory T cells' function	[52]
IL-6 receptor	Impairs central and naïve memory T cells' proliferation, survival, and effector function	[59]
CD44	Impairs Th1 cell survival, memory, effector function, and IFNγ production	[60, 61]

3.3. Identification on the Basis of Functional Alteration. CSCs can be functionally distinguished from SCs by the fact that they exhibit a sluggish rate of cell division, amplified drug, and radiotherapy resistance and display an activation of detoxification pathways which forms the basis for their identification as well.

Characteristic staining of retaining dyes like PKH, carboxyfluorescein succinimidyl ester (CFSE), or bromodeoxyuridine (BrdU) that mostly become dilute during the proliferation phase of the cell cycle can be delineated by the poor rate of cell division of CSCs especially in growth preparatory phase or G0. These dye retaining cells give rise to xenotransplants in a number of cancers of the breast, melanoma, pancreas, and glioma [62–66].

The incremented intensity of drug resistance has been found in CSCs due to detoxifying pathways. ABCB1, ABCB5, ABCG2, and ABCC1 which are the members of ATP binding cassette transporter family of proteins are active in CSCs and inactive during differentiation [67]. They function to pump out complex molecules from the cell cytoplasm, thereby, shielding the cells from exogenous toxins like various drugs utilized for chemotherapy. Peptides, lipids, proteins, polysaccharides, and a number of diverse hydrophobic drugs act as their substrates [68]. Targeting them with highly selective and specific inhibitor molecules remains a research niche that attracts the interest of cancer researchers all over the world [69]. Hydrophobic Hoechst dyes are also excluded/expelled by CSCs owing to this mechanism; a side population based on low dye levels is formed by CSCs which aids in their identification [70]. Certain ABCB proteins such as transporter associated with antigen processing (TAP protein) play an instrumental part in intracellular trafficking of peptides across the membranes with major association with dendritic cells (DCs) and major histocompatibility complex (MHC) class I function [71]. As a consequence, the off-target effect of tumor ABCB targeting may be lethal for generating proficient antitumor T-cell responses.

There is strong experimental and clinical evidence that CSCs are intimately involved in both intrinsic and acquired tumor resistance to anticancer treatments including radiotherapy. Unraveling the underpinning mechanisms that govern the maintenance of CSCs and their resistance to therapy continues to remain a daunting task. Radiation damages the DNA by causing single- or double-strand breaks or opting an indirect route through ROS and may selectively kill the relatively radiosensitive tumor cell populations leaving the therapy-resistant CSCs alive, thus leading to temporary or permanent cell cycle arrest via the selective repopulation from the surviving CSCs through DNA damage repair (DDR). DDR arbitrates senescence or cell death if the damage is irreparable [72]. The cell may die if the antioxidant defence mechanism is not able to bring out the cell from the state of oxidative stress caused by ROS. Intrinsic radioresistance in CSCs can be majorly attributed to both the two highly efficient pathways, namely, DDR and ROS operating in them. Extrinsic or acquired radioresistance may be ascribed to the localization of CSCs in hypoxic zones inside the tumor. Survival of CSCs is fostered to hypoxia-inducible factors (HIF) 1α and HIF2α which have been shown to activate Sonic Hedgehog, Wnt, and Notch pathways for stem cell renewal and multipotency. Also, an accumulating body of evidence demonstrates that CSC rich cancers respond negatively or less efficaciously to radiotherapy [73, 74]. Nevertheless, the impact of hypoxia is not inhibitory on immune cell function. HIF1α regulates long-term survival of activated DC. HIF2α turns on the cytolytic machinery of effector T cells that are hypoxia resistant. Effector T cells displaying glycolytic characteristics exert their effector function even in the areas with poor vasculature, may be where CSCs reside. Low-dose radiations harm naïve T cells unlike the memory T cells and effector T cells [75–77]. Blending radiotherapy with T-cell targeting of stem cells can be more fruitful stratagem which would impart promising synergistic effects.

CSCs may, however, be abolished by high-dose radiations as witnessed in prostate and lung cancers of the early stage. Partly, it may occur due to immunogenic cell death, which commences by uptake of tumor antigens and antigen cross-presentation by DC [78]. It can be contemplated if CSC death due to radiation is immunogenic or if radiation gives rise to specific effector and memory T cells leading to abscopal effect and ultimate failure to recur. Lots of scientific minds are engrossed to address this predicament of paramount clinical significance.

4. Immunomodulating and Immunoresistance Properties of CSCs

4.1. Immunomodulation. CSCs show immunomodulation mainly through immunosuppression of immune cells. *In vitro*

bioassays displayed an exemplary instance for the inhibition of proliferative T-cell responses and IL-2 production by CSCs in melanoma and glioblastoma. Treg frequencies were not increased in glioblastoma and increased in melanoma CSC-T-cell cocultures [79, 80]. Immunosuppression of antigen-presenting cells, Natural Killer (NK) cells, and T cells is exerted by tumorigenic growth factor- (TGF-) β, Interleukin-(IL-) 4, IL-13, and IL-10 secreted by CSCs [80–82].

Immune responses are weakened by cell surface molecules on CSCs like CD200. Experiments on breast cancer *in vivo* model have revealed that minimised T helper (Th)1 responses, elevated IL10 production, and attenuated neutrophil infiltration are credited to CD200 overexpression [83, 84]. Overexpression of programmed death ligand 1 (PD-L1) in cancers is associated with increased glycolytic behaviour. The high expression on CSCs triggers PD-L1 upregulation that is plausibly localization dependent or tumor type [85]. Melanomas do not express high eminent levels of PD-L1 but overexpression is a conspicuous feature in gastric, head, and neck and $CD133^+$ colorectal cancers [61, 79, 86, 87].

4.2. Immunoresistance. In contrast to non-CSCs, glioblastoma CSCs display downregulation of MHC class I. On the other hand, autologous T-cell responses were induced *in vitro* by these CSCs. Moreover, the vulnerability of CSCs to T-cell-mediated immune responses is boosted by IFN-γ-treatment [80]. Even though the feasibility of therapeutic vaccines in clinical settings lies in infancy, cancer vaccines that generate T-cell and antibody responses against tumor-associated antigens (TAAs) yield promising inferences in preclinical models. Vaccine specificity for CSCs antigens is majorly lacking which could also contribute to increased resistance to T-cell attack. Development of stem-like features like Nanog in surviving cancer cells subsequently after vaccination has been reported [88]. Silencing Nanog could aid in getting rid of T-cell resistance. This sheds light on the fact that stem-like features may build up in a cell population as a consequence of immunological stress; however, it does not show inherent SC resistance.

The evidence that purified CSCs are employed as vaccines brings an end to the entire dispute that cancer stem-like cells may not be inherently resistant to immune attack. T cells generated through this treatment provide better protection in the D5 melanoma model against pulmonary metastasis in compression to the vaccination using unseparated cells. This study along with others proves T-cell susceptibility and indicates the feasibility of CSCs targeting by T cells [51, 89].

5. Immunological Targeting of CSCs

Immunotherapy targeting tactics chiefly focus to hamper the immunosuppressive TME, disrupt immune-repressive regulatory networks, and activate cytolytic lymphocytes [90]. A gamut of strategies targeting immune responses in cancer cells and CSCs, in general, has been investigated. However, such immunotherapy aiming strategies in CSCs are in the budding phase in the preventive preclinical stage and need to be investigated further so that they can be taken from bench to bedside. Here, we are discussing some possible strategies to immunologically target CSCs.

5.1. Innate Immune Response to CSCs. An appropriate candidate for immunotherapy of hematologic and solid types of tumors is NK cells that are major effector cells for innate immunity [91, 92]. Nonetheless, the exact participation of NK cells in anti-CSC immune surveillance remains discordant. Wu and his coworkers scrutinized the immunogenicity of $CD133^+$ brain tumor stem cells (BTSCs) and showed that MHC I or NK cell-activating ligands fail to be expressed by a large proportion of $CD133^+$ cells, thereby making them defiant to innate and adaptive immune surveillance [93]. Wang et al. verified that the immune CSCs in breast cancer evade NK cell killing owing to the marginal expression of MICA and MICB (MHC class I-related chains A and B), two ligands for the stimulatory NK cell receptor NKG2D due to aberrant expression of oncogenic microRNA miR-20a [94]. On the other hand, a research group led by Castriconi accounted that a range of ligands of NK cell activation receptors are expressed by glioma stem cells (GSCs) which evokes optimal NK cell cytotoxicity. They deduced that GSCs fall prey to lysis mediated by both allogeneic and autologous IL-2 (or IL-15)-activated NK cells [95].

In another investigation by Tseng et al., it was inferred that NK cell-mediated cytotoxicity may harm primary oral squamous carcinoma stem cells (OSCSCs) more significantly as compared to their differentiated counterpart [96]. Similarly, allogenic NK cell recognition of colorectal adenocarcinoma CSCs was analyzed by Tallerico et al. and also showed that these CSCs are more predisposed to NK cells in contrast to their non-CSC counterpart. Elevated levels of ligands for natural cytotoxicity receptors in colorectal CSCs make them more vulnerable to NK cell killing as compared to non-CSCs [97].

Exceptional $\gamma\delta$ T cells comprise 1–5% of circulating lymphocytes and are of Vγ9Vδ2 phenotype and are part of innate effector group family. They are characterized by potent non-HLA restricted cytotoxicity against tumor units and identify and present antigens to $\alpha\beta$ T cells; hence, immunotherapy targeting $\gamma\delta$ T cells is grabbing the attention of the scientists worldwide [98, 99]. $\gamma\delta$ T cells target isopentenyl pyrophosphate (IPP) which is crucial for isoprenoid biosynthesis in eukaryotes [100, 101]. Cytolysis of sphere forming neuroblastoma cells sensitized to zoledronate is mediated by Vγ9Vδ2 T cells as demonstrated by Nishio and his group [102]. Similarly, zoledronate-sensitized human colon CSCs cause Vγ9Vδ2 T cells to exhibit an increased rate of proliferation, tend to secrete TNF-α and IFN-γ, and produce the apoptotic and cytotoxic molecules granzymes and TRAIL [103]. Increased CD69 expression by activated Vγ9Vδ2 T cells after zoledronate exposure was shown in a clinical study. Decreased expression of the lymphoid-homing receptors CCR7 and CXCR5 was observed in Vγ9Vδ2 T cells. On the contrary, upregulated peripheral tissue-homing chemokine receptors CCR5 and CXCR3 were also displayed by Vγ9Vδ2 T cells [102]. *In vitro*

these Vγ9Vδ2 T cells were found cytotoxic. These transferred Vγ9Vδ2 T cells homed predominantly to the spleen, lung, and liver and to the metastatic sites outside these organs [104]. This signifies that *in vitro* expansion of the autologous $\gamma\delta$T cells together with different antineoplastic drugs may be for the betterment of cancer treatment via CSC obliteration. Further investigations to substantiate direct targeting of CSCs by $\gamma\delta$ T cells are warranted. The dearth of clinical data relating to the use of nonspecific killer cells in the adoptive immunotherapy of cancers mediated by CSCs is the major lacuna in this area and needs to be addressed at the earliest.

5.2. Antigen-Specific Targeting by T-Cell Immunotherapy. The T-cell based immunotherapy needs to generate effector T cells followed by adoptive transfer of $CD8^+$ T cells back into patients. Here, we are describing the routes to generate CSC-specific T cells that include CSC-primed T cells and genetic engineering of T cells with chimeric antigen receptors (CARs).

Regarding CSC-primed T cells, $CD8^+$ T-cell response could be elicited by employing CSCs derived from breast, pancreatic, and head and neck cell lines [105]. For instance, an *in vitro* stimulus of human $CD8^+$ T cells secluded from peripheral blood of normal HLA-$A2^+$ donors with ALDH1A1 peptide-pulsed autologous DCs could trigger ALDH-specific $CD8^+$ T cells. Remarkably, animal survival after adoptive transfer in preclinical bioassays can be extended by these ALDH-specific $CD8^+$ T cells that can identify and abolish $ALDH^{hi}$ CSCs (decreased by 60%–89%) *in vitro* and restrain metastasis and xenograft growth *in vivo* [105].

Likewise, lung CSCs were isolated as $ALDH^{hi}$ population and exploited for lysate-pulsed DCs to stimulate $CD8^+$ T cells by cocultivation as demonstrated by Luo et al. [106]. Subsequently, noteworthy anticancer effects, ensuing in retarded tumor growth and increased survival, were revealed by these $ALDH^{high}$-$CD8^+$ T cells [106]. Moreover, HLA-I molecules and autologous cytotoxic T lymphocytes (CTLs) were expressed by CSCs that were purified as SP from bone malignant fibrous histiocytoma (MFH) *in vitro* coculture with SP cells [107]. Also, an important indication that the CTL clone specifically recognizes MFH CSC-specific antigens came from the observation that CTL clones are biased towards the recognition of SP cells rather than non-SP cells [107].

Nevertheless, it is imperative to note that CSC-primed T cells distinguished antigens are predominantly unidentified. Otherwise, CAR T cells symbolize a unique and a potential cancer immunotherapy. The past few decades have witnessed a furious attention of the scientific community towards applying this therapy in solid tumors which is driven by the profound success and advances in the treatment of hematological malignancies using the adoptive transfer of CAR T cells. T cells can be genetically crafted employing ex vivo gene transfer for specifically recognizing a TAA or for expressing the novel T-cell receptor, hence, arbitrating neoplastic cells. Consequently, CAR T cells can be engineered to target virtually any TAAs to recognize cell surface protein in an MHC-unrestricted manner [108].

In the preclinical experimental setting of solid tumors, CAR T cells have been perceived to target CSC-associated antigens, such as CSPG4 in many discrete types, EpCAM in prostatic carcinoma (PCa), CD133 in glioblastoma, and EGFRvIII and IL13Rα2 in gliomas [109–113]. Antitumor impacts of CAR T cells mediated by CSCs are highlighted through these studies although there is a paucity of studies in this area. Significantly, an elevated rate of toxicities has been observed for CAR *T* therapies targeting TAAs including ERBB2 in metastatic colon cancer and carbonic anhydrase IX (CAIX) in renal cell carcinoma apparently accredited to the common expression of targeted antigens by both cancer and normal cells [114, 115]. Isolation and expansion of T cells restricted to specific TAAs are a daunting task due to technical cumbersomeness. A meticulous approach towards recognition of CSC-specific antigens is expected to be vital in this approach for targeting CSCs. Propitious candidacy for focussing on CAR *T* therapy is of neoantigens that can be acknowledged skillfully by T cells without self-tolerance mechanisms.

Although the existing knowledge on CSC genomics is in its nascent phase, studies suggest the use of *in vitro* generated and expanded CSC-specific T cells for adoptive transfer into tumor-bearing hosts *in vivo* to target CSCs for eliminating the tumor. Theoretically, *in vitro* CSC-primed T cells may represent a newer and pragmatic immunotherapy to precisely focus on CSC [116] but practically this approach is still under the developmental stage.

5.3. CSCs Vaccines. The immunotherapeutic strategies are based on the activation of T-cell responses endogenously to target malignant cells via transferring TAAs to patients, which can be successfully achieved by vaccines such as whole cell, genetic, DC, and peptide. At present, the most efficacious and most interrogated strategy to prevent diseases is DC vaccination. Ex vivo induction of DCs, the professional antigen-presenting cells (APCs) from peripheral blood monocytes or marrow cells, pulsed with tumor antigens, maturated, and eventually administered to the patient. Comprehensive data from different experiments have thrown light on the use of DCs to initiate tumor-specific T-cell responses which epitomises a highly potential cancer vaccination approach [116–118]. In particular, DC-mediated tumor-specific immune responses have been fostered by CSCs that function as antigen sources [119, 120]. In principle, the employment of CSC lysates as an antigen source could facilitate synchronized targeting of diverse antigens and thereby be less prone to antigen loss due to tumor escape [116]. Also, non-CSCs are less effective in inducing antitumor immunity against CSC epitopes as compared to enriched CSCs that are immunogenic and better as an antigen source. For example, the fact that can be considered is that, in various syngeneic tumor models of immunocompetent mice, the CSC-DC vaccination notably circumvents lung metastasis and slows down the growth of squamous carcinoma compared to immunization with bulk

tumor cells [51]. The vaccine comprised of DCs pulsed with irradiated PCSC (prostate cancer stem cells) lines derived from the TRAMP (transgenic adenocarcinoma of the mouse prostate) tumors induces a more robust tumor-specific immune response than the response induced by DCs pulsed with differentiated prostate tumor cells. It has also been observed that the CSC-DC vaccine retards tumor growth [121].

In a study, cytotoxic T lymphocytes (CTL) antitumor response and activation of $CD8^+$ and $CD45^+$ T cells were also credited to the breast CSC-DC vaccine [122]. Fascinatingly, it has been observed that, in the postsurgery phase, CSC-DC vaccines are extremely efficacious when deployed in an adjuvant setting to eradicate microscopic CSCs, or as combinatorial therapy with radiation and/or chemotherapy in treating macroscopic tumors. This was observed in established murine melanoma D5 and squamous cancer SCC7 tumor models where CSC-DC was more efficient when compared to DCs pulsed with non-CSCs in curing microscopic tumors in [123]. Furthermore, $ALDH^{high}$ CSC-DC vaccines in the adjuvant setting were more promising than traditional DC vaccines as they reduced tumor relapse and lung metastasis coupled with enhanced host survival further and obstructing PD-L1 [124]. DNA vaccination is gaining hype as compared to conventional cell-based vaccines, as they involve injecting plasmids for direct antigen production culminating in a protective immunological response, thus preventing cancer [125]. Acceptability of DNA immunization and their aptitude to skilfully trigger antigen-specific T cells has been efficiently established by clinical trials. PAP and PSA have been acclaimed as DNA-based vaccines in human castration-resistant prostate cancer (CRPC) [126, 127]. In a study, Nishizawa et al. observed that, in comparison to immunization with the TAA survivin, immunization with CSC-specific DNAJB8 expression plasmids shows better antitumor immune response [128].

Recently, an experimental DNA vaccine has been developed against sternness-specific marker Sox2 and an antitumor effect also has been seen after immunization against murine lung TC-1/B7 cancer cells expressing oncogenic Sox2 [129]. In a nutshell, it can be contemplated that given the ease and theoretical applicability to target antigens, CSC-specific DNA vaccination holds promise to serve as a tool for immunotherapy.

5.4. Antibody-Based Immunotherapy. The past two decades have brought into limelight the significance and feasibility of monoclonal antibodies- (mAb-) based treatments as therapies against cancer treatment. Antibody-drug conjugates are powerful newer discovered weapons for the fight against cancer [130], and immunomodulatory antibodies, illustrated by anti-PD-L1, anti-PD-1, and immune checkpoints targeting anti-CTLA-4 antibodies, have also recently attained outstanding clinical success (discussed in immune checkpoints). The expression levels of some markers (Table 1) in CSCs considerably dissimilar to the other tumor cells, which provides promising targets for antibody-based immunotherapy. The endeavours targeting CSCs with specific antibodies have been highly fruitful in terms of therapy response. For instance, tumor progression is decelerated and apoptosis is triggered in leukemic stem cells by an anti-CD44 mAb [131, 132]. Similarly, human melanoma metastasis is lessened, animal survival in SCID mouse is enhanced, tumor growth reduced, and apoptosis commenced in murine breast tumors due to anti-CD44 antibodies [133, 134].

Certainly, targeting CSCs with antibodies has proved to be beneficial in improving treatment responses. Also *in vivo* tumor growth and *in vitro* proliferation of $CD133^+$ gastric and hepatocellular CSCs can be subdued by drug-conjugated anti-CD133 antibody [135]. A bispecific antibody consisting of CD133 and CD3 antibodies which were asymmetric in nature displayed a strong antitumor efficacy in a variety of tumors [136, 137]. Studies have revealed that antitumor capability in solid tumors can be made better with a CSC-specific antibody-incorporated liposomal nanoparticle delivery system loaded with drugs or a suicide gene [138, 139]. In yet another classic example, trastuzumab (HER2-targeting antibody) dramatically lessens the chances of breast cancer recurrence by targeting HER2, an important regulator of breast CSC self-renewal [140]. Similarly, other HER2-targeting agents, like monoclonal antibody pertuzumab and the immunotoxin conjugate ado-trastuzumab emtansine (TDM-1), have further upgraded the efficacy of human epidermal growth factor receptor (HER2) targeting in the clinical settings [141, 142].

Outstandingly, HER2 expression can also be targeted in GBM CSCs [143]. As the "CSC markers" may not necessarily be unique to CSCs, single-agent mAb therapy may adversely affect normal cells [130]. Combination therapy using an assortment of different antibodies targeting multiple CSC markers could potentially reduce doses of individual antibodies to accomplish the efficient abolishment of CSCs while reducing side effects due to huge concentrations of single anti-CSC mAbs.

5.5. Tumor Microenvironment Targeting Immunotherapy. There are few important factors present in the surrounding tumor microenvironment of CSCs such as hypoxia, chronic inflammation, inflammatory cytokines, and perivascular niches (role in the regulation of proliferation and differentiation) [144–146]. Stat3/NF-κB pathways in tumor and stromal cells are activated by inflammatory cytokines such as IL-1β, IL-6, and IL-8 to further secrete cytokines in a positive feedback loop that elicits angiogenesis, CSC self-renewal, and metastasis [144, 147]. In addition, the CSC population which coevolved in the tumor microenvironment are adjacent blood vasculature that forms a niche defined by severe enhanced angiogenesis and hypoxia [145, 146]. These facets of the tumor microenvironment have been deciphered as probable pharmaceutical targets of CSCs.

Recent studies have verified that reduced tumor growth is achieved via IL-6 and/or IL-8 cytokine signalling blockade [148, 149]. Repertaxin, a pharmaceutical commodity, a noncompetitive inhibitor of IL-8 and CXCR1 signalling,

could decrease tumor size and enhance chemotherapy efficacy in breast cancer model [150]. However, it has been seen that blocking single cytokines induces limited effects as both IL-6 and IL-8 are crucial for tumor growth and the expression of these genes combined with dismal prognosis in breast cancer patients. Hence, simultaneously inhibiting IL-6 and IL-8 expression was a more lucrative line of action to induce appreciable changes in tumor growth [151].

Tumor hypoxia is another fascinating means to hit the CSC niches. Hypoxia causes chemo- and radioresistance by activating the HIF pathway and upregulating HIF-1α, by mediating multiple biological effects of hypoxia in tissues. Albeit HIF pathway inhibitor molecules have been underway in clinical trials, they seldom become successful enough to cross the clinical trial stage [152]. Also, the CSC niche can be dislocated by targeting tumor vasculature. Limited success has been achieved from the clinical trials targeting angiogenesis by blocking the vascular endothelial growth factor pathway [145].

5.6. Immune Checkpoints. Immune checkpoints are known for declining autoimmunity by intervening coinhibitory signalling pathways [153]. In cancer, these inhibitory pathways are believed to be implicated in tumor immune resistance [154]. PD-1/PD-L1 axis and the cytotoxic T lymphocyte antigen 4 (CTLA-4)/B7 axis are the two instrumental pathways of immunoinhibition that have been identified to date. These negative mechanisms contribute to the development of a suppressive microenvironment rendering cell resistance to immune therapy [155, 156]. CSCs might secrete paracrine factors or direct cell-cell contact and jointly transform the immune cells in the CSC niche because physiologic stem cells have immunoprivilege and active immunoregulatory functions [3, 157–160]. Schatton et al. accounted that T-cell activation is downregulated by CSCs [79, 161]. They identified malignant melanoma initiating cells (MMICs), a novel type of CSCs, on the basis of the expression of ABCB5 (chemoresistance determinant) [161]. The human $ABCB5^+$ MMICs express PD-1 and B7.2 and low expression of PD-L1 in comparison to $ABCD5^-$ cells. The clinical benefit of PD1 and PD-L1 was witnessed in Hodgkin's disease, melanoma, and lung cancers [162–164]. A better-prolonged response was observed in a patient subset that was considerably more durable compared to targeted or cytotoxic therapies.

It is proposed that activated T-cell responses may be downregulated by tumor expression of PD-L1 through the PD-L1/PD-1 axis and its blocking results. Even though Schatton et al. suggested reduced expression of PD-L1 in MMICs [79], in another study, a better expression of PD-L1 was observed in CSCs of head and neck carcinoma [165]. It can be inferred from this that CSC subsets may reduce T-cell response through the PD-1/PD-L1 axis. However, future clinical trials on immune checkpoint blockade are mandatory for the establishment of these results. Also, the synergistic effects of immune checkpoint therapies with CSC-targeting immunotherapies are suspected to magnify the clinical applicability of each approach.

6. Limitation and Challenges

It has been found that owing to stemlike features like low immunogenicity and intrinsic conventional therapy resistance, CSCs are demonstrated to be participants in the processes of tumor progression, maintenance, metastasis, and relapse. Consequently, CSCs form a crucial target to treat residual disease and circumvent the process of recurrence. Immunotherapy is a pragmatic promising tool as evidenced by the results of a number of clinical trials on cancer patients. However, in the clinical trials, it has been shown that the objective response rate varies significantly and a durable response is often confined to a small patient population. It is important to note that the results of the current mono-immunotherapies in solid malignancies are generally unsatisfactory. One of the fundamental reasons for this inadequacy might be due to the presence of CSCs that are not efficiently targeted by the available regimens for immunotherapy. The concept of CSC-specific immunotherapy remains in its budding phase, although it has established utility in a few preclinical and clinical trials (Table 3); however, challenges still exist. In order to fabricate more promising strategies and novel therapeutics, genomic, immunological, and biological characterization of CSCs along with immune cell interactions in the TME is warranted. Despite the fact that considerable knowledge has been accumulated on human CSC properties, most of our current understanding is attributed to derived from xenograft studies in immune-compromised nude mice. In the future, employing humanized mice, immunodeficient strains with engrafted human immune systems, may help to throw light in this area [166]. Yet, another hurdle is the heterogeneity in the population of CSCs and the property of plasticity of cancerous cells. CSCs are acknowledged as heterogeneous [2] revealed by the fact that different subpopulations of CSCs sometimes express different phenotypic markers in a single cancer type. For example, a previous research group has confirmed that prostate CSCs are mainly PSA-/lo but this is rather heterogeneous inhabiting a vast array of tumorigenic cell subsets that can be prospectively purified out using distinctive markers [167].

Data amassed from different laboratories worldwide ascertains that the association between various CSC subsets within a similar cancer type remains largely unidentified, and if CSC subpopulations share common immunological features is also an area to be explored. Hence, there may be a possibility of a situation that a CSC-specific immunological treatment leads to the eradication of a particular subset of CSCs not all. Furthermore, CSCs and non-CSCs can be thought to exhibit diverse plasticity (not discussed in this review but reviewed in [2]). This tumor cell plasticity is a major impediment in the establishment of long-lasting and more effective targeted cancer therapies, because therapeutic annihilation of present CSC populations might be then followed by their regeneration from non-CSC origin within the tumor due to treatment pressures [2].

It is gripping to see that various agents aim at targeting TME components that demonstrate clinical relevance in the treatment of cancers, as our present knowledge about

Table 3: CSC-targeting strategies and their effect in different types of cancer.

Type	Cancer types	Effects of the CSC-targeting strategies	Reference
CSC-primed T cells	Head and neck	ALDH1A1-specific $CD8^+$ T cells distinguish and eradicate ALDHhi CSCs in *in vitro* bioassays, retard xenograft growth and metastases in *in vivo* bioassays, and prolong survival	[105]
	Lung	$ALDH^{high}$-$CD8^+$ T cells resulted in the inhibition of tumor growth and prolonged survival, hence, bestowing more considerable antitumor effects	[106]
CSC-lysate DC vaccine	Squamous cell Carcinoma/Melanoma	CSC-DC vaccine that was administered in the adjuvant setting after localized radiation therapy of established tumors resulted in a reduction of tumor growth, and vaccination significantly inhibited tumor growth, abridged $ALDH^{high}$ CSC frequency in primary tumors, and metastases through stimulation of humoral immune responses against CSCs	[123]
		SCC growth was regressed compared to immunization with bulk tumor cells and lung metastasis of melanoma cells was appreciably curtailed	[51]
		In the adjuvant setting, simultaneous PD-L1 blockade further enhanced local tumor recurrence and spontaneous pulmonary metastasis and also increased survival of the host	[125]
	Prostate	Tumor regression was witnessed in TRAMP mice, tumor growth was delayed in mice challenged with prostate CSCs, and tumor-specific immune response was induced that was stronger than differentiated tumor cells	[121]
	Glioblastomas	Antigen-specific T-cell responses against CSCs were elicited and survival in animals was improved	[120]
	Breast	Migration of DCs to the spleen activated $CD8^+$ and $CD45^+$ T cells; in turn, CTL antitumor responses were induced	[122]
CSC-mRNA-DC vaccine	Glioblastomas	Seven patients vaccinated with an mRNA-DC vaccine exhibited a common immune response	[172]
DNA vaccine	Renal cell carcinoma	Stronger antitumor effects were observed in immunization with DNAJB8 expression plasmids in contrast with immunization with the tumor-associated antigen survivin, which was expressed in both CSCs and non-CSCs	[128]
NK cells	Glioblastomas	Neural stem cells derived from tumor specimens were prone to attack by lysis mediated by both autologous IL-2 (or IL-15) activated NK cells but resisted freshly isolated NK cells	[95]
	Pancreatic/Breast/ Glioblastomas	CSCs isolated from an array of human cancer cell lines *in vitro* and dissociated primary cancer specimens ex vivo were preferentially targeted by allogenic activated human NK cells	[173]
mAb	Liver/Pancreatic	The growth of hepatic and pancreatic cancer cells was inhibited *in vitro* and *in vivo* and CD133 high CSCs were targeted by CIK cells bound with anti-CD133/ anti-CD3 bispecific antibodies	[137]
	Melanoma	Human melanoma metastasis was inhibited and the survival of tumor-bearing animals was prolonged by anti-CD44 antibodies	[133]
	Breast	Murine breast tumor growth was inhibited and apoptosis was induced by anti-CD44 antibodies	[134]
CSC-CAR T	Glioblastomas	Patient-derived GBM CSCs were annihilated in an orthotopic tumor model and *in vitro* by anti-CD133 CAR T cells	[112]
	Prostate	Significant antitumor efficacy was exhibited by EpCAM-specific CAR T cells *in vitro* and *in vivo* systems	[113]

the TME is limited. Notably, current research studies have advocated that conventional therapies of cancer are more prone to augment CSCs and restructure the TME which may modify the immunotherapy responding ability of CSCs [3]. For instance, chemotherapy enhanced the frequency of the CSCs in the tumors and downregulated the expression of HLA1 molecules in HNSCC and PCa [168, 169] that may cause immunoresistance. Collectively, a CSC epitomises a continuously reshaping target, as it keeps on developing along with tumor development and progression, particularly, under the influence of treatment. It can be concluded that the area of immune targeting of CSCs holds noteworthy promise in curing patients with cancer. While therapies that efficiently and selectively get rid of CSCs do not have clinical applicability, researches on various immunotherapeutic strategies to target CSCs (Table 3) are in progress and many of them have displayed efficacy in diminishing tumor growth and metastasis in preclinical and clinical settings.

Like all the monotherapies, mono-immunotherapy is improbable to treat cancer, and stratagem that merges both conventional therapies and CSC-specific immunotherapies would be attractive and promising tactics to combat the deadly disease cancer. Combinatorial approaches can reduce drug resistance and cancer cell plasticity and help attain efficacious treatment outcomes as compared to the

monotherapies. Theoretically speaking, the immunogenicity of CSCs can be incremented by the inhibition of negative immunoregulatory pathways and through the upregulation of APM and HLA-I components via a combination of treatment strategies with radiotherapy, chemotherapy, IFNs, and/epigenetic therapies [8]. For example, CAR T cells specific to CSCs along with other therapies could be effective in increasing their antitumor effect [170]. It has been reported that epigenetic drugs alter the expression levels of genes related to the immune system on either tumor cells and/or tumor-associated immune system cells in a way that replenishes the immunogenicity and immune recognition capability. For example, APC functions improved T-cell activation promoted by HDAC6 inhibitor ricolinostat in NSCLC cells, as a result of modulation of expression of MHC molecules, while CD4+FOXP3+ Treg cell suppressive functions are pacified and immune-mediated tumor growth arrest is facilitated and these are attributed to JQ1, a BET bromodomain inhibitor [171].

Henceforth, epigenetic therapy in combination with immunotherapy may represent a novel paradigm in cancer care and therapeutic intervention. In the coming future, meticulous assessment of these strategies either alone or coalescing with myriad treatments is required to shed light on the establishment of novel antineoplastic immunotherapy treatment regimes.

7. Conclusion

Owing to their intrinsic stemness that renders CSCs therapy resistant and repeatedly immunocompromised, they are believed to be vitally involved in tumor maintenance, progression, recurrence, and metastasis. Hence, targeting CSCs is indispensable for treating residual disease and for circumventing relapse. The results of clinical trials registered on http://clinicaltrials.gov website which are using immunotherapy to target CSCs are not yet available; therefore, it can be said that various immunotherapeutic strategies to target CSCs are at the developmental stage. These approaches include targeting of CSCs with immunological methods such as CSC-DC vaccine, targeting the tumor microenvironment, anti-IL-6 mAb, inhibition of CSC-mediated immune-suppression, and blocking through anti-PD-1/PD-L1 mAbs. As an obligation and a mandatory step, rigorous tests need to be undertaken to check these strategies either alone or in combination to further verify their therapeutic worth. However, immunologic targeting of CSCs symbolizes a novel potential approach in cancer therapeutics which we hypothesize will be more efficient in amalgamation with conventional modalities and agents having immunomodulatory property.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

High Aldehyde Dehydrogenase Levels Are Detectable in the Serum of Patients with Lung Cancer and May Be Exploited as Screening Biomarkers

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Objectives. Since early detection improves overall survival in lung cancer, identification of screening biomarkers for patients at risk represents an area of intense investigation. Tumor liberated protein (TLP) has been previously described as a tumor-associated antigen (complex) present in the sera from lung cancer patients. Here, we set out to identify the nature of TLP to develop this as a potential biomarker for lung cancer screening. *Materials and Methods*. Beginning from the peptide epitope RTNKEASI previously identified from the TLP complex, we produced a rabbit anti-RTNKEASI serum and evaluated it in the lung cancer cell line A549 by means of immunoblot and peptide completion assay (PCA). The TLP sequence identification was conducted by mass spectrometry. The detected protein was, then, analyzed in patients with non-small cell lung cancer (NSCLC) and benign lung pathologies and healthy donors, by ELISA. *Results*. The anti-RTNKEASI antiserum detected and immunoprecipitated a 55 kDa protein band in the lysate of A549 cells identified as aldehyde dehydrogenase isoform 1A1, revealing the molecular nature of at least one component of the previously described TLP complex. Next, we screened blood samples from a non-tumor cohort of 26 patients and 45 NSCLC patients with different disease stages for the presence of ALDH1A1 and global ALDH. This analysis indicated that serum positivity was highly restricted to patients with NSCLC (ALDH $p < 0.001$; ALDH1A1 $p = 0.028$). Interestingly, the global ALDH test resulted positive in more NSCLC samples compared to the ALDH1A1 test, suggesting that other ALDH isoforms might add to the sensitivity of the assay. *Conclusion*. Our data indicate that ALDH levels are elevated in the sera of NSCLC patients, even with early stage disease, and may thus be evaluated as part of a marker panel for non-invasive detection of NSCLC.

1. Introduction

Despite various treatment approaches such as surgery, radiotherapy, and chemotherapy, lung cancer remains the most common cancer-related cause of death in the world with a 15% 5-year survival rate of about [1–3]. Non-small cell lung cancer (NSCLC) represents the most frequent histology and accounts for 80–85% of newly diagnosed cases. The standard of care for functionally operable early-stage and resectable stage IIIA NSCLC is surgery which possesses a potential for cure. Nevertheless, only 20% of NSCLC are diagnosed at early stage and can be resectable; thus, early detection strategies remain an unmet clinical need [4–6].

So far, numerous studies investigated mainly the potential effects of chest X-rays and low-dose helical computed tomography (CT) for imaging alongside with sputum cytology on lung cancer detection. Although these studies showed encouraging results about stage distribution in favor of earlier stage disease, better surgical resection of the tumors, and also an increased survival rate, an improvement on overall mortality could not be determined [7–16]. Serological markers such as Carcinoembryonic antigen (CEA), neuron-specific enolase (NSE), and Cyfra 21-1 are serological markers used for the monitoring of treatment response in lung cancer patients, but their application as screening biomarkers is still a challenging question [17–19]. Theoretically, an ideal biomarker should be 100% sensitive and specific, a goal that is almost never accomplished. One strategy potentially increasing both parameters is to putting different biomarkers together into a screening marker panel. This approach, along with other noninvasive methods, may allow for further improvement of NSCLC screening [20].

In 1983, a tumor-associated antigen was isolated from NSCLC and named tumor liberated protein (TLP) by Tarro et al. and immunohistochemically localized in small and large granules into the cytoplasm [4, 21–25]. Given that it was also detected in the lumen of atypical glands and in the bronchial secretions of some specimens, TLP could be considered a secretory product of cancer cells. It has been shown that when TLP is isolated and purified from a patient's tumor and reintroduced into the body, it boosts an immune response in the host [4]. Starting from the partial sequence analysis of this protein, corresponding antigenic peptides have been synthetized and used to generate antisera in rabbits. Among the four TLP-derived peptides identified by Tarro, anti-RTNKEASI rabbit sera reacted specifically with NSCLC tumor extracts and sera from lung cancer patients [26]. TLP was detected in the sera of NSCLC patients above all at the early stage of disease.

In this study, we show that TLP (or a component of this putative complex) corresponds to aldehyde dehydrogenase (ALDH) isoform 1A1 (ALDH1A1). ALDHs are a broad family of intracellular enzymes that are involved in cellular detoxification, differentiation, and drug resistance processes by means of the conversion of exogenous and endogenous aldehydes to carboxylic acids [4, 6, 27–30]. Numerous studies have investigated the biological role of ALDH in cancers including breast cancer, colon cancer, head and neck, papillary thyroid carcinoma, and mainly lung cancer, where they have given supportive evidence for the correlation between ALDH activity and lung cancer stem cells [4, 31–37]. Moreover, Cao et al. showed that ALDH1A1 levels were elevated in the sera of NSCLC patients. The combination of serum ALDH1A1 and CEA significantly increased the screening sensitivity of single CEA test [38]. Here, we show that ALDH isoforms other than 1A1 may be released into the blood of NSCLC patients, and thus, screening sensitivity may be even more improved by employing an isoform-unspecific global ALDH assay without apparently lowering specificity. Diagnostic sensitivity and specificity will, however, have to be prospectively validated in larger cohorts of patients with early-stage NSCLC and in healthy subjects at risk for NSCLC as well as in other cancer patient cohorts.

2. Materials and Methods

2.1. Patient Characteristics and Materials. Blood samples from 45 newly diagnosed NSCLC patients, 17 patients with benign lung pathologies, and 9 healthy donors were collected during routine diagnostic workup. The work has been carried out in accordance with the declaration of Helsinki. All patients consented to the use of their biological material for this investigation as approved by the Landesärztekammer Hamburg (ethics committee) (project number PV4382). Patients with lung cancer stages I-IIIA were considered as early-stage disease, as previously published [30]. Clinical characteristics (type of lung cancer, patient age, sex, smoking, tumor histology, tumor stage, and secondary diagnosis) of this cohort are displayed in Table 1.

2.2. Antigen Synthesis and Antibody Production. The production of two different rabbit polyclonal anti-RTNKEASI immune sera and the synthesis of TLP-derived peptide RTNKEASI [26] were both conducted at Rockland Immunochemicals Inc. (Gilbertsville, PA, USA) and at BioGenes GmbH (Berlin, Germany). We further purified the anti-RTNKEASI immune serum produced at Rockland by means of chromatography against the RTNKEASI peptide.

2.3. Cell Culture. The human cell lines MRC-5, A549, Hela, CA46, HL60, PC3, and MCF-7 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air under the following conditions: CA46 cell line in Glutamax RPMI 1640; PC3 cell line in Glutamax HAM's F-12; MRC-5, HL60, MCF-7, A549, and Hela cell lines in Glutamax Dulbecco's Modified Eagle's Medium. All the media (Gibco, Life Technologies) were supplemented with 10% fetal bovine serum (Biochrom GmbH, Berlin, Germany) [39].

2.4. Western Blot. We prepared cell pellets and lysates from MCF-7, CA46, HL60, PC3 MRC-5, A549, and Hela cell lines as described previously [39, 40].

Table 1: Baseline characteristics of all patients and healthy controls.

	Healthy controls ($n = 9$)	Benign lung disease ($n = 17$)	Early-stage lung cancer ($n = 25$)	Advanced-stage lung cancer ($n = 20$)
Age in years, mean ± SD	42.7 ± 18.0	53.1 ± 18.3	68.5 ± 8.2	62.3 ± 8.8
Male, no. (%)	2 (22.2)	10 (58.8)	12 (48.0)	10 (50)
Smoker, no. (%)	0	11 (64.7)	20 (80.0)	15 (88.0)*
Smoking in pack-years, mean ± SD	0	28.4 ± 29.5	43.6 ± 30.6	47.3 ± 22.05
Type of lung cancer, no. (%)				
NSCLC			25 (100.0)	20 (100.0)
Adenocarcinoma			15 (60.0)	13 (65)
Squamous cell carcinoma			8 (32.0)	2 (10)
Large cell carcinoma			2 (8.0)	1 (5)
UICC classification, no. (%)				
IA			5 (20.0)	
IB			8 (32.0)	
IIA			6 (24.0)	
IIB			5 (20.0)	
IIIA			1 (4.0)	
IIIB				1 (5.0)
IV				15 (75.0)
Type of benign lung diseases, no. (%)				
COPD		8 (47.1)		
Cystic fibrosis		4 (23.5)		
Other benign lung diseases**		5 (29.4)		

NSCLC: non-small cell lung cancer; COPD: chronic obstructive pulmonary disease. *Smoking status of 3 patients unknown. **Other benign lung diseases comprise precapillary pulmonary hypertension ($n = 2$), interstitial lung disease ($n = 1$), sarcoidosis ($n = 1$), and mucoid impaction ($n = 1$).

Cell culture supernatant from A549 cells (5×10^6) was treated on ice with 10% trichloroacetic acid (TCA) and then incubated with ice-cold 90% acetone at −20°C. Subsequently, the sample was centrifuged and the pellet dried at 65°C for 30 minutes.

The isolated proteins were mixed in Laemmli sample buffer (20% glycerol, 4% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl, 200 mM dithiothreitol (DTT), and 0.01% bromophenol blue) and boiled before loading into SDS-PAGE gel. Finally, the proteins were blotted onto membranes and blocked in nonfat dry milk 8% + PBS + 0.1% Tween-20 before hybridization with the anti-RTNKEASI serum diluted 1 : 1000 in PBS + 0.1% Tween-20 + BSA 5% buffer or β-actin 1 : 20000 (Biolegend, San Diego, CA), overnight at 4°C. After primary antibodies, the membranes were probed with a secondary antibody (1 : 30000 in PBS + 0.1% Tween-20 + BSA 5%), and then, proteins were visualized by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK).

2.5. PCA. The anti-RTNKEASI serum (1 : 1000) was subjected to preincubation with peptide RTNKEASI (500-fold molar excess) or KDSGNEQTFLPP as a control peptide, before hybridization with blotted A549 sample for 2 hours, according to Rockland procedure. The membranes were probed with a secondary antibody, and immunoreactive bands were detected by enhanced chemiluminescence.

2.6. 2D Polyacrylamide Gel Electrophoresis. Protein isolation from cell lines was performed as described previously [41]. For the first dimension isoelectric focusing, we used 7 cm immobilized pH gradient (IPG) dry strips with a linear pH 4–7 gradient (GE Healthcare). Solubilized proteins (50 μg) were put onto the strips, rehydrated, and incubated over night [41]. Proteins were resolved by the PROTEAN IEF system (Bio-Rad) (voltage gradient at 20°C with a current limit of 50 μA) under the following conditions: 4 h at 250 V, 8000 V linear gradient to 15000 V·h, and rapid 8000 V to 75000 V h, for a total of 90 kV·h [41]. After equilibration in buffer 1 (130 mM DTT, 6 M urea, 20% glycerol, 0.05 M Tris-HCl, 2% SDS) and buffer 2 containing iodoacetamide in place of DTT, the first dimension strips were positioned to the upper part of 8% acrylamide (Applichem GmbH, Darmstadt, Germany), for performing the second dimension [41]. The separated proteins were stained by silver or blotted onto the membrane and probed with the pre-serum or anti-RTNKEASI serum.

2.7. Protein Identification by LC-MS/MS. The gel spots were treated with DTT (10 mM, 56°C, 30 min) and with iodoacetamide (55 mM, at room temperature, 20 min, in the dark). Subsequently, the proteins were digested by 13 ng/μl trypsin (sequencing grade modified trypsin, Promega) in 50 mM ammonium bicarbonate, 37°C, 16 h. Tryptic digested peptides were extracted in 65% acetonitrile (ACN)/5% formic acid (FA), vacuum-dried, and dissolved in 15 μL 0.1% FA. Tryptic digested peptides were separated on an UltiMate 3000 Rapid Separation liquid chromatography (RSLC) system (Dionex, Thermo Scientific, Bremen, Germany) coupled online via electrospray-ionization (ESI) to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, Bremen, Germany). The samples were loaded with a flow rate of 5 μl/min on a trapping column (Acclaim PepMap

μ-precolumn, C18, 300 μm × 5 mm, 5 μm, 100 Å, Thermo Scientific, Bremen, Germany; nanoACQUITY-UPLC Symmetry C18 trap column, 180 μm × 20 mm, 5 μm, 100 Å; buffer A: 0.1% FA in HPLC-H_2O; buffer B: 0.1% FA in ACN) with 2% buffer B. The loaded samples on the trapping column were washed firstly for 5 min with 2% buffer B (5 μl/min) and subsequently the peptides were eluted (200 nl/min) onto the separation column. Tryptic digested peptides were separated on a reversed-phase C18 column (Acclaim PepMap 100, 75 μm × 25 mm, 2 μm, 100 Å; Thermo Scientific, Bremen, Germany; nanoACQUITY-UPLC column, BEH 130 C18, Waters; 75 μm × 250 mm, 1.7 μm, 100 Å) with a flow rate 200 nl/μm with a binary buffer system of solvent A (0.1% FA in HPLC-H_2O) and solvent B (0.1% FA in ACN). The peptides were eluted with a gradient of 2–30% Solvent B in 30 min. MS analysis was performed in the positive ion mode and was programmed to acquire by data-dependent mode (DDA). The full scans were acquired in the Orbitrap mass analyzer of Fusion with a resolution of 120,000 FWHM at m/z 200 on the MS level over a m/z range from 400 to 1500 (maximum injection time: 50 ms and automatic gain control target: 4e5). The fragmentation was carried out with an intensity threshold of 1e4, and the fragmented ions were accumulated in the linear ion trap in the rapid mode. Only precursors with charge states between +2 and +5 and the most intense precursors were selected for fragmentation. The top intense ions were isolated to a target value of 1e4 with a maximum injection time of 50–150 ms. The raw data were processed with Proteome Discoverer, v1.4.1.14 (Thermo Scientific). Protein identification was performed by using UniProt FASTA database.

2.8. ALDH and ALDH1A1 ELISA Assay. Serum samples from patients with NSCLC and benign lung pathologies and healthy donors were collected and stored at −80°C. The sera were subsequently assayed according to the manual instructions of the global ALDH ELISA kit with a monoclonal antibody specific for different ALDH isoforms (Bluegene, Biotech, Shanghai, China) and the ALDH1A1 ELISA kit with a monoclonal antibody specific for the isoform 1A1 (Cloud-Clone Corp. Houston, TX, USA). Three independent sera patients' samples are analyzed. Data are expressed as means ± SD of 3 independent experiments.

2.9. Statistics. Data were presented as means ± standard deviation (SD). We used backward stepwise linear-regression modelling to analyze differences in the ALDH- and ALDH1A1 serum levels, respectively, between patients with and without NSCLC under consideration of age, sex, and smoking (in pack-years). Correlation coefficients were according to the method of Pearson. All analyses were carried out using IBM® SPSS® version 22 or GraphPad Prism™ version 5 software. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Peptide RTNKEASI Mimics a 55 kDa Protein Highly Expressed in Lung Cancer Cell Line A549. To identify the complete protein sequence or major component of TLP, we produced two polyclonal antibody sera (BioGenes GmbH, Berlin, Germany; Rockland Immunochemicals, PA, USA) by using the peptide epitope mimic RTNKEASI derived from TLP as previously published [25]. Compared to the preimmune sera, both anti-RTNKEASI sera detected one differential protein band in the NSCLC cell line A549 at 55 kDa (Figures 1(a) and 1(b)).

Based on these results, we performed a peptide competition assay (PCA) with limiting concentrations of detection antiserum and an excess of blocking RTNKEASI or control peptide. A partial extinction of the 55 kDa protein band in the presence of the specifically blocking RTNKEASI peptide compared to the control peptide confirmed that the 55 kDa band was specifically recognized by RTNKEASI-directed antibodies from the antiserum (Figure 1(c)).

In order to analyze specificity of the 55 kDa protein for lung cancer, we subjected protein lysates from a variety of tumor cell lines and normal lung tissue to western blot analysis with the anti-RTNKEASI serum. Our western blot results showed very high levels of this 55 kDa protein in the lung cancer cell line A549, whereas all other cell lines showed either no or faint bands at 55 kDa (Figure 2(a)).

We also analyzed the supernatant from A549 cell line to verify cell ability to secrete ALDH outside. Our western blot showed a specific band at 55 kDa with a considerable amount of this protein corresponding to ALDH, as confirmed by mass spectrometry analysis (Figure 2(b) and Table 2).

3.2. Mass Spectrometric Identification of ALDH1A1 as Parental Antigen Mimicked by Peptide RTNKEASI. In order to identify the 55 kDa protein by mass spectrometry (MS), we conducted a two-dimensional (2D) gel electrophoresis of protein extracts from A549 and MCF-7 (negative control) followed by western blotting and detection with the anti-RTNKEASI serum (Figure 3). We observed two neighboring spots at the same molecular weight of 55 kDa but with slightly different isoelectric points, 6.54 and 6.73 respectively, which exhibited reactivity with the anti-RTNKEASI serum. These spots were neither detected by the preimmune serum nor by the anti-RTNKEASI serum in the control cell lysate (Figure 3). These two spots were matched to and excised from 2D protein gels followed by MS. Our results showed that the 55 kDa protein corresponds to ALDH isoform 1A1 which is highly expressed in different tumors including NSCLC. This result was also confirmed by MS of one dimensionally (1D) separated protein extracts from A549 cells, A549 cell culture supernatant, and immunprecipitations with anti-RTNKEASI serum from the A549 lysate (Table 2). Sequence alignment of ALDH1A1 with the peptide RTNKEASI revealed no linear matches, suggesting that this peptide only structurally mimics ALDH1A1.

3.3. Detection of ALDH1A1 and Global ALDH in Sera of Patients with Lung Cancer and Benign Lung Pathologies.

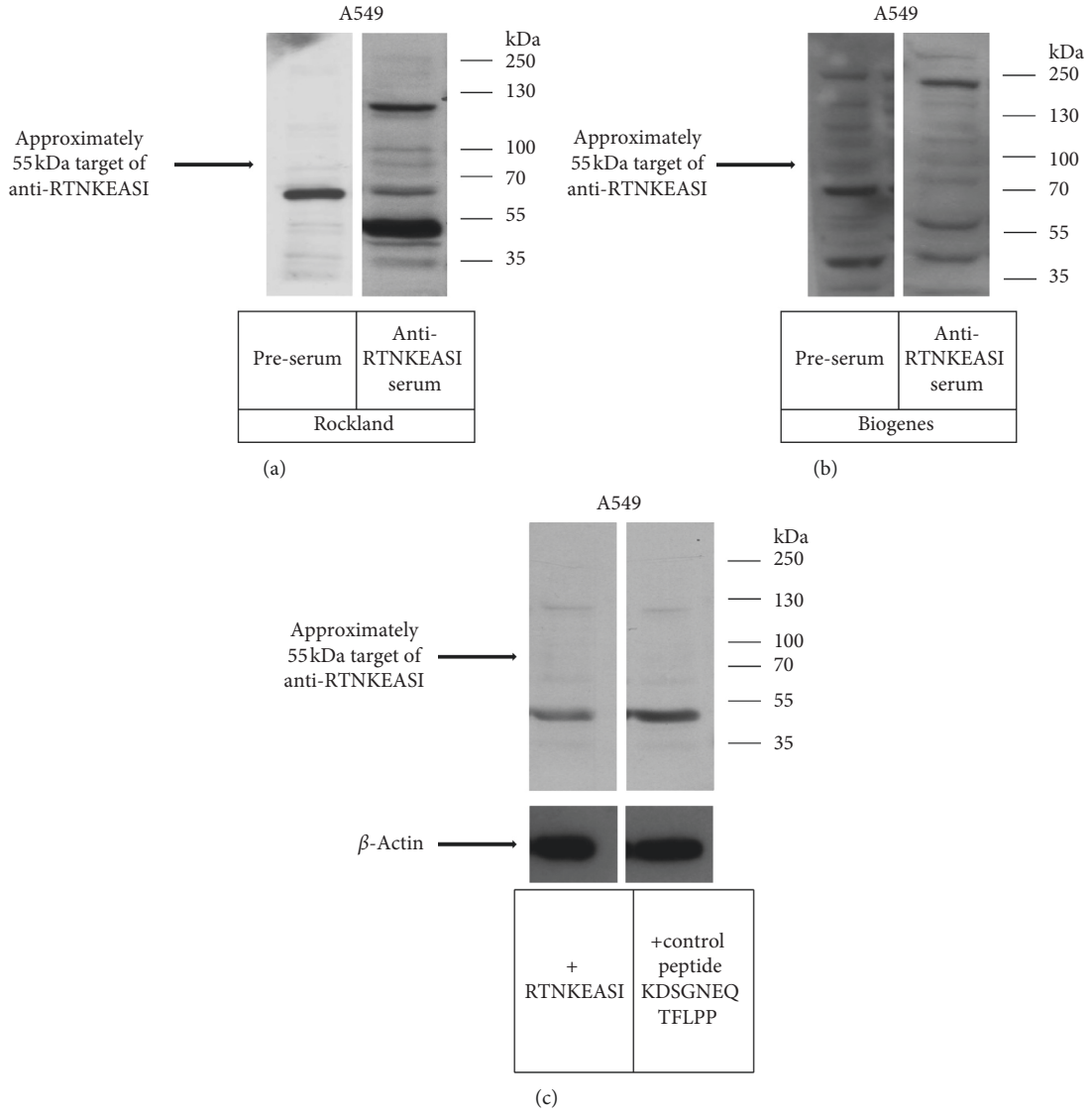


Figure 1: Western blot analysis of the polyclonal anti-RTNKEASI serum in A549 cell line. SDS gel was loaded with protein extract from non-small cell lung cancer A549 cell line followed by electrophoresis and immunoblotting. The blots were incubated with the preimmune serum and the polyclonal anti-RTNKEASI serum produced in rabbits from Rockland (a) or BioGenes (b). The 55 kDa target of anti-RTNKEASI appears only in sera of animals after immunization, and it is absent in the presera from the same rabbit. These results were confirmed with both Rockland and BioGenes antibodies. A PCA was performed by incubating the corresponding blots with the polyclonal anti-RTNKEASI serum from Rockland, pretreated with or without the peptide RTNKEASI (c) and also β-actin, as endogenous control. The intensity of the band approximately at 55 kDa detected by the secondary anti-rabbit horseradish peroxidase-conjugated was reduced after the preincubation of the antibody with the peptide RTNKEASI.

To explore the utility of serum ALDH1A1 as potential biomarker for lung cancer, we tested ALDH1A1 protein levels in sera from 25 NSCLC patients with early-stage disease, 20 NSCLC patients with advanced stage disease, 17 patients with nonneoplastic pulmonary diseases and 9 healthy donors. The baseline characteristics of this cohort are shown in Table 1. We compared ALDH serum levels in NSCLC patients with benign lung pathologies and healthy donors groups as negative controls to set the threshold of background and normalize the measurements, according to previous studies [42–44]. We found ALDH1A1 serum levels above a sensitivity threshold of 10 ng/ml (reflecting the estimated background noise) in only three of 45 NSCLC patients (6.7%), while all other NSCLC patients showed ALDH1A1 serum levels comparable with the control cohorts (Figures 4(a) and 4(b)). However, after precluding age, sex, and smoking (in pack-years) as nonsignificant parameters associated with the concentration of ALDH1A1 in the backward stepwise linear-regression analysis, overall, ALDH1A1 serum levels differed significantly between the lung cancer and the no-lung cancer patient groups. The mean difference in the ALDH1A1 concentration between no lung cancer and lung cancer is 2.10 ng/ml, 95% CI: 0.23 ng/ml to 3.98 ng/ml; $p = 0.028$.

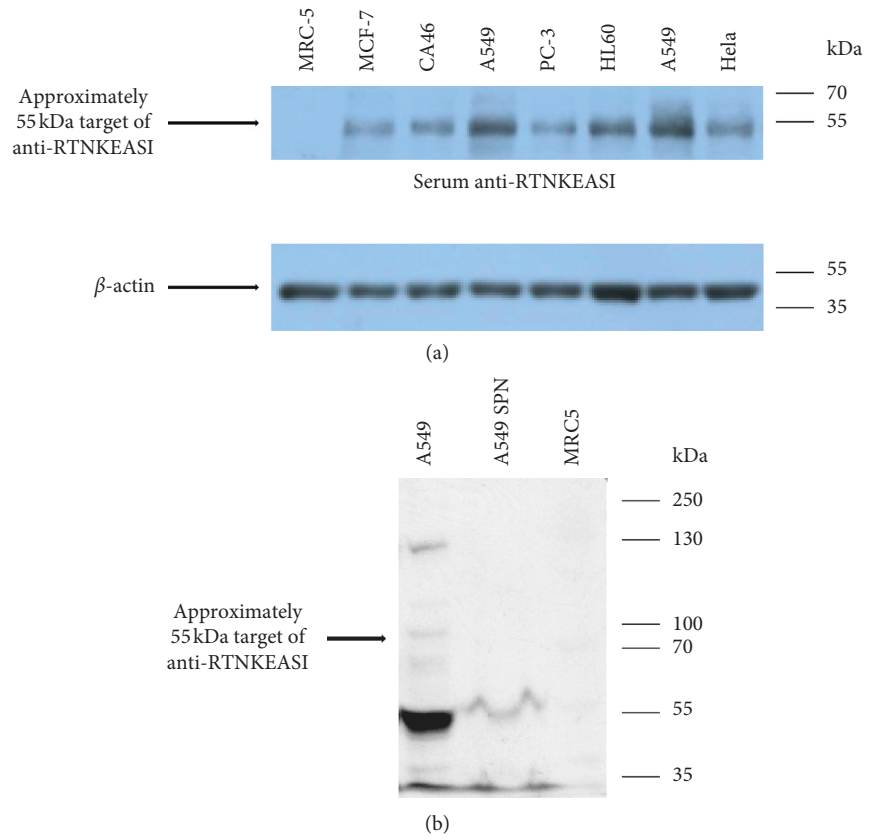


Figure 2: Western blot analysis for the polyclonal anti-RTNKEASI serum in cancer cell lines and cellular supernatant. (a) Protein extracts from non-small cell lung cancer A549, normal lung tissue MRC-5, Burkitt lymphoma CA46, leukemia HL60, breast cancer MCF-7, cervical carcinoma Hela, and prostate cancer PC-3 cell lines were loaded into SDS gel and subjected to electrophoresis and immunoblotting with the polyclonal anti-RTNKEASI serum from Rockland. High 55 kDa protein levels were identified in A549 cells and a lower amount in Hela, PC-3, CA46 and HL-60 cell lines whereas no protein levels were observed in MCF-7 cell line from breast cancer and in MRC-5 from normal lung tissue. An anti-ß-actin antibody was used for a loading control. (b) Protein extracts from cellular supernatant of non-small cell lung cancer A549 and cell lysate from A549 and MRC-5 cells were loaded into SDS gel and subjected to electrophoresis and immunoblotting with the polyclonal anti-RTNKEASI serum from Rockland. A specific band at 55 kDa was detected in the cellular supernatant sample with respect to cell lysate from A549; MRC-5 was used as negative control.

Since the MS analysis had also revealed matches with other ALDH isoforms (such as ALDH3A1), we hypothesized that these might add to the sensitivity of the assay. To this end, we performed further ELISA testings using a global ALDH assay without isoform specificity (Figures 4(c) and 4(d)). Remarkably, 33 out of 45 serum samples (73.3%) showed positivity for global serum ALDH (>10 ng/ml). Only one patient from the cohort of patients with benign lung diseases showed ALDH serum positivity above this threshold, and all healthy donors were serum ALDH-negative. Interestingly, of 25 patients with early-stage NSCLC, 15 patients (60%) showed elevated ALDH levels. Overall, serum ALDH levels were significantly elevated in the cohort of patients with NSCLC compared to patients without lung cancer. The mean difference in the ALDH concentration between no lung cancer and lung cancer is 13.90 ng/ml, 95% CI: 8.45 ng/ml to 19.35 ng/ml; $p < 0.001$. All the statistical analysis values are reported in Supplementary Materials (Table 1S and 2S).

According to the method of Pearson, neither ALDH1A1 nor ALDH concentration correlated significantly with the UICC stage ($p = 0.113$ and $p = 0.359$, respectively).

4. Discussion

Worldwide, NSCL patients have the highest mortality between patients with solid tumors, and their prognosis is tightly stage-correlated. Nevertheless, conventional methods for the diagnosis of NSCLC have high costs and produce potentially false-positive outcomes. Thus, the discovery of highly sensitive, specific, noninvasive, and cost-effective lung cancer biomarkers to use in association with conventional approaches may increase the sensitivity of NSCLC screening [4, 6, 45–47].

In this study, we show that the previously described TLP corresponds to ALDH1A1 and potentially other ALDH isoforms, which are highly expressed in NSCLC tissues [28, 33, 35–38, 46–49]. Since the peptide RTNKEASI did not linearly match ALDH isoform amino acid sequences, we concluded that it may structurally mimic these ALDH isoforms.

In our blood-based ELISA assays, we show that ALDH and, in accordance with Cao et al. [38], ALDH1A1 protein levels are statistically higher in patients with NSCLC compared to our nontumor cohort. However, only a small

Table 2: Mass spectrometric identification of aldehyde dehydrogenase as 55 kDa target of the anti-RTNKEASI serum.

Gene symbol	Protein description	Unique peptides	Score	MW (kDa)
55 kDa band from A549 cell lysate excised from 1D gel				
ALDH1A1	Retinal dehydrogenase	26	687	55.4
ALDH3A1	Aldehyde dehydrogenase, dimeric NADP-preferring	15	407	50.8
UGDH	UDP-glucose 6-dehydrogenase	3	140	55.7
GSR	Glutathione reductase mitochondrial	4	134	56.8
PDIA3	Protein disulfide-isomerase A3	3	93	57.1
ALDH2	Aldehyde dehydrogenase, mitochondrial	3	83	56.9
55 kDa band from A549 supernatant excised from 1D gel				
ALDH1A1	Retinal dehydrogenase 1	29	742	55.5
G6PD	Glucose-6-phosphate 1-dehydrogenase	26	599	59.7
ALDH3A1	Aldehyde dehydrogenase, dimeric NADP-preferring	10	303	50.8
55 kDa band from A549 immunoprecipitation excised from 1D gel				
ALDH1A1	Retinal dehydrogenase 1	28	351	55.5
G6PD	Glucose-6-phosphate 1-dehydrogenase	12	223	59.7
ALDH3A1	Aldehyde dehydrogenase, dimeric NADP-preferring	3	70	50.8
55 kDa spot 1 from A549 lysate excised from 2D gel				
ALDH3A1	Aldehyde dehydrogenase, dimeric NADP-preferring	20	515	50.4
CCT2	T-complex protein 1 subunit beta	18	100.8	57.5
ALDH1A1	Retinal dehydrogenase 1	12	57.4	54.8
ALDH1B1	Aldehyde dehydrogenase X, mitochondrial	11	52	57.2
55 kDa spot 2 from A549 lysate excised from 2D gel				
ALDH3A1	Aldehyde dehydrogenase, dimeric NADP-preferring	19	494.8	50.4
ALDH1A1	Retinal dehydrogenase 1	19	120.45	54.8

MW: molecular weight.

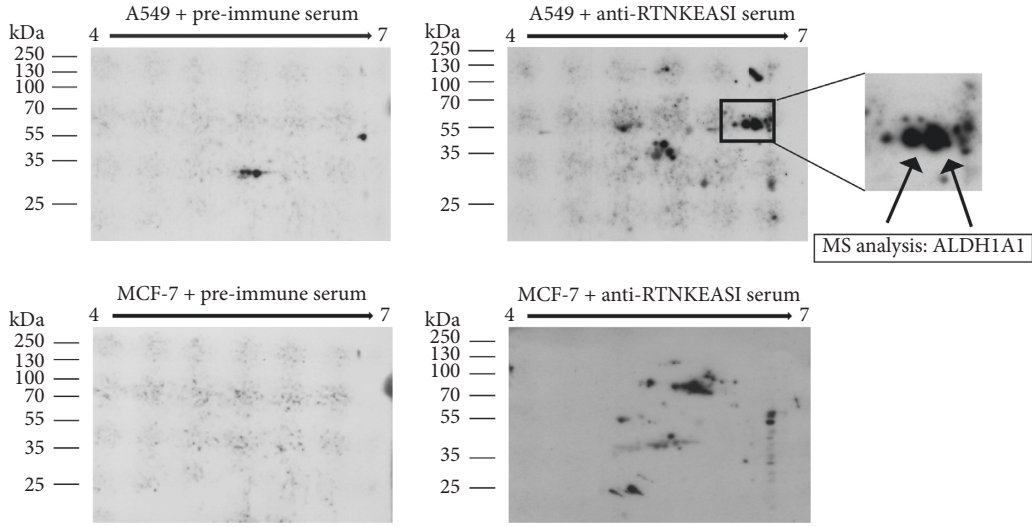
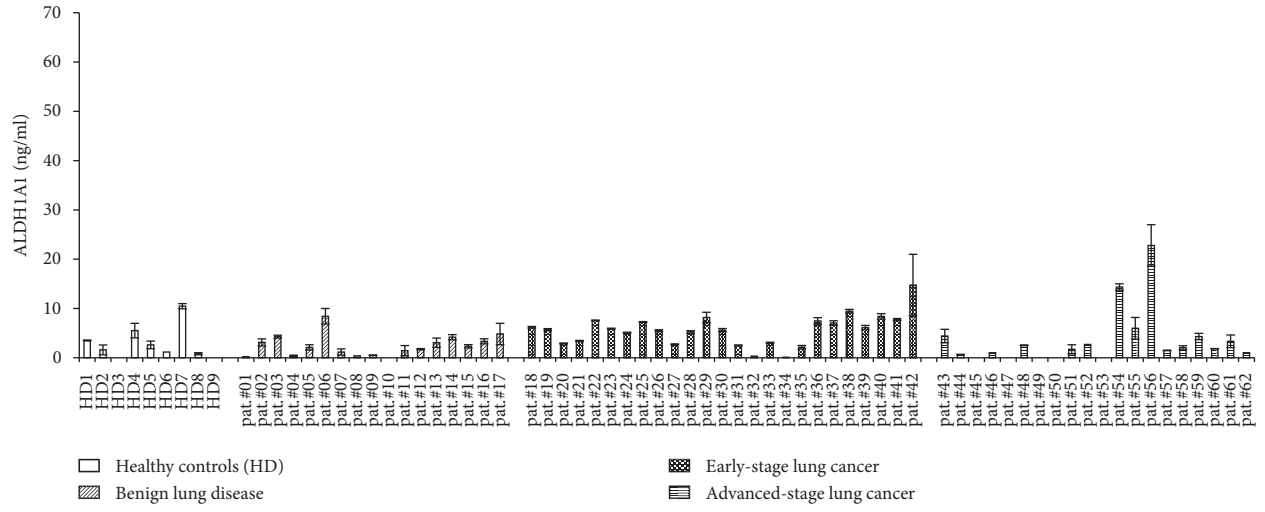
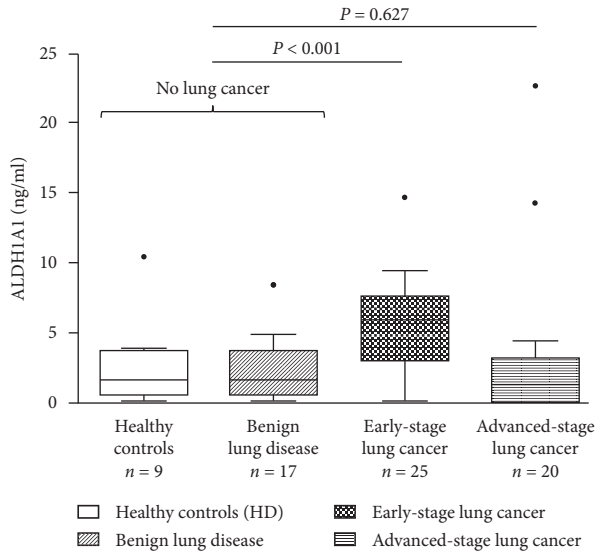


Figure 3: 2D gel electrophoresis and western blot analysis with cell lysates from A549 and MCF-7 cell lines using the polyclonal anti-RTNKEASI serum and preimmune serum. Protein extract from A549 and MCF-7 cells were loaded in 2D gel followed by electrophoresis and transferred to PVDF for immunoblotting with the polyclonal anti-RTNKEASI serum or preserum. A rectangle marks the location of the ALDHs detected with the polyclonal anti-RTNKEASI serum in A549 cells; the reactive spots indicated with arrows were absent in MCF-7 cells and in the blot incubated with the preserum. These protein spots at 55 kDa, excited and subjected to MS analysis, correspond to ALDH1A1.

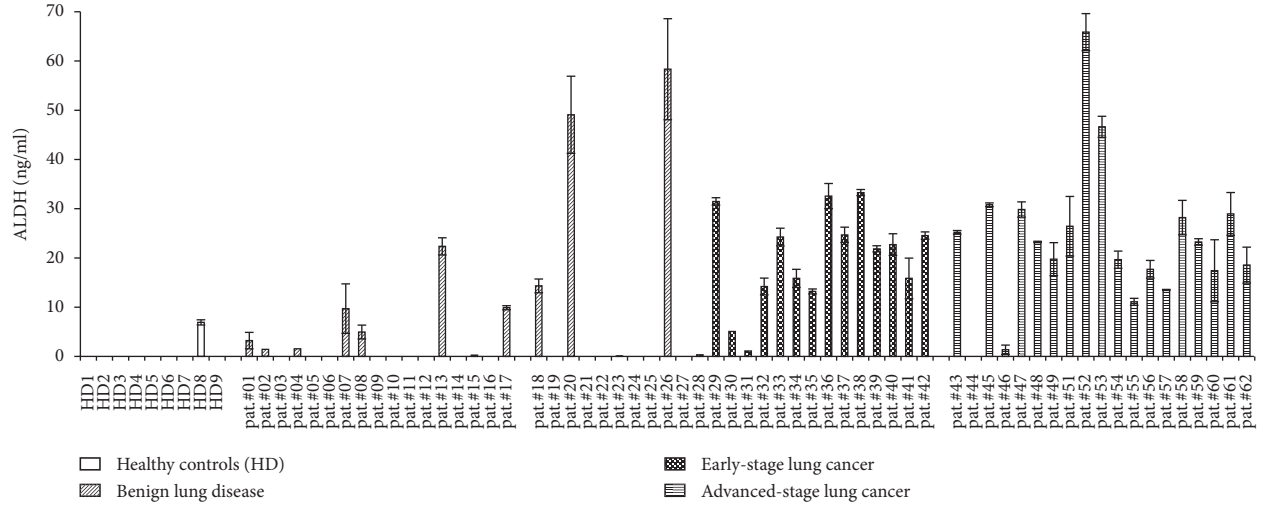
percentage of NSCLC patients (6.7%) display high ALDH1A1 serum levels, whereas sensitivity of the global ALDH test seems encouraging (73.3%). This suggested that other ALDH isoforms are also released in the sera of NSCLC patients potentially adding to the sensitivity of this global assay. This finding is in accordance with previous studies showing that several ALDH isoforms are involved in NSCLC [38]. ALDH3A1 is highly expressed in two types of NSCLC, adenocarcinoma and squamous cell carcinoma [48, 49]. ALDH3B1 expression was also found to be upregulated in a high percentage of human tumors, particularly in lung cancer [50]. Given the limited sample sizes of our cohorts, predictions on sensitivity and specificity of this marker are unreliable. However, it seems that the global ALDH test may



(a)



(b)



(c)

Figure 4: Continued.

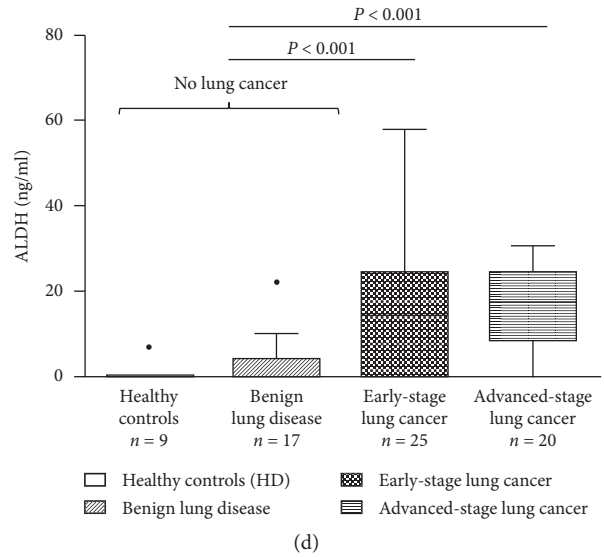


Figure 4: ELISA assay for sera (a, b) ALDH1A1 and (c, d) ALDHs. Sera samples from patients with NSCLC and benign lung pathologies and healthy donors were collected and subjected to an enzyme-linked immunosorbent assay with a monoclonal antibody recognizing specifically the isoform ALDH1A1 and all ALDH isoforms, respectively. (a) and (c) show individual data points, expressed as means ± SD of 3 independent experiments, while (b) and (d) show summary statistics. Boxes and whiskers represent median and (interquartile) range. A backward stepwise linear-regression modelling was used to analyze differences in ALDH1A1 and ALDH concentrations between patients with and without lung cancer after precluding age, sex, and smoking (in pack-years) as nonsignificant independent covariables.

not significantly decrease specificity as compared to the ALDH1A1-specific test.

ALDH1A1 and ALDH3A1 expression levels in normal pneumocytes are significantly higher in tobacco smokers versus nonsmokers [48]. Although we did not identify any significant association between ALDH levels and smoking status across the cohorts, the higher expression levels in smokers may explain the trend towards higher ALDH levels in patients with benign lung pathologies (including a high percentage of patients with chronic obstructive pulmonary disease) compared to our nonsmoking healthy donors.

Our very small percentage of ALDH1A1-positive NSCLC patients (6.7%) clearly contrasts with previously published work by Cao et al. [38] who detected a much higher percentage of ALDH1A1-positive NSCLC patients (55%). This may be due to differences in ALDH1A1 kits, which presumably contain monoclonal antibodies with different specificity for ALDH1A1 but may also be explained in part by differences in the patient cohorts or different cut-off levels. Moreover, our results showed that some patients positive with ALDH1A1 were not with global ALDH, probably due to a potential degradation of these samples as global ALDH was performed later in time.

In conclusion, elevated ALDH serum levels can be detected in the vast majority of patients with early- and advanced-stage disease. Therefore, serum ALDH should be evaluated as part of a marker panel for noninvasive detection of early NSCLC in a larger cohort of patients at risk.

Abbreviations

TLP: Tumor liberated protein
ALDH: Aldehyde dehydrogenase
NSCLC: Non-small cell lung cancer
CT: Computed tomography
CEA: Carcinoembryonic antigen
NSE: Neuron-specific enolase
ALDH1A1: Aldehyde dehydrogenase isoform 1A1
PCA: Peptide completion assay
DTT: Dithiothreitol
PVDF: Polyvinylidene difluoride
CHAPS: 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
(ACN): Acetonitrile
FA: Formic acid
RSLC: Rapid separation liquid chromatography
DDA: Data-dependent mode
LC/MS: Liquid chromatography-mass spectrometry.

Data Availability

All data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

MB and AR designed the study. AR collected the data. MV and AR analyzed the data. MV performed the statistical analysis. MB, AR, and MV interpreted the results. MB and AR drafted the manuscript with critical revisions from SL, SH, CB, and MR. SH conducted the mass spectrometry analysis. All authors approved the final version.

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Supplementary Materials

The first table describes the backward stepwise linear-regression analysis of the ALDH and ALDH1A1 ELISA assays, and the second table shows the mean values obtained in the related assay. (*Supplementary Materials*)

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Research Article

Durable Clinical Benefit in Patients with Advanced Cutaneous Melanoma after Discontinuation of Anti-PD-1 Therapies Due to Immune-Related Adverse Events

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Introduction. Anti-PD-1 therapies, pembrolizumab and nivolumab, are currently the standard of care for treatment of patients with metastatic melanoma. Treatment is usually continued until toxicity or disease progression. Though these therapies are well tolerated, some patients discontinue them due to immune-related adverse events (irAE). Discontinuation of therapy brings challenges to their management due to limited treatment options and lack of long-term prognostic information for these patients. Herein, we reviewed patients at our institution to analyze their clinical outcomes. *Materials and Methods*. Charts of 1264 consecutive patients enrolled between 8/1/2012 and 7/31/2017 at Melanoma Skin & Ocular Tissue Repositories at Holden Comprehensive Cancer Center at the University of Iowa Hospitals and Clinic were reviewed. Eligible patients were those who received single-agent anti-PD-1 therapy and subsequently discontinued it due to irAE. Reviewed data included patient demographics, prior medical history, baseline disease parameters, and outcomes. Kaplan-Meier survival analysis was done to determine progression-free survival (PFS) and overall survival (OS). *Results*. Overall 169 patients with advanced, unresectable, or metastatic cutaneous melanoma received anti-PD-1 therapy of which 16 (9.5%) white, non-Hispanic patients with median age of 64.5 (range 35 to 81 years) discontinued treatment due to irAE. Fifteen patients received pembrolizumab and one received nivolumab. The median duration of treatment was 4.7 (range 0.7 to 11.5) months. Median follow-up was 30.3 (range 4.6 to 49.4) months. Median PFS was 24.6 months and median OS was not reached. Durable clinical benefit (time to progression or next treatment of more than 6 months from last treatment) was observed in 13 (81.2%) patients. At the time of analysis, 8 patients had progressed and 4 patients died (all-cause). *Discussion*. Our results suggest that advanced melanoma patients discontinuing anti-PD-1 therapy due to irAE usually experience durable clinical benefit. However, caution is needed with these agents in patients with underlying autoimmune diseases.

1. Introduction

Monoclonal antibodies targeting programmed cell death 1 protein (PD-1) have shown to improve progression-free survival (PFS) and overall survival (OS) in patients with metastatic melanoma [1]. The advent of anti-PD-1 antibodies along with antibodies targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and therapies targeting BRAF mutation has provided multiple options to treat patients with metastatic melanoma. Due to these therapies, the median overall survival of metastatic melanoma has improved from 6 months to more than 3 years [2–4]. Currently, two monoclonal antibodies targeted against PD-1 have been approved as first-line agents for the treatment of metastatic melanoma [1].

PD-1 inhibitors can lead to durable responses [1, 5] and have better toxicity profiles as compared to CTLA-4 inhibitors and targeted therapies [1, 3, 4]. However, approximately, 86% of patients experiencing treatment-related toxicities (all grades) and severe (grade 3 or higher) toxicities are in the range of 17 to 22% [3, 4]. Treatment discontinuation due to

immune-related adverse events (irAEs) is estimated to occur in 15% to 25% of patients [3, 4]. These patients lack effective therapies as many of them do not have actionable mutation, and even in patients with BRAF mutation, the median PFS with BRAF-MEK inhibitors is low (11 to 15 months) with a high rate of toxicities [1, 6, 7]. Therefore, there is a need to understand the long-term prognosis of patients who undergo treatment discontinuation due to irAE to guide management decisions.

2. Materials and Methods

After approval from the Institutional Review Board, data of 1264 patients enrolled at Melanoma Skin & Ocular Tissue Repositories at Holden Comprehensive Cancer Center at the University of Iowa Hospitals and Clinics from 8/1/2012 to 7/31/2017 was reviewed. Patients with unresectable, advanced, or metastatic cutaneous melanomas who discontinued anti-PD-1 therapies due to irAEs were identified and their charts were reviewed in detail. Reviewed data included demographics (gender, race, and ethnicity), mutational status, prior treatment regimens including radiation therapy, melanoma metastases to brain and liver, and irAEs. Identified patients were followed till 02/26/2019. Progression (clinical or radiological) and responses were determined by iRECIST [8] and clinic notes. Outcomes with anti-PD-1 therapies including PFS, time from treatment discontinuation to progression, and OS were collected. Common Terminology Criteria for Adverse Events Criteria Version 4.03 were used to grade irAE [9].

2.1. Statistical Analysis. Baseline clinical and disease characteristics were summarized as medians and ranges for continuous variables and as numbers and percentages for categorical variables. Kaplan-Meier survival analysis was used to determine PFS and OS. Time was calculated from initiation of anti-PD-1 treatment to progression or, new treatment for PFS, time from last treatment to next treatment or progression for clinical benefit and to death due to any cause for OS. Durable clinical benefit was defined as time of 6 months or more to progression or next treatment from last therapy. Survival curves were drawn using GraphPad Prism Version 7.04 (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Baseline Characteristics. Overall 169 patients with advanced, unresectable, or metastatic cutaneous melanoma received anti-PD-1 therapy, of which 16 (9.5%) patients discontinued treatment due to irAEs. All patients who discontinued treatment were white and non-Hispanic. The median age was 64.5 (range 35 to 81) years. Ten (62.5%) patients were male and six (37.5%) were female. Eight (50%) patients had a BRAF mutation. Four (25%) patients had brain metastasis and one (6.3%) had liver metastasis. Six (37.5%) patients had prior treatment with ipilimumab for metastatic disease. Table 1 provides the baseline demographics and clinical profile of all patients.

3.2. Treatment. Fifteen (93.7%) patients received pembrolizumab and one (6.3%) patient received nivolumab. Eight (50%) received anti-PD-1 therapy as the first line, seven (43.7%) as second, and one (7.3%) as the third line. Patient 6 received bevacizumab in addition to pembrolizumab after 6 cycles of single-agent pembrolizumab due to pseudoprogression in the brain. None of the patients received concomitant radiation with anti-PD-1 therapy. The median duration of treatment was 4.7 (range 0.7 to 11.5) months. Table 2 provides a summary of treatment duration, line of therapy, and outcomes of each patient.

3.3. irAEs. The median duration from initiation of treatment to development of irAE was 4 (range 0.5-11.5) months. Most commonly observed toxicities leading to treatment discontinuation included diarrhea and rash (4/16, 25% each) and arthritis (3/16, 18.7%). Other observed toxicities included colitis (2), neuropathy (2), pancreatitis (2), fatigue, nausea, nephritis, adrenal insufficiency, hypothyroidism, low mood, mouth sores, hepatitis, uveitis, and myasthenia flare (1 each). Ten (62.5%) patients experienced grade 3 or higher toxicities. Table 3 provides a summary of these toxicities and immunosuppressive agents used for their management. Apart from the mentioned toxicities, patient 1 developed grade 2 hypothyroidism on day 81 and patient 8 developed grade 2 pityriasis lichenoides after 2 cycles secondary to pembrolizumab. However, both did not lead to treatment discontinuation.

3.4. Outcomes. Median follow-up was 30.3 (range 4.6 to 49.4) months. Eight (50%) had complete response, five (31.2%) had partial response, two (12.5%) had stable disease, and one (6.3%) had progressive disease as best response to treatment. At the time of analysis, 8 patients had progressed and 4 patients experienced all-cause mortality, of which one death was unrelated to melanoma. Median PFS was 24.6 months and median OS was not reached due to durable disease control (Figures 1 and 2). Durable clinical benefit was observed in 13 (81.2%) patients.

Patients number 4 and 16 had a PFS of less than 6 months PFS (Figure 3). Patient 4 had underlying thymoma and patient 16 had myasthenia gravis. With pembrolizumab, they experienced severe neuroimmune toxicity and flare-up of myasthenia gravis, respectively, which caused rapid clinical deterioration. All patients except one received steroids (oral, topical, or ophthalmic), while two received steroid-sparing agents. Of the eight patients who progressed, three were retreated with pembrolizumab-containing regimen. Of these three patients, one developed pembrolizumab induced psoriasis, while the remaining two tolerated it without any significant side effects (Table 2).

4. Discussion

Prior studies have given conflicting evidence with regard to the association of PD-1-related irAEs with survival outcomes in melanoma patients. Freeman-Keller et al. reported OS

Table 1: Patient demographics and clinical profile at the time of starting anti-PD-1 therapy.

Pt. No.	Age (years)	Gender	Mutational status	Brain metastasis	Liver metastasis	Prior adjuvant therapy	Prior therapy for metastatic disease	Prior immune-related adverse event	Radiation therapy within prior 3 months	Prior relevant history or autoimmune disease
1	63	Male	BRAF V600E	Yes	No	Interferon	Ipilimumab, stereotactic radiosurgery	Colitis	Yes	None
2	61	Female	BRAF Negative	No	No	Radiation	Ipilimumab	Colitis, rash	No	Lichen planopilaris
3	72	Female	BRAF Negative	No	No	GM-CSF	Ipilimumab	Colitis	No	None
4	81	Male	BRAF V600K, GNAQ, RAC1, POLD1, TERT	Yes	No	No	Radiation, craniotomy and tumor resection, stereotactic radiosurgery	NA	Yes	Thymoma
5	64	Male	BRAF V600E	No	No	Radiation	Ipilimumab with talimogene laherparepvec	None	No	None
6	59	Female	NRAS	Yes	No	Interferon	Ipilimumab, craniotomy and tumor resection, radiation	None	Yes	None
7	66	Female	BRAF V600E	No	No	No	NA	NA	No	None
8	35	Male	BRAF V600E, PTEN, MET	No	No	Interferon	Ipilimumab with interleukin-2, ipilimumab	Diarrhea, nausea, vomiting, hyperbilirubinemia, acute kidney injury, oliguria, tachycardia	No	None
9	61	Male	TP53	No	No	No	NA	NA	No	None
10	77	Male	KIT p.W557G	Yes	No	Radiation	Stereotactic radiosurgery	NA	Yes	None
11	65	Male	NRAS, TP53, KIT, SF3B1, CDK2NA	No	No	No	NA	NA	No	None
12	67	Female	Not done	No	No	No	NA	NA	No	None
13	78	Male	BRAF p.L597S	No	No	No	Carboplatin with paclitaxel	NA	No	No
14	64	Male	BRAF V600K	No	No	Radiation, ipilimumab	NA	Rash	No	No
15	47	Female	BRAF V600E	No	No	Interferon	Vemurafenib with high dose interleukin-2, vemurafenib with decitabine, craniotomy, stereotactic radiosurgery	Rash, diarrhea	Yes	No
16	75	Male	BRAF negative	No	Yes	No	NA	NA	No	Myasthenia gravis

Table 2: Details of the line of anti-PD-1 therapy, duration of treatment, survival, and subsequent treatment on progression.

PN	Anti-PD-1 Therapy	Line of anti-PD-1 therapy	ECOG PS	Response	DOT (months)	PFS (months)	TPTD (months)	Overall Survival (months)	Subsequent therapy
1	Pembrolizumab	second	1	PR	4.1	24.6	20.5	39.3∗	Radiation, pembrolizumab with development of pembrolizumab induced psoriasis
2	Pembrolizumab	second	0	CR	4.6	20.2	15.6	49.0∗	Radiation, pembrolizumab with TLR9 agonist without immune toxicities
3	Nivolumab	second	1	CR	2.6	49.4^{+}	46.8^{+}	49.4∗	NA
4	Pembrolizumab	first	1	SD	2.3	3.0	0.7	4.6	Dabrafenib with trametinib
5	Pembrolizumab	second	0	PR	3.5	30.3^{+}	26.9^{+}	30.3∗	NA
6	Pembrolizumab	second	1	CR	11.5	36.3^{+}	24.8^{+}	36.3∗	NA
7	Pembrolizumab	first	1	PR	4.8	18.1	13.3	23.4	Radiation
8	Pembrolizumab	second	1	SD	4.8	7.3	2.6	28.3∗	Craniotomy and surgical resection, stereotactic radiosurgery, vemurafenib with cobimetinib
9	Pembrolizumab	first	0	CR	6.5	17.5^{+}	11.0^{+}	17.5∗	NA
10	Pembrolizumab	first	1	CR	6.9	21.9^{+}	15.0^{+}	21.9∗	NA
11	Pembrolizumab	first	0	CR	9.7	30.1^{+}	20.5^{+}	30.1∗	NA
12	Pembrolizumab	first	0	CR	0.7	23.0^{+}	22.3^{+}	23.0∗	NA
13	Pembrolizumab	second	2	PR	4.6	16.8	12.2	25.0∗	Radiation, dabrafenib with trametinib
14	Pembrolizumab	first	0	PR	5.0	19.3	14.3	31.8	Pembrolizumab with TLR9 agonist without immune toxicity, radiation, vemurafenib with cobimetinib
15	Pembrolizumab	third	1	CR	11.5	37.3^{+}	25.8^{+}	37.3∗	NA
16	Pembrolizumab	first	0	PD	1.3	1.6	0.2	5.4	Temozolomide

PN: patient number. ECOG PS: Eastern Cooperative Oncology Group Performance Status.
PR: partial response. CR: complete response. ∗Alive.
SD: stable disease. PD: progressive disease. $^{+}$Censored, no evidence of progression.
DOT: Duration of treatment.
TPTD: time to progression from treatment discontinuation.

Table 3: Details of immune-related adverse events leading to discontinuation of anti-PD-1 therapy and their treatment.

Patient Number	irAE leading to discontinuation of anti-PD-1 therapy	Time of first presentation of any grade irAE from initiation of therapy (months)	Immune suppressive agents for treatment of irAE
1	Grade 2 inflammatory arthritis and neuropathy	4.1	Prednisone
2	Grade 2 diarrhea	4	Budesonide
3	Grade 3 colitis and diarrhea	2.6	Prednisone, budesonide
	Grade 2 adrenal insufficiency	4.1	Hydrocortisone
4	Grade 3 sensorimotor polyneuropathy	2.3	Prednisone
5	Grade 1 diarrhea	2.8	Budesonide
	Grade 3 pancreatitis and colitis	3.5	Budesonide
6	Grade 3 rash	11.5	Dexamethasone, topical steroids
7	Grade 2 inflammatory arthritis	4.6	Prednisone, methotrexate
8	Grade 2 fatigue, nausea, diarrhea, arthritis	4.8	None
9	Grade 3 pancreatitis	6.5	Prednisone
10	Grade 2 nephritis	6.9	Prednisone
11	Grade 2 hypothyroidism, low mood, mouth sores, rash	9.7	Topical steroids
12	Grade 3 rash	0.5	Prednisone
13	Grade 3 hepatitis	2.1	Prednisone, budesonide
14	Grade 3 uveitis	3.9	Prednisone, ophthalmic prednisolone
15	Grade 3 rash	11.3	Prednisone, topical steroids
16	Grade 4 myasthenia flare	0.8	Plasma exchange, mycophenolate mofetil, prednisone, intravenous immunoglobulin, abatacept

IrAE: immune-related adverse events.

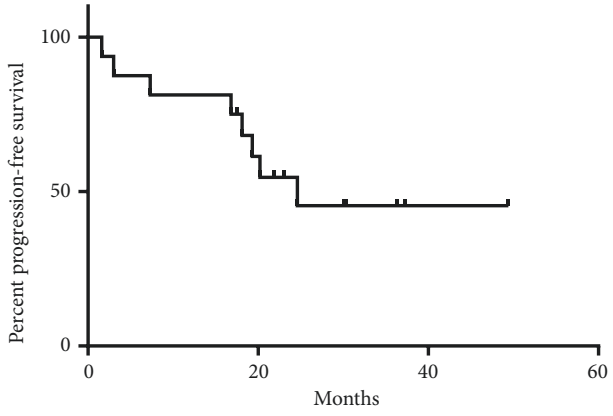

Figure 1: Kaplan-Meier curve for progression-free survival.

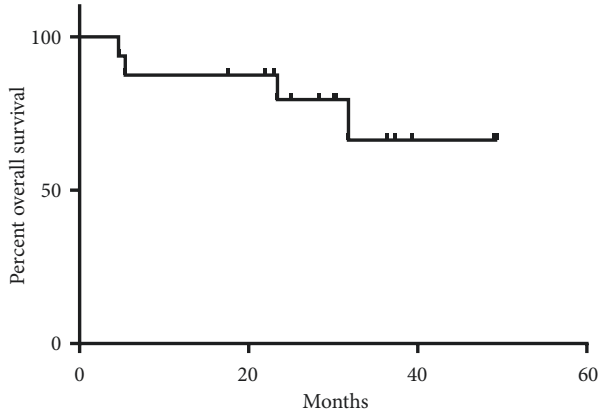

Figure 2: Kaplan-Meier curve for overall survival.

association with rash and vitiligo, while no survival benefit was seen with other irAEs including endocrinopathies, colitis, or pneumonitis. In the study, 12-week landmark PFS analysis was difficult to interpret due to exclusion of a large number of patients [10]. Another pooled analysis of 576 patients by Weber et al. reported that after exclusion of patients progressing before 12 weeks, there was no difference in PFS between patients without AEs and those with one to two AEs or between those with any-grade AE and all patients [11]. In another study by Indini et al., irAE was associated with improved PFS [HR 0.47 (95% CI 0.26, 0.86); p = 0.016] and OS [HR 0.39 (95% CI 0.18, 0.81); p = 0.007] on multivariable analysis in patients who received more than 2 doses of anti-PD-1 therapies [12]. Quach et al. reviewed single institution data of 318 patients treated with anti-PD-1 therapies with or without ipilimumab and reported a better response rate

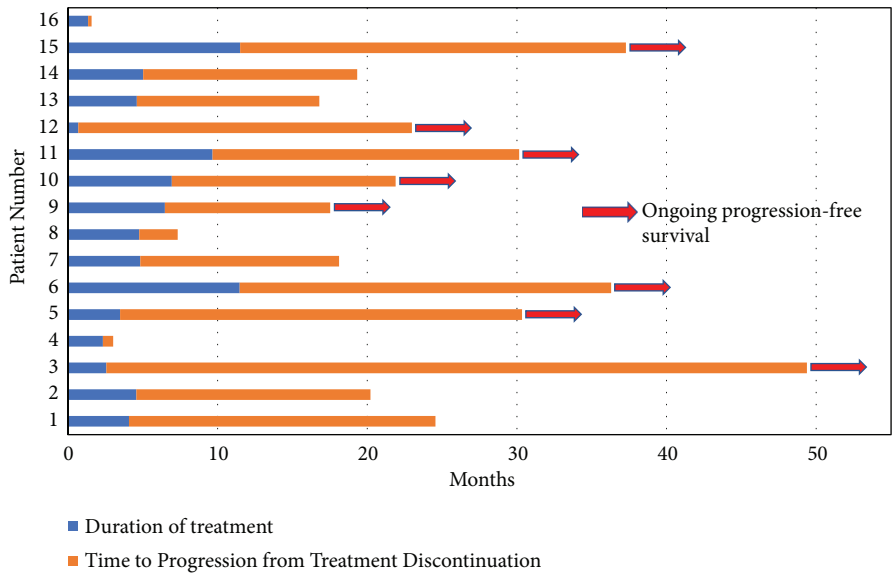

Figure 3: Swimmer's plot demonstrating duration of treatment and progression-free survival.

(60.0% vs. 28.6%; $\chi^2 p < .001$), median PFS (797 vs. 112 days; log rank $p < .001$), and median OS (1691 vs. 526 days; $p < .001$) in patients who experienced cutaneous side effects. Superior outcomes with regard to response rate, PFS, and OS were seen with vitiligo and rash as compared to pruritus [13].

In this study, we analyzed the impact on PFS and OS for patients who discontinued anti-PD-1 therapy due to irAEs. Complete response was seen in patients with irAEs from a wide spectrum and grade of toxicities including grade 2 and 3 diarrhea/colitis (2 patients), grade 3 rash (3 patients), grade 3 pancreatitis (1 patient), grade 2 nephritis (1 patient), and grade 2 hypothyroidism, low mood, mouth sores, and rash (1 patient). We found a durable clinical benefit in thirteen patients (81.2%) discontinuing PD-1 directed therapy due to irAEs; none of them progressed in more than one year and 6 (37.5%) in more than 2 years (Figure 3). The benefit was not seen in patients with an underlying autoimmune disease like myasthenia gravis or who are at a higher risk of developing it, like patient 4 with thymoma [14]. However, because of the small sample size, no definitive conclusion should be drawn; this finding needs to be explored further.

KEYNOTE-001 has reported durable complete remission after discontinuation of pembrolizumab in patients with complete remission [5]. However, we found durable benefit in patients with residual disease also. Though the mechanisms for this phenomenon are not clear, it is very much possible that PD-1 blockade may result in an adaptive memory immune response providing antitumor effect even after treatment cessation and translating as irAEs [15–17]. A similar study evaluated 19 patients with metastatic renal cell carcinoma who experienced an initial clinical response but after irAE discontinued PD-1/PD-L1 therapy. The median time on PD-1/PD-L1 therapy was 5.5 months; median TTP was 18.4 months and durable clinical benefit off treatment (TTP > 6 months) was observed in 68.4% (n=13) patients [15].

Pollack et al. reported that metastatic melanoma patients who discontinued CTLA4/PD-1 blockade due to irAEs can be rechallenged with anti-PD-1 therapies. The study showed a relatively higher rate of recurrent or different irAEs on resumption of anti-PD-1 therapies. They concluded that this approach can be used in selected patients [18]. We also found that patients who had to discontinue pembrolizumab due to irAE were able to be treated again with pembrolizumab-based therapies with manageable toxicities in two of the three patients. Our study has similar limitations as most retrospective studies including selection bias, chances of errors during data entry and confounding.

In summary, we present outcomes of 16 patients with metastatic melanoma who discontinued anti-PD-1 therapies due to immune toxicities. To the best of our knowledge, it is the largest series to date of real-world patients. Its strength includes a long-term follow-up and comprehensive analysis of each case which can help generate multiple hypotheses in combination with other relevant studies. Our results show durable clinical benefit in patients who discontinue anti-PD-1 therapies after irAEs. However, this needs to be confirmed in larger cohorts. We also need more comprehensive preclinical and clinical studies to determine how individual patient variables, cancer and immune system, interact to cause irAEs in only a select few patients while sparing a majority. We also need to develop predictive and prognostic novel biomarkers for anti-PD-1 therapies and also for irAEs.

Abbreviations

PD-1: Programmed cell death 1 protein
PFS: Progression-free survival
OS: Overall survival
CTLA-4: Cytotoxic T-lymphocyte-associated protein 4
irAE: Immune-related adverse events
TTP: Time to progression.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The authors received no specific funding for this work.

Conflicts of Interest

None of the authors have direct competing interest to the study. Umang Swami does not have any conflicts of interest. Varun Monga has research support from Immunocellular, Orbus Therapeutics, Deciphera, and NewLink Genetics. Aaron D. Bossler has received honorarium from Novartis and Roche. He is on advisory board of Novartis and Roche and has travel support from Novartis and Roche. Yousef Zakharia has institutional research support from NewLink. He is on the advisory board of Novartis, Pfizer, Roche Diagnostics, EISAI, Exelixis, JNJ, Castle Bioscience, and Amgen. Mohammed Milhem is on the advisory board of Genentech, BMS, EISAI, Novartis, EMD Serono, and Blueprint Medicines Corporation.

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Research Article

PKHB1 Tumor Cell Lysate Induces Antitumor Immune System Stimulation and Tumor Regression in Syngeneic Mice with Tumoral T Lymphoblasts

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Guest Editor: Shalini Gupta

Acute lymphocytic leukemia (ALL) is the most common pediatric cancer. Currently, treatment options for patients with relapsed and refractory ALL mostly rely on immunotherapies. However, hematological cancers are commonly associated with a low immunogenicity and immune tolerance, which may contribute to leukemia relapse and the difficulties associated with the development of effective immunotherapies against this disease. We recently demonstrated that PKHB1, a TSP1-derived CD47 agonist peptide, induces immunogenic cell death (ICD) in T cell ALL (T-ALL). Cell death induced by PKHB1 on T-ALL cell lines and their homologous murine, L5178Y-R (T-murine tumor lymphoblast cell line), induced damage-associated molecular patterns (DAMPs) exposure and release. Additionally, a prophylactic vaccination with PKHB1-treated L5178Y-R cells prevented tumor establishment *in vivo* in all the cases. Due to the immunogenic potential of PKHB1-treated cells, in this study we assessed their ability to induce antitumor immune responses *ex vivo* and *in vivo* in an established tumor. We first confirmed the selectivity of cell death induced by PKBH1 in tumor L5178Y-R cells and observed that calreticulin exposure increased when cell death increased. Then, we found that the tumor cell lysate (TCL) obtained from PKHB1-treated L5178YR tumor cells (PKHB1-TCL) was able to induce, *ex vivo*, dendritic cells maturation, cytokine production, and T cell antitumor responses. Finally, our results show that *in vivo*, PKHB1-TCL treatment induces tumor regression in syngeneic mice transplanted with L5178Y-R cells, increasing their overall survival and protecting them from further tumor establishment after tumor rechallenge. Altogether our results highlight the immunogenicity of the cell death induced by PKHB1 activation of CD47 as a potential therapeutic tool to overcome the low immunogenicity and immune tolerance in T-ALL.

1. Introduction

T cell acute lymphoblastic leukemia (T-ALL) is a hematological malignancy that affects mostly pediatric patients, as they account for 80% of the cases [1, 2]. It represents the second most common acute leukemia in adults, with a 5-year survival rate of about 30-50% [3–5] with a high risk of relapse [6]. The use of nelarabine for relapsed and refractory T-ALL only results in responses in a substantial minority of patients [7]. Among other treatments, allogeneic hematopoietic cell transplantation (HCT) is proposed in patients with high-risk or relapsed/refractory disease, and

γ-secretase inhibitors for patients with NOTCH1 mutations are currently in clinical trials. Multiagent chemotherapy is proposed for older and unfit patients. However, T-ALL treatments have lagged behind those proposed for B-cell ALL, and the development of new therapeutic approaches against this aggressive malignancy remains a challenge. Since the T-ALL high risk of relapse has been attributed to its low immunogenicity and immune tolerance [6], the immune system stimulation able to induce immunological memory against tumor cells appears as a challenging but promising goal.

With this aim, whole tumor cell lysates (TCLs) have been shown to be able to prompt antitumor immune responses in preclinical murine models for glioblastoma, breast, and ovarian cancer and in clinical trials for melanoma, prostate, and ovarian cancer [8]. These immune responses are correlated with damage-associated molecular patterns (DAMPs) induction and the availability of the tumor neoantigens, both of which are promoted in accordance with the specific cell death inductor [9]. DAMPs interact with dendritic cells' (DCs') receptors (CD91, Toll-like receptor 4, purinergic receptors, among others), promoting their maturation and increasing antitumor activity [10]. Thus, TCL can be used to induce an immunogenic response from DCs against multiple tumor antigens, triggering a polyclonal tumor-specific T cell response [11].

Cancer treatment with DCs pulsed with tumor antigens has proved effective antitumor responses in mesothelioma, glioma, and breast cancer [12–14]. However, the use of TCL as therapeutic vaccines has been also shown to be a useful strategy to elicit antitumor immune responses, while overcoming immunosuppressive mechanisms of the tumor microenvironment [8]. TCLs hold more promises as cancer vaccines than individual tumor-associated antigens (TAAs) because they can elicit immune responses to multiple TAAs [15]. However, the availability and types of neoantigens, the amount of DAMPs released, and the overall immunogenicity of the TCL strongly rely on the cell death inductor [9]. Thus, it is important to find effective cell death inductors that are able to provide an immunogenic TCL able to induce antitumor immune responses.

CD47 activation through coated [16–18] or soluble anti-CD47 antibodies [19, 20], or immobilized [16] or soluble peptides derived from the C-terminal domain of thrombospondin-1 [21, 22], is an effective way to induce cell death in different types of cancerous cells, even in cells coming from patients that are resistant to chemotherapy [21, 22]. Thus, a TCL obtained through CD47 activation might help to understand the implications of CD47-mediated cell death in the activation of antitumor immune responses. Recently, we have shown that treatment of T-ALL cells with the CD47-agonist peptide, PKHB1, induced immunogenic cell death (ICD) [23]. ICD was induced by PKHB1 in T-ALL cells, while it spares CD19 and CD3 lymphocytes [21], human and murine PBMCs, CD4 and CD8 T cells, and cells from murine lymphoid organs [23]. We found that PKHB1-treatment induces the exposure and release of several DAMPs (calreticulin (CRT), HSP70, HSP90, ATP, and HMGB1) in human T-ALL cell lines (CEM, MOLT-4) and their murine counterpart (L5178Y-R cells) [23]. *In vivo,* prophylactic vaccination experiments with PKHB1-treated cells prevented tumor establishment in immunocompetent BALB/c mice [23]. These results demonstrated that CD47 activation by PKHB1 was able to induce DAMPs release and provide neoantigens able to elicit an antitumor immune response that prevented tumor establishment. However, the therapeutic potential of this type of ICD was not studied.

In the present work we focused on determining whether the induction of ICD by PKHB1 has a therapeutic potential. Due to the immunogenicity of PKHB1-treated cells, we used the TCL obtained from PKHB1-treated L5178Y-R tumor cells (PKHB1-TCL) and focused on determining their ability to induce antitumor immune responses *ex vivo* and *in vivo* in an established L5178Y-R tumor developed in syngeneic BALB/c mice.

2. Material and Methods

2.1. T Cells and Dendritic Cells (DCs). This study was approved by the Animal Ethical Committee (CEIBA), of the School of Biological Sciences Number: 01/2015. All experiments were conducted according to Mexican regulation NOM-062-ZOO-1999.

The blood from sacrificed BALB/c mice was obtained by cardiac puncture. Peripheral blood mononuclear cells (PBMCs) isolation was performed by density gradient centrifugation using Ficoll-Hypaque-1119 (Sigma-Aldrich, St Louis, MO, USA). Murine CD3+ cells were isolated from total PBMCs by positive selection using magnetic-activated cell sorting (MACS) microbead technology with anti-CD3ε-biotin and anti-biotin microbeads (Miltenyi Biotech; >98% purity and >98% viability), as stated by manufacturer's instructions.

To obtain bone marrow-derived dendritic cells (DCs), after sacrifice, mice bone marrow was removed from femur and tibia of female BALB/c mice by flushing into RPMI-1640. Eluted cells were cultured for 5 days with 20 ng/mL of IL-4 and GM-CSF (R&D Systems, Minneapolis, MN, USA) until approximately 70% of the cells were CD11c+.

2.2. Cell Culture. L5178Y-R cell line (murine cancerous T lymphoblasts) was obtained from the ATCC. L5178Y-R, primary murine CD3+, and DCs were maintained in RPMI-1640 medium supplemented with 10% of fetal bovine serum, 2 mM L-glutamine, and 100U/mL penicillin-streptomycin (GIBCO by Life Technologies, Grand Island, NY, USA), and incubated at 37°C in a controlled humidified atmosphere with 5% CO_2. Cell count was performed using trypan blue (0.4% Sigma-Aldrich), a Neubauer chamber, and an optic microscope (Zeiss Primo Star) as proposed by the ATCC's standard protocols.

2.3. Cell Death Analysis. Annexin-V-allophycocyanin (Ann-V-APC 0.1μg/ml; BD Pharmingen, San Jose, CA, USA) and propidium iodide (PI, 0.5μg/ml Sigma-Aldrich) were used to assess phosphatidylserine exposure, cell death, and cell viability quantification, respectively, in a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) (total

population: 10,000 cells). Data was analyzed using FlowJo software (LLC, Ashland, OR, USA). 1X10^6 cells/mL were seeded and left untreated or treated for 2 h with 150 μM or 300 μM of PKHB1 (KRFY**VV**MWKK) or 150 μM of the control peptide 4NGG (KRFY**GG**MWKK) (as indicated) in serum-free media.

For cell death inhibitions we used the calcium chelator BAPTA (5mM), the antioxidant N-Acetyl Cysteine (NAC, 5 mM), the pan-caspase inhibitors Q-VD-OPH (QVD, 10 μM) and Z-VAD-FMK (Z-VAD, 50μM), the autophagic inhibitor Spautin-1 (SP-1, 15 μM), and the necroptotic inhibitor Necrostatin-1 (Nec-1, 50 μM). We pretreated the cells 30 minutes with the inhibitor before the treatment with PKHB1 (150 μM).

2.4. Calreticulin Exposure. L5178Y-R cells were plated (1x10^6 cells/mL), left untreated or treated with 300 μM of 4NGG or 150 μM or 300 μM of PKHB1, and incubated for 2 h. Cells were harvested, washed, and stained with Calreticulin-Phycoerythrin (Calreticulin-PE, FMC-75; Enzo Life Science, Farmingdale, NY, USA) antibody (1:1000) in FACS buffer. After 1 h in darkness at room temperature (RT), cells were washed and resuspended in 100 μL FACS buffer (PBS 1x and 2% of fetal calf serum) to be assessed by flow cytometry in a BD Accuri C6 flow cytometer (BD Biosciences) (total population: 10,000 cells). Data was analyzed using FlowJo software.

2.5. DCs Markers. DCs (1×10^6 cells/mL) were stained in 100 μL of FACS buffer with the indicated antibodies at RT for 30 minutes and then washed twice with PBS. The cell surface markers were evaluated by flow cytometry with the fluorescent label-conjugated antibodies, anti-CD11c-Alexa-fluor 488 (R&D Systems), anti-CD80-FITC, and anti-CD86-APC, from BD Biosciences (San Jose, CA, USA).

2.6. Cocultures. DCs-PKHB1 tumor cell lysate: DCs were resuspended in fresh medium at a concentration of 1×10^6 cells/mL. DCs were left untreated (control), or PKHB1-treated tumor cells were added at a concentration of 3×10^6 cells/mL to give a range of 1:3 DCs to PKHB1-treated tumor cells ratios. Coculture was left for 24 hours. Then the supernatant was removed, and the well was washed twice with PBS before doing the next coculture (with the addition of T-lymphocytes).

DCs-T-lymphocytes: Control DCs or DCs previously cocultured with PKHB1-TCL were maintained in fresh medium at a concentration of 1×10^6 cells/mL. Then, allogeneic BALB/c mCD3+ cells were added to each well at 3×10^6 cells/mL to give a range of 1:3 DC to CD3+ cells ratios. Coculture was left for 96 hours. Then, the lymphocytes were collected (by obtaining the supernatant), washed with PBS, and resuspended in fresh medium at a concentration of 5x10^6 cells/mL to be used in the next coculture (T- lymphocytes with cancer cells).

T-Lymphocytes-L5178Y-R cells: viable L5178Y-R cells were plated at a concentration of 1×10^5 cells/mL. Then, unprimed (previously cocultured with control DCs) or primed (previously cocultured with DCs-PKHB1-TCL) allogeneic BALB/c mCD3+ cells were added to each well at 5×10^5 cells/mL to give a range of 1:5 tumor to effector ratios. Coculture was left for 24 hours, before cytokine or calcein assessment.

2.7. Cytokine Release Assay. The supernatants from the indicated cultures were collected for IL-2, IL-4, IL5, and TNFα assessment (BD CBA Mouse Th1/Th2 Cytokine Kit, San Jose, CA, USA) by flow cytometry following manufacturer's instructions. IFNγ was assessed using an ELISA kit (Sigma-Aldrich) and using the Synergy HTTM (BioTek Instruments, Inc., Winooski, VT, USA) plate reader at 570 nm wavelength, following manufacturer's instructions.

2.8. Calcein Assay. L5178Y-R cells (1×10^6 cells/mL) were stained with (0.1μL/mL) of Calcein-AM from BD Biosciences (San Jose, CA) for 30 minutes and washed twice (PBS sterile). After this, T cells previously primed with DCs pulsed with PKHB1-TCL or with unpulsed DC were added in a 1:5 ratio. The L5178Y-R-T-lymphocytes' coculture was incubated at 37°C and 5% CO2 for 24 h. Finally, L5178Y-R-calcein negative cells were assessed in a BD Accuri C6 flow cytometer (BD Biosciences) (total population: 10,000 cells). Data was then analyzed using FlowJo software.

2.9. In Vivo Model. Six-to-eight-week-old BALB/c female mice were maintained in controlled environmental conditions (25°C and 12 h light/dark cycle) and were supplied with rodent food (LabDiet, St. Louis, MO, USA) and water *ad libitum*.

Tumor was established by subcutaneous injections of 2x10^6 L5178Y-R cells in 100 μL PBS, in the left hind. Tumor volume and mice weight were measured three times a week using a caliper (Digimatic Caliper Mitutoyo Corporation, Japan) and a digital scale (American Weigh Scale-600-BLK, USA), respectively. Tumor volume was determined with the following formula: tumor volume (mm^3) = $4\pi/3 * A(\text{length}) * B(\text{width}) * C(\text{height})$. When the tumor reached 100 mm^3, the first therapeutic vaccine of PKHB1-tumor cell lysate (PKHB1-TCL) was applied as follows:

L5178Y-R cells (5x10^6) were treated *in vitro* with 300 μM PKHB1 for 2 h (CC_{100}) in serum-free RPMI medium. Cell death was confirmed as previously indicated. Treated cells were inoculated subcutaneously in 100 μl serum-free media, in the right hind, twice a week. Controls were treated with 100 μl serum-free media.

For long memory assessment, we used six naïve mice (control) and six mice in complete remission after PKHB1-TCL treatment (tumor free >60 days). Both groups were injected with 2x10^6 living L5178Y-R cells in 100 μL PBS, in the left hind. The latter group was named PKHB1-TCL-Rechallenge. We then assessed tumor volume and survival, as described previously.

2.10. Statistical Analysis. Mice were randomly assigned to different groups for all *in vivo* studies. At least three independent experiments were repeated three independent times. Mann-Whitney tests and two-tailed unpaired Student's *t*-tests were performed using GraphPad Prism Software (San Diego CA, USA) and presented as mean values ±SD. The p values

were considered significant as follows: $p<0.05$, $p<0.01$, and $p<0.001$.

3. Results and Discussion

3.1. Calreticulin Exposure Correlates with Cell Death Induced by PKHB1. ICD is characterized by DAMPs exposure or release, and anticancer immune memory generation [24]. CRT exposure has been continuously reported as one of the principal DAMPs necessary for the correct maturation of DCs and antigen presentation [25, 26]. The activation of CD47 by PKHB1 induces CRT exposure in CLL cells [21]. Additionally, we recently reported that PKHB1 induces immunogenic cell death with DAMPs release (CRT, HMGB1, HSP79, HSP90, and ATP) and CRT exposure in T -ALL human cell lines and their murine counterpart, the L5178Y-R cell line (a murine T cell lymphoblastic tumor cell line) [23]. However, correlation between CRT exposure and cell death induced by CD47 was not established; for that purpose, here we assessed this feature using the L5178Y-R cell line.

First, we assessed cell death induced by the control peptide 4NGG, which does not bind to CD47 [21], and cell death induced by different concentrations of PKHB1. We found that 4NGG (300 μM) was not able to induce cell death in L5178Y-R cells, while PKHB1 induced a concentration-dependent cell death, reaching CC_{50} (cytotoxic concentration for 50% of the cells) at 150 μM and CC_{100} (cytotoxic concentration for 100% of the cells) at 300 μM.

Next, to evaluate the characteristics of the cell death induced by PKHB1, we used several cell death inhibitors. We have previously demonstrated that cell death induced through CD47 activation by PKHB1 is a fast and atypical caspase-independent and calcium-dependent mechanism [21, 23]. Thus, we assessed cell death using the calcium chelator BAPTA, as positive control of cell death inhibition by PKHB1, and the antioxidant NAC (N-Acetyl Cysteine) which inhibits several cell death modalities that involve ROS production [27]; as apoptotic pan-caspase inhibitors we used Q-VD-OPH [28] and Z-VAD-FMK (which also inhibits pyroptosis, [29]); we also used the autophagic inhibitor Spautin-1 [30] and the necroptotic inhibitor Necrostatin-1 [31]. In Figure 1(c) we can observe that that only the calcium chelator, BAPTA, was able to inhibit PKHB1-cell death. Effectively cell death induced by CD47 activation seems to be mostly cytoplasmic and mediated by calcium augmentation [21, 23], and due to the velocity of the process it seems to be a different mechanism of cell death from the commonly described to date.

Using 300 μM of PKHB1 for two hours induced 97% of cell death (defined as CC_{100}), and calreticulin exposure was observed for 90% of the cells. The PKHB1-tumor cell lysate of L5178Y-R cells (PKHB1-TCL) was generated with this CC_{100}. Figure 1 describes the PKHB1-induced CRT exposure in a PKHB1-concentration and cell death-dependent ways: indeed, the increasing number of Ann-V-APC/PI positive cells with increasing concentration of PKHB1 (Figures 1(a) and 1(b)) is correlated with an increasing CRT exposure (Figures 1(d) and 1(e)).

Calreticulin exposure and cell death have been shown to be correlated when using various agents inducing ICD [26], such as IMMUNEPOTENT CRP [32] and shikonin [33]; however in some cases, CRT has been reported to be exposed premortem [26]. We recently found that the CC_{100} was necessary for the highest release of HMGB1, HSP70, and HSP90 in L5178Y-R cells [23]. This observation led us hypothesize that since the PKHB1-TCL is rich in DAMPs, it might induce DCs maturation and antigen presentation to T cells promoting antitumor responses.

3.2. PKHB1-TCL Induces Maturation of Bone Marrow-Derived DCs. To determine if PKHB1-TCL was able to induce the maturation of DCs, bone marrow-derived murine DCs were left untreated (control) or pulsed for 24 h with the previously obtained PKHB1-TCL. We assessed cytokine production by PKHB1-TCL, but we did not find a significant release of TNFα, IFNγ, IL-5, IL-4, or IL-2 (Supplementary Table 1). After coculture, DCs were washed twice with PBS to remove any background noise given by the PKHB1-TCL. DCs cocultured with PKHB1-TCL show morphological changes (data not shown) and a significant increase in the expression of costimulatory molecules (CD80 and CD86) passing from 50% to 78%, while maintaining the expression of the DCs marker CD11c (Figures 2(a) and 2(b)). Furthermore, only DCs-PKHB1-TCLs show a significant increase in TNFα release in comparison with unstimulated DCs (Figure 2(c)).

Several types of TCL are able to induce DCs maturation at different degrees [8]; however most of them use LPS [33] or other adjuvants such as phytoextracts [34] and bacterial ghosts [35] in combination with the TCL. Our results show that PKHB1-induced cell death is able to promote DCs maturation and secretion of TNFα, even in the absence of other immune-stimulants. The mature DC phenotype was characterized by the expression of the endocytic receptor CD11c [36], CD80, and CD86 [37], which increased significantly ($p=0.0005$ and $p=0.0066$, respectively) in DCs cocultured with PKHB1-TCL. We can observe a slight nonsignificant decrease in the expression of CD11c (Figures 2(a) and 2(b)); this differentiation marker can be downregulated by dendritic cells after their activation by TLR agonists [38]. The secretion of TNFα has been associated with a mature phenotype, as it acts as an autocrine maturation factor for DCs [37]. Several TCLs are able to induce its secretion at several degrees, ranging from 20 pg/mL to 250 pg/mL [39, 40]. Here we found that DCs pulsed with PKHB1-TCL induced the secretion of TNFα at a 270 pg/mL concentration, indicating the efficient maturation of DCs by PKHB1-TCL.

3.3. PKHB1-TCL Induces an Antitumor T Cell Response. Once we determined that PKHB1-TCL was able to induce DCs maturation, we assessed if the pulsed DCs (DCs-PKHB1-TCL) were able to prime T cells. First, CD3+ cells were cocultured for four days with pulsed or unpulsed DCs, and we assessed TNFα, IFNγ, IL-5, IL-4, and IL-2 cytokine release. Table 1 shows that coculture of pulsed DCs with primary T-lymphocytes induces the release of TNFα, IFNγ, and IL-2, while IL-5 and IL-4 release were not detected. The secretion

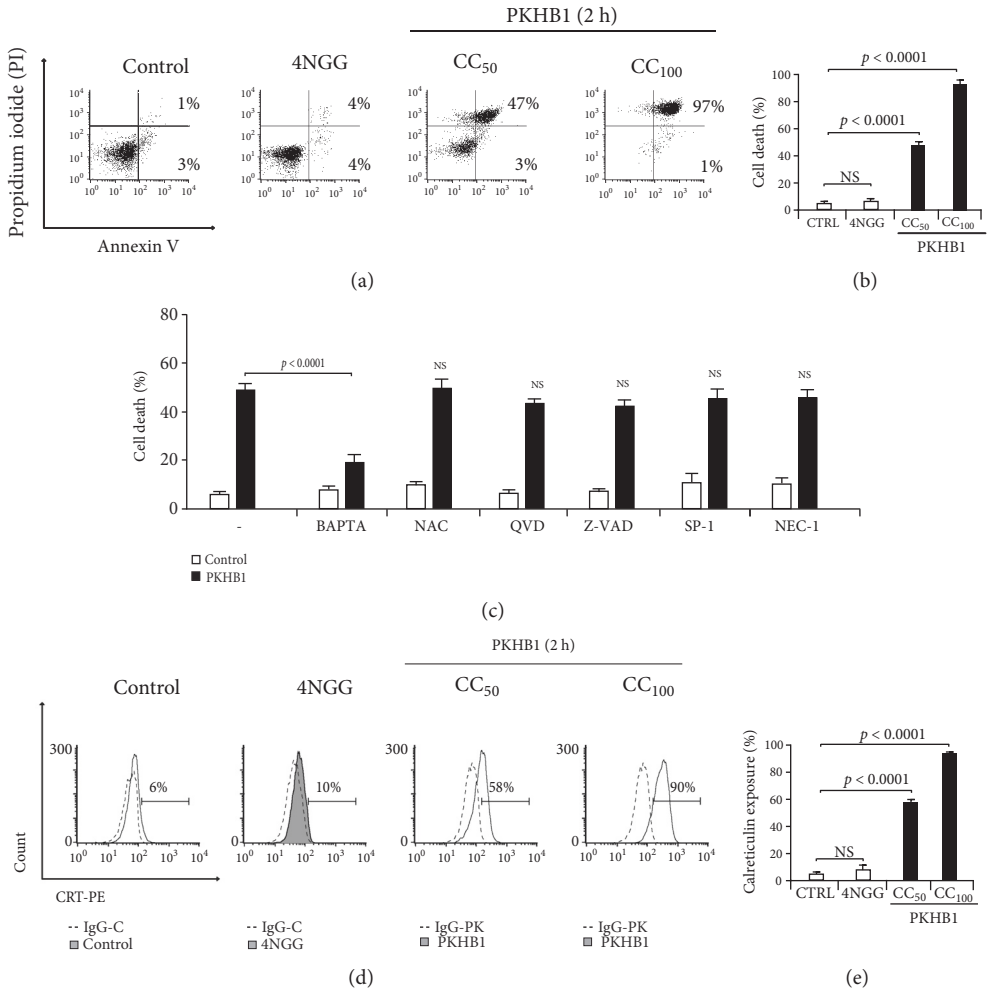

Figure 1: *PKHB1 induces cell death and calreticulin exposure in L5178Y-R cell line.* (a) Cell death was measured by Annexin-V-allophycocyanin (Annexin-V-APC) and propidium iodide (PI) staining and graphed. Dot plots of L5178Y-R cells untreated (control) and treated with control peptide 4NGG (300 μM) or CC_{50} (150 μM) and CC_{100} (300 μM) of PKHB1 for 2 h. (b) Graph represents the means (±SD) of triplicates of three independent experiments obtained as in (a). (c) Cell death induced by PKHB1 was assessed as in (a) with cells left without pretreatment (control) or pretreated (30 minutes) with BAPTA, N-Acetyl Cystein (NAC), Q-VD-OPH (QVD), Z-VAD-FMK (Z-VAD), Spautin-1 (SP-1), or Necrostatin-1 (Nec-1). (d) Calreticulin exposure induced by 4NGG (300 μM) and PKHB1 (CC_{50} and CC_{100}, 2 h) was measured using FACS in L5178Y-R cell line, and representative histograms are shown. (e) Graph represents the means (±SD) of triplicates of three independent experiments obtained as in (c).

of TNFα, IFNγ, and IL-2 is associated with a Th1 phenotype [41], which promotes an antitumor immune response [42].

Next, primed (cocultured with pulsed DCs-PKHB1-TCL) or unprimed (cocultured with unpulsed DCs) T-lymphocytes were collected and cocultured during 24 h with L5178Y-R cells (previously stained with calcein-AM). After 24 h of coculture, supernatants were obtained, and we assessed IFNγ, IL-4, and IL-2 cytokine release. A significant increase in IL-2 and IFN-γ release was observed in the supernatants of T-lymphocytes previously cocultured with DCs-PKHB1-TCL (Figure 3).

Once we observed that PKHB1-TCL induced IFNγ and IL-2 release, suggesting Th1 responses [41], we assessed antitumor cell cytotoxicity. For this purpose, we evaluated the loss of calcein in L5178Y-R cells. Results show that only T-lymphocytes cocultured with pulsed DCs-PKHB1-TCL induce a significant increase in the calcein negative L5178Y-R cells, in comparison with the T-lymphocytes cocultured with control DCs (not pulsed with PKHB1-TCL) (Figure 4). This confirms the correct antigen presentation by DCs-PKHB1-TCL and the T cell cytotoxicity against L5178Y-R cancer cells.

Detection of IL-2, INFγ, and TNFα in supernatants of DCs and T cell cocultures indicates the establishment of an efficient anticancer immune response. These observations are in agreement with the results observed in our cocultures of T cells with DCs-PKHB1-TCL. The secretion of these cytokines suggests a Th1 phenotype [41] which was confirmed by the loss in cell viability of L5178Y-R cells cocultured with primed T cells.

Several cytotoxic agents have been demonstrated to induce *ex vivo* antitumor T cell responses, such as bortezomib in myeloma [43] and doxorubicin in colon carcinoma [44]. Also the allogeneic off-the-shelf dendritic cell vaccine,

Table 1: TNFα, IFNγ, IL-5, IL-4, and IL-2 cytokine release (pg/mL) in cocultures of T lymphocytes with control or pulsed DCs.

	TNFα (pg/mL)	IFNγ (pg/mL)	IL-5 (pg/mL)	IL-4 (pg/mL)	IL-2 (pg/mL)
DCs-Control + T-lymphocytes	38.9 ± 14	0 ± 0	0 ± 0	0 ± 0	0.2 ± 0.2
DCs-PKHB1-TCL + T-lymphocytes	479.6** ± 156	974.33*** ± 115	0 ± 0	0 ± 0	3.5** ± 0.7

Bone marrow-derived murine DCs were left with medium alone (DCs-control) or pulsed (DCs-PKHB1-TCL) during 24h with a PKHB1-TCL. Then, DCs were cocultured during 4 days with T-lymphocytes, and the supernatants were collected to quantify TNFα, IFNγ, IL-5, IL-4, and IL-2 release, by FACS. Numbers represent the means (± SD) of triplicates of three independent experiments. **p<0.01; *** p<0.001.

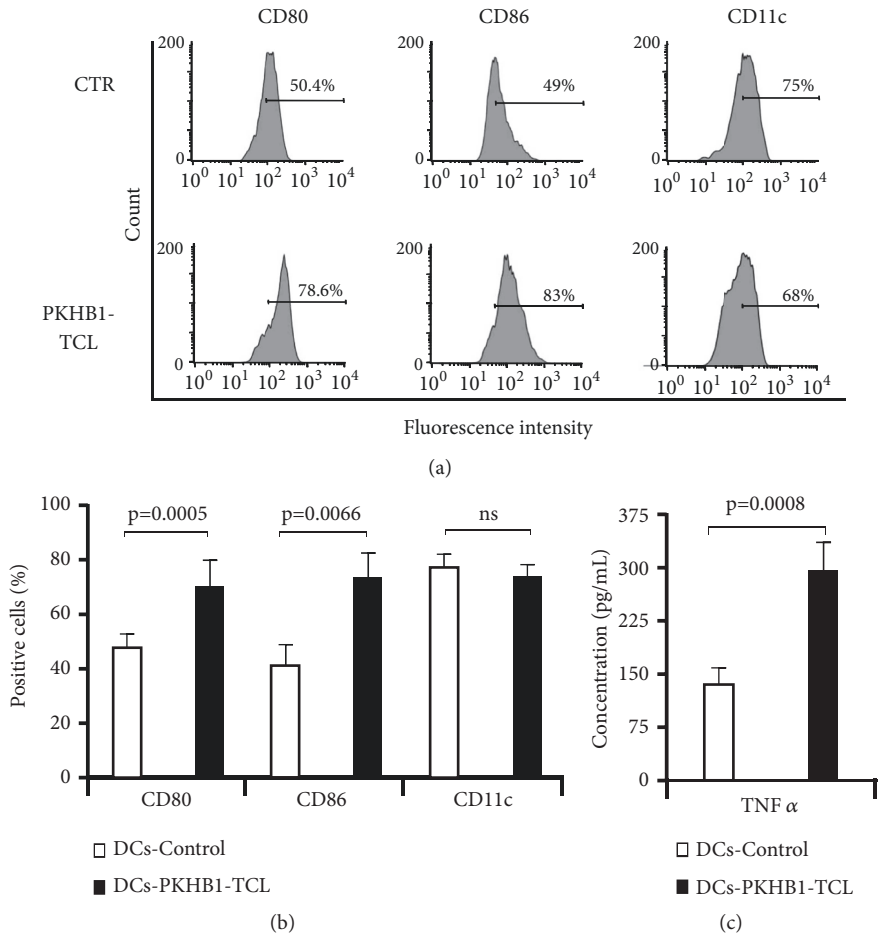

Figure 2: *PKHB1-tumor cell lysate induces the maturation of bone marrow-derived dendritic cells.* (a) Bone marrow-derived murine DCs were left only with medium (control) or pulsed during 24 h with a PKHB1-TCL. DCs were then stained to assess cell surface markers (CD11c, CD80, or CD86) by FACS, and representative histograms are shown. (b) DCs were treated as in (a) and the means obtained by FACS were graphed. (c) DCs were treated as in (a) and the supernatants were collected to quantify TNFα release, by FACS. Graphs represent the means (±SD) of triplicates of at least three independent experiments.

currently in clinical trials for acute myeloid leukemia [45], has demonstrated these responses *ex vivo* and *in vivo* in patients.

3.4. PKHB1-TCL Induces Tumor Regression. Once we established the *ex vivo* antitumor immune response induced by PKHB1-TCL, we assessed if the *in vivo* injection of PKHB1-TCL was able to diminish tumor growth and improve overall survival in syngeneic mice transplanted with L5178Y-R cells. First $2x10^6$ L5178Y-R cells were inoculated in BALB/c mice. When the tumor reached 100 mm^3, a mice control-group was left without treatment (Control; n = 7), and a second group was treated with PKHB1-TCL two times per week (PKHB1-TCL; n = 9) (Figure 5(a)). Tumor growth was measured: we observed that PKHB1-TCL-treated mice showed significantly diminished tumor growth after day 10 (7 days after the first treatment), which continued to decrease until no tumor was detected by day 30 (Figure 5(b)). Tumor growth diminution was reflected in overall mice survival, as PKHB1-TCL-treated

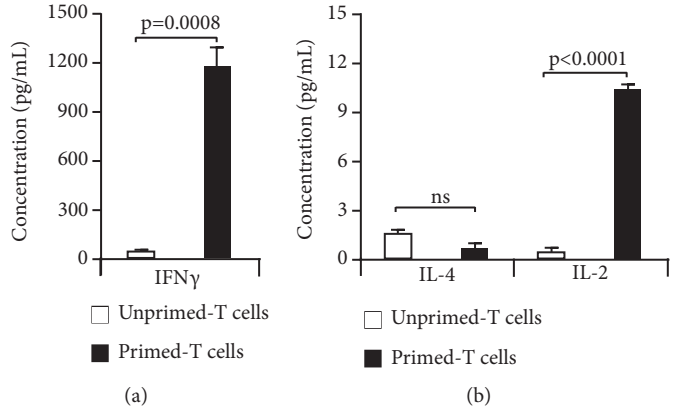

Figure 3: *IFN-γ and IL-2 secretion by unprimed or primed T cells cocultured with L5178Y-R cells.* (a) L5178Y-R cells were cocultured with unprimed T-lymphocytes (previously cocultured with unstimulated DCs-Control) or primed T-lymphocytes (previously cocultured with pulsed DCs-PKHB1-TCL) in a 1:5 tumor to effector ratio, for 24 h, and the supernatants were collected and assayed for (a) IFN-γ release by ELISA and (b) IL-4 and IL-2 release by FACS. Graphs represent the means (±SD) of three experiments performed independently.

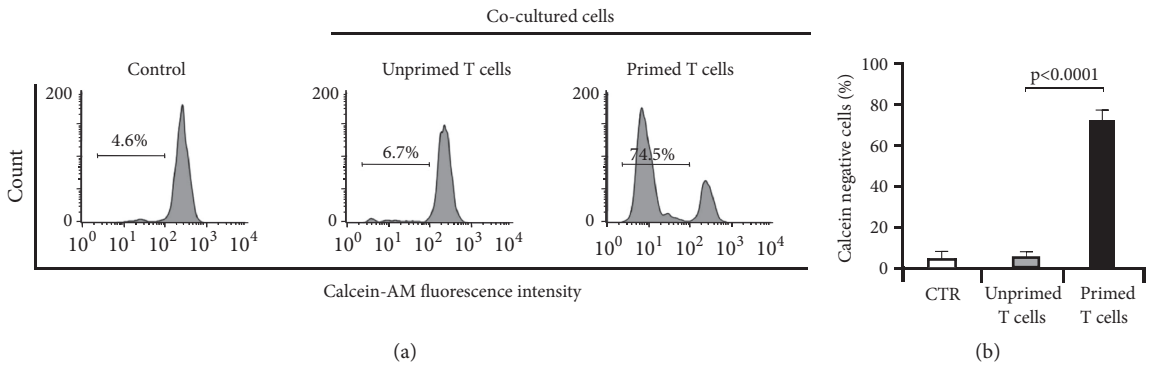

Figure 4: *Primed T cells' cytotoxicity in L5178Y-R cells.* (a) L5178Y-R cells were stained with calcein-AM and cocultured with unprimed T-lymphocytes (previously cocultured with unstimulated DCs-Control) or primed T-lymphocytes (previously cocultured with pulsed DCs-PKHB1-TCL) in a 1:5 tumor to effector ratio for 24 h. The percentage of L5178Y-R calcein negative cells was assessed by FACS; representative histograms are shown. (b) Graphs represent the means (±SD) of triplicates of three independent experiments obtained as in (a).

mice presented an 80% of survival over time (more than 150 days), while all control mice perished by day 11 (Figure 5(c)).

To assess immunological memory against tumor antigens after PKHB1-TCL-treatment, mice in complete remission (tumor free >60 days) were rechallenged with living L5178YR cells. Compared to naïve mice (control), in which a primary L5178YR cell challenge resulted in rapid tumor progression, those that were in remission were completely resistant to a rechallenge of L5178YR cells (Figure 6(a)). Furthermore, as no tumor developed, we observed a 100% of survival of the PKHB1-TCL-Rechallenged mice while all control mice perished by day 12 (Figure 6(b)).

It has been proved that other TCLs reduce tumor volume in different types of cancer, as, for example, combined with CpG in a glioblastoma mouse tumor model [46]. Additionally, in clinical trials TCLs have been tested in melanoma, prostate, mesothelioma, ovarian, and colorectal cancers [8]. These TCLs are usually produced using radiation, repeated freezing, and thawing, among others. Here we show that PKHB1-CD47 activation, which has been shown to effectively induce cell death in different types of cancer [21, 22], including cells coming from refractory patients [21, 22], can provide an immunogenic TCL which is able to promote an antitumor immune response, even in the absence of adjuvants.

Interestingly, we observed that tumor volume began to diminish 7 days after the first administration of PKHB1-TCL reaching tumor regression by day 28 (Figure 5(c)). This waiting time corresponds with the time needed for T cells to expand and activate an antitumor immune response [47].

Other types of TCL have been able to induce tumor regression, increasing the survival rate in patients with melanoma and prostate cancer [8]; however, they do so only in combination with adjuvants, such as the case of CpG oligodeoxynucleotides, which are TLR9-agonist [46].

We recently demonstrated that PKHB1 treatment of tumor-bearing mice induced long-term tumor prevention in 90% of the mice that presented complete tumor regression [23]. Here we demonstrated that PKHB1-TCL induces long-term immunological memory against tumor antigens, preventing tumor establishment in 100% of the mice after

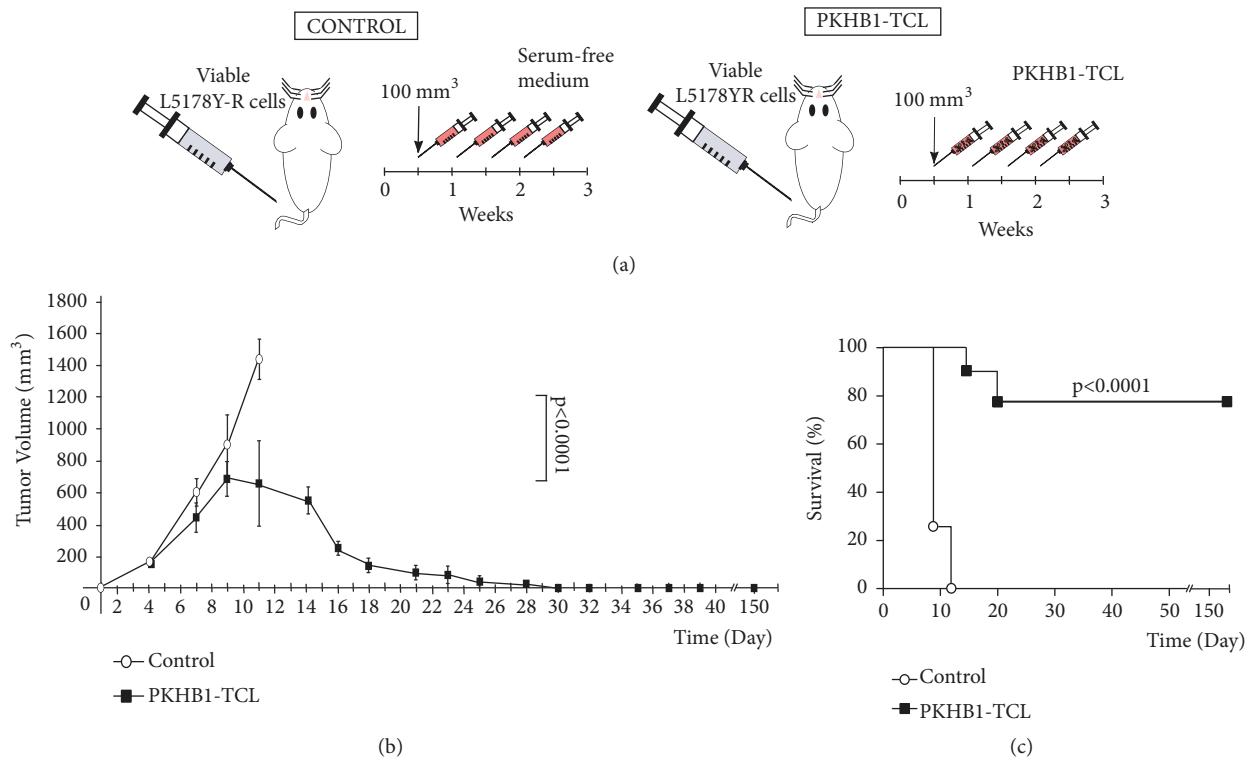

FIGURE 5: *PKHB1-TCL treatment in L5178Y-R-tumor-bearing mice induce tumor regression.* (a) Schema of PKHB1-TCL (5×10^6 CC_{100} PKHB1-treated L5178Y-R cells) treatment started when tumor reached 100 mm^3, and then PKHB1-TCL was administrated every 3 days for two weeks (for a total of four injections). (b) Graph indicates tumor volume (±SD) of untreated mice (control; n = 7) or PKHB1-TCL-treated mice (PKHB1-TCL; n = 9). (c) Kaplan-Meier survival curve of untreated mice (control; n = 7) or PKHB1-TCL-treated mice (PKHB1-TCL; n = 9) over time.

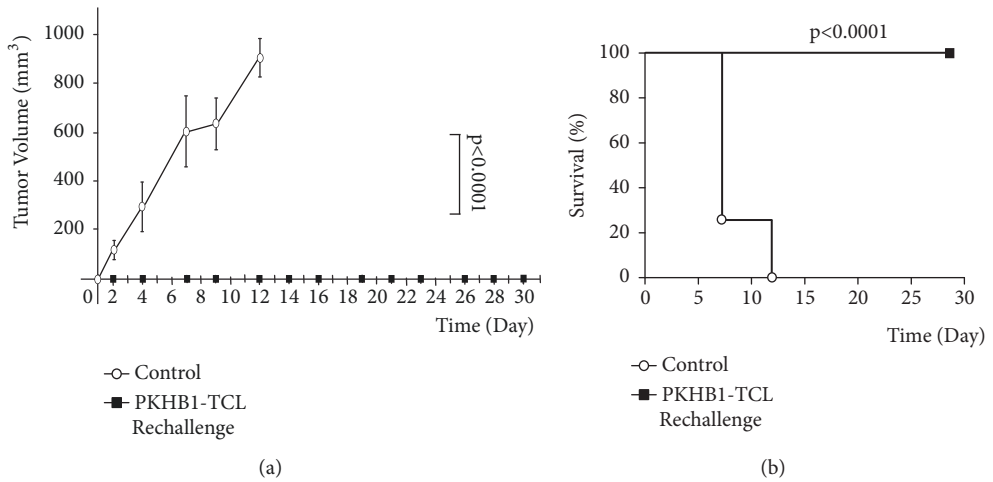

FIGURE 6: *PKHB1-TCL therapeutic vaccination induces long-term antitumor memory.* Mice in remission after therapeutic vaccinations were rechallenged with $2x10^6$ L5178Y-R viable cells. (a) Graph indicates tumor growth in control mice (control, n = 6) or mice in remission after a previous treatment with PKHB1-TCL that were rechallenged with living L5178Y-R cells (PKHB1-TCL-Rechallenge, n= 6). (b) Kaplan-Meier survival graph of mice treated as in (a) over time. Control: n = 6; PKHB1-TCL-Rechallenge, n= 6.

L5178YR cells rechallenge (Figure 6). This underlines the immunogenicity of CD47-mediated cell death, when administering a CD47 agonist peptide or CD47-killed cells. This long lasting immunological memory has been promoted also by a TCL obtained by repeated freezing and thawing and radiation-treated glioma cells, where nearly a 100% of survival was observed [48].

Although immunotherapy with pulsed DCs, primed T-lymphocytes, or CAR-T cells is the principal approach used to stimulate antitumor immune responses, here we demonstrate

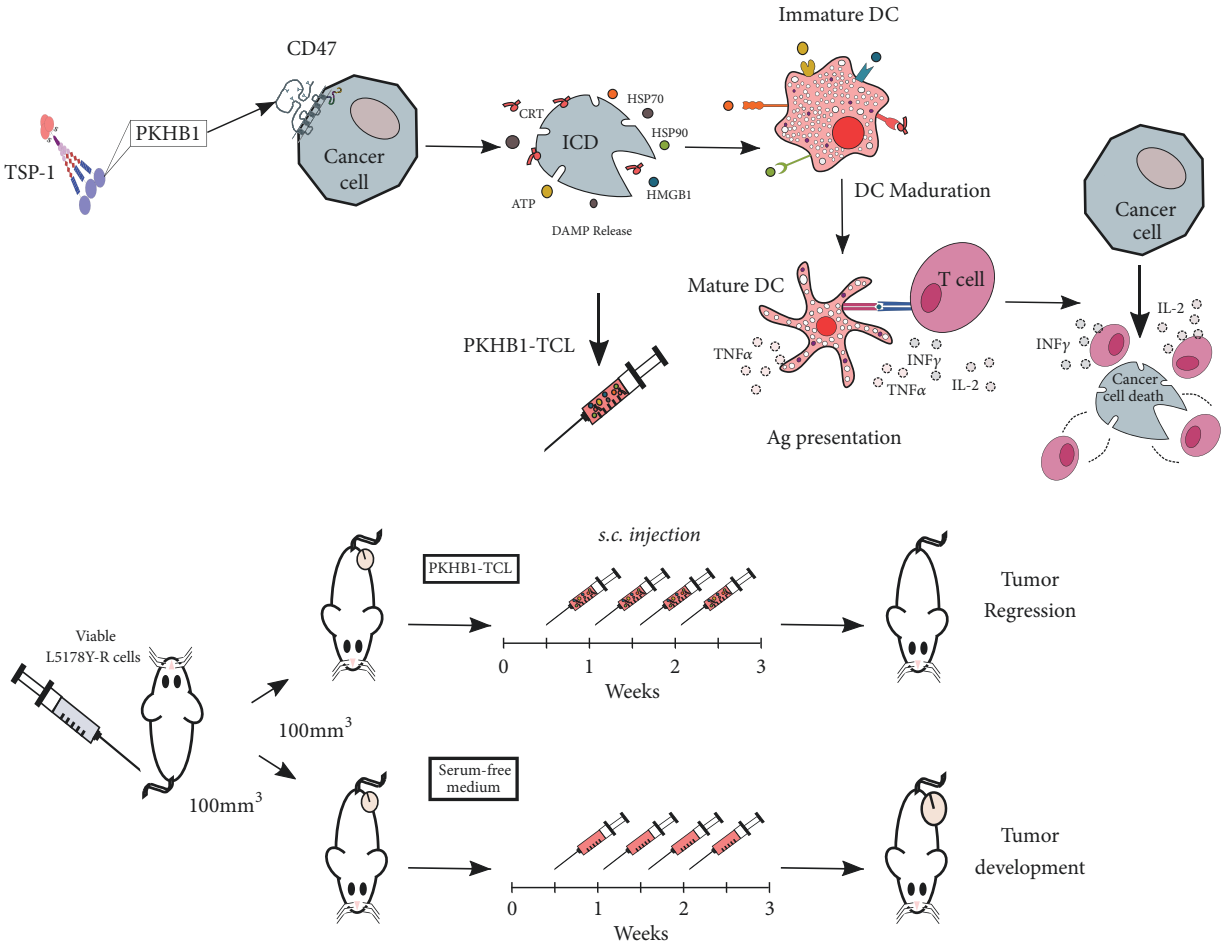

Figure 7: *Schematic representation of CD47-mediated immunogenic cell death in vitro, ex vivo, and in vivo.* PKHB1 induces selective ICD in L5178Y-R cell line leading to damage-associated molecular patterns (DAMP) release. DAMPs promote dendritic cell (DC) maturation and subsequence antigen presentation and T cell activation to induce cancer cell death. Moreover, PKHB1-treated cells administrated as a therapeutic vaccine induce tumor regression in syngeneic mice bearing L5178Y-R tumors. CRT, calreticulin; HMGB1, high-mobility group box 1; HSP, heat shock protein; ICD, immunogenic cell death; TSP-1, thrombospondin-1.

that the crude TCL obtained from PKHB1 treatment is able to induce tumor regression in 80% of the mice, while preventing tumor establishment in 100% of the rechallenged mice that survived after PKHB1-TCL-treatment.

4. Conclusions

In this work we determined that the ICD induced by the CD47-agonist peptide, PKHB1, has a therapeutic potential, as the PKHB1-TCL was able to induce antitumor immune responses *ex vivo* and *in vivo* in an established L5178Y-R tumor. This was done by promoting the maturation of DCs, which trigger T cell antitumor effects, including INFγ release and L5178Y-R cell cytotoxicity, leading to tumor regression (Figure 7). Additionally, PKHB1-TCL-treated mice developed long-term immunological memory. These results highlight the immunogenicity of the cell death induced by CD47 activation by PKHB1 as a potential therapeutic tool to overcome the low immunogenicity of cancer cells, such as T-ALL.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare the following competing financial interest(s): a patent application including results from this paper has been filed. The authors declare that no other conflicts of interest exist.

Authors' Contributions

Ana Carolina Martínez-Torres and Kenny Misael Calvillo-Rodríguez are main authors and equally contributed to this work.

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Supplementary Materials

Supplementary Table 1. TNFα, IFNγ, IL-5, IL-4, and IL-2 cytokine release by PKHB1-tumor cell lysate. L5178Y-R cells were treated for 2 h with PKHB1 (300μM) and the supernatants were collected to quantify TNFα, IFNγ, IL-5, IL-4, and IL-2 release, by FACS. *(Supplementary Materials)*

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Research Article

Production and Evaluation of an Avian IgY Immunotoxin against CD133+ for Treatment of Carcinogenic Stem Cells in Malignant Glioma: IgY Immunotoxin for the Treatment of Glioblastoma

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Background. Glioblastoma is the most common malignant tumor of Central Nervous System. Despite the research in therapeutics, the prognosis is dismal. Malignant glioma stem cells (MGSCs) are a major cause of treatment failure and increasing tumor recurrence. In general, cancer stem cells (CSCs) express prominin-1 (CD133), considered as a potential therapeutic target. In this study, we produced an avian immunotoxin directed against the subpopulation of CD133+ CSCs within a malignant glioma. We used the avian IgY because it has various advantages as increased affinity to mammal antigens and inexpensive obtention of large amounts of specific antibodies (approximately 1 mg/per egg). The design, production, purification and use of IgY anti CD133 immunotoxin constitute an original goal of this research. *Methods*. The immunodominant peptide of CD133 was designed to immunize hens; also, the extracellular domain of CD133 was cloned to probe the IgY antibodies. In parallel, a recombinant abrin A chain was produced in *E. coli* in order to join it to the Fc domain of the anti-CD133 IgY to conform the immunotoxin. This anti-CD133 IgY anti-tumor immunotoxin was tested *in vitro* and *in vivo*. *Results*. The cytotoxicity of the immunotoxin *in vitro* showed that IgY-abrin immunotoxin reduced 55% cell viability. After subcutaneous MGSCs implantation, the animals treated intraperitoneally or intratumorally with the IgY-abrin immunotoxin showed more than 50% decrease of tumor volume. *Conclusion*. Results showed that the IgY-abrin immunotoxin had cytotoxic activity against CD133+ MGSCs and provides a novel approach for the immunotherapy of glioblastoma.

1. Background

Glioblastoma (GBM) is the most frequent primary brain tumor in adulthood and the most aggressive astrocytoma. It is characterized by cellular heterogeneity, vascularization, and high capacity to infiltrate. Current mean overall survival after diagnosis is about 15 months [1]. One of the factors involved in the malignancy and resistance to treatment is the heterogeneous microenvironment conformed by a network of diverse cells. Among them, a subpopulation that share phenotypic properties with neural stem cells, cancer stem cells (CSCs) are key contributors to GBM progression due to

their ability for self-renewal and high proliferation [2]. CSCs are usually identified and isolated by stem cell markers, like the cell receptor prominin-1 (CD133) a penta-transmembrane glycoprotein [3]. As biomarker of GBM stem cells, CD133 is highly expressed. The expression of CD133 on CSCs makes this glycoprotein an adequate target to improve therapeutic efficacy of GBM.

The catalytic destruction of CSC cells would depend on the internalization of cytotoxic elements; in the case of CD133, it has been demonstrated that antibodies against this receptor are efficiently internalized [4]. In contrast to monoclonal IgG antibodies of mammalian origin, IgY polyclonal antibodies, the predominant immunoglobulin in birds [5], show diverse advantages, among them, a high recognizing capacity of mammal antigens and large quantity of IgY produced by hens immunized [6]. Production of IgY is reliably achieved and does not require bleeding of the host-producing antibodies because IgY antibodies can be isolated from the egg yolk. This isolation procedure is efficient and economical [7]. In the case of hens, around 10-20 mg of IgY per egg is produced [8]. Due to these advantages, we decided to produce an immunotoxin composed by IgY antibodies against CD133+ cells bound to a cytotoxin. An immunotoxin is an antibody conjugated to a toxin which joins a specific cell-surface receptor. Side effects to this therapeutic approach are greatly reduced [9]. Different toxins have been used to construct immunotoxins. We selected one characterized by its high cell lethality obtained with a low dose. The abrin is a toxin isolated from the seeds of the plant *Abrus precatorius*; it is similar to ricin and belongs to the group of type II ribosome-inactivating protein (RIP). Structurally, the toxin is composed by two chains, abrin A chain (31 KDa), which inactivates ribosomes into the cytosol, and the abrin B chain, which binds to the cell membrane by a terminal galactose receptor [10].

In this study, we constructed an immunotoxin, composed by purified IgY antibodies against the CD133 surface marker coupled to a recombinant abrin A chain. The CD133 is highly expressed in GSCs from malignant gliomas.

2. Materials and Methods

2.1. Design, Expression, and Purification of Recombinant CD133 and Abrin A Chain

2.1.1. Prominin 1 Antigen. (a) The analysis of immunogenicity algorithms, which consists of a combination of hydrophobicity, flexibility of the chain, and specific residues of protein CD133, showed that the most suitable sequence of the protein was that corresponding to amino acids 73 to 95 (CZEDTLRKFKQKAYESKIDYDKPET) conjugated to Keyhole Limpet Hemocyanin (KLH) to increase immunogenicity. This peptide was obtained from Aves labs to be used as antigen to immunize 2 hens. Also, a *E. coli* expressing AC133 (prominin 1, aa 20-108) was obtained from Bioclone Inc. (Bioclone Inc., San Diego, CA) which expresses the surface glycoprotein CD133, in order to confirm the specificity of the IgY purified anti-CD133 obtained from hens immunized with the CD133 peptide.

(b) The abrin A chain codifying sequence was achieved through bioinformatic analysis using the GenBank sequence CAA54139.1:EDRPIKFSTEGATSQSYKQFIEALRERLRGGLIHDIPVLPDPTTLQERNRYITVELSNSDTESIEVGIDVTNAYVVAYRAGTQSYFLRDAPSSASDYLFTGTDQHSLPFYGTYGDLERWAHQSRQQIPLGLQALTHGISFFRSGGNDNEEKARTLIVIIQMVAEAARFRYISNRVRVSIQTGTAFQPDAAMISLENNWDNLSRGVQESVQDTFPNQVTLTNIRNEPVIVDSLSHPTVAVLALMLFVCNPPN. The sequences selected to be cloned considered the optimal use of codons on *E. coli* BL21DE3pLysS (Novagen Cat. No. 69451-4) to guarantee the best expression of the recombinant constructs. Also, a Hind III/Xho I sequences were added on the 5′-3′ ends of the inserts to be ligated into HindIII/Xho I restriction sites of the expression vector *pET28a* (Novagen cAT. No. 69337-3). This plasmid confers kanamycin-resistance to cells, contains an IPTG-regulated promoter, and adds a 6-His tag to the recombinant protein to select the positive clones and purify the recombinant proteins (Supplementary Figure 1). Competent *E. coli* cells were heat-shock transformed with these plasmids and grown in Luria Bertani (LB) agar plus kanamycin (30 μg/mL) (LB-Kan). Positive colonies were pick-up and overnight cultured in liquid LB-Kan medium to obtain fresh recombinant cells. These transformed bacteria were induced to express abrin A chain adding IPTG 0.1 mM and cultures were allowed to grow 12 h at 37°C into a shaker as described elsewhere [11]. Then, cultures were centrifugated and the pellets were sonicated to obtain the bacterial lysate. These extracts were passed throughout affinity columns of HisPur™ Ni-NTA Purification Kit (Pierce Biotechnology lL 61105 USA Cat. No. 88229) to achieve the purification of the recombinants according to the manufacturer's instructions. Purification of abrin A chain was followed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and validated by Western blot assays.

2.2. Immunoreactivity of the IgY Immunotoxin Components. The specificity of purified IgY antibody for recognition of CD133 and the presence of abrin A chain were both analyzed by western blot (WB). The WB for CD133 was carried out with 30 μg of protein obtained from the culture lysate of *E. coli* expressing CD133 and *E. coli* expressing abrin A chain. According to standard blotting procedures, the lysates were loaded onto 12% SDS-polyacrylamide gel with Precision Plus Protein Standards (Bio-Rad). Gels were then transferred to nitrocellulose membrane (Pure Nitrocellulose Membrane 0.45 micron; Bio-Rad). The membranes were blocked for 1h with blocking buffer (0.5% BSA and PBS). For the CD133 protein, the membrane was incubated overnight with the purified anti-CD133 IgY as primary antibody, then the membrane was washed with 0.01M PBS/0.05% Tween and incubated for 1 h with rabbit anti-chicken IgG antibody (Jackson ImmunoResearh Laboratories, Inc. Code Number 303-035-003).

The WB for abrin A, the membrane was incubated with the primary antibody His-probe (H-15; Santa Cruz, USA), afterwards with mouse anti-rabbit IgG-HRP (Santa Cruz) secondary antibody. Membranes were washed with PBS and

developed by chemiluminescence with the ECL Plus Kit WB Detection System (GE Healthcare, Amersham, USA).

2.3. Immunization of Hens. Two hens (Gallus gallus, variety Hy Line Brown), 14 weeks of age, were immunized intramuscularly (IM), injecting 200 μg/ml of CD133 peptide in various zones of the pectoral region with subsequent reinforcements after 14, 28, and 56 days.

2.4. Isolation and Purification of IgY. IgY-antibodies directed to CD133 were isolated from the yolk of eggs from hyperimmunized hens [12]. Egg yolks were separated and diluted with PBS. Polyethylene glycol 600 (PEG, Sigma-Aldrich) was added progressively at concentrations of 3.5%, 8.5%, and 12% and centrifuged at 13 000 x g for 20 min at each concentration. The soluble fraction was collected.

Specific IgY anti-CD133 purification was carried out by affinity chromatography using sepharose columns (Pierce NSH-Activated, Agarose Spin Columns, Thermo Scientific). The binding buffer (0.1M sodium phosphate, 0.15M NaCl, pH 7.2) was added into NSH-activated agarose columns together with CD133 protein. Unspecific sites were blocked with blocking buffer (10 mM tris pH 7.5, 50 mM KCl and 20 mM EDTA). Then, the column was centrifuged two minutes at 1000 x g and incubated with PBS and antibodies IgY. Afterwards, the column was washed with 3 mL of Binding/Wash Buffer and centrifuged at 1000 × g for 2 minutes. Specific IgY anti CD133 were eluted by elution buffer. The pH of each fraction was adjusted to neutral adding 50μL of neutralization buffer.

2.5. Enzyme Linked Immunosorbent Assay (ELISA) of IgY Antibodies. The specificity and sensitivity of IgY antibodies obtained from preimmunized and immunized hens were evaluated by ELISA. An ovalbumin-conjugate of the peptide was first absorbed onto the ELISA plates at a concentration of 1 μg/ml in PBS. After overnight incubation at 4°C, 1:100 dilution of BlockHen ® (Aves Labs, diluted in PBS) was added to each well for two hours at room temperature to block non-specific sites. After thorough washing, wells on the plate were incubated with various concentrations of either purified immune IgY, purified pre-immune IgY, or affinity purified IgY. After overnight incubation at 4°C, the plate was washed and incubated one hour with HRP-labeled goat anti-chicken IgY (1:5,000 dilution, Aves Labs) at room temperature (with rocking). The plate was then washed, and HRP activity bound to the plate was determined using ortho-phenylenediamine and stable peroxide substrate buffer (Pierce). Finally, the plate was read by measuring the absorbance at 450 nm (ELx808 kinetic ELISA plate reader, Bio-Tek Instruments, Inc).

2.6. Conjugation of the Immunotoxin. The immunotoxin was constructed using the cross-linker SMPT (4-succinimidiloxicarbonil-α-[2-piridildithio] toluene, Thermo Scientific) as previously described [13]. SMPT is a heterobifunctional linker with two reactive groups, a sulfhydryl and an amino group. Briefly, SMPT was diluted in 5% dimethyl sulfoxide (DMSO), mixed with the antibody (1.6 mg/ml in PBS) and incubated 1 h at room temperature (RT). Unreacted SMPT was removed by dialysis with Slide-A-Lyzer Dialysis Cassette (10,000 MWCO. Thermo Scientific). The sample was then dialyzed overnight against PBS-10mM EDTA. Abrin A (1 mg/ml in PBS) was incubated 1 h with 2.5 mM dithiothreitol (DTT). After the IgY-SMPT linkage was obtained both, activated IgY and abrin A were mixed in a ratio of 2:1. After filter-sterilization through 0.22 μm filter, the link-up was carried out under nitrogen ambient conditions during 18 h at room temperature; 25 mg/ml cysteine was added for 6 h to inhibit the remaining pyridyl disulfide active sites. To purify the conjugated with A chain of abrin from the unconjugated antibody, the mixture was passed through a Cibacron blue 3GA agarose column.

2.7. Culture Conditions. The A-172, U-373, LN-18, U-87 (cell lines from human glioblastoma), C6 (cell line from rat malignant glioma), VERO (cell line from African green monkey kidney), and primary dermal fibroblasts were obtained from the American Tissue Culture Collection (ATCC). The cell lines were expanded using a permissive medium composed by 10% DMEM (Dulbecco's Modified Eagle Medium, GIBCO BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (GIBCO, BRL), 4mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. They were maintained in sterile conditions at 37°C and 5% of CO_2.

2.8. CD133+ Cell Sorting from C6 Cell Line. As C6 cells contain CD133+ MGSCs, we separated this subpopulation by separation columns as described by Otvos *et al.* [14]. Thus, separation of CD133+ MGSCs from the total C6 culture ($1x10^7$ cells) was made by magnetic sorting using CD133 MicroBead kit (MACS Miltenyi biotec®) in combination with LS Columns and miniMACS separator (MACS Miltenyi biotec®). The C6 cells were incubated with 100 μl of sterile PBS plus 10 μl of anti-CD133 IgY antibody (0.16 mg/ml) (Aves Labs Inc.) for 30 min. Cells were washed with 1 ml of sterile PBS and centrifuged 300 x g for 5 min. Then, C6 cells were incubated with 100 μl of sterile PBS plus 10 μl of goat anti-chicken IgY FITC-conjugated antibody (Aves Labs Inc.) (0.16 mg/ml) for 30 min. The pellet was resuspended in 100 μl of degassed buffer (sterile PBS pH 7.2, 0.5% BSA, 2 mM EDTA to dilute the autoMACS™ Rinsing Solution in 1:20 ratio), and 10 μl of anti-FITC-conjugated magnetic beads antibodies (Miltenyi Biotech), incubated for 10 min at 4°C in the dark, and subsequently washed with 2 ml of degassed buffer and centrifuged 300 x g for 10 min. After incubation with magnetic CD133 affinity beads, the suspension was added to magnetic-activated cell sorting columns. Cells negative for CD133 were washed through the column with DMEM F-12 medium with 1% penicillin/streptomycin, under magnetization. Columns were then removed from the influence of the MACS sorting magnet and CD133+ cells were eluted with DMEM F-12 medium supplemented with B27 (Miltenyi Biotec, Usa), 1% penicillin/streptomycin (Life Technologies), EGF 20 ng/ml (Miltenyi Biotec, UsaR), and 20ng/ml FGF (Miltenyi Biotec, USA).

2.9. Determination of CD133+ Cells. To determine the percentage of CD133+ cells of the MGSCs obtained by separation

with magnetic beads, cell culture spheroids in stem enriched medium and C6 cells were stained with anti-CD133 antibody and analyzed by flow cytometry. $5x10^5$ MGSC grown as neurospheres and adherent C6 cells, neurospheres were incubated with 200 μl of CD133/2-APC Miltenyi Biotec antibody-PBS (0.1ng/ml) for 30 min in the dark. The cells were then washed with 1 ml of PBS, centrifuged at 300 x g for 5 min, and fixed with 1% paraformaldehyde. The percentage of CD133+ was determined by flow cytometry (FACSCalibur Instrument BD Biosciences), evaluating 10,000 total events. The data were analyzed using the software Cell QuestPro and Flow Jo ver. 7.6.1. (Becton Dickinson, San Jose, CA, USA).

2.10. Cytotoxicity Assays. Cytotoxicity of abrin A chain, IgY antibody, and IgY immunotoxin were evaluated by crystal violet dye (Sigma-Aldrich). Human GBM cell lines A-172, LN-18, U373, C6 rat malignant glioma cells, VERO cells, and human fibroblasts were cultured in 96-well plates and treated either with IgY antibodies (34 μg/ml) or with abrin A (at concentrations ranging from 3.4 μg/ml to 34 μg/ml). Cell cultures were incubated 24 h at 37°C with 5% CO_2. Later, cell medium was removed, and the cells were washed with PBS and fixed with 1% PFA for 15 min, and 100 μl crystal violet (1% in PBS) was added. After 25 min at room temperature, the dye was removed and washed with PBS. Subsequently 200 μl of 10% acetic acid was placed for 5 minutes and the plates were analyzed in an ELISA reader at 570 nm.

The cytotoxicity of either IgY, abrin A, or the IgY immunotoxin in culture of MGSC was analyzed by the MTT assay (3-[4,5dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich). Cells seeded in 96-well plates and treated and were incubated for 24, 48, and 72 h. Subsequently, MTT reagent was added (0.5 mg/ml) and incubated for 4 h at 37°C; then, 100 μl of isopropanol containing 0.04N HCl was added; after overnight incubation at 37°C, the plates were analyzed in spectrophotometer at 570 nm.

2.11. In Vivo Assay in Experimental Malignant Glioma. An *in vivo* assay was performed to evaluate the glioma therapy with the immunotoxin. Subcutaneous implantation of MGSC enriched cells from the C6 cell line was made in nude male (nu/nu) mice of six weeks old that were fed *ad libitum* with sterile rodent diet and water. Those mice were maintained in microisolators at a regulated temperature (25 ± 2°C) and relative humidity of approximately 40-50%.

2.12. Subcutaneous Implantation of Tumoral Cells and Treatment with Immunotoxin. The implant was performed as described by Yin Zhu et al. [15]. One million MGSC cells resuspended in 100 ml of DMEM-F12 culture medium were implanted subcutaneously on the back of nude mice. Two weeks later, mice were separated into three groups of 5 mice: group 1 without treatment (control), group 2 treated with a single dose of the immunotoxin IgY-abrin A (1.34 μg/kg) by intra-tumoral route, and group 3 treated weekly with the immunotoxin dose 1.34 μg/kg (3 weeks) by intra-peritoneal route. Tumors were measured with a caliper at 14, 21, 28, and 35 days post implant. On day 35, mice were sacrificed.

2.13. Statistical Analysis. For descriptive purpose, continuous variables were summarized as arithmetic means and standard deviations (SD). One-way analysis of variance and post hoc Tukey test were conducted. Statistical Significance was determined with $p<0.05$ in a two-sided test. SPSS software package V 18.0 for Windows (SPSS Inc., Chicago, IL) was employed for data analysis.

3. Results

3.1. Recombinant CD133. To produce high-affinity antibodies against CD133, we designed an immunogenic peptide inside the extracellular domain of the protein using bioinformatic analysis of CD133 protein. The Hopp-Woods and Kyte-Doolittle (hydrophobicity), Emini (surface expression probability), Karplus-Shulz (chain flexibility), and Jameson-Wolf algorithms (the combination of attributes to determine antigenic index) showed that the amino acid sequence corresponding to region 73-95 of CD133 was the most antigenic (Figure 1(a)). Figure 1(b) shows the expression of the CD133 extracellular domain (aa 20-108) after IPTG induction in *E. coli* and its purification used after to probed IgY antibodies obtained after immunization of hens with the immunogenic peptide (Figure 1(c)).

3.2. Abrin A Chain. The correct insertion of the encoding sequence for the A-chain of abrin in the plasmid pET-28a was verified by Hind III and Xho I enzymes and running the plasmid DNA with the insert (Figure 2(a), lane 1) and the enzymatically digested plasmid (Figure 2(a), lane 2). After cloning, IPTG induction was performed and corroborated by 12% SDS-PAGE, where overexpression of abrin A was observed (Figure 2(b), lane 2).

Purification of the abrin A chain was made by TALON Ni+ affinity resin columns. The fractions obtained, as well as the total lysate of the proteins obtained from bacterial cultures, were analyzed by protein electrophoresis under denaturing conditions, by 12% SDS-PAGE. The overexpression of a 28 kDa band corresponding to the A chain of abrin was observed in the total lysate (Figure 2(c), line 1); purification of the protein was demonstrated in the fractions corresponding to the elution (Figure 2(c) lines 5, 6 and 7). Western blot (WB) showed the recognition of abrin A chain by the anti-histidine antibody (Figure 2(d)).

3.3. IgY Anti-CD133 Antibody. Eggs from immunized hens were collected between days 38 and 87. Obtention of polyclonal antibodies was achieved by the PEG precipitation technique; the specific polyclonal IgY anti-CD133 antibodies were isolated by affinity chromatography and analyzed in 12% polyacrylamide gel under denaturing buffer conditions. The immunization protocol used to induce the production of large amounts of anti-CD133 IgY antibody (approximately 1mg of specific antibody per egg from immunized hens). Figure 3(a) shows the presence of running bands corresponding to the heavy chain (65-68 kDa) and the light chain (25 kDa) of the IgY immunoglobulin.

Specificity of anti-CD133 IgY was demonstrated by Western blot; Figure 3(b) shows the binding from specific IgY

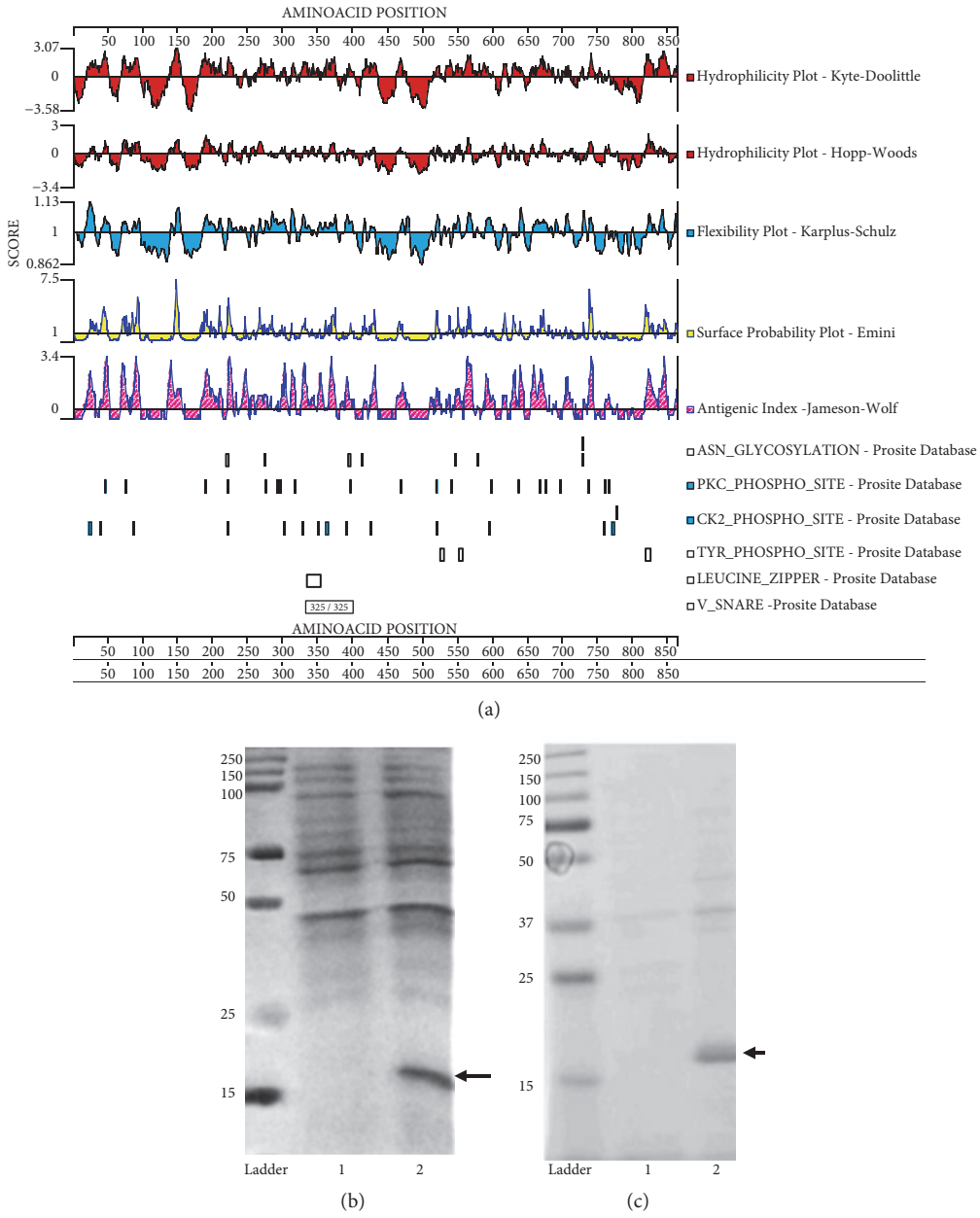

Figure 1: *Recombinant expression of CD133.* (a) Hydrophilicity, flexibility, surface probability, and antigenic index plots. Red plots show hydrophilicity and hydrophobicity of human CD133 protein along its amino acid sequence using Kyte & Doolittle and Hopp & Woods hydropathy scales. The blue plot shows the prediction of chain flexibility based on Karplus & Schulz method for predicting flexible segments from a given amino acid sequence. The yellow plot shows the prediction of the probability that a given region lies on the surface of a CD133 protein along its amino acid sequence. The pink plot shows the topological features of CD133 protein predicting potential antigenic determinants based on Jameson & Wolf H method. Additionally, (a) shows the asparagine glycosylation sites (ASN_GLYCOSYLATION), protein kinase C phosphorylation sites (PKC_PHOSPHO_SITE), casein kinase II phosphorylation sites (CK2_PHOSPHO_SITE), tyrosine phosphorylation sites (TYR_PHOSPHO_SITE), leucine zipper motif sites (LEUCINE_ZIPPER), and soluble N-ethylmaleimide-sensitive factor attachment protein receptor residing in the vesicle membrane (V_SNARE), predicted from Prosite Database. X-axes represent amino acid position along CD133 protein and Y-axes show the score of each analysis used. (b) Protein electrophoresis under denaturing conditions (15% SDS-PAGE); lane 1 shows the lysate of CD133-positive cells prior to induction with IPTG; lane 2 shows the overexpression of CD133 protein after IPTG with approximate weight of 18 kDa (arrow). (c) Elution of the CD133 protein purification in 15% SDS-polyacrylamide gel, the purified protein is observed (arrow).

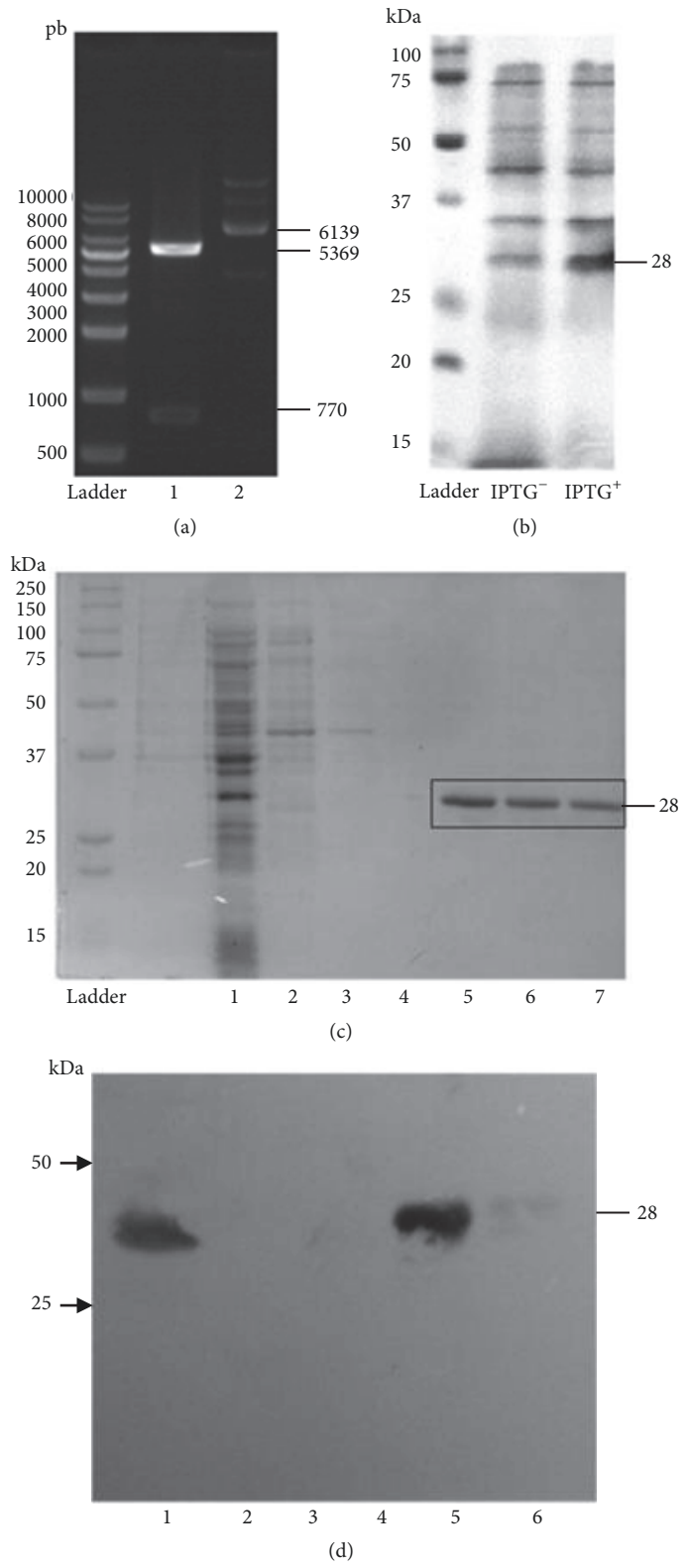

Figure 2: *Recombinant expression and purification of abrin A-chain*. (a) Agarose gel shows the pET-28a (+) plasmid digestion containing the abrin A chain insert. Lane 1 shows the plasmid digested by restriction enzymes HindIII and XhoI. Lane 2 shows the plasmid with the abrin A chain sequence insert. (b) 12% SDS-polyacrylamide gel shows the expression of recombinant abrin A chain by *E. coli* BL21DE3pLysS; fractions of cultures before and after induction with IPTG are shown (lanes 1 and 2, respectively). (c) A 12% SDS-PAGE shows the products of purification of abrin with IPTG: lane 1, proteins of total bacterial lysates; lane 2, proteins not adhered to the affinity column; lanes 3 and 4, products eluted after washes; lane 5, proteins eluted in the first fraction; lane 6, proteins eluted in the second fraction; lane 7, proteins eluted in the third fraction. (d) WB of abrin: lane 1 proteins of total bacterial lysates; lane 2 proteins not adhered to the affinity column; lanes 3 and 4, products eluted after washed; lanes 5 and 6, protein eluted from the Ni-column.

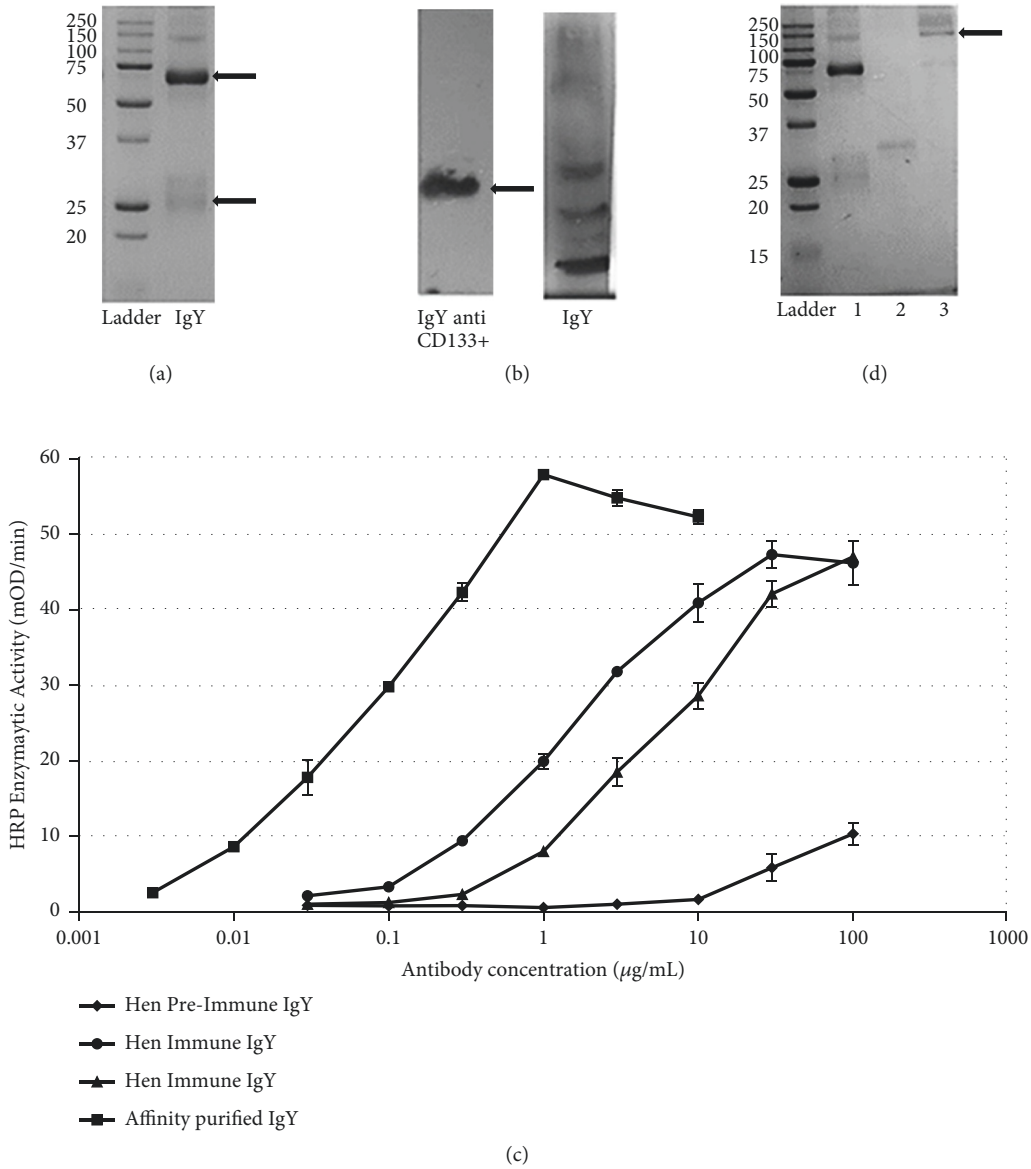

Figure 3: *Purification of anti-CD133 IgY and the Immunotoxin conformation*. (a) 12% SDS-PAGE shows the purification of anti-CD133 IgY, heavy chain (55-77 kDa), and light chain (23-26 kDa) of the immunoglobulin (arrow). (b) WB of bacterial lysate that expresses the protein CD133. The arrow indicates a band of 18 kD that corresponds to the protein recognized by the anti-CD133 IgY. The second film was incubated with non-CD133 IgY. (c) Specificity of IgY by ELISA: Over 10-fold difference was seen on the concentration of antibody recognizing the peptide sequence in the affinity purified IgY fraction as compared to immune IgY fractions. Half-maximal antibody binding occurred at IgY concentration of 50-100 ng/ml (as determined by A280). (d) Coupling of abrin with anti-CD133 IgY. Lane 1 shows specific anti-CD133 IgY purified; lane 2 shows abrin chain; lane 3 shows an increase in the molecular weight of Fc due to adequate union between abrin A chain and specific IgY antibodies (arrow).

anti-CD133 to an 18 kDa band which corresponds to CD133 protein from the Bioclone *E. coli* lysate. Western blot film shows the recognition of CD133 protein by the IgY anti-CD133 antibodies. Additionally, membranes incubated with CD133 depleted IgY antibodies recognize other proteins of no interest from *E. coli* (Figure 3(b)).

By ELISA analysis, we compared antibodies from pre-immune hens (as negative control), with immune antibodies (IgY fraction) from the same hen after injection of the peptide and affinity-purified antibodies from this IgY fraction. These data show that there were negligible amounts of antibody against the peptide in the pre-immune fractions. The Kd value of the affinity-purified antibodies was approximately 50-100 ng/ml; comparisons of the shift in IgY and affinity purified antibody preparations were approximately 10-fold, indicating the successful affinity purification (Figure 3(c)).

3.4. Antibody-Toxin Conjugate. The 12% SDS-PAGE was assayed to analyze the adequate binding between the specific IgY anti-CD133 and abrin A chain. On the polyacrylamide

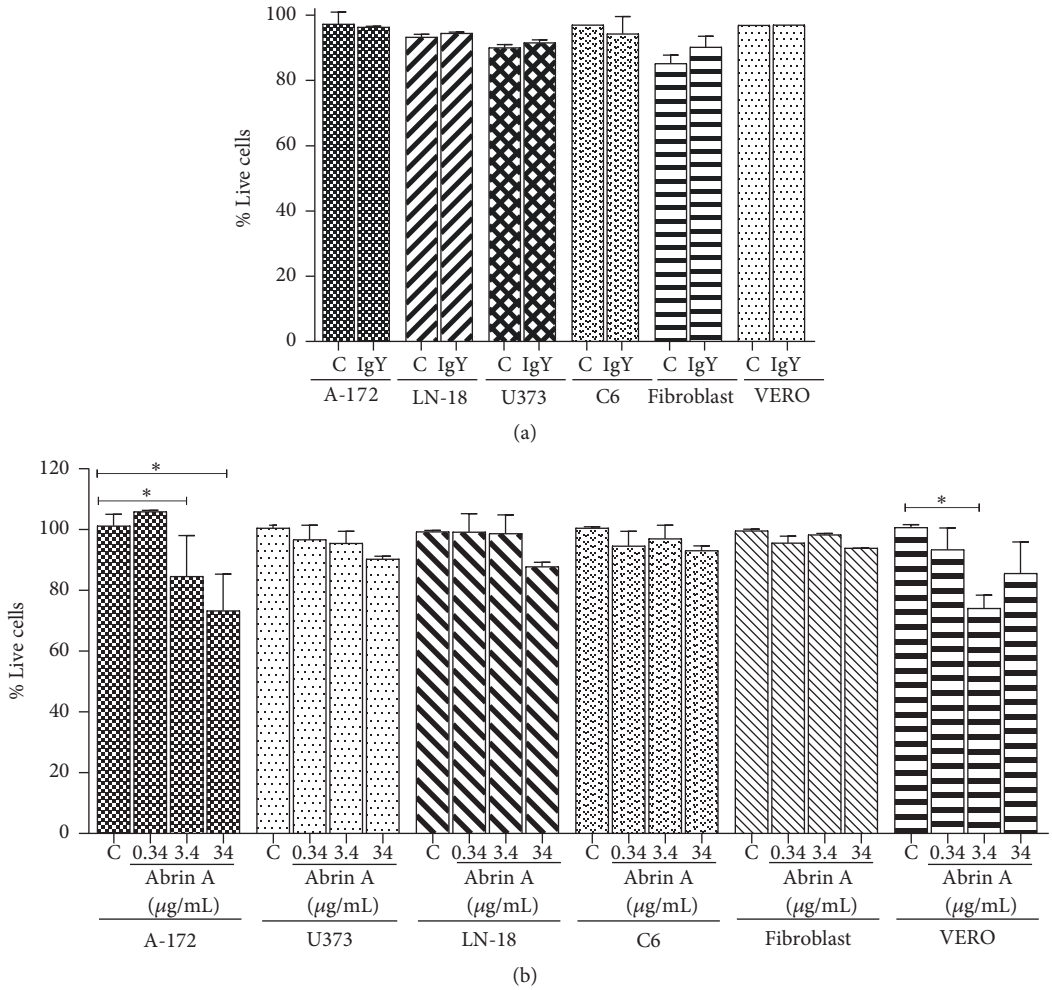

Figure 4: *Cytotoxicity assay*. (a) Evaluation of IgY cytotoxicity in GBM cells (A-172, U373, LN-18, 0and C6) and in fibroblast and VERO cell lines. Cell cultures were treated with IgY immunoglobulin (34 μg/mL) for 24 h. The graph shows no significant differences as compared to controls. (b) Evaluation of abrin cytotoxicity by violet crystal assay in GBM cells (A-172, U373, LN-18 and C6), significant decrease of cell viability was observed in the A-172 and VERO cells. Data are presented as mean values ± SD of three independent experiments. One-way ANOVA test followed by Tukey, $*$ $p < 0.05$ vs controls.

gel, a lower intensity of the heavy chain band (Figure 3(d), lane 2, 50-70 kDa band) was observed together with presence of a band of about 150 kDa representing the link-up between abrin A toxin and the IgY immunoglobulin heavy chain (Figure 3(d), last lane).

3.5. Effects of IgY Anti-CD133 and Abrin A on Malignant Glioma Cells. Malignant glioma cell lines (A-172, LN-18, U373, and C6), VERO cells, and fibroblasts were treated with the IgY antibody (34 μg/ml) for 24h; this treatment did not modify cell viability in any of the cell lines used (Figure 4(a)). Results showed that treatment of cells with abrin A since 3.4 μg/ml induced a decrease in cell viability in A-172 glioma cells and in VERO cells (Figure 4(b)).

3.6. Contents of MGSC in Malignant Glioma. Percentage of cells expressing CD133 was determined by flow cytometry. Eight percent of the population of C6 cell line was positive for CD133. Therefore, we purified this subpopulation with magnetic beads; these cells were cultured with DMEM-F-12 medium enriched with B-27, FGF, and EGF. After one week of culture, cells showed round morphology and growth in spheroids, here named glioma spheres (Figure 5(a)). Although the cells were cultured with enriched medium for stem cells, only 31% of this population was CD133+ (Figure 5(b)). Glioma spheres were used for cytotoxicity tests.

3.7. Cytotoxicity Assay over Glioma Spheres. Treatment with abrin A or with the immunotoxin induced glioma sphere disruption and cell death after 48h of treatment. Additionally, the immunotoxin produced early cell contraction and destruction after the first day compared to controls (Figure 6(a)). Treatment with immunotoxin after 24 h reduced significatively the cell viability (51%), while at 48 and 72 h the reduction on viability was 33% and 55%, respectively. Treatment with IgY did not induce significant changes in cell viability at any time of exposure, while treatment with abrin A at 24 h produced a significantly reduction in cell viability (33%

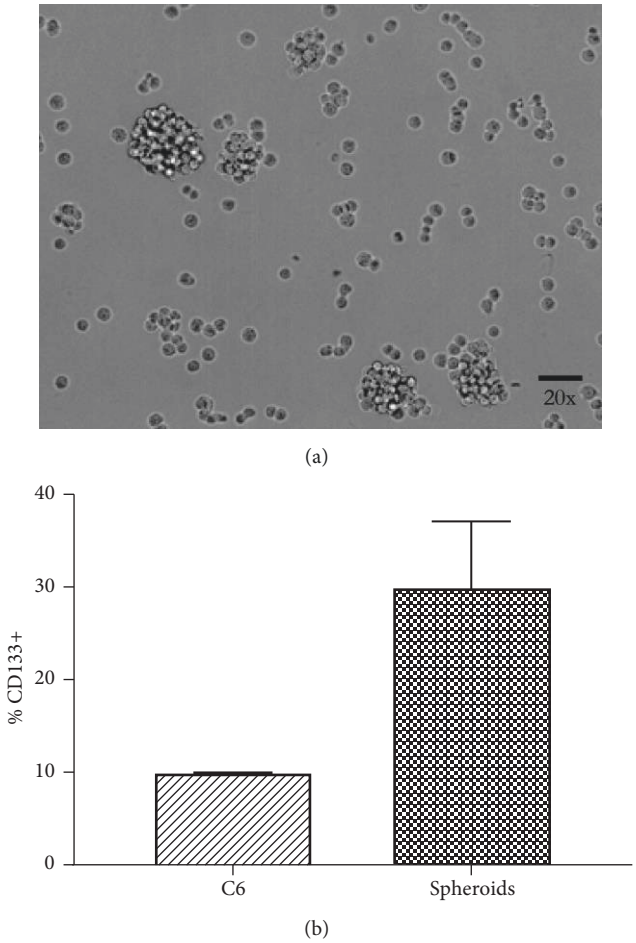

Figure 5: *Glioblastoma stem cells screening*. (a) Culture in enriched medium of stem cells showed sphere-forming cells that express CD133. (b) Flow cytometry showed that the CD133+ proportion was around 30% of MGSCs of C6 cells. Data are presented as mean values ± SD of three independent experiments.

in relation to controls) (Figures 6(a) and 6(b)). No changes were observed in CD133-cell population (data not shown).

3.8. Effect of IgY Immunotoxin In Vivo. The MGSC cultures enriched with stem medium possess capacity to generate subcutaneous tumors in nude mice. Effective growth is observed after day 15 post implantation. Tumor volume was measured for a period of 4 weeks after implant. The results of treated groups showed a favorable trend in reducing tumor volume (more than 75% tumoral reduction). The groups treated with the immunotoxin IgY-abrin (1.34 μg/kg) applied either with a single dose of intratumoral immunotoxin (IT) or with 3 intraperitoneal doses (one per week) induced a significant tumor reduction as compared with controls ($p = 0.027$ IT vs controls and $p = 0.021$ IP vs controls). In both treated groups, a similar reduction of tumor volume was observed, suggesting that the immunotoxin had an inhibitory effect after either local or systemic administration of the immunotoxin. The controls developed large tumor as compared to treated groups (Figure 7). No signs of toxicity were seen in both treated groups, after intratumoral or intraperitoneal administration of the immunotoxin. Also, all treated mice survived during all the experiment and no signs of toxicity were seen during the experiment.

4. Discussion

Since Paul Ehrlich's magic bullet concept an increase amount of antibody drug conjugates (ADCs) has been developed [16]. Bioconjugate technology has been dramatically growing in the past 10 years, allowing delivery of therapeutic agents into the tissue targeted. That technology usually relies on a bimodal agent, one used to target the cancer cells and the other the payload with therapeutic effect over the tumoral cell [17]. ADCs are relevant therapeutic agents because they are both selective and cytotoxic, because they are antibodies linked to a payload with antitumor activity [18]. The recent success of new formulations developed in ADCs to treat other solid tumors such as transtuzumab emtansine (T-DM1) for HER2-positive in breast cancer patients has been very encouraging even in brain metastasis [19, 20]. T-DM1 has gained recently attention as a possible therapy to treat glioblastoma because its capacity to pass blood brain barrier (BBB) from patients with brain metastasis and because HER-2 is also expressed in some glioblastomas [21, 22]. Another ADC's with recent success in the treatment of other malignancies are brentuximab vedotin (BV) (an antibody–drug conjugate targeting CD30) and inotuzumab ozogamicin (a humanized CD22 monoclonal antibody linked to the cytotoxic agent calicheamicin) used in Hodgkin lymphoma [23] and the treatment of B cell malignancies [24], respectively.

In GBM, the main ADCs trialed for treatment are immunotoxins due to the possibility of releasing the payload into the cytoplasm of the tumor cells with the consequent dead induction [25]. Experimental trials for treatment of GBM with immunotoxins have been directed at other therapeutic targets, such as transferrin receptor (TfR) taking advantage of the TfR endocytic pathway [26], fused human transferrin to a mutated diphtheria toxin (CRM107) by a stable and non-reducible thioether bond (Tf-CRM107); IL-13 receptor (Cintredekin besudotox) composed of human IL-13 and a truncated form of Pseudomonas exotoxin A [27]; IL-4 receptor as a chimeric immunotoxin constructed by fusion of mutein cpIL-4(13D) to a modified version of Pseudomonas exotoxin A (PE38KDEL) [28]; and EGFR. Actually, there are two immunotoxins with reports of phase I studies, one is an Anti-EGFR antibody conjugated to the toxic payload monomethyl auristatin F (MMAF) (ABT-414) [29] and the other an Anti-EGFR antibody conjugated to the maytansinoid DM1 (AMG-595) [30]. These studies reported that immunotoxins showed a relevant cytotoxic effect in patients with malignant brain tumors refractory to conventional treatment, without severe systemic or neurological toxicity [31].

In this context, there are no studies of immunotoxins against MGSCs even knowing they have active participation in tumorigenesis and growth which are also related to the high resistance of these cells to radiotherapy and chemotherapy [32]. However, recently Pfizer and Abbvie/Stemcentrx

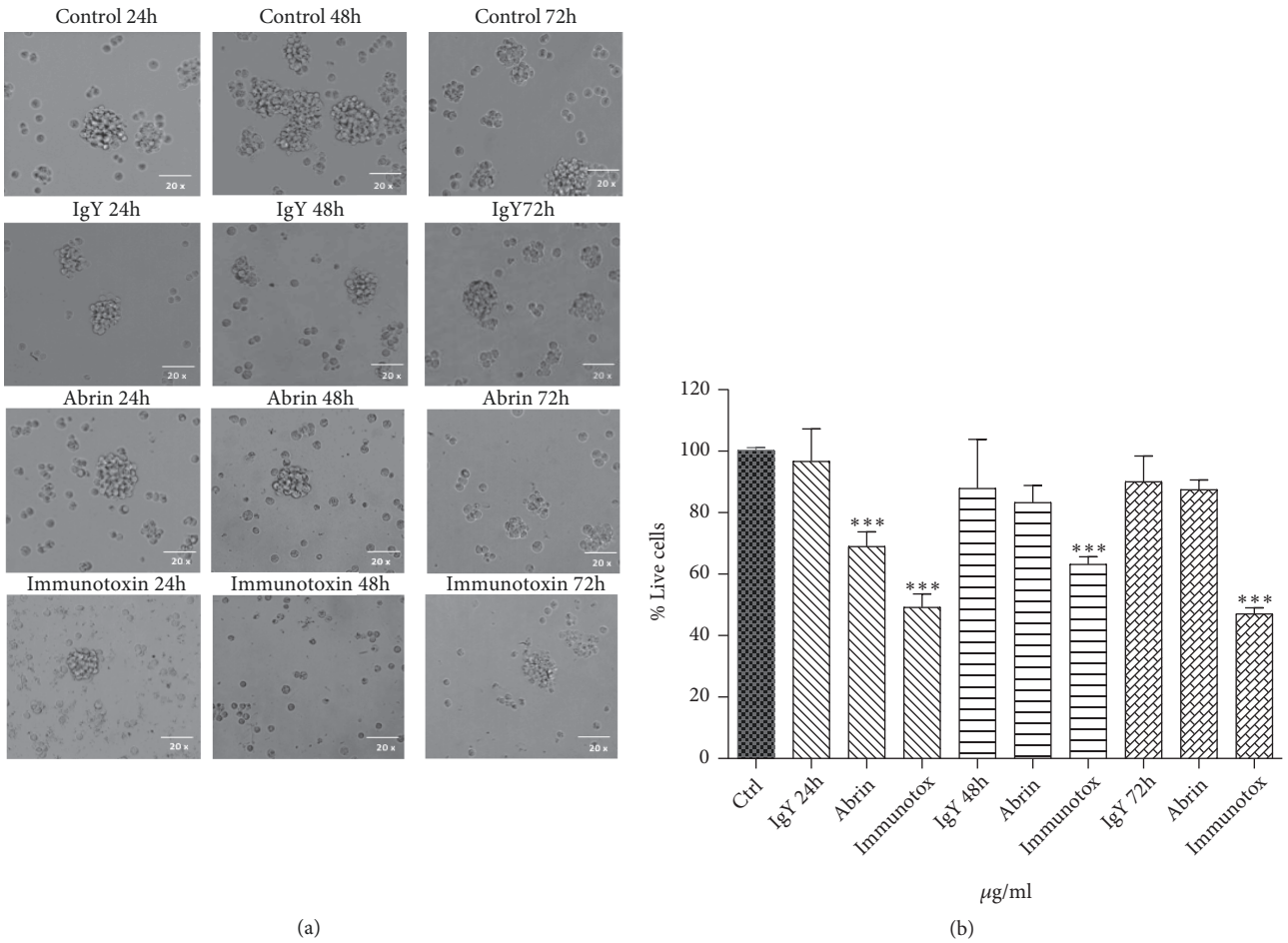

Figure 6: *Cultures of C6 cancer stem cells treated with abrin A chain, anti-CD133 IgY antibody, and immunotoxin* (34 μg/ml). (a) Microscopy images show a decrease in the number of cells and spheroids, as well as cellular damage induced by immunotoxin at 48h and 72h; in the case of abrin alone, changes were observed at 72h; antibody treatment did not induce significant differences. (b) Evaluation of cell viability by MTT assay. The graph shows a decrease of cell viability from 24 h to 72h of 55% treated with the immunotoxin. Data are presented as mean values ± SD of three independent experiments. One-way ANOVA test followed by Tukey, $*$ $p < 0.05$ vs control.

design, an ADC composed of a humanized anti-PTK7 and a cleavable auristatin microtubule inhibitor (Aur0101), showed capacity to target tumor-initiating cells (TICs) in triple-negative breast cancer (TNBC), ovarian cancer (OVCA), and non-small-cell lung cancer (NSCLC). Also, an AC133 biotinylated mAb linked to streptavidin–saporin was designed to targeting $CD133^{high}$ CSC-like cells from colon carcinoma and liposarcoma cells [33]. The same principle was used targeting all variants of CD44 [34]. However, this design was too complex in size (>700KDa) and no efficient penetration through solid tumors was seen. More recently, Bostad et al., with a whole size of the immunotoxin of 244 KDa, demonstrate an efficient targeting and uptake into endocytic vesicles in human colorectal adenocarcinoma WiDr ($CD133^{high}$) and breast cancer MDA-MB-231 (CD133+) cell lines [35].

Our study attempted to target selectively MGSCs subpopulation; therefore, we designed and tested a novel immunotoxin that combined the abrin toxin with IgY (immunoglobulin of avian origin). An immunotoxin against CD133+ cells has not been used before in glioblastoma; it is important to highlight that CD133+ cells show a peculiar strong resistance to various chemotherapeutic agents such as temozolamide, VP16, carboplatin, and taxol [36].

The research reported here includes original approaches for treatment of malignant glioma. This is the first study in which an immunotoxin against MGSCs was designed, using CD133 as the antigen selected for the generation of IgY antibodies. Epitope which has been proposed as tumor marker for the identification of MGSCs subpopulation and the most relevant cell population in malignant tumorigenesis. The MGSCs that express the CD133 protein have been associated with a high self-renewal ability and other tumoral-characteristics of malignant glial tumors [37]. Thus, we proposed the highly immunogenic region inside the CD133 protein as a therapeutic target for the design of a selective immunotoxin (Figure 1). The *Escherichia coli* strain BL21DE3, used for the expression of both, the CD133 protein and the A chain of abrin, has various advantages, among them, a high yield, molecular efficiency, and economical cultivation. The high yield of abrin A obtained by the recombinant

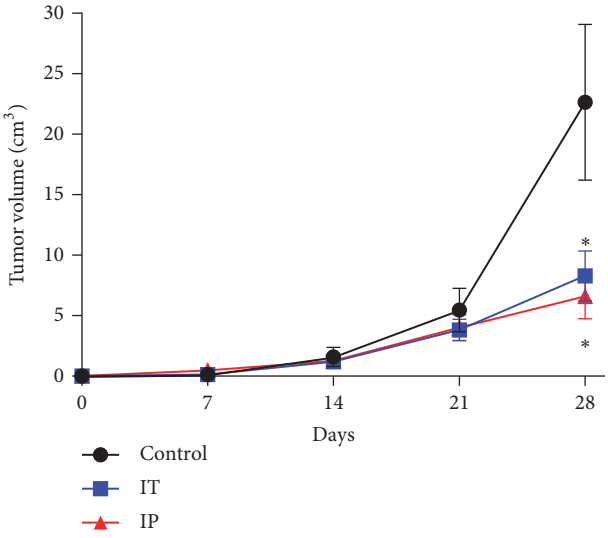

FIGURE 7: *Tumor growth kinetic.* The tumor volume of immunotoxin-treated mice (intraperitoneal (IP) and intratumoral (IT) route) and controls is shown in the graph. A decrease in tumor volume was obtained in those treated with the immunotoxin. Data are presented as mean values ± SEM. ∗ p = 0.027 IT vs controls and ∗ p = 0.021 IP vs controls.

techniques used in this study favors its exploration for therapeutic purposes. The A chain was selected because the main drawback to use complete abrin toxin to inhibit the growth of glioma cells is that the B-chain could bind d-galactose, which is present in both normal cells and glioma cells [38]. Whereas the A chain of the abrin toxin is a N-glycosidase which catalytically inactivates 60 S ribosomal subunits by cleaving a specific adenine residue at the position A4324 from the backbone of 26/28 S rRNA [39], this feature made A chain of the toxin a good candidate to be coupled to an antibody to produce an immunotoxin which would selectively bind against glioblastoma cells. The use of abrin A has been investigated in recent years due to its high toxicity at low concentration (0.2-10 μg/kg) [40]. Thorpe *et al.* [13] used abrin A-chain for an immunotoxin that showed antitumoral effects in lymphoma without relevant cytotoxicity in normal cells. Abrin A inhibits protein synthesis and promotes cell death [41], specifically apoptosis in cultured HeLa and Jurkat cells [42].

In this study, we purified and tested the A-chain of the abrin toxin. The overexpression of the chain A of abrin with IPTG by SDS-PAGE disclosed a band of 18 kDa corresponding to the abrin. The A-chain of abrin was expressed at high level without growth inhibition of the host *E. coli* (data not shown), which is consistent with reports indicating that abrin is toxic only to eukaryotic cells but not to bacteria's [43].The purification of this protein was efficient using Ni-NTA affinity columns (Figure 2). Measurement of the biological activity of the recombinant A chain of abrin in our studies demonstrated that the recombinant protein was biologically active. We tested the activity of the A chain of abrin in different cell lines and a large decrease of cellular viability of the A-172 cells was observed, suggesting that this cell line has greater endocytic capacity of the A chain of abrin than other glioma cells studied (Figure 5(b)). It has been reported that GBM expresses endocytic proteins indicating a receptor-mediated endocytosis capacity adequate for the internalization of the immunotoxin used in our investigation [44].

Monoclonal antibodies have been used for targeted therapies as immunotoxins [45]. In our experimental approach, we consider that IgY antibodies are the predominant immunoglobulin in birds [5]. High titers of IgY are obtained when hens are immunized; these antibodies also have a high affinity to mammal antigens due to phylogenetic differences between them [6]. Additionally, IgY obtention is efficient and economical as they can be collected from the egg yolk of immunized hens [6]. We were able to produce an anti-CD133 IgY antibody. Our results showed two bands, 25 and 70 kDa, corresponding to the light and heavy chains of IgY. Other proteins in the SDS-PAGE gel were observed which might correspond to vitelogenin excision products; however, these proteins do not interfere with IgY immunoglobulin-antigen coupling [46]. The IgY anti-CD133 obtained recognized specifically the CD133 protein; cytotoxicity tests confirmed that the avian immunoglobulin was nontoxic to various cell lines from human and rat malignant glioma, suggesting that the binding of anti-CD133 IgY antibody without the cytotoxic protein to these cells does not activate cell death pathways.

The construction of the immunotoxin was carried out by coupling the chain A of the abrin toxin with the IgY selective antibody against the CD133 protein. Treatment with the IgY immunotoxin produced in our laboratory induced a high percentage of cell death in MGSCs cultures, which in the case of our C6 cell cultures expressed 10% of cells bearing CD133.

Reports on the percentage of MGSCs in glioma cells are varied [47]; these variations could be attributed either to different isolation methods, to cell culture conditions, or to stem cell markers. Approximately 31% of the cells with stem-cell like properties in our spheroid cultures of MGSCs did bind to CD133 antibodies, of them, 55% were eliminated by the IgY immunotoxin treatment.

Immunotoxins have been used against solid and non-solid tumors, but better response has been obtained in the latter. Some alternatives have been developed to overcome difficulties, including the simultaneous use of immunosuppressants, reduction of molecule size, and humanization of immunoglobulin components [48].

The possible systemic toxic effect of the immunotoxin tested in this study against malignant glioma cells could be minor because it does not have the B chain of abrin, a galactose-specific lectin that facilitates cell entry [49] and because it is chemically bound by the SMPT crosslinker to IgY, which only recognizes CD133+ cells.

The stability of immunotoxins that used SMPT (a long-chain crosslinker for amine-to-sulfhydryl conjugation via NHS-ester and pyridyldithiol reactive groups) produced a strong, stable, and cleavable linkage *in vivo* that persists during ADC circulation in the bloodstream, compared with other linkers [50]. The specific cleavage into the endocytic vesicles releases the abrin A inhibiting ribosomal activity in CD133+ cells. Immunotoxins prepared with this reagent have

demonstrated high stability without affecting their toxicity in the target cells [50].

We conducted an *in vivo* study to determine the effect of IgY-abrin immunotoxin on the tumoral growth in nude mice with MGSCs of C6 subcutaneously implanted; our results showed a significant decrease in tumor volume when mice were treated with IgY-abrin immunotoxin IP and IT, suggesting that abrin has activity in the induction of apoptosis and inhibiting cell proliferation [51].

There are other studies targeting CD133+ cells in oral cancer [52], head and neck cancer [53], breast carcinoma [54], and colorectal cancer [55] with the objective to kill tumor-initiating cells; those studies show that targeting of CD133+ cells inhibits cell proliferation and tumor initiation and could reduce or eliminate established tumors.

As happened with antibodies produced in other species than human, the major limitation in clinical practice is immunogenicity. The probability that patients will start developing antibodies to the immunotoxin is highly expected, and thus the number of doses could be limited, even more when are combined with other check point modulating antibodies [56]. A possible limitation of our study might be the antigenicity of the IgY immunotoxin and possible production of blocking antibodies into the host when it was administered by systemic route; nevertheless, a single dose local injection was enough to diminish tumor size comparable to three systemic doses (Figure 7). Also, some limitations of systemic administration of immunotoxins in glioblastoma patients are as follows: limited amount into the tumor by their inability to efficiently cross the restrictive blood-brain barrier and the systemic toxicity to surrounding tissues producing suboptimal drug delivery even with large molecules as immunotoxins [57].

Studies with immunotoxins frequently imply an indirect question about pharmacokinetic and pharmacodynamic (PK and PD) parameters. Those studies are beyond the scope of our study; however, in case of brain tumors, there are few studies in this respect due to the lack of PD markers that can be employed in preclinical and clinical studies. Only one study reported results in solid tumors and no one in brain tumors [58]. In this study, we used a subcutaneous model to demonstrate specific targeted delivery of anti CD133 IgY-immunotoxin by the IP route. Also, it is difficult to get PK parameters in brain tumors due to the variable permeability of the BBB since permitting diffusion of high size molecules from the systemic circulation to the tumor as immunotoxins. The extend of the disruption of the BBB in brain tumors has not been well-quantified and it is different between patients. Nevertheless, convection-enhanced delivery technique allows enhanced distribution and concentration levels in the tumor that could be an option to avoid incomplete therapeutic drug dosing as a result of the BBB permeability.

Treatment with IgY immunotoxin by local delivery in clinical practice is a possibility to avoid the antigenicity and possible systemic toxicity with the same efficacy than systemic administration. Convection-enhanced delivery [59] could be an option to administered IgY immunotoxin, providing a low-cost alternative to treat glioblastoma and intrinsic pontine glioma patients.

5. Conclusion

Current therapy against GBM in humans is largely deficient. This therapeutic study directed against MGSCs cell subpopulation of malignant gliomas suggests that these cells might be responsible for some features of tumor dynamics as evasion of the immune response, activation of anti-apoptotic mechanisms, survival, self-renewal, and resistance to chemotherapy. The use of efficient immunotoxins against MGSCs could represent improvement of cancer therapy. The immunotoxin produced and tested in these experiments showed various original improvements, among them a convenient and economic source and cytotoxic effectiveness against CD133+ MGSCs. Further studies with similar therapeutic approaches could improve the therapeutic approach of human glioblastoma. An additional advantage of our proposal rests on the use of IgY immunoglobulin of avian origin in contrast with monoclonal antibodies; this a venue using an inexpensive biotechnological technique, due to the easy obtention of IgY from egg yolks, in contrast to the elevated costs and limitations of monoclonal antibodies. IgY antibodies might represent a convenient alternative for therapeutic experiments based on immunotherapy.

Data Availability

The raw datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval

All animal care and use of all experimental animals were performed in accordance with institutional ethical guidelines.

Conflicts of Interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. The development and use of this immunotoxin are currently under patenting (MX/a/2015/44554).

Authors' Contributions

Benjamin Pineda and Julio Sotelo were responsible for project coordination and management. Benjamin Pineda and Verónica Pérez-de la Cruz conducted the experimental and theoretical research design. Elda-Georgina Chavez-Cortez, Gustavo Vargas Felix, Edgar Rangel López, Carlos Martínez-Canseco, Julio Sotelo, Verónica Pérez-de la Cruz, and Benjamin Pineda reviewed, discussed, and wrote the manuscript. Edgar Rangel López assisted in the design, cloning, and expression of recombinant proteins (abrin and CD133). Gustavo Vargas Felix and Carlos Martínez-Canseco conducted the abrin purification. Gustavo Vargas Felix contributed to the production of polyclonal antibodies. Elda-Georgina Chavez-Cortez conducted the coupling and evaluation of

immunotoxin. Verónica Pérez-de la Cruz did the cell viability tests. All authors have read and approved the manuscript and concur with the submission.

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Supplementary Materials

Supplementary Figure 1. Schematic representation of the designed plasmid containing the recombinant sequence for abrin protein. It shows the main characteristics of the plasmid construct such as the appropriate antibiotic for selection of positive clones, the multicloning site for the insertion of the recombinant Abrin (770 pb) between the Xho I–Hind III restriction enzymes, the respective start and stop codon sequences for the transcription of the insert, and the 6-histidine residues added for the subsequent purification of the recombinant protein. *(Supplementary Materials)*

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Review Article

A Perspective Review on the Role of Nanomedicine in the Modulation of TNF-TNFR2 Axis in Breast Cancer Immunotherapy

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In the past decade, nanomedicine research has provided us with highly useful agents (nanoparticles) delivering therapeutic drugs to target cancer cells. The present review highlights nanomedicine applications for breast cancer immunotherapy. Recent studies have suggested that tumour necrosis factor (TNF) and its receptor 2 (TNFR2) expressed on breast cancer cells have important functional consequences. This cytokine/receptor interaction is also critical for promoting highly immune-suppressive phenotypes by regulatory T cells (Tregs). This review generally provides a background for nanoparticles as potential drug delivery agents for immunomodulators and further discusses in depth the potential of TNF antagonists delivery to modulate TNF-TNFR2 interactions and inhibit breast cancer progression.

1. Introduction

The term "nanotechnology" is a concept that has only emerged in the last decade with the prefix "nano" cited from the Greek word "nanos", indicating that something is dwarf-sized. Therefore, the term "nanotechnology" refers to a technology that uses very small particles invisible to the naked eye [1]. Before the 19th century, although the term nanotechnology had not yet been globally defined, the applications of nanotechnology were already used in the industrial field, [2]. During a meeting of American Physical Society in 1959, for the first time, Richard Feynman discussed the term of nanotechnology systematically, laying the foundations of the nanotechnology field [3]. Subsequently, at the end of 19th century and early of the 20th century, the field of nanotechnology experienced a massive expansion, when almost all industrialised countries started pursuing nanotechnology research in all fields including medicine [4]. Introduction of modern nanotechnology in the medical field aimed at better prevention, diagnostics, and therapy of diseases and was later called "nanomedicine".

Nanomedicine is a new science that emerged along with the establishment of technologies such as high resolution microscopes for biotechnology applications that allow investigations of nanomaterials (less than 100 nm) at cellular levels (Figure 1) [5]. Among several different nanomedicine platforms, nanotechnology-based drug delivery has received the greatest interest. Incorporating therapeutic drugs into nanomaterials and using these as carriers to target specific tissues, avoiding systemic side effects, remains a major challenge in

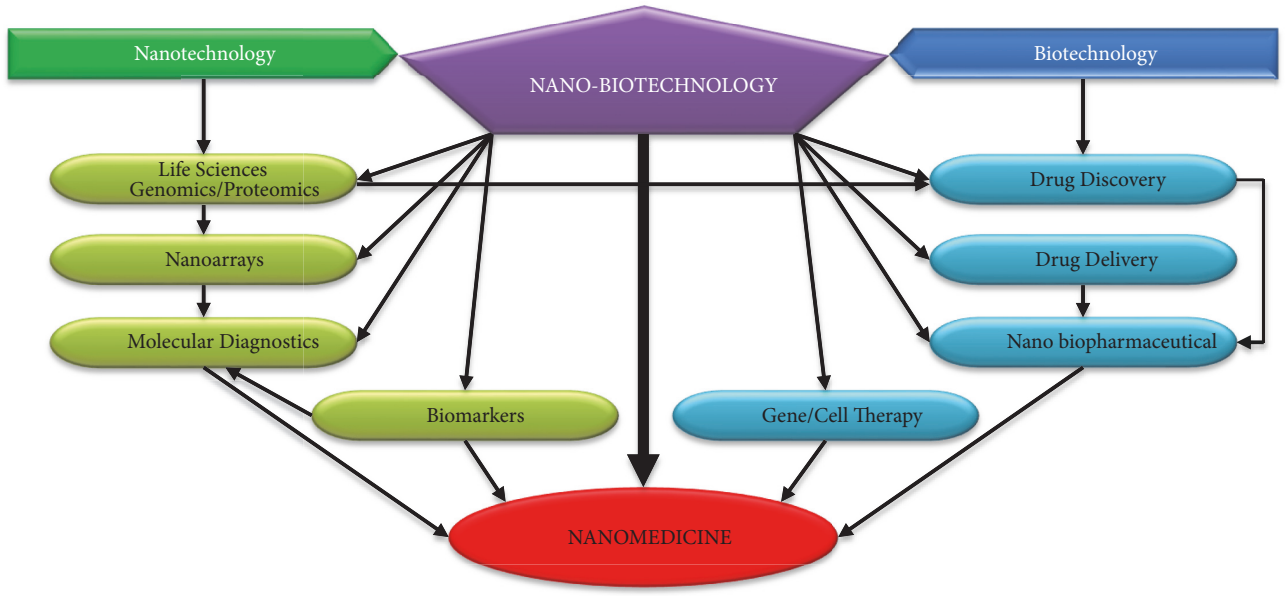

Figure 1: Illustration of how nanomedicine research is based on the applications of nanobiotechnology (adapted from Jain, 2008 [5]).

therapeutics [6, 7]. Many types of nanocarrier systems from diverse materials with distinctive physiochemical properties have been established for use in multiple diseases (Table 1), including the most common and explored type, liposomal drug carrier systems [8].

As cancer is one of the biggest health challenges facing humanity, a substantial amount of research has focused on nanomaterials as drug delivery agents to target cancer tissues, as illustrated by almost 12,000 manuscripts in the recent decade [33]. However, interest among the researchers in applying nanomedicine applications in different cancer types has varied with breast cancer receiving the least attention from nanomedicine, despite the fact that it is the most globally widespread cancer type with alarming rates of occurrence in many countries [34]. Furthermore, the majority of these studies used nanomaterials to target cancer cells with chemotherapy/drugs, while few studies focused on the use of nanomaterials to treat/control breast cancer in the context of immunotherapy. The most recent study used gold nanoparticles in breast cancer cells to deliver *Commiphora myrrha* and *Boswellia sacra* extracts to induce trisodium citrate dihydrate reduction which leads to cytotoxicity in breast cancer and normal cells. The study reported cytotoxicity in breast cancer cells, but no harm in normal breast cells [35].

Tumour necrosis factor (TNF) is generally considered a master proinflammatory cytokine [34]. During inflammatory processes (including the cancer microenvironment) TNF is one inflammatory mediator that is produced secreted firstly [37]. It fosters the generation of a cytokine cascade and promotes the production of other inflammatory mediators [e.g., transcription factors, interleukin (IL)-1, IL-6] [38, 39]. There are two types of TNF receptors (TNFR1 and TNFR2) localised at the cellular surface, which have unrelated intracellular regions [40]. A study in a model of inflammation-associated cancer revealed that TNFR2 is preferentially upregulated over TNFR1 and that treatment with the anti-TNF monoclonal antibody reduced the number and size of tumours [41]. Therefore, TNF-TNFR2 axis was implicated in the suppression of immune response and affects tumour progression and metastasis [42]. In the following sections, we will interpret a possible application of targeting TNF-TNFR2 interactions using a nanomedicine platform in breast cancer. This neutralisation of TNF as well as TNFR2 by using TNF antagonist drugs delivered through nanoparticles might be an effective therapeutic strategy on breast cancer cells. To the best of our knowledge, this is the first article discussing this hypothesis.

Table 1: The most well studied nanocarrier systems.

Type of nanocarrier	References
Liposomes	[8–12]
Dendrimers	[13–15]
Polymer-based platforms	[16–18]
Superparamagnetism nanoparticulates	[19, 20]
Gold nanoshells	[21–23]
Carbon-60 fullerenes	[24–26]
Nanocrystal	[27–29]
Silicon and silica-based nanoparticle	[30–32]

2. Nanomedicine and Breast Cancer

Cancer includes a range of diseases with alterations in the biological status of any nucleated cells, which causes malignant tumours with abnormal growth and division (neoplasia) [43]. It is one of the biggest challenges facing the world and is causing huge continuous losses without reaching effective-comprehensive solutions [43, 44]. Currently, both medical and research community have attempted an approach to

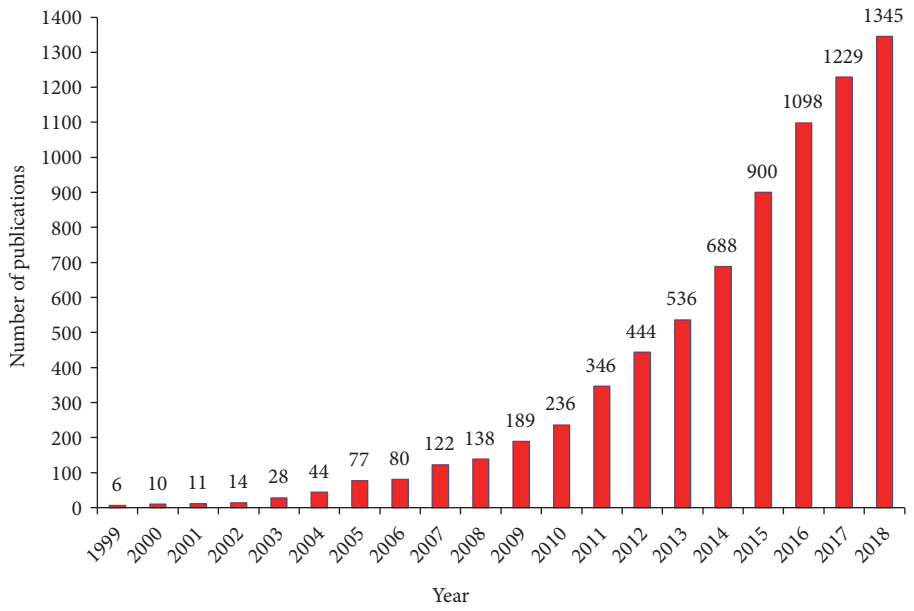

Figure 2: Annual publications regarding cancer nanomedicine research in the recent 20 years (applied on PubMed database on December 11, 2018, by using search terms: cancer nanomedicine/nanoparticles).

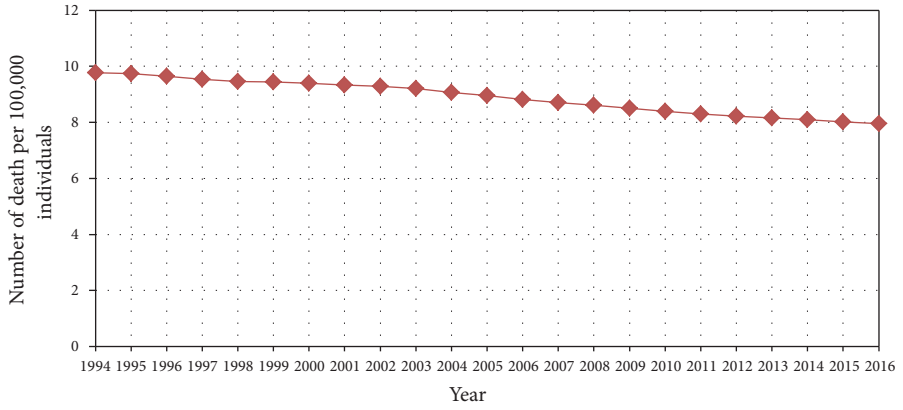

Figure 3: Global death rates caused by breast cancer between 1994 and 2016 [36].

nonconventional cancer therapies that can limit damage or loss of healthy tissues and be able to fully eradicate the cancer cells. Nanomedicine represents an efficient drug delivery system, which can deliver therapeutic agents directly to the targeted cancer cells only and minimize the dose-dependent side effects of drugs on nontarget sites [45]. By focusing on the targeted site, this could result in enhanced drug efficiency compared to conventional chemo/radiotherapy [46, 47]. Furthermore, the growing interest in utilizing this application for cancer research has been significantly increased year by year (Figure 2).

In 2016, the global prevalence of cancer ranged from 0.2 to 2 percent approximately [36]. Breast cancer was reported as the highest cancer prevalence with 0.12 percent, and until 2016 there were a total of 8.15 million breast cancer cases [36]. There was more than 20% increase in the global prevalence rate of breast cancer up to 10 years from 2008 to 2017 [48]. Moreover, breast cancer was classified in 2018 as the most common cancer among women, and the second most widespread cancer with more than 2 million cases diagnosed over the world [49]. According to the Avon Breast Cancer Foundation, in 2002, there were over 39,600 deaths caused by breast cancer among American women only [50]. Although breast cancer prevalence rates are increasing continuously, recent statistics have reported a decline in death rates (Figure 3) [36]. This decline could be due to increased awareness about the preventive measures and the periodic and early detection as well as treatment (Figure 4).

There are various complex classifications for breast cancer; the best one is the molecular phenotype classification that includes five different subtypes based on cancer genes expression such as molecular markers (Table 2) [51, 52]. The treatment and its effectiveness between various breast cancer types are different, and once metastasized, the effectiveness

Table 2: The biological subtypes of breast cancer.

Subtypes	Estrogen receptor (ER)	Human epidermal growth factor receptor-2 (HER2)	Ki-67 protein	Progesterone receptor (PR)	Comment
Luminal A Luminal B	Positive Positive	Negative Positive or Negative	Low High	Positive or Negative Positive or Negative	In comparing luminal A and B, luminal A is reported to be growing slower than luminal B, which means best prognosis in luminal A cancer; Ki-67 helps in monitoring how fast tumours grow.
Triple-negative (basal-like)	Negative	Negative	-	Negative	It is defined as basal-like breast cancer and is more common among young women especially with *BRCA1* gene mutations.
HER2-enriched	Negative	Positive	-	Negative	This cancer is growing faster than luminal cancers but with worse prognosis.
Normal-like	Positive	Negative	Low	Positive or Negative	Although 'normal-like' is similar to luminal A, its prognosis is worse than luminal A.

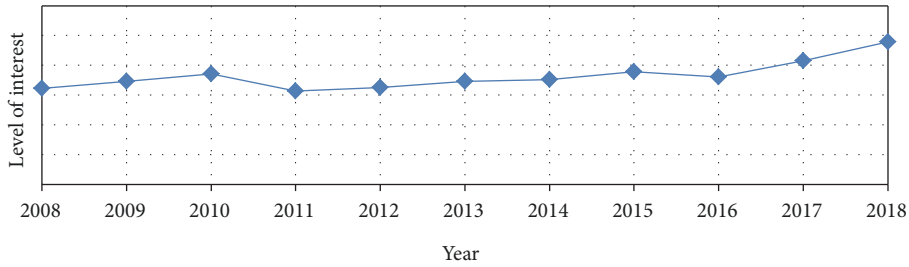

Figure 4: A search shows the levels of population awareness regarding breast cancer medications/treatments, over the recent 10 years. Applied on the Google Trends database up to December 11, 2018.

of all treatment strategies will be reduced [53]. Therefore, search for a more effective therapeutic option has been highly anticipated, especially in breast cancer.

In recent years, along with the extensive identification of molecular markers on breast cancer, several novel nanomedicine applications have been developed to specifically target these pathways (Table 3). Targeting breast cancer cells involves attaching specific molecules (ligands) on the surface of nanoparticles, and these ligands are able to recognize and bind only to complementary molecular markers found on the surface of targeted breast cancer cells [54]. Ligand-nanoparticle conjugate binds to the receptors (e.g., HER-2, EGFR, VEGFR, IGF-IR) expressed on the breast cancer, mediates internalization of nanoparticles through endocytosis, and releases the conjugated biomolecules by lysosomal degradation to the active sites of tumour cells [54]. As reviewed below, TNFR2, an immune checkpoint stimulator and oncogene, has more recently emerged as a potential new target for breast cancer therapeutics via its modulation on TNFR2 [55]. However, to date there is no study focusing on the development of nanomedicine targeting TNF-TNFR2 axis for breast cancer therapeutics. Generally, TNF-TNFR2 axis plays a significant role in the overall regulation of regulatory T cells (Tregs), providing protection for cancer cells by promoting their immune evasion in an immunosuppressive environment [55], besides activating myeloid-derived suppressor cells (MDSCs) to enhance tumour immune escape [56, 57].

3. TNF-TNFRs Interactions

TNF is a multifunctional cytokine secreted by various types of cells as well as being responsible for leukocyte recruitment, monocyte chemoattraction, and increased regulation of adhesion molecule expression and may also promote apoptosis [68]. TNF is expressed by immune cells including activated macrophages/monocytes, activated T cells, and natural killer (NK) cells and could be expressed by other nonimmune cells (e.g., fibroblasts and endothelial cells) [69]. The complexity of understanding the roles of TNF is partially due to the presence of different forms of TNF with equally different roles [70]. The membrane-bound form of TNF (mTNF) or pro-TNF is a transmembrane protein of 26 KDa which later can be converted to a soluble form of TNF (sTNF) which is released when mTNF is cleaved by TNF-converting enzyme (TACE) [71]. Although sTNF is the first to be in charge of the majority of responses, some studies have reported that mTNF has also the capacity to mediate similar responses, including some inflammatory responses, proliferation, B cells activation, and apoptosis [72]. Moreover,

Table 3: List of the studies on nanomedicine development in breast cancer therapeutic research.

Study	Experiment platform	Type of NPs	Conjugated biomolecules	Targeting pathway	Findings
Moses et al., 2016 [35]	*In vitro* on: MCF-7, MDA-MB-231 (breast cancer human cell lines) and MCF-10A (healthy human cells)	AuNPs	Extracts from *Commiphora myrrha* and *Boswellia sacra*	Inducing cytotoxicity	Cytotoxicity in both breast cancer cell lines was more aggressive without harm to healthy cells
Swanner et al., 2015 [58]	*In vitro* on MCF-7, MCF-10A, MDA-MB-231, 184B5, BT-549, and SUM-159 human cells	AgNPs	-	Oxidative stress and DNA damage	AgNPs led to selective cytotoxicity and radiation dose-enhancement effects in breast cancer cells as a self-therapeutic agent
Devulapally et al., 2015 [59]	*In vivo* (animal model) and *in vitro* on MDA-MB-231 human cells	PLGA-b-PEG polymer NPs	Antisense-miR-21 and antisense-miR-10b	Targeting metastasis and antiapoptosis by multitarget antagonisation of endogenous miRNAs	There was a substantial reduction in tumour proliferation at very low dose and 40% reduction in tumour proliferation compared to control
Shu et al., 2015 [60]	*In vivo* (animal model) and *in vitro* on MDA-MB-231 human cells	RNA NPs based on pRNA 3-way-junction (3WJ)	Anti-miR-21	Targeting metastasis and antiapoptosis by multitarget antagonisation of endogenous miRNA	Confirming the potential role of RNA NPs in miRNA delivery in cancer therapeutics
Liu et al., 2014 [61]	*In vivo* and *in vitro* (animal model) on SUM149, BT549, and MCF-10A cells	PEG-PLA NPs	siRNA	Targeting of cyclin-dependent kinase 1 (CDK1) by siRNA induces decrease of cell viability, enhances cell apoptosis	Tumour progression has been suppressed in mice without causing any systemic toxicity, and without activating the innate immune response
Deng et al., 2014 [62]	*In vivo* (animal model) and *in vitro* on MDA-MB-231 human cells	Hyaluronic acid-chitosan NPs	DOX and miR-34a	Suppressing the expression of anti-apoptosis proto-oncogene Bcl-2 and non-pump resistance in tumour cells by DOX. Also, miR-34a plays an intracellular role via targeting Notch-1 signaling which leads to inhibition cancer cell migration	The delivery of miR-34a and DOX has effects on tumour suppression
Deng et al., 2013 [63]	*In vivo* and *in vitro* on MDA-MB-468 animal model cells	Layer-by-layer nanoparticles	siRNA	Targeting of multidrug resistance protein 1 by siRNA enhances DOX efficacy and led to decrease in tumour volume	Increase of DOX efficacy led to decrease of tumour volume with no observed toxicity compared to the control treatments
Wang et al., 2011 [64]	*In vitro* on SK-BR3 and MDA-MB-468 human cells	AuNCs	Herceptin	Targeting and nuclear localization in ERBB2 overexpressing breast cancer cells	AuNCs were able to enter the cell nucleus and promoted the competency of Herceptin drug
Dreaden et al., 2009 [65]	*In vitro* on MDA-MB-231 and MCF-7 human cells	Plasmonic AuNPs	Tamoxifen-PEG-Thiol	Targeting estrogen receptor positive breast cancer cells	A high degree of perinuclear and cytoplasmic localization of the targeted particles, with increased potency and selective intracellular delivery of tamoxifen
Au et al., 2008 [66]	*In vitro* on SK-BR-3	Gold nanocages	Anti-HER2	Targeting of the epidermal growth factor receptor which is overexpressed on breast cancer cells	Optimal parameters of nanocages required to achieve cellular damage and increase percentage of dead cancer cells
Gradishar et al., 2005 [67]	Clinical trial on metastatic breast cancer patients	Albumin NPs	Paclitaxel	Paclitaxel is a chemotherapy drug, and it works based on antineoplastic/cytotoxic mechanism.	Nanoparticle albumin-bound paclitaxel demonstrated greater efficacy and a favourable safety profile compared with standard paclitaxel.

NPs: nanoparticles; AuNPs: gold nanoparticles; AgNPs: silver nanoparticles; DOX: doxorubicin; PLGA: poly(lactic-co-glycolic acid); PEG: poly (ethylene glycol); AuNCs: gold nanoclusters.

it was reported that the biological action of mTNF is based on cell contact-dependent signals. For example, mTNF has been shown to mediate inflammatory responses in astrocytes, but not in neurons, whereas both cell types sTNF have similar proinflammatory effects [73].

Both sTNF and mTNF are regulated by binding with their two receptors localised at the cellular surface. TNFR1 (p55) is encoded by *TNFR1* gene located on chromosome 12p13.31, consisting of 10 exons, and codes for a 55/60 kDa membrane receptor. TNFR2 (p75) is encoded by *TNFR2* gene located on chromosome 1p36.22, consisting of 10 exons, and codes for a 75/80 kDa membrane receptor [74]. These two receptors mediate different biological activities from TNF [75]. Studies have shown that the affinity of TNF for TNFR1 is lower compared to TNFR2; therefore, TNFR1 binds preferentially to high TNF concentrations and vice versa for TNFR2 [76, 77]. TNFR1 is expressed in nearly all nucleated cells, although in low levels [78]. Also, TNFR1 has been reported to be the primary mediator of TNF-induced apoptosis, linked to an intracellular region of TNFR1 called "death domain (DD)" that activates the nuclear factor kappa B (NF-κB) pathway [79]. Activation of NF-κB pathway plays a key role in the expression of genes that are responsible for encoding antiapoptotic proteins and several proinflammatory cytokines, including TNF, IL-6, and IL-1 [80]. On the other hand, studies showed that mTNF preferentially binds to and activates TNFR2, while sTNF binds to and activates TNFR1 [81]. Furthermore, TNFR2 participates in activation of B cells, enhances apoptosis by TNFR1, and plays a key role in other proinflammatory responses, including proliferation of T cells [80].

Upon binding of TNF to TNFR1, TNFR1 interacts with receptor-interacting protein 1/2 (RIP1/2) and TNFR1-associated DD protein (TRADD) to build a receptor complex [82] that induces Fas-associated DD protein (FADD), resulting in apoptosis [83]. However, TNFR1 is also able to induce other adaptor molecules which enhance cell survival, including cellular inhibitor of apoptosis protein 1/2 (cIAP1/2) and TNFR-associated factor 1/2 (TRAF1/2). These antiapoptotic signals by both cIAP1/2 and TRAF1/2 are acquired via downstream activation of NF-κB pathway [84]. On the other hand, studies found that TNF and TRAF3 are necessary for activated T cells [85]. The expression of the full-length isoform of TRAF3 lacking exon 8 (Traf3DE8) allows the activation of noncanonical NF-κB pathway by the deactivation of the NFκB-inducing kinase (NIK)-TRAF3-TRAF2 axis, which results in aggregation of NIK in activated T cells [84]. Noncanonical NFκB signalling pathway in turn regulates expression of some chemokines needed in adaptive immunity and structuration of the secondary lymphoid organ, such as B cell chemoattractant (CXCL13) [86]. Although the process(es) that drives the differential regulation of the alternatively spliced form of TRAF3 is not totally clear yet, some studies have reported that T cell-specific TRAF3$^{-/-}$ mice were able to double the number of normal TNFR2-expressing Tregs [87]. Tregs, positive for CD4, CD25, and Foxp3, primarily suppress excessive inflammation [88], and expression of TNFR2 on Tregs identifies them as highly suppressive Tregs [89]. Therefore it could be highly beneficial to use TNFR2 as a potential target in cancer therapeutics [89–92].

The implication of TNF in almost all steps of tumourigenesis has been reported, both as an angiogenic and antiangiogenic factor, depending on the TNF doses and nature (soluble and membrane-bound) [93]. Since TNFR1 and TNFR2 differ in their cytoplasmic domain, they trigger distinct signalling pathways [i.e., proapoptotic (TNFR1) and prosurvival (TNFR2)] upon interaction with TNF [94]. In recent years, several studies on different types of tumours have reported a high expression of TNFR2, resulting in enhanced proliferation, angiogenesis, and migration of several tumour types [95]. This enhancement of tumourigenesis by TNFR2 is coordinated through the stimulation of NF-κB or AKT serine/threonine kinase 1 signalling pathways, which in turn regulate DNA damage and repair of poly (ADP-ribose) polymerase (PARP) protein [96]. Moreover, preclinical studies found that blocking TNFR2 is sufficient to reduce the development of TNF-activated cells [97] as well as to increase TNF-associated cancer cell death [98]. TNFR1 shows high affinity to both soluble and membrane-bound TNF, while TNFR2 is only fully activated by mTNF [99]. Due to their different structure, their regulation through signalling pathways (MAPK and NF-kB) would induce different effects. TNFR1 is responsible for apoptosis while TNFR2 is responsible for cell proliferation and survival [100]. However, under some conditions, prolonged cell stress or disease condition, shift of TNFR2 to TNFR1 apoptotic signalling could occur [39]. All together these findings partially elucidate the role of TNFR2 in development of cancer and its differential function compared to signal kinase activation through TNFR1.

4. An Implication of TNF-TNFR2 in Breast Cancer

Numerous studies have explored the association of TNF and its receptors in breast cancer progression as well as the therapeutic possibilities. However, only a few investigated the impacts of TNFR2 expression in breast cancer [101]. In 2008, for the first time Rivas et al. studied the implication of TNF and its receptors (TNFR1 and TNFR2) on the molecular mechanisms and intracellular pathways of breast cancer proliferation [97]. This study showed that TNF enhances proliferation of breast cancer cells via the activation of p42/p44 mitogen-activated protein kinases (MAPK) pathway by binding to both TNFR2 and TNFR1. In addition c-Jun N-terminal kinase (JNK) and phosphoinositide 3-kinase (PI3K)/AKT pathway activation was also involved while NF-kB transcriptional activation was acquired by TNFR1 activation only [97]. However, in 2017, Yang and his colleagues showed that TNFR2 was implicated in promoting the progression of breast cancer via stimulation of AKT signalling pathway [95]. This signalling pathway protects cancer cells against DNA damage, which in return enhances breast cancer cell proliferation, cancer-associated fibroblast (CAF) induction, angiogenesis, and carcinogenesis [95]. In another study, Yang and his colleagues were able for the first time to confirm that there was a positive association between TNFR2 expression and its prognosis in terms of size of tumour, higher pathological

grade, and advanced clinical stage [102]. They reported that the expression levels of TNFR2 in breast cancer cells were positively associated with doxorubicin (anthracycline type of chemotherapy) resistance; overexpression of TNFR2 significantly promoted doxorubicin resistance, while less expression of TNFR2 significantly dampened doxorubicin resistance, while in turn this regulated the DNA damage and repair PARP protein [95]. In 2018, Nie et al. used two types of antibodies: a TNFR2-blocking and a CD25-targeted approach as a combination treatment in a colon cancer mouse model and breast cancer mouse model, resulting in the inhibition of cancer progression in both models [103]. As per our knowledge, no study examined the expression of TNFR2 in breast normal cells, while we found only one study reporting that it was detected at low levels in normal vascular endothelial cells [104].

As TNFR2 exists without DD, it can enhance proliferation and activation of Tregs via 3 main pathways, namely, NF-kB, activator protein 1 (AP1), and MAPK pathways [105], therefore avoiding the immunosuppressive effect of TNF which is similar to cancer cells survival pathways [100]. Studies demonstrated that Tregs expressed higher levels of TNFR2 than any other T cells, and these high expression levels by Tregs were correlated with the most suppressive population [89]. Moreover, a study performed by van der Most et al. in 2009 [106] used cyclophosphamide to downregulate Tregs during chemotherapy for cancers, as Tregs depletion could be used to enhance the effectiveness of chemotherapies. They also reported that the drug gemcitabine depleted cycling Tregs concurrently with downregulation of CD4+ CD25+ T cells [106]. In addition, few studies also showed that TNFR2 also inhibits the antitumour role of effector T cells (Teffs) and decreases cancer immune responses [107]. Torrey et al. proved that targeting TNFR2 could be an effective treatment as TNFR2 antagonistic antibodies inhibit proliferation of both cancer cells and tumour-infiltrated Tregs while inducing the expansion of Teffs [108]. Furthermore, study in both colon and breast cancer models shows that combination of immunotherapeutic stimulants with TNFR2-blocking antibodies not only inhibits the proliferation of cancer cells but also decreases the number of Tregs and the surface abundance of TNFR2 on Tregs, thus enhancing the effectiveness of treatment [103]. However, to date in addition to their impact on both Tregs and Teffs activities in breast cancer microenvironment, no study has examined the effectiveness of nanomedicines targeting TNF receptors for ligand-nanoparticle conjugate or using TNF antagonists (e.g., biomolecules) as a potential therapy for breast cancer in humans.

It has previously been shown that TNF antagonism (anti-TNF) is a successful therapeutic option that has been applied in various inflammatory cases, including inflammatory bowel disease (IBD), spondyloarthritis (SpA), psoriasis, and rheumatoid arthritis (RA) [109]. TNF antagonism prevents ligand triggering of TNF-TNFRs signalling and thus blocks TNF's cytotoxicity and inflammatory capacity [110]. Currently, there are five approved TNF antagonists used to treat symptoms in inflammatory disorders, including Etanercept, Infliximab, Adalimumab, Certolizumab Pegol, and Golimumab [109]. Among them, Etanercept is a novel TNFR2:IgG1 fusion protein that was developed and approved by FDA in 1998 [111], and it is the only TNF antagonist that is a nonmonoclonal antibody and does not contain a fragment crystallisable (Fc) portion, which means that it is unable to encourage complement activation, antibody-dependent cell-mediated cytotoxicity (ADCC), or apoptosis [112].

Anti-TNF biology functions by mopping up excess soluble TNF and reducing the endocrine activity of these cytokines. They would bind to TNF complexes to block cell-to-cell contact and/or trigger reverse signalling, lastly acting as agonists on Fc receptor (FcR)-expressing cells as they are fused to human IgG1 [110]. However, most of the previous studies on TNF antagonism focused on the inflammatory cases and particularly on rheumatoid arthritis; consequently there are no experimental studies on breast cancer in this context. As inflammation is known as a significant component in cancer progression and the microenvironment of cancer is controlled by inflammatory cells [113], we estimate that TNF antagonism is able to modify breast cancer cells' signalling cascades inducing cell division, migration, differentiation, or death depending on their expression markers and secreted cytokines.

Taken together, these findings suggest that targeting TNF-TNFR2 interaction with pharmacological agents, in an attempt to reduce the number and function of Tregs while enhancing the function and number of Teffs, could provide stronger immune responses against cancer cells and serve as a promising cancer therapeutic approach [55, 114]. On the other hand, studies have also shown that TNF-TNFR2 axis enhances the activation of myeloid-derived suppressor cells (MDSCs) and Tregs suppressive cells that promote tumour immune escape [56, 57]. Furthermore, TNFR2 accelerates the programmed death of macrophages for clearing cancer cells [115]. Thus, TNFR2 plays both direct and indirect role in cancer progression (Figure 5) [116]. We can summarize the pathways of these roles as follows: (1) direct effect of TNF in cancer progression modulated by TNF-TNFR2 axis breast cancer cells itself and (2) indirect effect of TNF in cancer progression modulated by TNF-TNFR2 on Tregs and MDSC which ultimately increase tumourigenesis, tumour invasion, and metastasis. TNF-TNFR2 effects are more prominent on Tregs compared to Teffs as these receptors are preferentially expressed by Tregs.

5. Nanomedicine in Targeting TNF-TNFR2 Axis

As we discussed before, nanomedicine platforms offer a variety of potentially efficient solutions for the development of immunotherapeutic agents that can be exploited for breast cancer treatment [117]. As mentioned before, the first study back in 2008 utilized conjugate gold nanocages with antiepidermal growth factor receptor (anti-HER2) monoclonal antibodies to target breast cancer cells. The targeted cells with the immuno–gold nanocages responded directly to pulsed near-infrared laser irradiation and the mortality rate of cells increased in line with increasing time of exposure till 5 min and became fixed. This study provided significant

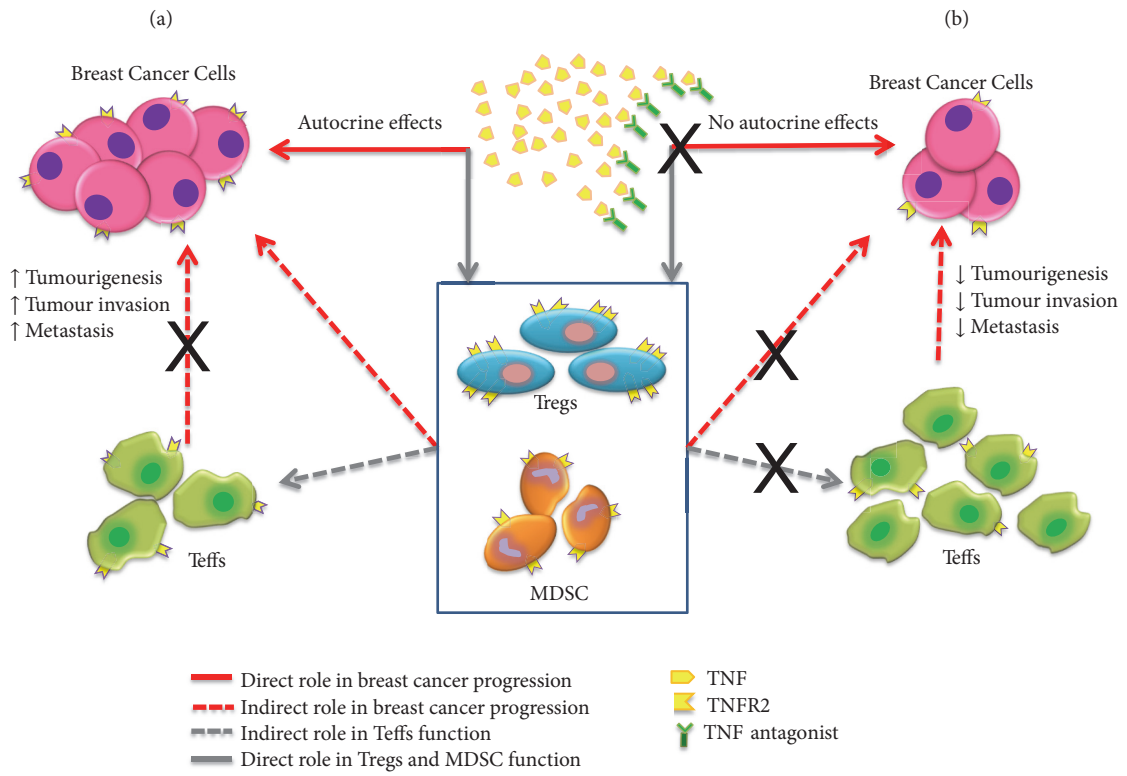

Figure 5: Role of TNF-TNFR2 in the progression of breast cancer and the potential role of TNF antagonists in competing with TNFR2 by mopping up excess soluble TNF and binding on the membrane-bound TNF. (a) TNFR2 is expressed on immune cells and tumour cells in cancer microenvironment. Instead of apoptosis, TNFR2 induces malignant transformation and tumour proliferation by sTNF that activates TNFR2 to enhance Tregs, cancer cells, and MDSC. Therefore, TNFR2 is implicated in enhancing tumour progression either by maintaining cancer microenvironment (immune responses) and enhancing cancer immune evasion, or by inducing cancer cells survival and proliferation [116]. TNFR2 was implicated in promoting the progression of breast cancer via stimulation of AKT signalling pathway which protects against DNA damage and, consequently, enhances proliferation, CAF induction, angiogenesis, and carcinogenesis. Further, a positive association had been reported between TNFR2 expression and its prognosis in terms of size of tumour, higher pathological grade, advanced clinical stage, and dampened doxorubicin resistance [95, 102]. (b) We hypothesized that TNF antagonists would modify breast cancer cells' signalling effects that lead to division, migration, differentiation, or death by assessing their expression markers and secreted cytokines.

details regarding the best dosage of immuno–gold nanocages and other information about the parameters of the laser irradiation in breast cancer treatment [66]. Although there are numerous applications of nanoparticles in modulating immune response [39], only a few experimental studies have developed different nanomedicine applications in breast cancer therapeutic research (Table 3). As mentioned earlier, to date there is no study investigating the involvement of nanoparticles in TNF antagonist or in regulating TNF-TNFR2 interactions on breast cancer. Polystyrene nanoparticles, which exhibit various immunological effects in the lung [118], have been used to selectively activate lung TNFR2+ cells, preferentially TNFR2 expressing Tregs. This aforementioned study is the first to show that TNFR2 can be targeted by nanoparticles for therapeutics application in lung diseases [119]. Thus, nanoparticles are expected to serve as an efficient tool to deliver therapeutic agents (including TNF antagonist) or even to directly regulate TNF-TNFR2 interactions in breast cancer cells. Therefore, we hypothesized that the utilization of nanoparticles with specific ligand would alter the function of breast cancer cells (e.g., uptake capacity or downregulation of membrane-bound TNF) and mediate TNF-TNFR2 signal that leads to distinct immunological effects, such as expansion of breast cancer cells, cytokines secretion, or survival capacity (Figure 6).

6. Future Directions

Studies have demonstrated that TNF-TNFR2 axis is implicated in the suppression of immune response and affects tumour progression and invasion by its oncogenic roles, which results in enhanced proliferation, angiogenesis, and migration of breast cancer. This receptor is also responsible for enhancing the proliferation and activation of Tregs and MDSCs, thus promoting tumour immune escape. Hence, neutralisation of TNF as well as TNFR2 by using TNF-antagonist drugs might be an effective therapeutic strategy for breast cancer cells. However, no studies to date have investigated the modulation of TNF antagonists targeting TNF-TNFR2 axis and their immunoregulation on breast cancer cells, while the potential of nanoparticles to mediate these effects in breast cancer is still unknown. The field of

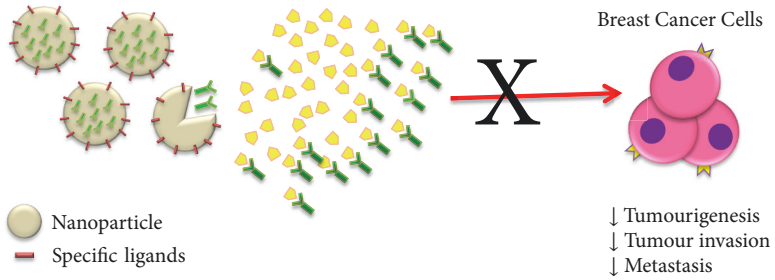

Figure 6: Nanoparticles are expected to serve as an efficient tool to deliver TNF antagonists or even to directly regulate TNF-TNFR2 interactions in breast cancer cells that leads to immunological cascades as observed in Figure 5(b).

nanomedicine has provided the possibility of targeting the TNF-TNFR2 axis to not only deliver therapeutic drugs to targeted sites but also restore the immune response to suppress the cancer cells. Based on accumulating evidence suggesting that tumour progression is governed not only by genetic changes intrinsic to cancer cells but also by environmental factors, future studies might use nanoparticles as a model for inert environmental stimuli. In summary, nanoparticles have the potential to be used as drug delivery vehicle in the future for nanomedicine development in breast cancer therapy. Therefore, future studies should investigate how the presence of nanoparticles with specific characterisation would alter the function of breast cancer cells via TNF-antagonist effects by TNF-TNFR2 signal and their contribution to distinct immunological effects.

Abbreviations

TNF: Tumour necrosis factor
TNFR: TNF receptor
mTNF: Membrane TNF
sTNF: Soluble TNF
TACE: TNF-converting enzyme
Tregs: Regulatory T cells
Teffs: Effector T cells
NK: Natural killer
IL: Interleukin
HER: Human epidermal growth factor receptor
ER: Estrogen receptor
AuNPs: Gold nanoparticles
AgNPs: Silver nanoparticles
BRCA: Breast cancer
CDK: Cyclin-dependent kinase
DOX: Doxorubicin
HA: Hyaluronic acid
CS: Chitosan
DD: Death domain
NF-κB: Nuclear factor kappa B
RIP: Receptor-interacting protein
TRADD: TNFR-associated DD
FADD: Fas-associated DD
cIAP: Cellular inhibitor of apoptosis protein
TRAF: TNFR-associated factor
PARP: Poly (ADP-ribose) polymerase
MAPK: Mitogen-activated protein kinases
JNK: c-Jun N-terminal kinase
PI3K: Phosphoinositide 3-kinase
CAF: Cancer-associated fibroblast
MDSCs: Myeloid-derived suppressor cells
AP: Activator protein
IBD: Inflammatory bowel disease
SpA: Spondyloarthritis
RA: Rheumatoid arthritis
Fc: Fragment crystallisable
FcR: FC receptor
ADCC: Antibody-dependent cell-mediated cytotoxicity.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Review Article

Γδ T Cell-Based Immunotherapy in Melanoma: State of the Art

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Metastatic melanoma is still associated with a poor prognosis, and there is increasing interest in immunotherapy alone or in combination with other adjuvant therapies. Γδ T lymphocytes play a pivot role in the immune response against cancer, but while γδ-based immunotherapy is already a clinical reality for several solid tumors, data on melanoma are still limited and fragmented. This systematic review presents preclinical and clinical evidence for a role of γδ T lymphocytes in immunotherapeutic strategies for advanced melanoma and discusses research state of the art and future perspectives. Current strategies focus on in vivo stimulation, and ex vivo adoptive therapy and vaccination; results are promising, but further studies are needed to better investigate the interactions in tumoral microenvironment and to improve clinical efficacy of immunotherapeutic protocols.

1. Introduction

Metastatic melanoma is still associated with a poor prognosis. Current therapeutic protocols are often ineffective: chemotherapy shows a low efficacy (15%), short duration of response (6 months), and no significant improvement in overall survival; BRAF-targeted therapies are highly effective in metastatic BRAF mutated melanoma (about 60%), but show a short-lived response, while immunotherapy shows a low frequency but extremely durable tumor response [1–3].

In the last years, there is increasing interest in immunotherapy alone or in the combination with targeted therapy [2]. Gamma delta T (γδ) lymphocytes are of particular interest due to their potent antitumoral effect via cytotoxicity and relative ease of culture in vitro. Promising reports from clinical trials support their use as immunotherapeutic agents, either via adoptive transfer of ex vivo expanded γδ T cells or in vivo activation with aminobisphosphonates, but data concerning melanoma are still limited [4–7].

This systematic review presents preclinical and clinical evidence for a role of γδ T lymphocytes in immunotherapeutic strategies for advanced melanoma and discusses research state of the art and future perspectives.

2. Materials and Methods

A systematic literature search was conducted in the PubMed database for articles published between November 01, 2008, and October 31, 2018. The following key words were used: "(melanoma[Title/Abstract]) AND (Vgamma9Vdelta2[Title/Abstract] OR gammadelta[Title/Abstract] OR gamma delta OR γδ [Title/Abstract]) and immunotherapy[Title/Abstract]". Article selection was performed according to the following criteria for inclusion and exclusion:

(i) inclusion criteria: preclinical or clinical research papers concerning the potential immunotherapeutic role of γδ T lymphocytes in advanced melanoma;

(ii) exclusion criteria: papers in language other than English, reviews.

Two reviewers independently screened all search results, abstracts, and full texts. Further search included relevant

Table 1: Characteristics and main results of selected studies.

Paper	Type of paper	N of patients/animals and study design			Results
		Patients (n)	Animals (n)	Laboratory research	
Harrer D.C. et al. BMC Cancer (2017)	Research	NA	NA	Isolated $\gamma\delta$ T cells transfected through mRNA electroporation with a gp100/HLA-A2-specific TCR and an MCSP-specific CAR.	Zoledronic acid-mediated expansion of $\gamma\delta$ T cells directly from PBMC is more efficient than expanding MACS isolated $\gamma\delta$ T cells RNA-transfected $\gamma\delta$ T cells responded to melanoma cells with antigen-specific cytokine secretion and tumor cell lysis, and retained their intrinsic cytotoxic activity towards melanoma cells after electroporation
Yang J. et al. Fron. Oncol (2017)	Clinical	n. 8 patients with stage III in-transit melanoma treated with IL-BCG	NA	NA	Vγ9Vδ2 T cells play a role in IL-BCG-induced melanoma regressions.
Hodgins N.O. et al, Journal of Controlled Release (2016)	Research	NA	NSG mice L-ZOL (toxicity assessment) L-ALD + $\gamma\delta$ T cells (efficacy assessment)	*In vitro* cell lines A375Ppuro	(i) In vitro, zoledronate and alendronate + Vg9Vd2 T-cells determined a significant and dose-dependent reduction in tumour cell viability. (ii) In vivo, combined injection of alendronate and $\gamma\delta$ T cells delayed tumour growth in an experimental metastatic lung mouse model
Wang X. et al. Cancer Cell. (2015)	Research	NA	C57BL/6j (B6; H2Kb), BALB/c (H2Kd), IFN-γ−/−, Prf1−/−, and Rag2IL2Rg doubly deficient mice. MyD88KO mice. IL-36R −/− mice (C57BL/6-Il1rl2<tm1Hblu>)	B16 and 4T1 cells primary lymphocyte culture from C57BL/6 mice.	(i) IL-36γ effectively promoted IFN-γ production by $\gamma\delta$ T and NK cells. (ii) Tumoral expression of IL-36γ greatly inhibited tumor growth and metastasis in vivo, mainly through IFN-γ. (iii) IL-36γ can boost the efficacy of tumor vaccination
Nieda M. Experimental Dermatology, (2015)	Research	NA	NA	PBMCs from HDs and Pts with metastatic melanoma (stage IV). Vγ9Vδ2 (or CD8$^+$) T cells stimulated with autologous CD56 high$^+$ IFN-DCs or mIL-4DCs in the presence of zoledronate (1 lM) and IL-2 (1000 U/ml) for 10–14 days.	CD56 high$^+$ IFN-DCs efficiently promote the expansion of CD56$^+$ Vγ9Vδ2 T cells in the presence of zoledronate and IL-2.

Table 1: Continued.

Paper	Type of paper	N of patients/animals and study design			Results
		Patients (n)	Animals (n)	Laboratory research	
Conlon K.C. et al, JCO. (2015)	Clinical	n. 11 Patients with metastatic Melanoma were treated with rhIL15 and frequency and cytokines released were analyzed (phase I study).	NA	NA	There was rhIL15-mediated activation of monocytes, NK and $\gamma\delta$ T cells. No objective remissions in all patients, with best response being stable disease.
Gehrmann U. et al, Cancer Res. (2013)	Research	NA	C57Bl/6, Vα14-Ca^{-}/$^{-}$ and CD1d^{-}/$^{-}$ mice. Exosomes loaded with αGC + OVA were used to treat the tumor-bearing mice and evaluate tumor growth.		(i) Exosomes loaded with αGC and OVA[Exo(αGC-OVA)] induce an early iNKT-cell response, dendritic cell, MZB cell activation as well as NK- and $\gamma\delta$ T-cell activation and proliferation *in vivo* (ii) Exo(αGC-OVA) also decrease tumor growth and induce T-cell infiltration in a mouse melanoma model
Lança T. et al, Journal of Immunology. (2013)	Research	NA	C57BL/6 (B6) mice. B6.TCRd^{-}/$^{-}$, B6.Ccl2^{-}/$^{-}$, B6.Ccr2^{-}/$^{-}$ Transplantable B16 melanoma model was used to profile chemokines in tumor lesions and assess their impact on $\gamma\delta$ TIL recruitment in vivo.		Cytotoxic $\gamma\delta$ T cells infiltrate B16 lesions and delay tumor growth *in vivo*. B16 lesions in TCRd-deficient mice would accumulate chemokines normally consumed by $\gamma\delta$ T cells during tumor infiltration, the CCR2 ligands: CCL2 and CCL12 were significantly overexpressed in TCRd-deficient mice. Human Vδ1 T cells (but not Vδ2) express CCR2 and migrate toward CCL2 *in vitro*.
Kunzmann V. et al, J Immunother. (2012)	Clinical	n. 21 Patients with metastatic renal cancer, metastatic MM or AML were enrolled to receive zoledronic acid plus IL-2 with 58 treatment cycles being administered.	NA		All patients showed an expansion in circulating $\gamma\delta$ T cells *in vivo* after zoledronate and low-dose IL-2. Quantitative analysis of cytokine serum levels demonstrated that *in vivo* activation of $\gamma\delta$ T lymphocytes by zoledronate plus IL-2 induced a significant increase in the proinflammatory cytokine IFN- γ. Serum VEGF levels negatively correlated with the clinical benefit.

Table 1: Continued.

Paper	Type of paper	N of patients/animals and study design			Results
		Patients (n)	Animals (n)	Laboratory research	
Nicol AJ et al. British Journal of Cancer. (2011)	Clinical	Phase I trial metastatic cancer patients (n=18) (n=7 melanoma). $\gamma\delta$ T cells were expanded ex vivo and adoptively transferred in combination with zoledronate administration	NA	NA	(i) Combination therapy with Vγ9Vδ2 T cells and zoledronate is well tolerated. (ii) Better results in patients not pretreated with zoledronate. $\Gamma\delta$ T cells had an activated effector memory phenotype, expressed chemokine receptors predictive of Vg9Vd2 homing to peripheral tissues and were cytotoxic in vitro against tumour targets, but most patients progressed despite therapy. (iii) the percentage of Tregs in the blood correlated with poor $\gamma\delta$ T cells expansion

Abbreviations: Melanosomal membrane-protein glycoprotein 100 (gp100), T-cell receptor (TCR), protein melanoma-associated-chondroitin-sulfate-proteoglycan (MCSP), chimeric antigen receptor (CAR), peripheral blood mononuclear cell (PBMC), magnetic-activated cell sorting (MACS), intralesional (IL) *Mycobacterium bovis* bacille Calmette–Guérin (BCG), Zoledronate (ZOL), liposomal ZOL (L-ZOL), liposomal ALD (L-ALD), interleukin (IL), interferon gamma (IFNγ), natural killer (NK), healthy donor (HD), patient (Pt), dendritic cells (DC), recombinant human interleukine 15 (rhIL15), invariant NKT (iNKT), alpha-galactosylceramide (aGC), ovalbumin (OVA), exosomes (EXO), C-C motif chemokine ligand (CCL) and receptor type 2 (CCR2), vascular endothelial growth factor (VEGF).

references from selected articles. Data on type of paper, number of patients or research animals, laboratory tests, and results were extrapolated from selected articles.

Data were analyzed to summarize current evidence on the following questions:

(i) What is the potential role for a $\gamma\delta$-based immunotherapy in advanced melanoma?

(ii) What are the possible targets and strategies for a $\gamma\delta$-based immunotherapy in advanced melanoma?

(iii) Is there any difference in possible immunotherapeutic approach for BRAF mutated melanoma?

3. Results and Discussion

Initial search retrieved 14 articles. Nine articles fulfilled inclusion and were selected based on full text review. One further relevant article was identified and a total of 10 papers were included in the study. Figure 1 presents a detailed flow diagram of our literature search strategy and results. Studies' design and results are summarized in Table 1.

Literature review revealed that strong preclinical and limited clinical evidence supports the role of $\gamma\delta$ T cells in immunotherapeutic strategies for advanced melanoma. Clinical data on $\gamma\delta$–based immunotherapy in melanoma are limited, with only 3 clinical studies on a total of 24 patients evaluating different immunotherapeutic protocols for advanced melanoma [8–10] and most studies being performed on melanoma cell lines. Investigated strategies include in vivo stimulation, or an ex vivo adoptive therapy or vaccination. Some authors propose direct stimulation through nitrogen-containing bisphosphonate ± IL-2, IL-36, IL-15, or intralesional-BCG, while others proposed a vaccination strategy through dendritic cells or dendritic cell–derived exosomes; adoptive cell therapeutic approach includes RNA-transfection of $\gamma\delta$ T cells with a chimeric antigen receptor or an $\alpha\beta$ T cell receptor (Figure 2).

Future prospective on the development of $\gamma\delta$-based immunotherapy for melanoma will be discussed on the basis of selected studies and other relevant literature—mainly descriptive—on $\gamma\delta$ T cells and melanoma.

Vδ1 and Vδ2 T cells are the main represented subpopulations of $\gamma\delta$ T cells, which account for up to 10% of circulating lymphocytes in adult and healthy humans. Vδ2 T cells are best represented in the peripheral blood and lymphoid organs, Vδ1 T cells, instead of mucosal and epithelial tissues. Vδ2 T cells recognize metabolites of host mevalonate and microbial nonmevalonate pathway (phosphoantigens) [11], opposite to Vδ1 T cells which have some candidate antigens of stressed and tumor cells, a different immune response bound to different pathogens. Furthermore, all $\gamma\delta$ T cells share characteristics of both adaptive and innate immunity, posing tricking questions still debated by immunologists about their belonging to one branch or the other of immunity. Nevertheless, new discoveries are regularly published which describe features of $\gamma\delta$ T cells belonging to both branches of immune system [12, 13]. Notably $\gamma\delta$ T cells have also the

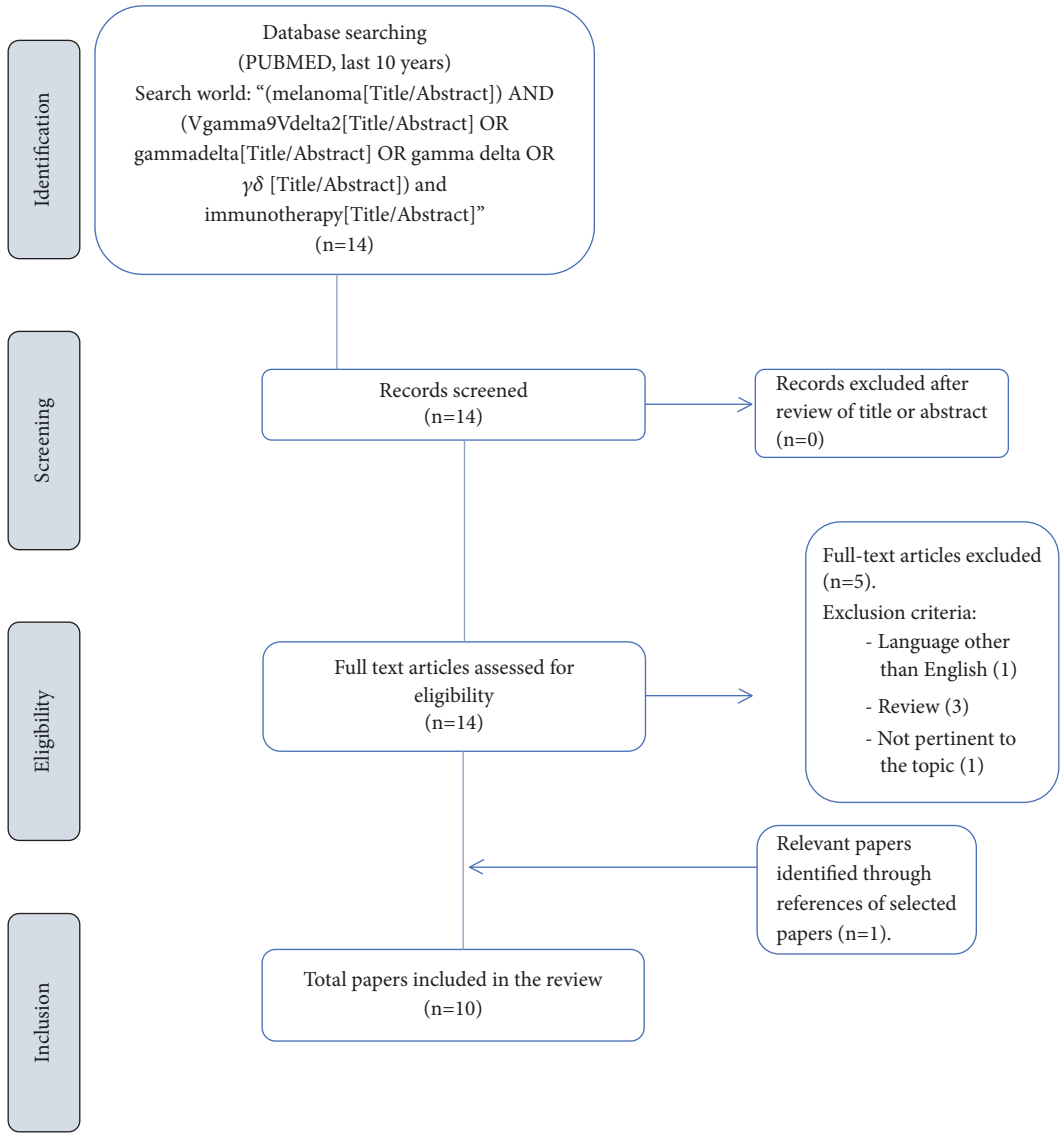

Figure 1: Flow chart of search and selection strategy.

capacity to effectively kill tumor cells [14] with granzyme and perforin, and there are some hints about phagocytosis too [15]. Furthermore, they are activated in a MHC-unrestricted way, whose expression is typically lost in cancer cells that try to escape to immune-surveillance. Then, their killing ability with the freedom from the MHC system makes $\gamma\delta$ T cells a very interesting candidate for immunotherapies.

Several studies have shown that $\gamma\delta$ T cells are an important component of tumor-infiltrating lymphocytes and have a positive prognostic role in patients affected by different types of cancer; a recent analysis of ~18,000 transcriptomes from 39 human tumors identified tumor-infiltrating $\gamma\delta$ T cells as the most significant favorable cancer-wide prognostic signature [16–18]. In melanomas, $\gamma\delta$ T cells, expressing either Vγ9/Vδ2 or Vδ1 TCRs, have been found within tumor-infiltrating lymphocytes (TILs) [19–21]. Previous studies from our group showed that $\gamma\delta$ T cells are the most represented subset of melanoma-infiltrating lymphocytes, displaying an activated phenotype and a strong in vitro cytotoxic activity toward melanoma cells. We showed a correlation among $\gamma\delta$ TILs and melanoma stage, and an inverse correlation of peripheral $\gamma\delta$ T with mortality and relapse rates in metastatic melanoma [21, 22]. Wistuba-Hamprecht K. et al. also showed an interesting correlation between Vδ2+ high frequencies and Vδ1+ low frequencies with favorable overall survival of melanoma patients treated with ipilimumab [23].

While $\gamma\delta$-based immunotherapy is already a clinical reality for other solid tumors, research on $\gamma\delta$ T cells and melanoma is mostly at a preclinical descriptive stage, and their immunotherapeutic potential has not been deeply investigated. $\Gamma\delta$ immunotherapeutic strategies have already proved their efficacy in several preclinical and clinical studies on other solid and nonsolid tumors. Most studies focused on the role of N-BPs (nitrogen-containing bisphosphonates) ± IL-2, which proved to be able to induce immunologic and clinical responses, potentially providing a substantially

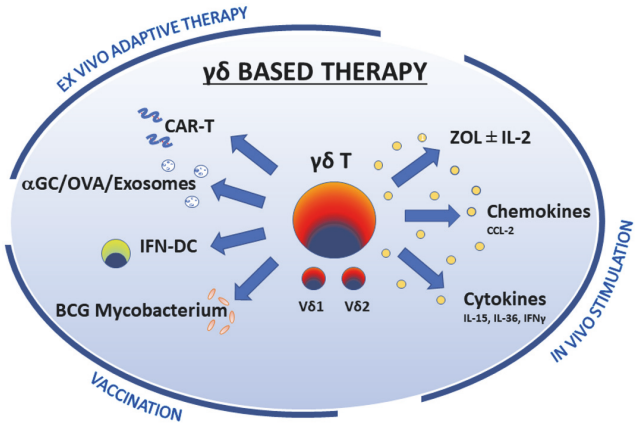

Figure 2: Schematic representation of the main preclinical and clinical research lines on $\gamma\delta$ T cell-based immunotherapy in melanoma.

increased window for more specific/targeted approaches to be administered [4–7].

Several studies in this review focused on the well-known sensitizing role of N-BPs on $\gamma\delta$ T cells.

Hodgins et al. evaluated the efficacy of zoledronate and alendronate to improve the Vγ9Vδ2 T cells effect both in vitro and in vivo. In vitro, both zoledronate and alendronate proved to be able to sensitize melanoma cells to Vγ9Vδ2 T cells, whose subsequent addition determined a significant and dose-dependent reduction in tumor cell viability; liposomal alendronate was 5 times less potent than liposomal zoledronate in vitro and caused less toxic side effects in an in vivo mice model. In vivo, combined injection of alendronate and $\gamma\delta$ T cells delayed tumor growth in an experimental metastatic lung mouse model; tumor cells viability correlated with IFN-γ concentration, suggesting that cell kill was due to the activation of $\gamma\delta$ T cells. They also showed that free N-BPs can sensitize cancer cells more efficiently than their liposomal formulations, which on the other hand show an enhanced passive accumulation and retention within solid tumors [24].

Nieda et al. focused on $\gamma\delta$ stimulation with zoledronate and IL-2, mediated by particular dendritic cells, differentiated from CD14 monocytes in the presence of interferon-α (IFN α) and granulocyte/macrophage-colony stimulating factor (GM-CSF), known as IFN-DCs (interferon dendritic cells). They showed that IFN-DCs exhibited a stronger capacity to stimulate autologous CD56+ Vγ9$\gamma\delta$T cells highly producing IFNγ in the presence of zoledronate and interleukin (IL)-2. Also, this stimulation increased the number of cytotoxic CD8+ T cells through the expansion of CD56+ Vγ9$\gamma\delta$T cells and the authors proposed the CD56 high+ IFN-DCs as hypothetical vaccine in immunotherapies for melanoma patients [25].

Two in vivo studies on stimulation of $\gamma\delta$ T cells with zoledronate reported limited clinical efficacy and suggested combination strategies for improving clinical outcomes. In a clinical phase II study, 21 patients with solid tumors, of which 6 had malignant melanomas, were selected and treated with a combination of zoledronate and low-dose IL-2. The treatment protocol was generally well tolerated, with mild adverse effects. All patients showed an in vivo expansion in circulating $\gamma\delta$ T cells after the first cycle. There was a significant increase in the proinflammatory cytokine IFN-γ with a positive correlation with expanded $\gamma\delta$ T cells. Although there were no objective tumor responses (according to the RECIST criteria) within both cohorts of solid tumors, 1 patient with melanoma with prior progressive disease experienced disease stabilization. Also, higher baseline and increasing VEGF serum levels during the first week of treatment with zoledronic acid correlated with poor prognosis. These data indicate a possible role for in vivo $\gamma\delta$ T cell stimulation, but limited efficacy, and suggest the need for combining immunotherapeutic approaches with anti-VEGF compounds in future clinical trials [26]. Also Nicol et al. pointed out a safe and tolerable profile of zoledronate, but limited clinical efficacy, in their phase I study on metastatic patients with solid primary tumors, of which 7 had melanomas; $\gamma\delta$ T cells were expanded ex vivo and adoptively transferred in combination with zoledronate administration. $\Gamma\delta$ T cells had an activated effector memory phenotype, expressed chemokine receptors predictive of Vγ9Vδ2 homing to peripheral tissues, and were cytotoxic in vitro against tumor targets, but most patients progressed despite therapy. Better $\gamma\delta$ expansion was achieved in patients not pretreated with zoledronate. Authors also observed that the percentage of Tregs in the blood of melanoma patients was significantly higher than controls and correlated with poor $\gamma\delta$ T cells expansion, and suggested that depletion of Tregs from patient mononuclear cells may be a prerequisite for successful $\gamma\delta$-based adoptive therapy [9]. Of note, further evidence for a clinical role of zoledronate for in vivo stimulation in melanoma patients came from Laggner et al., who reported a case of regression of pulmonary and bony metastases in a patient with malignant melanoma following palliative treatment with systemic zoledronate and localized radiotherapy to the bone [27].

Other authors tested a different $\gamma\delta$ T cells stimulation strategy which relies on injection of *Mycobacterium bovis* bacille Calmette-Guérin (BCG). In a study on 8 patients with stage III in-transit melanoma, epidermic injection of BCG into metastatic melanoma sites showed a 90% regression of injected tumors and 17% regression of uninjected tumors, likely through Vγ9Vδ2 T cells recruitment on melanoma sites. However, even this approach did not produce durable responses due to the transient nature of the BCG-induced inflammation [8].

The complex and intricate interactions between tumor cells, tumor microenvironment, and tumor-infiltrating immune cells result in a balance between tumor-promoting and tumor-controlling effects, and $\gamma\delta$ T cells functions are often diverted or impaired by immunosuppressive signals originating from the tumor microenvironment. Chemotactic signals, as well as chemokines/cytokines, could orchestrate this balance. For this reason, other researchers concentrated on the stimulating role of cytokines on $\gamma\delta$ T cells. Wang et al. showed that IL-36γ effectively promoted IFN-γ production by $\gamma\delta$ T and NK cells; also, tumoral expression of IL-36γ greatly inhibited tumor growth and metastasis in an in vivo mouse melanoma model, mainly through IFN-γ. Based on their results, the authors proposed

IL-36γ as immunotherapeutic weapon to boost the efficacy of tumor vaccination [28]. In another preclinical study in a mouse melanoma model, Lança et al. showed a protective role for CCR2/CCL2 through the recruitment of $\gamma\delta$ T cells. Chemokine CCL2 and its receptor CCR2 are involved in the accumulation of $\gamma\delta$ TILs, which produce IFN-γ and display potent cytotoxic functions. Also, CCL2 directed $\gamma\delta$ T cell migration toward tumor extracts in vitro, while the lack of $\gamma\delta$ TILs in TCRδ-deficient, but also in CCR2-deficient, mice enhanced tumor growth in vivo. They also demonstrated that human Vδ1 T cells, but not their Vδ2 counterparts, express CCR2 and migrate to CCL2, and opened new perspectives on CCL2 as promising target for manipulation of Vδ1 T cells in cancer immunotherapy [29]. Conlon et al. investigated instead the role of recombinant human (rh) IL15 in a clinical phase I study on 11 patients affected by metastatic melanoma and observed an rhIL15-mediated activation of monocytes, NK, and $\gamma\delta$ T cells, with no objective remissions in all patients, with the best response being stable disease, reinforcing the idea that combination therapies could be better immunotherapeutic options [10].

Harrer et al. explored a novel approach for adoptive T cell therapy based on mRNA electroporation of melanoma-specific antigen receptors into $\gamma\delta$ T cells. They proposed a GMP protocol for the expansion and mRNA transfection of $\gamma\delta$ T cells and gp100/HLA-A2-specific TCR or MCSP (melanoma-associated chondroitin sulfate proteoglycan)-specific chimeric antigen receptor (CAR). Zoledronate-mediated expansion of $\gamma\delta$ T cells directly from PBMC proved to be more efficient than expanding MACS isolated $\gamma\delta$ T cells; expanded $\gamma\delta$ T cells could be efficiently transfected using mRNA electroporation. They also observed that RNA-transfected $\gamma\delta$ T cells responded to melanoma cells with antigen-specific cytokine secretion, specifically lyse melanoma cells, and retained their intrinsic cytotoxic activity toward melanoma cell lines after electroporation [30].

Other emerging therapeutic agents in cancer immunotherapy are exosomes, which started to be used thanks to their ability to improve innate Th1 immunity and to amplify adaptive immune response. Gehrmann et al. tested the effect of the ligands a-galactosylceramide (aGC) and antigen ovalbumin (OVA) exosomes in a melanoma-bearing mice, demonstrating that antigen-loaded exosomes treatment induces $\gamma\delta$ T cell activation and proliferation, reduction in tumor growth, and T cell infiltration in vivo, besides inducing OVA-specific CD8 β T cell responses and boosting CD4β T and B cell responses. They also show that antigen-loaded exosomes are more potent than soluble antigens in inducing adaptive immunity, envisaging a role in future immunotherapeutic approach [31].

Despite increasing interest in the combination of BRAF-targeted therapies and immunotherapy, preclinical and clinical data concerning this approach are still limited, and we did not identify any specific study on BRAF mutated melanoma and $\gamma\delta$ T cells. In a murine melanoma model, Koya et al. showed that vemurafenib did not significantly alter the expansion, distribution, or tumor accumulation of the adoptively transferred lymphocytes with a genetically modified TCR, while it paradoxically increased their in vivo cytotoxic activity and intratumoral cytokine secretion [32]. Wilmott et al. also reported an increase in CD4(+) and CD8(+) TILs with BRAF inhibitors in metastatic melanomas [33]. These data provide support for conducting trials that combine BRAF inhibitors with immunotherapy in the hope of improving and prolonging clinical responses, although Hooijkaas A et al. suggested instead that vemurafenib may negatively affect the immune activity within the tumor [3]. Therefore, the potential effect of targeted therapy on the tumor microenvironment needs further insight and consideration in the design of clinical trials combining targeted therapy and immunotherapy. Also, interaction between BRAF-targeted therapy and $\gamma\delta$ T cells immune response should be specifically addressed.

Future research should also focus on tumor microenvironment and immune check points as target for a $\gamma\delta$ T cell-based immunotherapy and on the role of certain subset of $\gamma\delta$ T cells, which have been reported to be protumoral such as $\gamma\delta$ T17 cells, $\gamma\delta$ Tregs, and Vδ1 T cells [34].

4. Conclusions

Overall, current knowledge strongly suggests that $\gamma\delta$ T cells should be regarded as prominent tool for enhancing antitumor response in melanoma and points out different possible immunotherapeutic strategies. However, data are still fragmented and limited and focus on in vivo stimulation, and ex vivo adoptive therapy or vaccination. Results of current research are promising, but further studies are needed to better investigate the interactions in tumoral microenvironment and to define immunotherapeutic protocols with improved clinical efficacy.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Review Article

Immune Checkpoint Inhibition in Classical Hodgkin Lymphoma: From Early Achievements towards New Perspectives

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Immune checkpoint inhibition (ICI) became one of the major breakthroughs in cancer treatment over the past decade and entered into therapy within standard oncohematology practice. ICI has demonstrated impressive response rates as salvage therapy in relapsed/refractory (R/R) classical Hodgkin lymphoma (cHL) and is now being tested as an adjunction to chemotherapy in the frontline settings. CHL exquisite sensitivity to PD-1/PD-L1 axis inhibition relies on a particular biological background. By contrast, non-Hodgkin lymphomas (NHL) have demonstrated heterogeneous response rates using ICI. These observations highlight discrepancies between various types of lymphomas in terms of genetic alterations, immune microenvironment interactions, and disease phenotype. This review aims to focus on cHL immune escape mechanisms, focusing on cHL biological sensitivity to PD-1 blockade. We will summarize the available data issued from clinical trials on ICI in cHL and its safety profile. Going beyond the current use of monoclonal antibodies (mAb) targeting immune checkpoints in clinical practice, we will offer an overview of new combinatory therapeutic perspectives where cHL immunotherapy may be considered.

1. Introduction

Accounting for a tenth of lymphoma cases, classical Hodgkin lymphoma (cHL) is characterized by peculiar histologic and immunologic features [1]. A striking inflammatory infiltrate surrounding rare multinucleated giant cells were originally reported by Dorothy Reed more than one hundred years ago. This observation already highlighted the intriguing immune repercussion of cHL where authors noticed tuberculin anergy in affected patients [2]. CHL-associated cellular immunosuppression, which translates into an increased infectious risk that may precede disease by several years, was also further supported by the necessity of delivering irradiated blood products to avoid the risk of transfusion-associated graft-versus host disease (GVHD) in these patients [3]. Although considered a curable disease in almost 80% of cases, relapse cases of cHL are still challenging. Rescue and intensive chemotherapies followed by autologous hematopoietic stem cell transplantation (auto-HSCT) can put into remission about half of the patients [4].

The subset of patients necessitating further treatment in the cases of second relapse or refractory disease is considered for allogeneic HSCT (allo-HSCT). In this situation, a survival plateau has been difficult to reach at least until recently [5].

The impressive results of nivolumab (a fully human IgG4 monoclonal antibody against PD-1) in R/R cHL led to its FDA approval in 2016 [6, 7]. Demonstrating an objective response rate (ORR) of 66.3% in the Checkmate 205 trial, nivolumab's unprecedented performance made it a robust alternative bridge therapy to allo-HSCT [8]. CHL exquisite sensitivity to PD-1 blockade relies on lymphoma cell genetic alterations and particular tumor microenvironment (TME) inflammatory phenotype. In an attempt to optimize the first-line treatment of cHL, PD-1 blockade is now being tested as an adjunct to doxorubicin, vinblastine, and dacarbazine ("AVD" regimen) in Europe and USA in both early and advanced stages (NCT03004833 and NCT03033914 trials), respectively.

Preliminary results have shown high response rates with an acceptable safety profile in the frontline setting with patients achieving complete responses (CR) in 67-80% of cases [9, 10]. High expectations regarding PD-1 blockade in cHL reside in its potential to decrease treatment-related toxicity of current intensive chemotherapy regimen, such as bleomycin-related pulmonary toxicity, and to challenge the place of adjuvant radiotherapy in affected young populations [11]. Avoiding bleomycin may reduce the rate of fatal pulmonary toxicities, which has been reported in 4-5% of cHL patients in a systematic review [11]. Anti-PD-1 mAb are also being studied as an upfront treatment in patients unsuitable for standard therapy (NCT03331731).

Results of PD-1 blockade efficacy in R/R NHL are more variable: it is effective to treat primary mediastinal B-cell lymphoma (PMBCL) [12], Grey-Zone lymphoma [13], CNS primary diffuse large B-cell lymphoma (CNS-DLBCL), and primary testicular lymphoma (PTL) [14], where PD-1 inhibition significantly affects response rates. Heterogeneous immune escape pathways' expression and variable immunosuppressive properties of NHL explain in part these disparities [15].

Focusing on cHL as a paradigm for its high sensitivity to ICI, this review brings insights into the biological background behind its effectiveness. It also reflects on ICI current place in patient care and provides an overview of the strategies being foreseen to boost its effects in the future.

2. cHL Microenvironment and Immune Escape Mechanisms

CHL is a malignancy issued from giant, often multinucleated cells, called Reed-Sternberg (HRS). These cells constitute less than 5% of the tumor bulk, and they grow and survive with the help of interactions with and within a heterogeneous background of inflammatory cells. Germinal center B-cells are considered to be the origin of HRS cells [16].

In the era of polychemotherapy and radiation therapy, the various subtypes of cHL, namely, nodular sclerosis, lymphocyte-rich, lymphocyte-depleted, and mixed cellularity subtypes, demonstrating the heterogeneity of their underlying biology, do not translate into direct consequences for patient care [17]. The latter is mainly driven by disease stage and other risk features [18, 19]. However, the underlying biology of cHL offers now new prognostic markers and may regain the interest of clinicians in this new era of immunotherapy. Collectively, these histological subtypes share a variable number of infiltrating lymphocytes, generally forming the main component of the tumor cell environment, monocyte-macrophages, eosinophils, neutrophils, plasma cells, and mesenchymal stromal cells (MSC), such as endothelial cells and fibroblasts associated with a variable degree of tissue extracellular matrix. These infiltrative components are necessary to promote HRS growth. This has been demonstrated by HRS absence of growth in *ex vivo* experiments and indirectly by substantial difficulties to establish cell lines where interstitial cells are lacking [20]. Reciprocally, HRS cells shape their microenvironment to benefit in return from growth and survival signals provided by surrounding inflammatory cells. Directly and indirectly, HRS attract surrounding cells via chemokine secretion. This is valid for neutrophils (IL-8), eosinophils (CCL5, eotaxin), macrophages (CCL5), mast cells (CCL5), T regulatory lymphocytes (CCL5, CCL17, CCL20, CCL22), and type 2 helper T-cells (CCL17, CCL22). These environmental cells provide not only survival/support signals for HRS, but also, for part of them, immune escape signals [21–24].

Main HRS survival signals lead to NF-kB pathway hyperactivation [25]. They originate from CD40, CD30, TACI, and BCMA receptors engagement by their respective ligands located on the surface of surrounding CD4+ T-cells, eosinophils and mast cells (i.e. CD40L and CD30L), and/or secreted molecules from myeloid-derived cells and neutrophils for BAFF and APRIL, respectively [26, 27].

To counteract immune tumor rejection, HRS shape the TME to induce immune tolerance (Figure 1). HRS cells are coated with a large variety of immune checkpoint ligands and transmembrane receptors mediating exhaustion of infiltrating cytotoxic and type 1 helper T-cell subsets. Cytotoxic T-lymphocyte antigen 4 (CTLA-4), membrane-bound TGF-β, and PD-L1 (B7-H1) expression are some of the main Treg contact mediators acting in this exhaustion process. Among these, PD-1/PD-L1 axis constitutes one of the major escape mechanisms in cHL, as demonstrated by its dense coating on HRS cells [28], which is linked to a high prevalence of 9p24.1 chromosomic amplification, a loci bearing PD-L1/2 genes [29]. A similar amplification is also frequent in some groups of NHL such as PMBCL, CNS-DLBCL, and PTL cases, thus explaining in part their higher relative sensitivity towards ICI when compared to other B-cell NHL. Chromosomal 9p24.1 copy number gains and gene amplifications also affect nearby JAK2 locus, which further intensifies PD-L1 overexpression through JAK/STAT signal pathway activation. Latent membrane protein 1 (LMP1) encoded by the inserted EBV genome mimics CD40 signaling and therefore amplifies PD-L1/L2 overexpression, through AP-1 and JAK/STAT3-mediated epigenetic control. This observation sustains a reciprocal positive biological feedback between avoidance of viral clearance and immune escape of HRS cells [30–32]. PD-1-PD-L1/2 ligation triggers T-cell phosphatase activation and consequent dephosphorylation cascade of several proteins implicated in T-cell receptor (TCR), and PI3K-AKT-to-NF-kB signaling pathways. Tyrosine phosphatases are actually recognized as important immune checkpoint modulators, and active research on their potential inhibition to boost adoptive T-cell therapy is ongoing [33]. Consequently, IL-2 and IFNγ secretion is also repressed, thus inhibiting T-cell cytolytic activity and cell proliferation. Stanford's pathology department recently published their evaluation of PD-L1/-L2 expression on 702 immunostained B-/T-lymphoma samples [28]. This study confirmed the high prevalence of PD-L1 positivity in cHL samples (over 80% in cHL and 75% in nodular lymphocyte-predominant HL (NLPHL), resp.). In this series, all except one PD-L1 positive cHL sample (40 over 41) were also Epstein-Barr (EBV) positive by EBER in situ hybridization, in opposition to three over the nine PD-L1-negative cHL samples.

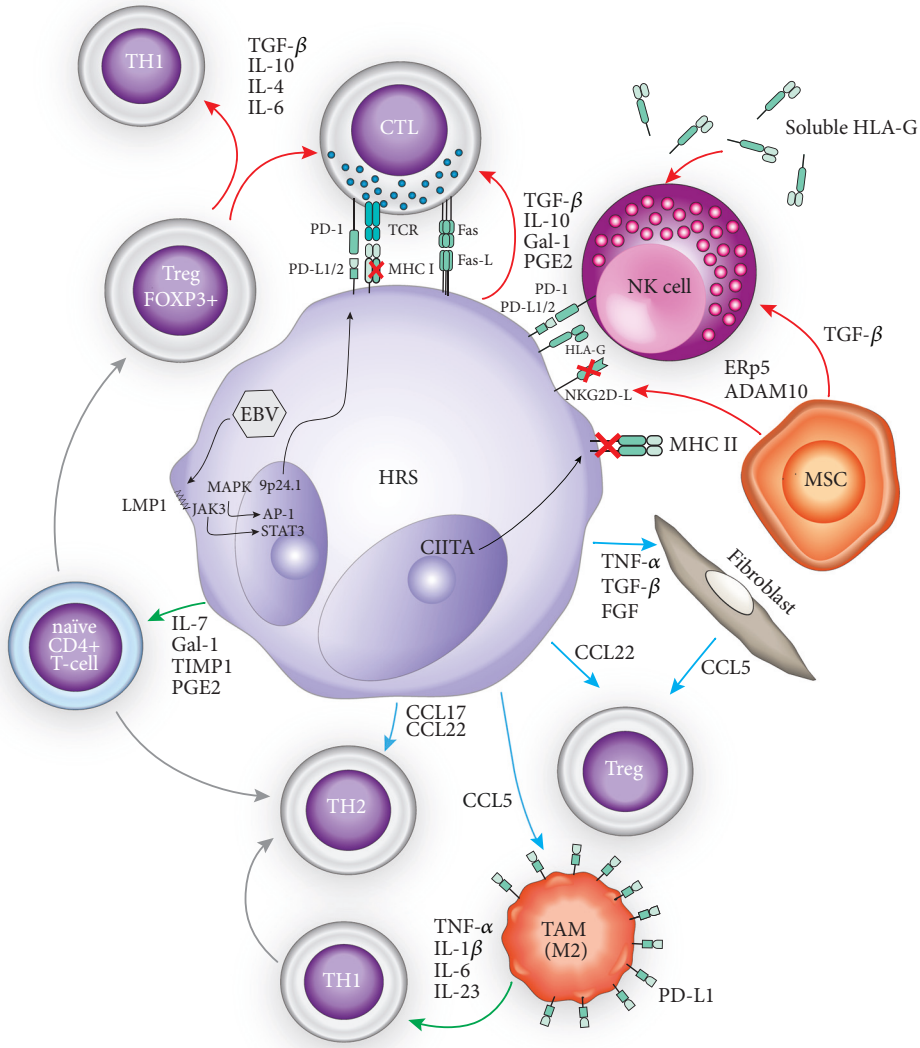

Figure 1: *Immune-escape mechanisms of cHL TME*. Chemokine secretion by HRS plays a central role in the TME immunosuppressive state of cHL. They allow differentiation of infiltrating naïve CD4+ T-cells into regulatory FOXP3+ and Th2 T-cells and provide CTL inhibitory signals through TGF-ß, IL-10, galectin-1 (Gal-1), tissue inhibitor of metalloproteinase 1 (TIMP1), and prostaglandin E2 (PGE2). HRS also attract Tregs/Th2 T-cells from the systemic circulation, through the secretion of CCL22 and CCL17, respectively, while at the same time promoting their expansion through the secretion of Gal-1, TIMP1, and PGE2. Fibroblasts also contribute to Treg chemoattraction through CCL5 secretion. In the same line, TAM promote the differentiation of Th1 cells towards the Th2 phenotype. Nevertheless, recent evidence challenges this concept of predominant Th2 polarized TME and evidenced an increase in activated Th1 T-cells in TME of cHL patients. EBV latent infection also plays a key role, through the production of LMP1 which activates the MAPK and JAK/STAT3 pathways leading to transcriptional activation of the 9p24.1 locus with consequent PD-L1/2 overexpression [5]. Even in EBV negative cHL, PD-L1 (and, to a lesser degree, PD-L2) expression at the surface of HRS cells is still high. PD-1-PD-L1 interaction triggers the inhibition of CTL function. TAM display a high surface PD-L1 expression, thus promoting PD-1-PD-L1 axis immune escape. EBV- cHL displays also a decrease in MHC class I expression, in comparison to their EBV positive counterparts mainly through B2-microglobulin subunit downregulation. MHC class II expression can also be impaired through epigenetic silencing, in the subset of mutated/translocated CIITA cHL. In this context, it is presumed that the PD-1/PD-L1/2 axis mediates immune escape, in first instance, by dampening NK cell activity. NK function is further downregulated by TGF-ß secreted by HRS and mesenchymal MSC. MSC also edit the surface expression of NKG2D-L through the enzymatic activity of secreted ERp5 and ADAM10. HRS cells also express Fas ligand at their surface, thus promoting apoptosis of interacting Th1 and cytotoxic T-cells [88]. Red arrows: "inhibition signal"; green arrows: "differentiation signal"; blue arrows: "chemoattraction" signal.

Table 1: Summary of immune escape mechanisms in cHL and alternative therapeutic strategies in development.

Immune escape mechanisms in cHL	Therapeutic agents with immunomodulatory properties tested in recruiting/active clinical trials in R/R cHL
Downregulation of MHC class I and II expression	Epigenetic modifiers in combination with immune checkpoint inhibitors∗: Decitabine + anti-PD-1 mAbs (NCT03250962)
Surface PD-L1/2 overexpression	JAK/STAT inhibitors in combination with immune checkpoint inhibitors: Ruxolitinib + anti-PD-1 mAbs (NCT03681561) Combinatorial immune checkpoint blockade: Ipilimumab + Nivolumab (NCT02408861, NCT02304458) Anti-LAG3 mAb (MK-4280) + anti-PD-1 mAb (NCT03598608) Brentuximab + Nivolumab +/- Ipilimumab (NCT01896999)
CTL anergy through PD-1-PD-L1/2 interaction (HRS / TAM).	Adoptive cell therapy: Chimeric Antigen Receptor (CAR) CD30-targeting T-cells (NCT01316146, NCT01192464, NCT02690545, NCT02917083, NCT02259556, NCT03602157, NCT03049449) Bi-specific chimeric antibody constructs: INBRX-105 (PD-L1-CD137) provides a combination of PD-L1 blockade with concomitant T-cell co-stimulation through CD137 (4-1BB) agonism (NCT03809624)
NK cell inhibition mediated by TGF-ß and NKG2D-L interaction (HRS / MSC).	Bi-specific chimeric antibody constructs: AFM13 (CD30-CD16A) recruits NK cells via binding to CD16A as immune effector cells (NCT02321592) AFM13 + anti-PD-1 mAbs (NCT02665650)
CTL inhibition through TGF-ß, IL-10, Gal-1, TIMP1 and PGE2. Stimulation of CD4 T-cells differentiation towards Treg and Th2 phenotype through TGF-ß, IL-10, Gal-1, TIMP1 and PGE2. Chemo attraction of Treg and Th2 through CCL5 (fibroblasts), CCL17 and CC22 (HRS).	Immunomodulatory agents: Lenalidomide + anti-PD-1 mAbs (NCT02875067, NCT03015896, NCT01953692) Ibrutinib + anti-PD-1 mAbs (NCT02940301).
Th1 and CTL enhanced apoptosis through Fas ligand surface expression (HRS).	Induction of immunogenic cell death (ICD) of tumor cells with chemotherapy in combination with immune checkpoint inhibitors: Bendamustine + anti-PD-1 mAbs (NCT03343652) Bendamustine + Gemcitabine + anti-PD-1 mAbs (NCT03739619)

∗It should be noted that the trials involving inhibitors of deacetylase (HDACi) in cHL revealed a limited efficacy with significant hematological and electrolytic toxicities, rendering their future development difficult in the absence of predictive biomarkers [89, 90].

CTLA-4 is another immune checkpoint located on the surface of T-cells which dampers the priming (early) phase of immune response. This function makes CTLA-4 a natural synergistic partner of PD-1/PD-L1 inhibition. Indeed, in addition to impeding CD28 costimulatory signaling in barring T-cells, by competing for its ligands (CD80/CD86), CTLA-4 interaction impacts also the NF-kB pathway leading to IL-2 production impairment [34, 35]. Its constitutive presence on Tregs also offers the possibility to target directly a main actor of TME immunosuppression [36]. Recent success in the treatment of melanoma, clear-cell renal cancer, and non-small cell lung cancer brings high expectations regarding ongoing trials combining both PD-1-PD-L1 and CTLA-4 inhibition in relapsing cHL (Table 1) [37–39].

Immunochemistry studies showed a high prevalence (>90%) of additional immune checkpoint regulators such as lymphocyte activation gene-3 (LAG3), T cell immunoglobulin, and mucin domain-containing protein-3 (TIM3) expressions. Expressions were found mainly on T-cells composing the TME of cHL. This was in opposition to PD-L1 displaying almost universal positivity on HRS cells. TIM3 was present in only one-third of samples included in a study assessing samples from 25 cases of cHL, while HRS were weakly LAG3-positive in a single case [40]. The implications of these findings are still unclear, even though they highlight a presumed significant role of these additional immune checkpoints within the TME component. LAG3 (CD223) is a cell surface receptor expressed by Tregs, activated B/T-cells, and antigen-presenting dendritic cells, which binds with high affinity to the major histocompatibility complex (MHC) class II. Functional consequences of its ligand engagement are cell context-dependent, promoting Treg function enhancement on the one hand, and suppressing effector T-cell function on the other hand [41, 42]. However, LAG3 signaling and intervening mechanisms of CD4/CD8 T-cell downmodulation are still poorly characterized [43]. TIM3 is an inhibitor receptor implicated in the exhaustion of cytotoxic and Th1 tumor infiltrating T-cells, although as for its former counterpart the underpinning mechanisms are still to be fully characterized [44, 45]. One of its ligands, Galectin-9, is a mediator Th1 cell death [46]. Blockade of these regulatory components is under active clinical research with several ongoing trials in solid and hematologic neoplasms, since they displayed synergism with PD-1 blockade in a preclinical setting (Table 1).

HRS also occults surface MHC class I and II in about 2/3 of cases, playing so, on both of the two-signal dependency of T-cell activation [47]. ß2-microglobulin transcription repression constitutes the main mechanism of MHC class I downregulation and seems inversely correlated with EBV status. MHC class II is also downregulated at a transcriptional level in the subset of Class II Transactivator Type I (CIITA) mutated and/or translocated cHL. MHC class II expression negativity is found in 15% to 40% of cases [48, 49]. Altogether, one-third of cHL display no expression of both MHC. These observations led to the assumption that PD-1 blockade efficiency in cHL is not primarily related to reinforce CTL immune rejection. Instead, a more pronounced effect on reversing Natural Killer cells (NK) inactivation is presumed, by impairing the interaction of PD-1 with PD-L1 located on the surface of HRS and tumor-associated macrophages (TAM). The latter are main providers of surface PD-L1 in the cHL TME because of its high density of expression on their surface [50].

NK cells are important mediators of antitumor surveillance. However, HRS cells are resistant to Fas receptor-mediated death and even in the absence of most MHC class I molecules are able to avoid NK cell activation. This is mediated by the expression of surface ß2-microglobulin-free HLA-G subunits [51]. HLA-G is mainly expressed in the placenta and plays a crucial role in its immunotolerance. A soluble form of the latter is also secreted by HRS cells and impairs NK cell extravasation and tissue migration. Finally, HLA-G can induce Treg differentiation. HRS cells display low levels of surface NKG2D ligand through secretion of proteolytic enzymes such as ERp5 (a disintegrin) and ADAM10 (a metalloproteinase domain-containing protein 10), also produced by MSC [52]. A soluble form of the NKG2D ligand is also presumably responsible for the internalization and subsequent downregulation of its receptor on circulating NK cells, thus inducing a systemic cellular dysfunction [53, 54]. TGF-ß secreted by MSCs further reinforces the downregulation of NKG2D receptor on the surface of NK and cytotoxic T-cells [55]. Several of the beforementioned characteristics of cHL immune escape, as, for example, a high number of infiltrating TAMs and a lack of MHC class I expression, negatively impact disease outcome [47, 56]. A summary of these immune escape mechanisms, together with treatment strategies under clinical investigation to overcome them, is provided in Table 1.

3. Immune Checkpoint Inhibition in cHL

3.1. Clinical Outcomes. The preclinical observation of PD-L1 overexpression in cHL led to the evaluation of ICI administration on disease evolution. To date, seven prospective clinical studies with ICI in cHL have been published (Table 2). In the first phase 1 study published in 2015 (Checkmate 039), 23 patients with R/R cHL were treated with nivolumab 3mg per kilogram of body weight every 2 weeks until complete response, tumor progression, or excessive toxicity [7]. Eighteen patients had relapsed after auto-HSCT and/or received brentuximab vedotin (BV) (an antibody-drug conjugate directed against CD30 and linked to microtubule-disrupting agent monomethyl auristatin E, MMAE) before relapse. High-grade adverse events (G3-4) occurred in 12 patients. Four patients had a complete response (CR) and 16 patients had a partial response (PR). The progression-free survival (PFS) at 24 weeks was 86%. Following this study, the same group of investigators performed a phase 2 study (Checkmate 205) including 243 patients. The latter was composed of three cohorts divided according to their treatment history: patients who did not receive BV (cohort A, n=63), patients treated with BV after auto-HSCT (cohort B, n=80), and patients who were treated with BV before and/or after auto-HCT (cohort C, n=100). The initial results from cohort B were published in the Lancet Oncology in 2016 [8]. Seven patients had a CR and 46 patients had a PR, with a PFS of 76.9% at 6 months. The results of the extended follow-up of the three cohorts were published in 2018 [57]. The overall response rate (ORR) was 69% (95% CI, 63% to 75%). Forty patients had a CR and the median PFS was 14.7 months. Response rates were similar across the three cohorts. The updated results of Checkmate 205 were presented in December 2018. Actualized ORR was 71% with 21% of patients achieving CR (Cohort A 32%, Cohort B 14%, Cohort C 20%) [58].

It should be emphasized that the Checkmate 205 study protocol was amended in July 2014 to allow patients to continue treatment beyond investigator-assessed progression if protocol-predefined criteria were met, including stable performance status and perceived clinical benefit. Patients treated beyond initial progression (TBP) were required to discontinue in the event of further progression (>10% further increase in tumor burden) [8]. Cohen reported on the 80 patients TBP over the 130 patients with progressive disease in the Checkmate 205 study. Amongst 67 evaluable patients TBP, 37 experienced stable or reduced target tumor burdens, despite the appearance of new lesions [59].

Finally, a small phase 2 study on nivolumab in relapsed cHL after treatment with BV on 17 patients with or without previous auto-HSCT was performed in Japan [60]. CR and PR occurred in four and nine patients, respectively, with a PFS of 60% at 6 months.

Another anti-PD-1 mAb, pembrolizumab, was studied in a phase 1 trial published in 2016 (Keynote 013) [61]. Thirty-one patients, all previously treated with BV with 22 of them having also received an auto-HSCT before relapse, were treated with pembrolizumab 10 mg per kilogram of body weight every 2 weeks. Five patients had a CR and 15 a PR, with a PFS of 69% and 46% at 24 and 52 weeks, respectively. Thereafter, a phase 2 study was conducted (Keynote 087), but with a dose of pembrolizumab of 200mg once every 3 weeks, based on its pharmacokinetic properties [62]. Patients were divided in three cohorts: those relapsing after auto-HSCT and subsequent BV (Cohort 1, n=69), those who were ineligible for auto-HSCT because of refractoriness to salvage chemotherapy and BV (Cohort 2, n=81), and those relapsing after auto-HSCT but without subsequent BV (although 41.7% received BV before transplantation) (Cohort 3, n=60). Among the 210 included patients, the ORR was 69% with 22.4% of CR. PFS at 6 months was 72.4%. There were no significant differences between the three cohorts. The updated results presented at ASH 2018 showed an ORR of

Table 2: Clinical trials of immune checkpoint inhibitors in cHL.

Study	Year	Drug, dose	Design	Phase	Clinical setting	No. of patients	ORR, %	CR, %	PFS
Ansell et al. (Checkmate 039) [7]	2015	Nivolumab, 3 mg/kg iv every 2 weeks until complete response, tumor progression, or excessive toxic effects	P	I	R/R	23	87	17	86% at 24 weeks
Armand et al. Checkmate 205 updated results (ASH 2018) [58]	2018	Nivolumab, 3 mg/kg iv every 2 weeks until disease progression, death, unacceptable toxicity, withdrawal of consent, or study end.∗ Patients in cohort C discontinue nivolumab after 1 year in persistent CR and could resume treatment if they relapsed within 2 years of the last dose	P Composed of 3 cohorts	II	R/R	243	69	16	Median PFS 15 months
			Cohort A (no exposition to BV)			63	65	29	Median PFS 17 months
			Cohort B (treatment with BV after auto-HSCT)			80	68	13	Median PFS 12 months
			Cohort C (treatment with BV before and/or after auto-HSCT failure)			100	73	12	Median PFS 15 months
Maruyama et al. [60]	2017	Nivolumab, 3 mg/kg iv on Day 1 each 14-day cycle.	P	II	R/R	17	81.3	23.5	60% at 6 months
Armand et al. (Keynote 013) [61]	2016	Pembrolizumab iv at a dose of 10 mg/kg every 2 weeks	P	I	R/R	31	65	16	69% at 24 weeks, 46% at 52 weeks

Table 2: Continued.

Study	Year	Drug, dose	Design	Phase	Clinical setting	No. of patients	ORR, %	CR, %	PFS
Zinzani et al. Keynote 087 updated results (ASH 2018) [63]	2018	Pembrolizumab, 200 mg iv every 3 weeks without premedication for a maximum of 24 months or until documented confirmed disease progression, intolerable toxicity, or investigator decision	P Composed of 3 cohorts	II	R/R	210	71.9	27.6	Median PFS 13.7 months
			Cohort 1 (progression after auto-HSCT and BV)			69	76.8	26.1	Median PFS 16.4 months
			Cohort 2 (progression after salvage chemotherapy and BV, but ineligible for auto-HSCT because of chemoresistant disease)			81	66.7	25.9	Median PFS 11.1 months
			Cohort 3 (progression after auto-HSCT, without BV)			60	73.3	31.7	Median PFS 19.4 months
Shi et al. (ORIENT-1) [68]	2019	Sintilimab, 200mg iv once every 3 weeks, until disease progression, death, unacceptable toxicity or withdrawal of consent, for a maximum of 24 months	P	II	R/R	92	80,4	34	77.6% at 6 months
Herbaux et al. [71]	2017	Nivolumab, 3 mg/kg iv once every 2 weeks without premedication until disease progression or unacceptable toxicity as assessed by investigators	R	-	R/R after allo-HSCT	20	95	42	58.2% at 12 months
Haverkos et al. [72]	2017	Nivolumab 3mg/kg iv every 2 weeks (n=28) or Pembrolizumab 200mg iv every 3 weeks (n=2)	R	-	R/R after allo-HSCT	30	79	50	Median PFS 591 days

BV = brentuximab vedotin, CR= complete response, HSCT= hematopoietic stem cell transplantation, IV = intravenously, NA = Not Available, ORR= overall response rate, P= prospective, PFS= progression free survival, R= retrospective, R/R= relapsed or refractory disease.

∗Amendment in July 2014: patients continued treatment beyond progression if protocol-predefined criteria were met (i.e. stable performance status and deriving perceived clinical benefit). Patients treated beyond initial progression were required to discontinue in the event of further progression (>10% further increase in tumor burden).

71.9%, with 27.6% of CR. Median PFS was 13.7 months. It seems also that cohort 2 (those with chemoresistant disease) had smaller ORR (66.7% [95%CI 55.3-76.8] vs. 76.8% [95%CI 65.1-86.1] in cohort 1 and 73.3% [95%CI 60.3-83.9] in cohort 3) and shorter PFS than the two other cohorts (11.1 months [95%CI 7.6-13.8] vs. 16.4 [95%CI 11.3-27.6] and 19.4 months [95%CI 10.8-22.1], resp.) [63].

Based on these studies, nivolumab and pembrolizumab received FDA [6, 64, 65] and UE [66, 67] approvals for the treatment of patients with cHL who relapsed or progressed after auto-HSCT and posttransplantation BV.

More recently, another anti-PD-1 mAb, sintilimab, was tested in a phase 2 trial (ORIENT-1) [68]. Ninety-two patients with R/R cHL were treated with sintilimab (200mg intravenous once every 3 weeks) until progression, death, unacceptable toxicity, or withdrawal of consent. ORR was 80.4%, with 34% CR and a PFS of 77.6% at 6 months.

Of note, exclusion criteria in all the abovementioned studies comprised allo-HSCT. Indeed, two murine models raised the concern of increased GVHD-related mortality due to ICI exposure after allo-HSCT [69, 70].

Recently, a retrospective study on 20 patients treated with nivolumab after allo-HSCT was published [71]. Six patients experienced acute GVHD, and 2 patients deceased. Noteworthily, all of these patients had already suffered a previous episode of acute GVHD. In this cohort, nivolumab did not induce chronic GVHD and no flare phenomena was noted in four patients with previously documented chronic GVHD. In this study, nine patients showed a CR under nivolumab and 10 had a PR, with a PFS at 12 months of 58.2%. Another retrospective study on 31 lymphoma patients (30 of them having cHL) treated with nivolumab (n=28) or pembrolizumab (n=2) for relapse after allo-HSCT found an ORR of 77% (15 CR, 8 PR), but with 8 (26%) GVHD-related deaths after anti-PD-1 therapy [72]. After initiation of anti-PD-1, 17 patients developed GVHD (6 acute, 4 overlap, and 7 chronic). Median PFS was 591 days.

The impact of anti-PD-1 treatment on the risk of subsequent GVHD when administered before allo-HSCT is also a matter of debate. In a retrospective study published in 2017, Merryman et al. described a cohort of 39 patients with lymphoma (31 patients with cHL), who received pembrolizumab or nivolumab and subsequently underwent allo-HSCT (median time of 62 days, range 7-260, between end of immunotherapy and allo-HSCT) [73]. They found a 1-year cumulative incidence of grade 3-4 acute GVHD of 23% and reported a PFS of 89%. An analysis of circulating lymphocyte subsets in 17 patients showed, in comparison to controls, decreased numbers of PD-1+ T cells and lower ratios of T-regulatory cells to conventional CD4+ and CD8+cells, suggesting a possible long-term implication of prior ICI treatment on the immune system after allo-HSCT. A recent review summarized the published data on the impact of prior or subsequent anti-PD-1 therapy on GVHD in patients with lymphoma (the majority of whom were cHL) treated with an allo-HSCT [74]. Among the 107 patients who received ICI before allo-HSCT, acute and chronic GVHD surged in 56% and 29% of patients, respectively. Mortality risk from GVHD was 11% in this study. One hundred and seventy-six patients treated with ICI after allo-HSCT were reported. The rates of acute and chronic GVHD were 14% and 5%, respectively, with a mortality risk from GVHD of 7%. In the absence of prospective data, recommendations for management of ICI before or after allo-HSCT are currently based on expert opinions [71]. These recommendations advocate empirically a 6-week interval between completion of anti-PD-1 therapy and allo-HSCT. In addition, these recommendations advise the use of reduced intensity conditioning regimen before allo-HSCT in this heavily pretreated patient population.

3.2. Safety Profile. Adverse events consecutive to ICI are distinctly different from the ones related to conventional chemotherapy. The blockade of the immunological checkpoints with mAb can trigger autoimmune complications that can affect any organ. These so-called immune-related adverse events (irAEs) vary in incidence and spectrum of affected organ systems depending on the agents used. The incidence of irAES of any grade is variable and can affect up to half of patients under anti-PD-1 therapy, such as those in the ORIENT-1 trial [68]. The incidence of high-grade irAEs among the 651 patients with R/R cHL included in different clinical trials is summarized in Table 3. The most frequent grade 3-4 adverse events according to common terminology (CTCAE v.4.0) were gastrointestinal under the form of enterocolitis (13%, 2%, and 5% in patients treated with nivolumab, pembrolizumab, and sintilimab, resp.), pulmonary (2.1% in total; including pneumonitis, dyspnea, and respiratory infections), and hepatic. Other reported high-grade adverse events encompassed general symptoms, such as fever and fatigue (1.2%), but also mucocutaneous (1%), cardiovascular (0.4%), endocrine (0.3%), rheumatological (0.3%), and renal and electrolyte (0.3%) disorders. These trials did not report fatal cases of irAEs and toxicities leading to treatment discontinuation were rare.

Hematologists should be aware of such possible complications in order to initiate early and adapted immunosuppressive treatments [75]. Most irAEs are reversible, except in the case of endocrine dysfunction, and are treated effectively by delaying the administration of the ICI and proper immunosuppressive treatments including corticosteroid and/or biological agents targeting key inflammatory cytokines such as interleukin-6 and tumor necrosis factor α [76].

3.3. Patients Not Responding to Anti-PD-1 Therapy. The radiological interpretation of tumor response is challenging in the context of immunotherapy. The assessment of tumor response using fluorodeoxyglucose-positron-emission tomography (FDG-PET) may lead, in some cases, to premature discontinuation of anti-PD-1 therapy, due to misleading imaging findings suggestive of disease progression. The immune activation and abundance of T-cell infiltration related to checkpoint inhibition has been linked to a phenomenon called "tumor flare" or "pseudoprogression" under the form of new lesions, or lesions increasing in size and metabolic activity [77]. A new biopsy or repeated imaging is advocated in these cases, mostly if the patient is experiencing clinical benefit from immunotherapy, before deciding to

Table 3: Adverse Events linked to immune checkpoint inhibitors in cHL.

Agent	Reference / Phase	No. of patients	Most Common AE	Grade 3 or 4 AEs	Comments
Nivolumab	Ansell et al. (Checkmate 039) [7]	23	*78% Drug-related adverse events of any grade; the most common (>10%)*: (i) Rash (22%) (ii) Thrombocytopenia (17%) (iii) Pyrexia (13%) (iv) Fatigue (13%) (v) Diarrhea (13%) (vi) Nausea (13%) (vii) Pruritus (13%)	*22% Drug-related adverse events of grade 3*: (i) Myelodysplastic syndrome (ii) Pancreatitis (iii) Pneumonitis (iv) Stomatitis (v) Colitis (vi) Gastrointestinal inflammation (vii) Thrombocytopenia (viii) Increased lipase levels (ix) Decrease lymphocyte level (x) Leukopenia	(i) Two patients discontinued treatment because of drug toxicity. (ii) AE reversible in all the patients except the 2 who discontinued.
	Younes and al. (Checkmate 205) [8]	80	*89% Drug-related adverse events of any grade; the most common (>10%)*: (i) Fatigue (25%) (ii) IRR (20%) (iii) Rash (15%) (iv) Arthralgie (14%) (v) Pyrexia (14%) (vi) Nausea (13%) (vii) Diarrhea (10%) (viii) Pruritus (10%)	*21% Drug related grade 3*: (i) Neutropenia (5%) (ii) Increased lipase levels (3%) (iii) Increased ALT (3%) (iv) Increased AST (3%) (v) Abdominal pain (3%) (vi) Dyspnea (1%) (vii) Pneumonia (1%) (viii) Hepatitis (1%) (ix) Rash (1%) (x) Arthritis (1%) (xi) Syncope (1%) *4% Drug-related AE grade 4*: (i) Increased lipase (3%) (ii) Decreased neutrophil count (1%)	AE leading to discontinuation: (i) Treatment related autoimmune hepatitis (1 patient) (ii) Treatment related increased ALT and ASAT concentrations (1 patient) (iii) Death from multi-organ failure (1 patient) not treatment-related.
	Armand et al. (Checkmate 205) [57]	243	*Drug-related AE any grade*: (i) Fatigue (23%) (ii) Diarrhea (15%) (iii) Infusion-related reaction (14%) (iv) Rash (12%) (v) Pruritus (10%) (vi) Nausea (10%) *Immune-mediated AE*: (i) Hypothyroidism /thyroiditis (12%) (ii) Rash (9%) (iii) Hepatitis (5%) (iv) Pneumonitis (4%) (v) Hyperthyroidism (2%) (vi) Diabetes mellitus <1%	*Drug-related AE grade 3-4*: (i) Lipase increased (5%) (ii) Neutropenia (3%) (iii) ALT increased (3%) (iv) AST increased (2%)	7% discontinue treatment because of drug-related AE: (i) Pneumonitis (2%) (ii) Autoimmune hepatitis (1%)

Table 3: Continued.

Agent	Reference / Phase	No. of patients	Most Common AE	Grade 3 or 4 AEs	Comments
	Maruyama and al. [60]	17	*Most common adverse events* (i) Pyrexia (41.2%) (ii) Pruritus (35.3%) (iii) Rash (35.3%) (iv) Hypothyroidism (29.4%)	*23.5% grade 3 or 4 AEs*: (i) Anemia (ii) Lymphopenia (iii) Thrombocytopenia (iv) Pyrexia (v) Hepatic function abnormal (vi) Pneumonia (vii) Hyponatremia (viii) Fulminant type 1 diabetes mellitus (ix) Interstitial lung disease (x) Rash	6 serious AE in 3 patients (all judged drug-related): (i) Pyrexia (ii) Hepatic function abnormal (iii) Hyponatremia (iv) Fulminant type 1 diabetes mellitus∗ (v) Interstitial lung disease∗ (vi) Rash ∗ Led to treatment discontinuation
Pembrolizumab	Armand et al. (Keynote 013) [61]	31	*68% Drug-related AE* (i) Hypothyroidism (16%) (ii) Nausea (13%) (iii) Diarrhea (16%) (iv) Pneumonitis (10%)	*16% Drug-related grade 3 AEs*: (i) Colitis (ii) Increased ALT and AST levels (iii) Nephrotic syndrome (iv) Joint swelling (v) Back pain (vi) Axillary pain	(i) Two patients discontinued due to grade 2 pneumonitis and grade 3 nephrotic syndrome
	Chen and al. (Keynote 087) [62]	210	*TRAE grade 1 or 2*: (i) Hypothyroidism (11.9%) (ii) Pyrexia (10%) (iii) Fatigue (8.6%) (iv) Rash (7.6%) (v) Diarrhea (6.2%)	*6.4% TRAE grade 3/4*: (i) Neutropenia (2.4%) (ii) Dyspnea (1%) (iii) Diarrhea (1%) (iv) Pyrexia (0.5%) (v) Cough (0.5%) (vi) Fatigue (0.5%) (vii) Hypothroidism (0.5%)	(i) 4.3% discontinued because of TRAEs (ii) 12.4% experienced TRAEs resulting in treatment interruptions.
	Zinzani et al. Keynote 087 updated results (ASH 2018) [63]	210	*72.9% TRAE of any grade*: (i) Hypothyroidism (14.3%) (ii) Pyrexia (11.4%) (iii) Fatigue (11%) (iv) Rash (11%)	*11.9% TRAE grade 3/4*: (i) Neutropenia (2.4%) (ii) Diarrhea (1.4%)	6.7% of patients discontinued treatment due to TRAE
Avelumab	Chen et al. [91]	31	*Treatment-related AE of any grade*: (i) IRR (26.7%) (ii) Nausea (20%) (iii) Rash (20%) (iv) Fatigue (13.3%)	*36.7% grade ≥3 AEs* – details NA	2 patients (6.7%) discontinued treatment due to IRR

Table 3: Continued.

Agent	Reference / Phase	No. of patients	Most Common AE	Grade 3 or 4 AEs	Comments
Sintilimab	Shi et al. (ORIENT-1) [68]	96	*93% treatment-related AE of any grade*: (i) Pyrexia 41% (ii) Hypothyroidism 20% (iii) Increased ALT 14% (iv) Pneumonitis 11% (v) Infusion reaction 9% (vi) Rash 11% (vii) Increase AST 8% (viii) Decreased platelet counts 10% *54% immune-related AE*	*18% treatment-related AE grade 3 or 4* *11% Drug-related serious adverse events*: (i) Pneumonitis 3% (ii) Lung infection 3% (iii) Infusion reaction 2% (iv) Upper respiratory tract infection 1% (v) Liver function abnormality 1% (vi) Decreased platelet counts 1% (vii) Peripheral neuropathy 1% (viii) Hyperthyroidism 1%	3% (3 patients) discontinued treatment due to adverse events (i) 1 patient with grade 2 pneumonitis (ii) 1 patient with grade 4 liver function abnormality (iii) 1 patient with grade 3 pneumonitis and grade 4 decreased platelet count.

IRR = infusion related reactions; AE = adverse event; TRAE = treatment-related AE; NA = not available.

stop the treatment. Some patients showing these pseudoprogressions may experience late responses and even long-lasting clinical benefit from anti-PD-1 therapy. The Lugano Classification lymphoma response criteria have been refined in 2016 to address this specific issue [78]. They determined the imaging criteria suggestive of pseudoprogression (in the absence of clinical deterioration) and classified these scenarios as "indeterminate responses" necessitating additional tests in order to identify a true progressive disease. If such an eventuality is confirmed, the treatment of patients who do not respond to anti-PD-1 therapy is a main concern. Rossi and colleagues retrospectively described the treatment of 30 patients with cHL highly pretreated and who failed anti-PD-1 therapy. Seventeen patients were treated with chemotherapy alone (group 1) and 7 with chemotherapy and anti-PD-1 (group 2). ORR was 59% and 86%, respectively. This observation suggests that anti-PD-1 therapy could resensitize tumor cells to chemotherapy-induced death [79].

4. New Perspectives

Future aim for patients with Hodgkin's disease, as well as with other lymphoma types and more broadly with cancers, is to provide them with more efficient treatment where side effects remain as minor as possible and manageable. In this endeavor, ICI has opened a new way to treat patients, at the price of awakening autoimmunity.

It can be foreseen that efficacy of actual checkpoint inhibition, targeting PD-1/PD-L1 (and CTLA-4), could be ameliorated when we contemplate and consider using some of the myriad of checkpoint molecules involved in the interactions of immune cells capable of killing lymphoma cells [80]. Such analysis has inherent complexity that resides not only within the number of checkpoints implicated, but also in their cell specificity and time-dependent expression.

One could foresee that combining ICI with classical chemotherapy, especially in heavily treated patients, would reduce the number of antigen-presenting and immune effector cells implicated in antilymphoma immune response awakened by ICI, and globally weakening ICI. Actually, ICI are prescribed mostly in heavily treated patients, but implementing ICI earlier in the course of the disease could be more beneficial in terms of treatment efficacy.

The Specificity of ICI efficiency resides in large part within the mutational burden of the tumor leading to neoantigen formation and presentation to immune cells. The antitumor response could be boosted with tumor-derived vaccines where patients are simultaneously receiving ICI. This alternative has been evaluated in preclinical models with success and is evaluated nowadays in some cancers, including lymphomas.

Finally, combining ICI with active and passive immunization could be most beneficial. This can be done by immunizing patients with tumoral neoantigen, or by transferring to patients tumoral neoantigen-specific cells (modified or not) followed by ICI to boost the antilymphoma response. Such an avenue is currently evaluated using T cells specific for EBV in combination with PD-1-PD-L1 blockade in EBV^+ lymphomas, including EBV^+ Hodgkin's disease (NCT02973113).

Immune checkpoint inhibitors are all implicated in classical as well as antilymphoma immune responses. A global analysis of ICI distribution, both spatially and with time, could help to design ICI administration with better specificity (i.e., targeting IC preferentially expressed in lymphoma versus healthy tissues) and efficacy (time and length of treatment). Such an analysis has already been undertaken without always-clear conclusions. This is probably due to the complexity of the antilymphoma immune response parameters. This complexity is best illustrated by the observations that ICI are even dependent on gut microbiota [81, 82]. In the future, using a more complete picture of biomarkers would help.

Additionally, one could envisage using chemotherapeutic drugs with known immunomodulatory activities to enhance the efficacy of immune checkpoint inhibition, such as cyclophosphamide and anthracyclines. These chemotherapies can elicit an immunogenic cellular death (ICD) characterized by apoptotic bodies exposing calreticulin and releasing adenosine triphosphate (ATP) and high-mobility group box 1 protein (HMGB1) which act as an "eat me" signal towards adaptive immune cells [83–85]. Interestingly, hypomethylating agents have also been proposed to prime ICI. Interesting immunomodulatory properties of Bruton kinase inhibitors (BTK) such as ibrutinib have been pointed out in lymphoma and myeloma xenograft models [86]. Through the off-target inhibition of ITK (interleukin-2-inducible T-cell kinase), ibrutinib is able to polarize T-cell response towards à Th1 tumor rejection-prone phenotype. This property is also amenable to ICI treatment combinations and is now being tested in R/R cHL (NCT02940301).

Combining radiotherapy with ICI has the potential to boost specifically the antilymphoma efficiency of ICI while preserving the development of an immune response. Indeed, radiotherapy leads to lymphoma cell death, liberates immune-activating chemokines and cytokines, activates dendritic cell and antigen presentation, and induces effector cells (such as CTL and NK cells) activation and proliferation [87]. ICI would boost the efficiency of these effector cells, resulting in preferentially local antilymphoma efficacy. In the context of cHL known to be most sensitive to radiotherapy, this combination may be highly synergistic and may conduct therapeutic strategies using lower doses of irradiation than those in current protocols.

Last, but not least, the education of medical specialists will have to be adapted according to these novel combination therapies in terms of both available therapies and follow-up exams. One example of the latter is that response is classically evaluated using radiological exams such as scanner in combination with FDG-PET. Such an exam cannot differentiate between residual lymphoma cells and activated and proliferating immune cells around and within the tumor. This can lead to a false positive evaluation of patient under ICI treatment.

5. Conclusion

Since the advent of modern oncology in the mid-fifties, cHL was the standard-bearer of chemotherapy and radiotherapy early successes. Thanks to these achievements, cHL is

nowadays considered a curable disease. However, a subset of patients still suffer from relapses, and despite being considered a disease of young adults, senior populations also display a peak of incidence. These relapsing/refractory patients pose a challenge for oncohematologists, regarding the ways to achieve high response rates and cures in the young patient population, but also to limit toxicities while assuring the best quality of care in the older one. PD-1 inhibition is revealed to be a valuable therapeutic option in these situations, with a high response rate and a reasonable toxicity profile. However, practitioners should not underestimate autoimmune toxicities and should be aware of the need to initiate immediate interventions if they occur. With immunotherapy being a new treatment modality, clinicians should be trained to recognize these adverse events. Many questions are still open, such as if ICI will indeed be able to decrease long-term toxicities of current standard treatment protocols, replace radiotherapy, or continue as salvage therapy, but also regarding the interpretation of treatment responses. In this new era of immunotherapy, Hodgkin's disease is once again colliding with the history of oncology, as a paradigm of ICI sensitivity and a model for therapy development.

Conflicts of Interest

Authors declare that they have no conflicts of interest.

Authors' Contributions

Diego De Goycoechea, Gregoire Stalder, and Filipe Martins contributed equally to the review.

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Review Article

The Combination of Stereotactic Body Radiation Therapy and Immunotherapy in Primary Liver Tumors

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Treatment recommendations for primary liver malignancies, including hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA), are complex and require a multidisciplinary approach. Despite surgical options that are potentially curative, options for nonsurgical candidates include systemic therapy, radiotherapy (RT), transarterial chemoembolization (TACE), and radiofrequency ablation (RFA). Stereotactic Body Radiation Therapy (SBRT) is now in routine use for the treatment of lung cancer, and there is growing evidence supporting its use in liver tumors. SBRT has the advantage of delivering ablative radiation doses in a limited number of fractions while minimizing the risk of radiation-induced liver disease (RILD) through highly conformal treatment plans. It should be considered in a multidisciplinary setting for the management of patients with unresectable, locally advanced primary liver malignancies and limited treatment options. Recently, the combination of immunotherapy with SBRT has been proposed to improve antitumor effects through engaging the immune system. This review aims at shedding light on the novel concept of the combination strategy of immune-radiotherapy in liver tumors by exploring the evidence surrounding the use of SBRT and immunotherapy for the treatment of HCC and CCA.

1. Introduction

1.1. Primary Liver Tumors. Primary liver cancer is the seventh most common cancer world-wide, with around 841,080 newly diagnosed cases in 2018 [1]. It is the third leading cause of cancer deaths in the world, with an estimated 781,631 liver cancer deaths occurring in 2018 [1]. It is also the fifth largest contributor to cancer mortality in the United States [2]. Although patients diagnosed at early stages have a relatively good prognosis, the majority of patients are diagnosed at later stages. The 5-year survival rate for all Surveillance, Epidemiology, and End Results (SEER) stages combined is 18%, and it drops to 2% in patients presenting initially with late stage disease [2, 3]. The two most common subtypes of primary liver tumors are HCCs that arise from hepatocytes and intrahepatic cholangiocarcinoma (IHCs) that arise from epithelial cells of the intrahepatic bile ducts [4].

1.2. Hepatocellular Carcinoma: Epidemiology and Prognosis. HCC accounts for 75 to 85% of primary liver cancers world-wide [1]. Its prevalence is highest in Eastern and Southern Asia and among males [5]. Recently, although the incidence has been declining in high-risk regions, the incidence in lower-risk areas including India, Europe, and North America is on the rise as rates of hepatitis C, obesity, and diabetes continue to increase. For instance, it has doubled from 2.6 to 5.2 per 100,000 populations over the period between 1990 and 2014 [6, 7].

HCC is the second most frequent cause of cancer death in men and the sixth leading cause of cancer death in women [1, 8]. Although surgical resection, liver transplantation, and ablation offer a potential for cure, only 20% of patients with HCC are suitable for primary surgical management at the time of diagnosis [9, 10]. The remaining 80% are diagnosed at advanced stages when curative treatments become nonfeasible [11, 12]. In fact, most patients with HCC often present with locally advanced, unresectable disease, when the tumor has already extended or invaded major vasculature. The absence of effective therapies in such cases contributes to the poor prognosis of HCC, with a 5-year

survival rate and a median overall survival (OS) that are less than 5% and 1 year, respectively [13–15]. Patients with advanced HCC are therefore offered nonsurgical approaches such as chemotherapy, targeted therapy, immunotherapy, TACE, RT, or percutaneous ethanol injection (PEI) [16–19]. Not only does the dismal prognosis of HCC patients stem from the advanced stage at presentation, but also it arises from high recurrence rates. In fact, nearly 80% of tumors recur 5 years following hepatic surgery [20].

1.3. Intrahepatic Cholangiocarcinoma: Epidemiology and Prognosis. The pathogenesis of IHC seems to be related to chronic inflammation and the resulting oxidative stress created in bile ducts [21]. IHC constitutes around 3% of gastrointestinal cancers [22]. It is the second most common primary hepatic malignancy in the United States following HCC, with around 5000 newly diagnosed cases per year [1]. The relative incidence was higher in men than in women over the period from 2008 to 2012 [22]. Several epidemiological studies show that while the incidence of extrahepatic cholangiocarcinoma (EHC) has decreased or stabilized, that of IHC continues to increase and has doubled among Asians as compared to African-Americans and Caucasians [22, 23].

The 5-year survival in IHC patients is less than 10%. The dismal prognosis is due to advanced stages at time of diagnosis, limited treatment options, and very high rates of recurrence and metastases [24]. Surgical resection remains the only potentially curative treatment option and is rarely feasible except in early stages of IHC [25]. Unfortunately, however, less than 20% of patients with IHC are candidates for surgical resection at the time of diagnosis. The remaining 70% have unresectable or advanced diseases requiring systemic therapies such as chemotherapy [26–28]. Such nonoperative therapies have significant limitations and the median survival for patients with inoperable disease remains poor (7 to 12 months). Even among patients who are surgical candidates, recurrence rates are as high as 52%, and 5-year postresection survival rates range from 8% to 44% [23, 27–31]. Whether primary or recurrent, most patients survive about 6 months in the absence of any treatment [23, 27, 29, 30, 32].

2. Management: Evolving Paradigms in Immunotherapy and Radiotherapy

Treatment recommendations for primary liver tumors are complex and require a multidisciplinary approach. Despite surgical options that are potentially curative, options for nonsurgical candidates include systemic therapy (immunotherapy, tyrosine kinase inhibitors [TKIs], and chemotherapy), external-beam radiation therapy (EBRT), TACE, and percutaneous tumor ablation ([RFA], microwave ablation [MWA], PEI, and cryotherapy). Here we focus on evolving RT and immunotherapy approaches.

3. Radiotherapy

Historically, EBRT has not played a substantial role in the treatment of liver malignancies secondary to the limited tolerance of the whole liver to radiation. Over the past decade, the approach to liver cancer patients has been affected by a paradigm shift that has revolutionized RT [37–39]. Consequently, RT has become the preferred treatment option for inoperable patients with tumors situated near the main portal vein, inferior vena cava, or the hilum of the liver [40]. Such tumors can cause liver failure related to vascular or biliary compromise, and surgical resection is not an ideal alternative given the location.

3.1. Radiotherapy and the Immune Response. It is well established that RT has direct cytotoxic effects on cancer cells and can generate a robust antitumor immune response through effects on both the tumor and its microenvironment. This occurs via a variety of mechanisms including enhanced tumor antigen presentation and upregulated major histocompatibility complex (MHC) class I expression [41]. The high doses of radiation used in SBRT increase tumor-cell lysis at the level of the localized treatment site and release tumor-associated antigens (TAA) in the process. The released TAAs are taken up by professional antigen presenting cells (APC), including dendritic cells (DC) and macrophages [42, 43]. Proinflammatory cytokines can then activate the APCs that migrate in turn to tumor-draining lymph nodes. Here, CD8+ cytotoxic T-cells are activated to provide antitumor immunity [44]. In addition to enabling the mobilization of T-cells against cancerous cells with the help of released TAAs, radiation results in the translocation of calreticulin (CRT) to the tumor-cell surface [45, 46]. This serves as a signal to activate macrophages and DCs which internalize CRT-expressing tumor cells.

Emerging clinical data suggest that RT may have systemic effects that go far beyond the locally irradiated target [47, 48]. Such abscopal effects refer to the ability of radiation delivered to a local site to minimize or eradicate metastases at distant sites, outside of the treatment field [49]. This nonspecific eradication of distant tumors and metastases can be accounted for by the systemic increase in the levels of proinflammatory cytokines and chemokines released from both the immune cells and tumor tissues, following exposure to radiation [43].

3.2. Stereotactic Body Radiation Therapy. SBRT is a highly specialized form of EBRT that delivers high doses of precisely targeted radiation in a few fractions to a tumor and minimizes radiation dose to adjacent normal tissue structures [50, 51]. It maximizes the cell-killing effect on the target, while at the same time minimizing injury in adjacent normal tissues. This hypofractionated image-guided RT is typically utilized for small tumors that require precise targeting. It is made possible by major improvements in patient immobilization, positioning accuracy, organ motion assessment, and radiation planning techniques [52].

SBRT is now in routine use for the treatment of lung cancer, and there is growing evidence supporting its use in primary liver tumors. Data reveal 1-year local control (LC) rates exceeding 90% following the use of SBRT in HCC [53] and hepatic metastases [54]. This has rendered SBRT the

focus of many studies that assess its safety and efficacy in primary hepatic tumors [55]. The use of high-dose ablative radiation is currently under evaluation in HCC in a phase III trial (NCT01730937) by the Radiation Therapy Oncology Group.

3.3. Stereotactic Body Radiation Therapy versus Percutaneous Tumor Ablation. Percutaneous tumor ablation, typically by RFA or MWA, is usually performed in patients with early stage, unresectable HCC. A retrospective study from the University of Michigan highlighted the relative efficacy of using SBRT as compared to percutaneous tumor ablation in patients with unresectable HCC [56]. Freedom from local progression (FFLP) rates at 1 and 2 years was lower with RFA than with SBRT for all tumors, whether less than or more than 2cm in size. The rates of grade 3 or higher acute toxicity were higher with RFA (11%) than with SBRT (5%). An ongoing trial at the Durham VA Medical Center is currently comparing the use of SBRT and MWA in surgical candidates who decline surgery or nonoperative, early stage HCC cases [NCT03402607].

3.4. Stereotactic Body Radiation Therapy versus Transarterial Chemoembolization. A large single-institution comparison of TACE and SBRT outcomes was performed on 209 patients with small HCC tumors (2.3-2.9cm) [57]. 84 patients with 1 to 2 tumors underwent TACE to 114 tumors, and 125 patients with 1 to 2 tumors underwent SBRT to 173 tumors during the period from 2006 to 2014. While no OS differences were noted, 2-year LC rates were higher with SBRT than with TACE (91% versus 23%). In addition, grade 3+ toxicity rates were higher in the TACE arm than in the SBRT arm (13% versus 8%). This suggests that SBRT can be used as a safe alternative to TACE.

Whereas TACE remains the most common locoregional treatment to serve as a bridging modality in HCC patients undergoing liver transplantation, the best one remains unclear. In a randomized phase II trial, 29 HCC patients with Child-Pugh Class (CTP) A/B liver cirrhosis who were planned for liver transplantation were randomized to either SBRT or TACE from 2014 to 2016 [58]. 12 patients received SBRT for a median total dose of 45Gy delivered over 5 fractions, and 15 patients received 2 TACE treatments. SBRT was shown to reduce hospitalizations and to be equally effective in TACE as a bridge to liver transplantation. The ongoing TRENDY trial [NCT02470533] is based on the hypothesis that the time to progression is more favorable after SBRT than after TACE in HCC patients ineligible for surgery or RFA. Results are expected to be available in April 2020. SBRT, therefore, represents a noninvasive, potentially curative modality that can be utilized in the definitive treatment of patients with HCC and as a bridge for patients potentially eligible for transplantation. While the 2016 NCCN guidelines listed EBRT as a viable option for the treatment of advanced HCC, current 2018 NCCN guidelines implement the role of SBRT in both resectable and nonresectable cases planned for transplantation [59].

In order to evaluate the combination of SBRT and TACE, a retrospective study of patients with HCC that are larger than 3cm in size was conducted [60]. Patients treated with SBRT following TACE experienced a median survival that is 13 months longer than that of patients treated with TACE alone. They tolerated SBRT well with no instances of significant morbidity being noted. The favorable survival outcomes resulting from the combination therapy support the notion that the strengths and weaknesses of SBRT and TACE are complementary. The improved survival mainly stems from improved LC rates and local recurrence rates in the combination group. Many ongoing studies are currently evaluating the combination of TACE with SBRT [NCT01918683, NCT02507765, NCT02513199, and NCT02794337] and comparing SBRT to TACE [NCT02182687 and NCT02470533].

3.5. Studies Evaluating the Use of Stereotactic Body Radiation Therapy for Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma. Despite its relatively recent adoption into clinical practice, SBRT use in liver tumors was first described in the early 1990s in a pilot study by Blomgren et al. [61]. Nine patients with HCC and one patient with IHC were treated with one to three fractions of 5 to 15Gy. Objective response (OR) rates were 70%, with around 50% of tumors having been shrunken or disappeared at time of evaluation and around 80% of tumors having not progressed on follow-up after 1.5 to 38 months. A number of retrospective studies and large single-institution phase I and II prospective trials evaluating the use of SBRT in the treatment of primary liver tumors followed.

One retrospective study by Sanuki et al. included 185 liver tumors ranging in diameter from 0.8 to 5cm and used SBRT doses ranging from 30 to 40Gy in 5 fractions [62]. 3-year LC and OS rates were 91% and 70%, respectively.

A phase I trial included 41 patients (31 HCC and 10 IHC) with unresectable tumors that have a median liver tumor size of 173mL [11]. After completing an SBRT treatment with a median dose of 36Gy in 6 fractions, the median survival for all patients was 13.4 months. 1-year survival and 1-year infield LC rates were 51% and 65%, respectively. No RILD or treatment-related grade 4 or 5 toxicities were noted within the first 3 months after treatment. The promising results from this phase I trial laid the foundation for further phase II and III studies of six-fraction SBRT in this setting.

A phase II trial from Princess Margaret Cancer Centre included patients with CTP Class A disease and used SBRT doses ranging from 24 to 54Gy in six fractions [53]. Results revealed a median OS of 17 months and a one-year LC rate of 87%. Grade 3 or higher toxicities were noted in 36% of patients and primarily consisted of asymptomatic lab abnormalities.

At the University of Michigan, an individualized dose allocation strategy using hyperfractionation was developed for liver cancer treatment and was evaluated by a phase II study. It included 46 IHC patients who were treated with conformal hyperfractionated RT with concurrent hepatic arterial fluorodeoxyuridine. Compared with historical

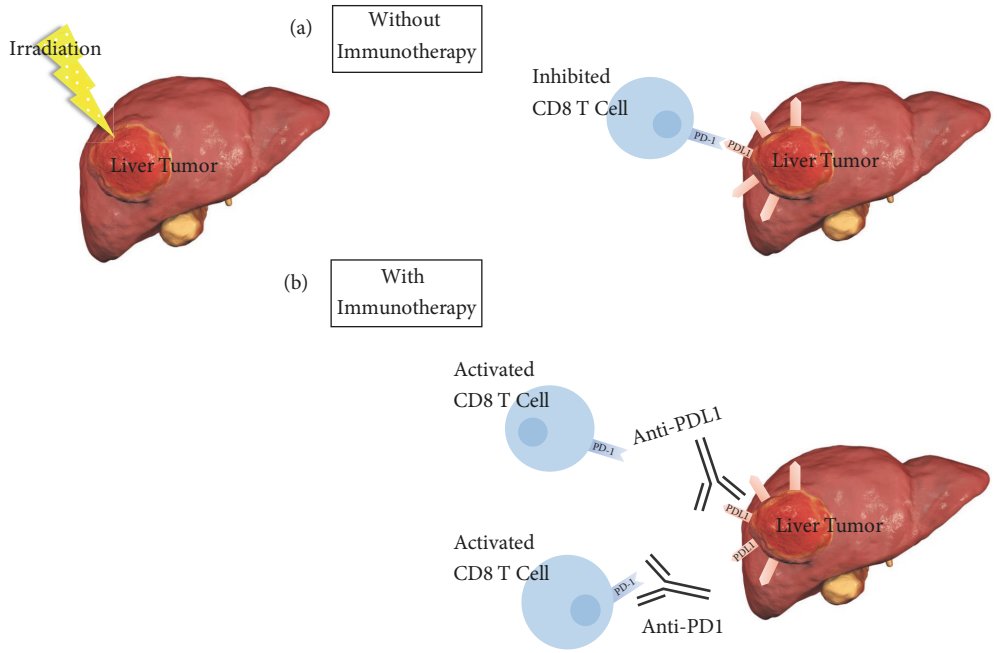

Figure 1: Irradiation of liver tumors with and without immunotherapy. (a) Liver tumors usually acquire radio-resistance through programmed-death ligand-1 (PD-L1) upregulation after radiation since the PD-L1/ programmed-death-1 (PD-1) axis induces CD8 T-cell exhaustion and results in tumor escape from the host immune response. (b) In order to overcome limitations of PDL1 expression upregulation and to reduce the rate of tumor recurrence in primary liver tumors, an immune-based treatment approach targeting PDL1 or PD-1 might be of help in harnessing an immune response to effectively kill liver tumor cells and reduce the rate of tumor recurrence.

controls, patients had significantly improved OS with a median survival of 13.3 months [63].

Therefore, based on initial promising data, SBRT seems to be a safe, effective, and noninvasive treatment option for carefully selected patients with unresectable tumors that are not amenable to other treatments.

3.6. Combining Stereotactic Body Radiation Therapy with Immunotherapy. Although there is evidence that RT alone provides the necessary signals for the cross-priming of cytotoxic T lymphocytes against tumor antigens, the adjuvant effect of RT appears to be relatively weak, and abscopal responses to RT alone are extremely rare. Also, despite the antitumor effect induced by RT, tumors often develop resistance via immune-escape mechanisms that promote recurrence [64, 65]. In HCCs, for example, frequent resistance to RT is acquired, often resulting in recurrence [66]. This radio-resistance usually occurs through programmed-death ligand-1 (PD-L1) upregulation after radiation [67, 68]. The PD-L1/ programmed-death-1 (PD-1) axis then induces T-cell exhaustion and results in tumor escape from the host immune response, as illustrated in Figure 1.

In order to overcome limitations created by the PD-L1/PD-1 interaction and to reduce the rate of tumor recurrence in primary liver tumors, novel therapies are required. Recent discoveries in tumor immunology, paralleled by technological advances in RT, have provided a promising role for combining SBRT with immunotherapy to augment and sustain the proimmunogenic antitumor effects seen with SBRT alone.

4. Immunotherapy in Liver Tumors

Since immune checkpoint inhibitors (ICIs) were first reported in 2010 and 2012, they have translated into a significant OS advantage in comparison to established therapies in metastatic melanomas and nonsmall-cell lung cancer (NSCLC) [69]. Their role is currently being evaluated in some gastrointestinal cancers. Given the background of chronic inflammation in the pathogenesis of many primary liver tumors, the use of immune-based treatment approaches might have a role in releasing the brakes created by the tumor on the immune system [70, 71]. Also, in order to overcome limitations of PD-L1 expression upregulation, an immune-based treatment approach targeting PD-L1 might be of help in harnessing an immune response to effectively kill liver tumor cells and reduce the rate of tumor recurrence. The two most actively studied inhibitory immune checkpoint receptors are cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and PD-1.

(1) Anticytotoxic T-Lymphocyte Associated Antigen-4 Agents. CTLA-4 receptor is exclusively expressed on regulatory T-cells, naïve T-cells, and activated T-cells, and it acts as a regulator of immune cells [72–74]. Through its binding to CD80 and CD86 located on APCs, it promotes immunosuppressive effects of regulatory T-cells [75, 76]. It is currently the second most common checkpoint receptor to be targeted.

The anti-CTLA-4 antibody, Tremelimumab, was evaluated in a phase I trial that included 21 patients with inoperable HCC tumors [77]. 17.6% and 76.4% of patients had partial

response (PR) and stable disease (SD), respectively, and 45% of patients experienced SD for more than 6 months. Although the use of Tremelimumab was well tolerated, it has not been approved yet by the FDA. The results of an ongoing phase I clinical trial combining Tremelimumab with RFA or TACE are still pending.

(2) Antiprogrammed-Death Ligand-1 and Antiprogrammed-Death -1 Agents. PD-1 receptor belongs to the CD28 superfamily and is expressed on regulatory T-cells, B-cells, and myeloid derived suppressor cells (MDSCs) [78]. It transmits coinhibitory signals and limits tumor-infiltrating lymphocytes (TILs) and T-cell proliferation in peripheral tissues. This results in effective immune resistance in the tumor microenvironment [79, 80]. Most data suggest that TILs are established prognostic markers in melanomas and breast cancers, and a recent study has shown that this applies to liver tumors as well [81, 82]. For instance, the presence of CD8+CD45RO+ TILs in surgical specimens obtained from EHC patients has been associated with prolonged OS [83].

PD-L1 is undetectable in most normal tissues and is inducible by inflammatory cytokines, especially types I and II interferon (IFN) [70]. It is frequently expressed on the surface of tumor cells. One study included tumor tissue samples from 37 patients with CCA and analyzed them immunohistochemically for markers including PD-L1 [84]. Almost 94% of samples were positive for PD-L1. Another study that included EHC tumors showed that 12% of tissue samples and 30% of tumor-associated macrophages were positive for PD-L1 [85]. This increased expression of PD-L1 in CCA cells was shown to be associated with poor prognosis [86]. In another study done on HCC patients by Shi et al., increased PD-1 expression in circulating and tumor-infiltrating CD8+ T-cells was also associated with poor disease progression [87].

Many phase I/II trials have shown promising outcomes with the use of the humanized anti-PD-1 antibody, Nivolumab, in patients with advanced melanoma, lung cancer, and renal cell carcinoma (RCC) [88]. This was followed by several trials evaluating its efficacy in HCC patients. Interim analysis results of a phase I/II trial (CA209-040) were presented at the 2015 ASCO meeting in Chicago, and they were promising considering the poor characteristics of recruited HCC cases [89]. For instance, the use of Nivolumab in a range from 0.1 to 3 ml/kg was associated with a 62% OS rate, a 19% response rate, a 5% complete response (CR) rate, and a 67% disease control rate at 12 months. Also, despite having terminated Nivolumab therapy several months after the attainment of CR, the two patients who attained CR within 3 months maintained this response for longer than 18 months. Such durable responses were observed at all dose levels of Nivolumab in all cohorts.

Results also showed that all participants, whether infected by HBV or HCV or not, encountered tumor size reduction [89]. One patient continued to have reduction in tumor size from around 10 to 2cm over a period of 48 weeks, and another patient had a marked drop in alpha-fetoprotein level from 21,000 to 283ng/mL. Many cases had a large number of multiple HCCs disappear after 6 weeks of therapy.

The report also indicated that Nivolumab monotherapy had a favorable safety profile in HCC similar to that seen in other types of cancer [89]. In fact, a dose-escalation study revealed that Nivolumab can safely be administered up to a dose of 3 ml/kg in HCV- or HBV-infected individuals and a dose of 10 ml/kg in the uninfected group. The only CTCAE grade IV adverse event noted was an elevation in lipase levels. Grade III increases in aspartate amino transferase and alanine amino transferase levels were seen in 11% and 9% of patients, respectively.

Many ongoing trials are assessing the efficacy of combining Nivolumab with gemcitabine/cisplatin on one hand, or Ipilimumab on the other, as a first-line therapy in advanced, unresectable CCA [90]. A single institution study is evaluating the efficacy of combining Nivolumab (n=4) or Pembrolizumab (n=10) with the multikinase inhibitor, Lenvatinib, as a second-line therapy in advanced biliary tract cancer cases who have failed prior anticancer therapy. Results from the interim analysis of the latter study were promising and showed a median PFS of 5 months and an OR rate of 21.4%. Also, 21% (n=3) of patients had PR and 79% (n=11) had SD [91].

A phase II trial evaluated whether testing for mismatch-repair (MMR) deficiencies in treatment-refractory cases of IHC might be of help in identifying those who might benefit from PD-1 pathway blockade [92]. Out of the 4 patients with MMR-deficient, metastatic, and treatment-refractory IHC who were treated with Pembrolizumab, one (25%) had CR and 3 (75%) had SD. In the interim analysis of the phase II KEYNOTE-028 trial (NCT02054806) that evaluated the role of Pembrolizumab in advanced CCA, almost 34% (n=8) of patients with positive PD-L1 expression had PR or SD [93]. It remains unclear, however, whether mutational tumor burden or PD-L1 expression is a better predictive biomarker in CCA.

Ongoing trials will provide us with more details about the role of targeted immunotherapy in primary liver tumors. Antibodies targeting PD-1, PD-L1, or CTLA-4 are expected to be approved and implemented in the setting of HCC and IHC in the very near future.

5. Rationale for the Combination of Immune Checkpoint Inhibitors and Radiotherapy

Immunotherapy has emerged as an attractive therapy for refractory cancers. Although results obtained in melanoma and NSCLC patients were beyond what is achievable with conventional therapies, it is expected that such responses will only occur in a subset of HCC or IHC patients who have a high tumor mutational burden [94]. In order to complement the therapeutic effect of immunotherapy in primary liver tumors, there is a need for a combination strategy. Emerging data demonstrate that one strategy to bolster the systemic antitumor immunity in response to immunotherapy is to combine it with RT [95]. Similarly, an abundance of studies suggest that the immunomodulatory effects of RT can be leveraged when combined with immune-based approaches [96].

RT results in the immunogenic death of tumor cells. It primes tumor-specific T-cells and induces the production of IFN-beta by T-cells, thereby enhancing MHC class 1 expression on parental and resistant cancer cells [97]. This restores the responsiveness of resistant tumors to anti-PD-1 therapy. The addition of anti-PD-1 antibodies to RT is therefore expected to promote the proimmunogenic tumor microenvironment.

5.1. Synergistic Effects of the Combination Therapy in Preclinical Studies. Many preclinical studies demonstrate a synergistic effect when RT and CTLA-4 or PDL-1 inhibitors are combined [67]. In a study by Yoshimoto et al., mice exposed to the combination of RT and anti-CTLA-4 antibody had improved antitumor immunity and prolonged tumor growth delay (from 13.1 to 19.5 days) when compared to those exposed to RT alone [98]. Vanpouille-Box et al. assessed the use of radiation-induced vaccination in mouse tumor models and observed improved survival following treatment with PD-1 and TGFβ blockade but not with TGFβ blockade alone [99]. Exposure of mice with intracranial gliomas to the combination therapy resulted in a markedly increased survival benefit when compared to those exposed to either treatment alone [100]. Tumors of these mice had the highest level of cytotoxic T-cells and the lowest extent of regulatory T-cell infiltration among all groups.

Local RT and systemic PD-L1 blockade augment T-cell responses not only in the primary tumor but also at distant sites [67]. This refers to the abscopal effect that was first described in the 1950s by Mole et al. [101]. It is defined as the regression or disappearance of lesions outside of the irradiated field. This phenomenon has rarely been observed in routine clinical practice following the administration of RT alone [102]. With the advent of immune modifiers, however, the abscopal effect has been increasingly reported in preclinical models since radiation-induced systemic abscopal responses can be facilitated with additional immune manipulation [67, 99, 103–110].

Combining RT with anti-PD-1 antibody treatment has consistently produced abscopal effects on secondary tumors that are distant from the irradiated primary site in mouse models of melanoma, colon cancer, RCC, and breast cancer [99, 107]. Interestingly, a study done by Demaria et al. suggested that adding CTLA-4 blockade to RT (12Gy) in the treatment of 4T1 mice with primary mammary carcinomas inhibited the formation of lung metastasis [111]. A subsequent study from the same group combined a hypofractionated regimen (3 × 8Gy) with the anti-CTLA-4 therapy, and immune infiltrates and abscopal effects were found to be more pronounced than when either modality was used alone [108].

The timing of RT relative to immunotherapy may be another important consideration when combining RT with immunotherapy [95]. This question has not been addressed thoroughly in the preclinical models. In a study on a mouse model of breast cancer in which a combination of CTLA-4 blockade and RT was used, the antibody was administered at different time points with the best abscopal response being seen when the first dose of antibody was given during RT [108].

5.2. Mechanisms That Improve Antitumor Immune Responses and Abscopal Effects

(1) Amelioration of Cancer-Cell Type I Interferon. It is now established that the cyclic GMP-AMP Synthase (cGAS) - Stimulator of Interferon Genes (STING) pathway plays an important role in improving the antitumor immune response triggered by RT and immunotherapy. It induces IFN which is required to achieve optimal DC recruitment and cross-priming of effector T-cells [112–114]. Crosspresentation corresponds to the mechanism used by DCs to process and present tumor antigens to CD8+ T lymphocytes [115]. This is mainly mediated by a specialized subset of DCs that is dependent on the basic leucine zipper ATF-like transcription factor-3 (BATF-3) transcription factor and sp2/soluble FLT-3 ligand (sFLT-3L) growth factor for development [116]. Data suggests that this BATF-3 DC subset is essential for the therapeutic effects of anti-PD1 and anti-CD137 monoclonal antibodies by means of crosspresentation of tumor antigens [117].

Deng et al. demonstrated that these DCs activate the cGAS-STING signaling axis following the exposure of tumor cells to RT which results in the accumulation of irradiated tumor cell derived DNA. cGAS senses these RT-generated double-stranded DNA fragments and catalyzes the reaction between GTP and ATP responsible for the formation of the second messenger, cyclic GMP-AMP (cGAMP). The latter binds to the STING adaptor protein, triggering the phosphorylation of interferon regulatory factor 3 (IRF-3) by TANK-binding kinase 1 (TBK-1). IRF-3 then transports to the nucleus where transcription of inflammatory genes is triggered and an increase in type I IFN and other immune modulatory molecules results. Type I IFNs induced by RT have been shown to mediate the antitumor immune response and increase the frequency of CD8+ T-cells in tumor-draining lymph nodes [118].

In a preclinical study on mice bearing a B16 melanoma, those that were cGAS-deficient had a lower response to anti-PD-L1 treatment than wild-type controls [119]. cGAS knockout mice had lower numbers of tumor-specific CD4+ and CD8+ T-cells following immunotherapy when compared to wild-type ones. Interestingly, intramuscular injections of cGAMP in cGAS-deficient mice enhanced the effect of anti-PD-L1 treatment.

(2) Reduction of Tumor Microenvironment Immunosuppression. Many studies show that the reduction in MDSC levels observed with the combination therapy might be critical in achieving an abscopal effect. In an experiment performed by Deng et al. on a mouse flank tumor model, substantial tumor regression was noted in the combination treatment group and was thought to be due to the concomitant increase in cytotoxic T-cell infiltration and the dramatic reduction in MDSCs [67]. In a patient receiving both, anti-CTLA-4 agents and palliative RT, an abscopal regression of a distant unirradiated tumor was immediately preceded by a sharp

reduction in the proportion of MDSCs in the peripheral blood mononuclear cell population [109]. Demaria et al. suggested that the abscopal effect is an immune-driven phenomenon caused by T-cells within the irradiated tumor microenvironment [103]. RT primes antitumor cytotoxic T-cells that are usually unable to overcome suppressive effects of the tumor microenvironment without the help of immune modulators. Anti-CTLA-4 and anti-PD-L1 work through separate mechanisms to liberate T-cells from immunosuppression and drive the immune response [33, 120]. Kaminski et al. postulated that cytokines released by these activated T-cells have a major role in generating an abscopal effect [121]. For instance, tumor necrosis factor (TNF) is responsible for the direct elimination of MDSCs both, locally and systemically. In vitro cytotoxicity assays correlate TNF release by activated cytotoxic T-cells with apoptosis of MDSCs [67]. In addition, immunofluorescence staining studies performed on tumors treated with a combination of RT and anti-PDL1 show that MDSCs stained positive for cleaved caspase 3, an apoptosis marker [67]. This interaction between cytotoxic T-cells, TNF, and MDSCs has been verified in in vivo studies in which the expression of exogenous TNF was abrogated in mouse tumor models using an adenoviral vector [122, 123].

5.3. Dose Dependence of the Abscopal Effect. In an attempt to elicit greater antigen release and further improve the efficacy of immunotherapeutic agents, many preclinical studies tested the effect of combining them with hypofractionated regimens, particularly SBRT. The trials to date have used a variety of different doses and fractionations ranging from 15 to 75Gy in 1 to 15 fractions. The choice of optimal radiation dose and fractionation schema is related to the resulting abscopal effects.

A number of studies have demonstrated dose dependence of abscopal effect. Inferior abscopal effects have been noted with a single 20Gy dose of radiation as compared with regimens of 8Gy in three or 6Gy in five fractions [108, 124]. In a recent report in Nature Communications by Vanpouille-Box et al., mice with bilateral TSA tumors were exposed to RT to one tumor and were followed for responses in both irradiated and nonirradiated tumors [112]. In the absence of anti-CTLA-4, a single dose of 20 or 30Gy achieved comparable infield control to that of a regimen of 8Gy delivered in 3 fractions; however, only mice treated with 8Gy in 3 fractions were found to achieve abscopal responses and complete durable regression of their irradiated tumors upon the addition of anti-CTLA4. Of note, such responses were abrogated upon the depletion of CD8+ T-cells.

Gene expression analysis of cells from the irradiated tumors revealed an IFN type I gene signature following exposure to 8Gy in 3 fractions but not following a single dose of 20 or 30Gy [108]. This work explains the dependence of the abscopal effect on dose size and fractionation. Interestingly, several studies suggest a link between the cGAS-STING axis, RT dose per fraction, and RT's synergy with immunotherapy. In multiple murine and human carcinoma cells tested, cytosolic double-stranded DNA accumulated with increasing dose size per fraction up to a critical threshold of 10 to 12Gy. After this cutoff value, the abscopal effect rapidly decreased. Single doses in excess of 10 to 12Gy were found to induce Trex1, the exonuclease that degrades cytoplasmic DNA, thus precluding the activation of the cGAS/STING pathway [112]. In this way, IFN induction does not occur resulting in the absence of RT-induced abscopal effects with doses beyond 10 to 12Gy per fraction [125]. This leads to a decreased synergy between RT and immunotherapy [112, 126]. The work of Vanpouille-Box et al. proposed the challenge of delivering sufficient dose per fraction to generate enough dsDNA to trigger the cGAS/STING pathway, while at the same time preventing Trex1 induction [127]. This opens a new chapter in the debate of the choice of optimal dose and fractionation [128].

5.4. Safety and Efficacy of the Combination Therapy. The promising preclinical data on the combination therapy in mouse tumor models have resulted in a number of analyses reporting the safety and efficacy of this strategy in humans. In a retrospective study by Hubbeling et al., no significant difference was reported between RT-related adverse events observed in metastatic NSCLC patients who received prophylactic cranial RT combined with PD-1/PD-L1 inhibitors as compared to events observed in patients who received only cranial RT [129]. Park et al. demonstrated that in the clinical setting, RT and anti-PD-1 treatment resulted in a near complete regression of the primary tumors and a 66% reduction in distant tumors via abscopal responses [107]. Similarly, Postow et al. reported that palliative RT (28.5Gy in three fractions delivered over 7 days) given concurrently with maintenance Ipilimumab treatment in a patient with melanoma caused regression of the targeted lesion as well as marked abscopal effects [109].

To examine the feasibility and efficacy of RT combined with immune checkpoint blockade, several studies have been conducted as summarized in Table 1. In a phase I clinical trial of 22 patients with multiple melanoma metastases [33], a single lesion was irradiated with 6 to 8Gy delivered over two or three fractions, followed 3-5 days later by four cycles of Ipilimumab. Evaluation of the nonirradiated lesions by CT imaging using Response Evaluation Criteria in Solid Tumors (RECIST) demonstrated that 18% of patients had a PR as best response, 18% had SD, and 64% had PD. The median PFS and OS at median follow-up of 18.4 and 21.3 months were 3.8 and 10.7 months, respectively.

In another prospective clinical trial, 22 patients with stage IV melanoma were exposed to palliative RT (8Gy in 3 fractions or 4Gy in 10 fractions) five days following treatment with 4 cycles of Ipilimumab [34]. RT to 1-2 disease sites were initiated within 5 days after starting Ipilimumab. Patients had ≥1 nonirradiated metastasis measuring ≥1.5 cm for response assessment. Combination therapy was well tolerated without unexpected toxicities. Eleven patients (50.0%) had clinical benefit from therapy at median follow-up of 55 weeks, with 14% of them having CR, 14% having PR, and 23% having SD.

In another clinical trial by Tang et al., 35 patients were treated with SBRT (12Gy in 4 fractions or 6Gy in 10 fractions) either concurrently (1 day after the first dose of Ipilimumab)

Table 1: List of studies on the combination of SBRT and immunotherapy in many cancers.

Author	Disease	N	RT	ICI	Schedule	Abscopal Effects
Twyman Nature [33]	Melanoma	22	6Gy x 2-3 8Gy x 2-3 (One Site)	Ipilimumab 3mg/kg/3w x4	Ipilimumab 3-5 days after RT	PD: 64% SD: 18% PR: 18% CR: None
Hiniker IJROBP 2016 [34]	Melanoma	22	8Gy x 3 4Gy x 10 (1-2 Sites)	Ipilimumab 3mg/kg/3w x4	RT within 5 days of Ipilimumab	SD: 23% PR: 14% CR: 14%
Tang CI. Can Res 2017 [35]	NSCLC, colorectal cancer (CRC), RCC, Others	35	12Gy x 4 6Gy x 10 (1 Site)	Ipilimumab 3mg/kg/3w x4	RT 1 day after Ipilimumab or 1 week after 2nd Ipilimumab	PR: 10% SD: 23% CR: None
Luke JCO 2018 [36]	Ovarian, Endometrial, CRC, Others	73	30-50Gy (3-5, 2-4 Sites)	Pembrolizumab 200mg/3 weeks until progression, death, or toxicity	Pembrolizumab 7 days after SBRT	PD: 38 SD: 21 PR: 8 CR: 1

or sequentially (1 week after the second dose) [35]. Among 35 patients who initiated Ipilimumab, response outside the radiation field was assessable in 31 patients. Three patients (10%) exhibited PR, seven patients (23%) had SD lasting ≥6 months, and none had CR. Of note, clinical benefit was associated with increases in CD8+/CD4+ T-cell ratio, peripheral CD8+ T-cells, and proportion of CD8+ T-cells expressing PD1.

One of the largest prospective phase I studies (Abstract 20) to determine the safety and efficacy of SBRT in combination with Pembrolizumab in patients with metastatic solid tumors who progressed on standard treatment included doses ranging from 30Gy in 3 fractions to 50Gy in 5 fractions. Pembrolizumab therapy was initiated 7 days after the final SBRT treatment. According to data presented at the 2018 ASCO-SITC Clinical Immune-Oncology Symposium, SBRT prior to Pembrolizumab treatment was well tolerated, and the OR rate was 13.5% in the 68 patients who had imaging follow-up. Some abscopal responses were seen, whereby 26.9% of patients had a reduction of at least 30.0% in any single nonirradiated lesion and 13.5% of patients had a reduction of at least 30.0% in the aggregate sum of nonirradiated lesions [36]. A phase III trial (CA184-043) evaluated the use of RT followed by Ipilimumab or placebo in metastatic castration-resistant prostate cancer cases who progressed on Docetaxel chemotherapy. Post hoc analyses of subgroups revealed a trend toward improved OS in the Ipilimumab study arm with a p value of 0.053 [130].

Currently, at least 12 ongoing prospective clinical trials are evaluating the safety and efficacy of the combination of RT and immunotherapy in metastatic NSCLC [131]. Similarly, there are at least 21 clinical trials investigating the combination of RT with other immune-stimulating agents in pancreatic adenocarcinoma [132]. The results of these clinical trials, expected in the next few years, will greatly enhance our understanding of the potential for SBRT to synergize with ICIs to provide clinically meaningful improvements in patient outcomes [131].

As such, current data related to combination therapy in primary liver tumors are based on results from either preclinical animal models, which are inherently limited in their applicability to the clinical setting, or preliminary results from ongoing trials. Although results seem promising, implementation in clinical practice would be premature, as robust hypothesis-testing clinical trials are required to determine appropriate approaches of integrating these modalities.

6. Conclusion

While SBRT alone and immunotherapy alone have shown promise as effective therapies in patients with primary liver tumors, the combination of SBRT and PD-L1, PD-1, or CTLA-4 blockade has not been tested in these tumors [133]. It is expected that such an approach would result in improved therapeutic outcomes similar to those obtained in metastatic solid tumors, including melanomas and NSCLC. Many questions remain with regard to the optimal way to harness ionizing radiation in combination with immunotherapy, and how to best select patients for this approach [134]. We look forward to the results of the clinical trials presented in this review in hopes that outcomes can be improved for primary liver tumors.

List of Abbreviations

APC: Antigen Presenting Cells
BATF-3: Basic Leucine Zipper ATF-like Transcription Factor-3
CRT: Calreticulin
CTP: Child-Turcotte-Pugh
CCA: Cholangiocarcinoma
CR: Complete Response
cGAMP: Cyclic GMP-AMP
cGAS: Cyclic GMP-AMP Synthase
CTLA-4: Cytotoxic T-lymphocyte Associated Antigen-4
DC: Dendritic Cell
EBRT: External-Beam Radiation Therapy
EHC: Extrahepatic Cholangiocarcinoma

FFLP: Freedom from Local Progression
HCC: Hepatocellular Carcinoma
ICI: Immune Checkpoint Inhibitor
IFN: Interferon
IRF-3: Interferon Regulatory Factor 3
IHC: Intrahepatic Cholangiocarcinoma
LC: Local Control
MHC: Major Histocompatibility Complex
MWA: Microwave Ablation
MMR: Mismatch-Repair
MDSC: Myeloid Derived Suppressor Cell
NSCLC: Nonsmall-Cell Lung Cancer
OR: Objective Response
OS: Overall Survival
PR: Partial Response
PEI: Percutaneous Ethanol Injection
PD-1: Programmed-Death-1
PD-L1: Programmed-Death Ligand-1
RILD: Radiation-Induced Liver Disease
RFA: Radiofrequency Ablation
RT: Radiotherapy
RTPCR: Real-Time Polymerase Chain Reaction
RCC: Renal Cell Carcinoma
RECIST: Response Evaluation Criteria in Solid Tumors
STAT-3: Signal Transducer and Activator of Transcription-3
sFLT-3L: Sp2/Soluble FLT-3 Ligand
SD: Stable Disease
SBRT: Stereotactic Body Radiation Therapy
SRS: Stereotactic Radiation Surgery
STING: Stimulator of Interferon Genes
SEER: Surveillance, Epidemiology, and End Results
TBK-1: TANK-Binding Kinase 1
TACE: Transarterial Chemoembolization
TAA: Tumor-Associated Antigens
TIL: Tumor-Infiltrating Lymphocyte
TNF: Tumor Necrosis Factor
TKI: Tyrosine Kinase Inhibitor.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this review paper.

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Review Article

Long-Term Survival, Quality of Life, and Psychosocial Outcomes in Advanced Melanoma Patients Treated with Immune Checkpoint Inhibitors

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Immune checkpoint inhibitors have become a standard of care option for the treatment of patients with advanced melanoma. Since the approval of the first immune checkpoint (CTLA-4) inhibitor ipilimumab in 2011 and programmed death-1 (PD-1) blocking monoclonal antibodies pembrolizumab and nivolumab thereafter, an increasing proportion of patients with unresectable advanced melanoma achieved long-term overall survival. Little is known about the psychosocial wellbeing, neurocognitive function, and quality of life (QOL) of these survivors. Knowledge about the long term side-effects of these novel treatments is scarce as long-term survivorship is a novel issue in the field of immunotherapy. The purpose of this review is to summarize our current knowledge regarding the survival and safety results of pivotal clinical trials in the field of advanced melanoma and to highlight potential long-term consequences that are likely to impact psychosocial wellbeing, neurocognitive functioning, and QOL. The issues raised substantiate the need for clinical investigation of these issues with the aim of optimizing comprehensive health care for advanced melanoma survivors.

1. Introduction

Up to 2010, no medical therapy investigated in a randomized clinical trial had shown to significantly improve overall survival (OS) for patients with unresectable advanced melanoma [1]. Less than half of all patients diagnosed with metastatic melanoma (AJCC stage IV) survived for more than 1 year and only 20% of all patients were alive after 3 years. However, prior to the development of the currently available life-prolonging medical therapies, a small percentage of patients with advanced melanoma experienced long-term survival for more than 5 years. The characteristics of this small subpopulation have never been fully elucidated. Patients with natural indolent evolution of metastatic disease and cases suspect of "spontaneous immune mediated remission" (often coincident with the development of vitiligo) are likely to have contributed to this historical "tail of the survival curve" for stage IV melanoma. In addition, complete resection of oligometastatic stage IV disease can occasionally provide durable remission in a small proportion of patients, but identifying these patients prospectively on objective clinical or histopathological characteristics has not been achieved and requires further investigation. Finally, durable remissions and long-term survival following conventional cytotoxic chemotherapy (e.g., dacarbazine, temozolomide) have also been reported in exceptional cases, most often after a complete response (CR) had occurred [2].

In the 1980s, it was established that a small percentage of patients with favorable baseline characteristics who were treated with high-dose interleukin-2 (IL-2) could achieve a durable complete remission. In a comprehensive review of the outcome of 270 patients with unresectable melanoma (8 clinical trials conducted between 1985 and 1993), receiving IL-2 administered at a high dose resulted in a complete response

(CR) in 6% and a partial response (PR) in an additional 10% of patients. A CR seemed a prerequisite for durable progression-free survival (PFS) as the median response duration in patients obtaining a PR was limited to 5.9 months. These IL-2 treatment regimens were associated with substantial toxicity with grade 5 adverse events (AE) occurring in 2% of patients. The two baseline predictive factors for response to high-dose IL-2 therapy were the performance status and whether patients had received prior systemic therapy. Combination regimens of IL-2, interferon-α (IFN-α), and cisplatin-based combination chemotherapies, while showing high overall response rates with some durable remissions, failed to significantly improve survival rates for patients with advanced melanoma and were subsequently abandoned [3].

Since 2010 effective systemic therapies have become available that improved OS of patients with advanced melanoma. Effective new therapies target the T-cell inhibitory immune checkpoint receptors (including the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and Programmed Death 1 (PD-1) receptors on lymphocytes), or the MAPK-signaling pathway in patients with $BRAF^{V600}$ mutant melanoma, as well as more recently talimogene laherparepvec (T-VEC, the first approved oncolytic virotherapy for cancer offering a survival benefit in patients with stage IV-M1a). Since 2010, all phase III studies conducted with these new agents have reached their primary endpoint, demonstrating improved OS and thereby revolutionizing the treatment options for patients with unresectable advanced melanoma.

2. Ipilimumab

The first systemic treatment ever to significantly improve OS for patients with unresectable advanced melanoma was the CTLA-4 blocking monoclonal antibody (mAb) ipilimumab. This drug was approved in 2011 based on the study outcomes of two randomized phase III trials. The first trial, CA184-002, compared ipilimumab (administered at a dose of 3 mg/kg intravenously [IV] every 3 weeks for a total of four consecutive doses) to a gp100 vaccine or the combination of both in HLA-2 positive patients with pretreated advanced melanoma [4]. For patients, with stable disease after at least 12 weeks of treatment, and who subsequently were diagnosed with progression of disease, reinduction with ipilimumab was allowed. The objective tumor responses according to the Response Criteria in Solid Tumors (RECIST) criteria ranged from 5.7% to 11.0% in the ipilimumab treatment arms. The median OS was improved to 10.0 months for the ipilimumab monotherapy-arm as compared to 6.4 months for the peptide vaccine-alone arm (HR 0.68; $p < 0.001$) (Figure 1). Combination of ipilimumab with the gp-100 vaccine provided no benefit over ipilimumab alone (Table 1).

In a second pivotal phase III study (CA184-024), ipilimumab (administered at a dose of 10 mg/kg every 3 weeks for a total of four consecutive doses and subsequently once every 12 weeks) was combined with dacarbazine chemotherapy (850 mg/m^2) and compared with dacarbazine plus placebo. Median OS was improved for ipilimumab plus dacarbazine (11.2 months) as compared to dacarbazine alone (9.1 months; HR 0.72; $p < 0.001$) (Table 1, Figure 1). The co-administration

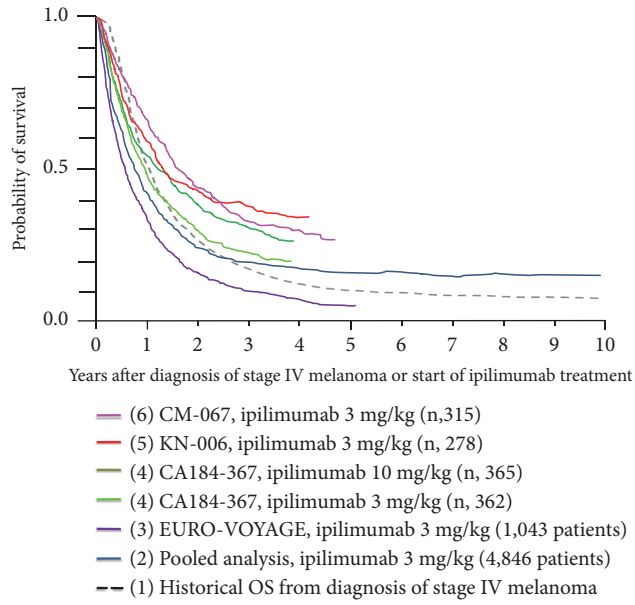

Figure 1: *Overlay of Kaplan–Meier curves indicating the probability for overall survival (OS) for patients treated with ipilimumab as first line of immunotherapy*, representing (1) the historical probability for OS for patients diagnosed with stage IV melanoma prior to the availability of life-prolonging medical treatment options (dashed black line) [1]; (2) a pooled OS analysis including individual patient survival data from 1,861 patients with metastatic melanoma from 12 clinical investigations of ipilimumab and 2,985 patients with metastatic melanoma from a US ipilimumab EAP (total n = 4,846) (dark blue line) [9]; (3) interim results from EURO-VOYAGE, a multicenter, observational, retrospective study of 1043 patients with advanced melanoma who participated in the EU ipilimumab EAP (purple line) [69]; (4) intention-to-treat population (365+362 patients) of the CA184-367 study comparing ipilimumab at 10 mg/kg (dark green line) to 3 mg/kg dosing level (light green line) [8]; (5) intention-to-treat population (278 patients) on the ipilimumab arm from the Keynote-006 trial (red line) [13]; (6) intention-to-treat population (315 patients) on the ipilimumab arm from the Checkmate-067 trial (pink line) [72].

of ipilimumab with dacarbazine significantly increased the incidence of grade 3 or 4 toxicity hepatic toxicity (grade 3 or 4 AEs occurred in 56.3% of patients treated with ipilimumab plus dacarbazine, as compared with 27.5% treated with dacarbazine and placebo) and hepatotoxicity in particular (grade 3 or 4 elevations in liver-function values noted in 17.4 to 20.7% of the patients) [5].

Additional evidence for the long-term beneficial survival effect from ipilimumab came from a large randomized phase II trial in pretreated patients comparing the 0.3, 3, and 10 mg/kg dose levels, indicating a dose-dependent outcome in terms of objective tumor response rate and survival, but also a dose-dependent increase in toxicity [65]. In 2011, ipilimumab received approval by the competent authorities in Europe, the US, and Australia for the treatment of advanced melanoma at a dose of 3 mg/kg administered every 3 weeks for a total of four consecutive doses. The label did not include a reference to the possibility of retreating patients who responded to the initial four doses. Although only a small proportion of

Table 1: Key features of referenced clinical trials with immune checkpoint inhibitors for advanced melanoma.

Name clinical trial Phase	Number of patients	Treatment plan	Primary endpoint	Median OS (95% CI)	Median progression-free survival PFS (95% CI)	Overall survival rates OS
CA184-002 Phase III [4]	676	G1:IPI 3 mg/kg + gp100 G2: IPI G3: gp100 Dose: Every 3 weeks for four cycles Mode: IV	OS	IPI + gp100: 10.0 mos. (8.5-11.5) IPI: 10.1 mos. (8.0-13.8) gp100: 6.4 mos. (5.5-8.7)	IPI + gp100: 2.76 mos. (2.73-2.79) IPI: 2.86 mos. (2.76-3.02) gp100, 2.76 mos. (2.73-2.83)	IPI + gp100, IPI, gp100: 1 yr.: 43.6% vs 45.6% vs 25.3% 2 yrs.: 21.6%, 23.5%, 13.7%
CA184-024 Phase III [5, 6]	502	G1: IPI 10 mg/kg + dacarbazine 850mg/m^2 G2: dacarbazine + placebo Dose: weeks 1, 4, 7, and 10, followed by dacarbazine monotherapy every 3 weeks until week 22. Mode: IV	OS	IPI + dacarbazine: 11.2 mos. (9.4-13.6) Dacarbazine + placebo: 9.1 mos. (7.8-10.5)	Median values for PFS were similar in the two groups at week 12	IPI + dacarbazine, dacarbazine: 1 yr.: 47.3% vs. 36.3% 2 yrs.: 28.5% vs. 17.9% 3 yrs.: 20.8% vs. 12.2% 5 yrs.: 18.2% vs. 8.8%
Expanded access program EURO-VOYAGE [7]	1034	IPI 3 mg/kg	OS	6.8 mos. (6.1-7.4)	Median PFS 2.6 mos. (2.6-2.7)	3 yrs.: 10.9 % 4 yrs.: 8%
CA184-367 III [8]	727	G1: IPI 3 mg/kg G2: IPI 10 mg/kg	OS	IPI 3 mg/kg: 11.5 mos. (9.9-13.3) IPI 10 mg/kg: 15.7 mos. (6-17.8)	IPI 3mg/kg: 2.8 mos. (2.8-2.8); IPI 10 mg/kg, 2.8 mos. (2.8-3.0)	IPI 3 mg/kg, IPI 10 mg/kg: 1 yr.: 47.6% vs. 54.3% 2 yrs.: 31.0% vs. 38.5% 3 yrs.: 23.2% vs. 31.2%
Pooled analysis from Phase II and Phase III [9]	1861	The majority of patients had received IPI 3 mg/kg or 10 mg/kg	OS	11.4 mos. (10.7-12.1)		3 yrs.: 22% for all patients, 26% for treatment-naïve patients and 20% for previously treated patients

Table 1: Continued.

Name clinical trial Phase	Number of patients	Treatment plan	Primary endpoint	Median OS (95% CI)	Median progression-free survival PFS (95% CI)	Overall survival rates OS
KEYNOTE-001 Phase Ib [10–49]	655	PEMBRO 2 mg/kg every 3 weeks, PEMBRO 10 mg/kg every 3 weeks or PEMBRO 10 mg/kg every 2 weeks until disease progression or intolerable toxicity	CR	23.8 mos. (20.2-30.4)	8.3 mos. (5.8-11.1) in all treated patients 16.9 mos. (9.3-35.5) in treatment naïve patients	3 yrs.: 42% in all treated patients; 51% in treatment-naïve patients 4 yrs.: 37% in all treated patients; 48% in treatment-naïve patients 5 yrs.: 34 % in all patients, 41% in treatment naive
KEYNOTE-006 Phase III [13]	834	PEMBRO 10mg/kg every 2 weeks PEMBRO 10 mg/kg every 3 weeks IPI 3 mg/kg every 3 weeks for four cycles	PFS and OS	Median OS was not reached in the resp. PEMBRO arms IPI: 16.0 mos.	PEMBRO every 2 weeks, 5.5 mos. (3.4-6.9); PEMBRO every 3 weeks 4.1 mos. (2.9-6.9); IPI 2.8 mos. (2.8-2.9)	PEMBRO every 2 weeks, PEMBRO every 3 weeks, IPI: 1 yr.: 74.1% vs 68.4% vs 58.2% 2 yrs.: 55% vs. 55% vs. 43%
Checkmate-067 Phase III [14]	945	NIVO 3 mg/kg or NIVO 1mg/kg + IPI 3 mg/kg every 3 weeks for 4 doses followed by NIVO 3 mg/kg every 2 weeks or IPI 3 mg/kg every 3 weeks for 4 doses	PFS	NIVO, 37.6 mos. (29.1 to not reached); NIVO + IPI not reached; IPI 19.9 mos. (16.9-24.6)	NIVO, 6.9 mos. (5.1-9.7); NIVO+IPI, 11.5 mos. (8.7-19.3); IPI, 2.9 mos. (2.8-3.2)	NIVO, NIVO+IPI, IPI: 3 yrs.: 52% vs. 58% vs. 34% 4 yrs.: 46% vs 53% vs 30%

CI, confidence interval; CR, complete response; gp100, glycoprotein 100 peptide vaccine; IPI, ipilimumab; NIVO, nivolumab; OS, overall survival; PEMBRO, pembrolizumab; PFS, progression-free survival; mos., month; yr., year.

the CA184-002 study population was retreated at the time of first progression following an initial favorable response to ipilimumab, this may have contributed to the long-term (≥3 year) survival results on ipilimumab monotherapy [66] (Figure 1).

Novel features of ipilimumab therapy included an increased potential for long-term survival benefit in a small proportion of patients, the occurrence of new adverse events (AEs), the so-called "immune-related AEs" (irAE), and the atypical kinetics of treatment response [67, 68]. A consistent finding across these clinical trials investigating ipilimumab was the absence of a measurable impact on OS in the first 3 to 4 months of treatment. With longer followup a moderate improvement of the median OS outcome became apparent, and the long-term probability for survival after 3 years or longer (the so-called "tails of the survival curves") was not reconverting, indicative of the fact that 10-15% of the ipilimumab treated population derived a highly durable survival benefit as compared to the control population. Mature survival data were reported in an updated report of survival rate of the CA184-014 trial and a pooled analysis of 1861 patients from 10 prospective and two retrospective studies: 5y-OS rate was 18.2% (95% CI, 13.6% to 23.4%) for patients treated with ipilimumab plus dacarbazine versus 8.8% (95% CI, 5.7% to 12.8%) for patients treated with placebo plus dacarbazine (P = .002) (Table 1). An "inflexion-point" on the curve followed by a plateau in the survival curve began at approximately 3 years [6] (Figure 1).

These findings were confirmed in a pooled analysis including 1861 patients from ten prospective and two retrospective studies, including a majority of patients receiving ipilimumab according to the 3 mg/kg (n = 965) or 10 mg/kg (n = 706) dose levels (Table 1). Twenty-two percent of the patients were alive at 3 years, and a plateau on the survival curve became apparent 3 years after the start of treatment [9]. A second analysis of OS data with a total of n = 4,846 patients (including an additional 2,985 patients from an expanded access program) further confirmed a survival plateau at 21% from 3 years on (Figure 1).

Following approval of ipilimumab in 2011, a phase III trial (CA184-367), was conducted to address the unresolved question regarding the optimal dosing of ipilimumab (3 vs. 10 mg/kg); 727 patients without prior exposure to BRAF or PD-1 inhibitors were randomly assigned (1:1) to ipilimumab with either dose level [8]. The median number of doses of ipilimumab administered was four in each arm, with retreatment being pursued in a minority of patients (6% and 9% of patients in the 10 and 3 mg/kg arms, respectively). The median OS was superior for patients treated on the 10 mg/kg arm (15.7 versus 11.5 months; HR 0.84; p = 0.04) (Table 1). No difference in the probability for survival was evident during the first 6 months of followup. Thereafter the curves separated and a distinct 2- and 3-year survival rate was observed between both dose levels of ipilimumab (Figure 1). Treatment-related AEs in the 10 mg/kg arm were more frequent as compared to the 3 mg/kg arm (79% all-grade and 34% grade 3 to 5 AEs, as compared with 54% and 14%).

More recently, the effectiveness of ipilimumab was examined in a systematic retrospective analysis of 1034 patients with advanced melanoma who were included in a European Expanded Access Program (EURO-VOYAGE). A median OS of 6.8 months was found and the 3- and 4-years OS rates were, respectively, 10.9 and 8% and thus were apparently lower than what had been reported before (Table 1) [69]. These results indicate that the level of the "tail of the survival curve" remains dependent on the baseline characteristics of the investigated population, with an important role for baseline covariables as determinants for durable survival for patients treated with ipilimumab (Figure 1) [7, 70, 71].

3. Anti-PD-1 Therapies

Since 2015, ipilimumab has been replaced as the preferred first choice immunotherapy for advanced melanoma by PD-1 blocking mAb. Pembrolizumab and nivolumab were approved in the EU, US, and Australia as first-line immunotherapy for advanced melanoma based on phase III trials demonstrating a significant improvement of both PFS and OS as compared to ipilimumab [74, 75]. Notwithstanding the relative short followup of up to 3-4 years for these study populations, superior survival rates have been reported at every land-mark analysis [72, 76]. Moreover, followup of patient populations treated on phase I trials with nivolumab and pembrolizumab have also demonstrated the potential for durable survival gains after up to 5 years of followup [10, 11]. Anti-PD1 therapies are associated with a lower incidence of immune-related AEs as compared to ipilimumab [12].

3.1. Pembrolizumab. In the KEYNOTE-006 phase III trial, patients with unresectable stage III or IV melanoma had been randomly assigned (1:1:1) to one of two dose regimens of pembrolizumab (10 mg/kg every 2 or 3 weeks) or one regimen of ipilimumab (3 mg/kg every 3 weeks for a total of 4 consecutive doses) [13] (Table 1). Pembrolizumab treatment was continued for a maximum duration of 2 years. After a median followup of 22.9 months, median OS was not reached in either pembrolizumab group and was 16.0 months with ipilimumab (hazard ratio [HR] 0.68, 95% CI 0.53-0.87 for pembrolizumab every 2 weeks vs. ipilimumab p=0.0009; and 0.68, 0.53-0.86 for pembrolizumab every 3 weeks vs. ipilimumab; p=0.0008) with a 24-month OS rate of 55% for pembrolizumab treated patients and 43% in the ipilimumab group. The 33-month PFS-rate was 31 vs. 14% and OS-rate 50 vs. 39% for the pooled pembrolizumab arms vs. the ipilimumab group. After a median followup of 45.9 months (range: 0.3-50.0) the 4-year OS rates were 42% in the pooled pembrolizumab groups and 34% in the ipilimumab group (Figure 2). One hundred and three patients (19%) received the maximum duration of 2 years of pembrolizumab treatment and only 14% of the patients experienced progressive disease (median followup of 20.3 months) [13].

In an open-label phase 1b clinical trial (KEYNOTE-001) patients received pembrolizumab 2 mg/kg or 10 mg/kg every 3 weeks or 10 mg/kg every 2 weeks until disease progression or intolerable toxicity. The median OS was 23.8 months in all 655 patients, with 3-year and 4-year survival estimates of 42% and

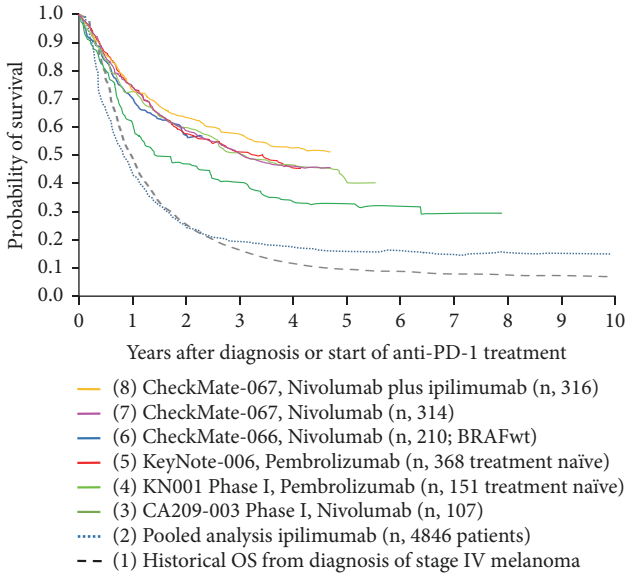

Figure 2: *Overlay of Kaplan–Meier curves indicating the probability for OS (OS) for advanced melanoma patients treated with anti-PD1 as first-line immunotherapy*, representing (1) the historical probability for OS for patients diagnosed with stage IV melanoma prior to the availability of life-prolonging medical treatment options (dashed black line) [1]; (2) a pooled OS analysis including individual patient survival data from 1,861 patients with metastatic melanoma from 12 clinical investigations of ipilimumab and 2,985 patients with metastatic melanoma from a US ipilimumab EAP (total n = 4,846) (blue line) [9]; (3) CA209-003 phase I clinical trial with nivolumab for pretreated advanced melanoma patients (dark green line) [11]; (4) treatment naïve patients (n: 151) treated in the Keynote-001 clinical trial with pembrolizumab (light green line) [10]; (5) treatment naïve patients (n, 368) from the Keynote-006 trial (red line) [13]; (6) nivolumab treated patients with BRAF V600 wild-type melanoma (n, 210) from the Checkmate-066 trial (blue line) [73]; (7) nivolumab monotherapy treated patients (n, 314) from the CheckMate-067 trial (pink line) [72]; (8) nivolumab plus ipilimumab treated patients (n, 316) from the CheckMate-067 trial (orange line) [72].

37% (Table 1). In the 152 treatment-naïve patients, the 3-year and 4-year survival estimates were 51% and 48%, respectively (Table 1). Recently the updated 5 years overall survival results have been published and an OS of 34% in all patients and an OS of 41% in treatment-naïve patients were found (Figure 2) [10, 76, 77].

3.2. Nivolumab. Similar survival outcome has been observed in another double-blind, phase 3 study, investigating nivolumab alone or nivolumab plus ipilimumab versus ipilimumab alone as first line therapy in 945 previously untreated patients with unresectable stage III or IV melanoma (Checkmate-067) [14]. Both nivolumab containing treatment arms significantly improved both PFS and OS as compared to ipilimumab and a superior PFS was obtained in the combination arm of nivolumab and ipilimumab (Table 1). However, treatment-related AEs of grade 3 or 4 occurred more frequently with upfront combination of nivolumab and ipilimumab (22.4% of the patients in the nivolumab monotherapy arm, 59.1% of those in the combination arm, and 27.7% of those in the ipilimumab monotherapy arm). After a minimum followup of 48 months, the median OS had not been reached in the combination group and was 36.9 months in the nivolumab monotherapy group, as compared with 19.9 months in the ipilimumab monotherapy group (hazard ratio for death with nivolumab plus ipilimumab vs. ipilimumab, 0.54 [$P < 0.001$]; hazard ratio for death with nivolumab vs. ipilimumab, 0.65 [$P<0.001$]). The OS rate at 4 years was 53% in the nivolumab-plus-ipilimumab and 46% in the nivolumab monotherapy arm, as compared with 30% in the ipilimumab monotherapy arm (Figure 2). The two groups including nivolumab had significantly longer survival compared to the ipilimumab group. In a descriptive analysis, the hazard ratio for death with nivolumab plus ipilimumab versus nivolumab monotherapy was not statistically significant (hazard ratio for death was 0.84 with a 95% CI, 0.67 to 1.05).

Comparable to patients treated with pembrolizumab, the hazard ratio for progression of disease decreased with time and the rate of PFS at 4 years was 37% in the nivolumab-plus-ipilimumab group and 31% in the nivolumab monotherapy arm, as compared with 9% in the ipilimumab monotherapy arm. In a descriptive analysis, the hazard ratio for progression or death was 0.79 (95% CI, 0.65 to 0.97) with nivolumab plus ipilimumab versus nivolumab indicating the potential for a stable survival plateau above 30% in both nivolumab treatment arms (Figure 2).

Long-term prediction of OS-rates in patients with advanced melanoma treated with anti-PD-1 mAb is currently only available for pretreated patient populations who participated in phase I clinical trial programs. The available data nevertheless are indicative that the OS probability curve is likely to reach a plateau. Thirty-four percent of patients treated with nivolumab in a phase I trial (CA209-003) were alive 5 years after initiating study treatment [49].

3.3. Real-World Outcome Data on Anti-PD-1 Therapy. In a poster presented at the SMR 2017 annual meeting real-world outcome data were reported on 189 advanced melanoma patients discontinuing anti-PD-1 treatment (pembrolizumab or nivolumab) in the absence of PD or treatment limiting toxicity [15]. Data were collected at 14 hospitals across Europe and Australia. Short-term outcome of patients that stopped therapy in absence of progression of disease or treatment limiting toxicity was encouraging, with a low-risk for PD (4% after a median FU of 35 weeks). Reintroduction of a PD-1-inhibitor in patients who progressed after discontinuation (n= 9 patients) indicated the potential for renewed antitumor activity. Additional reports, in line with these results on real-world outcome data, were recently reported by additional groups [16–19].

4. Long-Term Immune Related Adverse Events

The side effects of immune checkpoint blockade are often referred to as immune-related adverse events (irAE). The most common irAE occur in skin, liver, and gastrointestinal, pulmonary, and endocrine organs but autoimmune diabetes

and cardiovascular, renal, and musculoskeletal side effects are also reported [20, 21]. Most cutaneous, gastrointestinal, and hepatic AEs occurred within two months, whereas endocrine, pulmonary, and renal side effects appeared after 9 weeks [68]. Early diagnosis and treatment are believed to be important in mitigating the severity of irAEs [22]. Most of these irAE are reversible after treatment interruption and/or steroid therapy; however, the endocrine irAE (most commonly hypophysitis and thyroiditis) may necessitate life-long hormonal substitution [23, 24]. One study on 15 patients diagnosed with autoimmune hypophysitis induced by ipilimumab treatment reported that all patients had at least one hormonal defect at diagnosis [25]. In all patients clinical symptoms improved in the first month after starting glucocorticoid therapy. At the end of followup (median 33.6 months, range 7-53.5), 13 (86.6%) required long-term hormonal replacement with corticotropic deficiency persisting in all patients suffering from hypocorticism. No prospective study results are currently available on the long-term (>3 yrs) consequences of irAEs.

The high incidence of irAE observed with the combination of nivolumab (1mg/kg) and ipilimumab (3 mg/kg) has prompted the investigation of nivolumab or pembrolizumab combined with ipilimumab at a lower dose level of 1 mg/kg every 3 weeks [26, 27]. The CheckMate 511 study demonstrated a significantly lower incidence of treatment-related grade 3-5 AEs. However, longer followup is needed to address the long-term OS outcome as for ipilimumab in monotherapy, long-term OS is dose dependent.

5. Health Related Quality of Life in Melanoma Survivors

To date patient reported global health related quality of life (HRQOL) is measured using several valid instruments to assess different dimensions of HRQOL, such as psychological, social, physical, and spiritual aspects. HRQOL instruments can be generic, cancer specific, or cancer disease specific and measure only one or several dimensions. Commonly used scales to assess the global HRQOL in cancer patients include the European Organization for Research and Treatment of Cancer Quality of life Questionnaire (EORTC-QLQ-C30), the Impact of Cancer questionnaire (IOC), and the Generic Functional Assessment of cancer therapy (FACT-G) for which an additional melanoma scale was validated, the FACT melanoma (FACT-M) [28–31]. In the field of survivorship the EORTC-QOL survivorship questionnaire is currently in validation process [32].

Results from three randomized controlled trials (MDX010-20, KEYNOTE-002, and CheckMate 067) suggest that ipilimumab, nivolumab, and pembrolizumab, as a monotherapy, and the combination therapy of nivolumab plus ipilimumab or ipilimumab plus gp100 vaccine are well tolerated and either improve or maintain HRQOL as assessed with the EORTC QLQ-C30 scale, during the treatment induction phase [33–35]. However, there might be an underestimation of the influence of these treatments on the HRQOL because of low patient numbers in the later weeks of all studies due to disease progression, death, and AEs. In the KEYNOTE-006 it has been observed that the HRQOL assessed with the EORTC QLQ-C30 scale, in patients treated with pembrolizumab, was better maintained as compared to ipilimumab in patients with ipilimumab naïve advanced melanoma (Table 2) [34].

In a systematic review of 7 studies (4246 patients; 6 cross-sectional [36–44], and 1 prospective study [45]), it was found that determinants of lower HRQOL (either psychological, physical, or global) were marital status, age, sex, poor social support, melanoma severity at diagnosis, and comorbidities (Table 3) [46]. Dieng et al. found that HRQOL, measured with the Functional Assessment of Cancer Therapy (FACT-M), was correlated with fear of recurrence of disease in patients with metastatic melanoma (Table 3) [47]. A more comprehensive understanding of HRQOL can improve patient centered care in melanoma patients. In addition HRQOL assessment can be used as outcome measure for cancer research and help socioeconomic decision making. Therefore international consensus on how to assess HRQOL is mandatory, as well as the development and validation of melanoma specific assessment tools [46, 48]. In Table 4 an overview is given of the characteristics of the questionnaires used in the referenced trials.

6. Psychosocial Outcomes in Melanoma Survivors

With increasing numbers of advanced melanoma patients becoming long-term cancer survivors, even after discontinuing therapy, the issue of melanoma survivorship care becomes of relevance to more patients than ever before. Cancer survivorship has been extensively studied in other cancer indications [49]. In these studies, cancer survivors have been reported to suffer from mental and physical symptoms, fatigue, and neurocognitive dysfunction persisting after physical recovery from their disease. These mental and neurocognitive symptoms are associated with important psychosocial consequences such as delayed return to work, impaired family relationships, and reduced quality of life (QOL) [77, 78].

Only a few studies are focusing on psychosocial outcome in melanoma survivors, with all of them showing diminished wellbeing, high levels of distress, and fear for recurrence (Table 3) [36, 43]. Nevertheless, results of these studies are limited as they are all survey-based and mainly include patients with early stage melanoma and in a lesser extent nonmetastatic disease treated with adjuvant therapy. The following risk factors have been described to be related to higher distress in early stage melanoma patients: female gender, younger age, negative appraisal, and negative coping strategies (Table 3) [63]. Higher distress and fear of recurrence might be related to the necessity of continued self-examination, dermatological controls, and reduced sun exposure [37, 79]. Moreover, higher anxiety levels and fear for recurrence are associated with avoidance behavior in relation to dermatological controls [80]. The traumatic course of metastatic melanoma may also contribute to more difficult coping mechanism as compared to other cancer indications [46]. In accordance with these findings, Dieng explored

Table 2: Key features of referenced trials investigating Health Related Quality of life in patients treated with immune-checkpoint inhibitors.

First author	Study design	Study population and AJCC stage	Assessment of quality of life	Sample size	Response rate	Main conclusions on HRQOL
Revicki D. A. et al [33]	Phase III MDX010-20	Stage IIIc/IV pts. during treatment induction	EORTC QLQ-C30 at baseline and week 12	676 pts.: IPI + gp100: N=403 IPI alone: N=137 Gp alone: N=136	Baseline ≥ 95% Week 12: IPI + pg100: 62% IPI alone: 65 % Gp alone: 61%	IPI with or without gp100 does not have significant negative impact on HRQOL during the induction phase compared to gp100 alone.
Petrella T. M. et al [50]	Phase III KEYNOTE-006	Stage IIIc/IV pts. during treatment induction	EORTC QLQ-C30 EQ-5D at baseline and week 12	776 pts.: PEMBRO every 2 w.: N=270 PEMBRO every 3 w.: N=266 IPI 3 mg/kg: N=240	Baseline ≥ 98% Week 12: PEMBRO 2 w: 79% PEMBRO 3 w: 85% IPI: 74%	HRQOL was better maintained with PEMBRO than with IPI in patients with IPI-naive advanced melanoma.
Schadendorf D. et al [35]	Phase III Checkmate-067	Stage IIIc/IV pts. during first 12 months of treatment	EORTC QLQ-C30 EQ-5D at baseline there after resp. w. 1 and 5 of every 6 w. cycle during first 6 mos., and every 6 w. thereafter	945 pts.: NIVO: N=316 NIVO + IPI: N=314 IPI: N=315	Baseline ≥ 89% Week 13: NIVO: 78% NIVO + IPI: 53% IPI: 63%	Results of HRQOL data support the clinical benefit of NIVO monotherapy and NIVO plus IPI combination therapy in pts. with advanced melanoma. Differences in irAE between the 2 groups did not affect HRQOL.
Schadendorf D. et al [34]	Phase III KEYNOTE-002	Stage IIIc/IV pts. during the first 12 weeks	EORTC QLQ-C30	520 pts.: PEMBRO 2 mg/kg: N=176 PEMBRO 10 mg/kg: N=177 Chemotherapy: N=167	Baseline: Week 12 ≥ 93% PEMBRO 2 mg/kg: 69% PEMBRO 10 mg/kg: 75% Chemotherapy: 65%	HRQOL was better maintained with PEMBRO than with chemotherapy, supporting the use of PEMBRO in pts. with IPI-refractory melanoma

gp100, glycoprotein 100 peptide vaccine; IPI, ipilimumab; NIVO, nivolumab; PEMBRO, pembrolizumab; yr., year; w., week; pts., patients; HRQOL: Health Related Quality of Life; SF-36, Short Form 36.

Table 3: Key features of the referenced studies with the main findings on psychosocial outcome.

First author	Study design	AJCC stage Time (T) since diagnosis	Questionnaires	Sample size (response rate)	Main findings on HRQOL	Main findings on psychosocial outcome
Beutel M. E. et al [36] Fishbeck S. et al [43]	Cross-sectional Survey	Mainly stage I/II (41% staging was missing) T since diagnosis: 6 - 9 yrs. (70%), ≥ 10 yrs. (30%)	EORTC QLQ-C30 [28] Health Questionnaire Depression (PHQ-9) [51] Multidimensional General Anxiety disorder (GAD-7) [52] Illness specific support Scale (ISSS) [53]	1320 (52%)	Global HRQOL was comparable to general population Lower emotional, cognitive and social functioning and higher symptom burden compared to general population.	Increased depression and anxiety compared to the general population. 36% was in need of psychosocial support. Fear of recurrence of disease caused the highest burden.
Cromwell K.D. et al [45]	Prospective longitudinal study	Stage III T since diagnosis: 0-30 mos.	FACT-M [31] Lymphedema and Breast cancer questionnaire (LBCQ) [54]	277 (71%)	Lymphedema impacts HRQOL.	Lower extremity lymphedema pts. cope less effectively but improve over time Household chores and sleep are most impacted by lymphedema.
Palesh O. et al [37]	Cross-sectional survey	Stage unknown Median T since diagnosis 77 mos, range(0-336)	Non validated electronically administered survey	893 (18%)	-	Melanoma survivors experience continuing anxiety long after treatment. 30% of the pts. reported emotional distress. Long term survivors decreased use of skin protection and frequency of skin screening.
Schubert-Fritze et al [38] Schlesinger Raab A. et al [44]	Cross-sectional survey	Stage I/II T since diagnosis: 2 yrs.	EORTC QLQ-C30 [28] FACT-G [30, 55] Mental Adjustment to Cancer Scale [56]	1085 (61%)	Global HRQOL was comparable with the general population. Number of co-morbidities, age and lymphadenectomy increased the risk for worse global HRQOL, role functioning and worry about the future.	Doctor patient communication was correlated with emotional and social functioning. 42% of the pts. worried about recurrence of disease.

Table 3: Continued.

First author	Study design	AJCC stage Time (T) since diagnosis	Questionnaires	Sample size (response rate)	Main findings on HRQOL	Main findings on psychosocial outcome
Hamama-Raz Y et al [39, 40]	Cross-sectional survey	Stage I/II T since diagnosis: 5 yrs. (36%) ≥ 5 yrs. (64%)	Mental Health Inventory (MHI) [57] Cognitive Appraisal of Health Scale [58]	400 (75%)	Mean well-being score and mean distress score are similar compared to general population	Subjective factors, such as appraisal of the threat, may be more predictive than medical factors in coping with cancer. Men and women cope differently.
Waldmann et al [41]	Cross-sectional survey	Stage I/II (59%) Stage III (17%) Stage IV (1.9%) T since diagnosis: Q1: 15 mos. Q2: 39 mos.	EORTC QLQ-C30 [28]	762 (59%)	No clinical meaningful differences on global HRQOL between Q1 and Q2.	-
Holterhues C et al [42]	Cross sectional survey	Stage I/II (81%) Stage III (8%) Mean T since diagnosis: 4.6 (2.6) yrs.	Short Form Health Survey (SF-36) [59] Impact of Cancer scale (IOC) [60]	699 (80%)	Medical co-morbidity and female were the main predictors of impaired HRQOL. Impairment of HRQOL seems to be melanoma specific.	Time since diagnosis, tumor stage and co-morbidity were predictors of negative IOC scores. 85 pts. (35%) reported difficulties in obtaining life insurance, 98 (15%) obtaining mortgage.
Dieng M. et al [47]	Cross sectional survey	Stage 0/I/II Mean T since diagnosis: 7.6 (6.5) yrs.	FACT-M [31] Assessment of QOL-8 dimension scale (AQoL-8D) [61] Fear of cancer recurrence Inventory (FCRI) [62]	183 (89%)	High fear of recurrence was associated with a significant decrease of HRQOL. AQoL8D is an alternative to the FACT-M, more sensitive to changes in psychological health and fear of recurrence and can be used to asses utility based health status.	-

Table 3: Continued.

First author	Study design	AJCC stage Time (T) since diagnosis	Questionnaires	Sample size (response rate)	Main findings on HRQOL	Main findings on psychosocial outcome
Loquai C. et al [63]	Cross-sectional survey	Stage 0/I/II (81%) Stage III (13%) Stage IV (5%) T since diagnosis 0-2 yrs. (44%) 2-5 yrs. (26%) ≥ 5yrs. (31%)	Distress Thermometer (DT) with Problem List (PL) [64]	734 (71%)	_	52 % reported ≥1 emotional problem Presence of emotional problems, family problems and younger age were strongly associated with higher distress. DT and Pl reliable identify distressed melanoma patients.

Table 4: Description of the questionnaires used in the referenced studies.

Instrument	Goals	Cancer specific	Melanoma specific	Survivor-specific	Subscales	Remarks
EORTC QLQ-C30 [28]	Global HRQOL	yes	no	no	5 functional scales: physical, emotional, role, cognitive 9 symptom scales: fatigue, pain, nausea, dyspnea, appetite loss, insomnia, constipation, diarrhea 1 summary scale. 30 items	Possible lack of sensitivity for use in melanoma survivors to evaluate HRQOL [41]. Symptoms not specific for melanoma survivors. Not validated in cancer survivorship or in melanoma patients.
FACT-General [30]	Global HRQOL	yes	no	no	4 functional scales: physical, emotional, social, functional wellbeing. 27 items	Can be completed by the FACT-M scale.
FACT-Melanoma [31]	Global HRQOL	yes	yes	no	3 functional scales: physical, emotional, social, wellbeing. 27 items FACT-G + 24 FACT-M items	Melanoma specific with a specific post-surgery scale. Validated in all stages of melanoma.
Assessment of QLQ-8 [61]	Global HRQOL	yes	no	no	8 dimensions: 3 physical dimensions (independent living, pain, senses) and 5 mental dimensions (mental health, happiness, coping, relationships, self-worth). 35 items	Is sensitive to changes in mental and emotional health. May also be useful to capture the benefit of psychological interventions and to measure their cost effectiveness.
Impact of cancer (IOC) [29]	Global HRQOL	no	no	no	8 scales: physical functioning, vitality, social functioning, general health, bodily pain, physical and emotional role, mental health. 37 items	Adjustment to changes. Measures positive as well as negative impact of cancer. Not validated in cancer survivorship.

Table 4: Continued.

Instrument	Goals	Cancer specific	Melanoma specific	Survivor-specific	Subscales	Remarks
Lymphedema and Breast Cancer questionnaire (LBCQ) [54]	Symptoms and signs of lymphedema	yes	no	no	Assessment of 19 signs and symptoms. 59 items	Used in clinical practice to follow up lymphedema. Can be useful in melanoma survivors as lymphedema impacts on HRQOL and wellbeing [38, 44, 45]. Not validated in the melanoma survivorship setting.
PHQ-9 [51]	Depression	no	no	no	Assessment depressive symptoms. 9 items	Screening for depressive symptoms. Widely used in survivorship trails.
General anxiety disorder GAD-7 [52]	Anxiety	no	no	no	Screening for General Anxiety Disorder (GAD). 7 items	It is not yet known that GAD is present in metastatic melanoma survivors. Not validated in cancer survivorship.
Mental Adjustment to Cancer Scale [56]	Adjustment to cancer	yes	no	no	Measures fighting spirit, anxious preoccupations, helplessness and loneliness and fatalism. Updated scale includes also global adjustment to cancer. 40 items	Satisfactory measure of psychosocial outcome during the disease phase. Not validated in cancer survivorship.
Mental Health Inventory [57]	Psychological distress and wellbeing	no	no	no	Assessment of anxiety, depression, behavioral control, positive affect and general distress. Original 38 items, revised version with 18 items.	Allows screening of emotional distress as well as behavioral aspects. Widely used in the field of cancer. Not validated in cancer survivorship.
Fear of cancer recurrence (FCRI) [62]	Fear for cancer recurrence	yes	no	yes	Evaluates severity, triggers, psychological distress, coping strategies, insight and functional impairments. 42 items	Allows evaluating fear of recurrence of disease, which is in particular of interest in metastatic melanoma treated with immunotherapy in view of the high risk of recurrence, however not validated in melanoma setting.
Distress thermometer [64]	Distress	yes	no	no	Five categories: practical, family, physical and emotional problems, spiritual and religious concerns. 35 items	Useful and easy to use screening tool for emotional distress in clinical practice. Reliably identifies distress in melanoma patients [63].

the usefulness of psychoeducational intervention in patients diagnosed with stages 1-2 melanoma and found a substantial benefit compared to the patients who received standard of care [81].

Currently no data are available on the potential long-term emotional, physical and cognitive side effects of immune checkpoint inhibitors in patients with metastatic melanoma.

Moreover, an important subgroup of patients with brain metastasis is becoming survivors, which makes it imperative to study potential effects on neurocognitive functioning, especially because survivors who have previously been irradiated for brain metastases are at increased risk for focal postradiation necrosis of the brain [82]. Efforts to further comprehensively address these psychosocial, neurocognitive, and HRQOL issues are ongoing at present at our department. Preliminary observations indicate that a substantial fraction of these patients experience diminished HRQOL, persisting fatigue, severe emotional disturbances, and neurocognitive complaints [83, 84]. A multicentric study addressing HRQOL in long-term survivors following treatment with ipilimumab is currently ongoing in The Netherlands and Belgium.

In conclusion prospective investigation of the potential psychosocial, neurocognitive, and HRQOL issues is needed, in order to identify the care needs of advanced melanoma survivors. Optimizing patients' subjective wellbeing could potentially reduce the emotional, physical, and socioeconomic consequences of this devastating disease.

Conflicts of Interest

Anne Rogiers reports personal fees from BMS and MSD, outside the submitted work. Christian U. Blank reports personal fees from MSD, BMS, Roche, GSK, Novartis, Pfizer, GenMab, Pierre Fabre, and Lilly and grants from BMS, NanoString, and Novartis, outside the submitted work. Bart Neyns reports personal fees from BMS, MSD, Amgen, Pfizer, Roche, and Merk/Serono, outside the submitted work. Annelies Boekhout, Gil Awada, and Julia Schwarze declare that they have no conflicts of interest.

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Review Article

Immunotherapeutics in Multiple Myeloma: How Can Translational Mouse Models Help?

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Multiple myeloma (MM) is usually diagnosed in older adults at the time of immunosenescence, a collection of age-related changes in the immune system that contribute to increased susceptibility to infection and cancer. The MM tumor microenvironment and cumulative chemotherapies also add to defects in immunity over the course of disease. In this review we discuss how mouse models have furthered our understanding of the immune defects caused by MM and enabled immunotherapeutics to progress to clinical trials, but also question the validity of using immunodeficient models for these purposes. Immunocompetent models, in particular the 5T series and Vk*MYC models, are increasingly being utilized in preclinical studies and are adding to our knowledge of not only the adaptive immune system but also how the innate system might be enhanced in anti-MM activity. Finally we discuss the concept of immune profiling to target patients who might benefit the most from immunotherapeutics, and the use of humanized mice and 3D culture systems for personalized medicine.

1. Introduction

Multiple myeloma (MM) is a malignancy of plasma cells that reside within a supportive niche in the bone marrow (BM) [1, 2]. Monoclonal gammopathy of undetermined significance (MGUS) is a preceding, benign phase to MM, where a monoclonal paraprotein is detected in the peripheral blood but plasma cells account for less than 10% of BM haematological cells [3, 4]. Smoldering myeloma (SMM) is similarly asymptomatic, but plasma cells account for at least 10% of BM haematological cells. Patients are often diagnosed with MM when they develop end-organ features that include anaemia, bone fractures secondary to lytic lesions, hypercalcaemia, and/or renal disease [1, 2]. Acquired immune paresis complicates advanced disease due to residual hypogammaglobulinemia, B cell hypoplasia [5], the effects of cumulative chemotherapies [6–8], and an ageing T cell population [9, 10]. In end stage disease, plasma cells lose their dependence on the BM niche and can cause extramedullary disease with solid organ deposits and/or plasma cell leukaemia.

MM is a disease of older adults with a peak incidence in the 7th decade of life [11]. The increasing use of proteasome inhibitors and immunomodulatory drugs (IMiDs) over the last decade has made an impact on overall survival in MM patients [12, 13] but has transformed MM to a chronic palliative illness. As our knowledge of immunosenescence and T cell exhaustion within the chronic inflammatory environment of MM advances, evaluating the effectiveness of immunotherapeutics within a tumor microenvironment in an aged host is paramount. This review aims to encompass how mouse models can contribute to our understanding of the MM immune microenvironment and of the clinical use of immunotherapeutics and other novel agents in human MM.

2. Mouse Models of Multiple Myeloma

The two main types of mouse models used (Table 1) include

(1) immunodeficient xenograft models where mice lack immune subsets rendering them tolerant to the transplant of human MM cells (often referred to as "humanized"),

Table 1: Mouse models of multiple myeloma.

Model	Features	
Xenograft models		
SCID	Lack T and B lymphocytes	
NOD/SCID	SCID + no circulating complement and low NK cell function	
NSG (NOD/SCID/IL2Rγ^{null})	NOD/SCID + lack IL-2	
SCID-hu	SCID implanted with human fetal bone chips	
SCID-rab	SCID implanted with rabbit bone chips	
SCID-synth-hu	SCID implanted with 3D polymeric scaffolds coated with human BM stromal cells	
Immunocompetent models		
5T series	Syngeneic transplant of cell lines from spontaneously arising MM in aged C57BL/KaLwRij mice[188, 189]	
	5T2MM	Moderate, progressive disease course
	5T33MM	Aggressive, rapidly progressive disease course
	5TGM1	Cell line derived from 5T33MM
Vk*MYC	*Transgenic*: spontaneous AID-dependent activation of MYC in post germinal B cells[17] *Transplant*: syngeneic transplant of plasma cell lines from transgenic Vk*MYC mice	
Myc/Bcl-X_L	Bitransgenic offspring of hemizygous Myc transgenic mice and hemizygous Bcl-X_L mice[17]	
XBP-1	*Eμ*-directed expression of XBP-1 spliced isoform, a factor governing plasma cell development that has been reported to frequently be overexpressed in human MM[18]	
MOPC315.BM	Syngeneic transplant of plasmacytoma-resembling MM cells from granulomas in Balb/c mice injected intraperitoneally with mineral oil	

(2) immunocompetent mice that are either transgenically manipulated to develop a MM-like tumor or transplanted with MM cells from a syngeneic mouse.

2.1. Immunodeficient Models. SCID (severe combined immunodeficiency) and NSG (NOD/SCID/IL2Rγ^{null}) mouse models inoculated with human MM cells are widely used for drug discovery; however, their obvious disadvantage is that neither immunosurveillance nor the tumor microenvironment that supports myeloma cell survival is fully intact. Regardless, xenograft models are frequently used to assess antimyeloma therapies including monoclonal antibodies and vaccines.

2.1.1. Human MM Cell Lines. MM cell lines are derived from clones from humans with plasma cell leukaemia or other forms of extramedullary disease. These cells have evolved to survive outside of the bone marrow niche, have complex cytogenetic profiles, and are highly resistant to apoptosis. They have a rapid doubling time of 24-72 hours and are therefore very easy to culture. But for all these reasons, they do not accurately resemble most human myeloma cells, which are typically very difficult to culture outside of human BM. MM cell lines can be injected into mice intravenously via the tail vein, intratibially, or subcutaneously (SC). The former two methods are preferred as they more faithfully represent BM disease in MM, whereas SC injection imitates solitary plasmacytoma in an entirely separate immune compartment. Whilst cell lines are likely to be selective for highly effective antimyeloma therapies, their use in an immunodeficient setting does not recapitulate the typical immune myeloma microenvironment and may not allow an opportunity for immunotherapies to fully exert their effect.

2.2. Immunocompetent Models. The most commonly used immunocompetent mouse models of multiple myeloma in the literature are the 5T series and transgenic *myc*-overexpressing models (or syngeneic transplanted cell lines from these diseased mice).

The 5T series (including 5T2MM, 5T33MM, and 5TGM1) are cell lines derived from aged C57BL/KaLwRij mice that spontaneously developed plasma cell dyscrasia. On syngeneic transplant, recipients develop dissemination of the tumor, paraprotein, osteolytic bone lesions, and resultant hind limb paralysis [14, 15].

Knowledge of driver mutations in MM led to the development of the Vk∗MYC [16] and Myc/Bcl-X_L [17] *myc*-overexpressing mouse models of myeloma, and the Eμ-XBP-1s model [18]. All spontaneously develop MM-like tumor dissemination and paraprotein over a longer latency, with greater heterogeneity of disease than single clonal transplant. However, the time to disease is a greatly limiting factor at the bench. Syngeneic transplant of cells retrieved from diseased Vk∗MYC mice has similar MM-like disease but with faster kinetics [16], and the myeloma cells are responsive to most conventional therapies used in humans [19].

Less commonly used now are the plasmacytoma-resembling murine MM cell lines (MOPC315, J558, HOPC) that have been transplanted subcutaneously in syngeneic mice. These were obtained from granulomas arising from the intraperitoneal injection of mineral oil in Balb/c mice. The cell line MOPC315.BM has been derived from MOPC315 cells that exhibit bone marrow tropism [20].

3. The Tumor Microenvironment and Immune Dysfunction in MM

It is well established that MM cells influence the BM microenvironment to sustain tumor survival. This is achieved by pathologies that include osteoclastogenesis, increased angiogenesis, and immune editing. The role of xenograft and 5T murine models of MM to assess the efficacy of therapeutic agents for bone disease was reviewed recently [21].

Immunosurveillance describes the processes by which the immune system recognizes and eliminates foreign pathogens and tumor cells. This theory has been refined over the last 15 years to the concept of "immune editing", which is a dynamic process composed of three phases: (1) elimination, (2) equilibrium, and (3) escape [22]. In MM, the equilibrium phase is most noteworthy as it represents a therapeutic opportunity to utilize the immune system to slow or prevent disease progression. Immunosurveillance has been demonstrated in the Vk∗MYC mouse model, where immune control of MM was demonstrated via NK and $CD8^+$ T cells through CD226 (DNAM-1) interaction with its ligand CD155 on malignant plasma cells [23].

The development of immunosenescence, through which age-related changes of immune system lead to functional defects, may also contribute to loss of immunosurveillance with subsequent progression of tumors. These age-related changes include a drastic loss of thymic function and a decrease in the number and repertoire of naïve T cells in the 7^{th} decade [24, 25], coinciding with the peak incidence of MM. Simultaneously, there exists a chronic inflammatory state termed inflammaging: a sustained, low-grade increase in proinflammatory factors such as IL-6, IL-1, TNFα, and CRP [26]. This adds to the increased susceptibility of older humans to opportunistic infections, cancer, and autoimmunity [27]. Some of these changes are seen, and possibly accelerated, in malignancy and/or chronic viral infections, as discussed below.

3.1. Adaptive Immune System. This section concentrates on T cell pathology in MM that has been most intensively studied in the immunotherapeutics field. B cells have predominantly been evaluated in mouse models with regard to the oncogenic mutations that promote development into plasma cell malignancy. B cell hypoplasia has been described in human MM [5] and in the Vk∗MYC mouse model [28], and further study of how this might affect anti-MM T cell function is warranted.

3.1.1. T Cell Generation. Thymic involution leads to an age-associated decrease in the frequency of circulating naïve T cells in peripheral blood (PB) [10, 29], lymph nodes [30], and bone marrow (BM) [31]. Of additional relevance to the MM patient population, it has been shown that the human thymus is incapable of responding to a sudden decline in peripheral

T cells (i.e., after high dose chemotherapy or radiation) with a substantial increase in T cell output [32]. Studies with bone marrow transplant patients have shown that the thymus of the majority of patients over 40 years was unable to rebuild a naïve T cell compartment [33].

Despite the reduction in thymic output, overall T cell numbers are not affected due to compensatory proliferation of T cells in the periphery [34]. However, $CD4^+$ T cells do not proliferate to the same degree as $CD8^+$ T cells [33, 35] leading to a reduction in CD4:8 in MM patients [28, 36], which could be partly explained by the higher expression of CD122 (the β-chain of the IL-2/IL-15 receptor) on $CD8^+$ T cells [37] and increased availability of IL-15 in lymphopenic states [34]. Additionally, IL-7 dependent STAT-1 activation has been reported to limit homeostatic $CD4^+$ T cell expansion [38], and naïve $CD8^+$ T cells are particularly hyperresponsive to IL-15 because of lack of suppressor of cytokine signaling (SOCS)-1 [39]. The emergence of an oligoclonal T cell population with a limited TCR repertoire has been observed [40], as well as a senescence-associated secretory phenotype (SASP) that has low proliferative potential but retains the ability to produce cytokines and does not exhibit telomere shortening that is seen with ageing populations [40, 41].

The loss of naïve T cell populations is not paralleled in aged mice, where the thymus sustains the naïve T cell pool throughout their lifetime [42], and the CD4:8 ratio remains unaffected in diseased Vk∗MYC mice [28]. One study showed an impaired ability of aged mice to thymically recover T cells after irradiation [43], although in most cases this state is not replicated in MM mouse models because mice used in experimental models are invariably young adults. Of interest, it has been shown in the Vk∗MYC transplant model that $CD8^+CD44^+$ T memory cells were integral to MM control after BM transplant; however, mice transplanted with naïve ($CD44^-$) T cells had improved survival, indicating the importance of naïve T cell priming [44].

3.1.2. T Cell Differentiation. In addition to reduced thymic output of naïve T cells, chronic antigen exposure leads to alterations in the proportion of naïve: antigen-experienced T cells. This has been described in humans with ageing, persistent viral infections, and chronic malignancy. A major skewing towards a T cell population predominantly made up of effector memory T (T_{EM}) and $CD8^+$ T_{EMRA} cells has been demonstrated in human MM and replicated in Vk∗MYC mice with advanced disease in both the transplant and transgenic models [28]. This pattern has also been noted in another model of chronic B cell malignancy, the EμTCL1 mouse model of chronic lymphocytic leukaemia [45].

3.1.3. T Cell Exhaustion. T cell exhaustion refers to an altered T cell state that is manifested under conditions of chronic inflammation, such as chronic viral infection or cancer [46]. Exhausted T cells are not inert; but the loss of effector functions limits their ability to fully eradicate pathogens or tumor. $CD8^+$ T cells expressing inhibitory markers correlated strongly with disease progression after BM transplant in the Vk∗MYC mouse model [47]. Knowledge of inhibitory T cell signaling pathways has been instrumental in developing immunotherapeutics such as PD-1 and CTLA-4 inhibitors that are currently in human trials (see Therapeutics).

3.1.4. T Cell Polarization. Several groups have published evidence that there are increased numbers of Th17 cells in the PB and BM microenvironment of patients with MM compared to normal [48–50], and elevated levels of IL-17 [49, 50] and Th17-polarizing cytokines (IL-6, TGFβ, IL-23, and IL-1β) [49] in the BM. This has been proposed to be harmful in MM by promoting lytic bone disease [49, 51] and MM cell growth [50]. Others have suggested that the Treg/Th17 balance is the important factor, and lower Treg numbers carry a favorable prognosis [52]. Studies in Treg levels and activity have also been contradictory [53, 54], which is further confusing as to how to define Tregs by flow cytometry [52, 55, 56]. This remains an area for further exploration as greater understanding of the epigenetic factors involved in T cell polarization and the potential for plasticity between the subsets is developed [57].

In the mouse models, there is a notable Th1 response as evidenced by increased IFNγ production (predominantly by $CD8^+$ T cells) with advanced disease in Vk∗MYC mice [28, 58]. Transition from a Th1 to a Th2 response with increased IL-4 and IL-13 production has been described with advanced disease in the transgenic Vk∗MYC model [58], and Th2 cells provided no protection against disease in a 5TGM1 transplant model (and may even promote MM growth by promoting VEGF production) [59]. Th17 cells and IL-17 production was not significantly altered in Vk∗MYC mouse models [28], but it would be of interest to assess this in longstanding disease correlating with amount of bone disease and relative proportion of Tregs. Later work in the Vk∗MYC model has been suggestive of a pathological role for IL-17: IL-17A deficient donor grafts and inhibition of IL-17A with mAb improved MM control after BM transplant and, conversely, donor derived IL-17A promoted MM cell survival [44].

Increased Treg populations were described in the spleen, lymph nodes, bone marrow, and peripheral blood of 5T2 and 5T33 transplant mouse models, and these cells retained their suppressive function *ex vivo* [60]. In further analysis in the 5T2 model, it was evident that there are temporal differences in Treg accumulation, with changes being observed early in the spleen and peripheral blood but only at later stages of the disease in bone marrow.

3.2. Innate Immune System. Innate immune responses occur without prior exposure to antigen and memory T cell formation. Cells considered part of the innate immune response include granulocytes, antigen-presenting cells (APCs) such as dendritic cells (DCs), natural killer (NK) cells, and unconventional T cells such as invariant natural killer T (iNKT) cells and $\gamma\delta$ T cells. The latter make up a more substantial and diverse proportion of the murine immune system than in humans [61]. All of these cells have been described to be adversely affected in human MM [62–67] and are selectively discussed in more detail in Therapeutics.

Type I interferons are cytokines produced after immune cell recognition of pathogen-specific molecules via pattern recognition receptors such as Toll-like receptors (DCs can

be prolific producers). Release of type I interferons has numerous effects but is overall stimulatory to T cells by causing upregulation of MHC I and II on cells and hence increased peptide presentation. The consequences of drug-induced type I interferon production are discussed in DC Vaccines and Small Molecule Inhibitors.

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells that are increased in inflammatory states and play a pathological role in cancer by suppressing effector T cell function and promoting Treg expansion [68, 69]. They have been described as fundamental to MM-associated immunosuppression in the Vk∗MYC MM model and are driven by IL-18 that has emerged as a potential therapeutic target [70].

4. Matching Models with Human MM

4.1. Disease Stage. Chromosomal instability begins with MGUS, and cumulative chromosomal changes occur throughout the course of disease [71]. Secondary translocations, including dysregulation of c-myc, occur later in disease as the tumor becomes addicted to oncogenes [72] and escapes immunosurveillance. It would therefore seem logical that myc-overexpressing mouse models might better represent advanced disease, and those models that lack c-myc oncogene rearrangements (5TMM [73], XBP-1) might provide an opportunity to study the aetiopathogenesis of MM, in particular how MGUS transforms to MM. In all cases, it should be considered whether these models truly have an MGUS period, or whether they represent an initial state akin to smoldering MM with steady accumulation of tumor until mice exhibit symptomatic disease.

In our experience with the Vk∗MYC mouse model, it was important to interpret data in context of the amount of tumor burden and to take into account the differing disease dynamics of the transgenic and transplant models [28]. As an example, there have been contradictory reports in Vk∗MYC mice of either BM accrual of $CD4^+$ and $CD8^+$ T cells with increasing disease [58] or depletion of $CD8^+$ T cells with advanced disease [23] that can be accounted for by substantially different disease burdens in these cases. We found that immune dysfunction in Vk∗MYC mice with advanced disease was in keeping with relapsed/refractory multiple myeloma (RRMM) in humans [28], which certainly remains an area of need for novel therapeutics. Only using models with aggressive disease, however, could lead to agents being overlooked that work in indolent disease when there is a more functional immune system. Hence, if the focus of research is in preventing disease progression in the MGUS or smoldering phase of disease, aged transgenic mice with disease arising *de novo* are likely to provide a better model.

4.2. Cell Compartments. A valid criticism of translational studies is of the comparisons made between different cell compartments in mouse models and human samples. For obvious reasons, spleen and BM samples are not readily available from humans, and serial blood samples are most accessible for studies of immune cells. Where comparisons have been made between PB and BM mononuclear cells in human MM, $CD4^+$ subsets and associated cytokine profiles have been similar [28, 48–50], although PB contamination of BM samples does occur. There are differences in a few parameters however; for example, CD4:8 ratio is higher in PB than BM and there are fewer $CD4^+$ T_{CM} and more T_{EMRA} in BM than PB (which correlates with lower CD27 and higher CD57 expression in BM $CD4^+$ cells) [28].

Unlike humans (where extramedullary haematopoiesis is abnormal), the spleen is considered a haematopoietic organ in mice [74] and most frequently used for T cell analysis in studies because of ease of access and increased numbers of T cells retrievable. In many of the MM mouse models, hepatosplenic plasma cell infiltration and/or plasmacytomas occur and it is unclear whether this should be accepted as equivalent to BM infiltration or rather as true extramedullary disease.

5. Therapeutics

Much of the preclinical experimentation with immunotherapeutics has been performed in immunodeficient mice (Tables 2 and 3). Xenograft mouse models have proven useful in providing preclinical data for the use of novel immunotherapies in phase 1 human trials. Additionally, where drugs that looked favorable in the *in vitro* setting failed to yield sufficient clinical responses in phase 1 and 2 trials, returning to these models has helped provide evidence for combination therapies and phase 3 trials in humans. As already alluded to, xenograft models only provide proof of concept for the therapeutic efficacy of immunotherapeutics, and their effect in humans is often much more subdued than that in preclinical trials. Performing experiments in both immunodeficient and immunocompetent mice has been integral in elucidating the mechanism of action of novel agents (see Small Molecule Inhibitors).

5.1. Cellular Therapies. The oldest form of cellular therapy, stem cell transplantation, has been reviewed recently in MM [75]. However, chimeric antigen receptor (CAR)-T cells have really captured the scientific and public attention of late. Another approach to enhance anti-MM cytotoxic T cell activity is via dendritic cell (DC) vaccination, although DCs are significantly dysfunctional in MM patients [62, 63] that have repercussions for effective vaccination.

5.1.1. CAR-T Cells. CAR-T cells are cytotoxic T cells engineered to express receptors specific for a target antigen. In adoptive immunotherapy, millions of these cells are cultured in the laboratory and administered to the patient intravenously. For a broader review of the history and evolution of CAR-T cells in MM, readers are directed to other review articles [76, 77].

CAR-T constructs have been created for use in MM against B cell maturation antigen (BCMA), CD19, and kappa light chains. Whilst a 100% cure rate was achieved in xenograft murine models with anti-BCMA constructs [78, 79], only very modest effects have been achieved in phase

Table 2: Translational studies with immunotherapeutics targeting myeloma cells. Dara: daratumumab, len: lenalidomide, dex: dexamethasone, bort: bortezomib.

Target	Pre-clinical evidence	Phase 1/2 trials	Phase 3 trials
B2M	Anti-B2M Ab (xenograft) [190]	*Not progressed to human trials*	
BCMA	CD3-BCMA BiTE (xenograft) [125]	NCT02514239	
	CAR-T (xenograft) [78, 79]	11D5-3-CD828Z[80]	
	bb2121(contains 4-1BB) [81]	NCT02658929	
CD38	Daratumumab causes MM cell apoptosis in xenograft models [191]	Phase 1: GEN501[96] Phase 1/2: SIRIUS[97]	Dara/len/dex[98] Dara/bort/dex[99]
	α-radioimmunotherapy (5T33)[108]	*Not progressed to human trials*	
CD138	α-radioimmunotherapy (5T33)[104–107]	Phase 1 dosimetry study[110]	
	CAR-NK cells (NOD-SCID xenograft)[82]	*Not progressed to human trials*	
CS1	CAR-NK cells (NSG xenograft)[83]	*Not progressed to human trials*	
FcRH5	CD3-FcRH5 BiTE (xenograft)[192]	NCT03275103	
VLA-4	(5TGM1)[193]	*Not progressed to human trials*	

Table 3: Translational studies with immunotherapeutics targeting T and NK cells in the tumour microenvironment. Elo: elotuzumab; bort: bortezomib; dex: dexamethasone; thal: thalidomide; pom: pomalidomide; dara: daratumumab; CTD: cyclophosphamide, thalidomide, dexamethasone; NDMM: newly diagnosed multiple myeloma; RRMM: relapsed/refractory multiple myeloma.

Target	Pre-clinical evidence	Phase 1/2 trials	Phase 3 trials
CD137 (4-1BB)	Vk*MYC[23, 124] 5TGM1[123]	NCT02252263: Urelumab (+ elotuzumab)	
CS1(SLAMF7)	Anti-CS1, bortezomib (xenograft) [112]	Phase 1: Elo monotherapy[113] Phase 1: Elo/bort[117] Phase 2: Elo/bort/dex[118] Phase 1: Elo/len/dex[114] Phase 1b/2: Elo/len/dex[115] Phase 2: Elo/thal/dex[116]	NCT01335399 (ELOQUENT-1): Len/dex +/- elo in NDMM NCT01239797 (ELOQUENT-2): Len/dex +/- elo in RRMM[194] ACTRN12616001030460 (MM20): Elo/CTD vs CTD in RRMM
CTLA-4	CTLA-4 Ig (Vk*MYC)[23, 195]	NCT01592370 Arm 2: Ipilimumab (+ Nivolumab)	
KIR ligands	Anti-murine KIR mAb + len (xenograft)[120]	Phase 1: IPH2101 monotherapy[121] Phase 1: IPH2101 + len[122] NCT02252263: Lirilumab (+ Elotuzumab) NCT01592370 Arm 2: Lirilumab (+ Nivolumab)	
PD-1	PD-1 (Vk*MYC)[23]	Phase 1b: Nivolumab[134] Pembrolizumab/len/dex in RRMM[196] Phase 1/2: Pembrolizumab/pom/dex[135] NCT01592370 Arm 3/4: Nivolumab + dara +/- pom/dex NCT02036502 (KEYNOTE-023): Phase 1	NCT02576977 (KEYNOTE 183): Pom/dex +/- pembrolizumab in RRMM NCT02579863 (KEYNOTE 185): Len/dex +/- pembrolizumab in NDMM
PD-L1	PD-L1 (5T33)[132, 140]	NCT02685826: Durvalumab/len +/-dex NCT02616640: Durvalumab/pom +/-dex NCT02807454: Durvalumab +/-dara NCT02431208: Atezolizumab +/-dara	

1 trials in humans [80, 81]. Engineered NK cells specific to CD138 [82] and CS-1 [83] have also been effective *in vitro* and *in vivo* mouse models of human MM.

Because immunodeficient mouse models have been used in preclinical work, CAR-T cell-induced cytokine release syndrome (a not uncommon feature in humans) cannot be predicted for. This is an advantage in that significant morbidity and mortality might be avoided in the mice, but means that the human immune response is not being faithfully replicated: we are essentially measuring the ability of CAR-T cells to reach their target antigen in an *in vivo* system and perform cytolysis (as they would in an *in vitro* setting).

Treating MM patients with CAR-T cells remains a long way from widespread use clinically, mainly because of the cost but also the challenge of producing an effective and persistent T cell product from elderly and/or heavily pretreated patients. Studies of CD19 CAR-T cells in an NSG mouse model of lymphoma have shown that T_N and T_{CM} produce a superior CAR-T product to T_{EM} in terms of cytokine production ($CD4^+$) and cytotoxicity ($CD8^+$), and the potency of $CD8^+$ CAR-T cells is enhanced by their production in the presence of $CD4^+$ T cells [84]. It would therefore seem logical to collect and sort $CD62L^+$ T cells (i.e., T_N and T_{CM}) for CAR-T production from MM patients at first diagnosis, prior to T cell depleting therapies and subsequent further skewing of the T cell population, even if they are not used until relapse.

5.1.2. DC Vaccines. DC vaccines are produced from autologous *ex vivo* DCs generated from PB monocytes or BM progenitor cells that are exposed to MM-specific antigens. These can be derived from MM lysates or dying MM cells, or DCs can be transfected with MM-derived RNA or fused directly with MM cells. The goal of DC priming is, via enhancement of tumor-specific antigen presentation, to stimulate tumor-specific cytotoxic T cell activity and overcome T cell tolerance.

In MOPC-315 plasmacytoma-bearing mice, DC vaccine in combination with IMiDs controlled plasmacytoma growth [85]. Unfortunately this does not bear out in humans, where DC vaccines frequently show antigen-specific immune responses but do not demonstrate tumor regression [86, 87]. Returning to mouse models may yet provide an insight into how to improve clinical outcomes by enhancing DC function through choice of progenitor cell, cytokine stimulation or priming antigen, and timing and route of administration and by rescuing defective DC function (reviewed from a broader oncological perspective recently [88–92]). As an example, 5T33MM mice inoculated with α-GalCer-loaded DCs moderately prolonged survival [93]. Therapies that promote maturation of DCs and enhance type I interferon may also prove useful: for example, the novel Toll-like receptor agonist C792 inhibited plasmacytoid DC-induced MM cell growth in a xenograft model and enhanced the effectiveness of antimyeloma therapies [94].

A logical combination therapy with DC vaccines is checkpoint blockade (see Section 5.2.3), because PD-L1/2 expressed on DCs can be associated with suppression of effector T cells and expansion of Tregs [63]. It has been suggested that DC vaccination might be better utilized at a shorter interval after, or concurrently with, chemoradiotherapy to optimize immunogenic cell death, as suppressive immune cells are at their lowest at this time. Furthermore, their use in the posttransplant setting could be influential in the reemerging lymphocyte population. This is discussed further in the next section.

5.2. Monoclonal Antibodies (MoAbs). Monoclonal antibodies in the treatment of MM have been developed to target the plasma cell itself (Table 2) or to promote anti-MM immunity, whereby MoAbs target MM cell and immune cell interactions by acting as agonists or antagonists to key signaling receptors on NK and T cells (Table 3). Novel putative target antigens in MM are reviewed elsewhere [95].

5.2.1. MoAbs Targeting the MM Plasma Cell. Arguably one of the most exciting new drugs on the MM clinical scene is daratumumab, a human anti-CD38 IgG1k mAb. Xenograft mouse models were used to compliment *in vitro* data that daratumumab induced apoptosis of MM cells [96], and the drug has subsequently progressed from phase 1/2 trials [97] to promising results in phase 3 trials [98, 99]. Returning to xenograft models has further helped to establish mechanisms of action—in addition to antibody-dependent cellular cytotoxicity (ADCC), daratumumab induces programmed cell death via Fcγ receptor-mediated cross linking [100]. They have also been useful to provide evidence for the effectiveness of combination therapy with lenalidomide prior to phase 3 trials (in previously lenalidomide/bortezomib resistant MM) [101] and ATRA via upregulation of CD38 expression [102, 103].

Also utilizing plasma cell CD38 and CD138 expression, alpha-radioimmunotherapy delivers localized radiation by delivering α-particles to target cells and has been developed to treat low level residual disease in MM. Effectiveness with minimal toxicity has been shown in the 5T mouse model with an anti-CD138 mouse antibody [104–107] and an anti-CD38 mouse antibody [108, 109] coupled to bismuth-213. A small dosimetry study in humans has shown feasibility of this therapeutic approach with good biodistribution in the BM [110].

Elotuzumab is an agonist for the signaling lymphocytic activation molecule-F7 (SLAM-F7, a.k.a. CS1). It enhances NK cell-mediated ADCC of CS1-expressing myeloma cells via IL-2 and TNFα pathways [111]. Elotuzumab proceeded to phase 1 clinical trials after *in vitro* and *in vivo* studies indicated enhanced NK cell antimyeloma activity, which was further augmented in combination with bortezomib [112]. Whilst tolerated well by RRMM patients, this mAb was ineffective as monotherapy [113], but clinical responses were seen when combined with IMiDs [114–116] or bortezomib [117, 118]. It is likely that the timing of administration and choice of combination therapy are important, as coadministration of dexamethasone is profoundly immunosuppressive to NK cells [7]. Researchers are now returning to mouse models to support phase III trial combination therapies and to further evaluate mechanism of action.

5.2.2. Agonistic MoAbs. The cytotoxic functions of NK cells are regulated by a balance of expression of activating and inhibitory receptors, with the latter being known as killer cell immunoglobulin-like receptors (KIRs). The expression of ligands to KIRs is upregulated on MM cells, causing inhibition of NK cell activity [119]. IPH2101 is an anti-KIR human IgG4 mAb that prevents inhibitory KIR-ligand interaction against KIR2DL-1, KIR2DL-2, and KIR2DL-3. Initial *in vitro* experiments using IPH2101 in combination with lenalidomide showed synergistic anti-MM activity by enhancing NK cell function, and an *in vivo* tumor cell rejection model in C57BL/6J mice showed that a murine anti-KIR and lenalidomide had an additive effect [120]. Phase 1/2 clinical trials followed in humans with RRMM as monotherapy [121] and in combination with lenalidomide [122]. IPH2101 is no longer in development and has been superseded by another anti-KIR mAb lirilumab, which is in phase 1 trials in solid tumors.

Urelumab is an agonist for CD137, a costimulatory receptor target that is expressed on activated T cells, NK, and NKT cells. Activation with an agonistic mAb (4-1BB) exerted variable antimyeloma activity in Vk∗MYC mice [61, 109] and 5TGM1 mice [110]. In 5TGM1 mice, anti-CD137 mAb treatment led to a significant reduction in monoclonal paraprotein and extramedullary disease after 30 days of treatment, but had little effect on skeletal involvement [123]. It has also been trialed by two separate groups with two different transplant clones of Vk∗MYC: anti-CD137 mAb treatment with the Vk∗MYC 12653 clone showed a marked response in plasma cell infiltrate and paraprotein accompanied by a significant increase in survival [23], whereas the Vk∗4929 clone was virtually unaffected, even in combination with anti-CD40 antibody [124]. Of note, combination therapy with anti-CD137 and anti-CD40 prolonged survival in a minor proportion of treated mice who had a lower burden of disease at commencement of treatment: this highlights a problem with using transplant models with highly proliferative disease (as opposed to the indolent transgenic models), in that there may not be an opportunity for immunotherapies to be able to be shown to exert an effect. A phase 2 trial in RRMM patients with urelumab in combination with elotuzumab is underway (NCT02252263).

In order to promote immune synapse formation between T cells and tumor cells, bispecific T cell engager (BiTE) antibodies have been developed, which have had clinical success in lymphoma and acute lymphoblastic leukaemia. In myeloma, a xenograft model was used to provide *in vivo* data showing the efficacy of a CD3-BCMA BiTE [125], which is now in phase 1 studies in humans (NCT02514239). Other BiTEs in development include CD3-FcRH5, which has also progressed to phase 1 trial (NCT03275103), and an NK receptor binding BiTE CS1-NKG2D [126].

5.2.3. Antagonistic MoAbs. A MM cell line J558L was used in one of the first *in vivo* experiments with BALB/c mice to demonstrate the antitumor efficacy of PD-L1 blockade [127]. In the 5T33 mouse model, as has been reported in human MM patients [128–131], PD-L1 is overexpressed on MM cells and PD-1 expression is increased on T cells [132, 133]. After the success of PD1/PD-L1 pathway blockade in melanoma, these inhibitors were used in an array of cancers but with underwhelming responses in phase 1/2 trials in RRMM [134, 135], and there has been some critique about the appropriateness of PD-1 inhibition in MM patients [41]. Chronically exhausted T cells may not have the capacity to respond to checkpoint blockade owing to a stably differentiated epigenetic landscape [136–138]. Alternately, it has since been demonstrated in human MM that hyporesponsive $CD8^+$ T cell clones exhibit low expression of PD-1 or CTLA-4, suggesting that these cells are senescent rather than exhausted [139].

Returning to mouse models, inhibition of PD-1 had no effect on disease progression in Vk∗MYC [23]; however, in the 5T33 model, PD-1 was increased on T cells after autologous BM transplant and PD-L1 blockade increased efficacy of DC vaccine in combination with ASCT [132]. Further, PD-L1 mAb administered during the homeostatic proliferation phase after nonmyeloablative total body irradiation resulted in increased survival [140]. Immune checkpoint blockade with PD-1 blocking antibodies in the posttransplant setting also significantly improved disease control in Vk∗MYC mice [47].

To understand why PD-1 inhibition might be efficacious in these circumstances, it is important to note that PD-1 is not only upregulated in exhausted T cells but also as a normal process in effector T cells after activation of the T cell receptor. A balance between stimulatory and inhibitory signaling ultimately controls the magnitude of a T cell proliferation to antigen, and PD-1 facilitates apoptosis in $CD8^+$ T cells by increasing reactive oxygen species [141]. Therefore, utilizing PD-1 inhibition in the post-ASCT setting could represent a unique timepoint at which derepression of proliferating T cells could enable superior clearance of tumor by myeloma-specific T cell clones.

However, recent studies have suggested a more complex role of PD-1 in T cells. PD-1 signaling causes a metabolic switch from glycolysis to lipolysis and fatty acid oxidation that is critical for the development and maintenance of T cell memory [142, 143]. This might suggest that PD-1 inhibition at T cell activation might impair the subsequent development of T memory cells, but this has not been reported with mouse models of acute viral infection [144–146], and further investigations in the MM setting are warranted.

Ipilimumab targets cytotoxic T-Lymphocyte antigen 4 (CTLA-4), another inhibitory receptor that is upregulated early in T cell activation. Human trials with ipilimumab have been in solid cancers, largely advanced melanoma, with some success but there are concerning, and potentially severe, immune-related adverse effects. This reiterates a problem with checkpoint blockade in that reverting evolutionarily acquired mechanisms that prevent the expansion of autoimmune T cell clones can result in autoimmune complications.

T cell immunoglobulin and ITIM domains (TIGIT) have recently been described as another effective immune checkpoint target in the Vk∗MYC mouse model [47, 147].

5.2.4. Combination Therapy. It would seem a logical rationale to combine stimulatory and inhibitory checkpoint blockade,

or NK and T cell checkpoint blockade, to maximally antagonize tumor-induced immune suppression. Indeed, there are a number of ongoing human trials with PD-1/PD-L1 inhibitors in combination with other immunotherapies, and with checkpoint blockade combinations that combat both NK and T cell inhibition (Table 3). Unfortunately, phase 3 trials using pembrolizumab in combination with IMiDs and dexamethasone have been suspended because of fatalities related to immune-mediated pneumonitis in the pembrolizumab-receiving groups. This could dampen the pharmaceutical appetite for further trials in MM with this combination.

Further studies with mouse models have been supportive of combination therapies. In the Vk12653 (4-1BB-responsive) transplant model, CD137 agonist treatment both reduced the proportion of Tregs and increased $CD8^+$ effector frequency and function but also upregulated PD-1 and TIM-3 expression. Consequently, combination of CD137 mAb and anti-PD-1 early after BM transplant proved superior in MM disease control [44]. In the 5T33 mouse model, tumor-bearing mice treated with low dose whole body irradiation and combinations of immune checkpoint blockade (PD-L1 blockade with LAG-3, TIM-3, or CTLA4 blocking antibodies) had not only significantly improved survival rates, but also correlated with increased frequency of tumor-reactive T cells and elevated levels of inflammatory cytokines [133].

Ongoing work with checkpoint inhibitors is likely to concentrate on the timing of administration around other anti-MM therapies (particularly in the lymphopenic after BM transplant setting) and their use in combination with DC vaccines or oncolytic vaccines to optimize a specific anti-MM immune response. The potential to combine oncolytic vaccination (reviewed recently [148]) with immunotherapies to enhance immune surveillance was shown in a breast carcinoma mouse model with anti-4-1BB [149]. Specific to MM, the efficacy and safety of a vaccinia virus were established in a mouse xenograft model of MM [150], but has not yet progressed to human trials.

5.3. Immunomodulatory Drugs (IMiDs). Thalidomide, or its analogs lenalidomide and pomalidomide, is often used in combination therapy with proteasome inhibitors, alkylating agents, and/or corticosteroids in the treatment of human MM. IMiDs were first introduced as an antimyeloma therapy without fully comprehending their mechanism of action. It is now understood that the binding of IMiDs to cereblon (CRBN) [151] leads to the degradation of two zinc finger transcription factors: IKZF1 and IKZF3 [152, 153]. This inhibits MM growth as IKZF1 is required for plasma cell maturation and loss of IKZF1/3 leads to decreased *IRF4* and *MYC* expression [151, 154]. IMiDs achieved their name by being stimulatory to NK and T cells *in vitro*. This seems to be, in part, due to enhanced T cell IL-2 production, explained by the inhibition of IFZK1/3-mediated repression of the *Il2* promotor [152], although this effect is significantly abrogated by high dose steroid therapy [7].

It has long been appreciated that thalidomide does not have the same antitumor or antiangiogenic effect in rodents as that seen in humans [155]. Rodents have a point mutation in the substrate recognition protein of CRBN meaning that IMiDs cannot bind [156, 157] and therefore do not exert a direct antimyeloma effect in murine MM [19, 158]. To this end, several groups have developed humanized CRBN mouse models to further elucidate the *in vivo* immunomodulatory effects of IMiDs. It is possible that IMiDs have targets other than CRBN: there are a number of murine studies showing that lenalidomide enhances $CD4^+$ T cell [159] and NK cell [85] antitumor activity and, in CB17-SCID mice bearing subcutaneous MM.1S plasmacytomas, pomalidomide-resistant xenografts could respond to lenalidomide despite CRBN levels being low [160]. IMiDs have also been shown to exhibit synergistic effects in combination with tumor-antigen loaded DCs in the MOP-315 murine model of MM [85, 161].

5.4. Small Molecule Inhibitors. Small molecule inhibitors generally exert their antitumor effects by promoting tumor cell apoptosis or cell cycle arrest but, somewhat serendipitously in some cases, their off-target effects on the immune system are beginning to be comprehended. In fact, some would say that their full therapeutic effect may *depend* on a functioning immune system [162].

Histone deacetylase inhibitors (HDACi) exert their full effect in murine tumors when combined with traditional chemotherapy [162] or with CD137 and CD40 mAb (that promote APC function and thereby support cytotoxic T cells) [163]. The importance of host-derived IFNγ for the effectiveness of HDACi has been demonstrated utilizing immunocompromised and immunocompetent mouse models of adenocarcinoma, aggressive lymphoma [162], and breast carcinoma [164].

Combination therapies using HDACi with DNA methyltransferase inhibitors or IMiDs are increasingly being studied. Panobinostat in combination with azacitidine has shown efficacy in the transplant Vk∗MYC model, but the role of the immune system was not evaluated [165]. Quisinostat in combination with decitabine in 5T33MM diseased mice was also favorable and is, at least partly, attributable to a significant induction of a type I interferon response; decitabine in particular resulted in increased DC maturation [166]. In a leukaemia mouse model decitabine was also reported to deplete MDSCs [167], whether that bears out in the MM tumor microenvironment is yet to be proven.

We await the long-term outcomes of phase 2/3 trials using Vorinostat and Panobinostat in RRMM in combination with bortezomib and/or IMiDs. Of note, some HDACi have been reported to reduce cereblon and so might be expected to impair the efficacy of IMiDs in this setting [168].

The inhibitor of apoptosis (IAP) antagonist LCL161 competitively inhibits binding of cellular IAPs, which are frequently inactivated in MM. Contrary to expectations, LCL161 reduced tumor burden in Vk∗MYC aged transgenic mice and transplant models. This was, again, shown to be the result of type I interferon production by the MM cells that resulted in their increased phagocytosis by macrophages [169]. A phase 2 clinical trial in humans did not show any response to single agent LCL161. Returning to the transplant Vk∗MYC model, the combination of LCL161 and anti-PD-1 was curative in all mice that completed 2 weeks of treatment. Hence

combination therapy with LCL161 and PD-1 inhibition has been taken forward to phase 2 clinical trials (NCT03111992).

In a somewhat divergent approach to proinflammatory immunotherapies, bromodomain inhibitors (BETi), which are considered immunosuppressants due to their ability to reduce key proinflammatory cytokine and chemokine genes in sepsis [170], have been utilized in MM. The bromodomain inhibitor JQ1 resulted in rapid paraprotein regressions and improved survival outcome in transplanted Vk∗MYC mice, and it was shown to diminish IFNγ-induced PD-L1 expression on human and mouse tumor cell lines [171]. This is particularly relevant to myc-driven malignancies, as induction of PD-L1 may be partly due to the direct binding of MYC to the promoter region of CD274 (PD-L1) [172]. However, the JQ1 response was shown to be caused by the displacement of a BET protein from the transcriptional start site of CD274 and is therefore myc-independent [171]. Nevertheless, BETi may prove to have a role in selected human MM cells that have upregulated PD-L1 expression.

6. Future Directions

6.1. Personalized Care: Humanized Mice and 3D Culture Systems. Medical oncology is increasingly headed towards personalized care and, rather than a "one drug fits all" approach, it would be ideal to test the efficaciousness of immunotherapeutic drugs in an *ex vivo* model of an individual's tumor microenvironment prior to administration to the patient. Humanized mice may offer a conduit for this purpose, although it is not possible to incorporate a human thymus for normal T cell development: this may not be a disadvantage in the setting of MM due to the occurrence of thymic atrophy in immunosenescence.

Also promising are 3D tissue culture systems, which have the potential to be cheaper, less time consuming, and more ethically viable and have higher drug throughput than mouse models. The notable disadvantages currently (compared with mouse models) include the lack of vasculature and the challenges of maintaining plasma cells in an *in vivo*-like microenvironment alongside normal BM cell maturation. Several groups are making progress with replicating the BM microenvironment [173–179]: these generally involve either a tissue scaffold of osteoblasts [173, 174], crosslinked fibrinogen [176], or differentiated mesenchymal stromal cells [177] that can be combined with microfluidic chamber so that drug can be circulated similarly to capillary flow in the bone marrow. Increasing investment in these technologies over the last decade are likely to see improvements in the extracellular matrix scaffold and oxygen and nutrient distribution, as well as increased throughput and standardization of microscopic analysis and cell measurements.

6.2. Targeting Immunotherapies to Immune Profile. For some time it has been appreciated that evolving and cumulative genetic changes contribute to increased resistance of MM cells to apoptosis, the development of drug resistance, and poorer prognosis [71]. In some patients, clonal tides of MM can mean that therapies need to be switched depending on the dominant clone and its responsiveness [180]. In the same way, we should look to fitting treatments not only to the cytogenetic profile of the patients, but also to their immune profile.

Immune profiling can be performed by the assessment of T cell phenotype by flow cytometry; in one study, a putative immune signature by flow cytometry was associated with PFS and OS for MM patients treated with ASCT [181]. Features such as a reduced CD4:8, low proportions of circulating T_N and high proportions of T_{EM}/T_{EMRA} indicate immunosenescence and shifts in the T cell population due to iatrogenic lymphopenia, and are likely to correlate with poorer responses to immunotherapeutics. Individuals with an immune profile comparable to healthy donors (i.e., younger, newly diagnosed MM with less advanced or smoldering phenotype of disease) are likely to achieve the greatest benefit from immunotherapeutics, and targeting this group in clinical trials may result in superior trial outcomes and greater cost-effectiveness.

6.3. Immunotherapy in Immunosenescence. If the adaptive immune system is essentially considered terminally differentiated or "burnt out" in immunosenescent, heavily treated MM patients, is there a role for immunotherapies at all? In such patients, perhaps alternative approaches to replenishing an effective T cell pool should be evaluated such as "off the shelf" CAR-T cells (derived from young healthy donors). Notably, in the 5T33 mouse model, it was shown that T lymphocytes from younger mice were associated with better disease control [182]. Thymic regeneration techniques [183, 184], whilst still some way from being utilized clinically, represent another solution to the diminished naïve T cell population.

Other immunomodulatory approaches also need to be considered such as mimicking or enhancing $CD4^+$ T cell help [185]. The former might include cytokine support and agonists of costimulatory pathways such as CD27, and the latter utilizes innate immune signals to aid DC priming of $CD8^+$ T cells. Of note, NK-like T cells are more frequent at extremes of age and are correlated with healthy ageing [186, 187]—further understanding of their potential plasticity will help with the development of age-appropriate immunotherapies.

7. Conclusions

Mouse models will continue to be important for selecting drugs for clinical trials, as the actual efficacy and toxicity cannot be predicted *in vitro*. However, moving away from utilizing hardy human MM cell lines in immunocompromised mice and, instead, trialing immunotherapeutics in the immunocompetent mouse are likely to yield more informative preclinical information for both the use of immunotherapeutics and enhancing the performance of small molecule inhibitors. Importantly (and with particular relevance to combination immunotherapies), acknowledging the complimentary roles of the innate and adaptive immune systems, and dendritic cells as the interface between the two, will be integral in furthering the success of immunotherapies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Molecular Drivers of Potential Immunotherapy Failure in Adrenocortical Carcinoma

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Adrenocortical carcinoma (ACC) is a rare, highly aggressive cancer, often insensitive to conventional chemotherapeutics agents. Early diagnosis, followed by radical surgical resection plus/minus adjuvant mitotane therapy, is nowadays the only valuable option. Unfortunately, one out of four patients has metastatic disease at diagnosis and most of radically resected ACC patients are destined to recur with local or metastatic disease. Numerous efforts aimed at identifying molecular alterations crucial for ACC pathogenesis have been extensively conducted, with the hope to develop new treatments. Indeed, multiple genes and pathways have been identified as potentially targetable in ACC patients; however, despite the strong preclinical rationale, translational findings to clinical trials led to date to disappointing results. The immunotherapeutic intervention targeting T-cell checkpoint molecules has been proposed as well, but results obtained in early studies indicate that ACC patients would be unlikely to benefit from immunotherapy. Genetic alterations of different pathways involved in ACC carcinogenesis are also known substrates of resistance to immunotherapy. Among them, β-catenin gene CTNNB1 and TP53 gene are frequently mutated in ACC samples. Overactivation of the β-catenin pathway and loss of p53 protein function are potential tumor-intrinsic factors that, impacting on the ability of ACC cells to recruit dendritic cells, leading to T-cell exclusion, put this tumor among those that are potentially resistant to immunotherapy. Moreover, the steroid phenotype, which implies glucocorticoids hypersecretion in a subset of ACC, contributes to generating an immunosuppressive microenvironment. Here, we review clinical results of immunotherapy in ACC and we highlight molecular mechanisms driving immunotherapy failure in ACC, suggesting possible approaches to overcome resistance.

1. Background

Adrenocortical carcinoma (ACC) is a rare tumor derived from the adrenal cortex, with an estimated incidence between 0.7 and 2.0 per million population per year. ACC could occur at any age, but the peak of incidence is between 40 and 60 years, with higher prevalence in female (up to 60%) [1]. Despite intense efforts to improve management of ACC, both with preclinical and clinical studies, prognosis remains overall limited, although it has been recently recognized that ACC is a very heterogeneous disease and harbors a variety of morphological, clinical, and genetic variants that have a prognostic value [2, 3]. ACC is mostly sporadic, although it can be diagnosed within hereditary syndromes, such as Li-Fraumeni and Lynch syndromes, associated with specific germline mutations in TP53 gene or in various mismatch repair genes, respectively [4].

The disease stage at diagnosis is a key prognostic factor for ACC: 5-year survival for 60-80% in patients with stage I, up to 50% for locally advanced disease, dropping to a very low percentage (0-28%) in the case of metastatic disease [1]. Other prognostic factors at diagnosis are proliferation activity [5] and cortisol hypersecretion [6]. In regard to the management of ACC, current guidelines [1, 7] recommend the complete surgical resection of primary tumor as the only potential curative treatment, although it is a realistic approach only

in patients at stage I and II of disease and more rarely in those at stage III. In patients radically operated however, recurrence frequently occurs (30%-70% of cases) [1]. This is the reason why adjuvant mitotane, an adrenolytic drug [8], is prescribed in the majority of patients [1], although the efficacy of this drug in the adjuvant setting is supported by the results of a retrospective multicenter international study showing that postoperative mitotane treatment is associated with a significant reduction of the risk of relapse and death [9]. The management of patients with metastatic or inoperable disease (45% of patients at diagnosis [1]) requires systemic treatment which consists in either mitotane alone or mitotane plus etoposide, doxorubicin, and cisplatin (EDP-M) [10, 11]. Mitotane is the only drug approved to treat ACC, but its role is conditioned by the possibility of attaining therapeutic concentrations in the plasma [12]. However, its pharmacokinetics, safety profile, and adverse effects show high interindividual variability and strongly limit its efficacy [13]. The great majority of patients who received mitotane and EDP-M are destined to undergo disease progression. In these patients, a pharmacological approach that includes gemcitabine with capecitabine could be administered [14, 15], but this regimen has a limited clinical benefit. On these bases, there is a need of new treatment strategies.

In the past few years, molecular characterization of ACC identified genetic and molecular abnormalities and disclosed novel potential druggable molecular targets to develop new therapies. In particular, several comprehensive analyses of the genomic profile of ACC have been performed, showing a complex genomic landscape with the identification of recurring mutations in different genes such as ZNFR (20%), CTNNB1 (14%), TP53 (14%), and RB1 (11%) [3, 17, 18]. As previously mentioned, various genetic alterations are present in subgroups of tumors with different clinical characteristics and outcomes; of relevance, mutations in the CTNNB1 gene encoding for β-catenin and in the TP53 gene encoding for the tumor suppressor p53 proteins have been defined as poor prognostic factors for ACC [19]. Moreover, both mutations in CTNNB1 and TP53 genes have been shown to play a role in ACC carcinogenesis, as early and late events, respectively [20–22]. The Cancer Genome Atlas – Adrenal Cortex, that analyzed 91 cases for alterations in the ACC genome, reveals that, beside the cited molecular alterations, mutations in the PRKAR1A gene (8%) and the overexpression of IGF2 (90%) [3] can be observed as well. Analysis of results further indicates that copy number alterations likely play a critical role in ACC. Whole genome doubling is indeed observed in about 51% of ACC samples; in addition, hypoploidy or the loss of a significant amount of the genome is found in a high number of cases. The study further showed that the frequency of copy number changes is associated with an aggressive clinical course of the disease, supporting whole genome doubling as a key point of disease progression [3]. At the molecular level, in addition to p53 and Wnt-β-catenin pathways for which no specific molecular target agents are currently in use, other potentially druggable pathways have been identified in ACC patients, such as the Epidermal Growth Factor Receptors (EGFRs), Insulin Growth Factor-Receptor 1 (IGF-1R), and Vascular Endothelial Growth Factor Receptors (VEGFRs). However, in spite of the preclinical rationale, clinical trials testing drugs targeting these pathways led to disappointing results [23, 24].

2. Current Immunotherapy Trials in ACC

Current most successful immunotherapies against cancer are based on blocking key regulators of T cells (T-cell checkpoint molecules). These are inhibitory molecules with the ability to limit immune responses against tumors cells, such as Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) and programmed death receptor 1 (PD-1) or its ligand PD-L1 [16].

Multiple ongoing clinical trials have been designed to explore the role of anti-PD-1 or anti-PD-L1 drugs in numerous cancers, including ACC [25] (Table 1). However, initial clinical findings seem to be unsatisfactory, indicating that ACC patients would be unlikely to benefit from immunotherapy.

In particular, in the JAVELIN international, multicenter phase Ib trial, safety, pharmacokinetics, and clinical activity of the anti-PD-L1 monoclonal antibody avelumab were tested in patients with different metastatic solid tumors (ClinicalTrials.gov Identifier: NCT01772004). In this study, 50 ACC patients, previously treated with mitotane or platinum-based chemotherapy, were treated with avelumab (10 mg/kg IV, every 2 weeks) until progression, unacceptable toxicity or withdrawal. Results demonstrated, as expected, an acceptable safety profile, especially in patients with limited pretreatment. However, only a modest clinical activity was observed since a partial response was obtained in 3 of 50 patients (6%), while 42% and 46% of patients experienced stable disease and disease progression, respectively [26]. More importantly, however, the median progression-free survival and the overall survival of this patient population were 2.6 months (95% CI 1.4–4.0) and 10.6 months (95% CI 7.4—not estimable), respectively. Taken together, these results are similar to those obtained with the previously mentioned second-line combination regimen, gemcitabine and capecitabine. So, at least with the strategies available to date, immunotherapy seems to be not able to improve the current standard therapy in ACC.

3. Immunologic Properties of ACC

Several observations contribute to explaining the poor response of ACC to standard immunotherapies.

3.1. Immune Checkpoints Molecules. In order to preselect cancer patients most likely to benefit from immunotherapy, PD-L1 expression has been considered and likely reflects an immunoreactive tumor microenvironment [27]. A correlation between tumor PD-L1 expression and response to PD-1 therapy, in fact, has been provided for various cancer types, including melanoma, non-small-cell lung carcinoma, and renal cell carcinoma [27]. Accordingly, PD-L1 expression was investigated in 28 ACC tissues by immunohistochemistry, showing that a small percentage of tumors (10.7%) are positive for PD-L1 expression with a cut-off level of 5%

Table 1: Clinical studies investigating immunotherapy in ACC.

Drug	Target	Study phase	Patients	Results	Ref.
Avelumab	PD-L1	I	50	ORR: 6% OS: 10.6 months PFS: 2.6	[16]
Ipilimumab + radiotherapy	CTLA-4	I/II	Active, nonrecruiting	-	NCT02239900
Pembrolizumab	PD-L1	II	Recruiting	-	NCT02673333
Nivolumab + ipilimumab	PD-1/ CTLA4	II	Recruiting	-	NCT03333616

ORR: overall response rate; OS: overall survival; PFS: progression-free survival.

[28]. Therefore, according to this tumor intrinsic parameter, ACC could be poor, if not responsive to immunotherapy. However, the role of PD-L1 tumor expression as predictor of immunotherapy response is currently debating, due to positive ORRs reported in clinical studies in patients carrying PD-L1 negative tumors (ACC cases not included) [29]. For instance, nivolumab treatment is associated with clinical benefits in a number of tumors regardless of PD-L1 expression [29]. Taken together, these findings indicate that PD-L1 expression may not be the ideal biomarker of sensitivity also in ACC and that other markers of clinical efficacy and safety need to be identified.

3.2. Glucocorticoids. Another element of intrinsic immunoresistance in ACC is linked with hypercortisolism-secondary immune defects. Patients with cortisol-secreting ACCs are indeed characterized by suppression of T cell activity [30] and altered levels of circulating lymphocytes [31]. Recent insights into genomic characterization of ACC identify the so-called "steroid phenotype" based on differential expression of steroid synthesis pathway genes and clinically translating into patients with glucocorticoids hypersecretion. This subset of ACCs displays the lowest pathological immune scores in cancer stromal cells infiltrates among different human neoplasms. The clinical phenotype of ACC with steroid phenotype also correlates with the lowest overall survival. However, it must be noted that this lethal phenotype is also associated with a high proliferative score indicating that the immunosuppressive microenvironment induced by steroids contributes in part to determining the poorer prognosis, but that the intrinsic aggressiveness of these tumors also depends on other genomic alterations (see below) linked to proliferation [3]. Establishing the exact role of these two components in producing the patient clinical phenotype is beyond the scope of the present review.

Beside endogenous steroid hypersecretion in functioning ACCs, glucocorticoids are frequently prescribed as supplementation to treat adrenal deficiency in ACC patients treated with mitotane or following adrenal surgery. However, doses of steroid replacement and types of steroid used are much lower and different, respectively, compared to what is used for immunosuppressive or anti-inflammatory steroidal therapies [1]. Importantly, an ACC patient should not be excluded from immunotherapy trials because of steroidal replacement therapy nor glucocorticoids should be stopped in case of adrenal insufficiency.

With regard to immunotherapy, it should be kept in mind that despite the immunosuppressive "milieu" induced by glucocorticoids, dominant underlying biological properties of ACC tumors mostly contribute to the dismal prognosis of patients, wrongly inducing the impression of failure of immunotherapy because of glucocorticoids.

3.3. Genomic Alterations. The rationale to explain why ACC displays resistance to immunotherapy could be linked to the above-mentioned molecular alterations highly prevalent in ACC, namely, mutations in TP53 and CTNNB genes [17, 32, 33]. It is well known that despite the lymphocytic activation by checkpoint inhibitors, lack of spontaneous T-cell infiltration (non-T-cell-inflamed tumors) might result in immunotherapy ineffectiveness [16]. Interestingly, while the presence of multiple chemokines, such as the CXCL9 and the CXCL10, directly correlates with high number of infiltrating T cells [34], the specific lineage basic leucine zipper transcriptional factor ATF-like 3 lineage of dendritic cells (BATF3 DC) are considered the major source of these chemokines. Therefore, BATF3 DC appear to play a central role in orchestrating antitumor T-cell responses [16]. Several oncogenic pathways have been found to influence the local antitumor immune response by modulating BATF3 DC recruitment; among them, overactivation of β-catenin pathway has been associated with a reduced recruitment of BATF3 DC into tumor, leading to failure in chemokine release [35]. Evidence that upregulation of Wnt/β-catenin signaling is associated with T-cell exclusion has been provided for metastatic melanoma [35], bladder, head and neck [36], and colorectal cancers [37]. In addition to the Wnt/ β-catenin pathway, the inactivating mutations TP53 have been associated with defects in the ability of tumor cells to produce key chemokines required for BAFT3 DC recruitment [38]; p53 loss of function and lack of T-cell infiltration have been found in basal-like ER-negative breast cancers, but not in ER-positive breast cancer [39].

Therefore, either overactivation of the Wnt/β-catenin pathway or loss of p53 is potential tumor-intrinsic factors that, altering on the ability of ACC cells to recruit BAFT3

DC cell and leading to T-cell exclusion, likely indicate that this type of tumor is potentially resistant to immunotherapy. This point is strengthened by results reported on CTNNB1 expression and T-cell infiltration that have been investigated in a series of ACC tumors, showing that the increased CTNNB1 expression correlated with reduced infiltration in T cells [40]. Interestingly, high levels of CTNNB1 expression have been associated as well with increased cortisol levels [40] that likely contribute to the clinical resistance of ACC to immunotherapy [41].

Dysfunction of p53 due to mutations may contribute not only to carcinogenesis, but evidence indicates that it may also contribute immunologically to tumorigenesis and tumor progression, altering as well the immune-mediated response in the microenvironment. Indeed, in cancer cells with p53 dysfunction, restoring wild-type p53 drives immunological activity towards antitumor response [42]. Accordingly, in acute myeloid leukemia, there was also a significant increase in PD-L1 expression in patients with TP53 mutations when compared to wild-type TP53 patients [43]. Although these observations were made in tumors other than ACC and this hypothesis needs to be confirmed, it could be suggested that targeting immune escape mechanisms could establish sensitivity to the checkpoint inhibitors in ACC. Thus, combining the administration of immune checkpoint inhibitors with drugs targeting the Wnt-β catenin and TP53 pathways could be an attractive treatment paradigm to be explored.

4. Strategies to Overcome Immunotherapy Resistance in ACC

The combination approach suggested above could be intriguing in trying to overcome resistance in ACC. Unfortunately, despite several intensive studies, targeting both Wnt/β-catenin and p53 pathways is nowadays challenging, due to their important role in different physiological processes, which implies toxicity in case of effective inhibition. In regard to pathways, both active inhibitors of Wnt secretion and Wnt/receptor interactions, including antibodies and small peptides, are being tested in early-phase trials [44] and others are in preclinical development (for a review, see [45]). Among them, the OMP-54F28 agent, a fusion protein comprised of the cysteine-rich domain of frizzled family receptor 8 fused to the human immunoglobulin Fc domain, is able to bind to all Wnt ligands blocking Wnt signaling [46]. Moreover, LGK974, an inhibitor of the porcupine membrane-bound *O*-acetyltransferase, required for posttranslational acylation of Wnt and its subsequent secretion [47], inhibits Wnt signaling both in vitro and in vivo in different animal models [48]. A phase I trial to evaluate safety of LGK974 is ongoing [44]. Another phase I clinical trial, investigating toxicity and activity of the small-molecule CWP232291 that targets β-catenin degradation, is currently ongoing in the management of acute myeloid leukemia patients [44].

The above-mentioned drugs, however, are in their early phases of clinical development; thus, they will not be available soon. Thus, an approach to overcome the ACC resistance to checkpoint inhibitors could come from drugs already marketed for other therapeutic indications and that are endowed, as ancillary mechanism, with the ability to target this pathway. Preclinical experimental models could be strategic to shed light on this field. In line with this, we recently demonstrated that in the widely used ACC cell model, namely, the NCI-H295R cells, characterized by an abnormal β-catenin nuclear accumulation [49], both the CYP17A1 inhibitor abiraterone acetate that induces an increase of progesterone levels and progesterone itself induce cytotoxicity and partially reduce the nuclear accumulation of β-catenin [50, 51]. We are aware that this result, now under a deeper molecular characterization, was obtained with a preclinical in vitro approach, and we would like to stress on the fact that the clinical translation is not obvious. This observation, however, could stimulate further research in this direction, demonstrating as well the possible contribution of the Wnt/β catenin in the resistance to immunotherapy of ACC.

Concerning TP53, as already mentioned, it represents the most commonly mutated gene in cancer [52], leading to a great variability on the effects of mutation on p53 activity. Therefore, targeting functional variant mutant p53 requires a mutation-specific approach, ranging from the restoring of wild-type activity of the mutant p53 to the degradation of mutant protein [52, 53]. In ACC, TP53 mutations lead to the production of p53 protein that lacks its physiological function, appearing mostly in the late phase of tumor progression and associated with a poor outcome [2, 54]. Efforts in designing short synthetic peptides able to stabilize p53 or small molecules targeting key signaling interactions involving mutant p53 have been described, including gene therapy that uses viruses to deliver p53 to cancer cells [55]. Among the different strategies, the small-molecule APR-246, able to induce a conformational change toward wild-type like structure [56], has been shown to have strong cytotoxic effects in several cancer cell lines [57–59] and is currently under investigation in patients with various solid tumors [52]. However, these strategies are all in their early clinical development and none of them are currently available.

5. Other New Strategies and Neoantigens

Other recent observations point to immunotherapy as a valuable therapeutic approach for ACC. For example, the analysis of nonsynonymous mutations likely represents a useful predictive marker in selecting tumor types that are mostly likely to respond to the immune checkpoint therapy [60, 61]. The mutational load, in fact, is defined as the total number of somatic nonsynonymous point mutations that, by generating novel gene products detected by the immune subsystem as foreign, may trigger an anticancer response [60–63]. On this line, analyses of the mutational load in ACC tumors resulted in an intermediate mutational load value, thus suggesting that ACC could respond to immunotherapy [64].

According to previous conclusions, recent evidences underlined the potential value of microsatellite instability as determinant of immune responsiveness in ACC patients. While in a normal cell, the length of microsatellites is

maintained stable during multiple cell divisions by the mismatch repair (MMR) system, in cancer cells, the length of microsatellites can vary due to defects in the MMR system leading to the so-called "microsatellite instability" (MSI). Tumors with abnormal MMR processes and high MSI lead to additive mutations throughout the genome (e.g., "hypermutator" phenotype), a condition that is associated with response to immunotherapy [65]. Bonneville et al. recently found MSI in 4.35% of ACCs, a result which is inferior to that found in classical MSI-high-colon cancer (19.7%), but higher to the median value found across 39 tumor types (3.8%) [65]. Furthermore, high MSI is a constitutional characteristic of the Lynch syndrome, an autosomal dominant genetic condition associated with high risk of colon cancer as well as other cancers including ACC [66]. Recently, mutations in the MUTYH gene encoding for a DNA glycosylase involved in base excision repair (BER) of DNA damage have been described in two series of ACC patients. This finding further expands the mutational asset and MSI of ACC tumors and may, therefore, represent another potential predictive signature of immunotherapy efficacy different from MMR system [67].

The timing of an immune intervention could also play a role in determining its efficacy. Probably, immunotherapy has more chances to be effective in an advanced metastatic ACC rather than in an early one. Recent evidences have in fact highlighted that metastatic ACCs display a higher tumor mutation rate and tumor heterogeneity than primary tumors. Thus, this temporal and spatial heterogeneity could represent a potential advantage for immunotherapy [68].

Finally, the finding of the high expression of the Melan-A/MART1 in ACC [69] which is used as a marker for identifying lesions with adrenocortical origins [18] may also support the notion that ACC would have the chance to respond to immunotherapy against selected neoantigens. This melanoma-associated antigen, in fact, has been described as a human melanoma antigen recognized by autologous cytotoxic T cells [70].

6. Conclusions

Results obtained so far hardly lead to considering immunotherapy as a possible immediate therapeutic opportunity for ACC patients. Whether or not immunotherapy will offer a new hope for the management of ACC, however, needs to be further investigated, in particular in a combination therapy, that includes checkpoint inhibitors administered after or in association with chemotherapy molecular target therapies or radiation therapy [71, 72]. Several lines of evidence indicate in fact that the cytotoxic effects of chemo- and radiotherapy may function as immunogenic treatments by inducing expression or reexpression of tumor-associated antigens (TAAs) or by inducing additional new mutations and, therefore, inducing T-cell-specific immune responses [71, 72].

Furthermore, patients that could have the chance to receive clinical benefit from this approach need to be selected also through a molecular approach, in order to obtain a clinical efficacy, with a strict evaluation of the benefit/risk profile. Indeed, we recently proposed to test immunotherapy in ACC patients with altered MMR pathway concomitant with high levels of MSI [73]. Thus, in the future, the potential efficacy of immunotherapy also in the ACC setting will require an accurate patients' selection by means of a genetic approach and a multimodal treatment combining systemic antineoplastic therapies and/or radiotherapy and/or drugs inhibiting steroid synthesis and controlling hypercortisolism.

Conflicts of Interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of this review.

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Review Article

Current Status of Immunotherapy for Localized and Locally Advanced Renal Cell Carcinoma

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Systemic therapy strategies in the setting of localized and locally advanced renal cell carcinoma (RCC) have continued to evolve in two directions: as adjuvant therapy (to reduce risk of recurrence or progression in high risk localized groups), or as neoadjuvant therapy as a strategy to render primary renal tumors amenable to planned surgical resection in settings where radical resection or nephron-sparing surgery was not thought to be safe or feasible. In the realm of adjuvant therapy, the results of phase III randomized clinical trials have been mixed and contradictory; nonetheless based on the findings of the landmark S-TRAC study, the tyrosine kinase inhibitor Sunitinib has been approved as an adjuvant agent in the United States. In the realm of neoadjuvant therapy, presurgical tumor reduction has been demonstrated in a number of phase II studies utilizing targeted molecular agents. The advent of immunomodulation through checkpoint inhibition as first line therapy for metastatic RCC represents an exciting horizon for adjuvant and neoadjuvant strategies. This article reviews the current status and future prospects of adjuvant and neoadjuvant immunotherapy in localized and locally advanced RCC.

1. Introduction

Renal Cell Carcinoma (RCC) is common cancer globally, with approximately 400,000 people being diagnosed with RCC in 2018, a notable increase in incidence rates with time, and is among the top ten most common malignancies in the United States [1, 2]. Due to the widespread use of cross-sectional imaging, incidence of RCC has increased with most cases presenting as localized disease [3–5]. Despite such stage migration, the risk of recurrence remains high [6–9]. Poor prognosis of patients with recurrence and the risks associated with locally advanced resection or nephron-sparing surgery in the imperative setting for complex masses have served as an impetus to explore further approaches to improve outcomes.

The improved response rates and outcome in metastatic RCC ushered in the era of targeted therapies; both tyrosine kinase inhibitor (TKI) therapy and immune checkpoint inhibitors (ICI) have stimulated investigation into the utility of these agents as adjuvants in the setting of localized and locally advanced disease to reduce the risk of recurrence and improve survival [10–15]. Herein, we review and summarize the current status and future directions of adjuvant and neoadjuvant immunotherapeutic strategies in localized and locally advanced RCC, focusing on current literature and ongoing clinical trials in both areas.

2. Methodology

2.1. Literature Search. PubMed, MEDLINE, Cochrane Central Register of Controlled Trials, the American Society of Clinical Oncology, and ClinicalTrials.gov were searched with keywords including "neoadjuvant", "adjuvant", "immunotherapy", "targeted therapy", "immune checkpoint (anti-PD-1) inhibitors", and "renal cell carcinoma". Publications were included in the review if they were including patients with localized RCC. Articles other than English language, editorials, and case reports were excluded.

2.2. Assessment of Response. In adjuvant therapeutic investigations, survival endpoints included overall survival (OS), disease-free survival (DFS), and recurrence-free survival (RFS). These terms are defined as the interval of time from randomization to the first recurrence (locally or at distant

Table 1: Clinical criteria for adjuvant therapy or investigations and neoadjuvant therapeutic investigations.

Adjuvant Therapy	Neoadjuvant Therapy
Resected primary tumor	T1-4 NX/1 M0
and	or
pT2-3 N0 M0 (grades 2-4)	T1-4 NX/1 M1
or	or
pT4 N0 M0	Borderline resectable mass
or	or
pTany N1 M0	Facilitating nephron-sparing surgery
	or
	Downstaging IVC thrombus

metastatic sites), or the occurrence of secondary malignancies or death, and are generally used interchangeably [16]. Early investigations tended to focus on RFS as an endpoint, with more recent studies focusing on OS as the primary endpoint [17]. To assess tumor response in neoadjuvant investigations a number of criteria have been utilized to evaluate therapeutic effect: change in tumor size measured in greatest diameter, 2-dimensional product of tumor cross section based on cross-sectional imaging (WHO criteria) [18], Response Evaluation Criteria In Solid Tumors (RECIST) criteria [which defined partial response (PR) as ≥30% reduction in the primary lesion size, progressive disease (PD) as increase in tumor size ≥20% or presence of new lesions or stable disease (SD)] [19], and changes in tumor morphometric score, such as the RENAL (Radius Exophytic Nearness Anterior Location) nephrometry score, a system used for defining tumor complexity [20]. Table 1 demonstrates clinical criteria in which adjuvant and neoadjuvant agents have been investigated. In the adjuvant realm, these have been resected primary tumor and pT2-3 N0 M0 (grades 2-4), pT4 N0 M0, or pTany N1 M0. In the neoadjuvant realm, these are T1-4 NX/1 M0, T1-4 NX/1 M1, borderline resectable masses, facilitation of nephron-sparing surgery, or downstaging IVC thrombi resections.

3. Adjuvant Immunotherapy in the Management of Localized and Locally Advanced Renal Cell Carcinoma

In the TKI era, significant investigational efforts were conducted into the utility of these agents as adjuvants after extirpative surgery to reduce risk of recurrence and improve survival, with mixed and largely negative results. A summary of TKI trials is provided in Table 2. The first of these pivotal trials was the ASSURE trial (Adjuvant Sorafenib or Sunitinib in Unfavorable Resected Renal cancer) which enrolled 1943 patients with nonmetastatic high risk RCC with a study design to randomize according to a 1:1:1 ratio to receive Sunitinib 50mg, Sorafenib 800mg, or placebo for 1 year with a primary endpoint of DFS. The study ultimately found no difference in DFS between groups (HR 1.02, 97.5% CI 0.85-1.23) and was hampered by high rates of toxicity and discontinuation in the two treatment arms [36]. The PROTECT trial, which examined two doses of Pazopanib versus placebo, found a marginal benefit in DFS on secondary analysis in those patients receiving higher dose (800mg, HR 0.69 [95% CI 0.51-0.94 p=0.02]), and no difference in the lower dose group [37]. The ATLAS trial compared Axitinib with placebo randomizing 724 patients. The study was closed due to futility as there was no significant difference in DFS (HR=0.87; 95% CI: 0.660-1.147, p=0.321) overall [24]. It was the S-TRAC (Sunitinib Treatment of Renal Adjuvant Cancer) trial that was the first to show a significant improvement in DFS with this class of medications. A total of 615 high risk nonmetastatic patients were randomized to Sunitinib 50mg vs Placebo with a median follow up of 5.4 years. The study demonstrated an improved DFS of 6.8 v 5.6 years, HR 0.76, 95% CI 0.59-0.95, p=0.03. In this, like all the other studies, the toxicity associated with this class of medications was notable, as high as 60.5% [38].

Based on results of the S-TRAC study demonstrating a benefit in DFS, the United States Food and Drug Administration (FDA) approved Sunitinib as an adjuvant agent for high risk localized RCC in November 2017, the first such agent in RCC [15]. Indeed, regulatory approval has heralded a paradigm shift, which has been reflected in the recently updated NCCN guidelines that lists adjuvant therapy with Sunitinib as an option for patients with stage III disease, clear cell histology, and high risk for recurrence [39]. Still, there exist concerns regarding the reproducibility and relatively modest clinical benefit associated with TKI. In February 2018, for example, the European Medicines Agency rejected the use of Sunitinib in the adjuvant setting for high risk localized RCC for these reasons [29]. Nonetheless, enrollment in a clinical trial is still considered a preferred option for most patients at higher risk for recurrence after complete resection for localized RCC.

3.1. Immune Checkpoint Inhibitors. The emergence and success of immune checkpoint inhibition as a front-line therapeutic strategy for metastatic RCC has also heralded investigation of these agents as potential adjuvant agents [13, 40]. Indeed, the biologic rationale for immunotherapeutic adjuvant therapy is compelling, and perhaps more so than for TKI agents from a mechanistic standpoint. Clearance of circulating tumor cells or micrometastatic deposits by enhancement of the T1 immune tumor response by blockade of programmed death (PD)-1 receptor and programmed death-ligand 1 (PDL-1) may represent a more efficacious therapeutic pathway that antiangiogenic blockade [21], as has been demonstrated in management of clinical metastatic disease [13, 22]. Currently there are 4 clinical trials examining the potential of checkpoint inhibitors in localized RCC to reduce risk of recurrence: atezolizumab (1 trial, NCT03024996) [40], combination of nivolumab and ipilimumab (1 trial, NCT03138512) [23], pembrolizumab (1 trial, NCT03142334) [30], and durvalumab monotherapy or in combination with tremelimumab (NCT03288532) [41] (summarized in Table 3).

The IMmotion010 trial randomizes resected high risk clear cell or sarcomatoid RCC (pT3a+, high grade including M1 resected disease) to atezolizumab (PDL1 inhibitor) or placebo. The primary end point is RFS determined by

Table 2: Summary of adjuvant trials: completed and reported.

Trial	Design	Intervention	N	Inclusion Criteria (stage/grade/histology)	Results	Adverse Events
ASSURE, Haas et al. (2016) [21]	Randomized, Double-blinded, Placebo-controlled	Sunitinib or Sorafenib	1943	T1b N0 M0 (grade 3-4), pT2–pT4 N0 M0, pT(any) N1 M0; Clear Cell and Non-clear Cell	No difference in median DFS (HR 1.02, 97.5% CI 0.85-1.23)	Grade 3+ toxicities of sunitinib, sorafenib: hypertension (17%, 16%), hand-foot syndrome (15%, 33%), rash (2%, 15%), fatigue (18%, 7%)
PROTECT, Motzer et al. (2017) [22]	Randomized, Double-blinded, Placebo-controlled	Pazopanib	1538	pT2 N0 M0 (grades 3–4), pT3–4 N0 M0, pT (any) N1 M0; Clear Cell	No differences in median DFS (HR 0.86, 95% CI 0.70-1.06)	Increased ALT/AST lead to treatment discontinuation in 600 mg (ALT 16%/AST 5%) and 800 mg (ALT 18%/AST 7%) mg.
ATLAS, Gross-Goupil et al. (2018) [23]	Randomized, Double-blinded, Placebo-controlled	Axitinib	724	pT2–4 N0 M0, pT (any) N1 M0; Clear Cell	No difference in median DFS (HR 0.87, 95% CI 0.66-1.15, p=0.321)	Similar and serious adverse events between groups; more grade 3/4 (61% vs. 30%) for axitinib
S-TRAC, Ravaud et al. (2016) [24]	Randomized, Double-blinded, Placebo-controlled	Sunitinib	615	pT3 N0 M0 (grades 2–4), pT4 N0 M0, pT (any) N1 M0; Clear Cell	Improved median DFS (6.8 years v 5.6; HR 0.76, 95% CI 0.59-0.98)	Increased Grade 3 (48.4% vs. 15.8%); Grade 4 (12.1% vs. 3.6%) in sunitinib; Similar serious event rate.

Table 3: Summary of adjuvant and neoadjuvant immunotherapeutic trials: completed and reported.

Trial	Design	Intervention	N	Inclusion Criteria (stage/grade/histology)	Results	Adverse Events
Adjuvant Trials						
Jocham et al. (2004) [25]	Prospective, randomized	Autologous renal tumor cells	558	pT2–3b pN0–3 M0; Clear and Non-Clear Cell	Improved 5 year and 70 month PFS (HR 1.58, 95% CI 1.05-2.37; HR 1.59, 95% CI 1.07-2.36)	Local skin reactions
Wood et al. (2008) [26]	Prospective, randomized	Autologous tumor-derived protein	819	cT1b–4 N0 M0, cT(any) N1-2 M0; Clear and Non-Clear Cell	No difference in PFS at 1.9 median year follow-up (HR 0.92, 95% CI 0.729-1.169)	Local skin reactions
ARISER, Chamie et al. (2016) [27]	Randomized, Double-blinded, Placebo-controlled	Girentuximab	864	pT1b–2 (Fuhrman ≥3), pT3–4 N0, pT(any) N+; Clear Cell	No difference in DFS (HR 0.97, 95% CI 0.79-1.18) or OS (HR 0.99, 95% CI 0.74-1.32	Toxicity rate 21%, comparable to placebo
Neoadjuvant Trial						
Cost et al (2011) [28]	Retrospective	Sunitinib (12), bevacizumab (9), sorafenib (1), temsirolimus (3)	25	T3b+M1 (21)	25/0	12% downstage thrombus level; 4% upstage level; 4% altered surgical strategy

central radiologic assessment [40]. Checkmate-914 is a trial enrolling patients to a combination PD1 inhibitor + CTLA4 inhibitor (nivolumab with ipilimumab) or placebo for high risk clear cell RCC [23]. Keynote-564 is enrolling patient for adjuvant pembrolizumab (PD1 inhibitor) verses placebo for high risk patients with clear cell histology including M1 resected disease [30]. The RAMPART study recently began enrolling clear and nonclear cell patients to one of three arms: durvalumab with tremelimumab (PDL1 inhibitor + CTLA inhibitor), or durvalumab monotherapy, or placebo [41]. Current immunotherapeutic ongoing studies in the adjuvant setting are summarized in Table 4.

Table 4: Summary of adjuvant and neoadjuvant studies: ongoing or unreported.

Trial	Design	Agent	Planned Accrual	Inclusion Criteria (stage/grade)	Inclusion Criteria (histology)
Adjuvant Trials					
IMmotion010, (NCT03024996) [29]	Prospective, double-blinded, placebo controlled	Atezolizumab	664	Nonmetastatic	Clear cell, sarcomatoid
Checkmate-914, (NCT03138512) [22]	Prospective, double-blinded, placebo controlled	Nivolumab + Ipilimumab	800	pT2a – 4 N0 M0 (any), pT1-4 N1 M0 (any)	Clear cell
Keynote-564, (NCT03142334) [23]	Prospective, double-blinded, placebo controlled	Pembrolizumab	950	pT2 N0 M0 (grade 4 or sarcomatoid), pT3-4 N0 M0 (any), pT1-4 N1 M0, Resectable M1	Clear cell
RAMPART, (NCT03288532) [30]	Prospective, multicenter, double-blinded, placebo controlled	Durvalumab, Durvalumab + tremelimumab	1750	Leibovich Score 3-11	Any
Neoadjuvant Trials					
Merck Sharp Dohme Corp (NCT02212730) [31]	Prospective, open label, parallel assignment	Pembrolizumab	36	cT1b+ NX-0 M0	Any
Bristol-Myers Squibb (NCT02575222) [32]	Prospective, open label	Nivolumab	30	cT2a-T4 NX-1 M0, cT1-4 N1 M0	Clear cell
NCI (NCT02595918) [33]	Prospective, open label	Nivolumab	29	Stage I-III	Clear cell
Case Comprehensive Cancer Center (NCT02762006) [34]	Prospective, open label	Durvalumab, Tremelimumab	45	cT2b-4 NX-0 M0 cT1-4 N1, M0	Any
PROSPER, (NCT03055013) [35]	Randomized, double-blind, placebo controlled	Nivolumab	766	cT2 NX M0, cT1-4 N1 M0	Any
Roswell Park Cancer Institute (NCT02170389) [31]	Prospective, open label	RCC/CD40L RNA-Transfected Autologous Vaccine	4	pT1, NX-0, M0	Any

4. Vaccines and Targeted Immunotherapy

Tumor vaccines and targeted immunotherapy have been investigated in the adjuvant setting for RCC. This concept was first explored by Galligioni et al., which utilized autologous tumor cells and bacillus Calmette-Guerin, with negative results [25]. Variations on the same theme have been attempted with the same result [26, 27]. Jocham et al. published results of a randomized trial in 379 patients with pT2-3b N0-3 RCC to receive autologous renal tumor cell vaccine or no treatment and demonstrated decreased tumor progression in the treatment group at 5 years (HR 1.59, CI 1.07 – 2.36, p = 0.0304) [26]. More recently, in the ARISER study, girentuximab, a chimeric antibody targeting carbonic anhydrase IX (CAIX), was evaluated as adjuvant in 864 patients with high risk RCC. Girentuximab was well-tolerated, with toxicity rates comparable to placebo. Overall however, there was no significant difference between girentuximab and placebo for DFS (HR 0.97, 95% CI 0.79–1.18) or OS (HR 0.99, 95% CI 0.74–1.32) [42].

5. Neoadjuvant Therapy in Clinically Localized and Locally Advanced Renal Cell Carcinoma

Neoadjuvant therapy for RCC was initially implemented to accomplish reduction in metastatic disease prior to surgical debulking, facilitate more complex surgical resections, and

select patients with appropriate disease response to systemic therapy who may benefit from surgical debulking (Table 1) [43, 44]. Indeed, the paradigm of presurgical or primary systemic therapy in the setting of metastatic RCC for TKI has recently been solidified by publication of the SURTIME and CARMENA studies which suggested improvements in PFS with primary TKI prior to surgery and lack of improvement of outcomes in intermediate and high risk metastatic RCC in patients receiving primary cytoreductive nephrectomy, respectively [45, 46]. There have been 15 studies reported in the literature for indications of downstaging tumor size for resection of locally advanced disease (9 studies), facilitating partial nephrectomy (5 studies), and downstaging IVC thrombus level (4 studies). The first study assessing feasibility and efficacy of neoadjuvant therapy prior to resection of locally advanced disease was conducted by Thomas et al. who examined 19 patients with locally extensive primary tumors considered otherwise unresectable were administered Sunitinib (initial dose 50 mg daily) for one 4-week cycle. Analysis noted partial response in 16% (3/19) of patients (by RECIST criteria) with median size reduction of 24% and with 21% (4/19) eventually undergoing nephrectomy. Nonetheless, the authors also reported that 37% of patients experienced grade 3-4 toxicities. No unexpected surgical morbidity was found; however, the major complication rate was not reported [44]. Since then, others that have studied this outcome with various other TKIs have observed 11.8%-28% median reduction in tumor size. In the first prospective randomized double-blind placebo-controlled trial to assess downsizing effect of neoadjuvant TKI, Hatiboglu et al. randomized 12 patients in a 3:1 manner to sorafenib vs placebo and demonstrated median tumor volume reduction of 29% in the treatment group. Nonetheless, toxicity rates are significant as are high grade complications [47–50].

Another indication for investigation into the utility of neoadjuvant therapy has been to facilitate nephron-sparing surgery. The first study to focus on this particular aim was reported by Silberstein et al., who conducted a prospective pilot study and a retrospective multicenter review analyzing outcomes of neoadjuvant Sunitinib (50 mg daily for two 6-week cycles) in 12 patients (14 tumors) with clear cell RCC who had imperative indications for nephron-sparing surgery. The authors noted a mean tumor size reduction of 21.1% (7.1 to 5.6 cm) with 4/14 (28.6%) tumors having PR by RECIST criteria. Ultimately, partial nephrectomy was achievable in all patients without positive margins or requirement for dialysis. Nonetheless, the authors reported that 3/14 (21.4%) renal units experienced urine leaks, all of which resolved with conservative measures [51].

Others have studied the role of various other TKIs in facilitating nephron-sparing surgery and have had mixed results. Taken together the body of work in this area suggests that neoadjuvant TKI therapy for locally advanced disease or prior to partial nephrectomy may result in modest decreases in tumor size and complexity in a subset of patients; partial nephrectomy in this setting remains complex and requires surgical expertise in this area [28, 34, 35, 44, 50–56].

6. Neoadjuvant Therapy in the Management of Localized RCC: Future Directions

Similar to the advent of immunotherapeutic investigation for adjuvant therapy in localized RCC, a flurry of high quality studies are currently underway to examine the role of neoadjuvant ICI or combination ICI-TKI targeted therapy for advanced disease, particularly in the wake of the first positive trial demonstrating improved PFS using combination TKI and immune checkpoint inhibitors compared to TKI alone (13.8 vs 7.2 months PFS and response rate of 55.2% vs 25.5% favoring combination therapy) [31]. Currently, seven clinical trials in this arena are ongoing and are summarized in Table 4. Of these studies, 4 involve immune checkpoint inhibitors.

The anti-PD-1 receptor antibody pembrolizumab (1 study; NCT02212730) is currently enrolling (planned accrual of 36 patients) with any RCC histology and clinical cT1b or more, NX-0, M0 disease in a prospective, open label, parallel design [32]. Nivolumab, an anti-PD-1 receptor antibody, is also being studied in the neoadjuvant setting in both clear cell histology and any histology, in several ongoing prospective trials, open label trials, and one randomized double-blind placebo-controlled trial (NCT02575222, NCT02595918, NCT03055013) [33, 57, 58]. Yet another clinical trial involves an antibody directed against programmed cell death-1 ligand 1 (durvalumab/MEDI 4736) ± tremelimumab, an antibody directed against human T-cell receptor protein cytotoxic T-lymphocyte-associated protein 4 (CTLA4). This study is investigating patients with any RCC histology and local or locally advanced disease in a prospective, open label fashion [59]. Finally, an additional clinical trial that evaluated presurgical vaccine therapy was closed after enrolling 4 patients (NCT02170389); further investigation in this region is pending [60].

Utilization of systemic therapy to promote cytoreduction of primary tumors and to facilitate surgical excision should currently be considered to be investigational. Nonetheless, this concept has been borne out in a number of prospective phase II studies [50, 52, 53] and retrospective analyses [54]. The key question, of course, is whether primary systemic therapy facilitates planned surgical intervention that would otherwise not have been feasible. Rini et al. suggested that the partial nephrectomy rate in otherwise unfeasible nephron-sparing situations was 75% [53] and the senior author of the manuscript is the study chair of the largest study to date which will test the question of neoadjuvant therapy prior to imperative indication partial nephrotomy in situations where a partial nephrectomy is not otherwise suitable [61].

A counter argument is often made which is due to variability of surgeon experience and ability, what may be considered unfeasible by one surgeon may indeed be possible and safe by another. While we agree in the validity of this criticism, there nonetheless exists a subset of patients in whom a safe and efficacious nephron-sparing procedure or locally advanced resection is truly not be feasible, and with even mild cytoreduction, feasibility and efficacy of such a resection may be enhanced. The senior author of this manuscript bases his opinion on the fact that he has one of the largest series in the literature of large partial nephrectomies

(>7cm) performed, whether by open approach [62, 63] or by minimally invasive approach [64]. We recently also demonstrated efficacy of primary systemic therapy prior to nephrectomy with IVC thrombectomy [65] and believe that our results and those emerging from other groups suggest that concept of primary cytoreductive systemic therapy has merit should be investigated further.

7. Conclusion

Utility and efficacy of systemic therapy in the setting of localized and locally advanced RCC are areas of active investigation. Recent approval of Sunitinib as an adjuvant agent has changed the paradigm of management of patients in the United States, and advent of ICI therapy as first line agents for metastatic RCC is spurring further investigation into utility of immunotherapeutic agents or combinations in adjuvant and neoadjuvant settings.

Conflicts of Interest

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Research Article

Comparative Efficacy and Tolerability of Neoadjuvant Immunotherapy Regimens for Patients with HER2-Positive Breast Cancer: A Network Meta-Analysis

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This network meta-analysis addresses the need for evidence-based best-practice treatment regimens for HER2-positive breast cancer. We compared the relative efficacy and tolerability of currently available HER2-positive neoadjuvant immunotherapy regimens based on systematic searches of available randomized controlled trials (RCTs) data. Based on intention-to-treat principle, pathological complete response (pCR), overall serious adverse events (SAEs), and breast-conserving surgery (BCS) rate were analyzed using random-effect, Bayesian network meta-analysis, and standard pairwise meta-analysis. 16 RCTs (3868 patients) were included. Analyzed treatment regimens were as follows: chemotherapy+trastuzumab+pertuzumab (CTP), trastuzumab emtansine+pertuzumab (MP), chemotherapy+trastuzumab (CT), chemotherapy+pertuzumab (CP), trastuzumab+pertuzumab (TP), chemotherapy+trastuzumab+lapatinib (CTL), and chemotherapy+lapatinib (CL), and chemotherapy (C) alone. We found that, for the chance of achieving pCR, CTP was ranked first (SUCRA: 97%), followed by CTL, MP, and CT (SUCRA: 80%, 75%, and 55%, resp.). MP provided the safest regimen (SUCRA: 97%), then TP, C, and TPC (SUCRA: 82%, 76%, and 47%, resp.). CTL proved the most toxic therapy (SUCRA: 7%). No significant difference between neoadjuvant regimens was identified for BCS. Hormone receptor status did not impact ORs for pCR in any regimen. In conclusion, our findings support CTP as the optimum neoadjuvant regimen for HER2-positive breast cancer, with the best pCR and acceptable toxicity compared with CT. MP provides a therapeutic option for patients with poor performance status.

1. Introduction

Worldwide, breast cancer is one of the most common malignancies and the leading cause of death in females, with an estimated 1.7 million new diagnoses annually [1]. Among them, the overexpression of human epidermal growth factor receptor 2 (HER2, also called ErbB2) occurs in roughly 15-20% of breast cancers and is associated with aggressive proliferation and poor prognosis [2]. Until the past decade, increased understanding of the molecular events of HER2-positive oncogenesis has led to the development of a series of HER2-targeted drugs, which have revolutionized the standard of care for HER2-positive disease [3]. To date, four HER2-targeted agents, monoclonal antibody trastuzumab,

small-molecule inhibitor lapatinib, anti-HER2 heterodimerization domain antibody pertuzumab, and antibody-drug conjugate trastuzumab emtansine, have been approved for use in patients with metastatic HER2-positive breast cancer, and trials have been conducted, or ongoing, in both adjuvant and neoadjuvant settings.

Neoadjuvant systemic therapy (i.e., regimens commenced before surgery) was once reserved for local advanced breast cancer with the aim of downstaging and achieving operability [4], but it has been routinely delivered in primary operable (early) tumors [5, 6]. Importantly, the individual patient's response to neoadjuvant regimen, designated as pathological complete response (pCR) in the breast and axillary nodes at the time of surgery, is strongly correlated with improved overall survival (OS) and disease-free survival (DFS), particularly in triple-negative and HER2-positive diseases [7]. For this reason, the neoadjuvant approach using pCR as a surrogate endpoint has been adopted to accelerate the approval of new agents for high-risk early-stage breast cancers by the U.S. Food and Drug Administration (FDA) [8, 9] and European Medicines Agency (EMA) [10, 11]. Data from random controlled trials (RCT) has shown that regimens in neoadjuvant settings have similar OS and DFS compared with that in adjuvant trials, and more breast-conserving surgery (BCS) can be performed after neoadjuvant regimens because of tumor shrinkage, thus providing additional support for this approach [12, 13].

The current recommendation regarding neoadjuvant therapy options for HER2-positive breast cancer in National Comprehensive Cancer Network (NCCN) guidelines contains many regimens, including combinational therapies: chemotherapy+trastuzumab+ pertuzumab (CTP), trastuzumab+ emtansine+pertuzumab (MP), chemotherapy+ trastuzumab (CT), chemotherapy+pertuzumab (CP), trastuzumab+ pertuzumab (TP), chemotherapy+ trastuzumab+lapatinib (CTL), and chemotherapy plus lapatinib (CL) [14]. With the increasing number of new HER2-directed agents and combination regimens, there is an unmet need to define the optimum neoadjuvant regimens for HER2-positive breast cancer patients. The network meta-analysis enables indirect comparison by using a common comparator when a head-to-head comparison has not been made and combines direct and indirect comparisons to simultaneously compare different regimens with the preservation of randomization in individual trials [15]. Such a technique can improve the precision of the estimate (compared with direct evidence alone) and facilitate the quantification of the relative efficacy of regimens, even if no studies directly compare them [16, 17]. Although it is important to define optimal regimens for HER2-positive breast cancer patients using network meta-analysis, by far, only one network meta-analysis study has been published on the identification of the optimal regimen in patients with early-stage HER2 breast cancer in neoadjuvant setting (data was only updated until August 2012) [18]. A few other nonnetwork meta-analysis studies specifically compared two kinds of neoadjuvant agents using conventional pairwise comparisons [19–23]. Thus, an updated network meta-analysis study is undoubtedly needed.

In this study, we aimed to provide an updated and comprehensive view on the optimum neoadjuvant regimens for patients with HER2-positive breast cancer, through a random-effect network meta-analysis of all relevant randomized evidence comparing the relative efficacy and tolerability of the commonly used neoadjuvant regimens including CTP, MP, CT, CP, TP, CTL, CL, and chemotherapy alone.

2. Materials and Methods

2.1. Literature Search and Study Selection. Combining the search algorithms *Randomi∗*; *Breast cancer*; *Neoadjuvant*; *HER2/ERBB2,* a systematic search was conducted of articles published until April 2018 from MEDLINE, the Cochrane database, and EMBASE, with no language restriction (see full search terms in eTable 1 in Supplementary Materials). We regarded publications as eligible for inclusion if they were full manuscripts or abstracts of randomized trials that compared the benefits of two or more neoadjuvant regimens for HER2-positive breast cancer. We excluded retrospective or prospective observational cohort trials. Bibliographies of key articles in the field were hand-searched and reviewed for additional candidates. If multiple publications covered the same trial cases, only the most updated or most inclusive publication was included. Our meta-analysis adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [24].

2.2. Outcome Measure and Data Extraction. Our primary outcomes of interest included (1) pCR, defined as the FDA's Guidance for Industry [25], number of patients with no invasive cancer in breast and lymph nodes following completion of neoadjuvant therapy, and regimen-related serious adverse events (SAEs), defined as greater than or equal to grade 3 toxic effects according to the National Cancer Institute Common Terminology Criteria (NCICTC). We only assessed SAEs because grade 1-2 toxicity had lesser clinical significance and was not consistently reported. Secondary outcome was breast-conserving surgery rate (BCS).

Two investigators (W.D. and C.D.) separately selected trials and abstracted data with a prespecified information sheet. Extracted data included characteristics of the trials (acronym of the trial, inclusion period, publication year, country, trial design, randomization process, and stratification), characteristics of the patients (number of patients randomized, disease stage, median age, hormone receptor status, and node positivity), characteristics of the regimens (sequence, dosage, and duration), and outcomes (definition and number of patients using intention-to-treat principle whenever available).

Transitivity (i.e., the assumption that one can validly compare indirectly treatments A and B via one or more anchor treatments) is the fundamental premise underlying network meta-analysis [26, 27]. We examined whether the trials were sufficiently homogenous by comparing population baseline characteristics across the included trials [28].

2.3. Quality Assessment. Risk of bias of individual trials was separately assessed by the same investigators using

the Cochrane Collaboration's risk-of-bias tool outlined in chapter 8 of the *Cochrane Handbook for Systematic Reviews of Interventions, Version 5.1.0* [29]. Data and bias discrepancies were resolved by joint discussion to reach consensus.

2.4. Data Synthesis and Analysis. We initially performed standard pairwise meta-analyses to assess the available direct relative effects of the neoadjuvant regimens using STATA software version 14.0 (StataCorp, College Station, TX, USA). A random-effects model, which provides more conservative estimated effects, was applied [30]. Because all of the outcomes of interest were dichotomous variables, we calculated the summary effect sizes as odds ratios (OR) with 95% credible intervals (CrI). In these analyses, we used the I^2 index to assess the statistical heterogeneity, with values over 50% indicating significant heterogeneity [31].

To incorporate indirect with direct comparisons, we performed random-effects Bayesian network meta-analyses using Markov chain Monte Carlo methods in WinBUGS software version 1.4.3 (MRC Biostatistics Unit, Cambridge, UK) [16, 32]. This technique combined direct and indirect evidence of all relative treatment effects, provided estimates with maximum power, and allowed the ranking of the various neoadjuvant regimens based on the surface under the cumulative ranking (SUCRA) and the mean ranks [33, 34]. Analyses yielded 50,000 iterations with a burn-in number of 10,000 iterations and a thin interval of 50 to obtain the posterior distributions of the model parameters. Multiple chains (e.g., multiple initial values) were evaluated for each analysis. Convergence of iterations was evaluated by Gelman-Rubin-Brooks statistic [35]. To assess whether there was inconsistency between direct and indirect comparisons, we compared the pooled ORs from the network meta-analysis with corresponding ORs from standard pairwise meta-analysis [36]. Rank probabilities were calculated from proportions of Markov chain cycles. SUCRA for each regimen was calculated from a cumulative ranking probability that a regimen is above a certain ranking [37]. Statistical tests were two-sided and used a significance threshold of $p < 0.05$.

2.5. Small-Study Effects and Additional Analyses. We investigated the presence of small-study effects for each outcome by comparison-adjusted funnel plots; comparisons have been directed according to the effectiveness of neoadjuvant regimens, assuming that the more effective regimens are favored in small trials [38, 39]. Potential asymmetry would indicate a form of small-study effects depending on the defined direction, whereas symmetry in the funnel plot would indicate a lack of evidence of small-study effects.

Multiple sensitivity analyses were performed to assess the robustness of the findings. These were based on (1) exclusion of trials using different outcome definitions; (2) exclusion of trials using distinct types of chemotherapy drugs in neoadjuvant therapy; (3) exclusion of trials that did not administered chemotherapy concomitantly with HER2-targeted agents in neoadjuvant therapy; (4) exclusion of trials with high risk of bias in any domain assessed by the Cochrane risk of bias tool; and (5) exclusion of trials published as meeting abstracts.

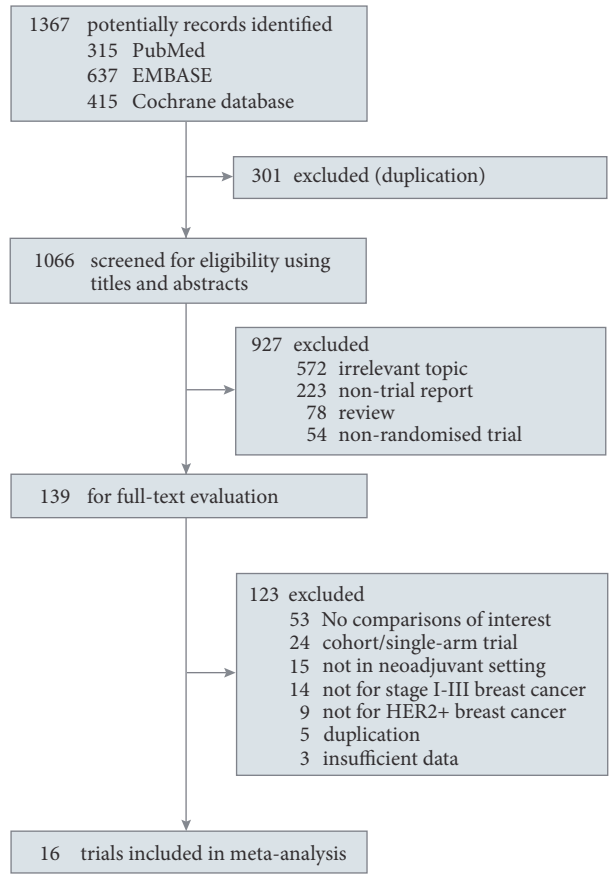


Figure 1: Summary of trial selection for network meta-analysis. HER2 indicates human epidermal growth factor receptor 2.

Additionally, we performed network meta-regression analysis adjusting for the percentage of hormone receptor–positive patients to assess whether the effects of neoadjuvant regimens on pCR were affected by hormone receptor status.

3. Results

3.1. Study Selection. Of the 1367 potential records that were initially identified by search strategy (Figure 1 and eTable 1 in the Supplementary Materials), 927 were discarded by eligibility screening of titles and abstracts. After further full-text evaluation for the remaining 139 records, 22 publications [40–61] pertaining to 16 distinct neoadjuvant trials were considered eligible for this meta-analysis, which comprised a total of 3868 patients (median number of patients per trial is 240; range: 29-615).

3.2. Baseline and Evaluation of Clinical Assumptions. The characteristics of the included trials and patients were presented in eTable 2 in Supplementary Materials. Of the 16 distinct trials, 13 were published as full manuscripts, and the other 3 [46, 47, 55, 59, 60] were in abstract form (of which data was supplemented by records presented on http://ClinicalTrials.gov). These trials mainly took place in

North America and Europe and were published or presented between 2005 and 2016. Most trials (12/16) recruited only women, 2 trials [59, 61] included both sexes, and the other 2 [40, 45] did not have a clear description of criteria about sex. This bias was unlikely to influence the results since the majority of participants were women. Eligible patients typically had previously untreated resectable, locally advanced, or inflammatory HER2-positive breast cancer (stage I-IIIC) with adequate baseline function of major organs. The proportion of hormone receptor-positive tumors ranged from 25% to 68% among trials.

The details of the treatment regimen and schedule are presented in eTable 3 in Supplementary Materials. Totally, these 16 trials covered 8 types of neoadjuvant regimens. All trials except two [49, 56] used HER2-targeted agents concomitantly with chemotherapy. In NeoALTTO [49], HER2-targeted agent alone was given for the first six weeks before combination therapy; in ABCGS-24 [56], chemotherapy was used alone for the first eighteen weeks. Over two-thirds of trials (11/16) used polychemotherapy that consisted of anthracycline plus taxane or carboplatin-docetaxel combination, while the others [45, 49, 52, 59, 61] used taxane monochemotherapy in neoadjuvant therapy.

Overall, we found no evidence of important discrepancies regarding trial design, population characteristics, and treatment schedules across the available direct comparisons. Therefore, the assumption of transitivity is likely to hold in the overall data-analysis.

3.3. Bias Assessment. Overall risk of bias was low in the included trials (eTable 4 in Supplementary Materials). Most trials (13/16) appropriately reported the method of random sequence generation, whereas in 2 trials [43, 48] there was high risk of bias in terms of allocation concealment. Due to the open-label design of all 16 trials, performance bias might exist [62]. We judged the adequacy of blinding by whether an outcome assessor was masked to treatment assignment, because it was critical to prevent detection bias in assessment of outcomes such as pCR. Nine of the 16 trials assessed the patients' response by a pathologist who was unaware to the treatment, while the other 7 [40, 43, 45, 46, 54, 55, 59] did not present a clear description. None of these trials had evidence of a definite high risk of bias in terms of attrition bias or reporting bias. Additionally, another source of bias was identified: three trials [40, 58, 61] were halted prematurely because of an apparent benefit of a treatment, and 2 [55, 58] had imbalanced baseline characteristics.

3.4. Meta-Analysis for Primary Outcomes

3.4.1. Pathological Complete Response. All sixteen trials reported data on pCR (3868 patients and 2422 events) and therefore were included in the analysis (Figure 2(a)). All trials except one [45] used pCR definition that there is no invasive cancer in both breast and lymph nodes at the time of surgery. The H2269s trial defined pCR as the absence of invasive cancer in breast only. Of the 28 comparisons included in network meta-analysis, 12 statistically significant differences were identified (Figure 3(a)). CTP was ranked first for the chance of achieving pCR (SUCRA: 97%), with nonsignificant different ORs of 0.66 and 0.63 compared with CTL and MP, and significant differences for the remaining regimens, with ORs ranging from 0.17 to 0.41 (key comparisons include CTP vs CT: OR, 0.41; 95% CrI, 0.20-0.84 and CTL vs CT: OR, 0.63; 95% CrI, 0.48-0.84) (Figure 3(a) and Figure S1A in Supplementary Materials).

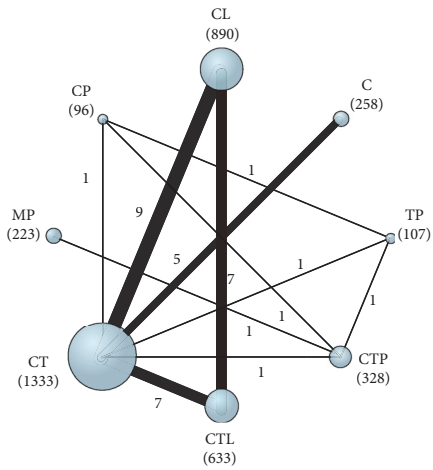
Sensitivity analysis with the removal of H2269s did not show any major change in terms of regimen effects or rankings (eTable 5 in Supplementary Materials). Meta-regression analysis on pCR adjusted for the percentage of hormone receptor–positive patients in each trial showed that ORs were not differed by the adjustment (Figure S2 in Supplementary Materials).

3.4.2. Serious Adverse Events. Data on neoadjuvant regimens-related overall SAEs were available in eleven trials (3306 patients and 1066 events) [42, 46, 48, 50–52, 54, 56, 57, 59, 61] (Figure 2(b)). One trial [42] did not report the number of patients with overall SAEs; the trial-specific OR was thus calculated with the sum of the individual serious toxic reactions. Network comparisons showed that MP was ranked as the safest regimen (SUCRA: 97%), with significant differences compared with all regimens except TP and chemotherapy alone (key comparisons include MP vs CTP: OR, 0.08; 95% CrI, 0.03-0.22 and MP vs CT: OR, 0.06; 95% CrI, 0.01-0.25) (Figure 2(b) and Figure S1B in Supplementary Materials). The regimen of CTP ranked fourth (SUCRA: 47%) for SAEs, with no significant differences compared with CT (OR, 1.14; 95% CrI, 0.47-4.26) or chemotherapy alone (OR, 3.20; 95% CrI, 0.78-13.35). There was also no significant difference between CTP and CTL (OR, 0.41; 95% CrI, 0.12-1.38). CTL was more likely to cause SAEs compared with all other regimens (SUCRA: 7%), with five significant differences being identified.

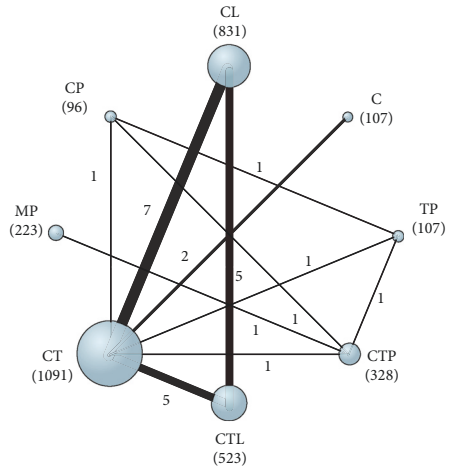
3.4.3. Ranking of Available Regimens. All the eight neoadjuvant regimens were ranked in Figure 4 according to both pCR value and overall safety profile (SAEs). CTP and MP lying in the lower left corner suggested being more favorable for the benefit and toxicity ratio with higher probability of being optimal treatments.

3.5. Meta-Analysis for Secondary Outcome

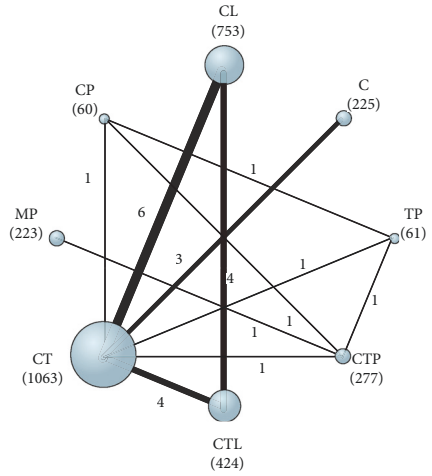
Breast-Conserving Surgery. Data from eleven trials (3086 patients and 1706 events) [42, 43, 48, 49, 51, 52, 54, 56–59] were included in the analysis of BCS (Figure 2(c)). The NeoSphere trial [52] only reported the number of patients who transformed to BCS candidates after neoadjuvant treatments, rather than the sum of the patients who underwent BCS. We thus calculated the trial-specific OR with the number of patients who were previously not candidates for BSC and the number of transformed ones for an evaluation of BCS conversion. Network comparisons showed that CP (SUCRA: 90%), CT (SUCRA: 63%), and CTP (SUCRA: 61%) were ranked as the top three regimens with the highest chance of BCS (Figure 2(c) and Figure S1C in Supplementary Materials). However, the findings should be interpreted with caution



(a) Pathologically complete response. 3868 patients and 2422 events (16 trials)



(b) Serious adverse events. 3306 patients and 1066 events (11 trials)



(c) Breast-conserving surgery rate. 3086 patients and 1706 events (11 trials)

Figure 2: Network diagrams of available treatment comparisons for each outcome. The size of the nodes is proportional to the number of patients (in parentheses) randomized to each treatment, and the width of the lines is proportional to the number of trials (beside the line) comparing the connected treatments. C indicates chemotherapy; CL, chemotherapy plus lapatinib; CP, chemotherapy plus pertuzumab; CT, chemotherapy plus trastuzumab; CTL, chemotherapy plus trastuzumab plus lapatinib; CTP, chemotherapy plus trastuzumab plus pertuzumab; MP, trastuzumab emtansine plus pertuzumab; TP, trastuzumab plus pertuzumab.

because all comparisons between the various treatments did not reach statistical significance.

Sensitivity analysis with the removal of NeoSphere trial did not change the rankings of BCS outcome (eTable 6 in Supplementary Materials).

3.6. Heterogeneity and Inconsistency. Comparison of results from pairwise meta-analysis and network meta-analysis is presented in eTable 7 in Supplementary Materials, the CIs of all ORs from network comparisons generally included CIs of corresponding ORs from pairwise comparisons, and the point estimates of ORs between the two meta-analyses were similar for each outcome, supporting that there was no important inconsistency between direct and indirect comparisons.

We found no evidence of significant difference between-trial heterogeneity in all comparisons, with the exception of CT versus CL for SAEs analysis (I^2 = 60 %) (eTable 7 in Supplementary Materials).

3.7. Small-Study Effects and Additional Analyses. As shown in Figure 5, the comparison-adjusted funnel plots appeared symmetrical for BCS outcome, but asymmetrical in primary outcomes, largely attributable to the spot located in the lower left corner for pCR that contributed by the H2269s trial [45] and the two outlying spots for SAEs contributed by the NeoALTTO trial [49], suggesting that these trials tended to favor active regimens over comparison-specific weighted average effect [39].

CTP							
0.66 (0.30-1.40)	CTL						
0.63 (0.36-1.10)	0.98 (0.37-2.46)	MP					
0.41 (0.20-0.84)	0.63 (0.48-0.84)	0.65 (0.27-1.63)	CT				
0.33 (0.15-0.67)	0.49 (0.21-1.13)	0.51 (0.20-1.28)	0.78 (0.34-1.71)	CP			
0.27 (0.12-0.58)	0.41 (0.31-0.55)	0.42 (0.17-1.09)	0.65 (0.50-0.83)	0.83(0.36-1.97)	CL		
0.19 (0.08-0.42)	0.29 (0.12-0.73)	0.30 (0.11-0.78)	0.46 (0.19-1.09)	0.59 (0.23-1.46)	0.71 (0.28-1.76)	TP	
0.17 (0.07-0.39)	0.26 (0.15-0.45)	0.27 (0.10-0.74)	0.41 (0.26-0.64)	0.52 (0.21-1.30)	0.63 (0.38-1.07)	0.89 (0.34-2.49)	C

(a) Pathologically complete response

MP							
0.37 (0.07-2.23)	TP						
0.26 (0.05-1.48)	0.71 (0.12-3.59)	C					
0.08 (0.03-0.22)	0.22 (0.05-0.86)	0.31 (0.07-1.29)	CTP				
0.07 (0.01-0.17)	0.18 (0.04-0.70)	0.25 (0.06-1.04)	0.82 (0.25-2.55)	CP			
0.06 (0.01-0.25)	0.16 (0.04-0.60)	0.22 (0.09-0.55)	0.71 (0.23-2.12)	0.87 (0.29-2.63)	CT		
0.04 (0.01-0.17)	0.10 (0.02-0.41)	0.14 (0.05-0.40)	0.46 (0.14-1.51)	0.57 (0.18-1.85)	0.64 (0.43-1.01)	CL	
0.03 (0.01-0.16)	0.09 (0.02-0.41)	0.13 (0.05-0.37)	0.41 (0.12-1.38)	0.51 (0.15-1.68)	0.58 (0.36-0.95)	0.90 (0.55-1.43)	CTL

(b) Serious adverse events

CP							
0.61 (0.23-1.59)	CT						
0.63 (0.24-1.66)	1.03 (0.37-2.74)	CTP					
0.58 (0.21-1.61)	0.95 (0.65-1.37)	0.92 (0.32-2.67)	CTL				
0.56 (0.18-1.71)	0.93 (0.54-1.58)	0.90 (0.29-2.87)	0.97 (0.51-1.88)	C			
0.49 (0.18-1.38)	0.81 (0.59-1.09)	0.78 (0.28-2.24)	0.85 (0.58-1.22)	0.87 (0.46-1.62)	CL		
0.45 (0.17-1.24)	0.74 (0.26-2.04)	0.73 (0.25-2.04)	0.77 (0.26-2.27)	0.80 (0.25-2.48)	0.92 (0.31-2.68)	TP	
0.41 (0.14-1.34)	0.68 (0.21-2.19)	0.67 (0.35-1.27)	0.71 (0.21-2.42)	0.73 (0.21-2.68)	0.92 (0.27-3.12)	0.84 (0.26-2.81)	MP

(c) Breast-conserving surgery rate

Figure 3: Pooled estimates for all possible treatment effects for each outcome (treatments were ordered by ranking). Effect estimates reflect comparison of the treatment in the row heading being compared to the column heading. Effect estimates of all outcomes are expressed as odds ratios (ORs) with 95% credible intervals. ORs with Bayesian p value less than 0.05 are in green. C indicates chemotherapy; CL, chemotherapy plus lapatinib; CP, chemotherapy plus pertuzumab; CT, chemotherapy plus trastuzumab; CTL, chemotherapy plus trastuzumab plus lapatinib; CTP, chemotherapy plus trastuzumab plus pertuzumab; MP, trastuzumab emtansine plus pertuzumab; TP, trastuzumab plus pertuzumab.

To assess the robustness of our findings, we performed additional sensitivity analyses based on exclusion of trials that did not use HER2-targeted drugs concomitantly with chemotherapy; exclusion of trials that used taxane monochemotherapy only; exclusion of trials that were considered high risk of bias in any bias domain; and exclusion of trials that presented as abstracts. These analyses did not affect the results of primary outcomes (eTables 8–10 in Supplementary Materials).

4. Discussion

The present network meta-analysis of 16 randomized controlled trials of 3,868 patients with breast cancer defined optimal neoadjuvant regimens for HER2-positive breast cancer patients by comparison of the relative efficacy and safety profiles of 8 commonly used neoadjuvant regimens, i.e., CTP, MP, CT, CP, TP, CTL, CL, and chemotherapy alone. To the best of our knowledge, this is the most comprehensive and updated study summarizing current randomized evidence on neoadjuvant regimens for HER2-positive breast cancer.

Our findings from this study highlighted the important updates on optimal neoadjuvant regimens for HER2-positive breast cancer patients. Firstly, our data suggested that the dual-HER2 blockade regimen of CTP is currently the most effective neoadjuvant regimen for the chance of achieving pCR, with little additional toxicity compared with CT or chemotherapy alone. This supports the use of neoadjuvant CTP as the first choice for patients with early-stage HER2-positive breast cancer to maximally translate into recurrence-free survival gains. In agreement with our finding, a recent conference report by Nakashoji et al. supported the notion that CTP has the highest probability of achieving pCR (SUCRA = 0.95) [63]. Secondly, although MP ranked only third in achieving pCR, they have the most favorable toxicity

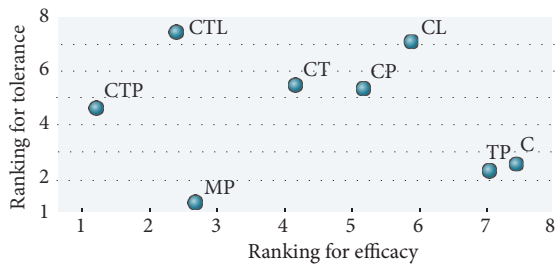


Figure 4: Ranking for pathological complete response and serious adverse events. C indicates chemotherapy; CL, chemotherapy plus lapatinib; CP, chemotherapy plus pertuzumab; CT, chemotherapy plus trastuzumab; CTL, chemotherapy plus trastuzumab plus lapatinib; CTP, chemotherapy plus trastuzumab plus pertuzumab; MP, trastuzumab emtansine plus pertuzumab; TP, trastuzumab plus pertuzumab.

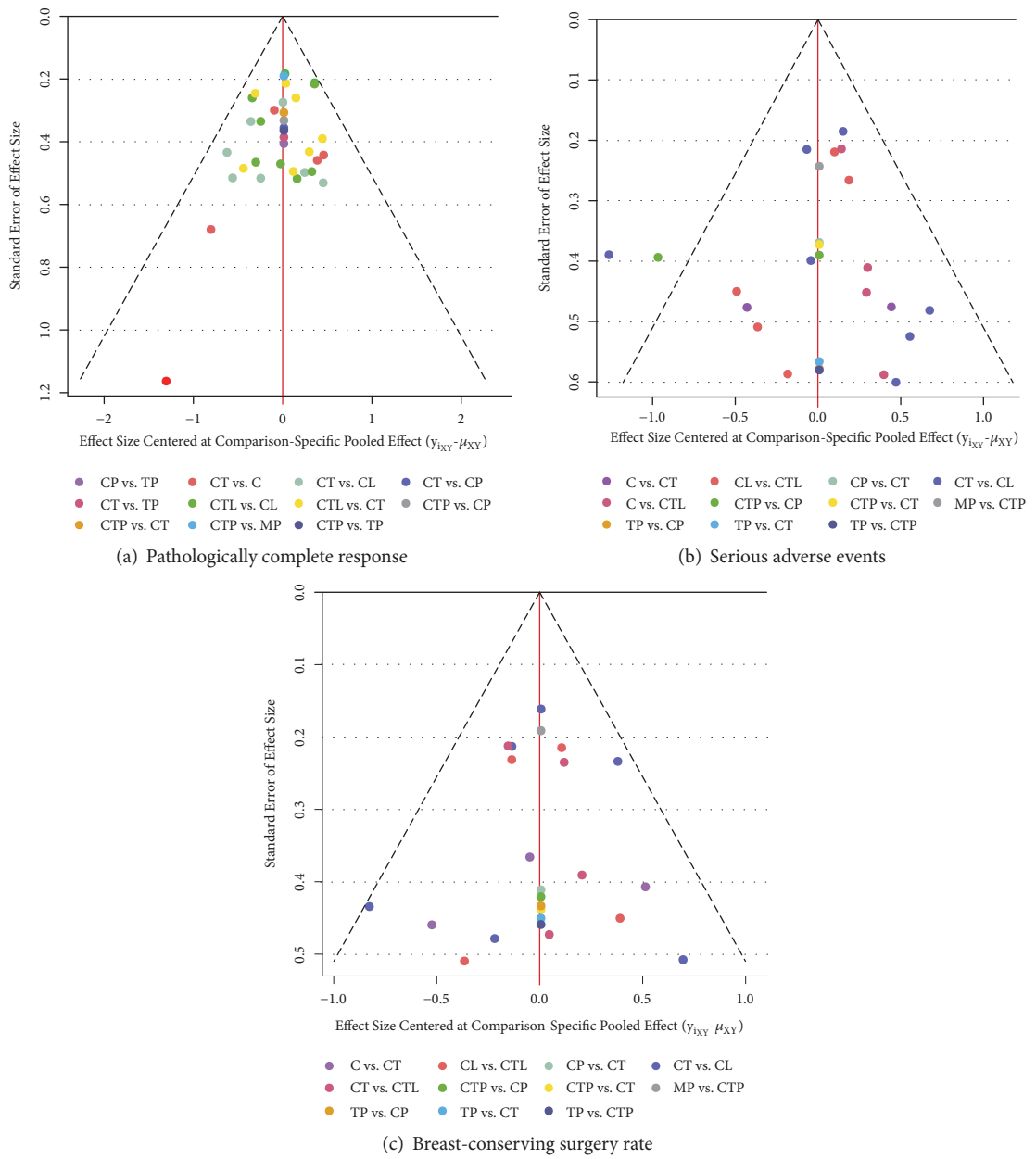
profile compared with other treatments and hence might be a suitable regimen option for patients unlikely to tolerate systemic taxane-based chemotherapy. Thirdly, our meta-regression analysis, which considered the potential effect of hormone receptor status on pCR, showed that the findings above were similar after adjustment for hormone receptor status. Finally, these findings are consistent and likely to be robust by assessment of multiple sensitivity analyses considering several patient-, treatment-, and trial-related factors.

More neoadjuvant trials included CTL as an experimental arm (7/16 in our meta-analysis) based on the results of several important preclinical studies [64–66] and adjuvant trials [67, 68] in HER2-postive population. However, most of these neoadjuvant trials (6/7) reported the increased number of grade 3-4 adverse events such as diarrhea, neutropenia, and hepatic toxicity when treated with CTL when compared with CT arm, leading the discontinuation rates to range from 15.3% to 54.5% of CTL arm even after dose adjustment [46, 49, 51, 54, 55, 61]. In our meta-analysis, CTL was ranked as the most toxic neoadjuvant regimen, with significant difference compared with CT. Indeed, a significant increase in the risk of overall SAEs was identified in CTL compared with CTP in an additional analysis using fixed-effect model (Figure S3), despite not being found in our random-effect analysis. The excess benefit (of the chance of achieving pCR) over risk (of experiencing serious adverse events) of CTL might be limited and more favorable in patients with high-risk breast cancer.

We noted no significant differences between neoadjuvant regimens with respect to breast conservation rate. None of the included trials, except for one [69], identified any significant differences for BCS. This trial showed that CTP improved BCS than MP. Nevertheless, our meta-analysis might be underpowered to provide definitive conclusions for ranking of regimen for BCS.

Our study extends findings from primary randomized controlled trials and previous pairwise meta-analyses by systematically synthesizing the entire body of relative and absolute efficacy and safety data. Our findings are partly in keeping with a previous network meta-analysis, reaching a similar conclusion that CTP was the most effective treatment [18]. However, there are several important differences between our study and the network meta-analysis by Nagayama and colleagues. Firstly, our study updated 6 recent randomized trials (LPT 109096, NSABP B41, TRIO-US B07, EORTC 10054, GALGB 40601, and KRISTINE) that were not included in the previous meta-analysis, increasing the sample size by more than a half (3868 versus 2247 patients), and thus providing greater statistical power and more precise estimates. Secondly, our study integrated evidence of a more recent treatment combination-MP into the analysis and, to our knowledge, for the first time represented the network-comparative evidence. Thirdly, rather than using per-protocol (PP) analysis in the previous meta-analysis, where patients who deviated from the protocol are excluded, our analyses were based on the 'intention-to-treat' principle (ITT). This means that all patients assigned to a group are taken into account, including those who deviated from the protocol for any reasons, for all outcomes when available. In conjunction with randomization, ITT approach is the best to guarantee that the groups of patients being compared have similar characteristics and usually best reflects the effects of treatment because it avoids the dilution due to noncompliance [70, 71]. Therefore, the findings from our study should be considered more conservative [71].

There are certain limitations in our study that merit further discussion. Firstly, same as in the previous study by Nagayama and colleagues, we did not perform meta-analysis on long-term outcomes such as OS and DFS/EFS, because the data accumulation for such outcomes was insufficient. As shown in Table 1, data on long-term outcomes were not available in most of the included trials. Secondly, the number of studies and the number of patients included (totaling 16 trials of 3868 patients) are relatively small. In addition, as shown in Table 1 and Figure 2, 6 out of 16 included studies (38%) included small sample arm/arms that had less than 100 participants (MD Anderson, H2269s, LPT 109096, CHER-LOB, TRIO-US B07, ABCSG-24, and EORTC 10054). As a result, the effect size estimated from those studies might be overestimated owing to lower methodological quality of small studies and possible publication bias [72–74]. Finally, our meta-analysis was based on summary statistics from published randomized trials rather than individual patient data. There might be some covariates at the individual patient level that might affect the treatment outcomes but were not reported. For example, our meta-regression analysis adjusting for hormone receptor status at the study level showed that the ORs on pCR were not different from those without the adjustment. However, such finding might potentially be subject to the ecological fallacy because individual trials did not report ORs comparing patients with and without hormone receptor positivity. Access to and examination of data from individual patients could resolve the problem of missing information on certain prognostic factors and increase the power of the meta-analysis.



(a) Pathologically complete response

(b) Serious adverse events

(c) Breast-conserving surgery rate

Figure 5: The comparison-adjusted funnel plot for each outcome. The funnel plot is a scatterplot of the treatment effect size vs its standard error. A funnel plot that is asymmetrical with respect to the line of the summary effect (vertical red line) implies that there are differences between the estimates derived from small and large studies. The studies are ordered from best to worst according to treatment effects. Missing (small) studies lying on the right side of the zero line suggest that small studies tend to exaggerate the effectiveness of higher-ranked treatments compared with lower-ranked treatments. Red line represents the null hypothesis that the study-specific effect sizes do not differ from the respective comparison-specific pooled effect estimates.

5. Conclusions

Our findings support that CTP is the currently optimal neoadjuvant immunotherapy regimen for HER2-positive breast cancer, due to the best chance of achieving pCR and relatively modest toxicity profile compared with other treatments. MP has the best tolerability and acceptable efficacy, which may be a therapeutic option for patients with poor performance status. CTL appears to be more toxic than other regimens, whose excess pCR benefits over toxicity were thus more likely achieved in patients with high-risk breast cancer. CP, CL, TP, and chemotherapy alone might not be considered as neoadjuvant therapeutic alternatives.

Data Availability

The data [3 supplementary figures and 10 supplementary tables] used to support the findings of this study are included within the supplementary information file submitted.

Table 1: Summary of Characteristics and Limitations of all Included Randomized Controlled Trials.

Study	Type	Design	Country	Clinical Stage	Cases (n)	Neoadjuvant treatment	Age	Arm	HER2+ %	HR+ %	Limitations of the Study	Ref
MD Anderson, 2005 & 2007	Peer reviewed	Open-label	United States	II-IIIA	42	CT C	52 48	23 19	100 100	56 58	Small sample size, unoptimal imaging modalities or cancer markers used, unclear description about building of outcome assessment, premature termination	[40, 41]
Pierga, 2010	Peer reviewed	Multicentre, open-label, phase II	France	II-III	120	CT C	47 47	62 58	100 100	55 63	Absence of long-term outcome	[42]
NOAH, 2010 & 2014	Peer reviewed	Multicentre, open-label, phase III	Europe and North America 6 counties	T3N1 or T4 or any T N2-3	235	CT C	NR	117 118	100	35 35	Unclear description about building of outcome assessment.	[43, 44]
H2269s, 2010	Peer reviewed	Open-label	United States	T2-4	29	CT C	50	15 14	100	NR	Small sample size, absence of HR status data, unclear description about building of outcome assessment, different pCR definition used, absence of long-term outcome	[45, 46]
LPT 109096 2011	Abstract	Multicentre, open-label, phase II	United States	T2-4, N0-2	100	CTL CT CL	49 51 51	33 33 34	100	NR	Full-text unavailable, small sample size.	[47]
GeparQuinto–GBG44 2012	Peer reviewed	Multicentre, open-label, phase III	Germany	T1 pNSLN+, T2cN+, T3-4,	615	CT CL	50 50	309 311	100 100	55 56	Unmasked allocation concealment, absence of long-term outcome	[48]
NeoALTTO, 2012 & 2014	Peer reviewed	Multicentre, open-label, phase III	International 25 countries	T2-4	455	CTL CT CL	50 49 50	152 149 154	100 100 100	51 50 52	More patients had to stop treatment due to side-effects in the lapatinib-containing groups	[49, 50]
CHER-LOB, 2012	Peer reviewed	Multicentre, open-label phase IIb	Italy	II-IIIA	121	CTL CT CL	49 50 49	46 36 39	100 100 100	61 58 62	Small sample size, absence of HR status data, absence of long-term outcome	[51]

Table 1: Continued.

Study	Type	Design	Country	Clinical Stage	Cases (n)	Neoadjuvant treatment	Age	Arm	HER2+ %	HR+ %	Limitations of the Study	Ref
NeoSphere, 2012 & 2016	Peer reviewed	Multicentre, open-label, phase II	International 19 countries	T2-4	417	CTL TP CT CP	50 49 50 49	107 107 107 96	100 100 100 100	47 47 47 48	Not been identified	[52, 53]
NSABP B41, 2013	Peer reviewed	Muticentre open-label, phase III	North America 3 countries	T2-T3, N0-N2a	519	CTP CT CL	NR	174 181 174	100 100 100	62 67 58	Unmasked allocation concealment, unclear description about building of outcome assessment,	[54]
TRIO-US B07, 2013	Abstract	Multicentre, open-label, phase II	United States	I-III	106	CTL CT CL	NR	58 34 36	100 100 100	NR	Unbalanced baseline characteristic, small sample size, absence of HR status data, absence of long-term outcome	[55]
ABCSG-24, 2013	Peer reviewed	Multicentre open-label, phase III	Austria	T1-4	93	CT C	50 48	44 49	100 100	41 38	Small sample size, key regimen is not used internationally, absence of long-term outcome	[56]
GEICAM, 2014	Peer reviewed	Multicentre, open-label, phase II	Spain	I-III or inflammatory	99	CT CL	49 48	50 52	100 100	60 56	Absence of long-term outcome, small sample size	[57]
EORTC 10054, 2014	Peer reviewed	Multicentre, open-label, phase IIb	Europe 5 countries	IIA-IIC	122	CTL CT CL	49 47 50	52 53 23	100 100 100	52 52 68	Unbalanced baseline characteristic, small sample size, premature termination, absence of long-term outcome	[58]
KRISTINE, 2016	Abstract	Multicentre, open-label, phase III	International, 11 countries	II-IIIC	444	MP CTP	NR	223 221	100 100	51 49	Full-text unavailable	[59, 60]
CALGB 40601, 2016	Peer reviewed	Multicentre, open-label, phase III	United States	II-III	295	CTL CT CL	48 50 50	118 120 67	100 100 100	59 59 58	Premature termination, absence of long-term outcome	[61]

Note. C indicates chemotherapy alone; CL, chemotherapy plus lapatinib; CP, chemotherapy plus pertuzumab; CT, chemotherapy plus trastuzumab; CTL, chemotherapy plus trastuzumab plus lapatinib; CTP, chemotherapy plus trastuzumab plus pertuzumab; HER2, human epidermal growth factor receptor-2; HR, hormone receptor; MP, trastuzumab emtansine plus pertuzumab; NR, not reported; TP, trastuzumab plus pertuzumab.

Disclosure

The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Supplementary Materials

The submitted compressed file (Suppl.zip) contains the following supplementary figures and tables: *Figure S1*. Treatment Rankings for Each Outcome; *Figure S2*. Meta-regression Analysis with Adjustment for Hormone Receptor Status for Pathological Complete Response; *Figure S3*. Pooled Estimates for Overall Serious Adverse Events Using Fixed-effect Model. *eTable 1*. Literature Search Strategy; *eTable 2*. Characteristics of Included Trials and Patient Populations; *eTable 3*. Neoadjuvant Treatments in Included Trials; *eTable 4*. Bias Assessment of Included Trials; *eTable 5*. Network Meta-analysis for Pathological Complete Response after Excluding H2269s Trial; *eTable 6*. Network Meta-analysis for Breast-conserving Surgery Rate after Excluding NeoSphere Trial; *eTable 7*. Comparative results from traditional pairwise meta-analysis and network meta-analysis; *eTable 8*. Network Meta-analysis for Primary Outcomes after Excluding the Trials That Did Not Used HER2-targeted Agents Concomitantly with Chemotherapy; *eTable 9*. Network Meta-analysis for Primary Outcomes after Excluding the Trials of High Risk of Bias; *eTable 10*. Network Meta-analysis for Primary Outcomes after Excluding the Trials Presented as Abstracts. *(Supplementary Materials)*

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Review Article

Current Challenges in Cancer Immunotherapy: Multimodal Approaches to Improve Efficacy and Patient Response Rates

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Cancer immunotherapy is a promising innovative treatment for many forms of cancer, particularly melanoma. Although immunotherapy has been shown to be efficacious, patient response rates vary and, more often than not, only a small subset of the patients within a large cohort respond favourably to the treatment. This issue is particularly concerning and becomes a challenge of immunotherapy to improve the effectiveness and patient response rates. Here, we review the specific types of available immunotherapy options, their proposed mechanism(s) of action, and the reasons why the patient response to this treatment is variable. The potential favourable options to improve response rates to immunotherapy will be discussed with an emphasis on adopting a multimodal approach on the novel role that the gut microbiota may play in modulating the efficacy of cancer immunotherapy.

1. Introduction

Cancer immunotherapy was voted "breakthrough of the year" by Science in 2013 primarily due to the success rates observed at the clinical level as well as the simple yet elegant approach of this treatment [1]. However, patients that are treated with immunotherapy have shown varying response rates among cancers and within cohorts with the same malignancy [2]. Varying response rates concerning this type of therapy may be attributed to the specificity involved in eliciting an immune response, overcoming the mechanisms that cancer cells employ to evade immune surveillance, and ensuring that the activated immune cells have access to the malignant tissues. There are several ways that the response rates can be improved including, but not limited to, identifying more specific biomarkers and immune checkpoint inhibitors. Also, better predictive tools and assays can identify patients that will best respond to immunotherapy. Conceptually, this treatment approach had existed since the late 1800s but was archived as "ineffective" when radiation and chemotherapy became the standard of care for many types of cancer [3]. Currently, immunotherapy is one of the most studied forms of cancer therapy in addition to supplemental chemotherapy. The approach to cancer immunotherapy involves harnessing the specificity and killing mechanisms of the immune system to target and extirpate malignant cells.

2. Anticancer Immunity and Immune Evasion Mechanisms

Normal anticancer immunity involves identifying and clearing early malignant cells that express tumor-associated antigens (TAAs). TAAs are presented in complex with human leukocyte antigens (HLA) on the surface of tumor cells [3]. A complex system of interactions involving dendritic cells (DCs), macrophages, plasma cells, cytokines, antibodies, and helper T cells all work in tandem to prevent tumor development [4]. In order for an anticancer response to be initiated, TAAs that are presented by DCs in context of HLA class I molecules to activate CTLs and in context with HLA class II molecules to activate CD4+ helper T cells [5]. Activated CD4+ Th1 and Th2 helper T cells secrete interleukin-2 (IL2) and interferons (IFN) which in turn are involved in the activation of CTLs. The cytokines involved in this CTL activation and response are mainly produced by Th2 cells. Additional complexity is that for CTLs to identify

tumor cells, the tumor cells must express TAAs on HLA class I molecules that initially generated the specificity of the CTLs [5].

During the tumor development, genetic mutations can also lead to the initiation of neoantigens that are recognizable by the immune system. However, once malignant cells are established, they are capable of evading this immune surveillance by turning off these antigens through a process called immune tolerance induction [4]. A second process known as immune evasion can occur when a tumor associates with its microenvironment to inhibit the antitumor response [4].

2.1. Cancer Evasion Mechanisms of Host Immune Response. Due to its high mutagenic capacity and keen survival capabilities, cancer cells use several mechanisms to evade the host immune response to reestablish their growth and continue to progress [6]. While many of these mechanisms are available for use in the "immune evasion toolbox," only a handful are proposed to be useful at any given time during cancer progression based on the specific mechanism that is most appropriate for tumor establishment [7]. Key evasion tactics include upregulation of checkpoint receptor ligands that essentially prevent tumor-infiltrating lymphocytes (TILs) from entering the tumor mass, upregulation of immune-suppressing cells including regulatory T-cells (Tregs), or induction of the production of suppressive cytokines such as IL-10 and TGF-β [7]. Other specific mechanisms include downregulating the facets of the antigen presentation system [7].

The establishment of the tumor microenvironment (TME) not only allows the tumor to develop but permits it to recruit components of the host immune system. These TME components primarily act as cellular barriers to prevent any infiltration by antitumor immune cells in addition to promoting tumor growth [7, 8]. The development of a thick stromal layer surrounding the cancerous mass creates a physical barrier that is characterized by several features known to promote cancer growth, including the development of hypoxic conditions and abnormal tumor neovascularization [4]. Not only does this prevent potential immune cells from penetrating the tumor mass but establishes the blood vessels to allow cells to metastasize to distant organs. Once established, tumors can evade the immune system until these mechanisms are overcome, namely, by immunotherapy approaches.

3. Individual Immunotherapy Approaches and Factors Contributing to Varied Effectiveness

Five key immunotherapy modalities are now clinically approved and can be delivered to patients [9], each with varying response rates as illustrated in Figure 1. These approaches can be further classified into two general categories: active and passive immunotherapies. An additional option is the combination of them [10]. The active approach involves directing the host immune system to TAAs on the surface of tumors. These antigens can be specific proteins or carbohydrates that are exclusively expressed or overly expressed in tumor cells.

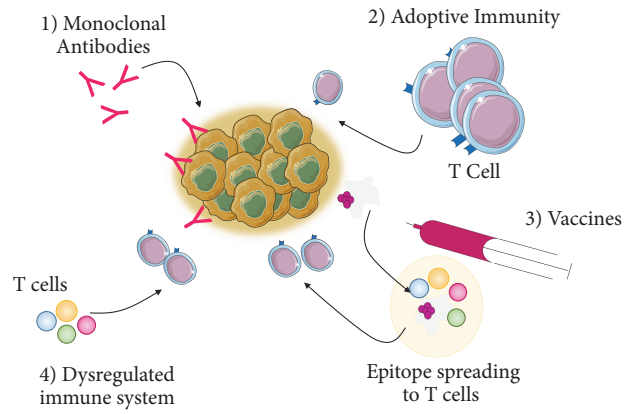

Figure 1: *Forms of Immunotherapy*. Currently available immunotherapy treatment options include (1) monoclonal antibodies, (1) adoptive immunotherapy, (3) vaccines, and (4) correcting a dysregulated immune system. These forms of immunotherapy are designed to either actively target a specific antigen on the tumor or enhance the host's immune system.

In contrast, passive immunotherapy involves enhancing the standard anticancer response by the immune system by using monoclonal antibodies, lymphocytes, and cytokines [10]. By extension, a combination therapy would involve one or more aspects of these two forms of immunotherapy. It is noteworthy that the delivery and effectiveness of immunotherapy are highly dependent on the cancer type, grade, predictive response rate, and expression of critical biomarkers [2]; however, patient response rates can still vary.

3.1. Monoclonal Antibodies and Their Varied Response Rates. The premise of monoclonal antibodies relies on targeting a specific antigen present on cancer cells, and it is a form of active immunity [11]. Monoclonal antibodies can either be unconjugated or be conjugated with therapeutic drugs that would produce a cytotoxic effect on cancer cells [11]. This type of immunotherapy has been used to treat many different types of cancers including breast, lymphoma, and colorectal cancer [7].

Based on the mechanism by which monoclonal antibodies exert their therapeutic effects, it is perhaps not surprising that response rates would vary. This method essentially targets a specific sequence, or epitope, of an antigen that is exclusively expressed on a tumor to induce cell death. One main reason for this variability in response rate is the fact that these monoclonal antibodies are highly specific. The "mono-" form of antibodies recognizes only one specific epitope [12] and, therefore, if there are other isoforms of the epitope due to mutations, monoclonal antibodies would be unable to recognize and bind to the antigen in question. Furthermore, the antigen that is being targeted would need to be present on the surface of cancer cells. Collectively, the specificity of monoclonal antibodies is one of the key contributors to the variation of response rates making immunotherapy ineffective.

3.2. Immune Checkpoint Inhibitors. Immune checkpoints function to prevent the advent of autoimmunity as a result of uncontrolled activation of T cells. Tumor cells can exploit this mechanism by deactivating tumor-infiltrating lymphocytes (TILs) and preventing them from targeting tumor cells [7]. For example, one critical immune checkpoint ligand is known as the programmed death ligand -1 or 2 (PD-L1 or PD-L2) and its receptor the programmed death-1 (PD-1) [7]. The PD-1 receptor is expressed on the surface of activated T cells and, upon binding with their ligand which is overexpressed on the surface of malignant cells, it leads the activated T cells to change their conformation to that of an inactive phenotype, rendering them ineffective [7].

Immune checkpoint inhibitors are a variant of monoclonal antibody immunotherapy that can block immune checkpoint receptors to allow T cells to be activated and clear tumor cells. This type of approach represents a form of passive immunity, which is designed to enhance the effectiveness of the immune system. Two currently approved checkpoint inhibitors are anti-PD-L1 and anti-cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) [4, 5] as depicted in Figure 2. These two forms of immune checkpoint blockade inhibitors have been successful in several cancers including metastatic melanoma [4]. The mechanism of action of CTLA-4 involves typically competing with the ligands CD80 and CD86 to bind to CD28 in order to deactivate T cells [3]. By preventing CTLA-4 from inactivating T cells, the proposed mechanism of action that results is that T cell activity is enhanced and at the same time Treg activity is diminished [3].

Similarly, blocking PD-1 leads to a response where Tregs are deactivated and activating antitumor T-cells. Unlike CTLA-4, the PD-1 expression is on tumor cells and other healthy cells throughout the body, a large portion of which are myeloid cells in the TME [3]. This approach is far more effective when initiating an immune response. However, response rates to this immunotherapy can vary among these cancers and within tumor cohorts of the same cancer type [2].

Recent reports have implicated the gut microbiota in modulating patient response to anti-PD-1 immunotherapy in patients presenting with melanoma [13]. The basis of the report is on the clinical results of a patient previously treated with antibiotics and had a weaker response rate to PD-1 inhibition when compared to those that had not been administered antibiotics [14]. This particular study investigated the effects of gut microbiota diversity and composition on the efficacy of PD-1 and added to a growing body of research implicating the microbiota on yet another aspect of cancer treatment.

More specifically, the results from the study showed that individuals who had a more diverse microbiota composition responded better to immunotherapy as evidenced by tumor shrinkage compared with individuals with a less diverse composition. Additionally, cancer patients that responded to immunotherapy had an increase in antitumor killer T cells and the difference in killer T cell quantity was correlated to the presence of species within the *Faecalibacterium* and *Clostridiales* phyla [13]. This evidence is suggestive of certain resident bacteria contributing to a positive response rate to immunotherapies while others may dampen or render it ineffective. Because each individual's microbiome is as unique as a fingerprint, it is logical that immunotherapy response rates may differ due to the composition of the gut at the time of immunotherapy.

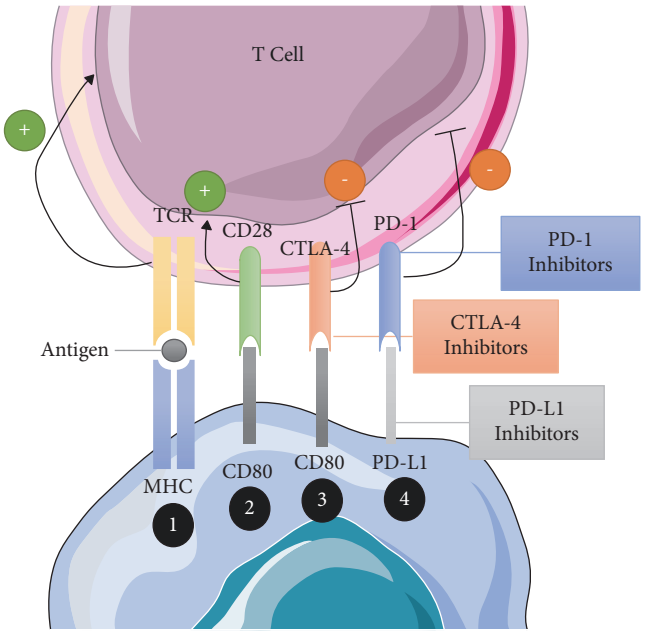

Figure 2: *Mechanism of Immune Checkpoint Inhibition.* MHC present antigens to the T-cell receptor in order to active T cells (1). Through interactions with the CD80 on tumor cells and the CD28 on T cells, T cells can be deactivated (2). Additionally, CTLA-4 competes with CD80 to deactivate T cells as well (3). Lastly, PD-L1 binds to the PD-1 receptor on T cells to deactivate T cells (4). Tumor cells employ the use of these mechanisms in order to prevent T cells from clearing malignant cells. By using inhibitors that prevent this interaction from occurring, T-cells remain active after identifying tumor cells and can clear them from the host. *Abbreviations:* CD; cluster of differentiation, CTLA-4; cytotoxic T-lymphocyte associated antigen-4, MHC; major histocompatibility complex, PD-1; programmed death-1, PD-L1; programmed death ligand-1, TCR; T-cell receptor.

3.3. Cytokines. Under normal immune responses, cytokines directly influence immunity, where they act to enhance or inhibit the effector cellular protein components of the immune system [4]. Although other proteins modulate the immune response, for simplicity, all proteins involved in immune modulatory activities will be broadly defined as cytokines throughout this report. As such, another mode of immunotherapy involves systemically infusing specific cytokines to enhance the immune response [4]. Currently, IFN-α and IL-2 are administered during cancer treatment [5]. More specifically, IFN-α has been characterized as an immune stimulator through the activation of DC and promotion of antigen presentation to elicit an immune response, enhancing the Th1 response, CTL activity, and the cytotoxic effects of NK cells [5]. IFN-α is also administered in combination with cancer vaccines to enhance therapeutic effectiveness. Similarly, IL-2 has been implicated in broadly

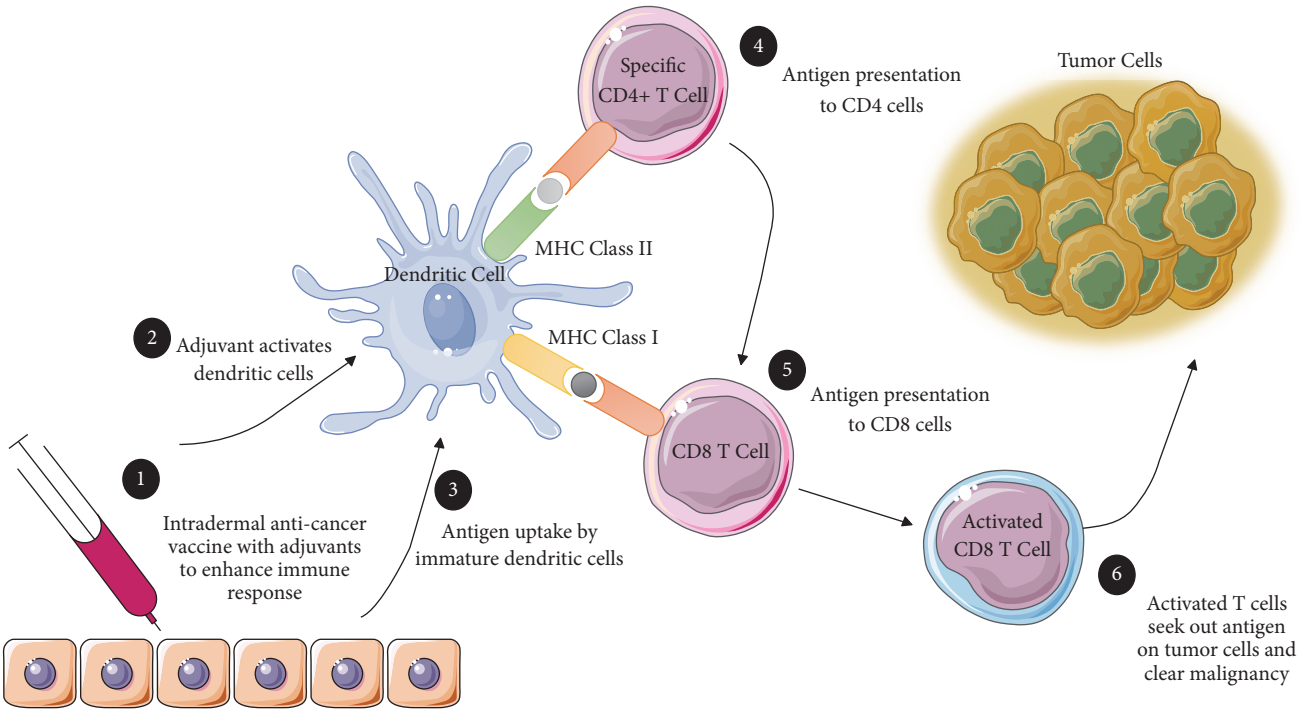

Figure 3: *Mechanism of Action of Cancer Vaccines*. As illustrated, cancer vaccines are administered through an intradermal injection (1) with adjuvants that activate dendritic cells (2). Immature dendritic cells take up the antigen; typically this antigen is uniquely expressed on tumor cells (3) and presents the antigen to CD4 cells (4) and CD8 cells (5). CD8 cells are then activated to seek out the antigen on the surface of tumor cells (6). *Abbreviations.* CD: a cluster of differentiation and MHC: major histocompatibility complex.

enhancing the antitumor effects of the immune system by increasing the activity of T cells, specifically tumor infiltrating cells and promoting NK cell activity [4].

While both of these types of cytokines have been used to enhance the immune system, one key caveat is that the patient would need to have a relatively robust immune system in order for these therapies to be effective. This caveat could contribute to the variation in response rates. As such, cytokines are usually used in combination with other forms of immunotherapy [4].

3.4. Cancer Vaccines. Similar to monoclonal antibody therapies, cancer vaccines are currently available as an immunotherapeutic treatment. This approach involves the conventional vaccination methods to induce an immune response. In brief, cancer vaccines that contain whole or fragments of cancer cells or antigens are designed to stimulate an immune response [11] as illustrated in Figure 3.

One main factor that contributes to differences in patient response rates to cancer vaccines is the specificity of the vaccine and whether the commercial form of the vaccine is produced with the same antigen present in the patient's tumor for the immune system to identify its presence in the body. For example, peptide-based vaccines are designed to respond to one tumor antigen in complex with its HLA [5] and thus would need to be administered to a minimal subset of patients that present this antigen. Another approach is immune- or dendritic cell-based vaccines which have shown promise in castration-resistant prostate cancer [5]. This approach involves extracting antigen-presenting cells (APCs), such as DCs, and activating them to the PA2024 prostate tumor antigen and then reintroducing the activated cells into the patient to specifically target prostate cancer cells expressing the antigen. This specific vaccine is made up of prostatic acid phosphatase (PAP), which is expressed in 95% of prostate cancers. PA2024 is delivered with granulocyte-macrophage-colony-stimulating factor (GM-CSF) in order to be taken up by APCs to active the host's T cells and, moreover, direct them to target exposed PAP on prostate cancer cells. A similar approach is applied to ovarian cancers which express the TAA CA-125 to activate DCs similarly as outlined for the prostate cancer studies. This approach assumes that a tumor is relatively homogenous and a large portion of the expressed antigen is presented to APCs. As such, this delivery strategy would significantly contribute to the efficacy of immunotherapies. Tumor heterogeneity is an issue that many cancer therapy strategies fail to consider when designing cancer vaccines. The approach requires a multimodal therapy to overcome tumor heterogeneity and is discussed in detail below.

3.5. Cell-Based Immunotherapy. Cell-based immunotherapy is a T-cell therapy that involves transferring natural T cells or genetically modified T cells that have been expanded *ex vivo* to target tumor antigens [7, 11] specifically. For example, these tumor antigens may include mutant proteins, tissue

differentiation antigens, and vascular antigens, to name a few [11]. Once infused into the patient, cytokines such as IL-2 are delivered in combination with the therapy to enhance the effects of these activated T cells [4].

One of the most promising cell-based immunotherapies is chimeric antigen receptor T therapy (CAR-T). This approach to immunotherapy involves genetically engineering T cells *ex vivo* to enhance their specificity and antitumor mechanism of action [4]. CAR-T cells are produced *ex vivo* and reintroduced into the patient to target cancer cells in a non-HLA dependent manner specifically. This therapy can circumvent immune evasion by cancer cells that preferentially lose their HLA molecules [4]. The premise of this immune therapy requires a tumor to express "chimeric-like" antigen in order to elicit a cytotoxic response.

4. General Variation in Response Rates of Immunotherapy

In the preceding sections, several forms of immunotherapies are currently available for patients; however, not all patients exhibit the same response rates. The variation of response rates reflects the therapeutic mechanism that is unique to each type of immunotherapy. In addition to the therapy-specific issues, other challenges contribute to the effectiveness of immunotherapy.

4.1. Pathophysiology and Tumor Microenvironment Affects Immunotherapy Access. Other components of the TME, such as tumor-associated macrophages (TAMs) and cytokines released to promote an immunosuppressive environment, also contribute to the efficacy of immunotherapy. This microenvironment makes delivery of therapeutic agents challenging, particularly concerning immunotherapy because they involve reactivating the immune system to penetrate the tumor. For example, pancreatic cancer is characterized by the development of an inflammation-driven desmoplasia, which forms a thick stromal microenvironment [15]. Therefore, not only would this barrier environment need to be disabled, but the inability of vaccines or activated T cells to penetrate the thick stromal layer would negatively affect response rates.

A recently discovered component of the microenvironment is the presence of bacteria inside pancreatic tumors that were shown to metabolize, inactivate, and potentially confer resistance to chemotherapy [16]. A similar process could potentially contribute to the varied effectiveness of immunotherapies and why response rates vary among patients with similar tumor profiles.

Lastly, perhaps the most crucial driver of differential response rates seen in cancer patients is the blood vessels and lymphatic vessels that feed into the regions surrounding the tumor. For example, mucosal tissues such as the lungs and the gastrointestinal tract are in direct contact with lymph and blood vessels and thus allow immunotherapy regimens to exert their therapeutic effects. In tissues where the TME has established minimal interaction with the surrounding physical barriers, it would be exceedingly difficult for an improvement to be observed and thus it represents another contributing factor in response rates seen in patients.

4.2. Development of Resistance. As with any therapy, the development of cancer resistance to the treatment is predictable; it is relatively inevitable given the highly proliferative nature of cancer cells. Immunotherapy is no different to any of the other cancer therapies. Acquired resistance in cancer cells is related to the plasticity of response mechanisms that allow cancer cells to alter their genetic pathways to compensate for changes in their immediate environment [7]. Some of these changes include epigenetic modifications and reactivation of alternative pathways [6].

One study investigated the genetic modifications in a tumor that allowed for the acquisition of resistance to the immune checkpoint inhibitor pembrolizumab [17]. Results from this study revealed that two patients had mutations in the *JAK1* or *JAK2* genes leading to a disruption in INFγ signalling and thereby reducing the expression of genes involved in T-cell mediated elimination of cancer cells. The genetic profile of a third patient showed a mutation in the *B2M* gene instead, which has properties in recognizing and clearing cancer cells. Based on these results, a key facet of individual response rates is related to the mutagenic nature of the type and stage of cancer and whether mutations in specific genes have taken place that renders immunotherapy ineffective.

4.3. Competency and Diversity of Individual Immune System. Given that immunotherapies involve the activation and amplification of the immune system, it is perhaps not surprising that differential response rates can also be the result of individual immune competency and diversity. Concerning competency, because immunotherapy is not considered a viable first-line treatment, many patients are either treated with chemo- or radiotherapy in combination with or before the administration of immunotherapy. The delivery of chemotherapy effectively reduces the competency of the immune system and would, therefore, affect patient response rates depending on the type of chemotherapy that was delivered. For example, if highly toxic chemotherapy is delivered to a patient presenting with an advanced stage of cancer and subsequently followed up with an immunotherapy, this could theoretically lead to reduced response rates as the immune system would have been compromised. Concerning immune diversity, recent reports have attributed the overall effectiveness of immunotherapy to the diversity of the HLA class I. These class-I molecules generally present intracellular processed proteins to CD8+ killer T cells, which specifically target cancerous cells expressing these processed tumor proteins [18]. Although this study considered the effectiveness of immune checkpoint blockage through anti-PD-1 or anti-CTLA-4 [19], the results can be translated to other forms of immunotherapy that involve antigen presentation like CAR-T therapy and cancer vaccines. Here, a greater diversity of HLA class I locus correlated with an overall survival following treatment [19]. The greater diversity of HLA class I molecules would be associated with an increased number of tumor

antigens that could be presented thus leading to increased survivorship following therapy. Differences in HLA diversity could lead to differential response rates of immune checkpoint blockade therapies as well as other immunotherapies. However, this particular aspect of the immune system would be more relevant as a novel predictive tool and will be discussed in detail below.

4.4. The Composition of Gut Microbiota. The gut microbiota is composed of trillions of bacteria, viruses, and fungi that colonize the human intestine beginning at birth and acts as a natural defensive barrier to infection. It is like an organ influencing virtually every vital bodily function [20, 21]. Recently the microbiota has been proposed to influence the efficacy of chemotherapies [22] and immunotherapies [13, 23], and this is an essential factor that may contribute to the variation in the effectiveness of immunotherapies as a whole. Although the studies have established a link between the gut microbiome and how it modulates response to immune checkpoint inhibitors, conceptually, this can also be applied to other forms of immunotherapy.

For example, the gastrointestinal tract consists of the gut microbiome and the mucosal immune system that are unique to the GI tract, and it has also been shown to contribute to a host's overall immunity [24]. Since many forms of immunotherapy are designed to reactivate the immune system or enhance its effects, the diversity and health of a patient's immune system would consequently correlate with the effectiveness of immunotherapy. By extension, therefore, the positive contribution of the gut microbiota in maintaining a healthy immune system would determine the effectiveness of immunotherapy.

5. Future Directions of Improving Response Rates to Immunotherapy

Although several critical mitigating factors contribute to the decreased effectiveness of immunotherapy, there are several areas in which additional research is currently being done that could improve response rates of cancer patients to therapy.

5.1. Identification of Additional Biomarkers. More conserved biomarkers that are expressed on the surface of tumor cells need to be discovered so that immunotherapies can be applied to a broader demographic of patients [2]. The goal of active immunotherapy is to target a specific sequence that is exclusively expressed on tumor cells, which are known as "neoantigens" or tumor-specific antigens (TSA) [25]. However, many of the antigens that are expressed on tumors are also expressed on healthy cells, which would render any therapy that has a nontumor specific antigen cytotoxic to healthy cells. Identifying TSA target for immunotherapy would likely yield increased effectiveness of treatment outcomes with minimal damage to healthy cells. One example of a potential target is the cancer testis antigens (CTAs) which are expressed more readily on cancer cells as opposed to healthy cells [2]. An additional facet of these antigens is that they elicit a robust immune response. CTAs are also expressed by cancer stem cells which are a very elusive subpopulation of the tumor that contributes to its ability to self-renew indefinitely even following therapeutic intervention. Therefore, identification of additional markers would help to circumvent the challenges posed by tumor heterogeneity because the probability of targeting more than one type of cell would be increased if the host immune cells are "taught" to recognize multiple types of antigens and launch a robust attack on the whole tumor.

5.2. Overcoming Resistance to Immunotherapies. One advancement of overcoming resistance to immunotherapy is the use of combination immunotherapy [17] or multimodal approaches. This approach would effectively increase the probability of antigens that are targeted by the immunotherapies and thus overcoming the compensatory nature of the cancer cells. An additional facet to this model could be supplemental immunotherapy using the epigenetic blockade in order to inhibit the processes that would typically regulate gene expression in response to therapy.

As such, specific epigenetic blockade regulators have shown some promise in enhancing the effectiveness of chemotherapy. For example, cancers may employ the use of DNA methyltransferases (DNMTs) for compensation purposes. DNMTs are required in order to alter the genetic profile of cancer cells in response to the changes in the surrounding environment [26]. Theoretically, DNMTs can upregulate proliferative pathways in order to compensate for the cytotoxic effects of current therapies, including immunotherapies. Because the activity of DNMTs is likely to be higher in cancer cells compared to normal cells, DNMTs could potentially be therapeutically targeted during or following current therapies to limit the compensatory activities of the cancer. Although this has not yet been tested in humans, a knockout study conducted on the DNMT, Dmnt-1 of leukemic stem cells, was able to demonstrate the validity of targeting epigenetic modulators. Knocking out Dmnt-1 shut down leukemogenesis and leukemic stem cell renewal without affecting normal hematopoiesis [27]. An *in vitro* study on the triple negative breast cancer MDA-MB-231 cell line employed the use of SAM (*S*-adenosyl-L-methionine), an inhibitor of demethylation of cells [28]. Results from this study demonstrated that blocking this activity inhibited the metastatic ability of MDA-MB-231 cells. Taken together, a method to overcome the resistance to immunotherapies could be through the use of inhibitors that can modulate the activity of DNMTs that exclusively promote genetic alterations that affect the ability of the immune system to identify and kill cancer cells.

5.3. Earlier Administration of Immunotherapy. Immunotherapies are traditionally given during the later stages as second-line treatment [2]. Currently, immunotherapies have not been proposed as viable first-line treatment options, which makes it exceedingly difficult for these therapies to be effective in patients whose immune system has been compromised as a result of the conventional therapies given. One solution

to this approach would be to deliver immunotherapies earlier than they are currently being done in the clinic so that the host immune system can have a robust response. Because the immunotherapy is generally administered following chemotherapy, residual tumor cells may no longer have the necessary antigens that currently acquired the immunotherapeutic target.

5.4. Personalized Approach to Overcoming Molecular and Physical Barriers to Immunotherapy. Due to the heterogeneous nature of many tumors as well as the unique pathophysiology of the characterized different cancers, personalized care is another solution that could potentially be used to modify response rates to immunotherapy [2]. Concerning molecular barriers, immunotherapy is only efficacious in a small subset of patients [2]. One proposed reason is that the approved immunotherapy drugs are designed to be active on a wide range of cancers, assuming they express the specific molecular profile that immunotherapies can identify and subsequently target. In addition to identifying additional targetable biomarkers, implementing a personalized approach by characterizing patient-specific tumors to test for a panel of biomarkers is an important avenue to consider. Once identified, candidate biomarkers that would elicit a robust response from the immunotherapy drug could potentially improve response rates.

Concerning physical barriers such as the dense stromal and immunosuppressive microenvironment, monoclonal antibodies that specifically target and deactivate these specific components can render the microenvironment immunosuppressive. This approach was attempted in preclinical animal studies modelling pancreatic cancer to determine a method to overcome the challenges present in the tumor microenvironment. For example, the C-X-C motif chemokine receptor 2 (CXCR-2) molecule was therapeutically targeted as a mode to overcome the immunosuppressive nature of pancreatic cancer [29]. The physiological mechanism of action of CXCR-2 is to act as a homing beacon for immune cells, specifically to attract neutrophils and myeloid suppressor cells [29]. In tumors, CXCR-2 is overexpressed on immune cells found in the tumor microenvironment of pancreatic cancer.

Additionally, this expression correlated with high levels of neutrophils and myeloid-derived suppressor cells in the tumor microenvironment contributing to pancreatic cancer progression. With impaired a CXCR-2 gene, there were decreased metastases, perhaps due to active T cells that were able to invade tumors. The principal mechanism of this study is that CXCR2 regulates T-cell infiltration. Given the immunosuppressive nature of the pancreatic cancer tumor microenvironment and the role played by CXCR-2, inhibiting this molecule could have important implications for immunotherapy. Although these applications were attempted by Steele et al. [29], many animals did not survive to receive immunotherapy. Nonetheless, this represents a promising avenue to consider in overcoming the critical challenges that contribute to various response rates.

5.5. Accurate Prediction of Immunotherapy Effectiveness. One area of therapeutic design to supplement conventional therapies is the use of the mutational status of cancer to assess the likelihood of a positive outcome with the targeted therapy. For example, patients that present with mutant KRAS nonsmall cell lung cancer have been well documented to be unresponsive to EGFR tyrosine kinase Inhibitor (TKI) therapy [30]. A similar predictive method is used when assessing the appropriateness of using various immunotherapies; this is especially important given the specificity of immunotherapy. Current predictive tools include characterizing the levels of PDL1 expression on a tumor to determine whether the immune checkpoint inhibition would be sufficient [3]. This approach is rational because individuals with higher rates of PD-L1 expression are more responsive to treatment [3]. As outlined above, HLA class-I diversity also acts as a predictive tool that could be used as an assay system to assess the response rates of patients treated with therapies that involve antigen presentation. Furthermore, characterizing the levels of HLA class-I diversity in tumors could be used in determining the efficacy of the treatment because this variation within cancer cells has also been linked to the treatment response rates. For example, patients' tumors that lacked HLA class-I diversity were linked with decreased survival [19]. By characterizing the HLA class-I diversity of both the tumor and the patients' immune system, predictability of immunotherapy could be determined with greater accuracy.

5.6. Re-Educating the Gut Microbiome to Enhance Immunotherapy Effectiveness. As outlined above, some factors contribute to the efficacy of immunotherapy and the response rates as a whole. One recently uncovered facet is the gut microbiota playing a crucial role in modulating the effects of immunotherapies. Because there are specific strains of commensal bacteria that can influence the response to immunotherapy, re-educating or diversifying the gut microbiota through the use of probiotics or prebiotics before or in conjunction with immunotherapies could lead to a robust response rate.

5.7. Probiotics. Probiotics are microbial food supplements that improve host gut microbiota balance. Thus far, the consensus has been that probiotics can enhance the host's immune response through several mechanisms [31]. The probiotics can promote the immune function by augmenting the mucosal barrier function, decreasing the mucosal transfer of luminal organisms and metabolites to the host, increasing the mucosal antibody production, and enhancing the epithelial integrity and direct antagonism of pathogenic microorganisms [32]. It is perhaps not surprising that the gut microbiota influences patient response to chemotherapies and immunotherapies. For example, a study by Vetizou et al. [33] demonstrated a correlation between the effectiveness of cancer immunotherapies and the composition of the gut microbiome, thereby implicating a more involved role for probiotics. In the report, CTLA-4 immunotherapy was used, and the findings from both preclinical mice studies and patients demonstrated a clear relationship between the

efficacy of CTLA-4 blockade dependence on the geodistribution of *B. fragilis* in the mucosal layer of the intestine and its association with *Burkholderiales*. These relationships included synergizing with TLR2/TLR4 signalling pathways.

As an extension of these findings, researchers have attempted to rebalance the gut microbiota to increase the effectiveness of anti-cancer treatments. Sivan et al. [34] demonstrated that the rate of tumor growth decreased through oral administration of the *Bifidobacteria* alone or in combination with anti-PD-L1 immunotherapy in mouse models of melanoma. These preliminary findings suggest that there is an important underlying mechanism that could increase the potency of immunotherapies. Additional sequencing of the 16S ribosomal subunit from mice that were treated with the probiotics followed by immunotherapy demonstrated that *Bifidobacteria* were associated with antitumor T-cell responses and that, in order to improve antitumor immunity, live *Bifidobacteria* may be an essential supplement to consider when treating patients with immunotherapy. Interestingly, there are standard components of commercial probiotic supplements recapitulated the same antitumor immunity effect.

5.8. Prebiotics. Concerning cancer treatment, chemotherapy significantly damages the intestinal microbiota by reducing the abundance of beneficial bacteria, including *Lactobacilli* and *Bifidobacteria*, while increasing potentially pathogenic bacteria (e.g., *Clostridia* and *Enterobacteriaceae*) [34]. As a potential treatment option, prebiotics has been proposed as a supplement to repair chemotherapy-induced intestinal dysbacteriosis. The concept of using prebiotics to target and alter the composition of the gut microbiota was first suggested in 1994 [35] and may have important implications for patient response to chemotherapy and immunotherapy.

Prebiotics are nonviable and indigestible compounds which increase the quantity of specific gut microbiota including *Bifidobacteria* and *Lactobacilli* [35]. In order to be classified as a prebiotic, a compound must be indigestible and not absorbed in the small intestine. However, it must also have the capacity to rebalance the gut microbiota to that of a healthier composition in addition to being selectively fermented by beneficial bacteria in the colon [36]. The latter leads to the production of short-chain fatty acids (SCFAs) in the colon [37]. Since the prebiotics not only modulate the gut microbiota *in vitro* (e.g., by promoting the proliferation of probiotics including *Lactobacillus plantarum L12* and *Bifidobacterium pseudocatenulatum B7003*), it also can improve the function of the bowel and immune system, the bioavailability of the metabolic health, and minerals. The prebiotics diminishes the risk and severity of the inflammatory bowel diseases (IBD) as well as the bowel syndromes unusually irritable bowel syndrome (IBS) [38]. Theoretically, if prebiotics are administered to patients and subsequently lead to the expansion of beneficial bacteria, they may have a positive implication in rebalancing the gut microbiota and could prime the host to respond favourably to immunotherapies.

5.9. The Novel Application of Nanotechnology to Improve the Efficacy of Immunotherapy. Applications of nanotechnology have primarily focused on revolutionizing diagnosis and improving the therapy of several types of cancer. These applications have included encapsulation of drugs into nanomaterials-based carrier systems, which may overcome their inherent limitations (e.g., their hydrophobicity and short half-lives) without the adverse effects on their therapeutic outcomes. Encapsulating therapeutics into the nanoparticles allows them to pass sequential physical and biological transport obstacles and to target the tumor tissue [39–43].

Recently, these applications have been extended to nanoparticles in fine-tuning the tumor microenvironment (TME) with the intention of rebalancing this environment and allowing for immune cell and immunotherapy transport [44]. In these studies, nanoparticles loaded with drugs, immunomodulatory substances, and oligonucleotides have been able to modulate regulatory T cell (Treg) populations indirectly. This approach is particularly significant because Tregs can act as a barrier for the effectiveness of the cancer immunotherapy [45, 46] where higher levels of Tregs have been correlated with more rapid cancer progression [47]. For instance, Kwong et al. [48] prepared liposomes anchored with anti-CD137 and IL-2Fc molecules in an *in vivo* melanoma study. The results from the study demonstrated that Treg levels were indirectly reduced, which could have critical applications in enhancing the immune response without added toxicity to the patient.

Doxorubicin is a chemotherapeutic agent used to treat breast cancer [49, 50] and has been shown to cause drug resistance [51]. Thus, an ideal doxorubicin-based tool for breast cancer should simultaneously overcome the drug resistance and inhibit the tumor-induced immunoresistance and immunosuppression [52]. Kopecka et al. demonstrated that aminobisphosphonate zoledronic acid (ZA) markedly reverses chemoresistance and immunoresistance in doxorubicin-resistant cell lines in-vitro [52]. However, administration of ZA as a free drug leads to reaching low intratumor mass, because it is intensely taken by bone [53]. To overcome this problem, Kopecka et al. encapsulated ZA within the self-assembling nanoparticles. Their results showed that the encapsulating of ZA resulted in the intratumor delivery of the aminobisphosphonate [54, 55] and enhancement of antiproliferative effects against tumors implanted in the immunodeficient animals [56, 57].

Furthermore, they investigated the impact of the nanoparticles encapsulating ZA in combination with doxorubicin on chemoresistance and immunoresistance of the breast tumors implanted in the immunocompetent mice. They observed that encapsulated ZA decreased IC_{50} of doxorubicin in human as well as murine chemoresistant breast cancer cells. It also restored the doxorubicin efficacy against a chemoimmunoresistant tumor implanted in the immunocompetent mice. Base on their findings, they suggested ZA loaded-nanoparticles as an ideal approach to simultaneously overcome the chemoresistance and immunoresistance in breast tumors.

Van Woensel et al. [58] also suggested that the intranasal nanoparticle encapsulating galactin-1 could be used as valuable adjuvant therapy in order to increase the efficiency of the immune-checkpoint blockade and chemotherapy. In the study, siRNA galactin-1-loaded chitosan nanoparticles were used to sensitize glioblastoma tumor microenvironment. Importantly, they found that both myeloid suppressor cells and Treg populations have been reduced. This approach is particularly significant because Tregs can act as a barrier to the effectiveness of the cancer immunotherapy where higher levels of Tregs have been correlated with more rapid cancer progression

Kwong et al. [48] encapsulated immunoagonists including anti-CD137 and interleukin (IL)-2Fc within nanoparticles. It is noteworthy that inflammatory toxicities limited systemic administration of the free forms of those immunotherapeutic drugs. Following intratumoral injection in the melanoma model, anti-CD137 and interleukin (IL)-2Fc loaded nanoparticles diffused into the tumor parenchyma and tumor-draining lymph nodes, while they were not able to enter the systemic circulation. The latter prevented the lethal inflammatory toxicities. Their data confirmed that the growth of simultaneously established distal tumors was inhibited significantly. They proposed that anti-CD137 and interleukin (IL)-2Fc loaded nanoparticles may have a synergistic effect in combination with the administration of well-tolerated immunotherapy agents, e.g., anti-CTLA-4 or anti-PD-1 which are known to enhance tumor regression in humans.

6. Future Directions in Improving Immunotherapies

Immunotherapy is now at the forefront of cancer treatment, but questions and challenges still remain around its efficacy, targeting, and toxicity. We have briefly detailed the latest developments in immunotherapy, including established and emerging targets and modalities, novel engineering strategies, combinations modalities, biomarkers, preclinical model approaches, strategies to mitigate toxicity, and clinical developments. Here, Figure 4 describes the overview of the factors contributing to varying response rates to immunotherapy and methods to overcome these barriers.

Examples in improving immunotherapies will come from the research on checkpoint inhibitors, adoptive T cell therapy, combinations, oncolytic viruses, manipulating the tumor microenvironment, and the gut microbiome. Technologies involved in novel gene editing with an understanding of cancer biology could unleash the full efficacy of chimeric antigen receptors T-cells (CAR-T) technology in both blood and solid tumors. Brown and Mackall [59] have recently highlighted our current understanding of resistance to CAR-T cell immunotherapy for leukaemia and lymphoma in revealing the barriers that must be addressed to increase efficacy of this novel class of therapeutics. The report identifies the key CAR-T advances and the their major problems such as the following: (a) CD19-targeted CAR-T cells produce excellent response rates in paediatric B cell acute lymphoblastic leukaemia (B-ALL) cases, but many of these patients will relapse, most often with CD19-negative leukaemia; (b) CD22-directed CAR-T cells produce high response rates in CD19 naive or resistant B-ALL but often relapse with $CD22^{low}$ leukaemia; (c) intrinsic gene programmes of memory versus exhaustion correlate with T cell fitness and determine response to CD19-targeted CAR-T cells in chronic lymphocytic leukaemia (CLL); and (d) loss of Tet methylcytosine dioxygenase 2 (TET2), an epigenetic modulator, prevented terminal T cell differentiation and enabled the progeny of a single CD8+ CAR-T cells towards complete remission in a patient with CLL.

The development of adoptive cell therapies across a wide range of indications includes CAR-T, T cell receptors (TCR), tumor infiltrating lymphocytes (TIL), and NK cells as well as new strategies for commercialization. The immunotherapy industry is currently dominated by antagonist antibodies such as PD-1 and CTLA-4. However, it is clear that antagonists alone are not enough to elicit good response rates in the majority of patients. Hence, there are latest developments in agonist immunotherapy with a rising interest in agonist targets including TNF receptors, inducible co-stimulator (ICOS), type 2 transmembrane glycoprotein receptor belonging to the TNF superfamily and expressed on activated T Lymphocytes (4 -1BB), Toll-like receptors, stimulator of interferon genes (STING), and V-domain Ig suppressor of T cell activation (VISTA). VISTA is a type I transmembrane protein that antagonizes the programmed death-ligands 1 and 2 (PD-L1 and PD-L2); it is produced at high levels in TILs, such as myeloid-derived suppressor cells and Tregs and its blockade with an antibody results in delayed tumor growth in mouse models of melanoma [60] and squamous cell carcinoma [61]. The review by Li et al. [62] discusses the antitumor properties of TLRs, RIG-I-like receptors (RLRs), and STING-mediated innate immune pathways, in addition to the promising innate immune targets for potential application in cancer immunotherapy.

Gao et al. [63] have provided evidence to suggest that an increase in immune cell infiltration may be insufficient to generate antitumor responses. Their data were the first evidence showing that VISTA is a compensatory inhibitory pathway in the clinical treatment using ipilimumab (monoclonal anti-CTLA-4) therapy. Blockade of other immune checkpoints such as PD1/PD-L1 and/or VISTA may be necessary to provide significant clinical benefit for patients with prostate cancer. Future studies will need to elucidate the role of VISTA as a potential resistance mechanism and determine whether VISTA can be targeted to improve antitumor responses in patients.

7. Conclusions

Cancer immunotherapy represents a new frontier in cancer therapies that have begun to show promise since their initial conceptualization. However, patient response rates continue to fluctuate for reasons that are not well understood but have been considered from multiple standpoints, including immune competency and diversity, differing antigen

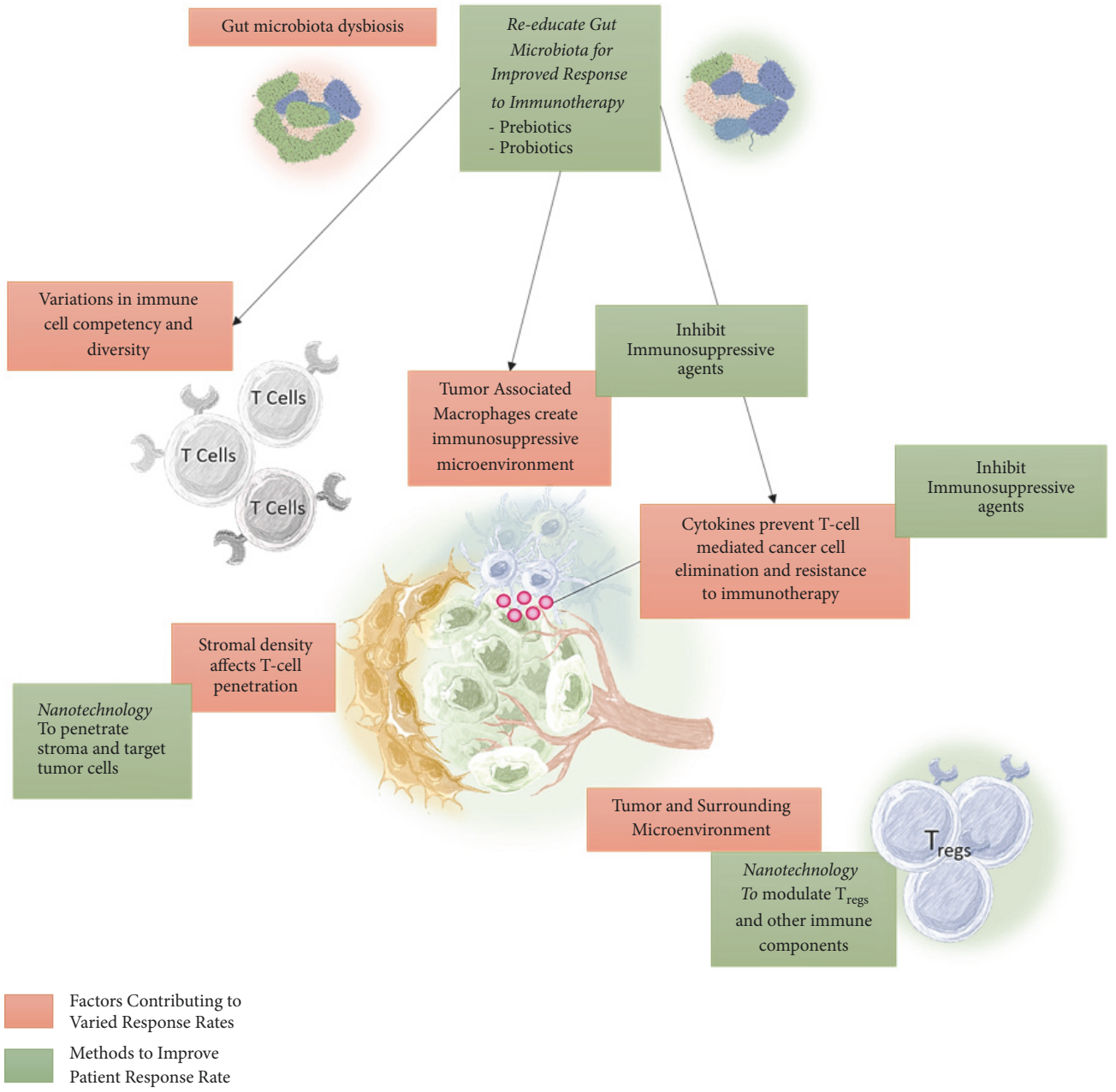

Figure 4: *Overview of the factors contributing to varying response rates to immunotherapy and methods to overcome these barriers*. Variations in immunotherapy response rates range from specific individual immune system diversity to the broad influence of the composition of the gut microbiota and are shown in red boxes. The proposed methods to overcome these barriers are indicated in green boxes. The gut microbiota can have overarching effects on patient response to immunotherapy due to the influence of the gut microbiota on the composition and function of the immune system.

specificity and expression levels, and more recently the role played by the gut microbiota. An improvement in the efficacy of immunotherapies will likely involve a more personalized and multimodal approach that cannot only target specific antigens that are present on a patient's tumor but is supplemented with agents such as epigenetic inhibitors and microbiota enhancers to elicit a more robust response. Thus, the complexity of the immune system and factors contributing to its activity are not well characterized, and additional research will require transdisciplinary approaches.

Disclosure

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

PD-L1 Expression in Human Breast Cancer Stem Cells Is Epigenetically Regulated through Posttranslational Histone Modifications

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Tumor progression through immune evasion is a major challenge in cancer therapy. Recent studies revealed that enhanced PD-L1 expression in cancer stem cells is linked to immune evasion. Understanding the mechanisms behind this PD-L1 overexpression in cancer stem cells is critical for developing more effective strategies for preventing immune evasion and increasing the efficacy of anti-PD-1/PD-L1 therapy. Tumorsphere formation in breast cancer cells enhanced epithelial to mesenchymal transition (EMT), which is evident by increased expression of mesenchymal markers. In this study, we analyzed CpG methylation of PD-L1 promoter in MCF-7 and BT-549 breast cancer cells and tumorspheres derived from them. PD-L1 promoter was significantly hypomethylated in MCF-7 tumorspheres, but not from BT-549 tumorspheres, compared with their cell line counterparts. The active demethylation of PD-L1 promoter was confirmed by the increase in the distribution of 5hmC and decrease in 5mC levels and the upregulation of TET3 and downregulation of DNMTs enzymes in MCF-7 tumorspheres, compared with the cell line. Additionally, we checked the distribution of repressive histones H3K9me3, H3K27me3, and active histone H3K4me3 in the PD-L1 promoter. We found that distribution of repressive histones to the PD-L1 promoter was lower in tumorspheres, compared with cell lines. Moreover, an overexpression of histone acetylation enzymes was observed in tumorspheres suggesting the active involvement of histone modifications in EMT-induced PD-L1 expression. In summary, EMT-associated overexpression of PD-L1 was partially independent of promoter CpG methylation and more likely to be dependent on posttranslational histone modifications.

1. Introduction

Breast cancer is the most common cancer in women accounting for 30% of all new cases reported, and it is a major cause of cancer-related death [1]. Recent advances in early detection and therapeutic interventions reduced the mortality rate remarkably [1]. Cancer immunotherapy has recently shown promising results for treating different cancers. Immune checkpoint inhibitors, as immunotherapeutic agents, showed promising outcomes with higher overall survival rate and progression-free survival, but unfortunately this has been achieved in a small fraction of cancer patients [2]. Even though therapy resistance, recurrence, and metastasis are still major challenges in breast cancer therapy and management, it has been reported that the presence of a subset of cells with unique features like self-renewal and differentiation called cancer stem cells (CSCs) could be a major contributor towards these challenges [3].

Numerous studies reported the overexpression of programmed death-ligand 1 (PD-L1) as a predictive biomarker for differentiating responders and nonresponders undergoing immune checkpoint inhibition (ICI) therapies targeting programmed cell death-1 (PD-1)/PD-L1 [4–7]. Moreover, PD-L1 overexpression plays a critical role in immune evasion through increase of T-cell apoptosis in many cancers [8]. The overexpression of PD-L1 can also act as a molecular shield to protect tumor cells from T-cell mediated killing [9]. Additionally, PD-L1 overexpression in MC38 murine colon cancer cells showed a direct suppression of $CD8^+$ TILs [10]. It has recently been reported that overexpression of PD-L1 in CSCs contributes to immune evasion through

EMT/β-catenin/STT3/PD-L1 signaling axis [11]. The expression of PD-L1 is regulated through multiple signaling pathways and transcriptional control. The genetic modifications for constitutive overexpression of PD-L1 in tumors could be explained due to the copy number alterations and potential oncogenic signaling pathways [12, 13]. We have recently reported that PD-L1 is overexpressed in human tumor tissues and dual inhibition of signal transducers and activator of transcription 1 (STAT1) and STAT3 can downregulate PD-L1 expression [14–16]. However, epigenetic mechanisms behind the regulation of PD-L1 are not fully disclosed.

Understanding the regulatory mechanisms involved in PD-L1 expression may open possibilities for the development of combination strategies to improve the efficacy of PD-1/PD-L1 blockade therapies. DNA promoter methylation studies in breast and colon cancer patients using paired normal and tumor tissues showed hypomethylation irrespective of their PD-L1 expression status [15, 16]. It has been reported that treatment of breast cancer cells with demethylating agent azacytidine induced an upregulation in PD-L1 expression [17]. These data collectively indicate that apart from DNA methylation, multiple regulatory mechanisms might be involved in the expression of PD-L1.

In this study, we investigated epigenetic regulatory mechanisms involved in the PD-L1 expression associated with epithelial to mesenchymal transitions (EMT) in human breast cancer stem cells. We found that PD-L1 expression was significantly upregulated in MCF-7 and BT-549 tumorspheres, compared with cell lines, and this upregulation was partially dependent on the PD-L1 promoter demethylation. Moreover, our results showed that less distribution of repressive histones in the PD-L1 promoter region and overexpression of histone acetylation enzymes can also contribute to the PD-L1 upregulation in tumorspheres, compared with cell lines. These data shed light on the possible epigenetic mechanisms involved in the upregulation of PD-L1 in tumorspheres.

2. Materials and Methods

2.1. Cell and Tumorsphere Culture. MCF-7 and BT-549 breast cancer cell lines (ATCC, USA) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma Aldrich, St. Louis, USA) in a humidified incubator at 37°C and 5% CO_2. For BT-549 cells, the cells were maintained in the media supplemented with 0.023 IU/ml insulin (Sigma Aldrich).

For the generation of tumorspheres, 80-90% confluent cell plates were trypsinized, washed, and resuspended in Cancer Stem Premium™ medium (ProMab Biotechnologies, Richmond, CA, USA). $1x10^4$ cells/ml were cultured in Cancer Stem Premium™ media in ultra-low attachment Nunclon Sphera plate (Thermo Scientific, Nunclon Sphera, Roskilde, Denmark). The cells were incubated for 5 to 10 days for tumorsphere formation. Media change was done by collecting the tumorspheres in 15 ml falcon tubes and allowed to settle by gravity. The pellets were washed with 1X PBS. Tumorspheres older than seven days were used for the subsequent experiments.

2.2. Flowcytometry. MCF-7 and BT-549 cells were trypsinized and washed with 1X PBS. The tumorspheres formed from MCF-7 and BT-549 were collected and washed with 1X PBS following gravity sedimentation. Trypsinization was done for five to seven minutes with thorough mixing on every 30 seconds. After trypsinization, the single cell suspension was mixed with an equal amount of complete media and cells were collected by centrifuging at 1600 rpm for three minutes. The cells were resuspended in complete RPMI-1640 media and sieved using 40 μm nylon cell strainers (STEMCELL technologies, Vancouver, Canada). $1x10^5$ cells were resuspended in 100 μl staining buffer (phosphate-buffered saline (PBS) with 2% FCS and 0.1% sodium azide) in FACS tube and stained with PD-L1-APC (clone MIH1; eBioscience, San Diego, USA). Data were acquired on BD LSRFortessa flow cytometer using BD FACS Diva software (BD Biosciences, Oxford, UK) and analyzed on FlowJo version 10 software (Tree Star Inc., Oregon, USA).

2.3. Western Blotting. The expression levels of EMT markers were measured using western blotting. Briefly, MCF-7 and BT-549 cells and tumorspheres were lysed on ice using 1X RIPA lysis buffer (ThermoFisher Scientific, Massachusetts, USA) containing protease inhibitor cocktail (Sigma Aldrich) and phosphatase inhibitors. Protein concentrations were measured using the Pierce™ BCA Protein Assay kit (ThermoFisher Scientific) according to the manufacturer's instruction. The absorbance was measured using the FLUOstar Omega microplate reader at 660 nm (BMG LABTECH, Ortenberg, Germany). Equal amounts of proteins were resolved in 10% acrylamide gel and blotted on nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). Membrane blocking was done by 5% skim milk or 5% Bovine serum albumin (BSA) in TBS-T. The membranes were then incubated overnight at 4°C with primary antibodies, β-actin, E-Cadherin, N-Cadherin, Vimentin, Snail, HDAC1, and HAT (p300/CBP). All primary antibodies were prepared at 1:1000 dilution in 5% skim milk or BSA in TBS-T. The membranes were then incubated with HRP-conjugated donkey anti-rabbit or Goat anti-mouse IgG (Invitrogen, California, USA) secondary antibodies (Dilution 1:10000) at room temperature for 2 h. Detection was performed using SuperSignal™ West Pico PLUS Chemiluminescent substrate (ThermoFisher Scientific, Massachusetts, USA) and images acquired using Molecular Imager® ChemiDoc™ XRS+ with Image Lab™ Software (Bio-Rad, California, USA). The densitometric analyses were performed using ImageJ software (National Institute of Health, Maryland, USA).

2.4. Quantitative Real-Time PCR. DNA and RNA were isolated using RNA/DNA/Protein purification plus kit (Norgen Bioteck Corp), as previously described [15]. 1μg RNA from each sample was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). PCR reactions were performed using QuantStudio 7 Flex qPCR (Applied Biosystems, California, USA) using Fast SYBER Green Master Mix (Applied Biosystems). Data were analyzed as previously described [15]. The absolute expression

of DNMTs and TETs in both cell lines and tumorspheres was checked by comparing the relative expression values of all genes normalized to relative expression normalized with β-actin. Primer sequences are provided in Table S1a.

2.5. CpG Methylation Analysis by Bisulfite Sequencing. The genomic DNA was extracted from tumorsphere and cell lines and treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) as previously described [15]. The sequences of M13-reverse primer used for sequencing are provided in Table S1c.

2.6. Chromatin Immunoprecipitation (ChIP) Assay. Cells and tumorspheres were subjected to ChIP analysis using Magna ChIP A/G chromatin immunoprecipitation kit (Merck Millipore, MA, USA) according to the manufacturer's instructions. Briefly, nuclear extracts prepared and sonicated using Covaris S2 system (Covaris, MA, USA) to obtain DNA fragments ranging from 100 to 200 bp. The assay was performed as previously described [15]. Primer sequences are provided in Table S1d.

2.7. Methyl-DNA Immunoprecipitation (MeDIP) Assay. Genomic DNA was prepared from the MCF-7 and BT-549 cells and tumorspheres. The DNA was sonicated using Covaris S2 system (Covaris, MA, USA) to obtain DNA fragments ranging from 200 to 400 bp. The sheared DNA immunoprecipitated using the 5hmC and 5mC mAbs. Isotype-matched control Ab was used to check nonspecific bindings. The immune complexes were precipitated using Dynabeads (Invitrogen). Relative enrichment of PD-L1 promoter region in the precipitated DNA fragments was analyzed by qPCR. Primer sequences are provided in Table S1d.

2.8. Statistical Analyses. The data were analyzed using the Shapiro-Wilk normality test with paired t-test/Wilcoxon matched-pairs signed rank test using GraphPad Prism 6.0 (GraphPad Software Inc., California, USA). The significances were represented as $***P<0.001$, $**P<0.01$, $*P<0.05$, and ns $P>0.05$. The data were represented as mean + standard error of the mean (SEM).

3. Results

3.1. The PD-L1 Expression Is Upregulated in Tumorspheres Enriched with Cancer Stem Cells. It has been reported that tumorsphere culture system provides a useful method for maintaining a CSC microenvironment [18]. In this study, we enriched the cells having stem cell property in MCF-7 and BT-549 cell lines using tumorsphere formation assay. Both MCF-7 and BT-549 cells showed typical tumorsphere formations (Figure 1(a)). We measured differential expression of the epithelial marker, E-Cadherin, and mesenchymal markers including vimentin, N-Cadherin, and snail to check the cancer stem cell properties of tumorspheres. Interestingly, we found that the expression of mesenchymal markers was upregulated, and epithelial marker E-Cadherin was downregulated in both MCF-7 and BT-549 tumorspheres, compared with cell lines (Figure 1(b)). The overexpression of mesenchymal markers shows epithelial to mesenchymal transition (EMT) happening in the tumorspheres. Moreover, we measured the mean fluorescence intensity (MFI) of immune checkpoint ligand, PD-L1, and found that PD-L1 was significantly upregulated in both tumorspheres, compared with cell lines (Figures 1(c) and 1(d)). mRNA analysis on cell lines and tumorspheres confirmed significant overexpression of PD-L1 in tumorspheres, compared with their cell line counterparts (Figure 1(e)). These data suggest that mesenchymal cells overexpress PD-L1, which might play an important role in immune evasion.

3.2. Tumorspheres Showed Distinct DNA Methylation Pattern for PD-L1 Promoter. The epigenetic changes involved in the PD-L1 expression during the EMT process were examined through PD-L1 promoter CpG methylation. CpG methylation plays a pivotal role in cancer initiation and progression [19]. This report prompted us to investigate the impact of DNA methylation on PD-L1 overexpression observed in tumorspheres. We analyzed 24 CpGs from the PD-L1 promoter to detect the CpG methylation landscape. Interestingly, we found that the PD-L1 promoter DNA methylation profile is different between MCF-7 and BT-549 tumorspheres. Tumorspheres from MCF-7 showed significant hypomethylation, compared with their cell line counterpart (Figures 2(a) and 2(c)), but there was no significant difference observed in between BT-549 cell line and tumorspheres (Figures 2(b) and 2(c)). PD-L1 promoter region was completely demethylated in both BT-549 cell line and tumorsphere (Figure 2(b)). These data suggest that overexpression of PD-L1 in tumorspheres is partially dependent on DNA methylation.

3.3. DNMTs Are Downregulated and TET3 Is Upregulated in MCF-7 Tumorspheres. The *de novo* DNMTs, DNMT3a, and DNMT3b are involved in the establishment of DNA methylation, whereas the TET proteins oxidize 5mC to generate 5hmC through active demethylation involving DNA repair machinery [20]. The balance between DNMTs and TETs can influence the gene expression through directly regulating the DNA methylation status [21]. The methylation/demethylation cycle was assessed in the breast cancer cells and tumorspheres through mRNA expression of DNMT3a, 3b, and TET1,2,3. Interestingly we found that, out of all three TETs, TET3 was increased in tumorspheres derived from both cell lines. The MCF-7 derived tumorspheres showed a decrease in DNMT3a and 3b suggests the involvement of DNA methylation-dependent epigenetic regulatory mechanism. Additionally, the increased levels of TET3 showed that a TET3 dependent active demethylation is active in MCF-7 tumorspheres (Figure 2(d)). The tumorspheres from BT-549 showed that both TETs and DNMTs were upregulated compared with the cell line. These data suggest that all cells were not following similar expression level of methylation/demethylation enzymes and promoter demethylation status for the upregulation of PD-L1 (Figure 2(e)). Moreover, the results were confirmed by evaluating

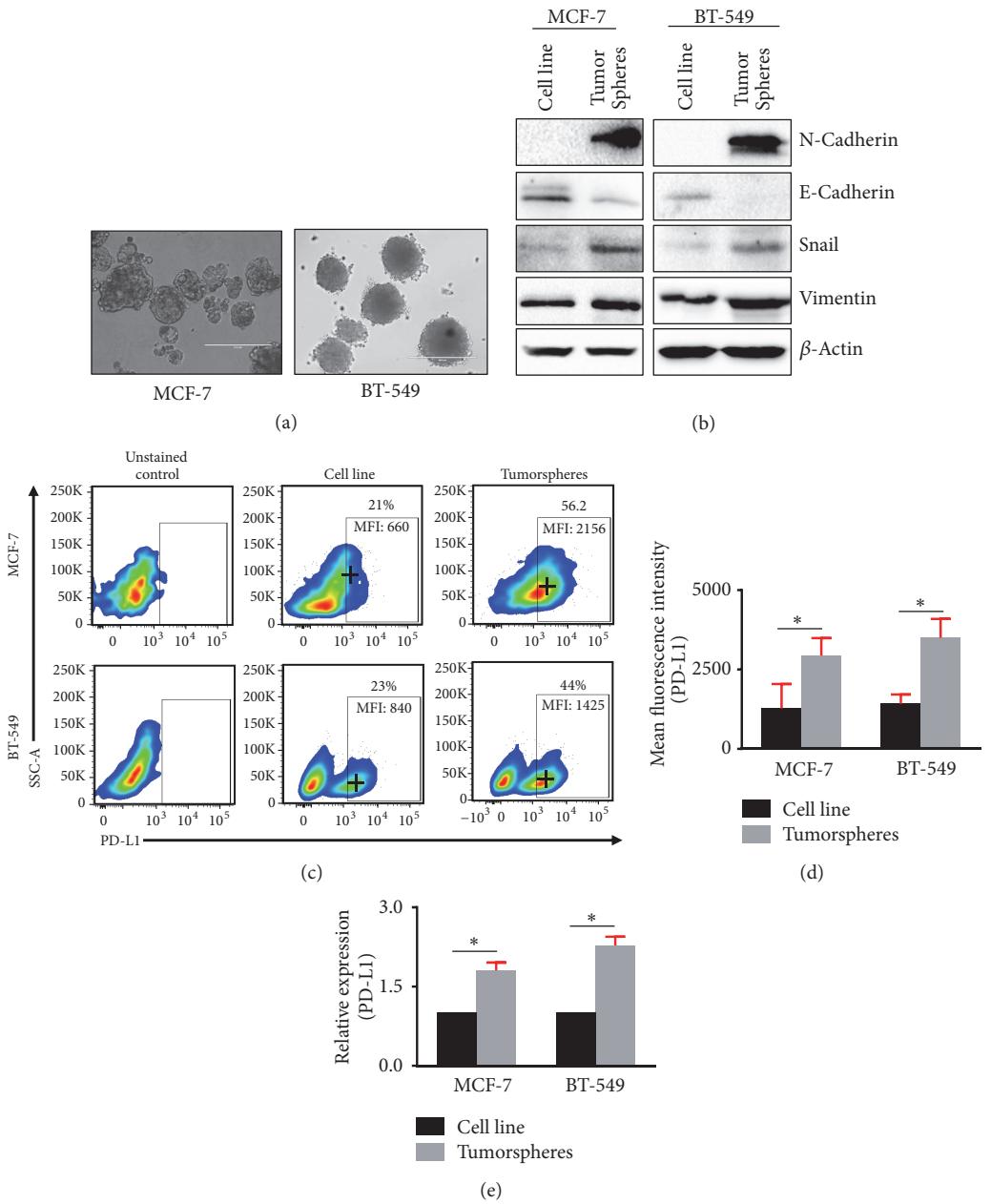

Figure 1: *EMT markers and PD-L1 expression in MCF-7 and BT-549 breast cancer cells and tumorspheres.* MCF-7 and BT-549 cells were cultured in Cancer Stem Premium™ media for 5-10 days. Representative image shows the tumorspheres formed from MCF-7 and BT-549 cell lines (a). Western blots show the expression of stemness markers in MCF-7 and BT-549 cell lines and tumorspheres (b). Representative flow cytometric plots show the expression of PD-L1 in MCF-7 and BT-549 cell lines and tumorspheres (c). Bar plots show the PD-L1 mean fluorescence intensity in MCF-7 and BT-549 cell lines and tumorspheres (d). Bar plots showing the relative expression of PD-L1 in MCF-7 and BT-549 cell lines and tumorspheres (e). All data were normalized to β-actin.

5hmC and 5mC levels in both cell lines and tumorspheres and found that MCF-7 derived tumorspheres enriched with cancer stem cells showed an increased 5hmC and decreased 5mC level, compared with cell line (Figure 2(f)), whereas tumorspheres from BT-549 show a significant decrease in both 5hmC and 5mC level, compared with the cell line. These data strongly recommend that active demethylation machinery is active in MCF-7 tumorspheres for the upregulation of PD-L1 expression but not in BT-549 tumorspheres.

3.4. Repressive Histones Regulate the Expression of PD-L1 in Tumorspheres. The epigenetic regulation of gene expression is not restricted to CpG hypomethylation but also depends on posttranslational modifications of histones. Histone modifications like methylation and acetylation are another epigenetic mechanism, which can regulate the chromatin organization [22]. To detect the role of histones in the regulation of PD-L1 expression, we checked the binding intensity of both repressive and active histone marks, including H3K9me3,

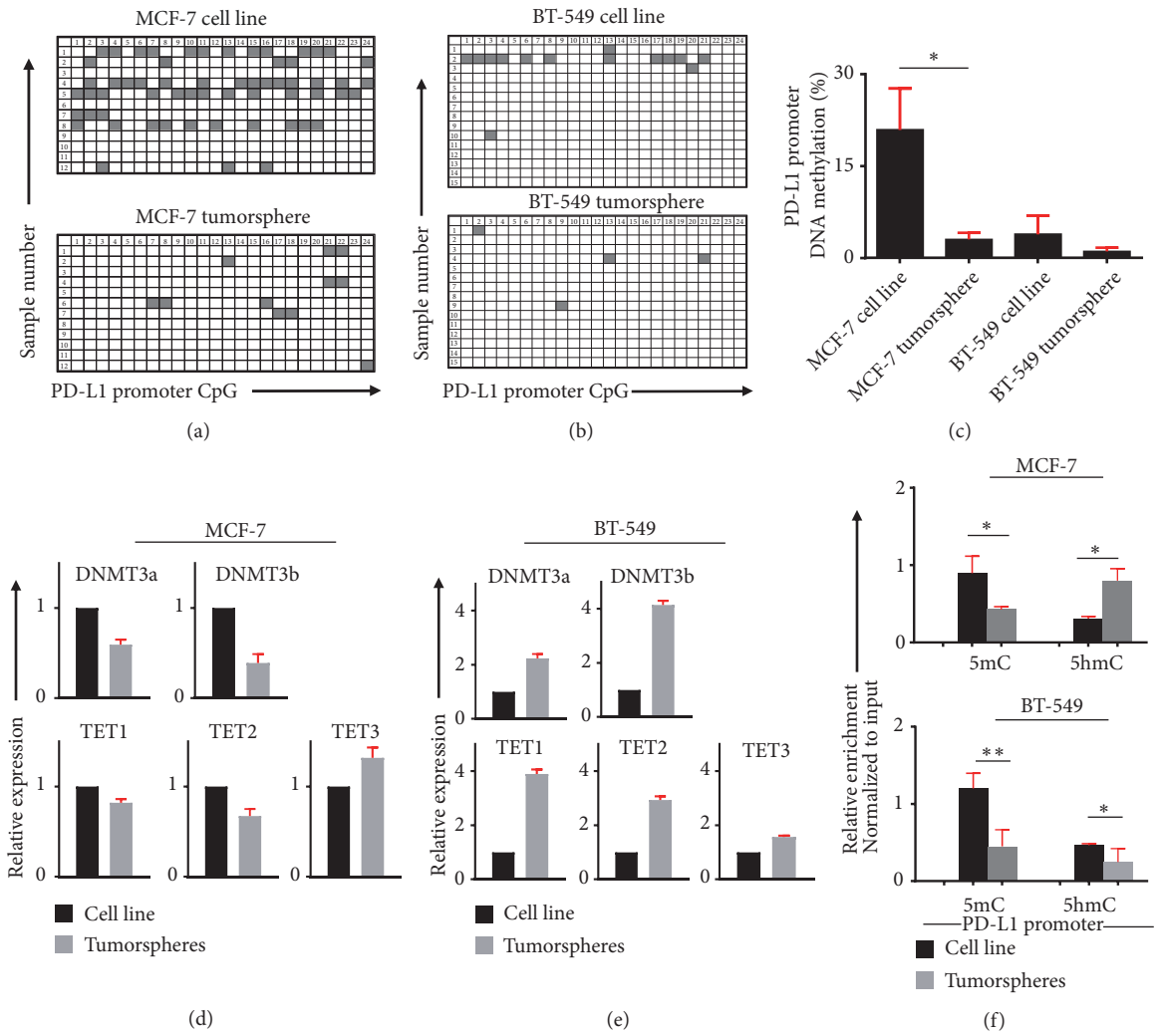

FIGURE 2: *Analysis of CpG methylation status and expression of methylation/demethylation enzymes in MCF-7 and BT-549 cell lines and tumorspheres*. Representative plots of PD-L1 promoter CpG methylation status analyzed by bisulfite sequencing of the genomic DNA isolated from MCF-7 (a) and BT-549 (b) cell lines and tumorspheres. Methylation status of individual CpG motif is shown by white (demethylation) or gray (methylation) colors. Bar plots show the methylation percentage of PD-L1 (c). Bar plots show the relative expression of DNMT3a, DNMT3b, TET1, TET2, and TET3 in MCF-7 (d) and BT-549 (e) cell lines and tumorspheres. All data were normalized to β-actin. Bar plots show the relative enrichment of 5-mC and 5-hmC in the PD-L1 promoter of MCF-7 (upper) and BT-549 (lower) cell lines and tumorspheres (f). Data are representative of two independent experiments.

H3K27me3, and H3K4me3 by keeping H3 as a positive control in the promoter region of PD-L1 in both breast cancer cells and tumorspheres. Despite the discrepancies in PD-L1 promoter CpG methylation and 5hmC pattern in tumorsphere-forming cancer stem cells, the histone modification showed a similar pattern in both cell lines. In both MCF-7 and BT-549 tumorspheres, the repressive histones H3K9me3 and H3K27me3 were significantly bound weakly to PD-L1 promoter, compared with their cell line counterparts (Figures 3(a) and 3(b)), whereas compared with repressive histones, positive regulatory histone H3K4me3 significantly binds more intensively to PD-L1 promoter in both tumorspheres and cell lines (Figure 3). These data suggest that, in tumorsphere-forming cells, the increase in PD-L1 expression is typically modulated through H3K9me3 and H3K27me3. Next, we checked histone acetylation machinery, as it is also an important regulator of chromatin anatomy. It has been reported that there is a dysregulation of HATs and HDACs involved in tumorigenesis [23]. HDAC1 is reported to be active under hypoxic conditions and in stem cells supporting their self-renewal [24]. We also observed a consistent overexpression of HDAC1 in tumorspheres derived from MCF-7 and BT-549 cells (Figures 3(c) and 3(d)). HAT (p300/CBP) is a transcriptional coactivator of histone acetyltransferase enzyme family that are responsible for epigenetic activation of EMT transcription factors, promoting breast cancer aggressiveness [25]. HAT was constitutively increased in the tumorspheres, compared with the cell line counterparts

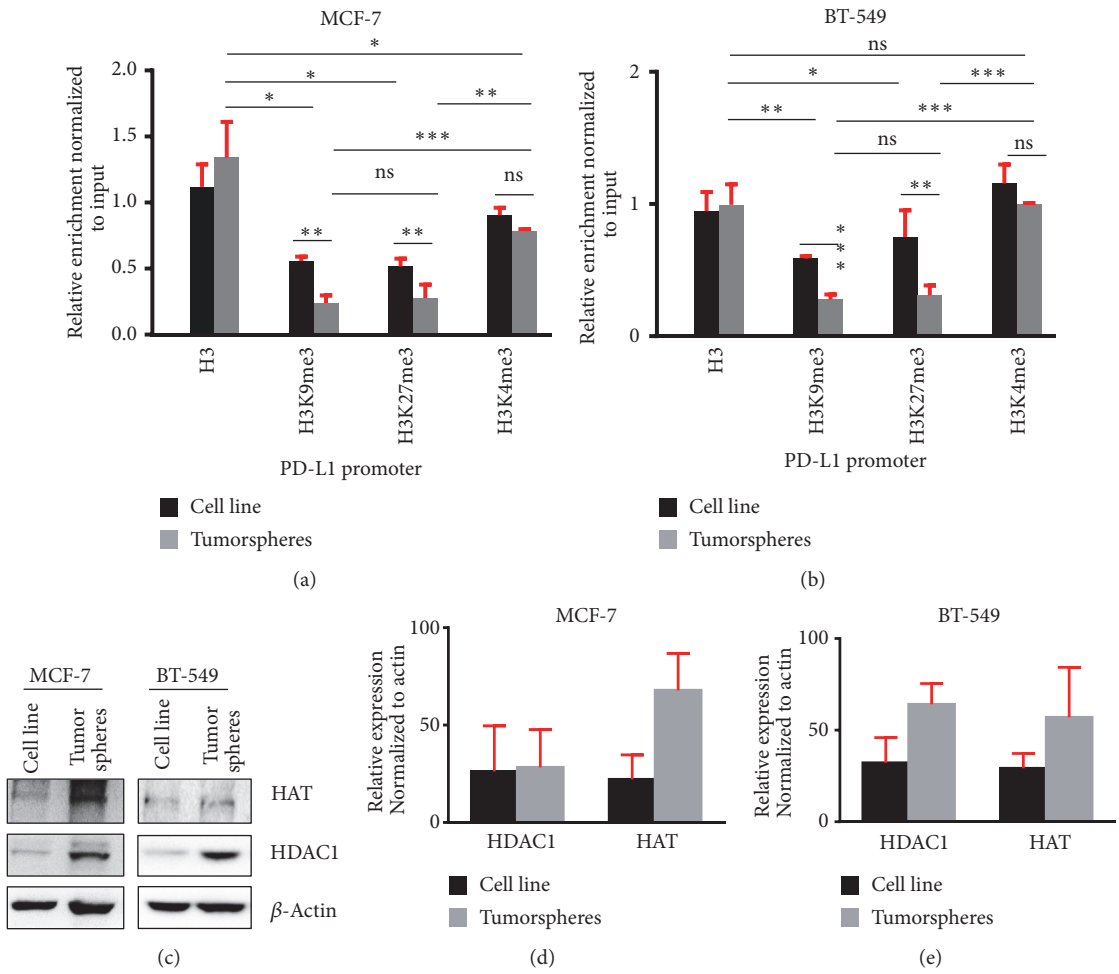

FIGURE 3: *Analysis of H3K9me3, H3K27me3, and H3K4me3 distribution in the PD-L1 promoter of MCF-7 and BT-549 cell lines and tumorspheres.* Chromatin prepared from MCF-7 and BT-549 cell lines and tumorspheres were precipitated using H3K9me3, H3K27me3, and H3K4me3 antibodies and IgG as negative control. qPCR was performed on the precipitated DNA using the PD-L1 primer and data were normalized to input. Bar plots show the H3K9me3, H3K27me3, and H3K4me3 distribution in MCF-7 (a) and BT-549 (b) PD-L1 promoters. Representative Western blots show the expression of HAT and HDAC in MCF-7 and BT-549 cell lines and tumorspheres (c). Bar plots show the HDAC1 and HAT in MCF-7 (d) and BT-549 (e) cell lines and tumorspheres. Data are representative of two independent experiments.

(Figures 3(c) and 3(e)). Altogether, our data suggest that both active histone acetylation and methylation play roles in the upregulation of PD-L1 in breast cancer stem cells.

4. Discussion

Cancer stem cells are the rare population of cells present in most of tumors, and these cells play critical roles in drug resistance, metastasis, recurrence, and immune evasion [26]. It has been reported that epigenetic silencing of antigen peptide transporter 1 (TAP1) gene in breast cancer stem cells promotes immune evasion [27]. Recent studies reported that expression of PD-L1 in the cancer cells is one of the major regulating factors for immune evasion [10, 28]. Also, EMT mediates immune evasion through the upregulation of multiple transcription factors and effector proteins. Moreover, upregulation of PD-L1 expression in CSCs made them resistant to peripheral blood mononuclear cells-mediated cancer cell killing *in vitro* [11]. Detailed mechanistic knowledge about the regulation of PD-L1 expression should help to avoid immune evasion as well as immunotherapy resistance. With this goal, we enriched the cancer stem cells through tumorsphere formation and the epigenetic regulatory mechanisms involved in the PD-L1 expression were investigated. We selected MCF-7 (luminal A subtype) and BT-549 (triple negative breast cancer, TNBC), which are known to maintain high degree of genetic mutations and epigenetic regulatory mechanisms [29]. However, role of tumor microenvironment in these epigenetic regulatory mechanisms and the expression of multiple proteins are the limiting factor of using cell lines.

PD-L1 expressions in cancer cells are regulated through multiple signaling cascades and mechanisms. We have recently reported that dual inhibitions of STAT1 and STAT3 constitutively inhibit PD-L1 expression in human breast cancer cells [14]. In addition to the Jak/STAT pathway, multiple other signaling cascades such as RAS/RAF/MEK/MAPK-ERK [30, 31], PI3K/PTEN/Akt/mTOR [32], EML4-ALK [33,

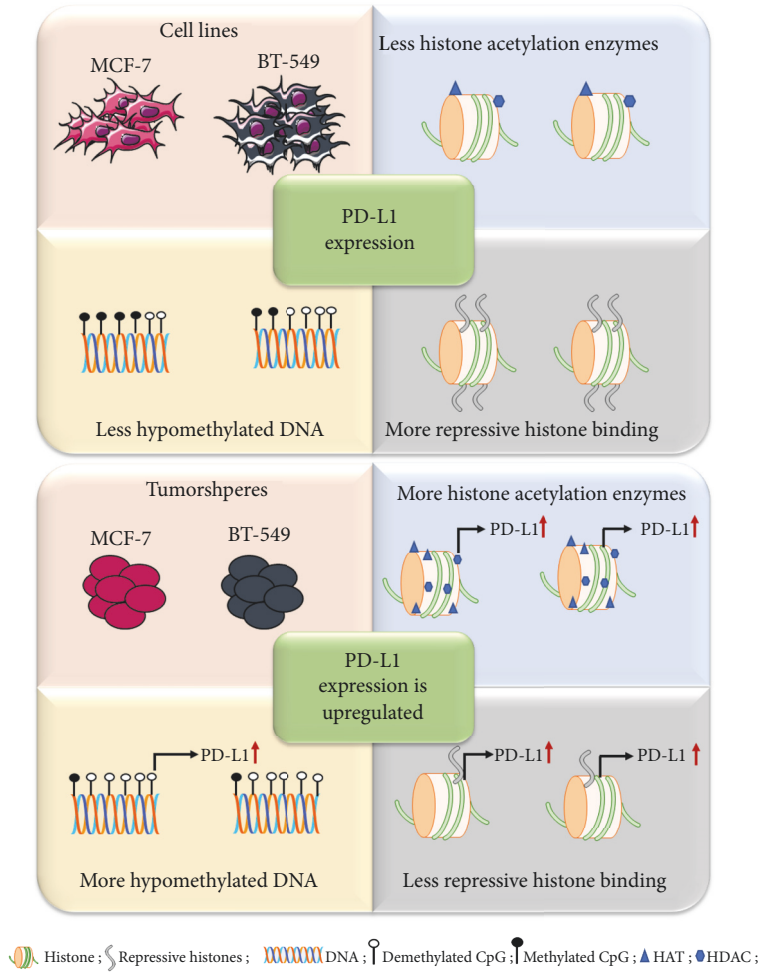

Figure 4: *A schematic diagram summarizing the epigenetic events involved in the regulation of PD-L1 expression in MCF-7 and BT-549 cell lines and tumorspheres.* The PD-L1 promoter CpG is hypomethylated in tumorspheres. Moreover, distribution of repressive histones (H3K27me3 and H3K9me3) in the PD-L1 promoter is decreased along with an upregulation of histone acetylation enzymes in tumorspheres, compared with cell lines.

34], and EGFR signaling pathways [35–37] were shown to have regulatory effects on PD-L1 expression in multiple malignancies [38].

Recently, we have reported that the overexpression of PD-L1 in breast and colon cancer tissues is independent of promoter CpG methylation and repressor histone tri-methylation [15, 16]. The promoter CpG methylation analysis of TNBC cell line BT-549 and the CSC enriched tumorsphere revealed similar methylation profile. This result suggests that there could be an additional epigenetic/transcription factor-mediated regulation for PD-L1 expression. The involvement of multiple regulatory mechanisms in the expression of PD-L1 during the EMT is also reported in nonsmall cell lung carcinoma [39]. In this study, we showed that the CpG methylation patterns in MCF-7 cell line and tumorsphere were different from BT-549. In MCF-7, a significant difference in DNA methylation pattern was detected with more hypomethylation in tumorspheres than the cell line. In addition to the CpG methylation, the expressions of DNMTs and TETs were also different between the two cell lines. Similar results were observed in the 5hmC and 5mC distribution. These indicate that cancer stem cells have different epigenetic regulatory mechanisms depending on the physiological and molecular status of cancer.

The posttranslational methylation of histones at the N-terminal tail has high importance in the protein expression. Histone 3 lysine 9 and 27 tri-methylation (H3K9me3, H3K27me3) leads to the inhibition of gene expressions [40]. ChIP-qPCR analysis on the PD-L1 promoter in both MCF-7 and BT-549 cell lines showed significantly decreased levels of H3K9me3 and H3K27me3 in tumorspheres, compared with the cell lines (Figures 3(a) and 3(b)). Interestingly, the H3K4me3 did not show significant change between cell lines and tumorspheres. This indicates that the upregulation of PD-L1 expression in cancer stem cells could be controlled through histone modifications. In addition to histone methylation, histone acetylation through HAT and HDAC has a major role in the gene expression through modulation of chromatin structure and enabling transcription factor binding, leading to the increased gene expression.

5. Conclusions

In this study, we report that epigenetic modifications including DNA methylation and posttranslational histone modifications (methylation and acetylation) regulated the expression of PD-L1 in breast cancer stem cells. Alterations in expression of methylation and demethylation enzymes were detected in the cell lines and tumorspheres. Moreover, histone modifications such as lysine tri-methylation and acetylation play significant roles in the upregulation of PD-L1 expression in CSC. The overall conclusion is graphically represented in Figure 4. Further studies are needed to validate the impact of DNA copy number variations in epigenetic regulations.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Table S1: Primer sequences used in this study. *(Supplementary Materials)*

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