COMBINATORIAL APPROACHES TO ENHANCE ANTI-TUMOR IMMUNITY: FOCUS ON IMMUNE CHECKPOINT BLOCKADE THERAPY

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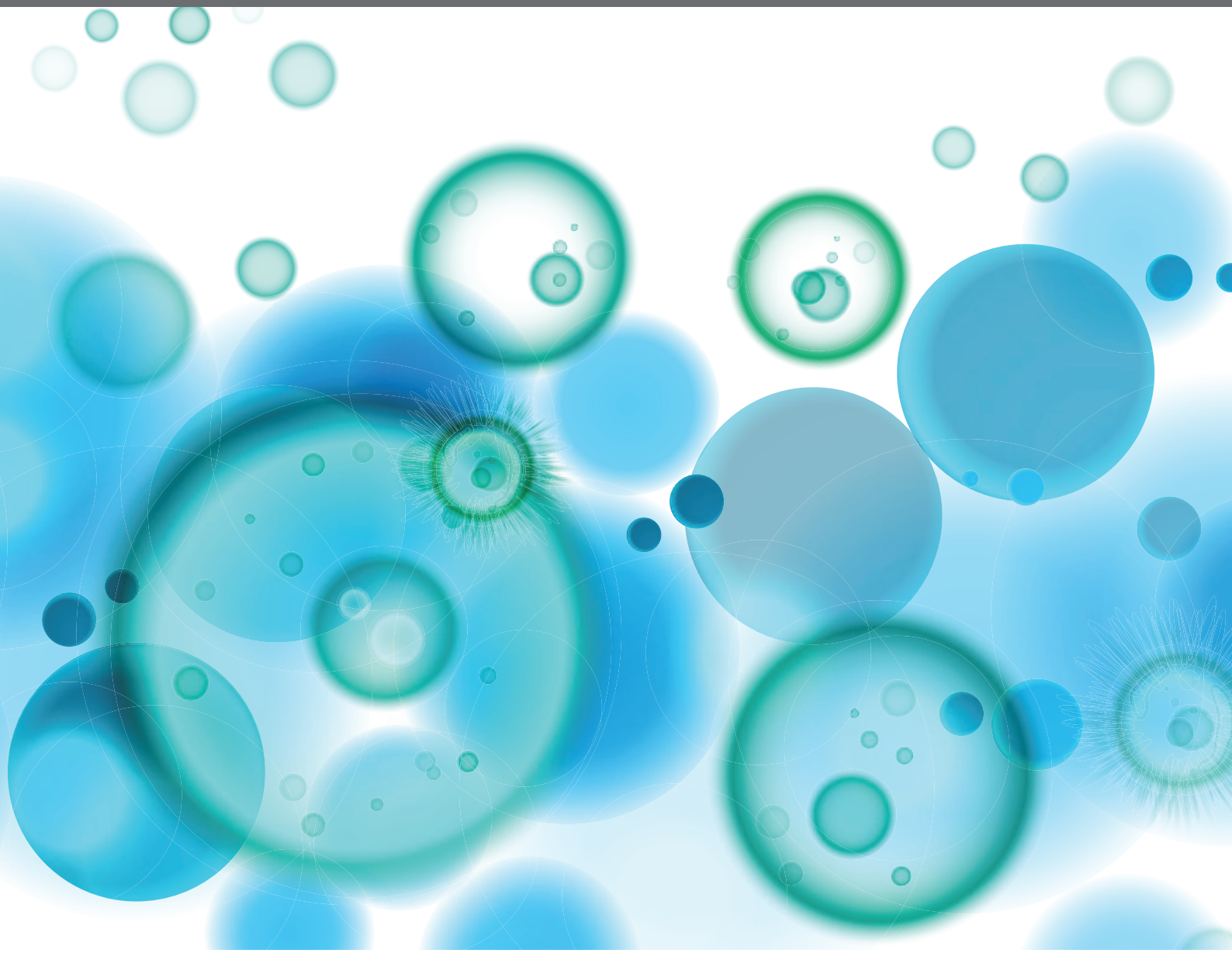

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COMBINATORIAL APPROACHES TO ENHANCE ANTI-TUMOR IMMUNITY: FOCUS ON IMMUNE CHECKPOINT BLOCKADE THERAPY

Topic Editors:
Patrik Andersson, Massachusetts General Hospital and Harvard Medical School, United States; Karolinska Institutet, Sweden
Christian Ostheimer, University of Oxford, United Kingdom; Martin Luther University Halle-Wittenberg, Germany

The immune system harbors great potential for controlling and eliminating tumors. Recent developments in the field of immuno-oncology has led to unprecedented clinical benefits for a broad spectrum of solid tumors. However, immunotherapy (IT) approaches currently have several limitations including (i) low response rate; (ii) development of resistance and (iii) causing severe immune-related adverse effects (IrAEs), which underline the importance of adequate patient selection. Importantly, IT holds promising synergistic potential when combined with standard-of-care chemotherapy, radiotherapy (RT) and anti-angiogenic therapy (AAT) as part of multi-modal oncologic treatment regimes. Published data suggest that there are potential synergy between RT and AAT, which ultimately could help potentiate the response to IT. However, the complex interactions between RT and IT and/or AAT remain poorly understood. Many research questions including optimal timing, scheduling and dosing, as well as patient selection and side effects of combined therapy approaches, remain to be addressed. This Research Topic aims to give a comprehensive overview of the current field with particular emphasis on the future outlook of RT and AAT as complementary approaches to improve IT in solid tumors.

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EDITORIAL
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Editorial: Combinatorial Approaches to Enhance Anti-tumor Immunity: Focus on Immune Checkpoint Blockade Therapy

Patrik Andersson[1,2] *and Christian Ostheimer*[3,4*]

[1] Edwin L. Steele Laboratories, Department of Radiation Oncology, Harvard Medical School, Massachusetts General Hospital, Boston, MA, United States, [2] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden, [3] Medical Science Division, Department of Oncology, Oxford Institute for Radiation Oncology, University of Oxford, Oxford, United Kingdom, [4] Department of Radiation Oncology, Martin Luther University Halle-Wittenberg, Halle, Germany

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Editorial on the Research Topic

Combinatorial Approaches to Enhance Anti-tumor Immunity: Focus on Immune Checkpoint Blockade Therapy

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Edited and reviewed by:
Catherine Sautes-Fridman, INSERM U1138 Centre de Recherche des Cordeliers, France

****Correspondence:***
Christian Ostheimer
christian.ostheimer@uk-halle.de

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INTRODUCTION

The advent of immunotherapy (IT), especially immune checkpoint blockers (ICBs), and its application in oncology has provided new hope for cancer patients. However, despite the rapid progress in the field of immunoncology, only a subset of patients currently benefit from these therapies. Many challenges remain to be resolved in order for IT to display optimal efficacy and good overall response rates in patients. First, many tumors have low tumor mutational burden (TMB), and therefore only produce limited antigens that can be recognized by endogenous T cells (1). Second, reduced antigen release or downregulation of antigen presentation machinery contributes to immune escape, leading to tumors with scarce numbers of infiltrating immune cells, indicating that reinvigoration of the pre-existing pool of anti-tumor T cells by ICBs may not be enough to induce tumor regression (2, 3). And third, even if the number and activity of T cells are successfully boosted by immunostimulatory therapies, the immunosuppressive tumor microenvironment (TME) restricts durable responses and contributes to treatment resistance (4). To overcome these limitations, new strategies are needed. Currently, several approaches exist where IT is combined with standard-of-care therapies, including radiotherapy (RT) and/or anti-angiogenic therapy (AAT) that are being evaluated in both preclinical and clinical settings. The aim of this article collection is to provide a comprehensive overview of recent developments and approaches in enhancing anti-tumor immunity with the focus on potential synergistic effects of RT and/or AAT with IT, ultimately supporting the rationale of combining IT with AAT and RT.

STRATEGY 1: INCREASE ANTIGEN PRODUCTION, RELEASE AND PRESENTATION

Recent findings indicate that high TMB is positively correlated to ICB responses across different types of tumors (5). DNA damaging therapies such as standard-of-care RT can be applied to induce reactive oxygen species (ROS), leading to immunogenic cell death and antigen release (6). Another

strategy involves using RT at sublethal levels to induce mutations to increase antigens, which would aid in immune recognition (7, 8). However, standard dosing of RT could be immunosuppressive by direct effects on lymphocytes and dendritic cells (DCs) (9). In contrast, recent evidence suggests that stereotactic body radiation therapy increases T cell activity and reduces inhibitory stroma in tumors (Menon et al.). It has been demonstrated that tumor-derived exosomes successfully delivered double-stranded DNA and induced IFN-mediated T cell responses more efficiently in irradiated mice (10). Furthermore, through increased recruitment and activity of DCs, owing to RT-induced expression of vascular endothelial cell adhesion protein 1 on the endothelium and CXCL16 in tumor cells (11, 12), RT could also directly promote T cell activation and priming. RT has been shown to stimulate the production of type I interferons (IFNs), leading to increased number of CD8α+ tumor infiltrating DCs and subsequent boost in antigen presentation and T cell priming (13–15). Interestingly, selecting the optimal dose seems to be crucial to determine the anti-tumor response. For example, high-dose RT (20 Gy x 2) prevented beneficial production of type I IFNs by induction of Trex1, which degrades double-stranded DNA released by radiation-induced tumor cell death (16). Nevertheless, it will be important to assess the immune response to RT in individual patients/tumors, which has recently been reviewed elsewhere (17).

Additionally, the TME seems to play an important role in antigen presentation by regulating DC function. Jiang et al. found that high tumor cell-intrinsic expression of FASN (fatty acid synthase) led to increased lipid accumulation in DCs, which reduced their antigen presenting capacity in an ovarian cancer model. As reported, blocking FASN increased T cell infiltration, hence, it would be reasonable to speculate that ICBs may be rendered more effective.

STRATEGY 2: VACCINATION

Instead of strategies aiming to increase the antigenicity of tumors by killing tumor cells or increasing their mutational load, vaccines can be utilized to take advantage of pre-existing alterations in tumors. Vaccines come in different flavors, including whole tumor cell lysates, synthesized proteins or peptides, viral vectors expressing tumor antigens, or DC-based vaccines. Utilizing pulsed mature DC-based vaccines could potentially overcome some of the immunosuppressive cues, which otherwise could reduce vaccine efficacy by limiting DC migration, maturation and antigen presentation (18). Unfortunately, only limited benefits with therapeutic vaccine monotherapy have been observed clinically. Even if the vaccines themselves successfully circumvent antigen presentation and priming of T cells, downstream obstacles of immunosuppression could still remain. Metabolic reprogramming in the TME could play an essential role in immunosuppression. For example, by depleting tryptophan and producing kynurenine, indoleamine 2,3-dioxygenase (IDO) promotes the generation of immunosuppressive regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (19). Experimentally, Moreno et al., show that treatment of HPV+ tumors with immunometabolic adjuvants (such as IDO inhibitors) could induce a therapeutic benefit of an otherwise ineffective HPV-16 vaccine. However, as Eleftheriadis elaborates on in his opinion piece, IDO inhibitors have so far failed clinically and researchers are currently trying to understand why.

These preclinical and clinical lessons collectively suggest that combinatorial approaches could offer great clinical benefits to boost vaccines. Mougel et al. discuss the rationale for combining vaccines with AAT or ICBs to overcome tumor-employed immune escape mechanisms, with a focus on current clinical efforts. In addition, van Gulijk et al. provide an overview specifically on DC-based vaccines and strategies to combine with chemotherapy, RT and ICBs.

STRATEGY 3: INCREASE T CELL INFILTRATION

Tumor tissues typically display limited number and heterogeneous distribution of T cells. Leukocyte infiltration is an active process that can be facilitated or hindered by the endothelium of blood vessels. Blood vessels are critical mediators of inflammation by providing a direct interface with which immune cells interact to gain access to tissues. Upon inflammatory cues, the endothelial cells lining the inner surface of blood vessels will express adhesion molecules and soluble mediators of leukocyte trafficking. In tumors, however, the immature nature of blood vessels can cause endothelial anergy, a state of lymphocyte tolerance characterized by repression of adhesion molecules, leading to failure of leukocyte trafficking (20–22). Klein provides a detailed review specifically on the tumor endothelium, with its implications for combination therapies using RT or IT. The endothelial-immune interface provides an opportunity for intervention, where AAT could be applied to increase the influx of anti-tumor immune cells. Strategies to normalize tumor vessels, with an overview on current preclinical and clinical efforts, and potential synergy with IT are discussed by Georganaki et al.. Furthermore, Amin and Hammers reviewed the clinical data of combining various AAT drugs with IT in advanced renal cell cancer patients, where AAT has shown particular benefits owing to high intrinsic VEGF-VEGFR signaling.

Alternative strategies could be employed to enhance T cell infiltration. For example, by performing gene expression analysis to look for correlations to immune profiles, Roszik et al. identified STAT3 as a promising target in cervical cancer. High STAT3 expression was inversely correlated with CD8+ T cell density, implying STAT3 as a promising target to enhance anti-tumor immunity (Roszik et al.). In fact, several clinical trials are investigating STAT3 inhibition. For example, one phase II trial specifically is testing the potential synergy of STAT3 inhibition with anti-PD1 (programmed cell death 1) in colorectal cancer patients (NCT03647839). Another promising approach is specific tumor cell-targeting by utilizing heat-shock-proteins (HSPs), which are overexpressed in various cancers and associated with

aggressive phenotypes and poor prognosis (23–26). Circulating levels of HSP70 could serve as prognostic markers (27). HSP70 for instance has been shown to successfully predict response after RT in advanced NSCLC (non-small cell lung cancer) and might serve as a therapeutic target to stimulate anti-tumor natural killer (NK) cell responses (28–30). Indeed, Shevtsov et al. observed a robust increase in infiltrating CD8+ T cells following adoptive transfer of *ex vivo* HSP70-activated NK cells in lung and glioma mouse models. Interestingly, survival benefits were further enhanced by the addition of anti-PD1 therapy (Shevtsov et al.). The exact underlying mechanisms for the described phenotype remain to be determined. However, NK cells can trigger cell death by both apoptosis and necrosis (31), which can lead to activation of the cGAS-STING (cyclic GMP-AMP synthase-stimulator of interferon genes) pathway. The subsequent production of type I IFN has been shown to drive infiltration of CD4+ and CD8+ T cells into tumors (32–34). Although highlighted in the context of RT, Goedegebuure et al. provide a schematic overview of the immune impact of cGAS-STING activation (Goedegebuure et al.: Figure 1). Paradoxically, RT-induced STING activation could also increase MDSCs via CCL2 production, thereby dampening CD8+ T cell activity (Darragh et al.: Figure 2). There are multiple ongoing clinical trials looking at RT and ICB therapies in NSCLC and head and neck squamous cell carcinoma patients, as reviewed by Nardone et al., which will provide important information on how to optimally design the treatment modalities with RT and IT. Interestingly, two recent phase II studies in NSCLC patients looking at adjuvant anti-PD1 therapy after RT, with or without other prior local ablative therapies, reported a promising although non-significant doubling in overall response rates (35) and an impressive increase in progression-free survival (36), thereby highlighting the potential of combining RT with IT.

STRATEGY 4: ALLEVIATE IMMUNOSUPPRESSION

Tumors are able to employ various resistance mechanisms to evade immune surveillance. The abnormal vasculature is one of the contributors of an immunosuppressive TME (37). The lack of perivascular coverage in tumor blood vessels and high interstitial fluid pressure in tumor tissues often result in malfunctioning or collapsed blood vessels. This results in tumor tissues experiencing high levels of hypoxia, which is one of the main drivers of immunosuppression (38, 39). While tumor cells can readily adapt to the low levels of oxygen, hypoxia also affects the phenotype of stromal cells and immune cells. For example, Tregs and MDSCs have been shown to gain further immunosuppressive capacity (40, 41), and macrophages polarize toward a tumor-promoting phenotype (TAMs) (42) under hypoxic conditions (Figure 6: Darragh et al.). By normalizing the vasculature using AAT, hypoxia can be reduced, and can thereby alleviate immunosuppression (43, 44). The increase in tissue perfusion and oxygenation will also increase the potential impact of RT by optimizing the generation of ROS. As reviewed by Goedegebuure et al., there is a reciprocal relationship where RT, in turn, can have a positive or negative impact on blood vessels and perfusion, depending on the dose and scheduling.

Although vessel normalization by AAT can indirectly improve the immunosuppressive TME, there are ways to directly target and reprogram the immune cells. Focusing on Tregs, Nagai et al. identified PRMT5 (protein arginine methyltransferase) as an interaction partner of FoxP3, a transcription factor important for Treg function. Pharmacological inhibition of PRMT5 led to reduced immunosuppressive activity in Tregs and inhibition of tumor growth (Nagai et al.). Another strategy to reprogram the immune compartment to an anti-tumor phenotype could be to provide IL-2, which would enhance cells such as CD8+ T cells and NK cells (45, 46). However, IL-2 therapy also stimulates Tregs (47), and has been limited by systemic toxicity (48). Mortara et al. reviewed the current efforts of using antibody-cytokine fusion proteins with IL-2 (so-called immunocytokines), designed to be tumor-targeting to overcome these previous limitations and hinder tumor progression by stimulating anti-tumor immunity. In addition to targeting the components of the adaptive immunity, several ongoing trials are investigating the therapeutic benefits of targeting cells of the innate immunity. Specifically, myeloid cells are strong contributors to immunosuppression, especially in glioma, which is the topic covered by Ding et al.. More generally, Dar et al. provided an overview of strategies to target the innate immunity to overcome resistance to RT, with a focus on the interplay between innate and adaptive immunity (see schematic summary in Dar et al.: Figure 1). Furthermore, Menon et al. focused on the stromal contributions to immune evasion and the immunomodulatory properties of RT as an important part of combinatorial treatment modalities. One debated potential effect of RT is the so-called radiation-induced "memory effect" by which prior RT is reported to enhance subsequent anti-tumor immune responses during, for example, ICB therapy. Retrospective analysis by Chen et al. in NSCLC patients suggested that previous RT improved the response to IL-2 infusion, which they attributed to a radiation-induced "memory effect".

STRATEGY 5: OVERCOME RESISTANCE

As with most therapeutic interventions, intrinsic or acquired resistance is the major obstacle for the success of RT, AAT, and IT. Knowledge of specific tumor-employed resistance mechanisms can offer a strong rational for combinatorial approaches. Darragh et al. discuss several TME-related resistance mechanisms upon RT. For example, RT-induced cell death leads to the release of ATP (adenosine triphosphate), which stimulates DC recruitment and activation (49). However, ATP is catabolized to adenosine by CD39/CD73, which is frequently upregulated on tumor cells and in the TME (50). In contrast to ATP, adenosine is immunosuppressive by limiting DCs and CD8+ T cells, and by simultaneously promoting Tregs and TAMs (Darragh et al.: Figure 4). The review by de Leve et al. highlights the therapeutic potential of targeting CD73/adenosine in cancer to improve RT responses.

To optimally target tumor cells, it has become clear that stem-like (so-called tumor-initiating) cells need to be specifically targeted as they represent a highly resistant population of cells (51, 52). Expression of SDF1 (CXCL12) and its receptor CXCR4 has been linked to stem cell niches where its signaling likely contributes to a stem-like phenotype (53). Hence, RT could greatly benefit from combination strategies with CXCR4-targeting approaches to eliminate resistant clones. The therapeutic potential of such combinations is reviewed by Eckert et al.. Another factor involved in stem cell renewal is TGF-β (transforming growth factor β), which also plays an important role in promoting immunosuppression and fibrosis. Blocking TGF-β by therapeutic antibodies has been shown to slow tumor progression, increase infiltration of T cells and synergize with ICB therapy (54, 55). Rossowska et al. took a different approach in which they modified MC38 tumor cells to secrete exosomes deprived of TGF-β1 (by expressing shRNA) and subsequently using those exosomes as treatment of wildtype MC38 tumors. In doing so, the authors observed a reduction in tumor progression, which was accompanied by increased anti-tumor immunity, thereby highlighting the therapeutic value of targeting TGF-β (Rossowska et al.).

As a concluding remark, antibodies targeting PD1/PD-L1 (programmed cell death ligand 1), with FDA approval in multiple indications have so far shown the most promise in patients. However, resistance is a major hurdle and we are only just beginning to understand the underlying mechanisms. Yao et al. report how anti-PD1 therapy can promote tumor cell proliferation if the tumor cells show intrinsic PD1 expression. In light of such findings, we need to carefully evaluate how to assess PD1/PD-L1 expression before stratifying patients for treatment. Ongoing clinical efforts are indicating that simultaneous targeting of several immune checkpoints, such as CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4), Lag-3 and Tim-3, could offer significant advantages over single ICB therapies. Khair et al. provide an exhaustive overview on this topic.

SUMMARY

One major concern when treating patients with ICBs, such as anti-PD1 and anti-CTLA-4 antibodies, is the high frequency of immune-related adverse events. This, along with lacking a reliable biomarker for patient stratification, underscores the need for multimodal therapy allowing for the use of lower doses and implementation of standard operating procedures to manage these side-effects without compromising efficacy. However, as is evident throughout the contributions in this article collection, several important outstanding questions remain to be fully addressed including optimal dosage, timing, and scheduling for these combinatorial approaches.

AUTHOR CONTRIBUTIONS

Both authors have actively participated in shaping the idea for the article collection, recruiting authors, and acting as editors for several of the contributions. PA and CO wrote the editorial together and made final edits.

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Ovarian Cancer-Intrinsic Fatty Acid Synthase Prevents Anti-tumor Immunity by Disrupting Tumor-Infiltrating Dendritic Cells

Li Jiang[†], Xuhong Fang[†], Hong Wang, Diyou Li and Xipeng Wang**

Department of Gynecology and Obstetrics, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

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Patrik Andersson, Harvard Medical School, United States

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Chao Wang, Brigham and Women's Hospital, United States
Amorette Barber, Longwood University, United States

***Correspondence:**
Li Jiang
jiangli@xinhuamed.com.cn
Xipeng Wang
xipengwang@hotmail.com

[†]*These authors have contributed equally to this work*

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Fatty acid synthase (FASN), the key metabolic enzyme of *de novo* lipogenesis, provides proliferative and metastatic capacity directly to cancer cells have been described. However, the impact of aberrant activation of this lipogenic enzyme on host anti-tumor immune milieu remains unknown. In this study, we depicted that elevated FASN expression presented in ovarian cancer with more advanced clinical phenotype and correlated with the immunosuppressive status, which characterized by the lower number and dysfunction of infiltrating T cells. Notably, in a mouse model, we showed that tumor cell-intrinsic FASN drove ovarian cancer (OvCa) progression by blunting anti-tumor immunity. Dendritic cells (DCs) are required to initiate and sustain T cell-dependent anti-tumor immunity. Here, our data showed that constitutive activation of FASN in ovarian cancer cell lead to abnormal lipid accumulation and subsequent inhibition of tumor-infiltrating DCs (TIDCs) capacity to support anti-tumor T cells. Mechanistically, FASN activation in ovarian cancer cell-induced the resulting increase of lipids present at high concentrations in the tumor microenvironment. Dendritic cells educated by $FASN^{high}$ OvCa ascites are defective in their ability to present antigens and prime T cells. Accordingly, inhibiting FASN by FASN inhibitor can partly restore the immunostimulatory activity of TIDCs and extended tumor control by evoking protective anti-tumor immune responses. Therefore, our data provide a mechanism by which ovarian cancer-intrinsic FASN oncogenic pathway induce the impaired anti-tumor immune response through lipid accumulation in TIDCs and subsequently T-cells exclusion and dysfunction. These results could further indicate that targeting the FASN oncogenic pathway concomitantly enhance anti-tumor immunity, thus offering a unique approach to ovarian cancer immunotherapy.

Keywords: ovarian cancer, FASN, tumor-infiltrating dendritic cells, immune response, immunity

INTRODUCTION

Ovarian cancer (OvCa) is the leading cause of death from gynecologic cancer worldwide (1, 2). Despite advances in surgery and chemotherapy over the past years, marginally progress has been made in improving overall survival in patients with OvCa (3). Therefore, new treatment modalities and paradigms are needed to significantly improve the prognosis of women diagnosed with OvCa.

Distorted cellular metabolism has been shown to support several steps during cancer development and is emerging as a hallmark of cancer (4, 5). Recently, it has become evident that metabolic reprogramming is an important regulator of tumor sustained growth (6). In addition to aberrant glucose metabolism, *de novo* fatty acid synthesis is obviously accelerated in human malignancies. Augmented lipogenesis provides one avenue for fulfilling the demand of cancer unrestrained growth (7–9). The increased lipogenesis is represented by significantly elevated expression and hyperactivity of numerous lipogenic enzymes (7). Fatty acid synthase (FASN) is the main enzyme involved in fatty acids synthesis that catalyzes the NADPH-dependent condensation of acetyl-coenzyme A (CoA) and malonyl-CoA to produce palmitate (9). Recent evidence showed that FASN plays a crucial role in the carcinogenesis process of various cancers including OvCa (10–13). Our previous report and others recent studies have been demonstrated that fatty acid metabolism contributes to ovarian cancer tumorigenesis, which indicated a "lipid addiction" phenotype for ovarian cancers (14–16). In cancer cells, FASN confers tumor growth and survival advantages, which appears to necessarily accompany the natural history of most human cancers. FASN expression in OvCa directly promotes tumorigenesis (14, 17), however, whether it also creates a tumor-permissive immune milieu is unknown.

A growing body of research indicates that ovarian cancer shuts down the immune system which would otherwise act as the first line of defense against the deadly tumor (18–22). Understanding the link between ovarian cancer cell intrinsic events and the immune response may enable personalized immune intervention strategies for OvCa patients. Recently, large-scale analyses show that $CD8^+$ TILs vary by histotype with high-grade ovarian cancers having the highest levels and a strong association with survival (20). It is well established that dendritic cells (DCs) are required to initiate and sustain T cell-dependent anti-cancer immunity. Newly, DC vaccines pulsed with autologous whole-tumor antigen has appeared as an important strategy for the mobilization of broad antitumor immunity and neoepitope-specific T cells (23). Ovarian cancer subverts the normal activity of infiltrating dendritic cells to inhibit the function of otherwise protective anti-tumor T cells (19). Re-programming or eliminating TIDCs abrogate OvCa progression (24). Several studies have also reported that metabolic reprogramming is an important regulator of the differentiation and function of dendritic cells (25). It is established that the function of dendritic cells in the tumor microenvironment is mediated by various tumor-derived factors. However, the detailed mechanism by which these factors affect DCs remains unclear. Recent several reports have revealed the importance of lipids in the function of immunosuppressive myeloid cells including dendritic cells in cancer and chronic inflammatory conditions (26–28). These data indicated that lipids could be a crucial factor in regulating the function of DCs. However, their source and the exact role of lipids in DCs of ovarian cancer activity remain unclear.

To specifically assess the effect of ovarian cell-intrinsic FASN activity in regulating the immune response, we first explore the link between ovarian cancer-intrinsic FASN expression and the accumulation of lipids in the tumor microenvironment of ovarian cancer. Moreover, we characterized the phenotype of lipid-laid DCs, and further investigated the mechanisms by which the tumor microenvironment would induce the uptake of exogenous lipids and enhance the metabolic reprogramming and dysfunctional activity of TIDCs. The results showed that upregulation of lipid accumulation in TIDCs characterized by defective profiling with impaired priming of anti-tumor T cells, which results from an increased uptake of lipids found at high concentrations in the tumor microenvironment with high FASN expression. Lipid accumulation in DCs results in inactivation of T cells, controlling a critical switch between immune stimulation and suppression. By contrast, selective inactivation of FASN partly rescues the dysfunction of dendritic cells induced by lipid accumulation.

MATERIALS AND METHODS

Animal Model

Female C57BL/6 mice (6–8 weeks old) and athymic C57 nude mice (6–8 weeks old) were purchased from Shanghai Laboratory Animal Center of China (Shanghai, China). All mice were maintained in a pathogen-free animal facility for at least 1 week before each experiment. For the ID8 model, 2×10^6 cells were used subcutaneously in C57BL/6 mice or nude mice. Cerulenin was obtained from Sigma (USA). *In vivo* experiments, treatment with cerulenin at 30 mg/kg was given i.p. at days 1, 4, and 7 after tumor inoculation in the cerulenin group. For the later treatment group, cerulenin was given i.p. at days 7, 10, and 13 at 30 mg/kg after tumor inoculation. For a collection of ascites, parental or different FASN expressed ID8 intraperitoneal ovarian tumors were generated as previously described (18). Briefly, 2×10^6 tumor cells were injected into wild-type C57BL/6 mice. Implanted animals progressively developed multiple peritoneal masses and eventually massive ascites in 1.5–2 months. Mice were weighed weekly to monitor malignant ascites accumulation and animals with severe abdominal distension were humanely euthanized. All animal experiments were undertaken with review and approval from the Animal Ethical and Experimental Committee of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine.

Clinical Samples and Database

Fresh ovarian tumor tissues, ascites, and autologous peripheral blood were obtained from 50 patients with ovarian cancer who underwent surgical resection at Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, China. Ascites from stage III or IV epithelial ovarian cancer patients was obtained either during debulking surgery or via ascites drainage. None of these patients had received chemotherapy or radiotherapy before surgery. All patients were diagnosed by pathological analyses based on the International Union against Cancer (UICC)-defined

Abbreviations: OvCa, ovarian cancer; TIDCs, tumor-infiltrating dendritic cells; DCs, dendritic cells; FASN, fatty acid synthase; TME, tumor microenvironment; TIMER, tumor immune estimation resource; FMOs, fluorescence minus one controls; MDSC, myeloid-derived suppressor cell; TCM, tumor-conditioned medium; TES, tumor explant supernatant; Tregs, regulatory T cells.

TNM criteria. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board and Ethics Committee of Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, China. In addition, we performed a bioinformatics analysis on the basis of microarray data retrieved from Oncomine online databases (29, 30) using a primary filter for ovarian cancer, sample filter to use clinical specimens and dataset filters to use mRNA datasets with more than 368 patients. Patients of all ages, gender, disease stages or treatments were included. We also used tumor immune estimation resource (TIMER) (31) to comprehensively investigate the molecular characterization of tumor-immune interactions in ovarian cancer.

Cell Culture

The mouse ovarian surface epithelial cell line ID8 was obtained from Millipore Sigma (SCC145, MA, USA) and cultured in DMEM:F12 (Gibco, Life Technologies, USA) containing 10% FBS (Gibco, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. All cultured cells were tested and found to be negative for mycoplasma contamination.

Immunohistochemistry

Standard immunohistochemically staining on human samples were performed using biopsies from ovarian cancer patients by the VECTASTAIN Elite ABC system (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. Anti-FASN polyclonal antibody (Abcam, USA), CD8-specific monoclonal antibody (CD8, Dako) was used as primary antibody. the number of CD8-positive T cells within samples (2.5 mm diameter) was counted using ImageJ cell counter and calculated as a number of $CD8^+$ T cells per mm^2. Samples with fewer than 50 $CD8^+$ T cells per mm^2 were considered T-cell-infiltrate low, whereas counts >50 per mm^2 were considered as T-cell high. All slides were scored by two observers blinded to the pathology and the clinical features.

The Generation of BMDCs and TIDCs

Murine bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6 mice bone marrow and cultured for 7 days with 20 ng/ml GM-CSF and IL-4 (R&D Systems, USA). Media was changed every 3 days. Mouse ID8 cells (5×10^5) were injected into the abdominal cavities of C57BL/6 mice in 100 μl of DMEM and allowed to grow 10–14 days. Tumor tissue was collected for tumor-infiltrated dendritic cells (TIDCs) separation. In some experiments, enrichment of dendritic cells from the peritoneal cavity of controls or tumor-bearing mice were incubated with 10 μl magnetic microbeads conjugated to an antibody against CD11c (Miltenyi) per 10^7 cells for 15 min at 4°C. Cells with magnetic beads were then removed from the cells suspension.

Tumor Supernatant Preparation and Collection

Tumor explants were prepared from freshly isolated subcutaneous tumors or peritoneal transplant tumor. ID8 tumor explants were removed after euthanizing the mice. Tumors were digested in 1 mg/ml collagenase Type IV (Roche), 0.25% DNase Type I (Roche) and 1% hyaluronidase at 37°C for 1 h. Tumor samples were then pressed through a 70 μm nylon filter (BD Biosciences) to create a single cell suspension. Cells were cultured in RPMI 1640 with 10% FBS and 1% penicillin plus streptomycin overnight. The cell-free supernatant was collected to prepare tumor explant supernatant (TES). ID8 cells were grown in a DMEM-complete medium. After 1 day, the medium was recovered and filtered through a sterile 0.22 μm syringe filter to prepare tumor-conditioned medium (TCM).

Lipid Staining

The sorted TIDCs or *in vitro* treated dendritic cells were cytospinned onto glass slides at 1,000 rpm, for 5 min. Slides were dried for 10 min, and then immediately were fixed with 4% paraformaldehyde (PFA). For lipid staining, the slides were stained with BODIPY 493/503 (ThermoFisher Scientific) at 0.5 μg/ml for 15 min at RT. Finally, slides were washed with PBS before nuclear staining with DAPI (Life Technologies) for 2 min, washed in PBS, dried, and then imaged with immunofluorescence microscopy.

Lipid Profiling Assay

For lipidomic analysis, supernatants from parental, $FASN^{low}$ (shFASN), or $FASN^{high}$ (shCtrl) ID8 malignant ascites or normal peritoneal fluid was harvested, and fatty acids and triacylglycerols were extracted and quantitatively analyzed via LC-MS at the Lipidomics Core Facility of Shanghai Jiao Tong University.

Isolation of Tumor-Infiltrating Cells

Mouse tumor samples were minced with scissors before incubation with 1 mg/ml collagenase (Roche) and 50 U/ml DNase I (Roche) in RPMI for 30 min at 37°C with agitation. Tumor samples were homogenized by repeated pipetting and filtered through a 70 μm nylon filter (BD Biosciences) in RPMI supplemented with 10% FCS to generate single-cell suspensions. Cell suspensions were washed once with complete RPMI and purified on a Ficoll gradient to eliminate dead cells. Cells from mouse spleens were isolated by grinding spleens through 40-μm filters. After red blood cell (RBC) lysis, all samples were washed and re-suspended in FACS buffer (PBS/0.5% BSA) or RPMI depending on further use.

Flow Cytometry Assay

Immune cells isolated from mouse tumors, spleens, ascites or *in vitro* cultured condition were pre-incubated with the anti-CD16/32 monoclonal antibody (Fc block, BD Biosciences) to block non-specific binding and then stained with appropriate dilutions of various combinations of fluorescently labeled antibodies for 30 min on ice. All experiments with BODIPY 493/503 staining were performed as previously described (32). For all fluorescence channels, positive, and negative cells were gated on the basis of Fluorescence minus One controls (FMOs). All flow experiments were performed on FACS Canto II machines (BD). All antibodies used for flow cytometry are listed

(**Supplementary Table S1**). All analysis was performed using FlowJo version 10 (FlowJo LLC).

Assessment of Dendritic Cell Antigen Uptake by Flow Cytometry

The endocytic activity of *in-vitro* generated BMDCs or TIDCs was assessed measuring the uptake of the fluorescent reporters DQ-OVA (ThermoFisher Scientific, USA) as previously described (33). DQ-OVA is a mannose receptor ligand consisting of naturally mannosylated OVA extensively labeled with the fluorochrome. Fluorescent detection will occur if DQ-OVA degrades by DCs. Briefly, 2×10^5 DC cells/ml were suspended in 100 μl complete conditioned medium and incubated with 25 μg/ml DQ-OVA at 37°C or at 0°C for 15 min. The incubations were stopped by adding 2 ml cold FACS buffer. The cells were washed twice with FACS buffer, and their fluorescence was analyzed using flow cytometry.

T Cells Stimulation

Mouse tumor single-cell suspensions were generated as described in the previous section. Cells were stained with anti-CD45-Alexa-Fluor-780 and anti-CD3-APC for flow cytometry sorting for $CD3^+$ T cells ($CD45^+CD3^+$) on FACSAria II Cell Sorter (BD). Dead cells were excluded using DAPI. The purity of flow-sorted populations was above 95%. 2.5×10^5 sorted $CD3^+$ T cells from the spleen of C57BL/6 mouse were stimulated on plates coated with 2 μg/ml anti-CD3 antibody (Biolegend) and soluble 1 μg/ml anti-CD28 antibody (Biolegend) in the T-cell medium for 8 h. Following indicated incubation, cells were harvested and suspended in TriZol Reagent (Invitrogen) for subsequent RNA isolation, and supernatants were stored at −80°C immediately and used for cytokine assay. For sorting $CD8^+$ naïve T cells, spleen cells of B6 mouse were stained with anti-CD45A-Alexa-Fluor-780, anti-CD3-APC, anti-CD8-PE antibodies for flow cytometry and $CD45A^+CD3^+CD8^+$ cell were sorted on FACSAria II Cell Sorter (BD). Dead cells were excluded using DAPI. The purity of flow-sorted populations was above 95%.

T Cells Suppression Assay

$CD8^+$ T cells were purified as described in the previous section and then labeled with 5 μM CFSE (Biolegend) in PBS for 6 min at 37°C. The CFSE-labeled $CD8^+$ T cells were then plated in complete RPMI media onto round bottom 96-well plates (2.5×10^4 cells per well) coated with 2 μg/ml anti-CD3 and 1 μg/ml anti-CD28 antibodies (Biolegend). Purified dendritic cells were added in indicated ratios (1:10) and plates were incubated at 37°C. The $CD8^+$ T cell proliferation is determined by Cell Trace Violet dye dilution measured by flow cytometry (FACS Canto II, BD) after 72 h.

Cytokine Quantification

Mouse IL-2 (R&D) and mouse IFN-r (R&D) were quantified from T cell culture supernatants using enzyme-linked immunosorbent assays (ELISA) following manufacturer's protocols (R&D Systems, USA). All ELISAs were done using 96-well high-binding Microlon 600 ELISA plates with a lower limit of quantification of 16.5 pg/mL (Greiner Bio-One, NC, USA) and plates were read using a Synergy HTTR microplate reader (Bio-Tek, USA).

Gene Expression

Fluorescent-activated cell sorting (FACS) was conducted on a FACS Aria-II (BD Biosciences), with 100,000 sorted cells flash frozen in liquid nitrogen as a cell pellet. For real-time PCR analysis RNA was prepared using RNA Isolation Kit (Invitrogen, USA). The quality and concentration of RNA were assessed with Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA). RNA reverse transcription used Prime-Script RT master mix (Takara, Japan). The RT-PCR analysis of indicated genes was performed using 7900 HT Real-Time PCR with SYBR Premix Ex Taq (Takara, Japan) in triplicate.

Statistical Analyses

Statistical analyses were performed with a Mann–Whitney test when comparing the means of two independent groups and two-way ANOVA when comparing more than two groups. $P < 0.05$ was considered statistically significant ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). All statistical analyses were done with GraphPad Prism 7.0 software.

RESULTS

FASN Was Upregulated in Human Ovarian Cancer and Negatively Correlated With Anti-tumor T Cell Infiltration

Oncomine data-mining analysis revealed that upregulation of FASN was presented in 9 of 20 cancer types, especially in ovarian cancer, bladder cancer, colorectal cancer, and prostate cancer (**Supplementary Figures S1A,B**). Consistent with our previous study (14), FASN was one of the top increased genes in human ovarian cancer (**Figure 1A**). Using Meyniel's dataset, we showed that the mRNA level of FASN was significantly increased in higher grade patients with ovarian cancer (**Figure 1B**). Similarly, the bioinformatics analysis from 308 OvCa patients showed that FASN mRNA levels were significantly upregulated in the patients with metastasis compared to non-metastatic patients (**Figure 1C**).

To explore the effect of FASN upregulation on tumor microenvironment (TME), we analyzed the expression of FASN in human ovarian cancer tissues by immunohistochemistry (IHC). We observed an inverse association between FASN expression in tumor cells and the infiltration of $CD8^+$ cytotoxic T cells (CTLs) (**Figure 1D**). We furtherly utilized the resource of Tumor-Immune Estimation Resource (TIMER) (31) to comprehensively investigate molecular characterization of tumor-immune interactions. TIMER analysis clearly showed a significant negative correlation between FASN gene and $CD8^+$ T cells in OvCa (**Figure 1E**). Immune signature genes have also been used to characterize immune infiltrates (34). We measured effector cell cytolytic activity using the transcript

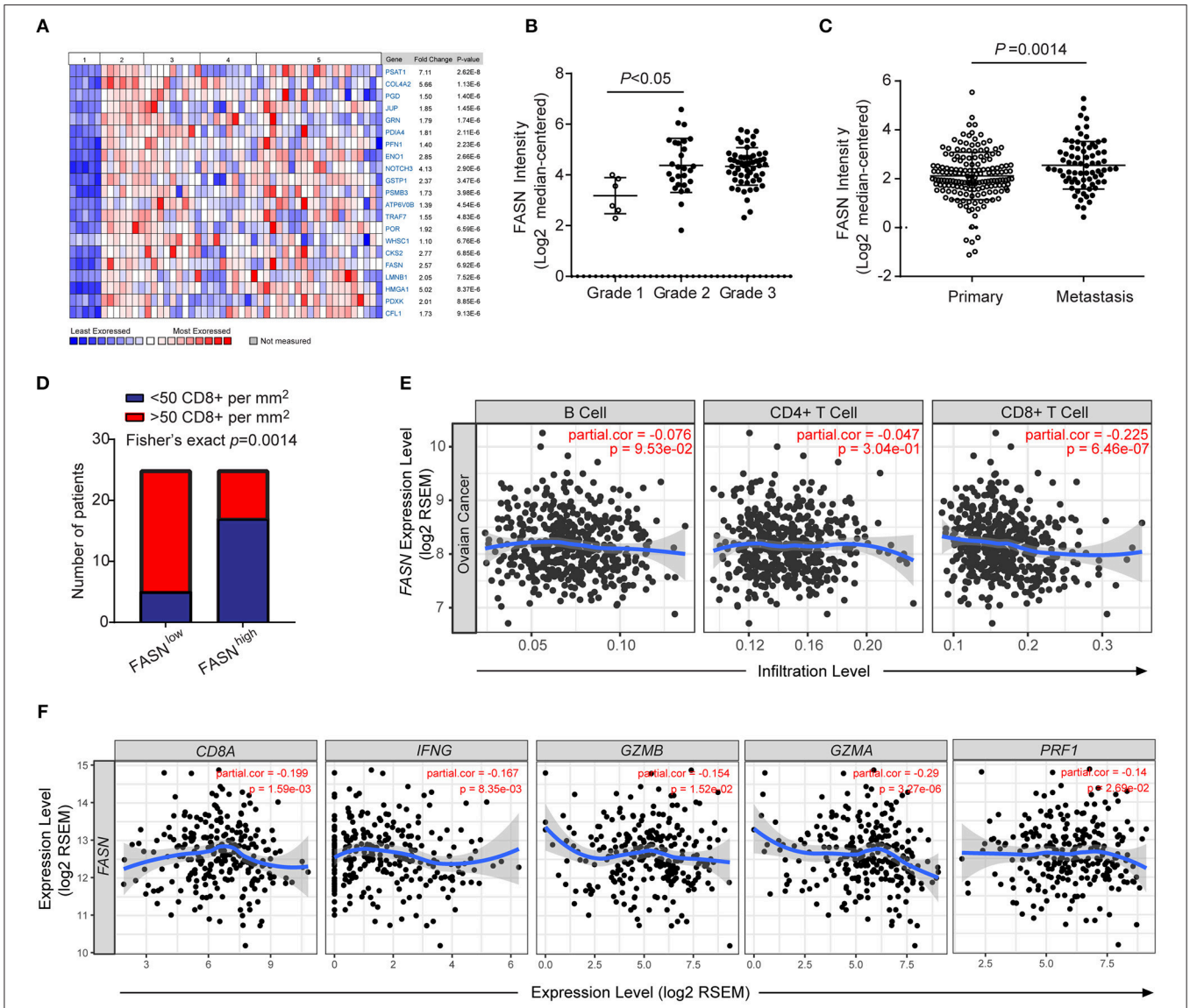

FIGURE 1 | FASN was upregulated in human ovarian cancer and negatively correlated anti-tumor functional T cell infiltration. **(A)** Heatmap depicted top genes list increased in different types of human ovarian cancer compared to the normal ovarian surface epithelium. 1. Ovarian Surface Epithelium ($n = 5$); 2. Ovarian Clear Cell Adenocarcinoma ($n = 7$); 3. Ovarian Endometrioid Adenocarcinoma ($n = 9$); 4. Ovarian Mucinous Adenocarcinoma ($n = 9$); 5. Ovarian Serous Adenocarcinoma ($n = 20$). **(B)** FASN expression was upregulated in a higher grade of human ovarian cancer. (Grade 1, $n = 6$; grade 2, $n = 27$; grade 3, $n = 58$). **(C)** The expression of FASN was increased in OvCa patients with metastasis (Primary patients, $n = 166$, metastatic patients, $n = 66$). **(D)** Correlation between the expression intensity of FASN and distribution of $CD8^+$ T cells in human ovarian cancer biopsies. Fisher's exact test with $n = 50$ (FASN low, $n = 25$; FASN high, $n = 25$). **(E)** The correlation of FASN mRNA expression with tumor-infiltrating B cells, $CD4^+$ T cells and $CD8^+$ T cells in human ovarian cancer based on TIMER analysis from TCGA datasets. The scatterplots showed the purity-corrected partial Spearman's correlation and statistical significance. **(F)** The correlation of FASN mRNA expression with gene signature of $CD8^+$ T cells (*CD8A, IFNG, GZMB, GZMA,* and *PRF1*) in human ovarian cancer based on TIMER analysis. The scatterplots showed the purity-corrected partial Spearman's correlation and statistical significance.

levels of five genes (CD8A, IFNG, GZMB, GZMA, and PRF1) to elucidate the association of FASN expression and immune evasion. Additional analysis revealed a significant negative correlation between FASN gene and CD8A transcripts, as well as related cytotoxic genes which were functional transcripts of the $CD8^+$ cytotoxic cells (**Figure 1F**). Bioinformatics analysis also revealed a negative correlation between FASN gene and dendritic cells transcripts (**Supplementary Figure S1C**). A similar observation was made in human prostate and bladder cancer which presented higher FASN expression (**Supplementary Figure S1D**), confirming its wide application. In sum, these data indicated an inverse correlation between ovarian cancer-intrinsic FASN level and tumor infiltration by $CD8^+$ CTLs in human ovarian cancer.

OvCa-Bearing Mice With Elevated FASN Expression Showed T Cell Exclusion and Defective Tumor-Infiltrating DCs

To investigate directly whether FASN signaling within tumor cells could adversely affect anti-tumor T-cell responses, we use an immunocompetent syngeneic mouse model transplanted mouse ovarian cancer cell ID8 with or without knockdown FASN (**Supplementary Figure S2A**). As expected, $FASN^{high}$ ovarian cancer grows faster and was more aggressive than $FASN^{low}$ ovarian cancer in the subcutaneous model (**Figure 2A**) or in the peritoneal model (**Supplementary Figure S2B**). Analysis of immune infiltrates revealed that $FASN^{high}$ ovarian cancer contained low $CD3^+$ T cells infiltration (**Figure 2B**). Additionally, the proportion of exhausted cells ($Eomes^+$T-bet^-) in $CD8^+$ populations was higher in $FASN^{high}$ tumors (**Figure 2C**), suggesting T-cell dysfunction in the tumor context. In contrast, the proportion of effector memory cells ($CD44^+CD62L^-$) in $CD8^+$ populations was lower in $FASN^{high}$ tumor than in $FASN^{low}$ tumor (**Figure 2D**). Tumor-infiltrating $CD4^+$ T cells and $CD8^+$ T cells displayed the similar phenotype in $FASN^{high}$ OvCa (**Supplementary Figures S2C,D**). However, $FoxP3^+$ regulatory T cells (Tregs) were detected with no difference (data not shown). Consistent with this phenotype, $CD3^+$ T cells sorted from $FASN^{high}$ tumors showed defective interleukin (IL)-2 and interferon (IFN)-γ production (**Figure 2E**, **Supplementary Figure S2E**). Our data suggested that tumor-intrinsic FASN prevents the early step of T-cell priming against tumor-associated antigens. Recent studies have reported that DCs commonly infiltrate ovarian tumors and promote malignant progression by preventing activation and expansion of tumor-reactive T cells (18, 19). Then, we sought to determine whether FASN might drive tumor growth by inhibiting DCs-dependent anti-tumor immunity. We didn't find the significant difference in the quantification of TIDCs or ascites-infiltrating DCs between $FASN^{low}$ and $FASN^{high}$ OvCa (**Figures 2F,G**). In consistent with the mouse model, based on TIMER analysis, human TCGA dataset also presented no significant difference between the distributions of DCs and each copy number status of FASN in ovarian cancer (**Supplementary Figure S3**). However, DCs expressing low levels of co-stimulatory molecules were present in tumor-draining LNs (DLNs) of mice with $FASN^{high}$ tumors (**Figures 2H,I**). Remarkably, we also found that sorted MHC-II^+CD11c^+ DCs from $FASN^{high}$ OvCa DLNs elicited comparable dysfunctional activity (**Figure 2J**). In contrast, DCs derived from the DLNs of $FASN^{low}$ OvCa did not impair the expansion of $CD8^+$ T cells (**Figure 2J**). Hence, these data indicate that DCs in the microenvironment of $FASN^{high}$ OvCa exhibit marked downregulation of costimulatory markers and dysfunctional activity.

Lipid Accumulation in Tumor-Infiltrating DCs Derived From the $FASN^{high}$ Ovarian Cancer Accounted for Its Defective Function

Recent evidence has reported that upon infiltrating the tumor microenvironment, myeloid cells including DCs increase the uptake of fatty acids that eventually leads to the upregulation of their immunosuppressive function (26, 27, 35). In an attempt to understand the mechanism of dysfunction by TIDCs in $FASN^{high}$ OvCa, we evaluated the distribution of lipid in TIDCs. After gating the TIDCs (**Supplementary Figure S4**), the lipophilic fluorescent dye BODIPY 493/503 was used to detect the lipid amount in DCs. Immunofluorescence microscopy of TIDCs from ID8 dissociated tumors 2 weeks after engraftment into mice revealed more BODIPY 493/503 in TIDCs than their control counterparts (**Figures 3A,B**). Flow cytometry analysis revealed a significantly increased lipid content in TIDCs from $FASN^{high}$ OvCa (**Figures 3C,D**), confirming the lipid accumulation in TIDCs from $FASN^{high}$ OvCa. We also measured the lipid content in $FASN^{high}$ OvCa as well as several DCs-containing compartments (spleen, lymph node, and peritoneal cavity). In tumor-bearing OvCa mice, DCs isolated from $FASN^{high}$ OvCa displayed higher levels of lipids than DCs from $FASN^{low}$ OvCa (**Figure 3E**). Given these observations in mice, we wondered whether human TIDCs derived from $FASN^{high}$ OvCa similarly have higher lipid accumulation. Indeed, we found that consistent with what was observed in mice, TIDCs have elevated lipid content in OvCa patients with higher FASN expression (**Figures 3F,G**).

The FASN-High Ovarian Tumor Microenvironment Is Rich in Lipids

Due to the high lipids in TIDCs from $FASN^{high}$ OvCa, we wondered if there was an increase of lipids in the tumor microenvironment of $FASN^{high}$ OvCa. The lipidomic analysis was analyzed in ascites of ID8 bearing mice collected 3 weeks after tumor implantation and normal peritoneal fluid from control mice. Analysis of lipid content by ESI-MS showed significantly higher concentrations of unsaturated fatty acids, saturated fatty acids (**Figures 4A,B**) and triacylglycerols (**Figure 4C**) in ascites of ID8 ovarian cancer-bearing mice. As expected, compared to $FASN^{low}$ OvCa bearing mice, there were significantly higher concentrations of unsaturated fatty acids, saturated fatty acids, and triacylglycerols in ascites of ID8 ovarian cancer-bearing mice with higher FASN expression (**Figures 4D–F**). To directly determine the source of the high lipid level, we analyzed the tumor cell conditioned medium (TCM). Analysis of lipid content also revealed that tumor-conditioned medium from ID8 cell with higher FASN expression had a higher level of triacylglycerol and fatty acids (data not shown). To test whether these lipids in tumor microenvironment would promote the conversion of DCs into metabolically inactive DCs, we generated DCs *in vitro* from bone marrow by using granulocyte-macrophage colony–stimulating factor and interleukin-4. *In vitro*–generated BMDCs were incubated with ascites derived from $FASN^{high}$ and $FASN^{low}$ OvCa. The result showed more than two-fold increase in the lipid level of BMDCs cultured with ascites from $FASN^{high}$ OvCa than $FASN^{low}$ OvCa (**Figure 5A**). We observed a similar effect with TCM from other ovarian tumors (data not shown). Analysis of DC-associated gene signature by RT-PCR also revealed that DCs generated in the presence of ascites with $FASN^{high}$ OvCa had decreased expression of costimulatory genes than $FASN^{low}$ OvCa

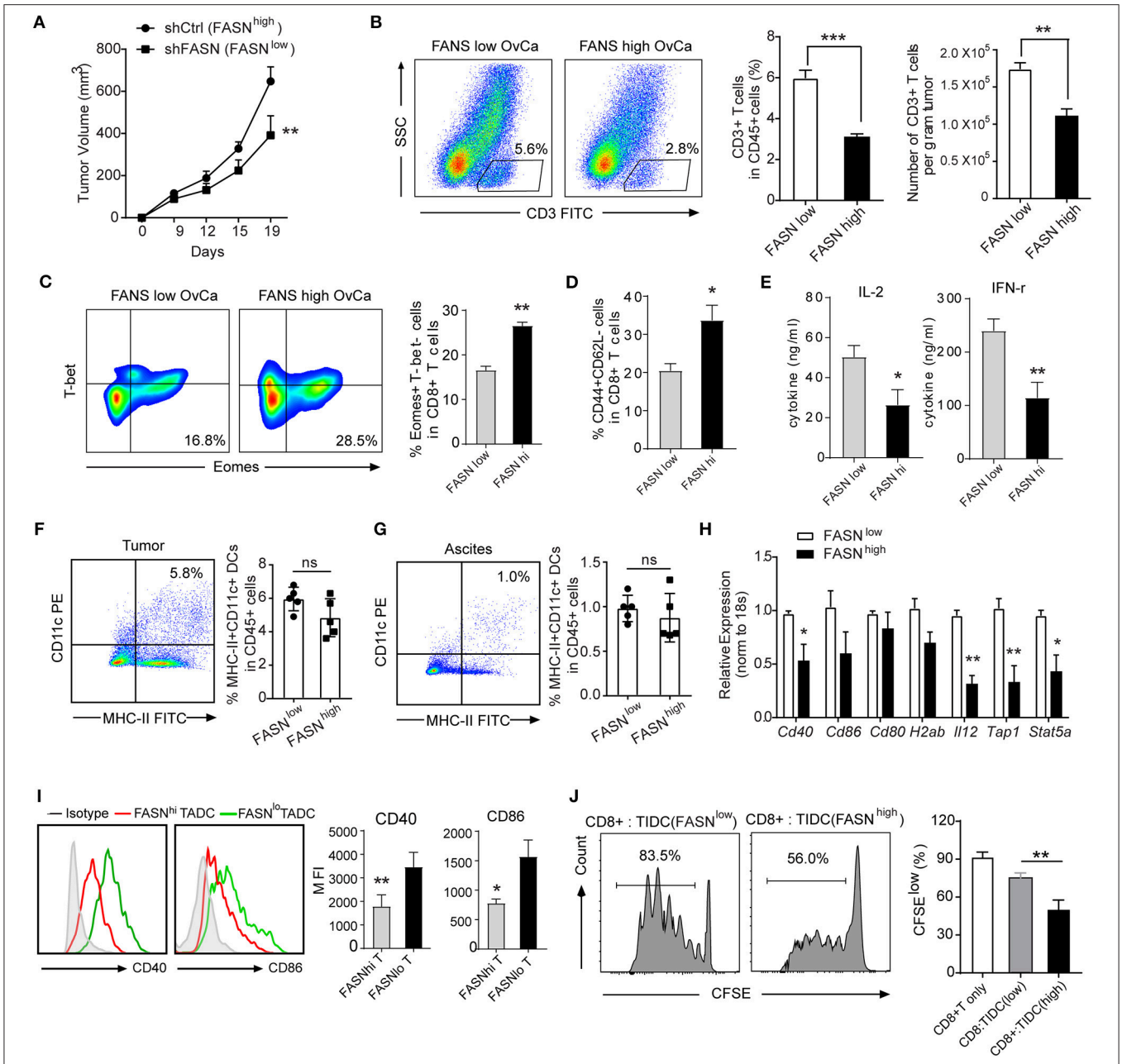

FIGURE 2 | Ovarian cancer with elevated FASN expression showed T cell exclusion and defective tumor-infiltrating DCs. **(A)** Tumor growth in synergetic mice after the inoculation of ID8 cell transfected with vehicle control (shCtrl: FASN^{high}) or knocking-down FASN (shFASN: FASN^{low}). **(B)** Representative example out of five for flow cytometry analyzing tumor-infiltrating CD3^{+} T cells in ID8 tumor-bearing mice with high (FASN^{high}) or low expression of FASN (FASN^{low}) on day 15. Statistical bar graph showed CD3^{+} T cells depicted as percentage living CD45^{+} cells and absolute numbers per gram tumor. $n = 10$. **(C)** Representative color contour plots examining Eomes^{+}T-bet^{-} exhausted CD8^{+} T cells from mice with shCtrl-ID8 or shFASN-ID8 tumor, and quantification of Eomes^{+}T-bet^{-} cells in CD8^{+} T cells was shown. **(D)** Quantification of tumor infiltrated CD44^{+}CD62L^{-} cell population in CD8^{+} T cell from tumor-bearing mice transplanted with shFASN-ID8 or shCtrl-ID8 ($n = 10$). **(E)** Analysis of cytokine IL-2 and IFN-r in the supernatant of sorted CD3^{+} T cells isolated from FASN^{low} or FASN^{high}-ID8 tumors with anti-CD3/CD28 stimulation ($n = 10$). **(F,G)** Flow cytometry analysis and quantification of MHC-II^{+}CD11c^{+} dendritic cells in the tumor **(F)** and ascites **(G)** of mice with the inoculation of shCtrl-ID8 (FASN^{low}) or shFASN-ID8 (FASN^{high}) ($n = 10$). **(H)** mRNA expression of indicated DCs associated genes in TIDCs sorted from ID-8 transplanted mice with FASN^{low} or FASN^{high}. The results were normalized to the level of expression of 18sRNA. **(I)** Representative flow cytometry analysis and quantification of CD40 and CD86 on MHC-II^{+}CD11c^{+} cells from tumor-bearing mice transplanted with FASN^{low} ID8 or FASN^{high} ID8 cells. ($n = 10$). **(J)** *In vitro* immunostimulatory activity of tumor-infiltrating CD11c^{+} cells purified from FASN^{low} or FASN^{high} ID-8 tumor-bearing mice. Representative histograms of CD8^{+} T cell proliferation at CD8^{+} to CD11c^{+} cell ratio 10:1 (left panel) and quantification of CD8^{+} T cell proliferation using CFSE dilution (right panel) ($n = 3$). Data presented as mean ± SEM; representative of at least 3 independent experiments; ns, no significance; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

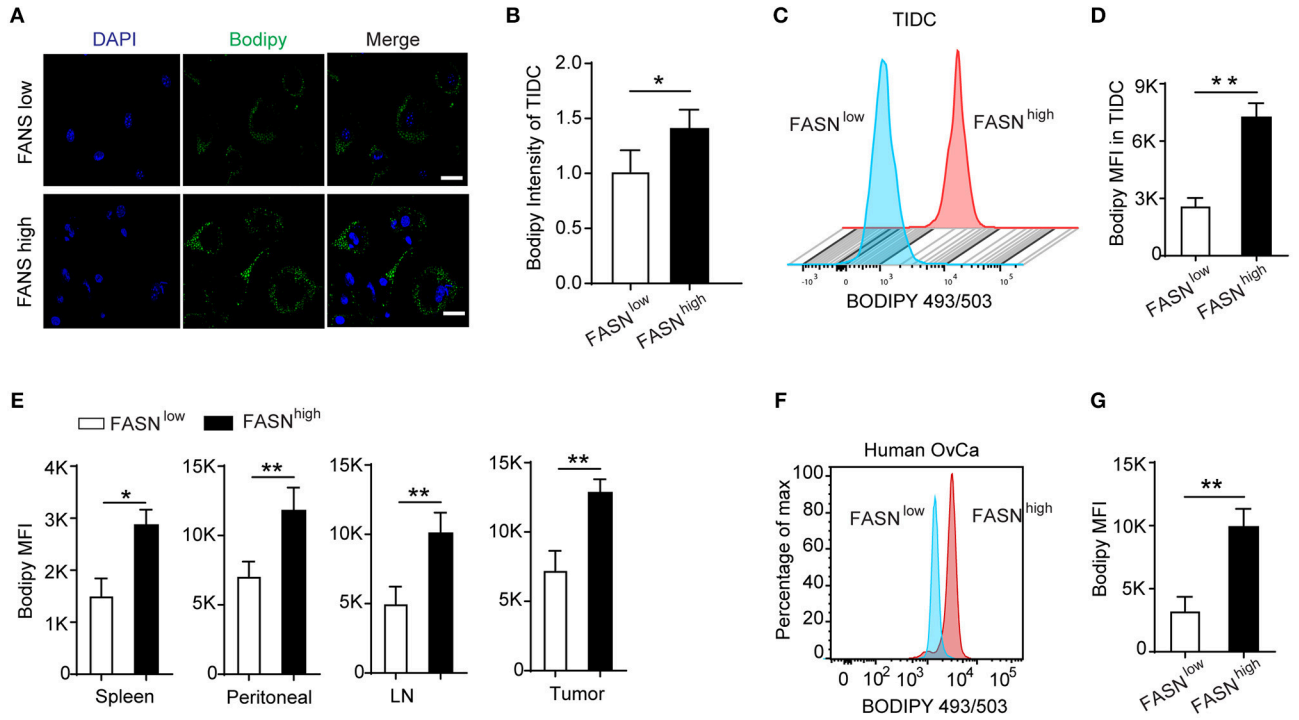

FIGURE 3 | Lipid accumulation in tumor-infiltrating DCs derived from the FASN^{high} ovarian cancer accounted for its defective function. **(A)** Staining of sorted tumor-infiltrating dendritic cells (TIDCs) from FASN^{low} or FASN^{high} ID-8 tumor-bearing mice with BODIPY 493/503. Representative images are shown; scale bar, 20 μm. Green, BODIPY 493/503; blue, DAPI. **(B)** Immunofluorescence intensity of BODIPY 493/503 in TIDCs was measured by Image J and quantification was shown ($n = 5$). **(C)** Flow cytometry analysis of lipid level in TIDC from FASN^{low} or FASN^{high} ID8 tumor-bearing mice. **(D)** Geometric mean fluorescence intensity (MFI) of BODIPY 493/503 in TIDCs was measured. Each group includes five mice. FASN^{low} and FASN^{high} ID8 tumor-bearing mice were evaluated in parallel in each experiment. **(E)** Quantification of lipid level in MHC-II^{+}CD11c^{+} DCs from spleen, ascites, lymph node, and tumor in FASN^{low} or FASN^{high} ID-8 tumor-bearing mice. **(F)** Flow cytometry analysis of BODIPY 493/503 staining of TIDCs from an individual with ovarian cancer showed human TIDCs also have high lipid level. **(G)** Cumulative results of lipid levels in TIDCs from individuals with OvCa. Data = mean ± SEM; representative of at least 3 independent experiments; $^{*}P < 0.05$; $^{**}P < 0.01$.

cancer (**Figure 5B**). Taken together, our data suggested that FASN upregulated lipid generation in the tumor microenvironment to induce the lipid accumulation of DCs.

FASN-High OvCa Ascites Induce DCs to Dampen Antigen Presentation and T Cell Activation

To investigate whether lipid accumulation in DCs has functional consequences, DCs was generated *in vitro* from bone marrow and educated with ascites from FASN^{high} or FASN^{low} ID8 tumor-bearing mice. To assess the ability of DCs to process antigen, a self-quenching conjugate of DQ ovalbumin (DQ-OVA) was used to show fluorescence upon proteolytic degradation, and the percentage and fluorescent intensity of uptake DQ-OVA were analyzed by flow cytometry. BMDCs educated by ascites from FASN^{high} OvCa tumor-bearing mice had a substantially lower DQ-OVA than from FASN^{low} OvCa mice (**Figures 5C,D**). We subsequently investigated whether lipid accumulation in DCs stimulates allogeneic T cells. Educated DCs with ascites was co-cultured with CD8^{+} T cell at 1:10 ratio with appropriate stimulation. DCs educated by ascites from ID8 FASN^{low} tumor-bearing mice had certain promoting effects on allogeneic T cell proliferation, whereas ascites derived from FASN^{high} OvCa inhibited the ability of DCs generated from wild-type BMPs to stimulate allogeneic T cells (**Figure 5E**). Similarly, DCs educated by TCM from FASN^{high} ID8 cells also had a substantially lower stimulatory effect for T cells proliferation than by TCM from FASN^{low} cells (**Figure 5F**).

Inhibition of FASN Suppress OvCa Progression by Inducing Anti-Tumor Immunity

To explore how FASN inhibition affects anti-tumor immune response *in vivo*, we analyzed tumor-infiltrating immune cells in ID8 mice treated with FASN inhibitor cerulenin, a small molecule antibiotic. Indeed, cerulenin treatment led to ID8 tumor growth regression in subcutaneous or intraperitoneal immunocompetent models (**Figure 6A**, **Supplementary Figures S5A,B**), but not in nude mice (**Supplementary Figure S5C**). In addition, *in vitro* assay indicated that the effect of cerulenin on DCs' function is minimal (**Supplementary Figure S5D**). Flow analysis showed significantly higher tumor-infiltrating CD4^{+} T cells and CD8^{+}

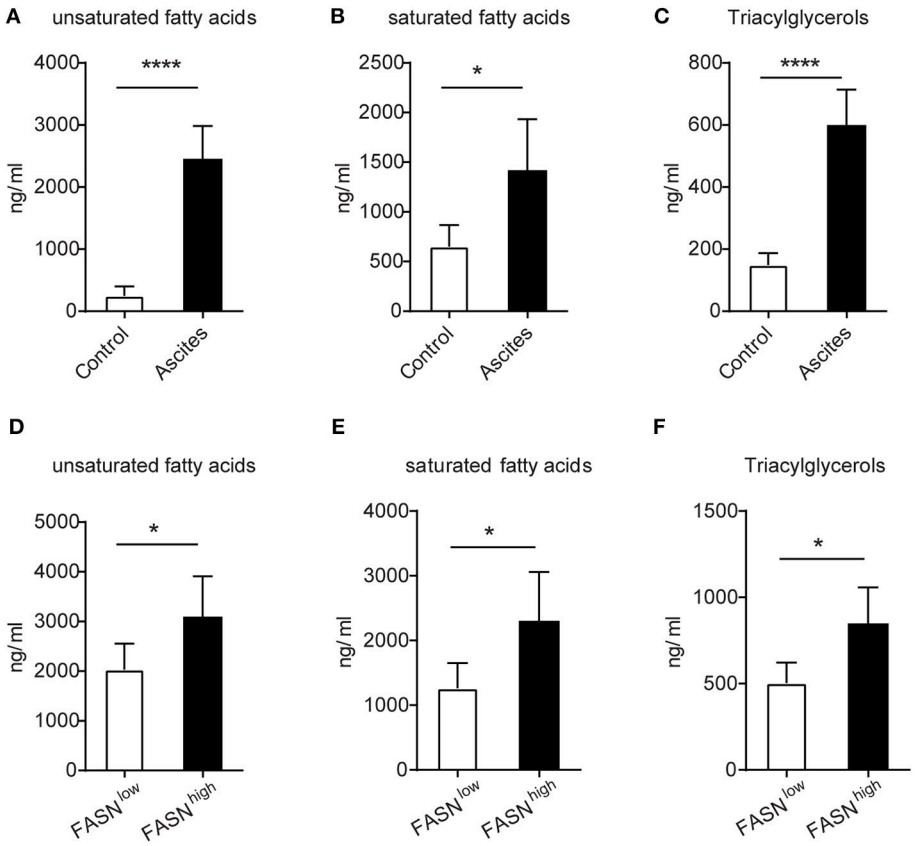

FIGURE 4 | The enrichment of lipids in the FASN-high ovarian tumor microenvironment. **(A–C)** The supernatants of malignant ascites were collected from ID-8 tumor-bearing mice after 3 weeks. The normal peritoneal fluid was collected from naive C57BL/6J mice. The amount of fatty acids and triacylglycerols were quantitatively analyzed via LC-MS. Depicted are total unsaturated fatty acids **(A)**, saturated fatty acids **(B)**, and triacylglycerols **(C)**. **(D–F)** C57BL/6J mice were intraperitoneally injected with 5×10^5 FASN^{low} or FASN^{high} ID8 cells, and supernatants of malignant ascites were collected after 3 weeks. Fatty acids and triacylglycerols were extracted and quantitatively analyzed by LC-MS. Depicted are total unsaturated fatty acids **(D)**, saturated fatty acids **(E)**, and triacylglycerols **(F)**. Data = mean ± SEM; representative of 2 independent experiments; $^*P < 0.05$; $^{****}P < 0.0001$.

CTLs in the tumor microenvironment of FASNi- treated ID8 mice (**Figures 6B,C**). Consistent with increased infiltration of cytotoxic T cell populations, we also observed more IFN-r$^+$ and Granzyme B$^+$ functional CD8$^+$ T cells and less Eomes$^+$ exhausted CD8$^+$ T cells in FASN inhibitor-treated OvCa tumor (**Figure 6D**, **Supplementary Figures S5E,F**). Furthermore, lipid content was significantly decreased in TIDCs derived from FASN inhibitor-treated ID8 mice compared to the untreated group (**Figure 6E**). To directly address this function of TIDCs, we sorted TIDCs from tumor tissue in ID8 transplanted mice with or without FASN inhibitor treatment. TIDCs isolated from FASN inhibitor-treated ID8 model displayed the enhanced capacity to induce the proliferation of CD8$^+$ T cells, compared with their control counterparts (**Figure 6F**). Taken together, our data show that OvCa-cell-intrinsic FASN activation can result in the exclusion of the host immune response, including lipid accumulation in tumor-infiltrating dendritic cells and subsequently the absence of a T-cell infiltrate and dysfunction within the tumor microenvironment (**Figure 6G**).

DISCUSSION

Here we conclude that ovarian cancer cell-intrinsic activation of an oncogenic FASN pathway can result in the exclusion of the host immune response, including the dysfunction of dendritic cells and the absence of a T-cell infiltrate within the tumor microenvironment. We uncover an unexpected role for tumor intrinsic FASN as a driver of DCs malfunction in the tumor microenvironment by lipid accumulation. We find that FASN signaling is a driver of immunosuppression and OvCa progression. The present study extends the investigation of the potential use of a FASN inhibitor for immunotherapy in mice. In this study, we also revealed the effect of cerulenin on growth inhibition of the syngeneic murine ID8 OvCa model by inhibiting immunosuppression.

Numerous studies have shown that many human cancer cells have high activities of FASN, and the cytotoxic effects of FASN have been described both *in vitro* and *in vivo* (17, 36, 37). However, the role of upregulated FASN in cancer cells and the detailed mechanisms of tumor cells killing by an inhibitor

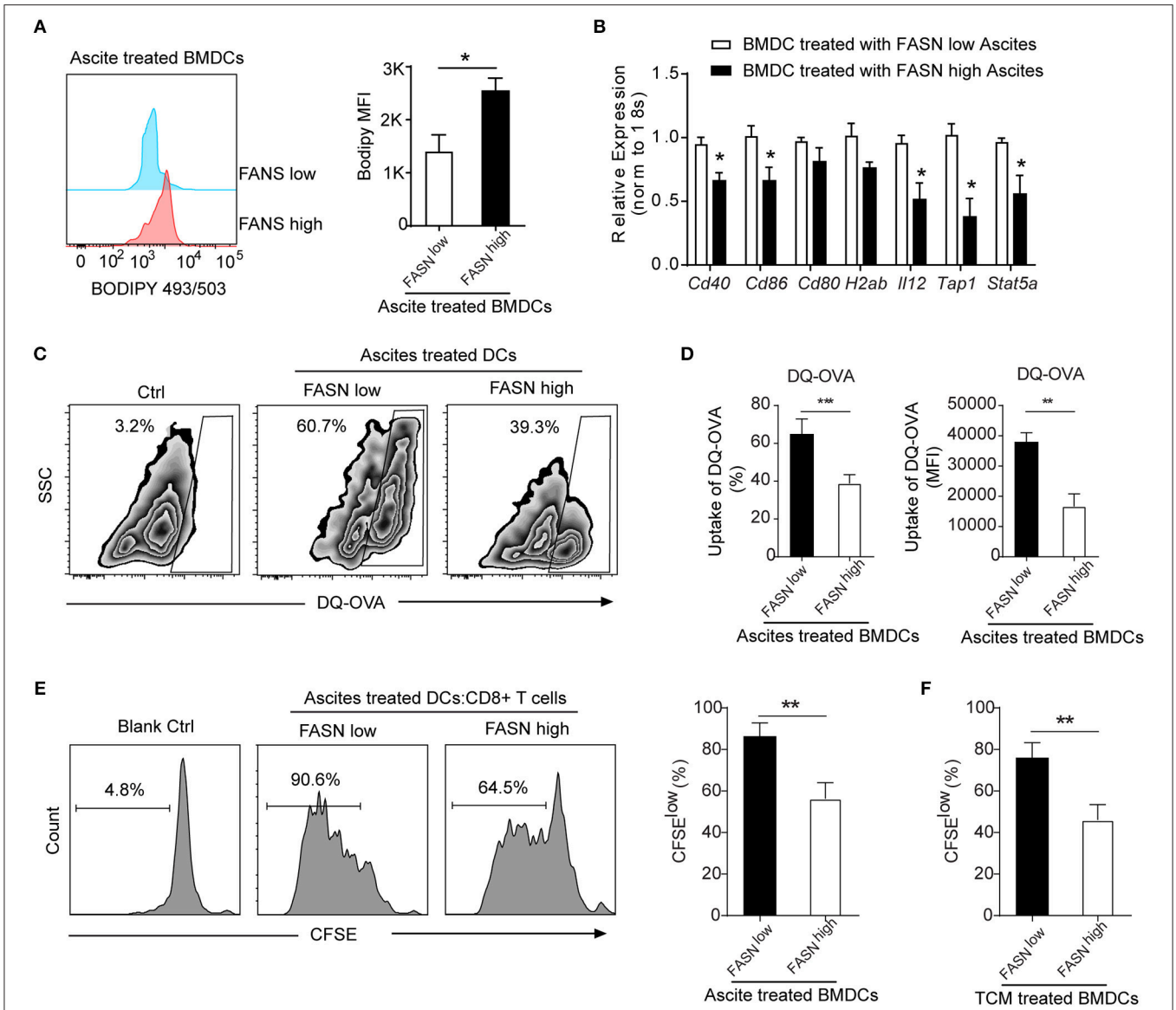

FIGURE 5 | Educated DCs by ascites exhibited defective antigen presentation and impaired priming of T cell activation. **(A)** Flow cytometric staining with Bodipy 493/503 in BMDCs cultured in the presence of 20% ascites harvested from FASN^{low} or FASN^{high} ID-8 tumor-bearing mice for 24 h. MFI average is depicted. Means ± s.d. are shown. **(B)** mRNA expression of indicated DCs associated genes in BMDCs cultured in the presence of 20% ascites harvested from FASN^{low} or FASN^{high} ID-8 tumor-bearing mice. The results were normalized to the level of expression of 18sRNA. **(C,D)** Uptake of DQ-OVA by BMDCs in the presence of 20% ascites harvested from FASN^{low} or FASN^{high} ID-8 tumor-bearing mice. Quantification of uptaking DQ-OVA was shown. Means ± s.d. of three mice per group are shown. **(E, F)** *In vitro* immunostimulatory activity of BMDCs treated with ascites **(E)** or TCM **(F)** harvested from FASN^{low} or FASN^{high} ID8 tumor-bearing mice. Representative histograms of CD8^{+} T cell proliferation at CD8^{+} to BMDCs ratio 10:1 (left panel) and quantification of CD8^{+} T cell proliferation using CFSE dilution (right panel) (n = 3). Data = mean ± SEM; representative of at least 3 independent experiments; ns, no significance; *P < 0.05; **P < 0.01; ***P < 0.001.

of FASN are still not fully understood. In this study, we have demonstrated that FASN was upregulated in human ovarian cancer and associated with the immunosuppressive microenvironment. FASN signaling has been confirmed as a regulator of multiple signaling pathways during tumor progression, including cell-cell adhesion, migration, proliferation and chemokine transcription (38–40). As such, FASN has been shown to be important in several cancer types, including ovarian cancer (14, 40). However, the role of FASN signaling in driving suppressive tumor microenvironment is less well understood. Recent reports have highlighted the importance role of lipids on the function of immunosuppressive myeloid cells including M2 macrophages, dendritic cells, and MDSC in inflammatory conditions and cancer (26, 27, 35, 41). In recent years, accumulation of lipids was implicated in the defective cross-presentation by tumor-associated DCs (42–44). This suggested that factors in tumor microenvironment could mediate the immunometabolic induction of DCs.

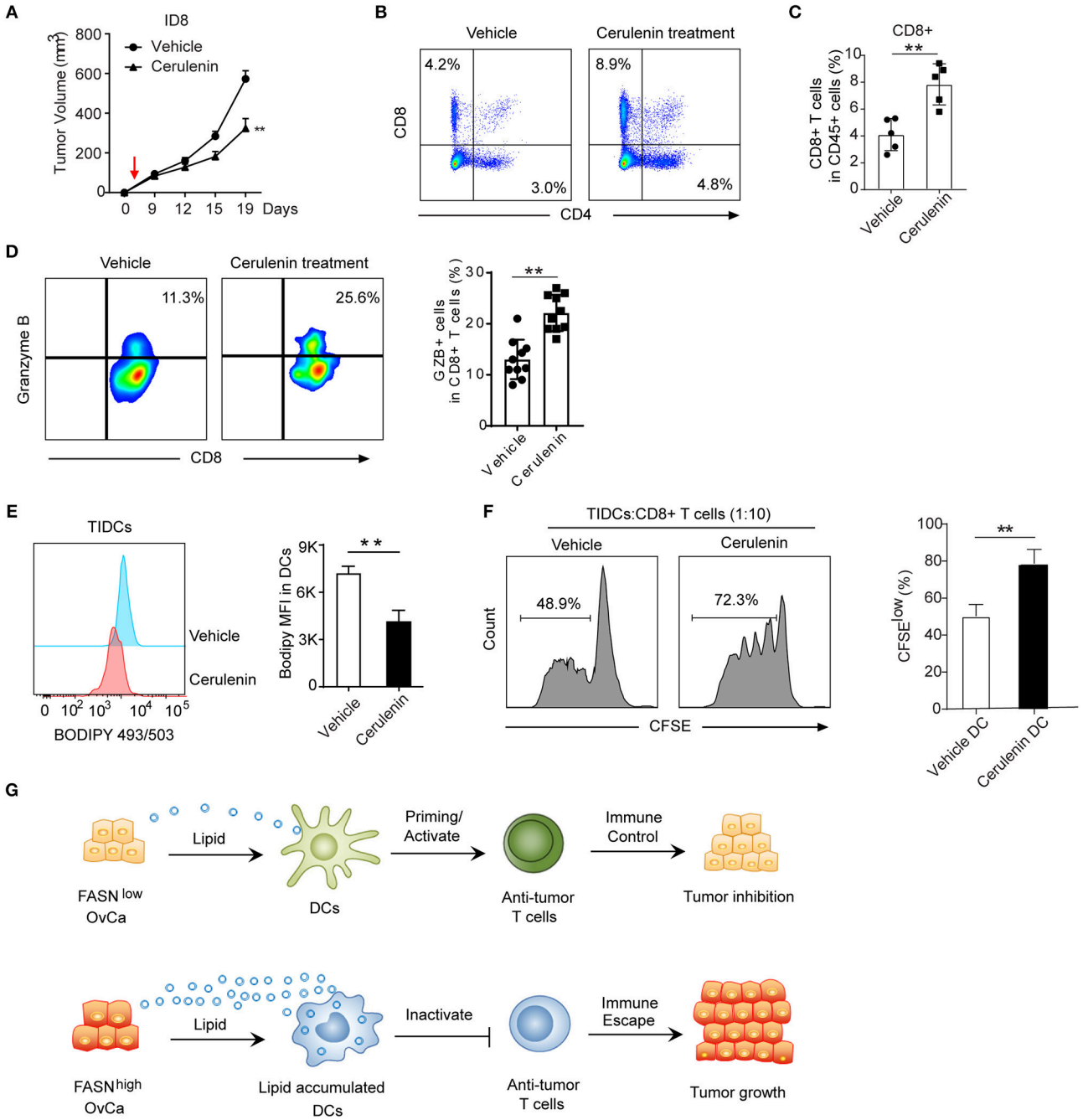

FIGURE 6 | Inhibition of FASN suppress OvCa progression by inducing an anti-tumor immune response. **(A)** Mean tumor volume of the subcutaneous ID8 tumor with the treatment of vehicle or cerulenin ($n = 10$). The red arrow indicated the start of cerulenin treatment. **(B)** Representative flow cytometric analysis of $CD8^+$ and $CD4^+$ T cells populations in ID8 tumors with or without the treatment of cerulenin at 15 days post-implantation. **(C)** Quantification of $CD8^+$ T cells population in ID8 tumors with or without the treatment of cerulenin at 15 days post-implantation ($n = 5$). **(D)** Representative flow cytometric analysis and quantification of Granzyme B expression on $CD8^+$ T cells population in ID8 tumor with or without cerulenin treatment at 15 days post-implantation. ($n = 10$). **(E)** Representative flow cytometric analysis and quantification of lipid level in TIDCs sorted from the ID8 tumor with or without cerulenin treatment ($n = 5$). **(F)** *In vitro* immunostimulatory activity of TIDCs sorted from the ID8 tumor with or without the treatment of cerulenin ($n = 5$). Representative histograms and quantification of $CD8^+$ T cell proliferation using CFSE dilution (right panel) were shown ($n = 3$). Data presented as mean ± SEM; $^{**}P < 0.01$. **(G)** Summary of crosstalk between $FASN^{low}$ or $FASN^{high}$ tumor cells and DCs-mediated adaptive immune response as described in the text.

Here, we identified that OvCa cancer-intrinsic FASN might initiate this process. OvCa-derived FASN produces abundance lipid that facilitates the uptake of lipids abundant in the tumor microenvironment, including free fatty acids and the triacylglycerol-carrying lipoproteins VLDL and LDL.

A substantial proportion of DCs in tumor-bearing hosts have an increased amount of lipids, specifically triglycerides. In this study we have tried to determine the mechanism of lipid accumulation in DCs and whether it has any functional consequences for these cells. Accumulation of lipids might be due to increased synthesis of fatty acids in $FASN^{high}$ OvCa cell and then result from increased lipid uptake from tumor microenvironment. Importantly, human cancer-associated DCs also express lipid transporters and therefore BMDCs cultured in $FASN^{high}$ OvCa conditioned media to develop into highly defective DCs. The uptake and accumulation of these lipids support the activation of immunodefective DCs. Several reports have demonstrated that dendritic cells in ovarian cancer correlated with clinical outcome in patients with ovarian cancer (45). In addition, ovarian cancer progresses involving the recruitment of immunostimulatory DCs that induce measurable T cell-mediated anti-tumor immunity (19). Our findings are also significant as we show an abundance of lipids in the tumor microenvironment of $FASN^{high}$ OvCa cancer that can be acquired by DCs. This observation supports previous reports that show increased levels of triglycerides, HDL-cholesterol in the circulation of ovarian cancer patients (46, 47). Moreover, the T-cell-inflamed tumor microenvironment phenotype appears to be predictive of clinical response to immune-based therapies (48). Immune escape among this subset appears to be a consequence of dominant effects of negative regulatory pathways such as PD-1, arguing that the clinical activity of anti-PD-1 is tipping the balance in favor of an ongoing immune response. By inference, tumor-intrinsic FASN activation may represent one mechanism of primary resistance to these therapies.

Taken together, our findings suggest a critical role of lipid uptake and accumulation in the metabolic and functional reprogramming in DCs of ovarian cancer. The regulation of these processes by tumor-derived FASN-dependent mechanisms provide an opportunity to simultaneously target the multiple immunosuppressive pathways harnessed by tumor-associated DCs. These findings indicate that tumor cell-intrinsic FASN signaling may be a driver of immune escape in ovarian cancer, and thus may be a target for combination with immunotherapy in ovarian cancer.

AUTHOR CONTRIBUTIONS

XW and LJ supervised the whole project, designed the experiments, analyzed data, and wrote the manuscript. LJ and XF performed the most experiments, analyzed data, and prepared the figures. HW and DL contributed to some experiments and provided the technical support. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02927/full#supplementary-material

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The Combined Use of Melatonin and an Indoleamine 2,3-Dioxygenase-1 Inhibitor Enhances Vaccine-Induced Protective Cellular Immunity to HPV16-Associated Tumors

Ana C. R. Moreno[1], Bruna F. M. M. Porchia[1], Roberta L. Pagni[1], Patrícia da Cruz Souza[1], Rafael Pegoraro[1], Karine B. Rodrigues[1], Tácita B. Barros[1,2], Luana R. de Melo Moraes Aps[1], Eliseu F. de Araújo[3], Vera L. G. Calich[3] and Luís C. de Souza Ferreira[1]*

[1] Vaccine Development Laboratory, Department of Microbiology, Biomedical Sciences Institute, University of São Paulo, São Paulo, Brazil, [2] Department of Clinical Chemistry and Toxicology, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil, [3] Department of Immunology, Biomedical Sciences Institute, University of São Paulo, São Paulo, Brazil

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Macarena, Spain

***Correspondence:**
Ana C. R. Moreno
carol@usp.br

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Immunotherapy has become an important ally in the fight against distinct types of cancer. However, the metabolic plasticity of the tumor environment frequently influences the efficacy of therapeutic procedures, including those based on immunological tools. In this scenario, immunometabolic adjuvants arise as an alternative toward the development of more efficient cancer therapies. Here we demonstrated that the combination of melatonin, a neuroimmunomodulator molecule, and an indoleamine 2,3-dioxygenase (IDO) inhibitor (1-methyl-DL-tryptophan, DL-1MT) improves the efficacy of an immunotherapy (gDE7) targeting human papillomavirus (HPV)-associated tumors. Melatonin or IDO inhibitors (D-1MT and DL-1MT) directly reduced proliferation, migration, adhesion and viability of a tumor cell line (TC-1), capable to express the HPV-16 E6 and E7 oncoproteins, but could not confer *in vivo* antitumor protection effects. Nonetheless, combination of gDE7 with melatonin or D-1MT or DL-1MT enhanced the antitumor protective immunity of gDE7-based vaccine in mice. Notably, expression of IDO1 in stromal cells and/or immune cells, but not in tumor cells, inhibited the antitumor effects of the gDE7, as demonstrated in IDO1-deficient mice. Finally, co-administration of gDE7, melatonin and DL-1MT further improved the protective antitumor effects and the numbers of circulating E7-specific $CD8^+$ T cells in mice previously transplanted with TC-1 cells. The unprecedented combination of melatonin and IDO inhibitors, as immunometabolic adjuvants, thus, represents a new and promising alternative for improving the efficacy of immunotherapeutic treatments of HPV-associated tumors.

Keywords: melatonin, 1-methyl-tryptophan, indoleamine 2, 3 dioxygenase, human papillomavirus, cancer immunotherapy

INTRODUCTION

Human papillomaviruses (HPV) are widely spread pathogens responsible for one of the most common sexually transmitted diseases worldwide (1). Since the role in genital malignancies is well established, HPV, particularly the genotypes associated with tumor onset, is considered a relevant public health concern, causing approximately half a million deaths worldwide every year (1). Virtually all cervical cancers and around 90% of squamous anal cancers can be attributable to HPV infection (2). Furthermore, the correlation between HPV infection and other anogenital and oropharyngeal cancers is steadily growing (3). HPVs comprise a diverse group, and more than 200 HPV genotypes has been identified. The classification into low-risk or high-risk HPV genotypes relies on the oncogenic potential during persistent infection in the cervical tissue (2). In this scenario, the constitutive expression of E6 and E7 oncoproteins, leading to cellular transformation and immortalization, is mandatory for the onset and maintenance of HPV-associated cancers by high-risk genotypes (4). HPV-16 infection is more prevalent than any other high-risk HPV genotype in most regions worldwide (5).

HPV vaccination could considerably reduce the morbidity and mortality of cancers causally associated with this virus. However, after 12 years, many populations worldwide have not been vaccinated (6). Unfortunately, in several countries, HPV immunization rates are significantly lower than rates of other childhood and adolescents immunizations (7). Furthermore, cervical cancer remains as one of the most frequent causes of cancer-related deaths among women throughout the world (1), and current treatment approaches vary according to the clinical stage of the disease (8). Regarding metastatic/recurrent cervical cancer, chemotherapy is considered the first-line approach (9). However, the performance of chemotherapy, as well as other therapeutic interventions, drops dramatically in more advanced tumors, a direct consequence of the establishment of an immunosuppressive milieu in the tumor microenvironment and peripheral systems marked by accumulation of inhibitory cytokines and dysfunctional immune cells (10). In this scenario, the development of efficient immunotherapies or adjuvants, that ameliorate the immune suppressive environment created by tumor cells and improve the performance of conventional treatments, represents a priority and a necessity (11).

In recent decades, several studies documented that melatonin, a natural antioxidant molecule largely distributed among living organisms, plays a fundamental role in neuroimmunomodulation (12). In addition to the regulation of the circadian rhythms (13), melatonin also affects a diversity of physiological processes including immune functions. More precisely, melatonin significantly enhances the differentiation of type 1 helper T cells (Th1) and IFN-γ production (12), important steps for the activation of tumor-specific $CD8^+$cytotoxic T cells. Additionally, melatonin has shown to have oncostatic and pro-apoptotic properties in a plethora of experimental tumor models and in different human tumor cell lines (14–16). Consequently, several studies have classified melatonin as a promising anticancer agent, including for combined therapies, with exciting potential to override the immunosuppressive environment associated with growing tumors. Concerning the challenge to overcome the tumor-mediated immunosuppression, melatonin showed inhibitory effects on the immunomodulatory enzyme indoleamine 2,3-dioxygenase-1 (IDO1) (16). Melatonin downregulates IDO1 at mRNA levels, as well as kynurenine production in skin cells and human melanoma cells (16). Moreover, melatonin synthesis can be stimulated by the racemic compound 1-methyl-DL-tryptophan (DL-1MT)(16), an inhibitor of IDO1 (17) and IDO2 (18).

It is well established that IDO1 expression suppresses innate and adaptive immune responses that, under certain circumstance, promote a tolerogenic microenvironment (19, 20). IDO1 acts in tryptophan degradation and kynurenine production that negatively regulates immune cells, leading to enhanced numbers of regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs) (20). A key issue about cancer immune escape mechanisms lies in the ability of tumor cells to edit their phenotype using extrinsic tumor suppressor mechanisms. Sustained by this phenomenon, immunotherapy raised as a significant therapeutic breakthrough against tumor induced immune suppression (21). Since IDO1 is an endogenous mechanism of immune tolerance *in vivo*, IDO1 inhibitors are emerging as experimental molecules in oncology (22). Indeed, small-molecules inhibitors of IDO1, like epacadostat, navoximod and indoximod (D-1MT enantiomer), are under phase II or II/III clinical trials (23, 24). However, based on recent negative results of ECHO-301/KEYNOTE-252 phase 3 trial in metastatic melanoma (clinical trial information: NCT02752074) (25), many trials, particularly those that use the IDO inhibitor epacadostat, needed to be halted. Still, researchers have shown that different therapeutic combinations may subvert the failure of clinical trials, pointing that IDO inhibitors should not be abandoned for cancer immunotherapy (clinical trial information: NCT01961115, NCT02077881) (26, 27).

In the present study, we evaluated the therapeutic potential of a novel immunotherapy focusing on three components: melatonin, 1MT and an HPV-16 therapeutic vaccine (gDE7) based on a recombinant protein generated after the genetic fusion of the HPV-16 E7-oncoprotein with the envelope glycoprotein (gD) of herpes virus simplex virus (HSV). The IDO inhibitors 1MT (enantiomers and racemic mixture) were chosen to compose our therapeutic approach based on their preclinical and clinical data as adjuvants of antitumor therapies (20, 22, 23, 27). The gDE7 vaccine (28, 29), as well as its DNA version (pgDE7h) (30), has shown excellent therapeutic effects in experimental conditions based in mice transplanted with TC-1 cells, a murine tumor cell line encoding the HPV-16 oncoproteins. We show here that IDO1 expression in immune cells and stromal cells, but not in tumor cells, impairs the antitumor effect of the gDE7 vaccine and, more relevantly, we demonstrated that combination of melatonin and an IDO inhibitor augmented the antitumor therapeutic effects of gDE7 and increased the activation of E7-specific cytotoxic $CD8^+$ T-cell responses. Our findings highlight the role of IDO1 as an important immunosuppression inducer that may impair the proper functioning of immunotherapy. Furthermore, we propose the unprecedented association of

melatonin and IDO inhibitors as immunometabolic adjuvants for cancer immunotherapy.

MATERIALS AND METHODS

Cell Culture

The TC-1 tumor cell line (31) was kindly provided in 2002 by Dr. T.C. Wu from Johns Hopkins University in Baltimore, MD, USA. The cells were cultured as previously described (29).

IDO1 Flow Cytometer Analysis

TC-1 cells were cultured until reach 90% of confluence, and then were harvested with trypsin and seeded in 96-well plates at a concentration of 5×10^5 cell/well. Cells were washed with MACS buffer [phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA], fixed and permeabilized with the BD Cytofix/Cytoperm™ Plus Buffer Set (#555028,) and intracellularly stained for 30 min at 4°C with either anti-Mouse IDO1 eFluor® 660 (#50-9473-82, eBioscience) or its isotype control rat IgG2b K eFluor® 660 (#50-4031-82, eBioscience) antibodies (mAbs). After washings, cells were suspended in MACS buffer for further flow cytometry analysis on the LSRFortessa™ (BD Biosciences). Data were analyzed using the FlowJo software (version 9.0.2, Tree Star) to determine the frequency of IDO1 positive cells.

Ehrlich Test

TC-1 cells (1×10^5) were seeded in 24-well plates and incubated in RPMI medium with 10% SFB for 24 h, until reaching 50% of confluence. After this period, fresh medium (1 mL) containing 1 mM of immunomodulators (D-1MT, L-1MT, DL-1MT, or melatonin) was added and the plates were incubated for 36 h. Culture supernatants were assayed for N-formyl-kynurenine as a measure of IDO activity. The supernatants (100 μL) were treated with 50 μL of 30% trichloroacetic acid (TCA), centrifuged for 10 min at 3,000 g, and the supernatant was incubated at 52°C for 30 min to hydrolyze N-formyl-kynurenine to kynurenine. A triplicate of each sample (80 μL) was aliquoted into a 96-well plate. Standards were prepared as serially diluted kynurenine from 1,000 μM in TCA-treated media. Freshly prepared Ehrlich's reagent (80 μL) (2 g of 4-Dimethylamino-benzaldehyde in 100 mL of glacial acetic acid) was added to each well and plate was incubated for 15 min at room temperature in the dark. The plates were read in a spectrophotometer at 492 nm.

Wound Healing Assay

TC-1 cells (5×10^5) were seeded in 24-well plates and cultured for 24 h until reaching 95% of confluence. The monolayers were then carefully scratched with the aid of a 200 μL pipette tip followed by the addition of fresh culture medium containing 1 mM of immunomodulators. Cells were photographed after appropriate incubation times using a light microscope.

Cell Adhesion Assay

TC-1 cells were cultured until reach 90% of confluence, and then were harvested with trypsin and washed with RPMI 1640 medium supplemented with 10% FBS and 50 U/mL penicillin/streptomycin. Cells were resuspended in fresh culture media containing 1 mM of immunomodulators, and then were seeded in 24-well plates at a concentration of 1×10^5 cell/well. Cells were incubated at 37°C and 5% CO^2 for 2 h. Next, the plate was placed on ice and cells were washed twice with ice-cold PBS to removing the non-adherent cells. Cells were then fixed in ice-cold methanol for 10 min and stained for 20 min with crystal violet solution (0.5% w/v, made in 25% methanol). Finally, plates were carefully rinse in distillated water until color no longer coming off in rinse. Cells were photographed in EVOS® FL Cell Imaging System (Thermo Fisher Scientific). The percentages of cells adhesion were measured by numbers of cells per field.

Cell Viability

TC-1 cells (1×10^5) were seeded in 24-well plates and incubated in RPMI medium with 10% SFB for 24 h, until reaching 50% of confluence. After this period, fresh medium, containing 1 mM of immunomodulators was added, and the plates were incubated for additional 24 h. Next, cell cytotoxicity was assessed using ethidium bromide (EB) incorporation in combination with acridine orange (AO) staining as described previously (32). Images were acquired in EVOS® FL Cell Imaging System (Thermo Fisher Scientific) and the numbers of death cells were counted per field.

Mice and TC-1 Tumor Cell Challenge

Wild type (WT) C57BL/6 mice, aged 8–10 weeks, were purchased from the Department of Parasitology of Institute of Biomedical Sciences and the Faculty of Veterinary Medicine of the University of São Paulo. The IDO1 gene ($IDO^{-/-}$) knocked mice were supplied by the animal facility unit of the Department of Immunology of the University of São Paulo. All procedures for manipulation, immunization and euthanasia were approved by the ethics committee for animal experimentation (protocol number CEUA 050/2014) and followed the standard rules approved by the National Council for Control of Animal Experimentation (CONCEA). The tumor cells transplantation was performed as previously described (29), at a concentration of 1×10^5 cells/100 μL/animal on 0 day. Mice were considered as tumor-bearing when tumors became palpable (7–10 day) and were sacrificed when tumors exceeded 15 mm in "L" diameter.

Immunization and Immunomodulators

The therapeutic gDE7-based vaccine was administered following a regimen of two subcutaneous doses with a week interval, as previously described (28). Each dose contained 30 μg of the gDE7 protein, diluted in saline solution (total volume of 100–200 uL) and inoculated in the right rear flank region of mice on 7 and 14 day. In addition to the vaccine, mice were also treated with 1MT and/or melatonin for 4 weeks every 48 h, starting on 9 day. The D-1MT isomer and the racemic mixture DL-1MT were given in concentrations of 10 mg/animal dissolved in a mixture of 0.5% tween-80, 0.5% methyl cellulose (33) and sterile milli Q water, being administered 100 μL/animal per gavage. Melatonin was given at a concentration of 0.2 mg/200 μL/animal, dissolved first in DMSO (1%) and subsequently in apyrogenic saline solution, being administered intraperitoneally.

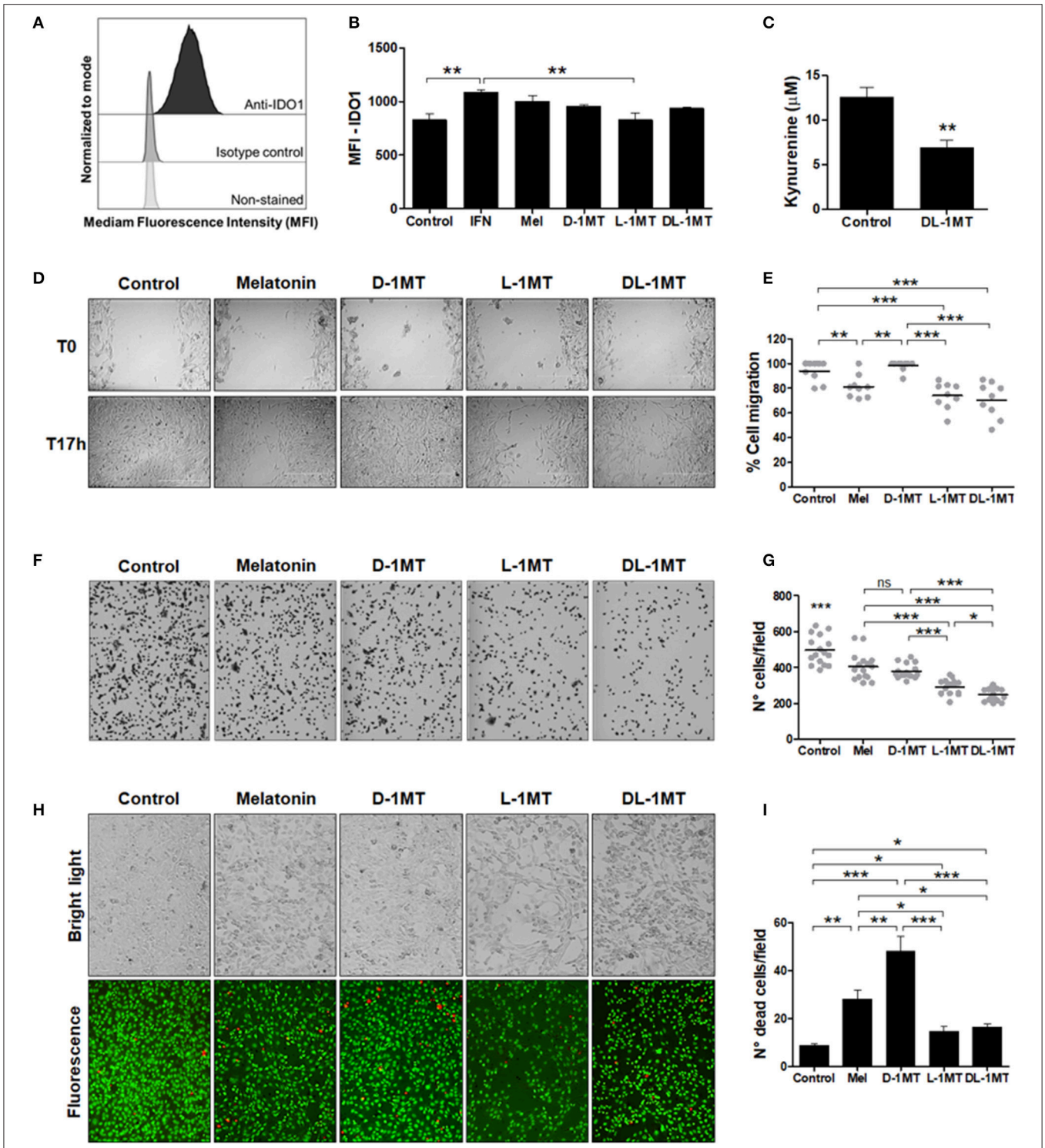

FIGURE 1 | IDO1 expression and the effects of melatonin and IDO inhibitors on TC-1 cells migration and adhesion. **(A)** IDO1 expression measured with anti-IDO1 antibody staining and flow cytometry analysis. Isotype control and non-stained cells were used as negative controls for IDO1 expression and cellular auto-fluorescence, respectively. **(B)** Median fluorescence intensity (MFI) of IDO1 expression in TC-1 cells measured by flow cytometry. Cells were treated with IFN-γ (50 u/mL), or melatonin (1 mM), or 1MT compounds (D-1MT, L-1MT, DL-1MT) (1 mM) for 24 h. Cells in culture media without immunomodulators (vehicle) are shown as reference controls. Data representative of two independent experiments performed in triplicates. **(C)** Ehrlich test performed to measure kynurenine concentrations in TC-1 cell supernatants after treatment with DL-1MT (1 mM) for 24 h. Data representative of two independent experiments performed in triplicates. Significance was

(Continued)

FIGURE 1 | determined by unpaired Student's *t*-test. **(D-E)** The effects of 1MT and melatonin (Mel) on TC-1 cells migration. **(D)** The bright-field microscopy imaging of TC-1 cells submitted to different stimulus at the beginning of the test (T_0) and 17 h later (T_{17h}). Cells kept in culture medium were used as a reference control. **(E)** Panels on the right indicate the quantification of the percentage of migratory cells through the cell layer wound healing assay after measurement of uncovered areas at the T_{17h} in relation to T_0. Data of three independent experiments performed in triplicates. **(F)** Representative bright-field microscopy images of adherent TC-1 cells 2 h after addition of the tested immunomodulators. **(G)** Adhesion of TC-1 cells in the presence of immunomodulators (1 mM) 2 h after seeding 24-well plates. Data representative of two independent experiments performed in triplicates. **(H)** Representative bright-field microscopy images (upper images) and fluorescence microscope images (botton images) to demonstrate cell density (cell proliferation) and cell viability, respectively, after cell growth in the presence of the different immunomodulators. **(I)** The graph represents the number of dead cells per field. Cells treated with culture media were included as controls. Data representative of two independent experiments performed in triplicates. All data are presented as means ± SEM. Statistical significance: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by ANOVA. (ns) Non-significant. When not signaled, * represents the statistical significance of one experimental group in relation to all others.

Blood, Spleen, and Tumor Microenvironment Analyses

Blood, spleen and tumor (when applicable) from naïve and tumor-bearing WT or IDO1$^{-/-}$ mice were used to analyze the frequency of different cell types. For blood samples, peripheral blood mononuclear cells were collected in heparin-containing vials. Cells were treated with ACK Lising Buffer (BioSource International) for lysis of red blood cells. Next, cells were washed with RPMI medium with 10% FBS (R10) and distributed in 96-well U-bottom plates for further staining. For spleen and tumor samples, mice were euthanized, and spleens and tumors were removed aseptically. Spleens were macerated with the aid of a syringe plunger, suspended in R10, filtered in a 70 μm cell strainer (Easy strainer Greiner Bio One) and treated with ACK lysis buffer. Subsequently, cells were washed and distributed in 96-well U-bottom plates for further staining. Tumor masses were minced with scissors and submitted to enzymatic digestion with 0.22 u/mL of collagenase D (#11088866001, Roche Diagnostics) at 37°C for 1 h, stirring gently every 10 min. After the incubation period, the enzyme was inactivated with 5 mM EDTA at room temperature for 5 min. Then, the samples gentle resuspended in R10 and filtered on a 70 μm cell strainer (Easy strainer Greiner Bio One). After centrifugation, the pelleted cells were resuspended in R10 and filtered on a 40 μm cell strainer. Then, cells were centrifuged, the pellet were resuspended in R10 and distributed in 96-well U-bottom plates for further staining. The following mAbs were used to discriminate different types of cells: anti-CD45-PerCP-Cyanine5.5 (#103131, Biolegend), anti-CD4-FITC (#553651, BD Pharmingen), anti-CD25-APC (#17-0251, eBioscience), anti-Foxp3-PE (#12-4771-80, eBioscience), anti-CD11b-Alexa Fluor 700 (#101222, BioLegend), anti-Gr-1-PE (#553128, BD Pharmingen), anti-Ly6C-Alexa Fluor 488 (#53-5932-82, eBioscience), anti-Ly6G-PE (#551461, BD Pharmingen), anti-CD11c-PE (#553802, BD Pharmingen), anti-MHC-II-FITC (#553605, BD Pharmingen), anti-F4/80-BV605 (#123133, BioLegend), anti-IDO1 eFluor® 660 (#50-9473-82, eBioscience), isotype control rat IgG2b K eFluor® 660 (#50-4031-82, eBioscience). For FoxP3 intracellular staining, we used the Foxp3 Transcription Factor Staining Buffer Set (#00-5523-00, eBioscience). For intracellular IDO-1 staining, we used the Intracellular Fixation and Permeabilization Buffer Set (#555028, BD Cytofix/CytopermTM Plus). Cells were characterized according to the following parameters: dendritic cells (CD45+, CD11chigh, MCH-IIhigh), macrophages (CD45+, MCH-II+, CD11b+, F4/80+), inflammatory monocytes (CD45+, CD11bint, Ly6Chigh, Ly6G- or CD45+, CD11bint, Gr1int), resident monocytes (CD45+, CD11bint, Ly6Cint, Ly6G-), MDSC (CD45+, CD11bhigh, Ly6Cint, Ly6G+ or CD45+, CD11bhigh, Gr1high), Treg (CD45+, CD4+, CD25+, FoxP3+). The gate strategy could be observed in the **Supplementary Figure S1**. Cells were acquired by LSR Fortessa™ (BD Biosciences) flow cytometer and data were analyzed using the FlowJo software.

Intracellular Cytokine Staining

Intracellular IFN-γ staining was performed as previously described (29). The mouse peripheral blood mononuclear cells were collected in heparin-containing vials14 days after the last gDE7 immunization (28 day).

Statistical Analysis

Statistical analyses were performed using Prism (GraphPad) software. The analysis was performed using the unpaired *T*-test, One-Way ANOVA or Two-Way ANOVA and the results confirmed through multiple comparisons by Tukey's test. Values of $p < 0.05$ were considered significant.

RESULTS

TC-1 Cells Express IDO

Usually IDO expression in murine tumor cells is observed after transfection of cells with IDO1 encoding viruses or after genetic manipulations (33). Here, we verified that, in contrast to other cell lines, the TC-1 cell line express IDO constitutively. IDO expression in TC-1 cells was demonstrated by flow cytometry using an isotype control antibody as a comparative control (**Figure 1A**). IDO in TC-1 cells was upregulated by IFN-γ but not by melatonin, D-1MT, L-1MT, and DL-1MT (**Figure 1B**). In addition, TC-1 cells accumulate kynurenine in culture supernatants, which decreased significantly in the presence of DL-1MT (**Figure 1C**). These results indicate that IDO is enzymatically active in TC-1 cells.

Melatonin and 1MT Have Direct Effects on TC-1 Cells Migration, Adhesion and Viability

We next evaluated whether melatonin and IDO inhibitors would have a direct effect on the *in vitro* growth of the TC-1 tumor cells. With this purpose, we carried out wound healing assays

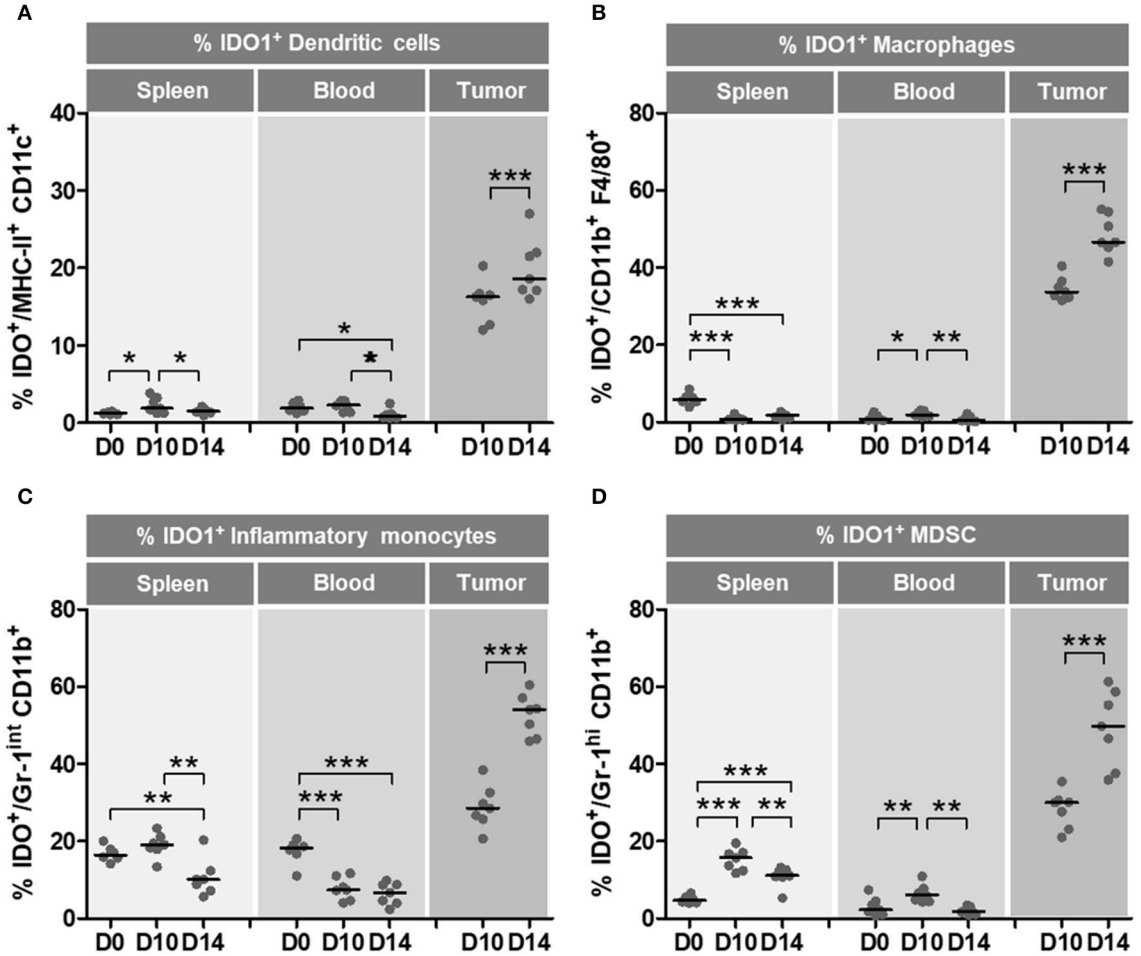

FIGURE 2 | Immune microenvironment cells increase IDO1 expression during oncogenesis. Wild-type (WT) C57BL/6 mice ($n = 7$) were subcutaneously injected with 1×10^5 TC-1 cells and tumors were evaluated at 10 and 14 day after the tumor cell transplantation. Naïve mice were used as a reference of physiological parameters. The frequencies of DCs, macrophages, inflammatory monocytes and MDSC were evaluated in spleens, blood and tumor tissues. IDO1 expression was measured by intracellular staining and flow cytometry analyses. **(A–D)** Frequency of IDO1-expressing immune cells at 0 day (D0–naïve mice), 10 day (D10) and 14 day (D14) after TC-1 transplantation. Parameter used to characterize the different cell populations: **(A)** DCs ($CD45^+$, $CD11c^{high}$, MCH-II^{high}), **(B)** macrophages ($CD45^+$, MCH-II^+, $CD11b^+$, $F4/80^+$), **(C)** inflammatory monocytes ($CD45^+$, $CD11b^{int}$, $Ly6C^{high}$, $Ly6G^-$), **(D)** MDSC ($CD45^+$, $CD11b^{high}$, $Ly6C^{int}$, $Ly6G^+$). Data representative of two independently performed experiments. Statistical significance: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by ANOVA.

for assessment of cell migration. As shown in **Figures 1D,E**, melatonin reduced the migratory behavior of TC-1 cells and similar effects were observed in cells treated with L-1MT and DL-1MT. Interestingly, D-1MT did not show any significant effect on migration of TC-1 cells. We also measured the attachment of the TC-1 cells to a plastic surface and all immunomodulators caused a partial impairment of the cell adhesion behavior when compared with untreated cells (**Figures 1F,G**). No difference was observed between cells treated with melatonin and D-1MT, which decreased cell adhesion by approximately 20%. The racemic mixture of 1MT isomers reduced approximately 50% of cell adhesion, whereas L-1MT decreased cell adhesion by approximately 36% (**Figure 1G**). Additionally, melatonin, L-1MT and DL-1MT decreased cell proliferation capacity while melatonin and D-1MT were more cytotoxic than L-1MT and DL-1MT (**Figures 1H,I**). Taken together, these results demonstrate direct effects of melatonin and 1MT derivates on TC-1 cell behavior.

IDO Expression in Immune Cells Increases in the Course of Tumor Growth and Impairs the Antitumor Effects of gDE7-Based Immunotherapy

To evaluate the role of IDO1 in the *in vivo* growth of TC-1 cells, expression of IDO1 was measured in DCs, macrophages, inflammatory monocytes and MDSCs from spleen, blood and tumor masses at 10 and 14 days after tumor cell transplantation in wild type mice. As shown in **Figure 2**, there was a substantial increase of IDO1 expression in immune cells at the tumor microenvironment over time. Comparing 0 and 10 day with day 14 post TC-1 transplantation, we observed a concomitant

decrease in the number of IDO1-expressing DCs (**Figure 2A**), macrophages (**Figure 2B**), inflammatory monocytes (**Figure 2C**) and MDSCs (**Figure 2D**) in spleen and blood of TC1-grafted mice, which suggests that these cells are migrating to the tumor site. These observations emphasize that IDO1 expression by immune cells in the tumor microenvironment contributes to the immunosuppressive environment that may affect the efficacy of immunotherapies.

To further evaluated the role of IDO1 in the growth of tumor cells and tissue-specific microenvironment, we grafted the TC-1 cells in IDO1$^{-/-}$ mice and measured the presence of macrophage, DCs and immunosuppressive cells in spleens and tumor tissues 21 post tumor cell transplantation (**Figures 3**). Interestingly, larger tumors were observed in IDO1$^{-/-}$ mice regarding the parental mouse strain, although no difference was observed in the spleens (**Figures 3A–D**). Moreover, although Treg cell population was increased in the spleen of the IDO1$^{-/-}$ mice, the frequency of these cells in the tumor microenvironment was the same in both mouse strains (**Figure 3E**). In contrast, the capability of DCs to migrate to the tumor site was reduced in IDO$^{-/-}$ mice (**Figure 3F**), while there was no difference in the frequency of macrophages frequency in the spleen and tumor from both mouse strains (**Figure 3G**). Interestingly, while IDO1$^{-/-}$ mice had a higher frequency of MDSCs in the blood and tumors than the C57Bl/6 mice, we observed a higher frequency of resident monocytes in the spleens and a higher frequency of inflammatory monocytes in both spleens and blood of IDO$^{-/-}$ mice (**Figures 3H–J**). However, at 21 day post TC-1 cell engraftment, no differences in the frequency of these cells were observed in the tumor microenvironment (**Figure 3J**). It is important to highlight that cells from IDO$^{-/-}$ mice did not express IDO1 (data not shown) and the only source of this enzyme was the transplanted TC-1 cells.

Subsequently, we evaluated the impact of IDO1 expression on the efficacy of gDE7-based immunotherapy in C57Bl/6 and isogenic IDO1$^{-/-}$ mice. Seven days after TC-1 injection, mice were immunized at a suboptimal conditions (28, 29). The vaccine was subcutaneously administered twice (7-day interval), starting 7 days after TC-1 cells challenge, a time point in which the tumors became palpable (**Figure 4A**). Although we observed a tendency of tumors to be higher in IDO1-deficient animals, there was no statistical difference concerning the tumor growth kinetics up to 35 days, in both WT and IDO$^{-/-}$ not immunized mice, when tumors achieve a diameter of approximately 13-14 mm (**Figure 4B**). In contrast, the protective immunity conferred by gDE7 was significantly enhanced in IDO$^{-/-}$ mice regarding WT mice (100% and 20% survival, respectively) (**Figures 4B–D**). These results demonstrated that although the absence of IDO1 did not impair TC-1 cells *in vivo* growth, IDO1 have a dramatic effect in the modulation of protective antitumor immune responses elicited by animals submitted to the immunotherapy.

Combination of Melatonin and 1-DL-MT Improves the Efficacy of gDE7-Based Immunotherapy

We next evaluated if association of a suboptimal vaccine regimen combined with melatonin and1-MT would enhance the antitumor immunity elicited in mice challenged with TC-1 cells. The treatment with immunometabolic adjuvants started 2 days after the first gDE7 dose (9 day) and finished on 36 day (**Figure 5A**). As previously shown, mice treated only with the vaccine (gDE7 group) showed partial tumor control (**Figure 5B**) and no tumor-free record (**Figure 5C**). Similarly, mice treated only with the immunomodulators did not exhibit significant tumor growth control (**Figures 5D,E**). Meanwhile, the combination of the immunotherapy and the immunometabolic adjuvants promoted a significant increase in the antitumor protective immunity (**Figures 5B,C,F**). Mice treated with gDE7 and melatonin, D-1MT or DL-1MT showed significant tumor growth delay compared with the gDE7-treated group (**Figure 5B**). Lower protective values were observed when the numbers of tumor-free mice were considered, with 10% tumor-free mice for animals treated with gDE7 and melatonin, or gDE7 and DL-1MT, and none for those animals treated with gDE7 or gDE7 plus D-1MT (**Figure 5C**). However, the combination of melatonin and DL-1MT, but not with D-1MT, synergistically enhanced the anti-tumor protective effects conferred by gDE7, leading to a complete antitumor protection in 60% of challenged mice (**Figures 5C,F**) at the end of the observation period. Collectively, these results demonstrated that melatonin improves the performance of a cancer immunotherapy when combined with DL-1MT.

Finally, we next evaluated the presence of circulating cytotoxic CD8$^+$ T cells, which have a pivotal role on the elimination of the tumor cells. The results indicated an increased number of IFN-γ producing CD8$^+$ T cells in gDE7-immunized mice treated or not with melatonin or 1MT compounds compared to non-immunized group (**Figures 5G,H**). Noticeably, E7-specific CD8$^+$ T cells isolated from mice treated with both melatonin and DL-1MT produced higher levels of IFN-γ in response to stimulation with the E7-derived peptide compared to the other immunization groups (**Figure 5H**). Taken together, the present data demonstrated that the association of melatonin and DL-1MT synergistically enhance the induction of protective immune responses and increase the antitumor immunity elicited in immunized mice.

DISCUSSION

Considering the increase association of IDO1 expression and HPV-induced malignancies, incorporation of two immunometabolic adjuvants, melatonin and IDO1 inhibitors, to an anti-cancer vaccine resulted in enhanced *in vivo* antitumor effectiveness without visually noticeable side effects usually observed with other anti-cancer treatments. The combination of IDO inhibitors to the immunotherapy clearly increased the anti-cancer effects by reducing the negative impact of IDO1 expression on the protective immunity induced by the vaccine. The incorporation of melatonin to the proposed immunization regimen was supported by previous evidences that, in addition to its oncostatic properties (34), it negatively regulates IDO1 expression and its synthesis can be driven by 1MT, a classical IDO1 inhibitor (16). The results demonstrate that the unprecedented combination of melatonin and IDO1 inhibitors (particularly DL-1MT) improves the

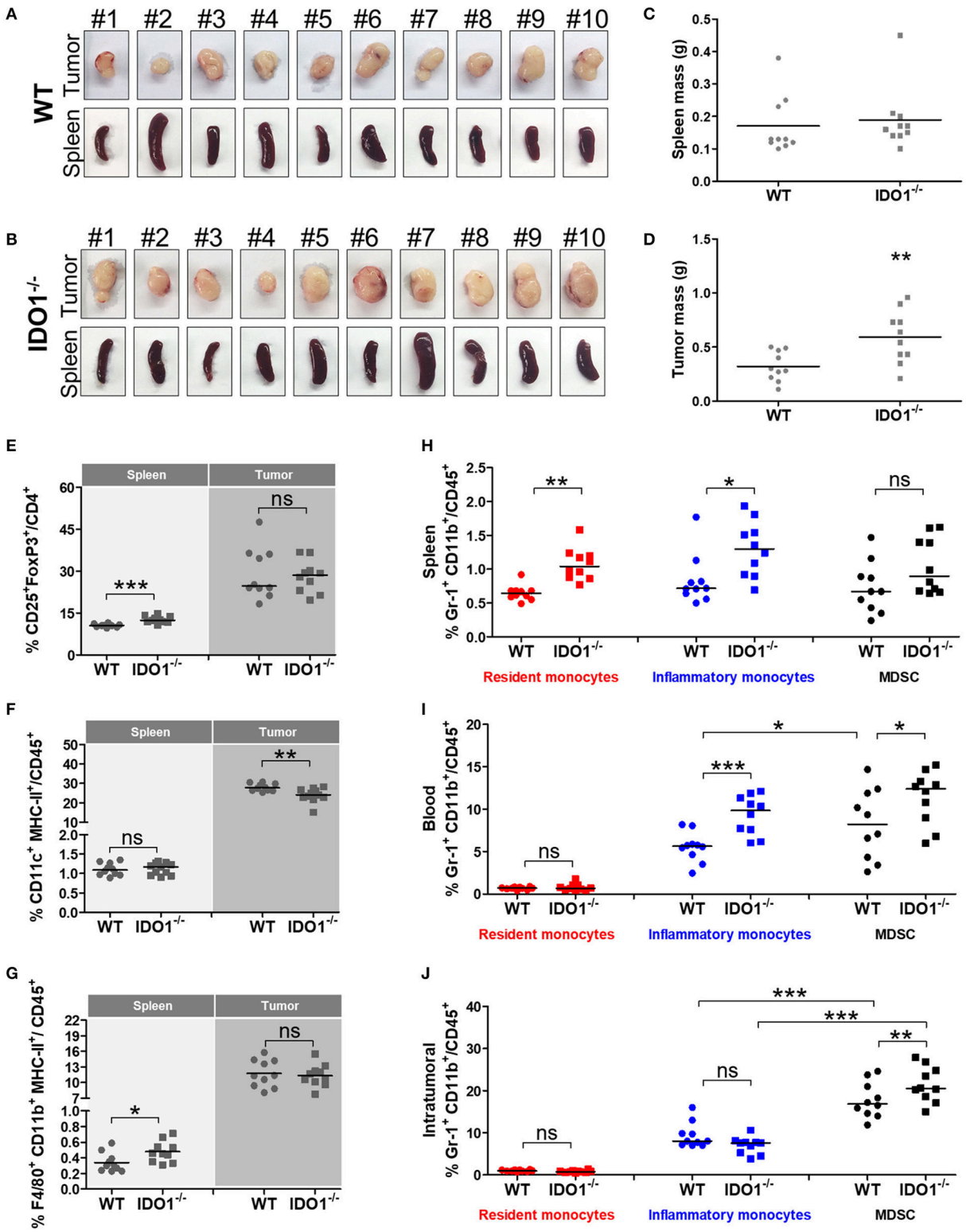

FIGURE 3 | The absence of IDO1 expression modifies the frequency of immunological cells in distinct mice tissues. Images of tumor masses and spleen from wild-type (WT) **(A)** and IDO$^{-/-}$ **(B)** mice subcutaneously injected with 1×10^5 TC-1 cells and euthanized 21 days later ($n = 10$ per group). Comparative analyses of **(C)** spleen and **(D)** tumor masses of WT and IDO$^{-/-}$ mice. Comparative analyses of **(E)** Treg, **(F)** DCs and **(G)** macrophages in spleens and tumor tissues of WT and IDO$^{-/-}$ mice. Comparative analyses of resident monocytes, inflammatory monocytes and MDSC in **(H)** spleens, **(I)** blood and **(J)** tumor tissues of WT and IDO$^{-/-}$ mice. Statistical significance: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by ANOVA or unpaired Student's *t*-test. (ns) Non-significant.

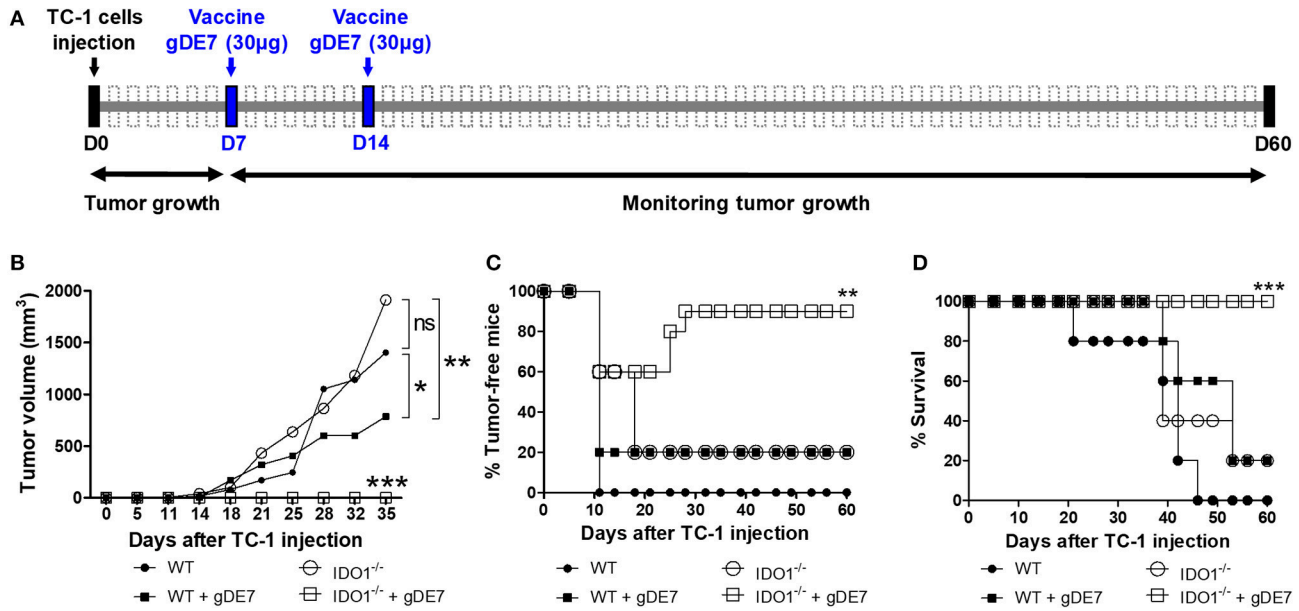

FIGURE 4 | IDO expression impairs the antitumor effects of gDE7-based immunotherapy. **(A)** Wild-type (WT) and IDO$^{-/-}$mice were subcutaneously injected with 1 × 10^5 TC-1 cells and immunized with two doses (7-day interval) of gDE7 (30 µg per animal) via subcutaneous (s.c.) route. Tumor growth was monitored until 60 day, when the animals were euthanized. Antitumor effects were measured by **(B)** tumor volume, **(C)** the percentage of tumor-free mice and **(D)** the percentage of mice survival. Vaccinated IDO$^{-/-}$ mice were significantly different than all other groups, highlighting that the gDE7-dependent antitumor effects are enhanced by inactivation of host IDO gene expression. Data from two identical experiments ($n = 5$) were pooled and analyzed by ANOVA or by Kaplan-Meyer test (exclusively for survival assay). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. (ns) Non-significant. When not signaled, * represents the statistical significance of one experimental group in relation to all others.

performance of the anti-cancer vaccine, leading to enhanced antitumor protection and activation of E7-specific CD8$^+$ T cell response. Altogether, the present evidences further support the beneficial effects of immunometabolic adjuvants to the treatment of tumors, particularly those associated with papillomaviruses, and support further investigations under clinical conditions.

A hierarchical profile between IDO1 expression have been observed in different cancer types, whereupon endometrial and cervical cancer had the highest and most frequent IDO1 expression (35, 36). In cervical cancer, IDO$^+$ cells were often located at the periphery of tumor nodules, surrounded by IFN-γ producing T lymphocytes (35, 36). The involvement of IDO1 in the mounting of an immunosuppressive microenvironment in HPV-associated cervical cancer was first reported in 2008 by Kobayashi and collaborators, who showed that the numbers of IDO1-expressing immune cells significantly increased from normal cervix condition to the cancerous state (37). Notably, by measurement of tryptophan and kynurenines metabolites in serum samples of cervical cancer patients, enzymatically active IDO1 was associated with a poor clinical outcome (38). Indeed, several pathological parameters, such as tumor size, lymph node metastasis and advanced disease stage, emphasize the role of IDO1 expression in promotion of tumor growth and highlight the potential positive impacts of IDO1 inhibition in the fate of tumor treatments (38). Regarding the contribution of IDO1 to an immunosuppressive milieu, microenvironment analyses revealed higher IDO1 expression in dermal DCs from grafted skin cells expressing HPV-16 E7 oncoprotein than nontransgenic control skin cells. In addition, treatment of mice engrafted with HPV oncoprotein-expressing cells with DL-1MT promoted skin graft rejection (39).

The increased frequencies of IDO1-expressing cells, such as DCs, macrophages, inflammatory monocytes and MDSC, in mice transplanted with TC-1 cells indicated that the use of IDO1 inhibitors would improve tumor growth control. We initially used IDO1-deficient mice (IDO1$^{-/-}$) to evaluate the influence of IDO1 expression on tumor growth. Interestingly, lack of IDO1 expression in the host did not impair the *in vivo* growth of TC-1 cells. This outcome has also been observed in the melanoma (B16F10 cells) tumor model (33) and in the azoxymethane-induced colon tumor model (40). Nonetheless, lack of IDO1 expression positively impacted the protective antitumor immunity elicited in mice immunized with gDE7. This phenomenon could be partially explained by the greater inflammatory potential of these animals when compared to WT mice, since IDO1$^{-/-}$ tumor-bearing mice showed higher frequencies of resident monocytes in spleens and inflammatory monocytes in both spleens and blood. Indeed, in a pulmonary model of paracoccidioidomycosis, the absence of IDO1 expression led to a higher influx of activated inflammatory cells into the lungs, which promoted an increased expansion of T cells (41). Moreover, IDO1 knockout mice showed increased pro-inflammatory cytokines expression and decreased Treg cells

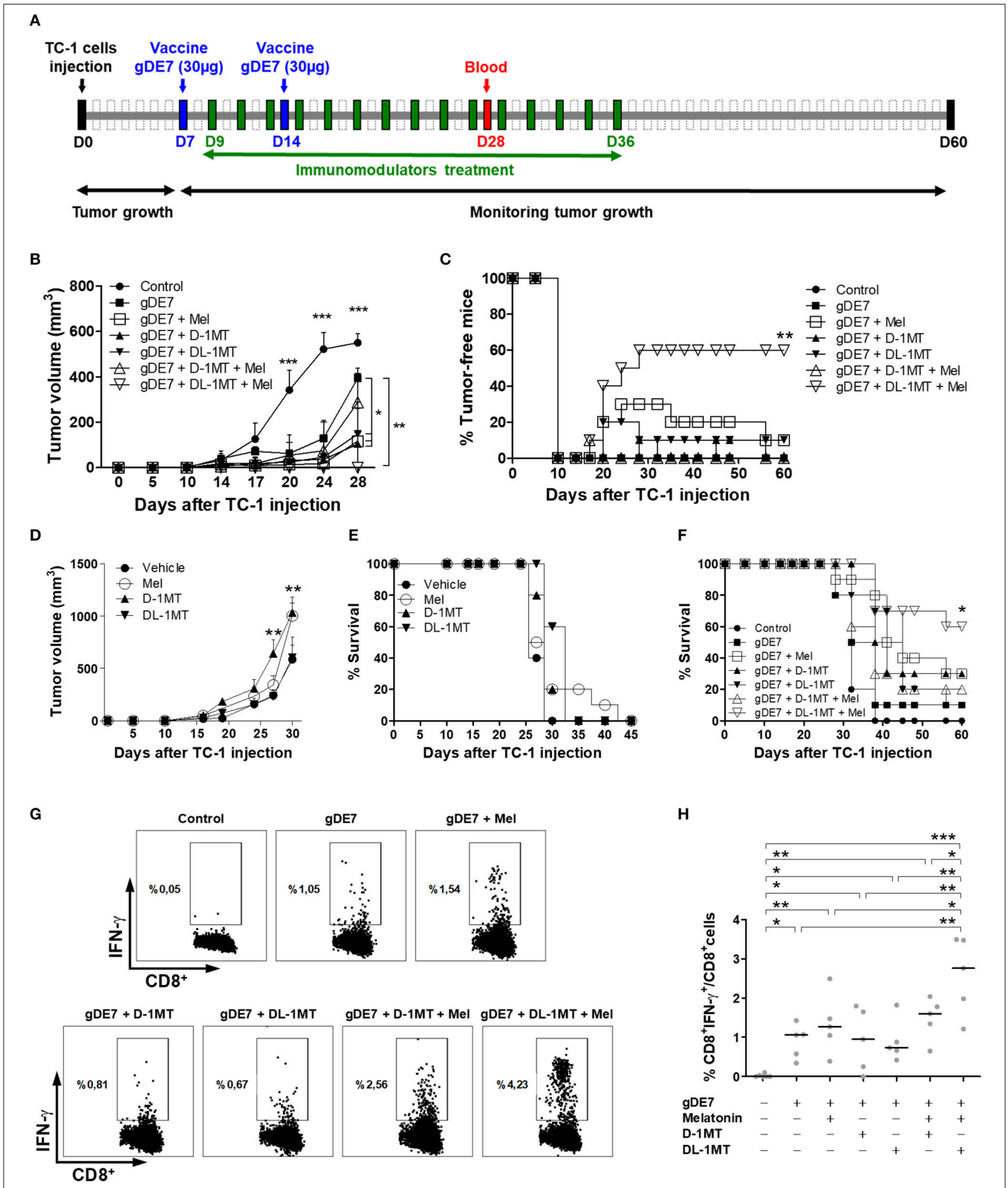

FIGURE 5 | Co-administration of melatonin and DL-1MT synergistically enhances the antitumor effects of gDE7-based vaccine in immunized mice. **(A)** WT mice were subcutaneously injected with 1 × 10^5 TC-1 cells and immunized with two doses (7-day interval) of gDE7 (30 µg per animal) via the s.c. route (day 7-D7

(Continued)

FIGURE 5 | and day 14-D14). The treatment with melatonin (0.2 mg per animal, intraperitoneally) or 1MT compounds (10 mg per animal through gavage) started 2 days after the first vaccine dose (day 9-D9) and every 48 h till 36 day (D36). Untreated and unvaccinated mice were considered control groups. The tumor growth was monitored for 60 days (D60). **(B,C,F)** An enhanced antitumor effect induced by gDE7 was observed after its combination with a single immunometabolic adjuvant. However, the association of melatonin (Mel), DL-1MT and gDE7 resulted in maximal antitumor effects regarding **(B)** tumor size, **(C)** tumor eradication (tumor-free-mice) and **(F)** mice survival. To analyze the effect of immunometabolic adjuvants on tumor growth, C57BL/6 mice were subcutaneously injected with 1×10^5 TC-1 cells and treated with 10 mg/animal of D-1MT or DL-1MT or 0.2 mg/200 μL/animal of melatonin for 4 weeks every 48 h, starting on 9 day. One group received apyrogenic saline and the 1MT vehicle as a control. Antitumor effects for each tested group was evaluated by **(D)** tumor volume and **(E)** the percentage of survival. The tumor growth was monitored until 45 day, when the animals were euthanized due to tumor size. **(G,H)** Blood samples were harvest at 28 day (D28) post TC-1 cells injection and analyzed for the frequencies of activated $CD8^+$ T-cells ($CD8^+$IFN-γ^+ T-cells) by flow cytometry. **(G)** Plots of circulating E7-specific IFN-γ producing $CD8^+$ T cells. **(H)** Percentages of circulating E7-specific $CD8^+$IFN-γ^+/$CD8^+$ T cells in each tested mice groups. Data from two identical experiments ($n = 5$) were pooled and analyzed by ANOVA or by Kaplan-Meyer test (exclusively for survival assay). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. (ns) Non-significant. When not signaled, * represents the statistical significance of one experimental group in relation to all others. Data represent means ± SD from one representative of two independently performed experiments ($n = 5$) with comparable results.

in a colon tumor mouse model (40). Thus, the present results add a new piece of evidence that IDO1-targeting therapy could improve antitumor therapies by reprogramming inflammatory cells.

Since cancer is a multifactorial disease that arises from alterations in different physiological processes, multidrug anti-cancer treatments may reach a better outcome at clinical conditions. Currently, passive immunotherapies, based on mAbs targeting different cellular checkpoint controllers, had changed the landscape of cancer treatment, such as those blocking cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) or its ligand PD-L1 (42). However, treatment of solid tumor still poses a challenge due to the frequent emergence of either innate or acquired resistance (43). Indeed, IDO1-expressing cells promote PD-L1 expression in DCs, which in turn activate Treg cells, while treatment with anti-PD-L1 or anti-PD-L2 or anti-PD-1 can reverse this effect (44). In addition, IDO1 expression has been associated with the neovascularization of tumor metastasis (45). In this scenario, the combination of IDO1 inhibitors with immunotherapies and other anti-cancer drugs seems to be particularly encouraging and emphasizes the relevant role of tumor biology knowledge in the development of more efficient therapies.

Regarding the translational use of IDO inhibitors, different clinical trials are ongoing. Recently, a failure on a phase 3 trial in metastatic melanoma, based on the combination of epacadostat (IDO1 inhibitor) with pembrolizumab (anti-PD-1 antibody), generated a disappointment in the so-called "second generation" of immuno-oncology drugs (clinical trial information: NCT02752074) (25). However, in another trial based on epacadostat plus a multipeptide melanoma vaccine, besides normalized serum kynurenine/tryptophan ratios in most patients, data indicated an enhancement of $CD8^+$ T cell infiltration in tumor milieu in patients with melanoma submitted to the combination therapy (clinical trial information: NCT01961115) (26). Interestingly, a phase 2 trial with another class of IDO inhibitor, the indoximod (D-1MT), plus gemcitabine and nab-paclitaxel showed promising results regarding the use of IDO inhibitors for patients with metastatic pancreas cancer (clinical trial information: NCT02077881) (27). In this trial, data indicate increased intra-tumoral $CD8^+$ T cell density in biopsies of responder patients submitted to the combination therapy. Overall, these data highlight the importance of the association of antitumor vaccines, and/or immuno-chemotherapy with IDO inhibitors.

We recently showed that the gDE7-based vaccine induces multifunctional E7-specific $CD8^+$ T cells with cytotoxic activity as well as expansion of effector memory T cells and activation of mouse and human specialized DC subset capable to promote antigen cross-presentation (29). In the present study, we observed that treatment with one metabolic adjuvant provided enhanced gDE7-mediated antitumor protection but only the combination of melatonin and one IDO inhibitor conferred complete tumor protection. Regarding IDO1 inhibitors, we observed a superior preclinical antitumor activity relative to DL-1MT in side-by-side comparisons to D-1MT, which is the isoform actually under clinical trials (23). Previous evidences indicated that D-1MT was more effective than DL-1MT as an anti-cancer agent and reversed the T cell suppression effect mediated by IDO1-expressing DCs (33). Similarly, recruitment and activation of tumor-infiltrating MDSCs and regulatory T cells, driven by expression of IDO1, could be successfully reversed by D-1MT in mice (19). On the other hand, the anti-cancer effects of DL-1MT has been attributed to capacity to abrogate the anti-proliferative effects of IDO1-expressing mesenchymal stromal cells (46). Additionally, DL-1MT can down-regulates expression of paxillin-family proteins and promotes activation of AHR-driven responses in mesenchymal stromal cells (47) rising a pro-inflammatory signature that may augment the efficacy of cancer immunotherapies. From the point of view of the direct effects of 1MT on TC-1 cells, it is important to notice that 1MT isomers and, its racemic mixture, showed distinct *in vitro* cellular effects. D-1MT proved to be more cytotoxic than the other compounds whereas DL-1MT notably impacted the adhesion of the TC-1 cells, a phenomenon that could be explained by its presumed impact on the disturbance of cytoskeleton proteins (44). Our data brings additional information about the effects of 1MT, showing that besides the modulation of the inflammatory responses, 1MT isomers have also significant effects on the cell behavior that may impact antitumor responses induced by the therapy.

Regarding the fact that 1MT (D-1MT or DL-1MT) can promote partial activation of $CD8^+$ T cells, the addition of melatonin to the combined immunotherapy led to increased frequencies of tumor-reactive cytotoxic T lymphocytes capable to clear tumor cells. Available evidence indicates that melatonin enhances human and mice T cells activation (12), and is

involved in the regulation immune functions by modulating T cells polarization (48). Therefore, melatonin has been used to synergize immune-activation with conventional cancer treatment modalities, emerging as an important anti-cancer molecule acting at different stages of tumor progression (49). In fact, melatonin acts as an anti-cancer inhibitory molecule targeting anti-proliferative signaling (16), angiogenesis (50), tumor evading mechanisms (48), tumor metastasis (51) and induction of cell death (52). Additionally, melatonin powerfully enhances cisplatin-induced cytotoxicity and apoptosis in cervical cancer HeLa cells *in vitro*, exhibiting cytotoxic, pro-oxidant, and pro-apoptotic actions in this cells line (52).

It is important to highlight that the therapeutic potential of both melatonin and 1MT were observed only when combined with gDE7-based vaccine. Treatment of tumor-bearing mice with melatonin or 1MT without co-administration of gDE7 did not generate significant antitumor protection. In fact, treatment with melatonin or D-1MT promoted faster tumor growth in mice implanted with TC-1 cells. Therefore, the present results demonstrate that the antitumor effects of melatonin and 1MT are restricted to conditions where the drugs are co-administered to animals in combination with an active immunotherapy, which emphasizes their metabolic adjuvant roles.

In conclusion, the present study demonstrates that the combination of melatonin and IDO1 inhibitors display synergistic effects when combined with a tumor-specific immunotherapy and, therefore, represents a new and promising perspective for the control of HPV-associated tumors, and possibly other cancer types.

AUTHOR CONTRIBUTIONS

AM: Conception and design, development of methodology, acquisition of data, analysis and interpretation of data, writing, review and/or revision of the manuscript, administrative, technical, or material support, and study supervision. BP: Acquisition of data, analysis and interpretation of data, writing, review and/or revision of the manuscript. RLP: Acquisition of data, writing, review and/or revision of the manuscript. PS: Acquisition of data. RP: Acquisition of data. KR: Acquisition of data. TB: Acquisition of data. LA: Writing, review and/or revision of the manuscript. EdA: Acquisition of data. VC: Administrative, technical, or material support, review and/or revision of the manuscript. LF: Conception and design, interpretation of data, review and/or revision of the manuscript, administrative, technical, or material support, study supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.01914/full#supplementary-material

Supplementary Figure S1 | Gating strategy for the evaluation of immune cells in blood, spleen and tumor microenvironment. Doublets were initially excluded from analysis by FSC and SSC parameters. Cells were gated by the expression of $CD45^+$ and subsequently separated according to cell type specific markers. Dendritic cells and macrophages were distinguished by $CD11c^{high}$ $MCH\text{-}II^{high}$ and $MCH\text{-}II^+$ $CD11b^+$ $F4/80^+$ expression, respectively. Resident monocytes were characterizedby the expression of $CD11b^{int}$ $Ly6C^{int}$ $Gr1^-$, inflammatory monocytes by the expression of $CD11b^{int}$ $Ly6C^{high}$ $Ly6G^-$ or $CD11b^{int}$ $Gr1^{int}$ and MDSC by the expression of $CD11b^{high}$ $Ly6C^{int}$ $Ly6G^+$ or $CD11b^{high}$ $Gr1^{high}$. Inflammatory myeloid cells were considered tolerogenic when IDO expression was detected intracellularly. For Treg cells analysis cells were separated by the expression of $CD4^+$ followed by gating on $CD25^+$ $FoxP3^+$. Finally, the antitumor specific response were caracterized by E7-specificIFN-γ^+ producing $CD8^+$ T cells.

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OPINION
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What May Constrain the Success of Indoleamine 2,3-Dioxygenase 1 Inhibitors in Cancer Immunotherapy?

*Theodoros Eleftheriadis**

Department of Nephrology, Faculty of Medicine, University of Thessaly, Larissa, Greece

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Patrik Andersson, Harvard Medical School, United States

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Francesca Fallarino, University of Perugia, Italy

***Correspondence:**
Theodoros Eleftheriadis
teleftheriadis@yahoo.com

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Indoleamine 2,3-dioxygenase 1 (IDO1) catalyzes the initial rate-limiting step of tryptophan degradation along the kynurenine pathway and suppresses T-cell immune response by two paths; the activation of general control non-derepressible 2 kinase (GCN2K) and aryl-hydrocarbon receptor (AhR). In the microenvironment of the immune response, tryptophan depletion activates GCN2K, which inhibits T-cell proliferation and induces T-cell apoptosis (1). From a teleological point of view, the selection of tryptophan depletion as an immunomodulatory mechanism is ingenious. Tryptophan is an essential amino acid not synthesized by human cells, its concentration in the body is the lowest among all amino acids, and its deprivation due to low intake appears only in 2 days (2). Thus, its depletion in the microenvironment of inflammation can emerge acutely. Interestingly, and indicating the specific role of the above immunomodulatory mechanism, IDO1-induced tryptophan depletion does not affect the other amino acid sensing system, the mammalian target of rapamycin complex 1 (mTORC1), in T-cells (3–5), which is in accordance with studies showing that mTORC1 is sensitive to the depletion of specific amino acids; more precisely of leucine, isoleucine, valine, and possibly arginine, but not of tryptophan (6). In parallel with IDO1-induced GCN2K activation, kynurenine, a derivative of tryptophan degradation, activates AhR, which induces naïve CD4+ T-cell differentiation into regulatory T-cells (7).

The immunosuppressive properties of IDO1 were discovered by the observation that its expression in the placenta contributes to a successful semi-allogenic pregnancy (8). Then it was revealed that inflammatory stimuli induce IDO1 expression in antigen-presenting cells, and the immunosuppressive role of this enzyme has been confirmed in experimental models of autoimmunity and transplantation (9–12).

Indoleamine 2,3-dioxygenase 1 is also expressed in many types of cancer, and the majority of studies suggest that this enzyme plays a significant role in the escape of tumors from immunosurveillance (13, 14). More precisely in various types of cancer, IDO1 expression has been confirmed, individually or in combination, in tumor cells, in interstitial cells in lymphocyte-rich areas, and in endothelial cells. In most cases, IDO1 expression seems to be the result of an ongoing immune response by infiltrating T-cells and other immune cells that produce interferon-γ (IFN-γ) (14, 15), a cytokine that induces macrophage and dendritic cell (DC) activation and IDO1 expression (13, 14, 16). The infiltrating immune cells fail to eliminate cancer cells because due to accumulated mutations they escape the initial immune response. The persisted immune response results in increased IDO1 expression by tolerogenic DCs, myeloid-derived suppressor cells, and tumor-associated macrophages. Tryptophan depletion and kynurenine production by IDO1 induce more immune cells to become tolerogenic and inhibit effector T-cells, whereas increase regulatory T-cells. Regulatory T-cells by expressing cytotoxic T-lymphocyte-associated-antigen-4 (CTLA-4) inhibit further effector T-cells and increase IDO1 expression in DCs closing a positive feedback loop of immunosuppression (16). However, in a subset of tumors IDO1 is expressed by cancer cells in the absence of any inflammation indicating that it may be the result of oncogenic events and may contribute to escape of tumor by immunosurveillance by preventing T-cell infiltration (14, 15).

Nevertheless, other studies question the role of IDO1 overexpression in the adverse clinical outcome of certain cancers. Ishio et al. found that the recurrence-free survival rate of patients with IDO1-positive hepatocellular carcinoma is significantly higher than that of patients with IDO1-negative hepatocellular carcinoma (17). Takao et al. showed that increased IDO1 protein is related to worse prognosis in patients with serous type, but not with clear cell or endometrioid type of ovarian adenocarcinoma (18). Riesenberg et al. revealed that the expression of IDO1 in tumor endothelial cells correlates with long-term survival of patients with renal cell carcinoma (19). Jacquemier et al. determined that high IDO expression is associated with morphological medullary features and has an independent favorable prognostic value in patients with basal-like breast carcinoma (20). Recently, Heeren et al. showed that in patients with early stage cervical cancer, a marginal IDO expression pattern in the tumor dominantly predicts a favorable outcome, which might be related to IFN-γ release in the cervical tumor microenvironment (21).

Most importantly, despite the initial experimental and clinical indications about the efficacy of IDO1 inhibitors in cancer immunotherapy (16), in the recently Incyte's phase III clinical trial, the addition of the IDO1 inhibitor epacadostat in a therapy with the programmed death 1 immune checkpoint inhibitor pembrolizumab, made no difference for the patients with metastatic melanoma receiving both drugs. This failure led three companies to the decision to suspend, cancel, or downsize 13 trials of IDO1 inhibitors in combination with immune checkpoint inhibitors (22).

There are some possible explanations for these disappointing results. First, IDO1 expression, confirmed by either immunohistochemistry or polymerase chain reaction, in a tumor does not necessarily mean that this enzyme is functional. For instance, IFN-γ induces both the expression of IDO1 and the production of nitrogen monoxide (NO) in macrophages, but the latter inhibits IDO1 enzymatic activity (23). Also, in an inflammatory environment, both NO and superoxide anion are produced resulting in the generation of peroxynitrite anion, which inhibits by nitration IDO1

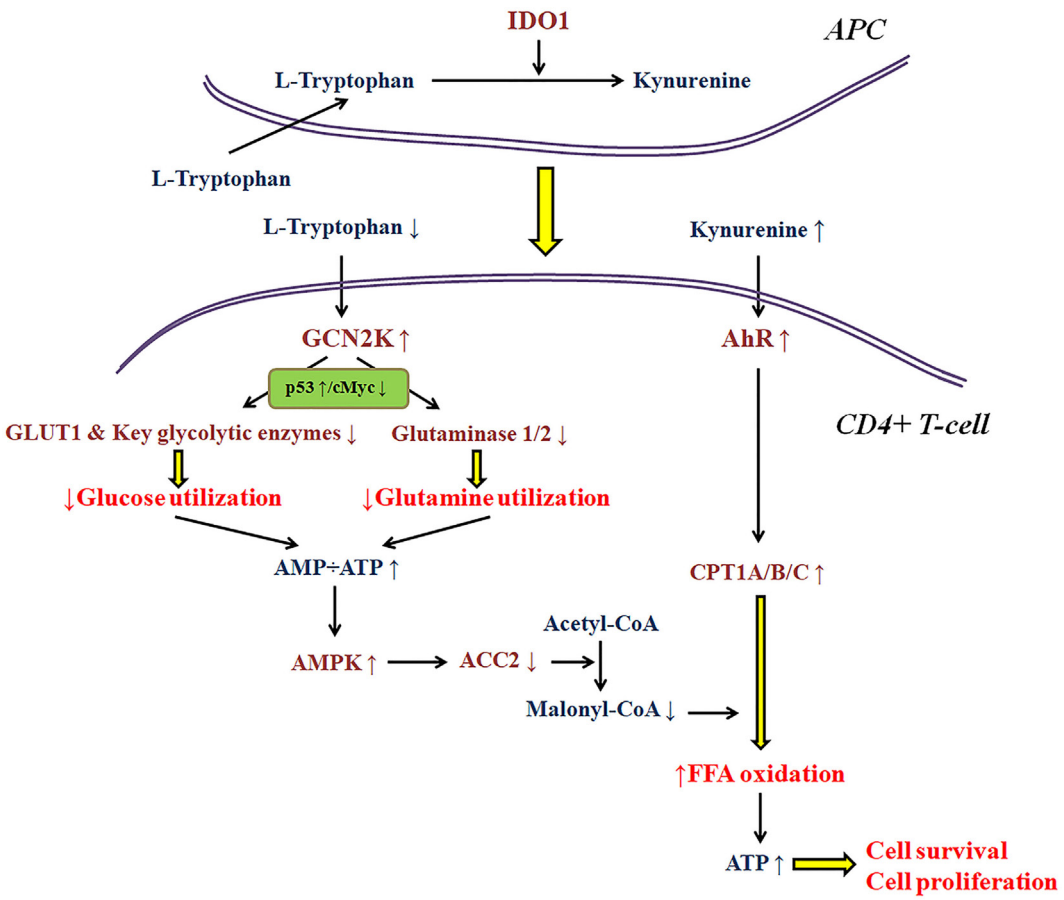

FIGURE 1 | A model about the effect of indoleamine 2,3-dioxygenase 1 (IDO1) on the utilization of the main energy sources by activated CD4+ T-cells. In the immune response microenvironment, IDO1 by degrading L-tryptophan along the kynurenine pathway activates general control non-derepressible 2 kinase (GCN2K) and aryl-hydrocarbon receptor (AhR). By upregulating the transcription factor p53 and downregulating the transcription factor c-Myc, activated GCN2K decreases the expression of glucose transporter 1 (GLUT1), key glycolytic enzymes, and glutaminases inhibiting the consumption of glucose and glutamine. The reduced utilization of these pivotal sources of energy by activated T-cells results in reduced ATP production. The latter activates AMP-activated protein kinase (AMPK), which phosphorylates and inactivates acetyl-CoA carboxylase 2 (ACC2) resulting in decreased production of the carnitine palmitoyltransferase I (CPT1) inhibitor malonyl-CoA. In parallel, activation of AhR increases the expression of all CPT1 isoenzymes. Since CPT1 controls free fatty acid oxidation, these IDO-induced alterations promote free fatty acid oxidation as an alternative fuel for ATP production, supplying the required energy for CD4+ T-cell survival and proliferation.

enzymatic activity without affecting its protein level (24). Moreover, phosphorylation of specific IDO1 tyrosine residues blocks its catalytic activity (25). Thus, assessing along with IDO1 expression its enzymatic activity by detecting in the tumors along with IDO1, proteins that are known to be modified or expressed after GCN2K or AhR activation would yield more accurate results about the role of this enzyme in the escape of tumors from immunosurveillance.

In addition, IDO1, by activating GCN2K, alters the metabolism of T-cells, inhibits their proliferation and induces apoptosis in a p53-dependent way (4, 26, 27). The transcription factor p53, also known as tumor suppressor p53, inhibits aerobic glycolysis, which characterizes rapidly proliferating cells, and induces cell cycle arrest and/or apoptosis (28, 29). Interestingly, activated GCN2K also increases p53 expression in nonimmune cells, such as human aortic endothelial and renal epithelial cells (30, 31). The fact that in most the tumors the p53 pathway is directly or indirectly inactivated (28), offers an advantage in cancer progression. IDO1 expressed by cancer cells or infiltrating immune cells by depleting tryptophan in the local microenvironment activates GCN2K in the T-cells that infiltrate the lesion inhibiting their proliferation and inducing apoptosis. On the contrary, due to the ineffective p53 pathway in the cancer cells, tryptophan depletion does not inhibit tumor growth. Acting in such a way, IDO1 contributes to the escape of cancer from the immunosurveillance. However, in the case of cancer with the intact p53 pathway, the IDO1 expressed by the infiltrating immune cells may be able to activate GCN2K in cancer cells and inhibit tumor progression in a p53-dependent way. In such a case, the administration of an IDO1 inhibitor may decrease the antitumor immune response. Interestingly, in an experimental study, IFN-γ exhibited its antiproliferative effects only in cancer cell lines in which it upregulated IDO1 expression with a consequent tryptophan deprivation; suggesting a possible direct antitumor effect of this enzyme in certain types of cancer. However, the p53 pathway was not assessed in the tested cancer cell lines (32). Thus, evaluation of the cancer p53 status before the administration of an IDO1 inhibitor may be vital.

Also, and despite the studies about the role of IDO1 in supporting tumor vessel formation (33, 34), the ability of activated GCN2K to induce p53 expression, and possibly cell cycle arrest or apoptosis, in endothelial cells (30), raises questions about the effect of IDO1 inhibition on the required for the tumor progression neoangiogenesis. Interestingly, expression of IDO1 in endothelial cells of renal tumors is associated with a better prognosis (19).

As regards the immunosuppressive properties of IDO1 *per se*, research in my laboratory, revealed that this enzyme affects T-cell fate at least in part by altering cell metabolism (3–5, 26, 35, 36). Thus, the availability of various nutrients in the microenvironment of the immune response may have a significant impact on IDO1 immunomodulatory properties. Most of the conclusions about the molecular pathways involved in the IDO1-induced immunosuppression were extrapolated under the strictly controlled conditions of cell cultures (1, 7). Nevertheless, if a free fatty acid is added in the culture medium, the trend for CD4+ T-cell differentiation toward a regulatory phenotype remains, but the antiproliferative and pro-apoptotic properties of IDO1 disappear (35, 36). The reason relies on the effect of IDO1 on T-cell metabolism. As depicted in more detail in **Figure 1**, depletion of tryptophan by activating GCN2K inhibits glucose and glutamine catabolism (3, 4, 26, 36). However, kynurenine by activating AhR induces free fatty acid β-oxidation, which refuels CD4+ T-cells with energy, allowing their proliferation and preventing their apoptosis (35, 36). Accordingly, two of the three ways by which IDO1 is supposed to suppress T-cell-mediated immune response may not take place if enough free fatty acids are present in the cancer microenvironment. In such a case, the gain in antitumor immunity by inhibition of IDO1 would be far less than the expected. The data about the concentration of free fatty acids in the various types of cancer are scarce.

TABLE 1 | Factors that may limit the anticancer effect of indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors.

The role of IDO1 in the escape of cancer from immunosurveillance may be overestimated	Most studies assessed only IDO1 expression but not its activity. However, certain conditions that may be present in the cancer microenvironment may inhibit IDO1 activity without affecting its protein level
In certain tumors, IDO1 may induce apoptosis of the cancer cells	In human lymphocytes, epithelial and endothelial cells, IDO1 by activating general control non-derepressible 2 kinase (GCN2K) induces p53-mediated apoptosis. Thus, in the minority of cancers with an intact p53 pathway, IDO1 expression in the infiltrating immune cells may be beneficial
In certain tumors, IDO1 may suppress neoangiogenesis	Although there are studies that support a positive role for IDO1 in tumor neoangiogenesis, the fact that GCN2K activation induces p53-mediated apoptosis in human endothelial cells raises questions. In renal carcinoma, the expression of IDO1 in endothelial cells signifies a worse prognosis
The immunosuppressive properties of IDO1 may be overestimated	Traditionally, it is thought that IDO1 suppresses T-cells proliferation, induces their apoptosis, and promotes their differentiation toward a regulatory phenotype. However, the presence of free fatty acids in the tumor microenvironment may abolish the antiproliferative and pro-apoptotic properties of IDO1

In conclusion, there are many aspects to be revealed about the role of IDO1 in the escape of cancer from immunosurveillance (**Table 1**). Along with tumor IDO1 expression, assessment of its activity may prevent overestimation of its role in the escape of cancer from immunosurveillance. In cancer with an intact p53 pathway, expression of IDO1 by the infiltrating immune cells may exhibit antitumor activity. Also, in an environment relatively rich in free fatty acids the immunosuppressive properties of IDO1 may be decreased considerably, and the gain in antitumor immunity from its inhibition may be less than the expected. The role of IDO1 in tumor neoangiogenesis remains to be better elucidated as well. Administration of IDO1 inhibitors may be beneficial to certain but not all cancers. Beyond tumor IDO1 expression, assessment of other factors such as IDO enzymatic activity, the status of the p53 pathway in the cancer cells, and the availability of free fatty acids in the tumor microenvironment, i.e., the application of a more personalized medicine, may help IDO1 inhibitors to find their place in cancer immunotherapy.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Therapeutic Cancer Vaccine and Combinations With Antiangiogenic Therapies and Immune Checkpoint Blockade

Alice Mougel[1,2], *Magali Terme*[1,3*] *and Corinne Tanchot*[1*]

[1] PARCC (Paris-Cardiovascular Research Center), INSERM U970, Paris, France, [2] UFR Science du Vivant, Université Paris Diderot, Sorbonne Paris Cité, Paris, France, [3] Faculté de Médecine, Université Paris Descartes, Sorbonne Paris Cité, Paris, France

Considering the high importance of immune surveillance and immune escape in the evolution of cancer, the development of immunotherapeutic strategies has become a major field of research in recent decades. The considerable therapeutic breakthrough observed when targeting inhibitory immune checkpoint molecules has highlighted the need to find approaches enabling the induction and proper activation of an immune response against cancer. In this context, therapeutic vaccination, which can induce a specific immune response against tumor antigens, is an important approach to consider. However, this strategy has its advantages and limits. Considering its low clinical efficacy, approaches combining therapeutic cancer vaccine strategies with other immunotherapies or targeted therapies have been emphasized. This review will list different cancer vaccines, with an emphasis on their targets. We highlight the results and limits of vaccine strategies and then describe strategies that combine therapeutic vaccines and antiangiogenic therapies or immune checkpoint blockade. Antiangiogenic therapies and immune checkpoint blockade are of proven clinical efficacy for some indications, but are limited by toxicity and the development of resistance. Their combination with therapeutic vaccines could be a way to improve therapeutic outcome by specifically stimulating the immune system and considering a global approach to tumor microenvironment remodeling.

Keywords: cancer vaccines, immunotherapies, combinatorial strategies, antiangiogenic treatments, immune checkpoint blockade

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***Correspondence:**
Magali Terme
magali.terme@inserm.fr
Corinne Tanchot
corinne.tanchot@inserm.fr

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INTRODUCTION

Therapeutic cancer vaccines are based on specific stimulation of the immune system using tumor antigens to elicit an antitumor response. Nevertheless, therapeutic cancer vaccines are still considered as a strategy that fails to demonstrate clinical benefits. Indeed, when compared to other newly developed immunotherapies such as immune checkpoint blockade (ICB) or CAR T-cell therapies, therapeutic vaccines still show very few outcomes in the establishment of clinical responses in advanced cancer patients.

Numerous improvements have been made in recent decades in therapeutic vaccination protocols that enhance the immune response elicited by the vaccination. FDA approval of the first therapeutic vaccine in 2010, the DC-based vaccine sipuleucel-T (Provenge®) in the treatment of

prostate cancer (1), brings to the force possible success for therapeutic vaccination strategies.

In this mini review, we discuss the results and limitations of vaccine approaches tested in clinical trials targeting different tumor antigens. After describing the rationale for combining therapeutic vaccines with either antiangiogenic therapies or ICB, we present examples of combinations in preclinical mouse models and in human clinical trials.

THERAPEUTIC CANCER VACCINES

Targeted Antigens

In the context of antitumor vaccine improvement, significant efforts have been focused on the choice of tumor antigen to target. Although numerous cancer vaccine strategies have been studied, targeted antigens remain at the heart of the discussions. They are classified into two main categories.

Tumor-associated antigens (TAAs) are expressed by tumor cells and also by normal cells such as overexpressed antigens (Her2/neu, survivin, MUC-1 ...), cancer testis antigens (MAGE-3, NY-ESO-1 ...), or differentiation antigens (Mart1, PSA, PAP ...). Although TAAs are expressed at a certain level by normal cells, their immunogenicity induces specific T-cell responses (2–4). However, a certain degree of self-tolerance can be applied to TAAs.

In the case of tumor-specific antigens (TSAs), such as oncogenic viral proteins in virally induced cancers or neoantigens generated by non-silent somatic mutations of normal proteins, such central thymic tolerance is bypassed, being regarded as foreign antigens by the immune system. Neoantigen-based vaccine strategies have shown specific anti-tumor immunity in numerous preclinical models and have been tested in early (Phase I) human clinical trials with very promising results (5–10).

Therapeutic Vaccine Strategies

Different therapeutic vaccine strategies have been developed including whole tumor cell, tumor-cell lysates or gene-modified tumor cells, protein- or peptide-based vaccines, RNA and DNA vaccines, viral vector engineered to express tumor antigen and DC-based vaccines loaded with DNA, RNA or peptides. These strategies are further detailed in specialized reviews (10–15).

Many preclinical and clinical trials using these different strategies have been performed and we will focus here on those that have reached phase II/III clinical trials (16, 17). Some examples are provided below and in **Table 1** (18–29).

Cancer vaccine strategies based on autologous dendritic cells (DCs) pulsed with tumor antigens have been widely studied (30, 31). For example, a phase III clinical trial testing peptide-pulsed DCs as a first-line treatment in advanced melanoma patients has been performed, but DC vaccination was ineffective compared to chemotherapy (18). A randomized phase II/III clinical trial is currently ongoing in glioblastoma patients to test a DC-based vaccine (NCT03548571). Many parameters such as DC-based vaccine administration, maintenance of DC viability and maturation as well as standardization of *ex vivo* generation can be limiting (32–34).

Improvement of vaccine platforms has also led to the use of viral vectors with modified viruses engineered to express both targeted antigens and immunomodulatory molecules. In this context, modified vaccinia virus of the Ankara strain (MVA) has been studied. A vaccine containing MVA expressing tumor antigen MUC-1 and immunostimulatory cytokine IL-2 (TG4010 vaccine) was tested in a phase II clinical trial in patients with metastatic renal cell carcinoma (mRCC) (19). Induction of an immunological response against MUC-1 was observed and safety established. Nevertheless, the trial showed no clinical benefit following vaccination. In the TIME clinical trial (phase II), TG4010 was tested in NSCLC patients with or without chemotherapy. Patients who showed a MUC-1-specific response ($n = 16$) had an improved clinical outcome with a median overall survival (OS) of 32.1 months vs. 12.7 months in non-responders ($n = 6$) (20).

Other vaccines have also emerged using this modified virus strategy, such as TroVax, an MVA expressing fetal oncogene 5T4 (MVA-5T4) studied in a phase III clinical trial in the treatment of renal cancer, though no clinical benefit was found (21). PROSTVAC (or PSA-TRICOM), a poxviral-based prostate-specific antigen (PSA) vaccine has been assessed in a phase II clinical trial in the treatment of metastatic castration-resistant prostate cancer (mCRPC) and was associated with a 44% reduction in the death rate and an 8.5 months improvement in median OS (22).

Thus, therapeutic vaccine strategies take many forms and importance is now also given to vaccine administration routes to enhance efficacy (35) as well as work done on vehicle delivery and adjuvants (12, 15, 36–38).

Therapeutic Vaccines in the Era of Combination Strategies

However, even though cancer vaccines have been greatly improved, overall they still fail to provide any clinical benefit as monotherapy in patients with advanced cancers. During cancer progression, tumors develop several mechanisms of immune escape such as tumor angiogenesis, recruitment of immunosuppressive cells, and over-expression of inhibitory molecules, all leading to an ineffective antitumor immune response (39, 40).

Due to their lack of migration and/or progressive exhaustion, tumor-specific T cells generated by vaccination do not act effectively against the tumor. Combination with strategies that counteract such immune escape mechanisms is therefore essential.

Therapeutic vaccine approaches have a major place in the arsenal of weapons developed to fight cancer and in many published studies have been combined with radio- or chemotherapy, with promising results, as reviewed elsewhere (41, 42).

In this mini review, we will concentrate on combinations of therapeutic vaccines and antiangiogenic treatments (AATs) or ICB, highlighting their synergistic potential and providing an update of preclinical and clinical results.

TABLE 1 | Some examples of phase II/III clinical trials testing therapeutic cancer vaccines.

Vaccine	Cancer type	Trial phase	Trial outcomes	ClinicalTrials.gov Identifier and References
DC-BASED VACCINE				
Peptide-pulsed DCs	Advanced melanoma	Phase III	DC vaccination was ineffective compared to chemotherapy	Schadendorf et al. (18)
VACCINE USING VIRAL VECTOR				
TG4010: MVA expressing MUC-1 and IL-2	mRCC	Phase II	Induction of an immunological response against MUC-1 has been observed and safety has been established. No clinical benefit following vaccination	Oudard et al. (19)
TG4010: MVA expressing MUC-1 and IL-2	NSCLC	Phase II (TIME clinical trial)	Patients showing a MUC-1-specific response ($n = 16$) had an improved clinical outcome with a median overall survival (OS) of 32.1 vs. 12.7 months in non-responders ($n = 6$)	NCT01383148 Tosch et al. (20)
TroVax: MVA expressing fetal oncogene 5T4 (MVA-5T4)	Renal cancer	Phase III	No clinical benefit	NCT00397345 Amato et al. (21)
PROSTVAC (or PSA-TRICOM): MVA expressing PSA	mCRPC (metastatic castration-resistant prostate cancer)	Phase II	44% reduction in death rate and an 8.5 month improvement in median OS	NCT00078585 Kantoff et al. (22)
TUMOR CELL-BASED VACCINE				
GVAX	Metastatic hormone-refractory prostate cancer	Phase I/II	Dose escalation study. The immunotherapy was well-tolerated. The median survival time was 35.0 months in the high-dose group, 20.0 months in the mid-dose group and 23.1 months in the low-dose group	NCT00140348 Higano et al. (23)
Allogenic GM-CSF secreting tumor	Pancreatic adenocarcinoma	Phase II	Vaccine combined with chemoradiation is safe. Median disease-free survival was 17.3 months and median OS was 24.8 months, which compares favorably with published data	NCT00084383 Lutz et al. (24)
PROTEIN/PEPTIDE-BASED VACCINE				
recMAGE-A3 protein + AS15 immunostimulant	Completely resected stage IB, II, and IIIA MAGEA3+ NSCLC	Phase III	Vaccination failed to increase the disease-free survival of surgically resected NSCLC patients	NCT00480025 Vansteenkiste et al. (25)
Rindopepimut with GM-CSF plus temozolomide	Newly diagnosed EGFRvIII+ glioma patients	Phase III	Rindopepimut did not increase survival in newly diagnosed glioblastoma patients	NCT01480479 Weller et al. (26)
IMA901: 10 different synthetic tumor-associated peptide	mRCC	Phase II	Vaccination was safe and well-tolerated. Median OS was 23.5 months for patients pretreated with cyclophosphamide	NCT00523159 Walter et al. (27)
Tecemotide (L-BLP25): MUC-1-derived lipopeptide	NSCLC (stage III)	Phase III	No significant difference in OS.	NCT00409188 Butts et al. (28)
Multiepitope vaccine composed of tyrosinase, gp100 and MART-1 peptides	High-risk resected melanoma	Phase III	No RFS or OS improvement in vaccinated patients	NCT01989572 Lawson et al. (29)

THERAPEUTIC CANCER VACCINES AND ANTIANGIOGENIC TREATMENTS COMBINATIONS

Rationale for Vaccine Combination With AATs

During carcinogenesis, tumors will increase the expression of pro-angiogenic molecules such as vascular endothelial growth factor (VEGF), which is involved in tumor angiogenesis. This process fosters access to nutrient and oxygen supplies for the tumor and allows proliferation and metastatic dissemination even though vasculature development is abnormal (43). On the other hand, the aberrant tumor vasculature actively suppresses anti-tumor responses by providing a physical barrier to T cell infiltration and limits therapeutic drug efficacy due to poor delivery to the tumor (40). Tumor angiogenesis also contributes to immune escape with its immunosuppressive roles (44–47). For example, VEGF is involved in inhibition of DC maturation, development of tumor-associated macrophages, increase in regulatory T cells (Tregs), accumulation of myeloid-derived suppressor cells (MDSCs), and expression of inhibitory checkpoints such as PD-1 on $CD8^+$ T cells (48).

Within this framework, numerous antiangiogenic molecules have been developed to repress tumor angiogenesis. AATs target many components of the tumor microenvironment (endothelial cells, tumor cells, DC, MDSC, Tregs….) resulting in a shift of cytokine and chemokine production favoring antitumor activities. These therapies lead to transient vascular normalization while dampening immunosuppression (40, 47, 49, 50). In this review, we will focus on drugs targeting the VEGF/VEGF receptor (VEGFR) pathway, such as bevacizumab (an anti-VEGFA antibody), tyrosine kinase inhibitors such as sunitinib, sorafenib or axitinib, which target VEFGR but also other pathways, and anti-VEGFR antibodies. These antiangiogenic drugs are currently the most used in the clinical setting. However, the benefits provided by these treatments are still limited and acquired resistance can appear (51, 52).

Considering the extensive tumor microenvironment remodeling induced by AATs, a combination of such strategies with therapeutic vaccines could help to enhance the immune response against tumors. By improving in quantity and quality the infiltration of T lymphocytes activated by the vaccine and by decreasing immunosuppression, AATs might act in synergy with therapeutic cancer vaccines.

Preclinical Studies Combining AATs And Vaccines

Several preclinical studies have sought to define the best timing of administration of therapeutic vaccines and AATs for an optimal synergy of action. These studies have yielded conflicting results.

In a first study, sunitinib was combined with a DC-based vaccine expressing IL-12 and pulsed with OVA-peptide (DC-IL12-OVA) in a B16-OVA tumor model. This combination improved therapeutic efficacy, increased type-1 antitumor T-cell recruitment in the tumor microenvironment and decreased immunosuppressive cells (MDSCs, Tregs) (53). Similar results in terms of efficacy were obtained using the tyrosine kinase inhibitor axitinib instead of sunitinib (54).

Bose et al. showed that the best therapeutic efficacy was achieved when sunitinib was administered alongside vaccine priming or boosting, whereas starting sunitinib treatment after vaccination did not lead to optimal efficacy (53).

Farsaci et al. studied the combination of sunitinib with a modified virus-based vaccine using rMVA containing transgenes for co-stimulatory molecules (B7-1, ICAM-1, and LFA-3) as well as for the carcinoembryogenic antigen (CEA) administered to CEA-transgenic mice bearing MC38-CEA tumors (55). Sunitinib was administered for 4 weeks followed by 2 weeks without treatment (comparable to schedules in cancer patients). In this context, vaccine was administered before, alongside or after the start of sunitinib treatment. Only situations where sunitinib preceded vaccination were associated with increased therapeutic efficacy when compared to sunitinib alone. The authors concluded that synergy between sunitinib and therapeutic vaccination happened after sunitinib preconditioned the immune system.

Finally, a study combining sunitinib with a protein-based vaccine using recombinant α-lactalbumin in a 4T1 mammary tumor-bearing mouse model highlighted the fact that sunitinib inhibited the priming phase of the active immunization protocol by reducing the number of $CD11b^+$ $CD11c^+$ antigen-presenting cells in draining lymph nodes and spleen when sunitinib is administered alongside vaccination (56).

Besides the importance of the timing of administration, the dose of antiangiogenic treatment is a key factor for vessel normalization in the tumor (47, 49). In a preclinical model of breast cancer, only low doses of anti-VEGFR2 antibody (DC101) enabled vascular normalization, and a combination of low-dose DC101 with irradiated tumor cell vaccine enhanced tumor control compared to vaccine alone (57).

Although there seems to be no consensus regarding treatment scheduling, some patterns do emerge. The nature of the vaccine strategy should be taken into account, as should the antiangiogenic drug properties, considering the large spectrum of immune responses considered.

Clinical Trials Combining AATs And Vaccines

Based on positive results in preclinical studies, the combination of AATs and therapeutic vaccines in human subjects is currently being assessed. In a phase II clinical study involving newly diagnosed intermediate and low-risk mRCC patients, sunitinib was combined with a personalized DC-based vaccine AGS-003 consisting of monocyte-derived DCs transfected with total autologous tumor RNAs and CD40L RNA. Vaccine administration was started at the beginning of the second cycle of sunitinib. Combination treatment induced immunological responses and prolonged survival (58). These encouraging results led to a phase III clinical trial, but AGS-003 failed to improve OS (NCT01582672).

The same lack of clinical response has been reported in a phase II clinical trial combining the multipeptide IMA901 with sunitinib in the treatment of mRCC (59).

Although progress has been made in the understanding of the impact of AATs, combinatorial approaches with cancer vaccines have failed to demonstrate clinical efficacy. To improve clinical outcomes, before considering vaccination we need a better understanding of immunological remodeling induced by antiangiogenic therapies and an assessment of the immunological stage of patients already on antiangiogenic drugs in the context of standard of care treatment. On the other hand, as mentioned above, attention should be paid to the treatment schedule and the doses of AAT. Clinical trials are usually not design to address this issue. To date, the combination of vaccines and antiangiogenic therapies has been less studied than other combinations, although numerous clinical trials are ongoing (some are listed in **Table 2A**) and hopefully will provide new insights into how such strategies might optimize synergistic effects.

THERAPEUTIC CANCER VACCINES AND IMMUNE CHECKPOINT BLOCKADE COMBINATIONS

Rationale for Vaccine Combination With ICB

As described before, the acquisition of immune checkpoint molecules by effector T cells in the tumor microenvironment render them progressively exhausted and unable to kill tumor cells. This has led in recent years to the development of ICB, which is now used in the treatment of many types of cancer.

Monoclonal antibodies (mAbs) directed against co-inhibitory molecules involved in T cell exhaustion or Treg cell function such as CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4) and the PD-1/PD-L1 axes (programmed cell death 1/programmed death-ligand 1) have revolutionized the treatment of an increasing number of cancers, including melanoma, lung cancer, renal cancer, bladder cancer, and Hodgkin's lymphoma (60).

Although success stories such as anti-CTLA-4 and anti-PD-1 mAbs have emerged, these strategies seem to work as monotherapies in a restricted number of patients and some limitations have emerged, with the development of acquired resistance (61, 62). It is important to highlight that poorly immunogenic tumors (also referred to as "cold" tumors), like pancreatic and prostate cancers, are not sensitive to checkpoint blockade (63). Accordingly, the rationale of combining ICB with therapies that increase the number of infiltrating tumor-specific T cells, such as vaccination, is often underlined (63, 64).

Preclinical Studies Combining ICB And Vaccines

Anti-CTLA-4 mAbs in combination with cancer vaccines have been tested in some preclinical studies. For instance, a GM-CSF-producing tumor cell vaccine combined with CTLA-4 blockade has important synergistic effects in reducing tumor size and increasing the antitumor immune response in a melanoma model (65) and in a prostate cancer model (66).

Wada et al. have demonstrated the importance of timing with the combination of anti-CTLA-4 mAbs and GM-CSF gene-transfected tumor cell (GVAX) vaccine in the prostate cancer model Pro-TRAMP (67), by showing that anti-CTLA-4 mAbs should be administered after vaccination to produce additive effects. They hypothesized that delayed CTLA-4 blockade could avoid compensatory expansion of the Treg compartment, which might affect generation of an effective antitumor response.

To improve treatment of pancreatic ductal adenocarcinoma in which checkpoint inhibitor monotherapies seem to be ineffective, Soares et al. have shown that combining anti-PD-1 with GVAX vaccine improved survival and enhanced T-cell activity in mice (68).

Similar conclusions have been reached with another non-immunogenic tumor model. The combination of DC tumor lysate-based vaccine with PD-1 mAbs resulted in long-term survival in mice bearing large established glioma tumors, while neither treatment alone improved survival (69).

In addition, a preclinical study revealed that PD-1 checkpoint inhibition combined with an adenoviral-based vaccine targeting HPV-E6/E7 protein in the context of $E6^+/E7^+$ tumor-bearing mice resulted in a more effective antitumor response (70). The authors also showed that treatment with vaccine alone upregulated the expression of several inhibitory immune checkpoint molecules, including PD-1 and LAG-3 on $CD8^+$ tumor-infiltrating lymphocytes. Another study reported increased PD-1 expression on $CD8^+$ tumor-infiltrating lymphocytes after vaccination and significantly enhanced tumor growth inhibition when vaccine was combined with anti-PD-L1 (71). These results strengthen the rationale for vaccine and ICB combination and suggest that multiple checkpoint inhibition could help enhance the synergy of action with vaccine strategies.

Following this idea, Duraiswamy et al. showed that dual blockade of PD-1 and CTLA-4 combined with GVAX vaccine resulted in the rejection of CT26 colorectal tumor in 100% of mice and of ID8-VEGF ovarian carcinoma in 75% (72). Murine studies therefore support the concept that anti-CTLA-4 and anti-PD-1 mAbs increase the frequency of activated T cells and the effector T cell to Treg ratio in vaccinated tumors. While studies have focused on the scheduling of anti-CTLA-4 mAbs with vaccination, this issue has been less studied for the combination of anti-PD-1 mAbs and vaccine.

Clinical Trials Combining ICB And Vaccines

Following preclinical studies, clinical trials have tested the efficacy of such combinatorial approaches in cancer patients.

In melanoma patients, peptide vaccines have been tested in combination with ipilimumab (anti-CTLA-4), but were not associated with improved outcomes compared to ipilimumab alone (73–75). Nevertheless, some of these peptide vaccines have not shown great immunogenicity when tested alone in preclinical trials.

But combinatorial trials have also yielded promising results. In a phase Ib study, ipilimumab was tested in combination with GVAX vaccine in patients with pancreatic adenocarcinoma. The combination of the vaccine with anti-CTLA-4 therapy improved OS compared to ipilimumab alone (76). In another study, the authors showed that antibody-mediated CTLA-4 blockade increases tumor immunity in some patients who were previously vaccinated with GVAX (77).

TABLE 2 | Some examples of ongoing clinical trials combining cancer vaccines and antiangiogenic therapies **(A)** or immune checkpoint blockade **(B)**.

(A) Ongoing clinical studies combining cancer vaccines and antiangiogenic therapies

Vaccine	Antiangiogenic therapy	Other therapy/adjuvants	Cancer type	Trial phase and status	ClinicalTrials.gov Identifier
Peptide vaccine (EGFRvIII, EphA2, Her2/neu peptide)	Bevacizumab		Glioblastoma	Phase II: active, not recruiting	NCT02754362
HSPPC-96 (personalized peptide-based vaccine)	Bevacizumab		GBM	Phase II: active, not recruiting	NCT01814813
Intuvax (allogenic cell-based therapy)	Sunitinib	poly-ICLC +/– KLH	mRCC	Phase II: active, not recruiting	NCT02432846
PF-06755990 (vaccine)	Sunitinib	Tremelimumab	Prostate cancer	Phase I: recruiting	NCT02616185

(B) Ongoing clinical studies combining cancer vaccines and immune checkpoint blockade

Vaccine	Immune checkpoint blockade	Other therapy /Adjuvants	Cancer type	Trial phase and status	ClinicalTrials.gov Identifier
NeoVax (neoantigen peptide)	Pembrolizumab	Radiation Therapy	Glioblastoma	Phase I: active, not recruiting	NCT02287528
Peptide vaccine	Pembrolizumab		Pancreatic ductal adenocarcinoma or colorectal adenocarcinoma	Phase I: recruiting	NCT02600949
DPX-Survivac (encapsulated peptide)	Pembrolizumab	Cyclophosphamide	Advanced ovarian, primary peritoneal or fallopian tube cancer	Phase II: recruiting	NCT03029403
pTVG-HP (DNA vaccine encoding PAP antigen)	Nivolumab	GM-CSF	Non-metastatic, PSA-recurrent prostate cancer	Phase II: recruiting	NCT03600350
GVAX (GM-CSF-secreting tumor cells)	Nivolumab	Cyclophosphamide	Pancreatic cancer	Phase I/II: recruiting	NCT02451982
PROSTVAC (poxviral vector expressing PSA)	Nivolumab		Pancreatic cancer	Phase I/IIa: recruiting	NCT02933255
PROSTVAC (poxviral vector expressing PSA)	Ipilimumab		Pancreatic cancer	Phase II: recruiting	NCT02506114
GVAX (GM-CSF-secreting tumor cells)	Nivolumab, ipilimumab	CRS-207, Cyclophosphamide	Pancreatic cancer	Phase II: recruiting	NCT03190265
Dendritic cell-based p53 vaccine	Nivolumab, ipilimumab		Relapsed small cell lung cancer	Phase II: recruiting	NCT03406715
Neoantigen DNA vaccine	Durvalumab		Triple negative breast cancer	Phase I: recruiting	NCT03199040
CDX-1401 vaccine (DEC-205/NY-ESO-1 fusion protein)	Atezolizumab	Guadecitabine	Recurrent ovarian cancer	Phase II: recruiting	NCT03206047

Bevacizumab, anti-VEGF-A mAb; sunitinib, tyrosine kinase inhibitor; pembrolizumab and nivolumab, anti-PD-1 mAbs; ipilimumab and tremelimumab, anti-CTLA-4 mAbs; durvalumab and atezolizumab, anti-PD-L1 mAbs.

In a phase I clinical trial, MART-1 peptide-pulsed DCs combined with tremelimumab (anti-CTLA-4) resulted in objective and durable tumor responses in melanoma patients (78). Of 16 patients, 4 had an objective response, 2 had a partial response, and 2 a complete response. TriMixDC-MEL, autologous monocyte-derived DCs electroporated with synthetic mRNA, combined with ipilimumab in advanced melanoma has shown an encouraging rate (38%) of highly durable tumor responses in a phase II trial, including 8 complete responses and 7 partial responses (79).

Viral vector-based vaccines combined with ICB have also shown great promise in human studies. The PROSTVAC vaccine combined with ipilimumab in treatment of mCRPC in a phase I dose-escalation trial proved safe, but clinical outcome has not been studied (80) and a randomized phase II trial is currently recruiting (NCT02506114). Aside from CTLA-4 blockade, PROSTVAC efficacy is also being assessed when combined with nivolumab (anti-PD-1) (phase I/II recruiting) (NCT02933255).

Clinical studies of a combination of anti-PD-1 antibodies and vaccines are still limited, but some early trials show encouraging results.

In a phase I study, patients with advanced solid cancers received p53MVA vaccine combined with pembrolizumab (anti-PD-1) (81). Clinical responses were observed in 3/11 patients, in whom disease remained stable for 30, 32, and 49 weeks, associated for two of them with an increased frequency and persistence of p53-reactive $CD8^+$ T cells.

In a single-arm, phase II clinical trial, 24 patients with incurable HPV-16–positive cancer were vaccinated with ISA 101, a synthetic long-peptide vaccine composed of overlapping HPV E6 and E7 peptides in combination with nivolumab (82). The overall response rate of 33% and median OS of 17.5 months are promising compared to the 16–22% overall response rate and median survival of ∼9 months with PD-1 inhibitors alone in similar patients (63).

Two phase I studies have shown that nivolumab in combination with peptide vaccines targeting differentiation antigens is safe and produces an immunological response in melanoma patients (83, 84).

Although clinical data on the combination of anti-CTLA-4 or anti-PD-1 mAbs and vaccines are still limited, some phase I or II clinical trials are ongoing (85). **Table 2B** lists ongoing clinical trials combining those mAbs but also involving PD-L1 blockade with therapeutic vaccines with or without more conventional therapies (radiotherapy, chemotherapy).

Regarding the complexity of assessing real clinical improvement in such combinatorial trials, an effort should be made in terms of trial design to determine whether the efficacy of vaccine-induced immune responses is improved when combined with immune checkpoint inhibition. It will also be necessary to evaluate immune mechanisms involved in the response to treatment in patients. Those parameters will then allow better scheduling for combination therapy, depending on the nature of the therapeutic vaccine and inhibitory molecules used.

CONCLUSIONS AND PERSPECTIVES

Although therapeutic cancer vaccines have been associated with past failures, the era of combinatorial strategies in the treatment of cancer prompts their reconsideration. Strategies have been optimized and immunologic enhancement due to vaccines is now accepted. The overwhelming immunosuppressive tumor microenvironment that reduces the clinical efficacy of vaccines can now be modified by different approaches. Combinations of cancer vaccines and antiangiogenic therapies or ICB have emerged and shown promising results. To date, very impressive results for those combinations described in mice have not yet been recapitulated in humans. However, studies in mice have mainly used sub-cutaneous tumor grafts growing rapidly and representing an early stage of the disease. Conversely, clinical trials mainly concern patients with advanced cancers, i.e., at a late phase of the disease when immunosuppressive mechanisms are induced. Consequently, we currently lack clinical data showing any breakthrough, a better understanding of the tumor microenvironment will allow us to consider new combinations. Questions remain concerning the timing of treatments, adjuvants, immunization routes, optimal immunogenic vaccines, and tumor remodeling. There is also a need to set up clinical trials in patients at early disease stages. Combinations including newly developed ICB or costimulatory pathways as well as other antiangiogenic strategies such as vaccines directly targeting angiogenic compounds could also bring new hope and lead to clinical success. Finally, in the near future, multiple therapies involving distinct but complementary aspects of antitumor responses may be considered as the combination of vaccines, antiangiogenic therapies and ICB.

AUTHOR CONTRIBUTIONS

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

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Combination Strategies to Optimize Efficacy of Dendritic Cell-Based Immunotherapy

*Mandy van Gulijk[1,2], Floris Dammeijer[1,2], Joachim G. J. V. Aerts[1,2] and Heleen Vroman[1,2]**

[1] Department of Pulmonary Medicine, Erasmus MC, Rotterdam, Netherlands, [2] Erasmus Cancer Institute, Erasmus MC, Rotterdam, Netherlands

Dendritic cells (DCs) are antigen-presenting cells (APCs) that are essential for the activation of immune responses. In various malignancies, these immunostimulatory properties are exploited by DC-therapy, aiming at the induction of effective anti-tumor immunity by vaccination with *ex vivo* antigen-loaded DCs. Depending on the type of DC-therapy used, long-term clinical efficacy upon DC-therapy remains restricted to a proportion of patients, likely due to lack of immunogenicity of tumor cells, presence of a stromal compartment, and the suppressive tumor microenvironment (TME), thereby leading to the development of resistance. In order to circumvent tumor-induced suppressive mechanisms and unleash the full potential of DC-therapy, considerable efforts have been made to combine DC-therapy with chemotherapy, radiotherapy or with checkpoint inhibitors. These combination strategies could enhance tumor immunogenicity, stimulate endogenous DCs following immunogenic cell death, improve infiltration of cytotoxic T lymphocytes (CTLs) or specifically deplete immunosuppressive cells in the TME, such as regulatory T-cells and myeloid-derived suppressor cells. In this review, different strategies of combining DC-therapy with immunomodulatory treatments will be discussed. These strategies and insights will improve and guide DC-based combination immunotherapies with the aim of further improving patient prognosis and care.

Keywords: DC-therapy, combination therapy, chemotherapy, radiotherapy, immune checkpoint inhibitors

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***Correspondence:**
Heleen Vroman
h.vroman@erasmusmc.nl

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INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) and mediate a critical role in the interface between the innate and adaptive immune system. DCs can be subdivided in different subsets including conventional DCs (cDCs) and plasmacytoid DCs (pDCs) that arise in the bone marrow and reside in peripheral tissues in an immature state. In addition, monocytes are able to differentiate into monocyte-derived DCs (moDCs) upon inflammatory conditions (1–4). Activation and maturation of DCs are induced upon exposure to environmental stimuli including damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), leading to enhanced expression of co-stimulatory molecules, cytokine production, reduced phagocytosing capacity, and improved T- and B-cell activation (5, 6). DC-mediated T-cell

activation is initiated by antigen presentation on major histocompatibility class (MHC) I and II and further guided by co-stimulation and secretion of cytokines (7–9). In addition to T-cell activation, DCs can activate natural killer (NK) cells by cell-cell contacts and secretion of pro-inflammatory cytokines such as type I interferons (IFNs) (10). However, in a tumor setting, DC functionality is often compromised as, for example, oncogenic mutations limit DC migration (11–14). In addition, factors secreted by cancer cells limit DC maturation by inducing overexpression of signal transducer and activation of transcription 3 (STAT-3) (15). This leads to insufficient antigen presentation, T-cell anergy and decreased T-cell proliferation, thereby restricting effective anti-tumor immunity (16–18).

Therefore, administering mature *ex vivo*-activated DCs loaded with tumor antigens may circumvent suppressive tumor-derived signals, thereby inducing effective anti-tumor immunity upon vaccination. For the past two decades, DC-therapy has shown to be safe, well-tolerated and capable of inducing anti-tumor immunity (19). However, response rates to DC-therapy are limited, with objective responses rarely exceeding 15% (20). Several mechanisms may contribute to the limited clinical efficacy besides suboptimal DC-therapy design, including downregulation of tumor-associated antigens (TAAs) and MHC molecules by tumor cells, restricted migration of DCs to lymph nodes (LN) and the inherent immune suppressive tumor microenvironment (TME) (21–26). The TME harbors a complex network of tumor tissue, stroma and immune cells including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T-cells (Tregs). These suppressive cells inhibit activation, proliferation and effector functions of infiltrating immune cells by the expression of co-inhibitory molecules and secretion of immunosuppressive cytokines (27–29). Conventional therapies, including chemotherapy and radiotherapy, or more recently developed immunotherapies such as immune checkpoint inhibitors are able to counteract the immunosuppressive environment of the tumor. Therefore, combining these therapies with DC-therapy could lead to synergistic effects and improve clinical responses. In this review, we will discuss current approaches of DC-therapy, promising combinations with chemotherapy, radiotherapy, and immune checkpoint inhibitors that are clinically applicable and future perspectives for novel combination therapies that can improve DC-therapy efficacy.

CURRENT APPROACHES OF DC-THERAPY

In order to obtain a sufficient number of DCs for administration, DCs are commonly generated from isolated $CD14^+$ monocytes or from $CD34^+$ hematopoietic progenitors isolated from peripheral blood, bone marrow or cord blood (3, 5). Culturing purified $CD14^+$ monocytes with granulocyte-monocyte derived growth factor (GM-CSF) and interleukin (IL) 4 will lead to differentiation into immature moDCs (30). Vaccination with these immature DCs loaded with tumor antigens characterizes first-generation DC-therapy and resulted in poor clinical results with a tumor regression of 3.3% (31). In second-generation DC-therapy, DCs are additionally matured by 'maturation cocktails' including Toll-like receptor ligands and cytokines which improved clinical results with objective response rates of 8–15% (31). Sipuleucel-T, the only US FDA approved DC-therapy for use in (prostate) cancer patients, can be positioned at the intersection between first- and second-generation DC-therapy as maturation is not achieved by maturation cocktails but rather by the fusion of GM-CSF to prostate antigen (32). In next generation DC-therapy, naturally-occurring DC (nDCs) subsets are employed as nDCs are superior over moDCs in terms of functionality and production costs and time. In addition, different DC subsets also induce different tumor-specific immune responses, as vaccination with murine cDC1s induced a prominent $CD8^+$ T-cell driven anti-tumor immune response that was beneficial in tumors with abundant Tregs whereas cDC2s induced a Th17-mediated anti-tumor immune response that was advantageous in tumors with TAMs (33, 34). Clinical trials using nDCs have shown that the usage of nDCs is safe, feasible and associated with promising efficacy, which indicates that this should be further investigated (35, 36).

DC Loading

DCs can be loaded with different sources of tumor antigens, such as mRNA, peptides, proteins or whole tumor cell lysate (5, 37). While peptides bind directly to MHC molecules, proteins and tumor cells must be phagocytosed and processed before presentation on MHC molecules can occur. Furthermore, loading of DCs with tumor-associated peptides enables the induction of specific T-cell responses, thereby minimizing the risk on side-effects. However, for most tumor types, TAAs are still unidentified. Loading the DCs with tumor lysate circumvents the requirement of identified TAAs and additionally initiates a broad spectrum of immune responses that is not restricted to cytotoxic T lymphocyte (CTL) activation. This can improve DC-therapy efficacy as objective clinical responses observed upon treatment with DCs loaded with tumor lysate (8.3%) are higher than treatment with DCs presenting defined antigens (3.6%) in a meta-analysis of 173 trials (38).

Route of Administration

To induce effective anti-tumor immunity, migration of DCs to lymph nodes is essential. Therefore, various administration routes have been exploited (intradermally, intranodally, intravenously, subcutaneously, and intratumorally), although to date the superior route of administration is still not established. Also the percentages of DCs that migrate successfully toward the lymph nodes is limited, with up to 4% of injected DCs reaching the lymph node after intradermal injection and 0–56% reaching the lymph node after intranodal injection (26). The migratory capacity can be improved by preconditioning the injection site with a potent recall antigen, tetanus/dipteria toxoid, which improved overall survival (OS) and progression free survival (PFS) in glioblastoma patients (39). In addition to improving migratory capacity, researchers have also targeted apoptotic pathways by promoting Bcl-2 or inhibiting BAK/BAX signaling in DCs to increase the lifetime of DCs and thereby enhance

bioavailability of the injected DCs, which resulted in improved activation of T-cells (40–43). However, despite these attempts to improve DC-therapy, combinatorial strategies are essential to prorogue suppressive mechanisms in the TME and to further potentiate the clinical efficacy of DC-therapy.

COMBINATION THERAPIES TO ENHANCE DC-THERAPY EFFICACY

Combination With Chemotherapy

Chemotherapeutics are traditionally designed to eradicate and eliminate malignant cells to lower tumor burden. However, more recent insights indicate that chemotherapy also has off-target immunological effects depending on the type of chemotherapy, such as immunogenic cell death (ICD) of tumor cells, thereby enabling the induction of anti-tumor immunity (44). ICD stimulates emission of DAMPs, including adenosine triphosphate (ATP), high mobility group box 1 (HMGB1), and calrecticulin (CALR), which initiates antigen uptake, maturation, activation, and recruitment of endogenous DCs in the tumor (45, 46). In addition, specific chemotherapeutics can directly deplete suppressive immune cells including Tregs and MDSCs (47–49). Due to the effects on tumor burden and the immunosuppressive TME, chemotherapeutics could have synergistic effects when combined with DC-therapy. For instance, tumor reduction by neo-adjuvant chemotherapy could improve DC-therapy, as DC-therapy is most effective in cases of low-tumor burden (31). In addition, depletion of immunosuppressive cells in the TME renders the TME more receptive for tumor-specific T-cell infiltration upon DC-therapy. Timing of chemotherapy administration may be crucial as potential synergistic effects of combination treatments depend on the interval and sequence of treatment administration (50). For instance, chemotherapy applied prior to DC-therapy with substantial intervals aims at tumor reduction whereas shorter intervals or concurrent combination therapy allow depletion of suppressive immune cells. In the following sections, combinations of well-studied chemotherapeutics with *ex vivo* antigen-loaded DCs will be discussed. A summary of the main characteristics of the studies is presented in **Table 1**.

Cyclophosphamide

Cyclophosphamide is an alkylating agent that has tumoricidal effects, thereby reducing tumor burden (72). In addition, cyclophosphamide initiates ICD and transient lymphoablation upon high doses, thereby resulting in depletion of suppressive immune cells and stimulation of anti-tumor T-cell responses. In contrast, low-dose cyclophosphamide improves tumor-specific immunity by Treg depletion (**Figure 1**) (47). In mesothelioma, melanoma and colon carcinoma murine models, administration of cyclophosphamide prior to DC-therapy prolonged survival compared to mice treated with monotherapy. This is likely caused by a cyclophosphamide-induced decrease in Tregs, and subsequent increase in T-cells, as observed in these studies (51, 52). Cyclophosphamide administration 3 days prior to DC-therapy was shown to induce T-cell responses to 3 melanoma gp100 antigen-derived peptides G154, G206-2M, and G280-GV in 6 out of 7 melanoma patients post vaccination (55). A reduction in Tregs was also observed in mesothelioma patients treated with concurrent combination of cyclophosphamide and DC-therapy but remained unaffected in a study with melanoma patients (56, 57). These differences could be explained by differences in sampling time, as reduction in Tregs was evaluated after the first cyclophosphamide treatment in mesothelioma patients (56), whereas in melanoma patients, these levels were assessed after 4 and 6 cycles of DC-therapy (57). Combining DC-therapy with cyclophosphamide also improves clinical efficacy, as patients with ovarian cancer that received cyclophosphamide concurrent with DC-therapy and bevacizumab, a VEGF-a blocking antibody, exhibited significantly prolonged survival compared to patients without cyclophosphamide treatment (58). These results were associated with reduced TGF-β levels, a cytokine that is abundantly produced by Tregs in ovarian cancer. Contradictory, combined DC-therapy with cyclophosphamide resulted in poor clinical responses in patients with metastatic renal cell carcinoma. However, as the DCs administered in this study were of allogeneic origin, the lack of clinical efficacy could be explained by the nature of the DCs administered (59). These results indicate that Treg depletion upon cyclophosphamide treatment is able to synergistically augment DC-therapy efficacy both in preclinical and clinical settings, depending on the tumor type and DCs applied.

Temozolomide

The alkylating agent temozolomide (TMZ) induces lymphoablation upon high doses whereas at low doses it primarily targets Tregs (**Figure 1**) (49). As this compound effectively crosses the blood-brain barrier, TMZ is mainly used to treat glioblastoma and melanoma, as the brain is a frequent metastatic site for melanoma (73, 74). In patients with advanced melanoma, administration of one TMZ cycle prior to each DC-therapy decreased circulating Tregs with 60.5% (60). Simultaneous administration of TMZ and DC/glioma cell fusions in recurrent and newly-diagnosed glioblastoma patients resulted in WT-1, gp100, and MAGE-A3-specific CTLs upon vaccination. In the newly-diagnosed patients, PFS and OS were improved compared to an international trial of TMZ monotherapy (61). However, in recurrent glioblastoma patients, where DC-therapy was followed by TMZ administration, combined treatment failed to improve 6-month PFS compared to a reference group with TMZ monotherapy (62). This could be due to reduced CTL numbers caused by TMZ-induced lymphoablation, thereby counteracting the effects of DC-therapy, as shown by a recent study (63). Interestingly, this study also illustrated that, in contrast to CTL numbers, NK cells in peripheral blood remained constant after concurrent combinations with TMZ. However, whether the effects observed on NK cells were associated with depletion of Tregs remains elusive. Furthermore, this indicates that TMZ administration before or during DC-therapy could enhance DC-therapy efficacy, whereas DC-therapy followed by TMZ may exert negative effects on DC-induced anti-tumor immunity.

TABLE 1 | Study characteristics of (pre)clinical studies.

Type of CTX	Cancer type	n[a]	Comparison group	Treatment schedule	Type of DC vaccine	Dosage CTX	Immunological response CTX[c]	Immunological response combination treatment[c,d]	Clinical response	References
PRE-CLINICAL										
Cyclophosphamide	Mesothelioma (AB1)	6	Untreated CTX + DC-Tx + CTX DC-Tx + CTX	CTX: day 3–10[b] DC-Tx: day 12[b]	Tumor lysate-loaded mature BM-derived DCs	0,13 mg/ml (drinking water)	↓ Tregs		Prolonged survival compared to untreated	(51)
	Melanoma (B16)	10	Untreated CTX DC-Tx	CTX: day 5[b] DC-Tx: day 9 and 23[b]	Tumor lysate-loaded mature BM-derived DCs	50 mg/kg body weight			Prolonged survival compared to monotherapy and untreated	(52)
	Colon carcinoma (CT26)	10	Untreated CTX DC-Tx	CTX: day 5[b] DC-Tx: day 9 and 23[b]	Tumor lysate-loaded mature BM-derived DCs	50 mg/kg body weight	↓ Tregs	↑ IFN-γ secreting lymphocytes	Prolonged survival compared to monotherapy and untreated	(52)
Gemcitabine	Pancreatic cancer (Panc02)	6–8	Untreated CTX DC-Tx	CTX 2 days prior and after DC-Tx for 5 weeks	BM-derived mature DCs loaded with Panc02 cells	25 and 50 mg/kg body weight			Prolonged survival compared to untreated (for both dosages)	(53)
	Pancreatic cancer (Panc02)	8	Untreated CTX DC-Tx	CTX: every 3-4 days until day 42 (start day 3) DC-Tx: day 3, 7 and 10[b]	Unloaded immature BM-derived DCs	120 mg/kg body weight	↓ MDSCs	↑ IFN-γ secreting lymphocytes ↑ CD8+ T-cells in tumor tissue	Prolonged survival compared to monotherapy and untreated	(54)
CLINICAL										
Cyclophosphamide	Melanoma	7		CTX: 3 days prior to first DC-tx. DC-tx: 6 vaccinations with 3-week intervals	gp100 antigen derived peptide-loaded mature autologous DCs	300 mg/m^2		• T-cell immunity against gp100-derived antigens 6/7 • Positive correlation DC derived IL-12p70 levels and time to progression		(55)
	Mesothelioma	10		7x CTX followed by 1x DC-Tx 4 days after CTX. Cycle repeated 3x	Tumor lysate-loaded mature autologous DCs	2 × 50 mg	↓ Tregs		Disease control in 8 patients	(56)
	Melanoma	22		7x CTX followed by 1x DC-Tx. Cycle repeated 6x	Mature autologous DCs transfected with p53, survivin and hTERT	50 mg		• Tregs and MDSCs unchanged • IFN-γ Immune response 6/17	PD: $n = 13$ OS: 10.4 mo SD: $n = 9$ PFS: 3.1 mo	(57)

(Continued)

TABLE 1 | Continued

Type of CTX	Cancer type	n[a]	Comparison group	Treatment schedule	Type of DC vaccine	Dosage CTX	Immunological response CTX[c]	Immunological response combination treatment[c,d]	Clinical response		References
	Ovarian cancer	22	DC-tx (+ bevacizumab) ($n = 10$)	CTX one day prior to each DC-Tx + bevacuzimab given 1x each 3 weeks Repeated 4-5x	Tumor-lysate loaded mature autologous DCs	200 mg/m^2		↑ Vaccine-specific T-cells ↑ IFN-γ serum levels ↓ TGF-β serum levels compared to no CTX	Improved OS compared to no treatment with CTX		(58)
	Renal cell carcinoma	22	DC-tx ($n = 12$)	CTX: 3 and 4 days prior to each DC-Tx DC-Tx: 3 vaccinations with monthly intervals	Tumor lysate-loaded mature allogeneic DCs	300 mg/m^2		• No proliferative or cytokine immune responses	No CTX PD: $n = 9$ SD: $n = 2$ MR: $n = 0$ LFU: $n = 1$ OS: 20.3 mo	CTX PD: $n = 4$ SD: $n = 1$ MR: $n = 2$ LFU: $n = 3$ OS: 23.2 mo	(59)
Temozolomide	Melanoma	21		14x CTX followed by 1x DC-tx. Cycle repeated 6x	Tumor lysate-loaded mature autologous DCs	75 mg/m^2		↓ Tregs	PD: $n = 10$ SD: $n = 6$ PR: $n = 1$ NT: $n = 3$	OS: 10 mo	(60)
	Glioblastoma	32		CTX: 5 days/28 in each cycle DC-Tx: 3x starting 2 weeks after CTX. Repeated 3x	DCs fused with glioma cells	150–200 mg/m^2		• WT-1, gp100 and MAGE-A3 specific immune responses 4/4	Recurrent OS: 18.0 mo PFS: 10.3 mo	Initial OS: 30.5 mo PFS: 18.3 mo	(61)
	Glioblastoma	14		CTX: 5 days/28 starting one week after 3rd DC-Tx Cycle repeated up to 6x DC-Tx: 3x each cycle with 2 weeks intervals.	Tumor cell-loaded mature autologous DCs	150–200 mg/m^2			PD: $n = 4$ SD then PD: $n = 3$ PR then PD: $n = 2$ NT: $n = 4$	OS: 23 mo PFS_{6mo}: 22%	(62)
	Glioblastoma	24		CTX: 5 days/28 starting after 3rd DC-Tx. Cycle repeated 6x DC-Tx: 1-4: 2-weeks intervals, 5-6: monthly intervals, 7: 8 weeks after 6th DC-Tx	Tumor lysate-loaded mature autologous DCs	75 mg/m^2		• Positive correlation activation NK cells and PFS	OS: 20.1 mo PFS: 10.5 mo		(63)

(Continued)

TABLE 1 | Continued

Type of CTX	Cancer type	n[a]	Comparison group	Treatment schedule	Type of DC vaccine	Dosage CTX	Immunological response CTX[c]	Immunological response combination treatment[c,d]	Clinical response		References
Gemcitabine	Pancreatic cancer	10		CTX: day 1,8 and 15 of a 28-days cycle DC-Tx: Starting one week after first CTX cycle. Given 3x biweekly	I, II or I/II-WT1 restricted peptide-loaded mature DCs	1,000 mg/m^2			PD: $n = 3$ SD: $n = 7$		(64)
Premetrexed and cisplatin	Mesothelioma	10		CTX: 4x each 3 weeks DC-Tx: 3x each 2 weeks starting 12 weeks after last CTX	Tumor lysate-loaded mature autologous DCs	Premetrexed: 500 mg/m^2 Cisplatin: 75 mg/m^2		↑ KLH-specific antibodies 10/10	PD: $n = 6$ SD: $n = 1$ PR: $n = 3$		(65)
Oxiplatin and capecitabine	Colon cancer	7		CTX: 1x oxiplatin followed by 14x capecitabine. Cycle repeated 8 times DC-Tx: 3x during first cycle of CTX	CEA peptide-loaded mature autologous DCs	Oxiplatin: 130 mg/m^2 Capecitabine: 2,000 mg/m^2		• CEA-specific T-cell response 4/7 • Proliferative KLH-specific CD4$^+$ T-cell response 7/7			(66)
Bortezomib and dexamethasone	Multiple myeloma	50	CTX ($n = 24$)	Bortezomib: day 1,4,8, and 11 Dexamethasone: day 1-2, 4-5, 8-9, 11-12 DC-Tx: 6x day 15-20 Cycle lasted 28 days. Repeated 3x	Autologous DCs/CIK	Bortezomib: 1.0-1.3 mg/m^2 Dexamethasone: 20 mg		↑ CD4/CD8 ratio ↑IL-2 and IFN-γ in PB ↓ IL-4, IL-5 and TGF-β in PB compared to CTX	Improved quality of life compared to no DC-Tx		(67)
Dacarbazine	Melanoma	6		CTX: 6x at 3-week intervals DC-Tx: 6x one day after CTX	Autologous IFN-DCs	1,000 mg/m^2		• Tyrosinase, NY-ESO-1 and gp100-specific immune response 2/3	PD: $n = 2$ SD: $n = 3$ NT: $n = 1$		(68)
Carboplatin and paclitaxel	Melanoma	9		CTX: day 1 of each cycle DC-Tx: day 8 and 22 of each cycle Cycle lasted 28 days Repeated 3x	WT1, gp100, tyrosinase, and MAGE-A2/A3 peptide-loaded mature DCs	Carboplatin: AUC5 Paclitaxel: 175 mg/m^2		• WT1-specific immune response 4/9	PD: $n = 4$ SD: $n = 4$ PR: $n = 1$	OS: 12 mo PFS: 2.3 mo	(69)

(Continued)

TABLE 1 | Continued

Type of CTX	Cancer type	n[a]	Comparison group	Treatment schedule	Type of DC vaccine	Dosage CTX	Immunological response CTX[c]	Immunological response combination treatment[c,d]	Clinical response	References
Docetaxel	Prostate cancer	40	CTX (n = 19)	CTX: 1x each 3 weeks. Repeated 10x DC-Tx: 2x in cycle 1-5 and 1x cycle 5-10	Mature autologous DCs transfected with PSA, PAP, survivin and hTERT	75 mg/m^2	• MDSCs and Tregs unchanged	↓ MDSCs (positive correlation with PFS) • Tregs unchanged	PFS without DC-Tx: 5.5 mo PFS with DC-Tx: 5.7 mo	(70)
	Esophageal cancer	10		CTX: day 1 of each cycle DC-Tx: day 15 and 22 of each cycle Cycle lasted 4 weeks. Repeated 3x	WT-1 peptide-loaded matured DCs	50 mg/m^2		• WT1-specific immune response 5/8	PD: n = 9 SD: n = 1 OS: 5 mo	(71)

AUC, area under curve; BM-derived DCs, bone marrow-derived DCs; CEA, carcinoembryonic antigen; CTX, chemotherapy; CIK, cytokine-induced killer cells; DC, dendritic cell; DC-Tx, dendritic cell therapy; gp100, glycoprotein 100; hTERT, human telomerase reverse transcriptase; IFN, interferon; IFN-γ, interferon gamma; IL, interleukin; KLH, keyhole limpet hemocyanin; LFU, lost to follow-up; MAGE, melanoma-associated antigen; MDSC, myeloid-derived suppressor cell; MR, mixed response; NK cells, natural killer cells; NT, not treated; OS, overall survival; PAP, prostatic acid phosphatase; PB, peripheral blood; PD, progressive disease; PFS, progression-free survival; PR, partial response; PSA, prostate-specific antigen; SD, stable disease; TGF-β, transforming growth factor beta; Tregs, regulatory T-cells; WT, wilms tumor gene.

[a]*For preclinical studies n is number mice/group, for clinical studies n is the total number patients.*

[b]*Days after tumor inoculation.*

[c]*Compared to baseline unless indicated otherwise.*

[d]*Immunological responses measured after combination treatment.*

Gemcitabine

Gemcitabine is able to improve anti-tumor immunity by depletion of MDSCs and Tregs (**Figure 1**) (47, 48, 75). Treatment of mice bearing pancreatic tumors with gemcitabine 2 days before and after DC-therapy prolonged survival compared to untreated mice, which was not observed for both monotherapies (53). Concurrent treatment of DC-therapy and gemcitabine in a murine pancreatic model delayed tumor growth and prolonged survival compared to both monotherapies. This could be dependent on MDSC numbers, as MDSC numbers were significantly reduced in spleens and tumors of mice treated with gemcitabine (54). However, in pancreatic cancer patients, despite decreased PD-1$^+$CTL numbers in responders, the concurrent combination did not result in decreased MDSC and Treg numbers in responders vs. non-responders (64). These results indicate that gemcitabine may enhance DC-therapy efficacy, however the mechanism of action warrants further investigation.

Combination With Other Chemotherapies

With the aim to reduce tumor burden, Hegmans et al. treated mesothelioma patients with premetrexed and cisplatin 12 weeks prior to DC-therapy, which resulted in immunological responses in all patients against keyhole limpet hemocyanin (KLH), a protein used to assess T-cell responses initiated by DC-therapy (65). As this trial has no control arm no conclusions on synergy can be made. Co-administration of oxiplatin, capecitabine and DC-therapy in colon cancer patients induced proliferation of KLH-specific CD4$^+$ T-cells in all patients as well (66). An effect on CD4$^+$ T-cells was also observed in multiple myeloma patients wherein treatment with DCs and cytokine-induced killer cells (CIK) combined with bortezomib and dexamethasone improved CD4$^+$/CD8$^+$ T-cell ratios compared to baseline and treatment with chemotherapy alone (67). Specific anti-tumor immunity with CTLs directed against gp100, tyrosine and NY-ESO was induced in 67% of the patients with advanced melanoma treated with the combination of DC-therapy and dacarbazine (68). In addition, in 44% of the patients with stage IV melanoma, a specific immune response against WT1 was induced upon treatment with DC-therapy and carboplatin and paclitaxel (69). However, combination with docetaxel failed to improve clinical responses in patients with esophageal cancer and did not result in improved PFS in patients with prostate cancer compared to docetaxel monotherapy (70, 71). These results indicate that combined treatment with chemotherapy and DC-therapy is feasible and safe, however further research should be conducted providing insight into the potential synergistical effects.

Combination With Radiotherapy

Ever since radiotherapy was found to affect non-radiated tumor lesions in a process called the abscopal effect, the immunomodulatory effects of this therapy have been more thoroughly appreciated. As radiotherapy induces ICD, one primary effect is the release of DAMPs and tumor-derived antigens, thereby initiating the activation and migration of DCs to the LN where DCs subsequently cross-present these antigens to T-cells and induce systemic anti-tumor immune

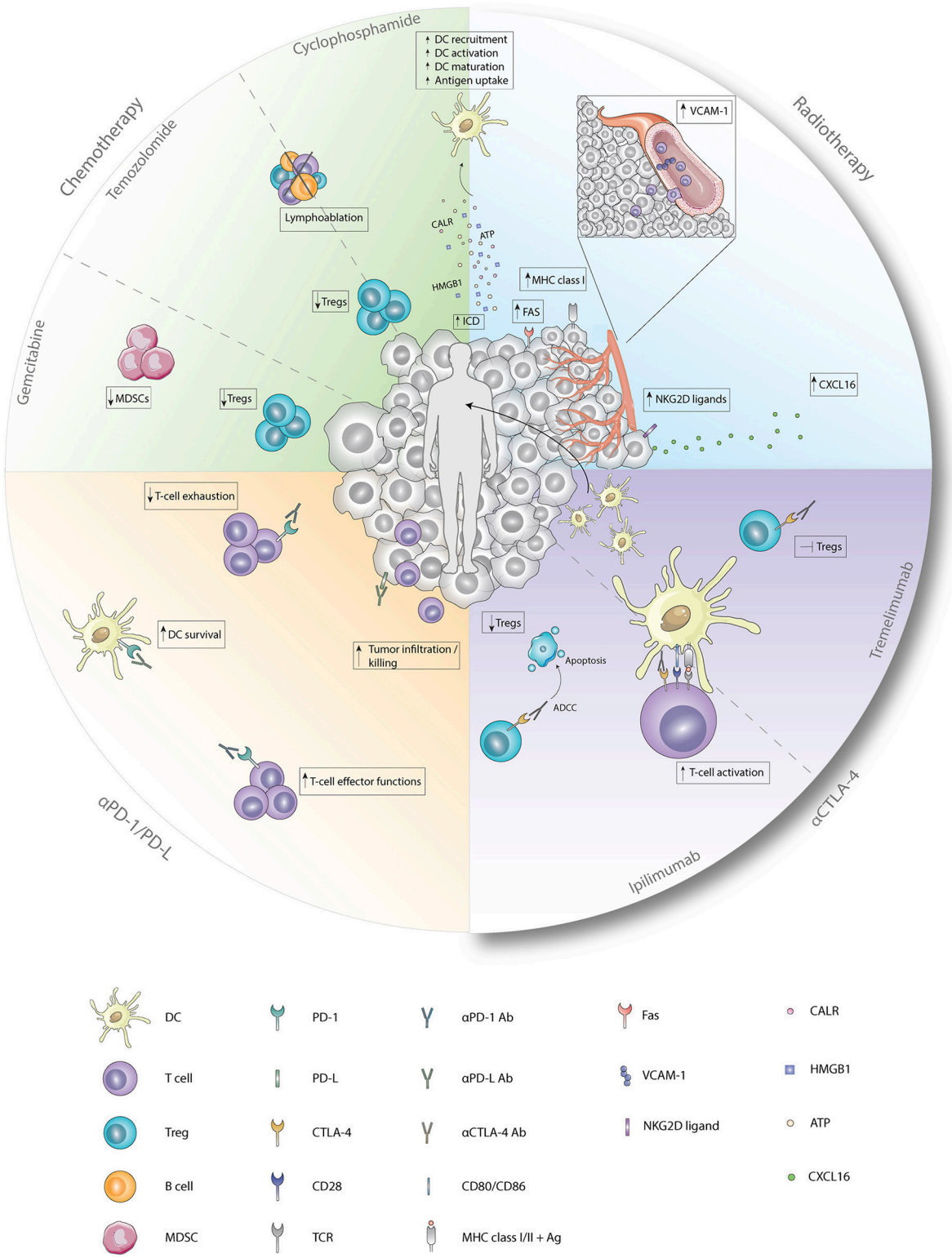

FIGURE 1 | Immunological effects of chemotherapy, radiotherapy, and checkpoint inhibitors. Cyclophosphamide induces ICD which enhances the recruitment, activation, maturation, and antigen uptake by DCs. In addition, cyclophosphamide and temozolomide deplete Tregs and induce lymphoablation upon treatment with low-dose or high-dose, respectively. Immunological functions of gemcitabine entail depletion of Tregs and MDSCs. Radiotherapy induces, besides ICD, enhanced expression of FAS, MHC class I, and NKG2D ligands on tumor cells and enhanced expression of VCAM-1 on endothelial cells. Furthermore, secretion of CXCL16 by tumor cells is increased after radiotherapy. Antagonistic CTLA-4 antibodies enhance T-cell activation by the preventing the binding of CD28 with CD80/86. Ipilimumab

(Continued)

FIGURE 1 | depletes Tregs by ADCC whereas tremelimumab inhibits functions of Tregs upon binding. Anti-PD1 antagonistic antibodies enhance T-cell effector functions while preventing exhaustion of T-cells. Blockade of PD-1 on DCs improves survival while blockade of PD-L on tumor cells results in improved tumor-cell infiltration and killing. Ab, antibody; Ag, antigen; ATP, adenosine triphosphate; CALR, calreticulin; CTLA-4, cytotoxic T-lymphocyte-associated antigen; CXCL16, chemokine ligand 16; DC, dendritic cell; Fas, first apoptosis signal; HMGB1, high mobility group box 1; MDSC, myeloid-derived suppressor cell; MHC class I/II, major histocompatibility complex class I/II; NKG2D ligand, natural killer group 2 member D; PD-1, programmed death 1; PD-L, programmed death ligand; TCR, T-cell receptor; Treg, regulatory T cell; VCAM-1, vascular endothelial cell adhesion protein 1.

responses (**Figure 1**) (76–80). The induction of systemic anti-tumor immunity was indeed observed when radiotherapy was combined with GM-CSF as it generated abscopal effects in some patients (81). In addition, the combination with Flt-3 ligand in a Lewis lung carcinoma murine model reduced metastases and prolonged survival (82). However, in settings of compromised DC functionality, intratumoral injection of exogenously-prepared unloaded DCs followed by radiotherapy could be advantageous. Induction of systemic immunity was observed in a squamous-cell carcinoma murine model, as combining radiotherapy with intratumoral DC administration increased the presence of CTLs in the tumor-draining LN (TDLN) compared to DC-monotherapy (83). In addition, reduced tumor burden and prolonged survival were observed compared to monotherapy in multiple preclinical models (84–88). In clinical trials with patients suffering from hepatocellular carcinoma and high-risk sarcoma, combining intratumoral injection of unloaded DCs with radiotherapy induced tumor-specific immunity in 70 and 52.9% of the cases, respectively (89, 90). In addition to induction of synergistic effects when combined with unloaded DCs, radiotherapy may also improve efficacy when combined with loaded DCs as it transforms irradiated tissue into an immunogenic niche by enhancing the expression of vascular endothelial cell adhesion protein 1 (VCAM-1) on endothelial cells, FAS, MHCI and natural killer group 2D (NKG2D) on tumor cells and increasing CXCL16 secretion, thereby promoting homing, infiltration and tumor killing by DC-induced lymphocytes (**Figure 1**) (91–96). In patients with stage I esophageal cancer, 1- and 2-year survival were significantly improved upon treatment with loaded DCs and radiotherapy as compared to radiotherapy alone. Addition of CIK to this combination failed to improve survival in patients with stage III/IV non-small-cell lung cancer (97, 98). These results indicate that combinatorial treatment has synergistic effects, but these depend on tumor type and stage, as improved efficacy is most prominent at early tumor stages.

Combination With Immune Checkpoint Inhibitors

In cancer, tumor cells and immune cells often overexpress co-inhibitory molecules, such as PD-1/PD-L1 and CTLA-4, which suppress anti-tumor immunity. Checkpoint inhibitors targeting these co-inhibitory molecules improve existing anti-tumor immunity when administered as monotherapy (99, 100). Additionally, combinations with DC-therapy may result in synergistic effects as expression of these co-inhibitory molecules could also limit durable DC-therapy effects by inhibiting DC-therapy induced T-cells as well as DCs directly.

PD-1/PD-L Blocking Antibodies

The PD-1/PD-L-axis exerts negative effects on TME-infiltrating immune cells by inhibiting T-cell effector functions, NK cells and inducing T-cell exhaustion (101–104). Additionally, PD-L1 expression on tumor cells also directly inhibits IFN-γ-mediated cytotoxicity by a STAT3/caspase 7 dependent pathway (105). Therapeutically targeting PD-1/PD-L1 could therefore render the TME more receptive for lymphocyte infiltration and sensitize tumor cells for cytotoxicity that could act synergistically upon combination with DC-therapy (**Figure 1**). Combining DC-therapy with PD-1 blockade reduced Tregs, induced IFN-γ secretion, while secretion of IL-10 by $CD4^+$ T-cells was decreased. In addition, cytotoxicity of CTLs improved when PD-1 was inhibited in a co-culture of tumor cells and T-cells isolated from mice treated with DC/myeloma fusions (106). *In vivo* investigation of DC-therapy combined with PD-1 blockade reduced tumor volume of mice with melanoma (107) and prolonged survival in murine models for glioblastoma (108) compared to monotherapy. These beneficial effects on anti-tumor immunity were also observed in a breast cancer murine model upon combinations with anti-PD-L1 antibodies (109). Additionally, this study investigated the combination of specific blockade of PD-L1 on DCs by *in vitro* incubation with antagonistic monoclonal antibodies (109).

PD-L1/2 are both expressed on DCs and are associated with suppression of effector CTLs and $CD4^+$ T-cells and induction of Treg expansion (110–117). Conversely, the expression of PD-1 on DCs negatively affects DC survival (118). This indicates that blockade of PD-1 or PD-L1 on DCs could enhance anti-tumor immunity *in vivo* via multiple ways. PD-L1 blockade on DCs improved maturation and proliferation of DCs during culture, inhibited tumor outgrowth and prolonged survival compared to mice treated with DCs on which PD-L1 was not blocked (109). These results underline the importance of PD-L1 expression on DCs in inhibiting anti-tumor immunity. Therefore, efforts are undertaken to establish DC-specific PD-L1 blockade, primarily by different RNA introducing techniques, such as small interference RNA (siRNA) or short hairpin RNA (shRNA). Preclinical data indicate that PD-L1 can effectively be silenced using these approaches without affecting viability, maturation or costimulatory molecule expression. In addition, silencing PD-L1 or PD-L2 specifically on DCs enhanced proliferation of tumor-specific CTLs and $CD4^+$ T-cells, augmented production of IFN-γ, tumor-necrosis factor alpha (TNFα), IL-2, IL-5, and IL-12 and promoted cytolysis of tumor cells *in vitro* (119–123). These promising data provide incentive to further investigate the combination of systemic PD-(L)1 blockade with DC-therapy and PD-L1 blockade on DCs.

CTLA-4

The antagonistic antibodies ipilimumab and tremelimumab are designed to target CTLA-4, an inhibitory pathway that inhibits activation of naïve T-cells by preventing the binding of CD28 on T-cells to CD80/CD86 on APCs, a mechanism widely exploited by Tregs (124, 125). In various murine models, ipilimumab was shown to induce antibody-dependent cell-mediated cytotoxicity (ADCC), thereby facilitating Treg depletion while tremelimumab inhibits effector functions of Tregs (**Figure 1**) (126, 127). However, recent clinical data question the Treg-depleting capacity of ipilimumab, as treatment with ipilimumab did not deplete Tregs in the TME of patients with melanoma, prostate cancer and bladder cancer (128). In a retrospective study with stage III melanoma patients that progressed after DC-therapy, administration of ipilimumab induced tumor-specific T-cell responses in 72% of the cases although this was not associated with improved OS (129). Clinical and CTL responses were also not associated in a clinical trial with 16 melanoma patients treated with MART-1 peptide loaded DCs and tremelimumab (130). However, most promising clinical results were obtained by a recent study, in which the overall response rate reached 38% in advanced melanoma patients. These patients were treated with ipilimumab combined with DCs electroporated with CD40L, CD70, and constitutively activated TLR-4 encoding mRNA and one of 4 melanoma-associated antigens (MAGE-A3, MAGE-C2, tyrosinase, or gp100) fused to an HLA-class II targeting signal (131). This indicates that combining DC-therapy with CTLA-4 targeting agents could lead to synergistic effects.

Combination With Other Immunomodulating Therapies

Recently, also other immunomodulatory therapies were approved that enable depletion of specific immunosuppressive cell types, such as macrophages that are depleted upon antibody or tyrosine kinase inhibition of the M-CSF-receptor. In line, we have previously combined DC-therapy with M-CSFR inhibitor treatment in murine tumor models and found improved survival compared to DC-monotherapy. In addition, numbers, proliferation and exhaustion state of CTLs were improved (132). Similar results were obtained when combining DC-therapy with a CD40-agonistic antibody, capable of converting macrophages to a proinflammatory phenotype, and further stimulating the $CD40^{+}$DCs (133). Besides macrophages, selective depletion of Tregs could enhance anti-tumor immunity. Results in a preclinical melanoma mouse model showed that depletion of Tregs using anti-CD25 antibodies prior to DC-therapy elicits long-lasting anti-tumor immunity, as most mice remained tumor-free after tumor rechallenge (134). Further investigation into these combinations in different (pre)clinical models could lead to promising novel combination strategies.

FUTURE PERSPECTIVES

Despite the clinical success of DC-therapy, clinical efficacy remains limited to a proportion of patients and integration of combinatorial approaches are therefore warranted to improve efficacy. Timing of these combinatorial approaches should be carefully considered as this will affect the potential synergistic mode of action. In addition, determining optimal combination therapies likely depends on multiple factors including patient's condition, tumor type, stage and composition of the TME. Therefore, characterization of tumor cells and immune cells present in the TME or peripheral blood of individual patients will help to select immunotherapies that most likely will work synergistically with DC-therapy. For example, treatment of tumors enriched with Tregs should entail combinations with Treg-depleting chemotherapeutics, whereas DC-therapy should be combined with PD-L1 antagonistic antibodies in tumors with high PD-L1 expression. Furthermore, careful characterization of the TME, and peripheral blood could provide novel insights for combination strategies.

CONCLUSION

Although combinations with DC-therapy have demonstrated beneficial effects contributing to anti-tumor immunity, the potential for further improvement remains. A major focus should be on the careful characterization of tumor and peripheral blood of each individual patient as this will be needed to tailor treatments and enhance efficacy on a personalized level. In addition, more controlled clinical trials should be executed to directly compare efficacy with monotherapy. Timing of treatment administration should be taken into consideration in these studies as it could affect the efficacy of combination therapies.

AUTHOR CONTRIBUTIONS

MvG and HV wrote the manuscript and generated the figure and table. FD and JA contributed to the revisions of the manuscript. All authors approved the manuscript for publication.

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MINI REVIEW
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The Tumor Vascular Endothelium as Decision Maker in Cancer Therapy

*Diana Klein**

Institute of Cell Biology (Cancer Research), University Hospital, University of Duisburg-Essen, Essen, Germany

Genetic and pathophysiologic criteria prearrange the uncontrolled growth of neoplastic cells that in turn initiates new vessel formation, which is prerequisite for further tumor growth and progression. This first endothelial lining is patchy, disordered in structure and thus, angiogenic tumor vessels were proven to be functionally inferior. As a result, tumors were characterized by areas with an apparent oversupply in addition to areas with an undersupply of vessels, which complicates an efficient administration of intravenous drugs in cancer therapy and might even lower the response e.g. of radiotherapy (RT) because of the inefficient oxygen supply. In addition to the vascular dysfunction, tumor blood vessels contribute to the tumor escape from immunity by the lack of response to inflammatory activation (endothelial anergy) and by repression of leukocyte adhesion molecule expression. However, tumor vessels can remodel by the association with and integration of pericytes and smooth muscle cells which stabilize these immature vessels resulting in normalization of the vascular structures. This normalization of the tumor vascular bed could improve the efficiency of previously established therapeutic approaches, such as chemo- or radiotherapy by a more homogenous drug and oxygen distribution, and/or by overcoming endothelial anergy. This review highlights the current investigations that take advantage of a proper vascular function for improving cancer therapy with a special focus on the endothelial-immune system interplay.

Keywords: neovascularization, angiogenesis, radiotherapy, anti-angiogenic therapy, vascular stabilization, immune escape

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***Correspondence:**
Diana Klein
Diana.Klein@uk-essen.de

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INTRODUCTION

New vessel formation is a hallmark of tumor growth and progression (1–3). Once a critical tumor mass (of approximately 1–2 mm^3) has formed, the metabolic demands of the growing cancer cells together with the diffusion limits of nutrients and oxygen foster the generation of a tumor-associated neovasculature (4). Known as the angiogenic switch, this process is regulated directly and indirectly by the tumor using a variety of pro- and anti-angiogenic signaling molecules, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), angiopoietins and thrombospondins, among others (5, 6).

In contrast to the normal, usually quiescent vasculature, tumor blood vessels were proved to be functionally abnormal because of their immature phenotype: the endothelial lining is patchy, the basement membrane is defective or discontinuous and respective vessel walls lack the mural elements (smooth muscle cells and pericytes); so they cannot actively respond to physiologic stimuli (**Figure 1**) (7, 8). Thus, there is relative imbalance between tumor tissue and the formation of adequate vascular structures, which finally results in tumor areas with an apparent oversupply in addition to areas with an undersupply of vessels. This complicates not only the efficient

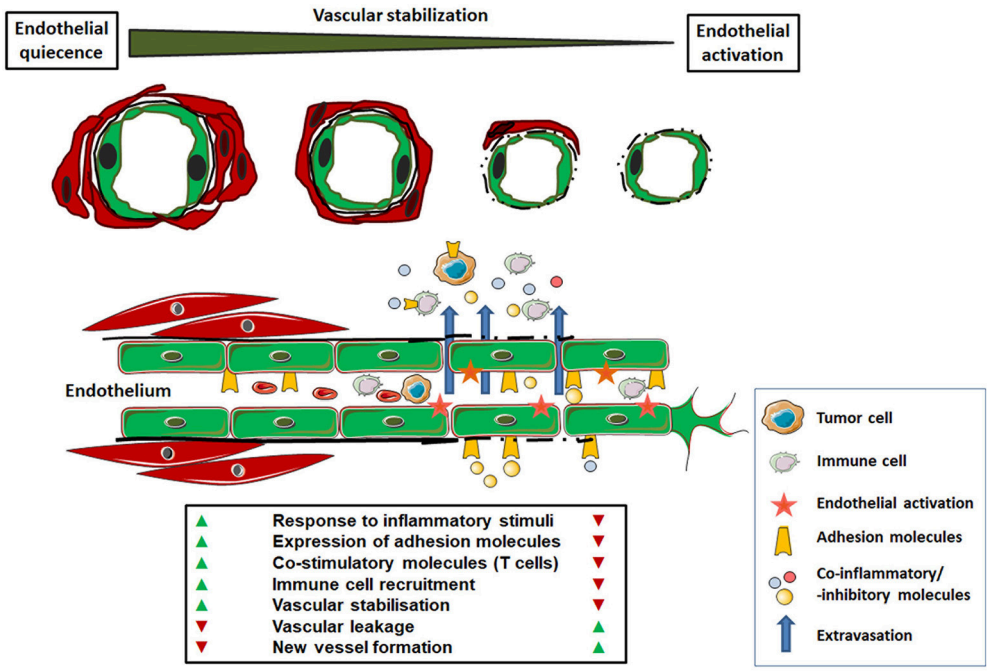

FIGURE 1 | Functional characteristics of normal versus tumor-associated endothelium. In the healthy state, mature endothelial cells (shown as green cells) are characterized by quiescence. A regular blood flow and pressure is achieved by vascular stabilization, which is the association and integration of vascular mural cells (smooth muscle cells and pericytes; shown as red cells). Thus, the normal endothelium provides an efficient barrier to liquids or cell extravasation. Upon activation, e.g., in response to inflammatory signals, normal endothelial cells can up-regulate cell adhesion molecules (selectins and integrins) for the capture, rolling and arresting of circulating immune cells prior tissue extravasation. The anergic tumor endothelium lacks that response to inflammatory stimuli. In response to tumor-secreted angiogenic factors the endothelium becomes activated. This activated and/or "angiogenic" endothelium phenotype is characterized by a missing or defective basement membrane and structural instability (lack of vascular mural cells), which leads to increased vascular leakage. In addition, these newly formed and functional abnormal blood vessels are chaotically organized which, together with endothelial anergy, limits the effective immune cell distribution and tissue infiltration. The altered expression of co-stimulatory and–inhibitory molecules with the potential to block anti-tumor immune cells further contributes to an immunosuppressive microenvironment within the tumor.

distribution of nutritions and oxygen but also the effective administration of cancer therapeutics. Even at the molecular level, i.e., regarding the expression of important signaling molecules, receptors or cell adhesion molecules in the tumor vascular bed, there is an imbalanced state between pro- and anti-oxidants, -inflammatory molecules, and -coagulation signals (9–11). As a result, tumor endothelial cells bear immune-regulatory properties: alterations in the immune cell attraction and activation, as well as in the expression of co-stimulatory and -inhibitory molecules can promote immune tolerance and thus generate an immune-privileged tumor microenvironment (12–14).

However the newly formed tumor vessels can remodel in terms of vascular maturation within the course of tumor progression (7, 10, 15, 16). Herein, a partial stabilization, which is achieved by the association and integration of vascular mural cells occurs particularly in the central areas of the tumors, which is associated with a significant reduction of vascular densities and augmented necrosis in these tumor regions (**Figure 2**) (7, 16, 17). The process of vascular remodeling within a tumor is influenced by the cancer therapy. Especially in anti-angiogenic therapy, angiogenesis inhibitors foster vascular stabilization and a partial normalization of the tumor vascular bed, which is supposed to improve the efficiency of the previously established therapeutic approaches, such as chemo-, radio-, and/or immuno-therapy (18–20). This review highlights the central role played by the tumor vascular endothelium for cancer therapy and summarizes the current strategies that take advantage of a proper vascular function for overcoming anti-tumor immunity and thus improving immunotherapy.

ENDOTHELIAL ACTIVATION AND DYSFUNCTION

One important physiological function of normal endothelial cells is quiescence of the inflammatory response and thus, participation in immune surveillance (21, 22). Quiescent endothelial cells fail to provide the requisite signals for leukocyte recruitment; but the cells can be activated to express adhesion molecules and to release chemokines that promote capture and transmigration of blood leukocytes into tissues. Endothelial cell activation can typically induced by multiple factors, including circulating inflammatory cytokines, such as tumor necrosis factors (TNF) and interleukins (IL), reactive oxygen species, oxidized low density lipoprotein, autoantibodies and traditional

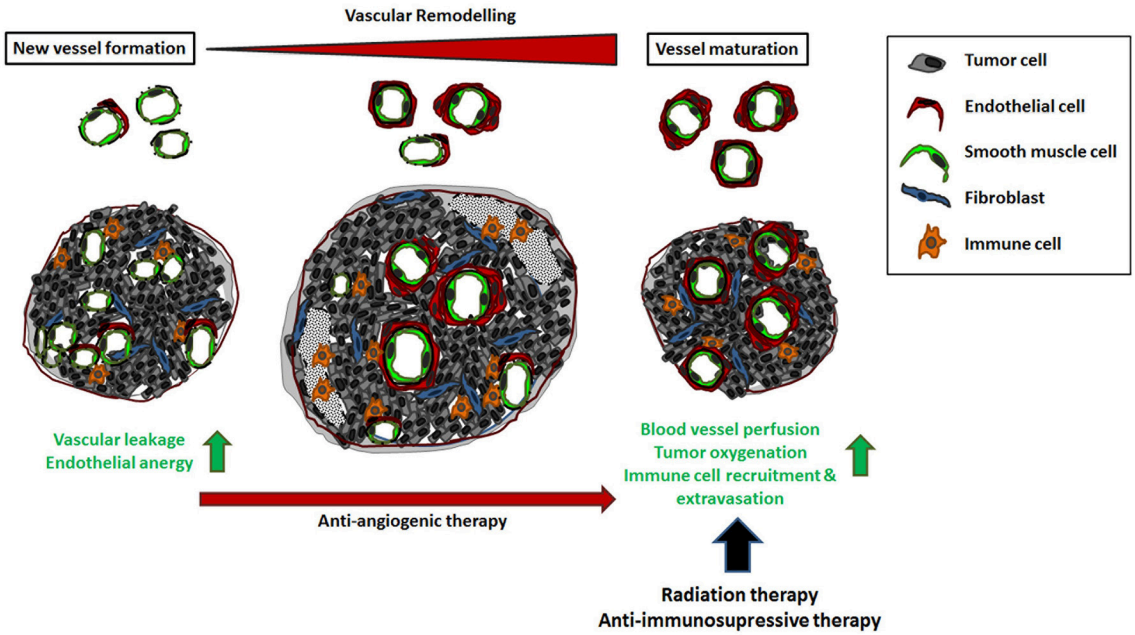

FIGURE 2 | The impact of vascular remodeling for cancer therapy. Tumor neovascularization supplies a high dense network of chaotically organized, immature and unstable vessels. Vascular dysfunction as well as the unresponsiveness to inflammatory stimuli results in an uneven blood flow and pressure as well as an inefficient distribution of blood stream components, including circulating immune cells. This complicates the effective administration of cancer therapeutics. In the course of tumor progression, these angiogenic vessels can mature by the association of vascular mural cells (vascular remodeling) that stabilizes the immature vessels resulting in normalization of the vascular structures. Vascular remodeling is dynamic and strictly regulated process; an ordered remodeling seems to be critical for proper vascular development, maintenance and stability of the vessel wall. The process of vascular stabilization is accelerated in cancer therapy when anti-angiogenic agents were applied. As a result, blood vessel perfusion and thus oxygenation as well as the efficient distribution of applied drugs are improved. In addition, vascular maturation and normalization restores the potential of the tumor endothelium to recruit and direct circulating immune cells to the tumor tissue.

risk factors directly and indirectly activate endothelial cells (21). The term activated endothelium implies a change in endothelial cell morphology (23). Endothelial activation was further specified as a change in surface molecules and in endothelial cell functions in response to cytokine treatment, and it was emphasized that these changes does not represent endothelial cell injury or dysfunction (24, 25). Components of endothelial cell activation are upregulation of surface antigens (e.g., HLA molecules) and leucocyte adhesion molecules (e.g., E-selectin, ICAM-1/2, and VCAM-1), pro-thrombotic endothelial cell changes (e.g., loss of the surface anticoagulant molecules thrombomodulin and heparan sulfate), cytokine production (e.g., IL6, IL8, MCP1), and changes in the vascular tone (e.g., loss of vascular integrity, expression of vasodilators, and NO). These components mutually interact in causing local inflammation (25). Endothelial activation also leads to an increase in angiopoietin-2, which is known to destabilize barrier function and promote inflammation (26). The recruited and extravasated immune cells appear then in vicinity of the activated endothelial cells, and can further become activated (23). Importantly, the phenotype of activated endothelial cell is reversible and can return to the quiescent, non-activated phenotype when the activating factors were removed (27–30). Prolonged activation of the endothelium can be associated with the loss of microvascular barrier integrity and subsequent vascular injury or progress to endothelial cell apoptosis (31).

THE TUMOR ENDOTHELIUM

Phenotypic differences at the molecular and functional levels have been identified for tumor and normal endothelial cells (32). Tumor secreted growth factors, and in particular VEGF, are the principal drivers of most the fundamental morphogenetic events involved in the induction of tumor vascularization including activation of the hitherto quiescent endothelium in terms of stimulating endothelial cell proliferation and migration (33). Many tumor types are characterized by a VEGF upregulation. Tumor hypoxia can also foster increased VEGF expression levels, which in turn perpetuates angiogenic processes (34). Tumor endothelial cell are very heterogeneous and thus vascular function of respective tumor blood vessels vary depending on the type of tumor and progression stage (35, 36). The newly formed blood vessels of tumors as well as of metastatic tumors are more immature with fewer pericytes. In general, tumor endothelial cells are characterized by a proangiogenic phenotype, with the upregulation of several angiogenesis-related genes, such as VEGFR1/R2 and matrix metalloproteinases (MMPs) to modulate the basement membrane and degrade the extracellular matrix allowing endothelial cell migration. The resulting tumor vascular bed is disorganized, tortuous, and the leaky phenotype of angiogenic tumor blood vessels that is accompanied by an irregular blood and heterogeneous permeability limits for the efficient distribution of blood components within the

tumor mass. Further on, the structural abnormalities like poorly interconnected endothelial cells, no regular associated mural cells, and abundance of vesiculo-vacuolar organelles contribute to the leaky, hyper-permeable phenotype, finally causing extravasation of intravascular fluids and plasma proteins (37, 38). Therefore, an markedly increase in the intra-tumor fluid pressure throughout the tumor is observed, while normal pressure values were found in the tumor's periphery or in the surrounding tissue (39, 40). The high tissue pressure within the tumor, together with mechanical stress from the proliferating cancer cells and the extra mass of generated matrix, is able to collapse tumor vessels, that means closing their lumen through compressive forces, leading to the collapse of the blood vessels and finally resulting in hypoxia (32, 38, 41). The compromised blood flow in tumor blood vessels further decreases oxygen and nutrient supply, causing physiological stress to the tumor. The physiological microenvironments of many macroscopic tumors were therefore characterized by high interstitial fluid pressure (interstitial hypertension), which besides nutrient deprivation and hypoxia in turn was associated with malignant progression, development of metastatic disease and a poor disease-free survival in a large number of cancer types (42–44).

Angiogenic growth factors were further shown to suppress the expression of adhesion molecules involved in leukocyte binding (e.g., ICAM-1/2, VCAM-1, E-selectin and CD34) in tumor endothelial cells, which then causes the unresponsiveness of tumor endothelial cells to inflammatory signals, a phenomenon called endothelial cell anergy that causes lymphocyte tolerance (45–48). Hence, the interaction of leukocytes with the endothelial cells lining the vessels is reduced, and thus intra-tumoral recruitment of effector T-cells, either induced or adoptively transferred, is impaired and subsequently fail to exert the anti-tumor effects necessary to eradicate the tumor (49, 50). This is one of the mechanisms tumors have developed to escape the immune surveillance (51). Concerning the mechanism, angiogenic growth factor like VEGF and bFGF inhibited the TNF-mediated activation of NF-KB. In addition, bFGF induced hyperphosphorylation of p38 MAPK on endothelial cells (52, 53). Promoter histone modifications were further shown to mediate tumor endothelial cell anergy, as adhesion molecule expression was shown to be epigenetically repressed in tumor endothelial cells, and that DNA methyltransferase and histone deacetylase inhibitors which have angiostatic activity could re-induce expression of the *ICAM-1* gene by reversal of histone modifications in the *ICAM-1* promoter, thereby restoring leukocyte-vessel wall interactions and leukocyte infiltration (51).

TUMOR ENDOTHELIUM-MEDIATED REGULATION OF THE IMMUNE RESPONSE: CLINICAL IMPLICATIONS FOR TARGETING THE TUMOR VASCULATURE TO IMPROVE IMMUNOTHERAPY

A functional vascular network is prerequisite not only for nutrients or oxygen supply but also for the immune cells to enter the tissues. The functional and structural abnormalities of tumor blood vessels together with the unresponsiveness of the endothelium to inflammatory stimuli caused by proangiogenic factors decrease the recruitment of immune effector cells into the tumor, thus limiting the effectiveness of cancer immunotherapies (54, 55). Given that the abnormal tumor vasculature contributes to the immune-suppressive tumor microenvironment, processes of vascular normalization in terms of vessel maturation were supposed to potentiate cancer immunotherapy by promoting immune cell infiltration into tumors and reducing the immune suppression within the tumors (55, 56).

Today, immunotherapy for activating therapeutic anti-tumor immunity has become a mainstay of cancer therapy (57, 58). Although the use of monoclonal antibodies directed against cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and the programmed cell death-1 (PD-1/CD279) T-cell receptor and/or its ligand (programmed death-ligand 1 (PD-L1/B7-H1/CD274) showed unprecedented durable responses in some patients with a variety of cancers, acquired resistance to immune checkpoint antibody blockades was commonly observed in most cancer patients (59, 60).

The different approaches being currently explored to increase recruitment of immune effector cells, include manipulating the expression of homing-associated molecules on T-cells and tumor endothelial cells. Concerning the first option, a successful approach to target or restore tumor-induced immunosuppression was made by adoptive cell therapy using tumor-reactive T-lymphocytes that resulted in objective tumor regression in >50% of treated patients (61). The potential to treat a wide range of solid cancers with autologous T-cells was further highlighted when re-directed T-cells expressing a non-MHC restricted chimeric antibody receptor (recognizing CD19 on B-cells) in refractory B-cell malignancies were successfully used to overcome dominant immunosuppression (62, 63). However, the success of such therapies again depends on applied agents (here the lymphocytes) in finding their desired place, leaving the bloodstream and subsequently infiltrating the tumor tissues (12, 64).

Thus, strategies addressing directly the vascular system to sensitize tumors or improve the therapeutic response in cancer therapy were established and already shown to exert beneficial effects in immune checkpoint blockade. In a very elegant preclinical study Elia et al. showed that a selective (pre)activation of the tumor endothelium with the cytokine TNF promoted intratumoral T-cell infiltration, and immune checkpoint blockade (65). The authors used low doses of NGR-TNF, a Cys-Asn-Gly-Arg-Cys peptide-TNF fusion product, in simultaneous combination with anti-CTLA-4 and anti-PD-1 antibodies to treat transgenic adenocarcinoma of the mouse prostate (TRAMP) mice with autochthonous prostate cancer and mice with orthotopic B16 melanoma. NGR-TNF administration was already used as a safe and therapeutic systemic administration to target TNF selectively to angiogenic tumor vessels which then altered the endothelial barrier function together with an upregulation of leukocyte-endothelial cell adhesion molecules, the release of pro-inflammatory cytokines, and the infiltration of tumor-specific effector CD8(+) T-cells. As a result, NGR-TNF enhanced the therapeutic activity of

adoptive and active immunotherapy, delaying tumor growth and prolonging survival (66, 67). Finally, the combined therapy had beneficial effects on endogenous immune surveillance, through depletion of regulatory T-cells and expansion of a fully functional, polyclonal repertoire of cytotoxic T-lymphocytes (65).

Proper vascular function as revealed by measurements of vessel perfusion was further used to predict the therapeutic response to immune checkpoint blockade (68). Here, the authors used clinically relevant mouse breast tumor models that were either sensitive or resistant to immune checkpoint blockade treatment (with anti–CTLA4 and anti–PD1 agents) and thus mirror cancer progression and therapy response in humans. A significantly enhanced vessel perfusion was observed mostly in treatment-sensitive tumors, which was accompanied by an accumulation of CD8+ T-cells and interferon-gamma production, strongly suggested that increased vessel perfusion reflects the successful activation of anti-tumor T-cell immunity by immune checkpoint blockade (68). Thus, the authors reported here a reliable and noninvasive indicator for predicting immune checkpoint blockade responsiveness which was related to proper vascular function of the tumor endothelium.

Conclusively, tumor endothelial cells are actively involved in immune cell exclusion and inhibition of lymphocyte activation, fostering an immunosuppressive intratumoral microenvironment that contributes to the tumor immune escape and severely impairs conventional cancer therapies (9, 14, 69). Hypothetically, tumors resistant to immune checkpoint blockade could become sensitive to such treatment again when the tumor endothelium specific alterations in leukocyte-endothelial adhesive interactions were normalized. In line with this idea, Huang et al. showed that synchronizing vascular normalization by antiangiogenic (anti-VEGFR2) therapy with T-cell activation induced by a whole cancer cell vaccine therapy enhanced anticancer efficacy in a CD8(+) T-cell–dependent manner in both immune-tolerant (MCaP0008) and immunogenic (MMTV-PyVT) murine breast cancer models (56). Even the administration of an antibody against mouse VEGF synergized with adoptive cell transfer-based immunotherapies (70). Herein, normalization of the tumor vasculature through disruption of the VEGF/VEGFR-2 axis increased extravasation of adoptively transferred T-cells into the tumor. Combining VEGF blockade with an additional blockade of angiopoietin-2 by a bispecific antibody provided superior therapeutic benefits in the melanoma cancer as well as in metastatic breast and pancreatic cancer models (71, 72). Neutralization of both angiogenic factors resulted in vascular regression of angiogenic blood vessels whereas the remaining blood vessels were normalized and facilitated the extravasation and perivascular accumulation of activated, IFNγ-expressing CD8(+) cytotoxic T lymphocytes (72). The perivascular T-cells in turn induced the expression of PD-L1 in tumor endothelial cells via IFNγ, which was utilized when additionally PD-1 blockade improved tumor control by the bispecific antibody in the different cancer models.

Using regulator of G protein signaling 5-deficient mice, a genetically induced vascular normalization mouse model, in which newly formed blood vessels were characterized by a mature and thus stabilized phenotype, it was further shown that tumor vessel normalization consequently reduced vascular leakiness and hypoxia within the tumors, leading to an influx of immune effector T-cells (22, 30). Herein, vessel maturation was accompanied by a restoration of endothelial cell anergy as adhesion molecules on the luminal surface of tumor endothelial cells were increased and more uniformly distributed. Furthermore, the use of anti-angiogenic therapy was shown to normalize the tumor vasculature and thereby improve cancer immunotherapies.

Instead then of starving tumors from their blood supply and achieving complete vessel regression, vessel normalization by anti-angiogenic therapy has gained more attention for generating more mature and regular functioning tumor blood vessels with increased vessel perfusion. This is supposed to improve distribution of circulating blood components, oxygenation, removal of suppressive metabolites, as well as distribution of therapeutically applied drugs (56). In addition, anti-angiogenic therapy mediated vessel normalization was shown to reverse endothelial cell anergy resulting in (re)sensitizing tumor blood vessels to inflammatory stimuli by inducing homing molecule expression and thus an improved T-cell-dependent anti-cancer immunity (12, 70, 73).

Improving the aberrant structural abnormalities and associated dysfunctionalities of tumor blood vessels, and thus lowering tumor hypoxia and enabling immune cell infiltration, by antiangiogenic therapy was shown to synergize with immunotherapies for more durable effects (74). In an preclinical study using the polyoma middle T oncoprotein breast cancer and the Rip1-Tag2 pancreatic neuroendocrine tumor mouse models it was shown that anti-angiogenic therapy can improve anti-PD-L1 treatment and further, the other way round that anti-PD-L1 therapy can sensitize tumors to anti-angiogenic therapy and prolong its efficacy (74). Herein, vessel normalization (as shown by reduced microvessel densities, increased diameters and a regular pericyte coverage) promoted lymphocyte infiltration and enhanced cytotoxic T-cell activity.

In addition, to tumor endothelial cell anergy that limits the adhesion and subsequent extravasation of recruited leukocytes, tumor-derived factors can further induce endothelial cell-mediated apoptosis of recruited immune cells, e.g., by induced death mediator Fas ligand (FasL, also called CD95L) expression which directly kills anti-tumor T-cells finally leading to an inefficient recruitment of effector CD8(+) T-cells into the tumor (12, 75). Within the tumor endothelium of breast, prostate, colon, bladder, renal cancers a selective expression of FasL was reported that was associated with scarce CD8(+) infiltration and a predominance of FoxP3(+) regulatory T-cells (76). As the induced FasL expression in tumor endothelial cells which acquired the ability to kill effector CD8(+) T-cells but not regulatory T-cells was mediated by tumor-derived VEGF, IL10 and prostaglandin E2 cooperatively, the authors proposed a "tumor endothelial death barrier" that contributes to the tumors immune escape cells (76). The tumor endothelium was also shown to express increased levels of PD-L1 under inflammatory conditions, which in turn was able to bind to PD-1 on activated lymphocytes to negatively control T-cell activation (77–79).

Another molecule which became of interest for activating therapeutic anti-tumor immunity is the interferon-inducible intracellular enzyme indoleamine 2,3-dioxygenase 1 (IDO-1), which catalyzes the initial and rate-limiting step in the degradation pathway of the essential amino acid tryptophan to kynurenine (80, 81). Kynurenines in turn induces proliferation, activation and recruitment of T regulatory cells and myeloid-derived suppressor cells that further suppress tumoricidal T-cells. Increased IDO-1 expression levels were already associated with tumor progression, poor prognosis, and a decreased overall survival (82, 83). IDO-1 expression can be found in different tumor cells, normal epithelial cells, monocyte-derived cells and in particular also in tumor endothelial cells (84–86). Of note, in some tumor entities, the tumor endothelial cells rather than tumor cells were shown to be responsible for increased IDO expression, e.g., in metastatic renal cell carcinoma (84). IDO-1 expression levels in tumor endothelial cells were further suggested being a predictive biomarker for the response to immune-based cancer therapy (86–88). For example, in colorectal cancer, IDO-1 expression by host endothelial cells was a negative prognostic factor for regression free survival, independent of disease stage (89). Therefore, an inhibition of the (endothelial-specific) IDO-1 signaling pathway could be a promising novel adjuvant therapeutic strategy for clinical application in immunotherapy.

However, the actively participation of tumor endothelial cells in the innate and adaptive immune responses is not limited to the ability to attract and direct a wide range of immune cells and elevate extravasation from the host circulation. Tumor endothelial cells are believed to have a role in antigen presentation (9, 13, 14). Endothelial cells were found to act as antigen presenting cells by constitutively expressing major histocompatibility complex I and II molecules and presenting endothelial antigens to T-cells resulting in T-cell activation (90, 91). Endothelial cells also were shown to express the co-stimulatory molecules CD80 and CD86 that are essential for activation of naïve T-cells, but following transplantation only activation of CD4(+) or CD8(+) T-cells was reported (92).

Conclusively these findings strongly argue for new therapeutic approaches including combinations of the anti-angiogenic treatments with immunotherapies in addition to the current standard regimens for cancers, particularly for those that do not respond to surgery, chemotherapy, or radiation.

TUMOR ENDOTHELIUM MEDIATED IMMUNOLOGICAL CONSEQUENCES IN THE CONTEXT OF RADIOTHERAPY

Tumor eradication or local cancer control for a better outcome are the main goals of radiation therapy. Endothelial cells act as critical determinants of the radiation response in tumors as radiotherapy generally fosters endothelial apoptosis, increased vascular permeability, and acquisition of a pro-inflammatory and -coagulant phenotype (93–95). The radiation sensitivity of vessels in general correlates with their morphology: capillaries and small vessels (like angiogenic tumor vessels) are extremely sensitive to ionizing radiation, whereas larger blood vessels seem to be less affected (96, 97). Radiation induces phenotypic changes of tumor endothelial cells (e.g., apoptosis or senescence) as well as wide range of microenvironmental changes by production and secretion of reactive oxygen and nitrogen species, growth and chemotactic factors, which in turn govern recruitment of immune cells (11, 98, 99).

As an apparent approach, sensitizing tumor endothelial cells to radiation-induced apoptosis resulted in a more pronounced tumor growth delay upon irradiations in preclinical animal models, which suggested that a therapeutic targeting at the level of the tumor vasculature could counteract radiation resistance (97, 100). In contrast, in an elegant preclinical study Moding et al. reported that radiosensitizing endothelial cells did not increase local tumor control of soft tissue sarcomas after stereotactic body radiation therapy (101). Furthermore, proangiogenic factors including VEGF can rapidly repress radiation induced ceramide generation, and subsequently endothelial apoptosis (102). Therefore, targeting endothelial cells aiming at achieving complete tumor starvation is not supposed to be curative. More likely, approaches that improve the vascular function and thus tumor oxygenation as well as the recruitment and activation of immune cells by tumor endothelial cells gained attraction also in radiation therapy to enhance the sensitivity of the tumors to ionizing radiation.

To improve blood perfusion and thus tumor oxygenation, again vascular normalization using anti-angiogenic-therapy was suggested (103, 104). According to this hypothesis Koo et al. recently showed that a combined radiotherapy and anti-angiogenic treatment (with the second-generation multi-targeted receptor tyrosine kinase inhibitor sunitinib malate, which inhibits PDGF and VEGF) showed synergistic effects in anti-cancer treatment using heterotopic human lung cancer xenografts (105). Herein, radiation induced extensive necrosis in the central portion of the tumors, as the immature tumor blood vessels were sensitive to radiotherapy. The resulting decreased vascular supply created then a hypoxic area and decreased the tumoricidal effect of radiotherapy by reducing the oxygen-free radicals. When radiotherapy was then combined with anti-angiogenic treatment that inhibits the formation of immature blood vessels, the tumor perfusion was maintained and tumor necrosis was reduced. This treatment combination resulted then in a more significantly suppressed tumor growth, as vessel normalization, which achieved an efficient tumor perfusion, significantly improved tumor oxygenation that is prerequisite for the tumoricidal effects of ionizing radiation (105). In line with these findings Zhu et al. could show that inhibition of hypoxia-induced angiogenesis limits the efficiency of radiotherapy (106). Radiotherapy-sensitive lung tumors were characterized by low levels of hypoxia inducible factor-1α and VEGF, which may reflect better oxygenated tumors with less angiogenic and thus more matured blood vessels. In contrast, high expression levels of the respective genes were detected in radiotherapy-resistant lung tumors which might be based on the hypoxic tumor microenvironment with more angiogenic tumor blood vessels (106). Conclusively, combining anti-angiogenic treatment with radiation therapy can achieve better tumor control as oxygen

is a potent radiosensitizer; this may result in the use of lower radiation doses, as thus minimizing treatment-related normal tissue toxicity (107).

In general, the pharmacological inhibition of pro-angiogenic factors triggers apoptosis of angiogenic endothelial cells in the immature and leaky tumor blood vessels leading to the selection for mature, non-leaky vessels, the so-called pruning effect. Within these matured and normal vessels endothelial anergy is restored. Jaillet et al. reported that ionizing radiation altered the glycosylation pattern of endothelial cells, in particular increased high mannose-type N-glycans and decreased glycosaminoglycans, which stimulated the interactions between irradiated endothelial cells and monocytes (108). Thus, targeting either the endothelium glycome may be considered as therapeutic target for modulating the inflammatory response or combining radiation therapy that seems to reduce endothelial anergy with anti-immunosuppressive therapy. Indeed, re-activation of the tumor vasculature was also shown to improve the therapeutic outcome of radiotherapy combined with immune-modulators. e.g., vessel specific-delivery of IL2, a cytokine known to stimulate the proliferation of cytotoxic T-cells, natural killer cells, and regulatory T-cells, resulted in an additive or synergistic anti-tumor effect when the administration of this immunocytokine was combined with radiotherapy (109). Tumor endothelium specific targeting was achieved by coupling IL2 to the small immune protein L19 that recognizes the extra domain B (ED-B) of fibronectin associated with tumor neovasculature. Of note, specifically addressing the tumor vasculature resulted in higher and thus more effective intratumoral local concentration of IL2 while reducing side effects, as the high doses used by systemic administration to reach an effective intratumoral dose of IL2 often leads to toxicity (e.g., capillary leakage) (109, 110).

In addition, preclinical and clinical evidence exists for the immuno-stimulatory properties of radiotherapy. Radiation treatment can foster immunogenic tumor cell death whereby danger-associated molecular patterns (DAMPs, e.g., calreticulin and adenosine triphosphate) were released which in turn can recruit and activate dendritic cells to process tumor antigens for naïve T-cells finally resulting in an anti-tumor immune responses (111, 112). Of note, radiation-induced tumor-targeted immunotherapy was shown to improve the therapeutic index and to extend the reach of immunomodulatory agents (113). In particular, radiation induced upregulation of VEGF expression was used to target 4-1BB/ CD137, a major immune-stimulatory receptor expressed on activated CD8(+) T-cells, to the irradiated tumor as well as to distant tumor lesions. This innovative method used radiation therapy to extend tumor-targeted immunotherapy also to VEGF low tumors. Radiation-induced tissue injury, which is known to trigger angiogenic processes, is accompanied by upregulation of VEGF expression, especially in lesions expressing low levels of VEGF. The agonistic 4-1BB oligonucleotide aptamer was conjugated to an aptamer that binds to VEGF (114). The administration of this conjugate after tumor irradiation was used to induce an optimal 4-1BB co-stimulation at the tumor site that in turn enhanced tumor immunity and inhibited tumor growth, while no toxicities classically associated with systemic administration of 4-1BB ligands was observed. Thus, systemically administered but specifically tumor-/ VEGF-targeted 4-1BB co-stimulation in combination with radiation elicited a potent antitumor immune response capable of controlling the growth of distant non treated subcutaneous and metastatic breast tumor lesions (113). This anti-tumor T-cell activation as a result from tumor-localized radiation-induced anti-tumor immune responses strongly argues for a synergistic effect of radiotherapy with immune checkpoint inhibitors (115).

CONCLUSION

The tumor vascular endothelium is a key cell compartment for the response of tumors to cancer therapy. The tumor initiated neovascularization for nutrients and oxygen supply prior tumor progression results in a structural and functional abnormal tumor vasculature, which contributes to a pro-tumorigenic and immunosuppressive environment altering the therapy response of tumor cells. In particular for clinically approved immunotherapies, such as immune checkpoint blockade and adoptive T-cell transfer, the functional abnormal tumor vasculature fosters therapy resistance by limiting an inefficient recruitment, distribution and infiltration of tumor eradicating immune cells. Therefore, tumor vasculature targeting agents in order to re-activate specifically the tumor endothelial cells in terms of vascular normalization provide promising strategies to optimize the efficacy of currently employed cancer therapies, especially immunotherapies.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Vascular Targeting to Increase the Efficiency of Immune Checkpoint Blockade in Cancer

Maria Georganaki[†], *Luuk van Hooren*[†] *and Anna Dimberg**

Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, The Rudbeck Laboratory, Uppsala, Sweden

Boosting natural immunity against malignant cells has had a major breakthrough in clinical cancer therapy. This is mainly due to the successful development of immune checkpoint blocking antibodies, which release a break on cytolytic anti-tumor-directed T-lymphocytes. However, immune checkpoint blockade is only effective for a proportion of cancer patients, and a major challenge in the field is to understand and overcome treatment resistance. Immune checkpoint blockade relies on successful trafficking of tumor-targeted T-lymphocytes from the secondary lymphoid organs, through the blood stream and into the tumor tissue. Resistance to therapy is often associated with a low density of T-lymphocytes residing within the tumor tissue prior to treatment. The recruitment of leukocytes to the tumor tissue relies on up-regulation of adhesion molecules and chemokines by the tumor vasculature, which is denoted as endothelial activation. Tumor vessels are often poorly activated due to constitutive pro-angiogenic signaling in the tumor microenvironment, and therefore constitute barriers to efficient leukocyte recruitment. An emerging possibility to enhance the efficiency of cancer immunotherapy is to combine pro-inflammatory drugs with anti-angiogenic therapy, which can enable tumor-targeted T-lymphocytes to access the tumor tissue by relieving endothelial anergy and increasing adhesion molecule expression. This would pave the way for efficient immune checkpoint blockade. Here, we review the current understanding of the biological basis of endothelial anergy within the tumor microenvironment, and discuss the challenges and opportunities of combining vascular targeting with immunotherapeutic drugs as suggested by data from key pre-clinical and clinical studies.

Keywords: angiogenesis, cancer, checkpoint blockade, PD-1, PD-L1, CTLA-4, VEGF, endothelial activation

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***Correspondence:**
Anna Dimberg
anna.dimberg@igp.uu.se

[†] *These authors have contributed equally to this work*

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INTRODUCTION

The field of cancer immunotherapy has made significant improvements during the last decade due to the development of new effective means to boost tumor immune responses and achieve long-term remission or even cures in patients that were previously deemed to be untreatable. A major breakthrough was the development of antibodies targeting negative regulators of T-cell activation, termed immune checkpoints. Ipilimumab, an antagonistic antibody targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) improved overall survival in metastatic melanoma patients in 2010 (1). Following the success of anti-CTLA-4 therapy, antibodies targeting programmed cell death protein 1 (PD-1), or its ligand PD-L1,

proved to be effective at improving overall survival in a wide variety of cancers (2–7). Importantly, a proportion of patients achieve long-term remission, highlighting the potential of immune checkpoint blockade to induce durable responses (8). The encouraging results of these studies has sparked an interest from the cancer research field and inspired further investigations into targeting of alternative immune checkpoint molecules.

While checkpoint blockade represents a breakthrough in cancer therapy, a majority of cancer patients do not respond and some tumor types appear to be intrinsically resistant. The treatment is designed to boost an ongoing immune response and is inefficient in cases where initial immune activation is lacking, including tumors that are devoid of infiltrating T-cells (3, 9). Development of therapeutic strategies to enhance immune cell recruitment may therefore increase the proportion of patients responding to immune checkpoint blockade. Circulating T-cells are recruited through expression of adhesion molecules and chemokines on the endothelial cells, collectively mediating capture, rolling, and transmigration of leukocytes from the blood stream into the inflamed tissue (10). In many types of cancer, constitutive stimulation by pro-angiogenic factors secreted in the tumor microenvironment renders the vasculature morphologically and functionally abnormal, constituting a barrier to efficient leukocyte recruitment. In this mini-review we summarize phenotypical differences between normal vessels and tumor vessels in mediating leukocyte recruitment, the molecular mechanisms that underlie these functional changes and current efforts to improve immune checkpoint blockade through vascular targeting.

IMMUNE CHECKPOINT BLOCKADE THERAPY RELIES ON EFFICIENT T-LYMPHOCYTE RECRUITMENT

Immune checkpoint blockade works through inhibiting negative feedback loops that downregulate T-cell activation following an initial immune response. T-cell activation and T-cell receptor signaling has recently been reviewed in detail (11, 12). T-cells remain naïve until they encounter licensed antigen-presenting cells (APC)s that present the correct peptide antigen on major histocompatibility complex (MHC) molecules together with the appropriate co-stimulatory molecules. T-cell activation requires recognition of the MHC-antigen complex displayed on an APC, engagement of co-stimulatory molecules such as CD28 on the T cell with B7 family members on the APC and stimulation by inflammatory cytokines. In response to T-cell activation, other co-stimulatory molecules such as ICOS and OX40 are expressed, but also molecules that instigate negative feedback loops to prevent over-activation of T-cells. One of those negative feedback molecules is CTLA-4, which competes with CD28 for binding to B7 family members expressed on the surface of APCs (13–16). CTLA-4 is also highly expressed on regulatory T cells, and antibodies targeting CTLA-4 have been suggested to deplete them from the tumor microenvironment through Fc effector functions (17). Although the relative importance of the immune checkpoint and regulatory depletion mechanisms for therapeutic efficacy is still under active debate (16), blocking CTLA-4 in cancer enhances T-cell activation, but can also lead to autoimmune responses.

After activation, which generally occurs in secondary lymphoid organs, T-cells circulate and extravasate through the vasculature at sites of inflammation to locate and kill target cells displaying the cognate peptide antigen on their MHC molecules. At the tumor site, T-cell activity can be hampered by several types of immunosuppression, including engagement of PD-1 expressed on T-cells by its ligand PD-L1 expressed on stromal cells and/or malignant cells (18, 19). Thus, anti-cancer immunity can be enhanced by antibodies that block the PD-1/PD-L1 interaction. Although manipulating T-cell activation status by blocking inhibitory receptors or enhancing co-stimulatory molecules has proven to be efficacious in boosting anti-tumor immune responses, these treatments strictly rely on efficient transport of lymphocytes from the site of T-cell activation to the tumor tissue. It is therefore not surprising that tumors that are not infiltrated by T-cells, and tumors where T-cell infiltration is only observed at the tumor border but not in the core, do not respond well to immune checkpoint blockade (3, 9). Several mechanisms contribute to regulating the inflammatory state, including the mutational landscape of the tumor, expression of chemokines, and checkpoint molecules and recruitment of immunosuppressive cells (20, 21). In cases where an immune response is correctly mounted but where lymphocyte recruitment to the tumor tissue is lacking, pharmacologically altering vascular phenotype to allow efficient leukocyte trafficking may sensitize resistant tumors for immunotherapy.

LYMPHOCYTE RECRUITMENT INVOLVES LEUKOCYTE/ENDOTHELIAL INTERACTION

Leukocyte recruitment by activated endothelial cells and subsequent migration through the vessel wall is mediated by direct molecular interactions between proteins expressed by leukocytes and endothelial cells (**Figure 1A**). This finely tuned process, known as the leukocyte adhesion cascade, involves leukocyte capture, rolling, adhesion, arrest, and transendothelial migration (10). This is enabled by up-regulation of adhesion molecules and chemokines on the surface of endothelial cells, denoted "endothelial activation." Leukocyte capture and rolling are mainly mediated by interaction between selectins expressed on endothelial cells (P-selectin and E-selectin) and leukocytes (L-selectin) to carbohydrate ligands including P-selectin glycosylated ligand 1. Firm adhesion of leukocytes is mediated through interaction of leukocyte integrins with endothelial adhesion molecules. For T-lymphocytes, firm adhesion is mainly induced by lymphocyte function-associated antigen (LFA)-1 and very late activation antigen (VLA)-4 binding to endothelial intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, respectively. Activation of integrins via inside-out signaling associated with chemokine stimulation triggers leukocyte arrest to the endothelium (10). Blood flow-derived shear stress contributes

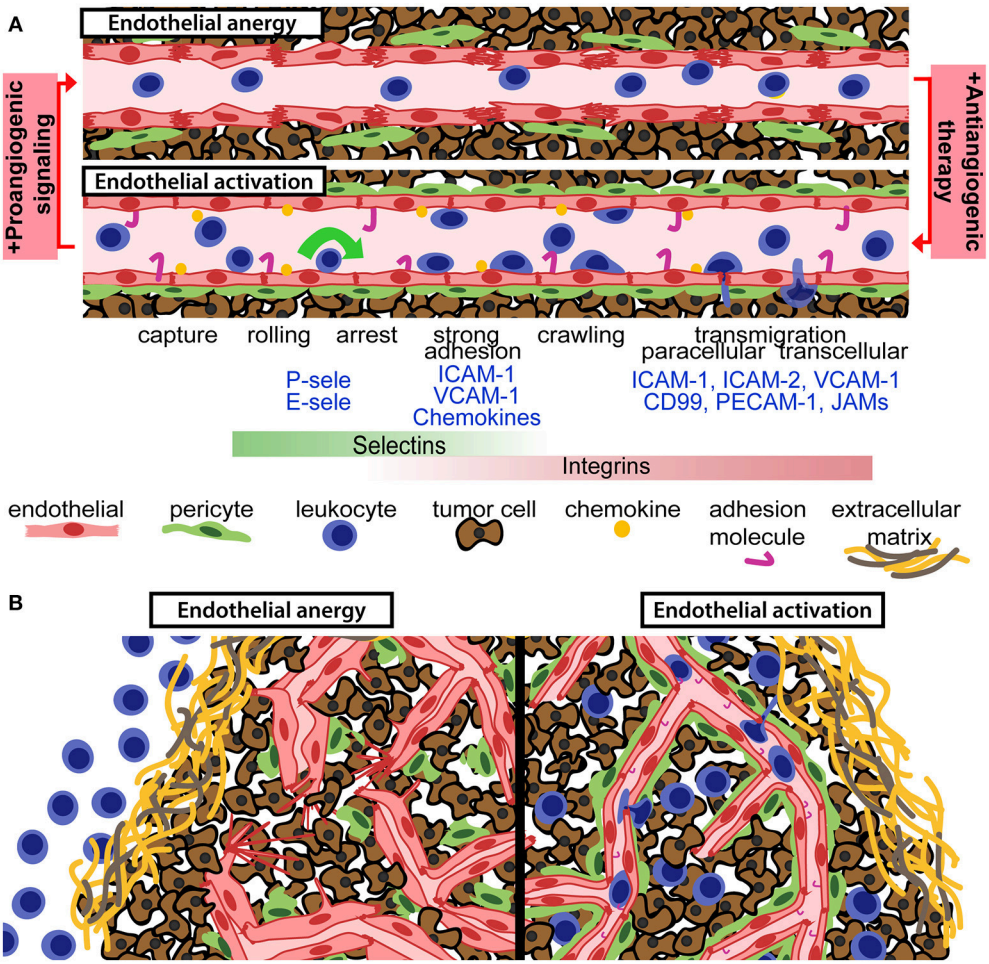

FIGURE 1 | Anti-angiogenic therapy can relieve endothelial anergy, improve vessel function and enhance T-cell infiltration. **(A)** Aberrant pro-angiogenic signaling in the tumor microenvironment gives rise to an anergic endothelium with reduced pericyte coverage, disrupted endothelial cell junctions, and suboptimal activation status. Anti-angiogenic therapy reverts those defects and permits for enhanced leukocyte recruitment, through the leukocyte adhesion cascade. Chemokines and adhesion molecules on the activated endothelial surface allow for leukocyte capture, rolling, arrest, and transendothelial migration into the tumor tissue. **(B)** Aberrant pro-angiogenic signaling in tumors is associated with dysfunctional and anergic tumor vessels, which are not capable of recruiting tumor-targeted leukocytes (left panel). Vascular targeting can relieve endothelial anergy, improve perfusion and increase the recruitment of leukocytes into the tumor microenvironment (right panel).

to efficient leukocyte capture and integrin activation through mechanical forces (22). Transendothelial migration can occur through either through paracellular or transcellular pathways (10, 23). Finally, leukocytes migrate through the basement membrane and pericyte layer to reach the inflamed tissue (10). Recruitment of lymphocytes to the tumor tissue strictly depends on efficient regulation of molecules required for cell-cell interactions during capture, rolling, adhesion, and transendothelial migration.

TUMOR ANGIOGENESIS RESULTS IN MORPHOLOGICALLY AND FUNCTIONALLY DISTINCT VESSELS

Tumors need access to capillary network to proliferate, and the ability of tumors to stimulate angiogenesis is recognized as one of the hallmarks of cancer (24). Angiogenesis is induced as a result of enhanced growth factor secretion in the tumor microenvironment, shifting the balance from predominantly angiostatic to pro-angiogenic signaling (25). This "angiogenic switch," observed as a shift from avascular to vascular tumors, can occur in dormant, and slow growing tumors and be associated with tumor progression to higher malignancy grades.

Several mechanisms can trigger neovascularization in tumors, including hypoxia, genetic alterations in tumor cells, expression of cytokines, and growth factorsm and recruitment of bone marrow-derived circulating cells (26–28). When proliferation of malignant cells results in a tumor mass that cannot be sufficiently oxygenated by pre-existing vasculature this leads to hypoxia. Hypoxia-induced stabilization of hypoxia-inducible factor (HIF)-1α triggers up-regulation of its target genes, including several pro-angiogenic genes such as vascular endothelial growth factor (VEGF) (29). VEGF secreted by

tumor cells diffuses through the tissue and activates its receptor VEGFR2 expressed on endothelial cells (30). Downstream of VEGFR2 activation, multiple intracellular pathways are induced that regulate cell division, survival, sprouting, and migration of endothelial cells (30). Several other pro-angiogenic growth factors contribute to tumor angiogenesis, including the family of angiopoietins and their cognate receptor TIE-2 and the fibroblast growth factor family (31). Some tumors harbor mutations of the gene coding for the von Hippel-Lindau protein, a crucial member of the ubiquitin ligase complex that degrades HIF-1α (32). These mutations stabilize HIF-1α, allowing expression of pro-angiogenic factors under normoxic conditions. Myeloid cells, including macrophages, neutrophils, and myeloid derived suppressor cells (MDSCs), can also stimulate vessel formation through expression of pro-angiogenic factors and/or matrix metalloproteases that release VEGF from extracellular matrix (33).

Physiological angiogenesis is a well-controlled process that is attenuated when the need for new vessels have been met, but tumor angiogenesis is deregulated and continuous due to excessive expression of pro-angiogenic factors (34). Tumor angiogenesis can give rise to disorganized vessels that are tortuous, dilated and poorly covered by pericytes (35). The tumor vasculature is often leaky due to endothelial junctional defects, blood flow is generally slow and perfusion is irregular (25). Gene expression analyses have shown that tumor vessels differ molecularly from their normal counterparts and have revealed a high level of vessel heterogeneity depending on the resident tumor tissue (36–40). Importantly, tumor vessels can have multiple phenotypes ranging from normal to dysfunctional and the morphology and functionality significantly differ depending on tumor type and anatomical site.

TUMOR BLOOD VESSELS ARE BARRIERS TO EFFICIENT LEUKOCYTE RECRUITMENT

Immune cells in the circulation are dependent on the vascular network to reach the tumor and kill malignant cells. However, functional abnormalities of tumor blood vessels represent difficult hurdles for leukocyte recruitment. The architectural defects of tumor vessels limit perfusion and alter sheer stress, and differential protein expression in tumor endothelial cells can dampen the immune response (34, 41–44). Tumor endothelial cell respond inefficiently to pro-inflammatory signaling, and fail to express sufficient levels of molecules involved in the leukocyte capture, adhesion and extravasation process (**Figure 1**). Downregulation or ineffective clustering of adhesion molecules on tumor endothelial cells limits T-cell infiltration and inhibit anti-tumor immunity (45–47). Reduced expression of adhesion molecules in tumor vessels has been observed in several types of human cancer (48–50). Endothelial activation is generally induced by binding of pro-inflammatory cytokines such as tumor necrosis factor (TNF)α and interleukin (IL)-1 to their endothelial receptors, leading to activation of the transcription factor nuclear factor-κB (NF-κB) and up-regulation of selectins, adhesion molecules and chemokines (51). Pro-inflammatory cytokines are abundantly expressed in many cancers, but pro-angiogenic factors present in the tumor microenvironment can suppress expression of adhesion molecules and chemokines that attract cytolytic T-cells and NK cells such as CXCL10 and CXCL11 (41, 52, 53). VEGF-induced signaling pathways can directly interfere with TNF-α-induced NF-κB activation, globally repressing TNF-α-induced gene expression in endothelial cells (53). Consistent with this, antagonizing VEGFR2 signaling sensitizes endothelial cells to TNF-α (54). However, the interplay between angiogenesis and inflammation is context dependent. TNF-α stimulation synergistically primes endothelial cells for VEGF-induced angiogenesis (55). Notably, VEGF stimulation can induce leukocyte infiltration in some systems, and pathways downstream of VEGF signaling can both induce and repress adhesion molecule expression (56–58). Nitric oxide and molecules such as epidermal growth factor-like domain 7 can also regulate adhesion molecule expression and clustering in tumors (59, 60). Another less studied feature of endothelial regulation of tumor immunity is the selective recruitment of immunosuppressive leukocytes through expression of specific adhesion molecules such as the common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) (41).

In addition to regulating leukocyte entry, tumor endothelial cells can alter the anti-tumor immune response by modulating immune cell activity or viability. This can occur as a response of endothelial cells to tumor-derived growth factors (61). The concept of a "tumor endothelial barrier" refers to molecules expressed on endothelial cells that inhibit promote T-cell arrest. An example of this is tumor endothelial upregulation of FasL in response to tumor-derived VEGF, IL-10 and prostaglandin E2, which has been shown to selectively kill effector CD8 T-cells but not Treg cells (44, 62). Endothelial cells can express several inhibitory molecules including immune checkpoint molecules [PD-L1, T-cell immunoglobulin domain and mucin domain (TIM3), B7-H3 and B7-H4], death receptor-ligands (TNF-related apoptosis-inducing ligand (TRAIL) and secreted immunomodulatory factors (IL-6, prostaglandin E (PGE) 2, IL-10, and TGF-β) (44, 62).The relative importance of endothelial expression of these molecules in immunosuppression and their regulation in tumor vessels need further investigation. Antigen presentation by endothelial cells suggests that they can function as potential antigen presenting cells (63). Whether tumor endothelial cells present antigen and if this is sufficient for activation of T-cells, or alternatively induces T-cell anergy, is still unknown. As discussed below, anti-angiogenic therapies can alleviate endothelial anergy and enhance T-cell recruitment in tumors (53, 64–67). Immunosuppressive molecules expressed on tumor endothelial cells represent new potential targets for novel combination treatments with immunotherapy.

SUCCESSES AND FAILURES OF ANTI-ANGIOGENIC THERAPY

The idea that anti-angiogenic therapy could block tumor progression by depriving the tumor cells of oxygen and

TABLE 1 | Selected studies combining anti-angiogenic therapy with immune checkpoint blockade in preclinical models and clinical trials.

Anti-angiogenic target	Immune checkpoint target	Cancer model	Survival	References
PRECLINICAL MODELS				
VEGF (B20-4.1.1)	PD-L1 (6E11)	SCLC	+ **	(81)
VEGFR2 (DC101)	PD-1 (RMPI-14)	Colon-26 adenocarcinoma	+	(82)
VEGF and ANG2 (Vanucizumab)	PD-1 (RMPI-14)	MMTV-PyMT, RIP1-Tag2, Melanoma, Neuroendocrine	+ ***	(83)
VEGFR-1,-2 and−3 (Axitinib)	CTLA-4 (9H10)	Melanoma	+	(84)
VEGFR2* (Sunitinib)	PD-1 (RMPI-14)	Colon cancer	+	(85)
VEGFR2 (DC101)	PD-L1 (10F.9G2)	Pancreatic cancer, breast cancer and glioblastoma	+	(86)
VEGF + ANG2 (10F.9G2 + CVX-241)	PD-L1 (10F.9G2)	Breast cancer	+/-	(87)
Anti-angiogenic target	**Immune checkpoint target**	**Cancer type**	**Trial**	**References**
CLINICAL TRIALS				
VEGFR-1,-2 and−3 (Axitinib)	PD-1 (Pembrolizumab)	Renal cell cancer	Phase 3	(88)
VEGF (Bevacizumab)	CTLA-4 (Ipilimumab)	Metastatic melanoma	Phase 1	(89) (90) (91)
VEGFR-1,-2 and−3 (Axitinib)	PD-L1 (Avelumab)	Advanced clear-cell renal cell carcinoma	Phase 1b	(92)
VEGFR-1,-2 and−3 (Lenvatinib)	PD-1 (Pembrolizumab)	Renal cell cancer	Retrospective	(93)
VEGF (Bevacizumab)	PD-L1 (Atezolizumab)	Metastatic renal cell carcinoma	Phase 1b	(94)

*Antibody clone or brand name in brackets. SCLC = small-cell lung cancer, * broad tyrosine kinase inhibitor, ** increased T-cell exhaustion, *** increased T-cell numbers and endothelial activation. Ongoing clinical trials are available at www.clinicaltrials.gov and were recently reviewed by Fukumura et al. (95).*

nutrients (68) led to intense research efforts and sparked numerous clinical trials. A number of anti-angiogenic drugs have been approved to date, several of which are antibodies or small tyrosine kinase inhibitors that target VEGF/VEGFR signaling (69). The first clinically approved drug was a humanized antibody targeting VEGF named Bevacizumab. Treatment with Bevacizumab slows tumor growth in patients with non-small cell lung and colorectal cancer, though with only a marginal improvement of long-term survival (70, 71). It has also been approved for patients with cervical cancer, glioblastoma, ovarian cancer and renal cell carcinoma (72). In breast, melanoma, pancreatic, and prostate cancer no improvement of overall survival has been observed (73).

Treatment of colorectal cancer patients with Bevacizumab results in an initial response with decreased tumor growth or even regression. However, relapse is common, associated with rapid rebound angiogenesis, and tumor regrowth is often more aggressive than before anti-angiogenic treatment (74). Several mechanisms have been proposed for the resistance to anti-angiogenic treatment, including co-option of normal vessels in the surrounding tissue, recruitment of pro-angiogenic myeloid cells and upregulation of alternative pro-angiogenic factors (25). Notably, anti-angiogenic treatment can increase invasiveness and promote metastasis formation in experimental models of cancer (75, 76). Although metastasis-promoting effects of anti-angiogenic therapy have not been observed in clinical studies, the pre-clinical work has cautioned the field and questioned how anti-angiogenic therapy should best be administrated (77).

ANTI-ANGIOGENIC THERAPY CAN IMPROVE THE EFFECT OF IMMUNE CHECKPOINT BLOCKADE

The importance of a functional vasculature for immune cell recruitment justifies efforts of combining immunotherapy with vascular targeting to improve vessel function and enhance up-regulation of adhesion molecules and chemokines. Inhibition of angiogenic signaling using sub-maximal doses of anti-angiogenic drugs may result in a normalization of vascular function and improve the efficacy of other anti-cancer drugs, as proposed by Jain (78). Anti-angiogenic therapy provides relief of continuous angiogenic signaling, which at sub-maximal doses can result in vessel pruning, maturation, and improved perfusion (69). For cancer immunotherapy, there is an added benefit that anti-angiogenic drugs enhance expression of adhesion molecules and chemokines involved in T-cell recruitment (53, 64–67). Therefore, combining immunotherapy with anti-angiogenic drugs may relieve endothelial anergy and induce lymphocyte infiltration into tumors that prior to treatment were of an immune-excluded phenotype (**Figure 1B**). Indeed, by combining adoptive T-cell transfer with anti-VEGF therapy in murine melanoma, tumor T-cell infiltration was increased and survival was prolonged (79). An important challenge in this concept is that the dosing of anti-angiogenic drugs is crucial for normalizing vessels and improving T-cell recruitment, and that the optimal dose may differ between patients (80). Nevertheless, the combination of immunotherapy and anti-angiogenic therapy has shown benefit in various therapeutic settings (**Table 1**).

Drugs targeting VEGF/VEGFR2 signaling have been observed to enhance the response to immune checkpoint antibodies in pre-clinical tumor models. The combination of anti-VEGF and anti-VEGFR2 antibodies prolonged survival in a murine model of adenocarcinoma in combination with PD-1 blockade (82). Similarly, an antibody targeting both VEGF and Angiopoeitin-2 improved responses to PD-1 inhibition in preclinical cancer models (83). The VEGFR inhibitor axitinib combined with anti-CTLA-4, but neither monotherapy, prolonged survival of mice bearing murine melanoma (84). This was associated with increased numbers of $CD4^+$ and $CD8^+$ T cells in the tumor after the combination treatment. In addition to their effect on vessel phenotype, therapies targeting pro-angiogenic factors can alleviate immunosuppression by directly affecting the immune cells. For example, the tyrosine kinase inhibitor Sunitinib can decrease MDSCs and Tregs (67, 96, 97).

The first phase I clinical trial combining anti-angiogenic therapy with immune checkpoint blockade was a study using Bevacizumab and ipilimumab (anti-CTLA-4). The combination therapy modulated tumor vessel morphology and induced endothelial activation, associated with increased infiltration of dendritic cells and cytotoxic T-cells in melanoma tumors (89, 98). Similarly, combining atezolizumab (anti-PD-L1) with Bevacizumab in patients with metastatic renal cell carcinoma resulted in enhanced trafficking of lymphocytes, and increased cytotoxic T cells (94). Following these promising results, several clinical trials with the same therapeutic rationale have been initiated (95, 98, 99).

FUTURE DIRECTIONS BEYOND NORMALIZATION AND ENDOTHELIAL ACTIVATION

An emerging concept is that vascular targeting in combination with immune checkpoint blockade may promote tumor immunity by inducing formation of high-endothelial venules (HEV)s. HEVs are specialized vessels found in secondary lymphoid organs that are adapted for lymphocyte trafficking (100). The combination of anti-VEGFR2 antibodies with PD-L1 antibodies induced formation of HEVs and improved T-cell infiltration in the polyoma middle T oncoprotein (PyMT) breast cancer model and the Rip1-Tag2 pancreatic neuroendocrine tumor model (RT2-PNET) (86). Formation of HEVs in glioblastoma models required further stimulation using a lymphotoxin β receptor agonistic antibody, resulting in enhanced T-cell infiltration and reduced tumor growth (86). Vessel normalization in combination with a vascular targeting peptide coupled to LIGHT, a ligand for the lymphotoxin β receptor, induced HEVs and tertiary lymphoid structures in Rip1-Tag5 pancreatic neuroendocrine tumors. Importantly, this therapeutic approach sensitized these tumors to anti-PD-1 and anti-CTLA-4 antibody therapy (101). These studies indicate that beyond normalizing vessels, transforming tumor vessels to HEVs can be of additional benefit in enhancing the response to cancer immunotherapy. Furthermore, HEVs may promote formation of tertiary lymphoid structures which have been associated with a beneficial response to cancer immunotherapy in several types of cancer (100, 102).

Current efforts in vascular targeting aim to improve the efficacy of cancer immunotherapy through inhibition of pro-angiogenic signaling. However, several immunosuppressive molecules that contribute to the tumor endothelial barrier are regulated through alternative pathways, and may be induced secondary to immune activation. This aspect has not yet been sufficiently explored. An increased understanding of the cross-talk between tumor cells, endothelial cells, and immune cells during immune checkpoint blockade therapy may lead to new combinatorial treatment regimens that enhance the abundance of activated T-cells in tumor tissue. This can ultimately increase the proportion of patients that respond to immune checkpoint blockade.

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REVIEW
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The Evolving Landscape of Immunotherapy-Based Combinations for Frontline Treatment of Advanced Renal Cell Carcinoma

Asim Amin[1,2]* *and Hans Hammers*[3]

[1] *Levine Cancer Institute, Charlotte, NC, United States,* [2] *Atrium Healthcare System, Charlotte, NC, United States,* [3] *University of Texas Southwestern Medical Center, Dallas, TX, United States*

Insights into the biology of advanced renal cell carcinoma (aRCC) and the development of agents targeting the vascular endothelial growth factor (VEGF) pathway have positively impacted the outcomes for patients with aRCC. With the recent approval of the dual immune checkpoint inhibitors (ICIs), nivolumab and ipilimumab, by the U.S. Food and Drug Administration (USFDA), and the European Medicines Agency (EMA), the era of VEGF monotherapy for untreated aRCC appears to be coming to an end for patients with access to the combination therapy. The frontline treatment options for renal cell carcinoma are evolving rapidly and will lead to the approval of other combination immunotherapies—especially those with VEGF inhibitors. Here we review the clinical data for dual immune checkpoint inhibition with nivolumab plus ipilimumab as well as the emerging data for ICI plus VEGF inhibitor combinations and discuss the challenges these will pose for the clinical practitioner.

Keywords: combination immunotherapy, VEGF inhibition, immune check point inhibitor, advanced renal cell carcinoma, immuno modulation

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Fudan University, China

***Correspondence:**
Asim Amin
asim.amin@atriumhealth.org

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INTRODUCTION

Historical Perspective

Immunotherapy with high-dose interleukin-2 (HD IL-2) (1, 2) had been the mainstay for treatment of advanced renal cell carcinoma (aRCC) in the United States until agents targeting the VEGF pathway became available in 2005. HD IL-2 was shown to elicit a response in 25% of patients with advanced clear renal cell carcinoma (3). **PR**oleukin **O**bservational Study to Evaluate the Treatment Patterns and **CL**inic**a**l Response in **M**alignancy (PROCLAIM), a US-based multicenter study designed to capture real-world clinical data for interleukin-2 in patients with metastatic melanoma, aRCC, or other malignancies showed that response after treatment with HD IL-2 was durable. The median overall survival was not reached at a median follow-up of 21 months; the 30-month survival rate for patients who achieved a complete response (CR), partial response (PR), or had stable disease (SD) was 100, 75, and 78%, respectively (4). Given the considerable toxicity associated with HD IL-2, the applicability and overall impact on kidney cancer was limited since it required patients to have an overall excellent level of fitness and specially trained staff to oversee administration. The advent of multi-tyrosine kinase inhibitors (TKIs) or antibodies targeting the VEGF axis clearly had a significant and broad impact on the natural history of advanced renal cell carcinoma; however, durable response is rare (5–14), and therefore the development of new options that are tolerable and have the potential for durable responses remains an area of active investigation.

Anti-tumor Immune Response

The generation of an effective anti-tumor immune response requires several critical events to happen in a well-orchestrated sequence. The initial step is presentation of tumor antigen/s by the dendritic cells/antigen presenting cells in the context of self major histocompatibility complex (MHC) molecules to the T cells. This occurs in the lymphoid tissues or the central immune environment. Recognition of the tumor antigens as non-self by the T cells results in generation of the first signal for an anti-tumor immune response to proceed. T cell activity is subsequently modulated by several proteins—immune checkpoints—expressed on the surface of T cells. These immune checkpoints can serve as both, "on" or "off switches" for the T cell. Blockade of the "off switches" or stimulation of the "on switches" can result in increased activity of the T cells and has been used for modulation of the anti-tumor immune response in the clinical setting (**Figure 1**).

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is an inhibitory immune checkpoint expressed on the surface of activated T cells. Engagement of CTLA-4 with B-7 family of molecules expressed on the antigen-presenting cells results in an inhibitory signal that switches T cells off. Ipilimumab is an anti-CTLA-4 antibody that blocks this interaction and prevents T cells from switching off (**Figure 1**) in the central immune environment. Tremelimumab is another anti-CTLA-4 antibody that is currently undergoing investigation. Additionally, T regulatory (Treg) cells, which are potent suppressors of the immune response, are known to express very high levels of CTLA-4 and are probably affected by CTLA-4 inhibition as well. The activated T cells then move to the tumor micro-environment where they encounter multiple inhibitory factors. Programmed death 1 (PD-1) is an inhibitory checkpoint expressed on activated T cells that when engaged with its ligands, PD-L1 or PD-L2 (expressed on some tumors, immune system cells, and normal cells), results in suppression of T cell activity. Blockade of PD-1/PD-L1 axis by anti-PD-1 antibodies (pembrolizumab and nivolumab) or anti-PD-L1 antibodies (atezolizumab, avelumab, and durvalumab) allow T cells to maintain their anti-tumor activity in the tumor micro-environment. Objective responses ranging from 20 to 60% have been demonstrated with ICIs in various tumors.

ICIs in aRCC

Nivolumab is an anti-PD-1 antibody that blocks the interaction between PD-1 and its ligands PD-L1/PD-L2, thereby preventing the cytotoxic T cells from "switching off" or getting "exhausted." In a randomized phase 2 study for patients who had previously shown progression after at least one line of anti-angiogenic therapy for their aRCC, nivolumab monotherapy at the doses of 0.3, 2, and 10 mg/kg administered intravenously every 2 weeks, showed an overall response of 20% (15). Treatment was tolerated well and the overall survival compared very favorably to what had been observed in prior phase 3 studies. A subsequent phase 3 (CheckMate 025) study compared nivolumab monotherapy to everolimus in patients with aRCC who had received prior treatment with a VEGF TKI. Statistically significant improvement in overall survival in favor of nivolumab (25 months, 95% CI 21.8-not estimable vs. 19.6 months with everolimus, 95% CI 17.6–23.1) led to the approval of nivolumab for this population of patients with aRCC (hazard ratio 0.73, 98.5% CI 0.57–0.93; $p = 0.002$). Grade 3–4 treatment-related adverse events were observed in 19% of the patients who received nivolumab and 37% who received everolimus (16).

Ipilimumab is an anti-CTLA-4 antibody that prevents activated cytotoxic T cells from "switching off" by blocking its interaction with the B7 family of molecules. In advanced melanoma, the combination of nivolumab plus ipilimumab showed substantial activity and was approved by the USFDA (17, 18). The recent approval of nivolumab plus ipilimumab for intermediate and poor-risk patients with aRCC based on the CheckMate 214 study (described below) in the first-line setting by the USFDA and EMA marked a new milestone and established the proof of concept for combination immunotherapy in aRCC (19), albeit with a modified dosing schema.

CHECKMATE 214—NIVOLUMAB PLUS IPILIMUMAB vs. SUNITINIB

In this phase 3 study, patients with aRCC were randomized in a 1:1 ratio to receive either nivolumab 3 mg/kg plus ipilimumab 1 mg/kg (N3I1) intravenously for 4 doses every 3 weeks followed by nivolumab monotherapy maintenance every 2 weeks or sunitinib at the dose of 50 mg orally once a day on a 4-week-on, 2-week-off schedule. The co-primary endpoints were objective response rate (ORR), progression free survival (PFS), and overall survival (OS) in intermediate and poor-risk patients. Secondary endpoints were ORR, PFS, and OS in the intention to treat (ITT) population and the incidence of adverse events (20).

One thousand ninety-six patients were enrolled in the study, 550 on the N3I1 arm and 546 on the sunitinib arm; 425 patients in the N3I1 arm and 422 patients in the sunitinib arm were intermediate and poor risk. In the intermediate and poor-risk patients, the ORR was 42% (95% CI 37–47) in the N3I1 arm, compared to 27% (95% CI 22–31) for those who received sunitinib ($p < 0.001$). Statistically significant improvement in overall survival was noted in favor of the N3I1 arm, compared to sunitinib (hazard ratio, 0.63; $p < 0.001$). At a median follow-up of 25.2 months, the median overall survival was not reached for the N3I1 arm (95% CI 28.2 months to not estimable), compared to 26 months for the sunitinib arm (95% CI 22.1 months to not estimable). The median duration of response in the N3I1 arm was not reached (21.8 months to not estimable) and was 18.2 months (14.8 months to not estimable) in the sunitinib arm. The median PFS was 11.6 months, compared to 8.4 months for N3I1 and sunitinib arms, respectively, and did not meet criteria for statistical significance (hazard ratio, 0.82; $p = 0.03$).

No new safety signals were noted; 93% of the patients who received N3I1 and 97%, who were treated with sunitinib, experienced an adverse event. Grade 3–4 events were observed in 46% of the patients in the N3I1 arm and 63% in the sunitinib arm. Treatment was discontinued in 22% of the patients in the N3I1 arm and 12% in the sunitinib arm, secondary to adverse events. There were 8 deaths in the N3I1 arm and 4 deaths in the sunitinib arm attributed to treatment. Around 35% of patients required treatment with high-dose corticosteroids (defined as 40 mg prednisone equivalents for at least 14 days).

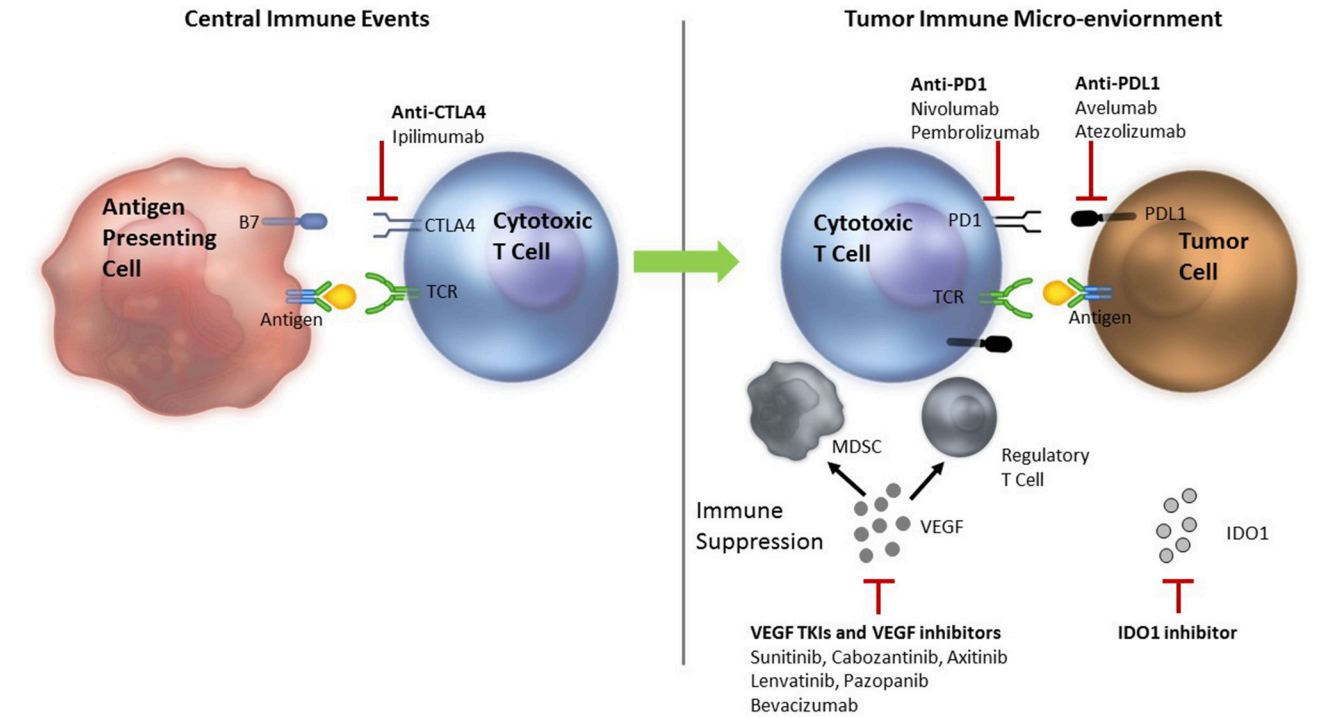

FIGURE 1 | Rationale for combining ICIs with VEGF inhibitors—Anti-tumor immune response modulation. TCR, T cell receptor; MDSC, myeloid derived suppressor cells; Treg, T regulatory; VEGF, vascular endothelial growth factor; PD-1, programmed death 1; PD-L1, programmed death ligand 1; CTLA 4, cytotoxic T lymphocyte antigen 4; TIM3, T cell immunoglobulin mucin receptor 3; LAG3, lymphocyte activation gene 3; ICIs, immune checkpoint inhibitors. Tumor antigen is presented to the cytotoxic T cells in the lymphoid tissues to initiate an anti-tumor immune response. The response is modified by several ICIs. VEGF inhibits dendritic cell maturation decreasing antigen presentation and inhibits T cells leading to their exhaustion. Primed T cells move to the tumor microenvironment where they encounter more immune suppression induced by VEGF that recruits MDSCs and Treg cells. VEGF also results in neo-angiogenesis that can alter the quality and quantity of infiltrate of the tumor immune microenvironment adding to immune suppression. Inhibition of VEGF by VEGF TKIs and anti-VEGF antibodies can reverse the VEGF induced immune suppression. Anti-CTLA-4 antibodies, ipilimumab and tremelimumab bind to the CTLA-4 inhibitory checkpoint and prevent the T cells from switching off. Anti-PD-1 antibodies nivolumab or pembrolizumab and anti-PD-L1 antibodies avelumab, atezolizumab or durvalumab bind to PD-1 and PD-L1 in the tumor, respectively to prevent T cells from switching off.

Based on the above results, the combination of nivolumab and ipilimumab was approved for the first-line treatment of intermediate and high-risk patients with aRCC by the USFDA and EMA.

RATIONALE FOR COMBINING ICIs WITH VEGF INHIBITION

VEGF and Tumor Immune Micro-Environment (TIME)

Tumor micro-environment is complex and not well-characterized. Interactions between the milieu of cytokines present in the micro-environment, phenotype of the immune cells, proteins expressed on the tumor cells, stromal components, and vascularity may all impact the outcomes for immunotherapy. VEGF plays a key role in aRCC and has been targeted successfully with significant therapeutic efficacy. The antitumor activity of VEGF TKIs/VEGF blockers has in most part been attributed to inhibition of neo-angiogenesis; however, the angiogenic activity also interacts with the immune status. Therefore, VEGF inhibition may modulate the host tumor immune micro-environment (TIME) and contribute to anti-tumor activity.

The presence of VEGF in the tumor micro-environment can lead to immune suppression via several mecahnisms. High VEGF levels lead to an abnormal vasculature in the tumors with high interstitial pressures that can decrease the immune cell traffic impacting the quantity and quality of the infiltrate. Based on the early data from evaluation of the immune infitrate in the tumor, the TIME has been classified as Binnewies et al. (21):

I-E TIME (Infitrated-excluded): Tumor-immune micro-environment characterized by exclusion of cytotoxic T cells from the core. Considered immunologically "cold tumors."

I-I TIME (Infitrated-inflamed): Tumor-immune micro-environment infiltrated with cytotoxic T lymphocytes expressing PD-1, leukocytes and tumor cells expressing PD-L1. Considered immunologically "hot tumors."

TLS-TIME (Infiltrated-inflamed tertiary lymphoid structures): A subclass of infiltrated–inflamed micro-environment displaying tertiary lymphoid structures/aggregates with every population of lymphocytes including naïve T cells, regulatory T cells, B cells, and dendritic cells.

The quality and quantity of immune cell infiltrate in the TIME can impact the response to immunotherapy with ICIs. Immunologically "hot" tumors respond more often than"cold" tumors.

Increased level of VEGF in the tumor can induce suppression of both innate and adaptive immune responses, e.g., VEGF has been shown to directly inhibit dendritic cell maturation (22, 23), increase recruitment of myeloid-derived suppressor cells (MDSCs) and Treg cells (24, 25), and decrease trafficking and efficacy of cytotoxic T cells (26). VEGF has also been reported to inhibit T cell development (27). VEGF-A in the tumor micro-environment was shown to increase expression of inhibitory checkpoints, PD-1, CTLA-4, TIM3, and LAG3, which was shown to be reversed by antibodies against VEGFR-2 (26). Elevated serum and tumor VEGF levels have been associated with poor disease-specific survival in patients with aRCC (28).

Inhibition of the VEGF axis by VEGF TKIs and anti-VEGF antibodies can potentially reverse the immune suppression induced by VEGF. In the preclinical renal cell carcinoma model (RENCA), combination of a murine anti-PD-1 antibody and suntinib showed synergistic activity and greater numbers of tumor infiltrating lymphocytes, compared with controls treated with each agent alone (29). In a clinical trial, patients with aRCC showed significant increase in the percentage of interferon gamma–producing T cells, decrease in IL-4 production, and decrease in Treg cells in the peripheral blood after receiving sunitinib 50 mg orally once a day for 28 days (30). Significant reduction in MDSCs was also observed, demonstrating reversal in immune suppression (31). Expansion of tumor infiltrating lymphocytes and reduction in MDSCs was observed in primary tumors from patients who received sunitinib prior to the surgery, compared to those who were treatment-naïve (32). Insight into how different TKIs may vary in their ability to modulate the TIME is still limited. While sunitinib did not appear to impact dendritic cell function, sorafenib was noted to inhibit generation of antigen-specific T cells due to dendritic cell suppression (33). Bevacizumab has been noted to promote activity and reverse the inhibitory effects of VEGF on dendritic cells (34, 35). In a clinical trial with the combination of ipilimumab plus bevacizumab in patients with advanced melanoma, activated vessel endothelium with extensive CD8+ cell and macrophage infiltration was observed, compared to controls who received ipilimumab alone (36).

Durable responses have been observed with immune checkpoint inhibitors (ICIs) in up to 40% of the patients with aRCC treated with a combination of PD-1/CTLA-4 inhibitors. Although there has been a significant improvement compared to historical controls, there remains an unmet need in this therapeutic area. Recognizing that there are big gaps in our knowledge, VEGF inhibitors by improving dendritic cell function, antigen presentation, normalization of the tumor vasculature with greater trafficking of immune cells, increased cytotoxic T cell infiltration, and decreased MDSCs and Treg cells could potentially reduce the immunosupressive effect in the tumor micro-environment; therefore, evaluating them in combination with ICIs appears to be a logical step. In this context, multiple efforts are underway; here we describe the immune checkpoint inhibitor–based combinations that are approved or are in advanced stages of development (**Tables 1**, **2**).

IMMUNOTHERAPY PLUS VEGF INHIBITOR COMBINATION STUDIES

CheckMate 016—Nivolumab Plus TKIs (Sunitinib/Pazopanib) or Ipilimumab

CheckMate 016 was the first trial to explore the safety and tolerability of combination immunotherapy in the setting of aRCC (37–39). This multicenter phase 1 study had 5 treatment arms that included the combination of nivolumab (N) with either TKIs, sunitinib (S) or pazopanib (P) or ipilimumab (I) at the following doses; nivolumab 1 mg/kg plus ipilimumab 3 mg/kg (N1I3), nivolumab 3 mg/kg plus ipilimumab 1 mg/kg (N3I1), and nivolumab 3 mg/kg plus ipilimumab 3 mg/kg (N3I3). The TKI combination arms had an initial dose escalation phase with a starting nivolumab dose of 2 mg/kg (N2) intravenously every 3 weeks with planned increase to nivolumab 5 mg/kg (N5) intravenously every 3 weeks in the expansion phase dependent on the maximum tolerated dose (MTD) assessed by the modified toxicity probabity interval (MTPI). Sunitinib and pazopanib in these arms were administered at the standard dose of 50 mg orally once a day on a 4-week-on, 2-week-off schedule, and 800 mg orally once a day, respectively. In the nivolumab plus ipilimumab arms, the combination therapy with N1I3, N3I1, or N3I3 was administered intravenously every 3 weeks for 4 doses in the induction phase followed by maintenance nivolumab monotherapy at the dose of 3 mg/kg, administered intravenously every 3 weeks. Primary endpoints for the study were safety and tolerability; secondary endpoints included ORR, duration of response (DOR), and PFS rate.

A total of 194 patients were enrolled in this study, 153 received treatment; 33 patients received N+S; 20 recieved N+P; 47 patients were assigned to both N1I3 and N3I1 arms; and 6 patients were treated on the N3I3. The N+S arm completed dose-escalation and expansion phases, while the N+P arm did not proceed to expansion given the early hepatic toxicity observed in the dose-escalation phase. All 6 patients in the N3I3 arm were censored at the time of analysis.

All patients (100%) assigned to the ICI+VEGF–TKI combination arms, N+S and N+P, experienced a treatment-related adverse event; the incidence of grade 3–4 events was 81.8 and 70%, respectively. The most common grade 3–4 adverse events were hypertension (18.2%, 10%), increased alanine aminotrasferase (ALT) (18.2%, 20%), increased aspartate aminotransferase (AST) (9.1%, 20%), diarrhea (9.15, 20%), and fatigue (9.1%, 15%) in the N+S and N+P arms, respectively. In the N+S arm, 39.4%, and in the N+P arm, 60%, of the patients required systemic corticosteroids for management of adverse events that were attributed to immune-mediated etiology. There were no deaths attributed to treatment; 39.4% of the patients in N+S arm and 25% in the N+P arm discontinued treatment due to adverse events.

TABLE 1 | Phase 1/2 immunotherapy based combination studies.

Study	Patients	ORR	PFS
CheckMate 016 (Phase 1)			
N+S	*n* = 33	54.5%	12.7 mon (95% CI, 11.01–16.66)
N+P	*n* = 20	45%	7.2 mon (95% CI, 2.79–11.07)
N1I3	*n* = 47	40.4%	9.4 mon (95% CI, 5.6–18.6)
N3I1	*n* = 47	40.4%	7.7 mon (95% CI, 3.7–14.3)
NCT01472081			
Pembrolizumab + Axitinib	*n* = 52	73%	20.9 mon (95% CI, 15.4–not evaluable)
(Phase 1/2)		(8% CR)	
NCT02133742			
IMmotion 150 (RP2)	*n* = 305		
Atezolizumab + Bevacizumab	*n* = 101	Atezolizumab + Bevacizumab	
vs. Sunitinib vs. Atezolizumab		ITT = 32%	11.7 mon (95% CI, 8.4–17.3)
		PD-L1+ = 46%	14.7 mon (95% CI, 8.5–25.1)
	n = 100	Sunitinib ITT = 29%	8.4 mon (95% CI, 7.0–14)
		PD-L1+ = 27%	7.8 mon (95% CI, 3.8–10.8)
	n = 103	Atezolizumab ITT = 25%	6.1 mon (95% CI, 5.4–13.6)
NCT01984242		PD-L1+ = 28%	5.5 mon (95% CI, 3.0–13.9)
JAVELIN Renal 100 (Phase 1b)	*n* = 55	ITT = 58.2% (5.5% CR)	Not available
First Line Avelumab + Axitinib		PD-L1≥1% vs. PD-L1- 65.9% and 36.4%	
NCT02493751		PD-L1≥5% vs. PD-L1- 67.9% and 50%	
Study 111 Pembrolizumab + Lenvatinib	*n* = 30	66.7%	17.7 mon (95% CI, 9.6–not estimable)
(Phase 1b/2) NCT 02501096	Treatment naïve *n* = 12	83%	
	Previously treated *n* = 18	50%	
	PD-L1 +	58%	
	PD-L1-	71%	
Nivolumab + Cabozantinib	*n* = 7		Not available
		54%	
Nivolumab + Cabozantinib + Ipilimumab (Phase1)	*n* = 6		

ORR, Objective response rate; PFS, Progression free survival; N+S, Nivolumab plus Sunitinib; N+P, Nivolumab plus Pazopanib; N1I3, Nivolumab 1 mg/kg plus Ipilimumab 3 mg/kg; N3I1, Nivolumab 3 mg/kg plus Ipilimumab 1 mg/kg; RP2, Randomized Phase 2.

In the ICI combination arms, N3I1 and N1I3, 93.6% of patients experienced treatment-related adverse event; 50% of these events were graded as 3–4. The incidence of grade 3–4 events in the N3I1 arm was 38.3% and in the N1I3 arm, 61.7%. The most common grade 3–4 adverse events were diarrhea (4.3, 14.9%), increased AST (4.3, 12.8%), ALT (4.3, 21.3%), and asymptomatic elevation of lipase (14.9, 27.7%), respectively. Any treatment with corticosteroids was required in 61.7% patients in the N3I1 arm and 83% of the patients in the N1I3 for management of immune-mediated adverse events. No deaths were attributed to treatment in either arm; 10.6 and 27.7% of the patients in the N3I1 and N1I3 arms, respectively, discontinued treatment due to adverse events.

The confirmed ORR was 54.5% in the N+S arm and 45% in the N+P arm. Responses were sustained; the median DOR in the N+S arm was 60.2 weeks (37.1–not reached) and 30.1 weeks (12.1–174.1) in the N+P arm. The median PFS was 12.7 months (11–16.7) for the N+S arm and 7.2 months (2.8–11.1) for the N+P arm. At a median follow-up of 50.0 months, the median OS was not reached (36.8–NR) in the N+S arm and was 27.9 months (13.3–47.0) in the N+P arm.

The confirmed ORR was 40.4% in the ICI combination arms, N3I1 and N1I3; 10.6% in the N3I1 arm achieved a complete response. The median PFS was 7.7 months (95% CI 3.7–14.3) in the N3I1 arm and 9.4 months (95% CI 5.6–18.6) in the N1I3 arm. The median OS was not reached in the N3I1 arm (95% CI 26 months to not reached) and was 32.6 months (95% CI 26 months to not reached) in the N1I3 arm.

The N3I1 combination arm was observed overall to have the most favorable toxicity profile and efficacy that led to the

TABLE 2 | Phase 3 immunotherapy based combination studies.

Study	Patients	ORR	PFS
CheckMate 214 (Phase 3)	Total $n = 1096$		
First line			
N3I1 → N vs. Sunitinib	Intermediate and poor risk	42% (9% CR)	11.6 mon (95% CI, 8.7–15.5)
	$n = 425$		Hazard ratio – 0.82 (99.1% CI, 0.64–1.05) $p = 0.03$
	Intermediate and poor risk $n = 422$	27% (1% CR)	8.4 mon (95% CI, 7.0–10.8)
N3I1 → N vs. Sunitinibn	ITT $n = 550$	39%	12.4 mon (95% CI,9.9–16.5)
			Hazard ratio – 0.98 (99.1% CI, 0.79–1.23) $p = 0.85$
			12.3 mon (95% CI, 9.8–15.2)
	ITT $n = 546$	32%	
NCT02231749			
IMmotion 151	ITT = 915		
(Phase 3)	PD-L1+ $n = 362$		
First Line			
Atezolizumab + Bevacizumab vs. Sunitinib	ITT $n = 454$	37% (CR 5%)	11.2 mon (95% CI, 9.6–13.3)
	PD–L1+ $n = 178$	43% (CR 9%)	11.2 mon (95% CI, 8.9–15)
	ITT $n = 461$	33% (CR 2%)	8.4 mon (95% CI, 7.5–9.7)
	PD-L1+ $n = 184$	35% (CR 4%)	7.7 mon (95% CI, 6.8–9.7)
			Hazard ratio for PD-L1+ patients – 0.74 (95% CI, 0.57–0.96), $p = 0.02$
NCT02420821			
JAVELIN Renal 101 (Phase 3)	Total $n = 886$		
First Line			
Avelumab + Axitinib vs. Sunitinib	ITT $n = 442$	51.4%	13.8 mon (95% CI, 11.1–not estimable)
	PD-L1+ $n = 270$	55.2%	13.8 mon (95% CI, 11.1–not estimable)
	ITT $n = 444$	25.7%	8.4 mon (95% CI, 6.9–11.1)
	PD-L1+ $n = 290$	25.5%	7.2 mon (95% CI, 5.7–9.7)
			Hazard ratio for PD-L1+ patients – 0.61 (95% CI, 0.47–0.79), $p < 0.0001$
			Hazard ratio for ITT patients – 0.69 (95% CI, 0.56–0.84), $p = 0.0001$
NCT02684006			

ORR, Objective response rate; PFS, Progression free survival; N3I1→ N, Nivolumab 3 mg/kg plus Ipilimumab 1 mg/kg followed by Nivolumab maintenance.

phase 3 CheckMate 214 trial, comparing this dose to standard sunitinib.

Pembrolizumab Plus Axitinib

An open-label, multicenter phase 1b study reported by Atkins and colleagues evaluated the combination of pembrolizumab at the dose of 2 mg/kg, administered intravenously every 3 weeks plus a starting dose of axitinib at 5 mg orally twice a day in treatment-naive patients with advanced renal cell carcinoma. The study was conducted in 2 phases, an initial dose-finding phase followed by an expansion phase. The primary endpoint was assessment of dose limiting toxicity (DLT) in the first 6 weeks. Secondary endpoints included assessment of adverse events, PD-L1 status, and antitumor activity including best overall response rate (BORR), DOR, PFS, and OS (40).

Eleven patients were treated in the dose-finding phase; 41 patients received treatment in the expansion phase. Of the 11 patients treated in the dose-finding phase, 3 DLTs were observed. MTD was determined to be pembrolizumab 2 mg/kg, every 3 weeks plus axitinib 5 mg orally twice daily, and used for the expansion phase. Grade 3–4 treatment-related adverse events were observed in 65% of the patients; the most common were hypertension (23%), diarrhea (10%), fatigue (10%), and increased ALT (8%). The most common possibly immune-related adverse events observed were diarrhea (29%), increased ALT (13%), hypothyroidism (13%), and fatigue (12%); 19% had grade 3 events.

Objective response was observed in 73% of the patients; 8% had CR, 65% had a PR, and 15% had SD. The median PFS was 20.9 months (95%CI 15.4—not evaluable). The median DOR was 18.6 months (95% CI 15.1—not reached). The median OS was not reached at median follow-up of 20.4 months.

The experience from this study led to the phase 3 KEYNOTE 426 study (NCT02853331), which compared the efficacy and safety of the combination of pembrolizumab plus axitinib to standard sunitinib, administered at the dose of 50 mg orally once

a day on a 4-week-on, 2-week-off schedule. This phase 3 study has completed accrual, results are not reported yet (41). A recent press release from the sponsor indicated that at the time of first interim analysis, the combination of pembrolizumab and axitinib met the primary endpoints of improved OS and PFS compared to sunitinib (www.mrknewsroom.com, accessed October 18, 2018).

Atezolizumab Plus Bevacizumab

Atezolizumab is a humanized IgG1 antibody that binds to PD-L1 and blocks its interaction with PD-1, preventing T cell exhaustion. A phase 1 study of atezolizumab with bevacizumab showed the combination to be safe with similar antitumor activity, as observed in historical controls. IMmotion 150 was a randomized phase 2 study that compared the efficacy of atezolizumab alone, atezolizumab plus bevacizumab vs. standard sunitinib. Atezolizumab was administered at a fixed dose of 1,200 mg intravenously every 3 weeks as monotherapy or with bevacizumab at 15 mg/kg intravenously every 3 weeks in the combination arm. Sunitinib was administered at the dose of 50 mg orally once a day on a 4-week-on, 2-week-off cycle. The primary objective was evaluation of PFS between the atezolizumab containing arms vs. the sunitinib arm based on the PD-L1 expression status (<1 or ≥1%) on the tumor-infiltrating immune cells (42).

A total of 305 patients were accrued at multiple sites between January 2014 and March 2015 in a 1:1:1 ratio. The patient demographics were well-balanced in the 3 arms. At a median follow-up of 20.7 months, the median PFS in the ITT population was 11.7 months (95% CI, 8.4–17.3) in the atezolizumab plus bevacizumab arm, vs. 8.4 months (95% CI, 7.0–14.0) with suntinib (hazard ratio 1.00; 95% CI. 0.60–1.45), and 6.1 months (95%CI, 5.4–13.6) with atezolizumab monotherapy (hazard ratio 1.19; 95% CI, 0.82–1.71 vs. sunitinib). In the PD-L1+ patients, the PFS was 14.7 months (95% CI, 8.2–25.1) for the combination, vs. 7.8 months (95% CI, 3.8–10.8) with sunitinib (hazard ratio 0.64; 95% CI, 0.38–1.08), and 5.5 months (95% CI 3.0–13.9) with atezolizumab monotherapy (hazard ratio 1.03; 95% CI 0.63–1.67 vs suntinib). In the ITT population, the ORR was 32% (CR 7%) for the combination, 29% (CR 5%) for sunitinib, and 25% (CR 11%) for the atezolizumab monotherapy. In the PD-L1+ patients, the ORR was 46% (CR 12%) for the combination, 27% (CR 7%) for sunitinib, and 28% (CR 15%) with atezolizumab monotherapy.

Treatment-related grade 3–4 adverse events were observed in 57% of the patients who recieved suntinib, 17% with atezolizumab, and 40% with the combination. There were 2 treatment-related deaths each in the sunitinib and atezolizumab monotherapy arms, and 3 in the combination arm.

The above experience led to the phase 3 study IMmotion 151 (NCT02420821), which compared the combination of atezolizumab plus bevacizumab vs. suntinib. The preliminary data for this study were presented at the American Society of Clinical Oncology (ASCO)—Genitourinary Conference 2018 (43). This study randomized 915 treatment-naive patients with aRCC in a 1:1 fashion to recieve atezolizumab 1,200 mg intravenously, with bevacizumab 15 mg/kg intravenously every 3 weeks or sunitinib 50 mg orally once a day on a 4-week-on, 2-week-off schedule. Patients were stratified by PD-L1 expression (<1 or ≥1%) on the tumor-infiltrating immune cells. Co-primary endpoints were PFS in PD-L1+ patients and OS in ITT patients. Secondary endpoints were PFS in ITT population, ORR, and DOR (20).

The ITT population included 915 patients; of these, 362 were PD-L1+. The median PFS for the PD-L1+ patients at median follow-up of 15 months was 11.2 months (95% CI, 8.9–15) in the combination arm vs. 7.7 months (95% CI, 6.8–9.7) for sunitinib (HR 0.74; 95% CI 0.57, 0.96, $p = 0.0217$). In the ITT population, the median PFS was 11.2 months (95% CI, 9.6–13.3) for the combination vs. 8.4 months (95% CI, 7.5–9.7) in the sunitinib arm (HR 0.83; 95% CI 0.70,0.97, $p = 0.0219$). The ORR was 43% in the PD-L1+ patients who received the combination vs. 35% for sunitinib. In the ITT population, the ORR was 37% for the combination vs. 33% for sunitinib. OS had not matured at the time of the analysis. Grade 3–4 adverse events were observed in 40% of the patients who received the combination, and 54% who received sunitinib; 12% in the combination arm and 8% in the sunitinib arm discontinued treatment secondary to adverse events. This study met the primary endpoint of improved PFS in the PD-L1+ patients treated with the combination of atezolizumab and bevacizumab compared to sunitinib and supports its use in the frontline setting for these patients.

Avelumab Plus Axitinib

Avelumab is a human IgG1 antibody that binds to PD-L1 on tumor cells and blocks its interaction with PD-1 expressed on T cells, thereby preventing the T cells from being switched off in the tumor micro-environment. Avelumab has been approved for the treatment of merkel cell carcinoma. The JAVELIN—Renal 100 was a phase 1b dose-finding study that assessed the MTD and safety of the combination of avelumab and axitinib in treatment-naive patients with advanced renal cell carcinoma. In the dose-finding phase, patients recieved axitinib 5 mg orally twice a day and were then initiated on avelumab 10 mg/kg intravenously every 2 weeks. Almost all of the patients in the dose expansion cohort were favorable or intermediate risk. The primary endpoint was evaluation of DLT for the combination in the first 4 weeks of treatment. Secondary enpoints included assessment of safety, ORR, DCR, DOR, PFS, and OS (44).

Of the 79 patients screened between October 2015 and September 2016, 55 were deemed eligible; 6 patients were treated in the dose-finding cohort, and 49 in the expansion cohort. The MTD for the combination was established to be avelumab 10 mg/kg intravenously every 2 weeks plus axitinib 5 mg orally twice a day. Ninety-six percent of the patients experienced at least one adverse event attributed to their treatment; 58% had grade 3–4 events. There was one treatment-related death, secondary to autoimmune myocarditis. Immune-mediated adverse events were observed in 42% of patients; 9% had grade 3–4 severity. Objective response was confirmed in 100% of the patients in the dose-finding cohort and in 53% of the patients in the expansion cohort for an ORR of 58%, 6% being complete responses. At a median follow-up of 52.1 weeks, DOR, PFS, and OS could not be assessed. PD-L1 expression was ascertained for 52 patients. Using

a 1% cutoff for expression of PD-L1 on the tumor-associated immune cells, the ORR was 63% for those with expression ≥1%, compared to 36% for those with expression <1%.

The phase 3 study (NCT02684006) JAVELIN—Renal 101 compared the combination of avelumab 10 mg/kg intravenously every 2 weeks plus axitinib 5 mg orally twice a day, vs. sunitinib 50 mg orally once a day on a 4-week-on, 2-week-off schedule for patients with treatment-naive aRCC (45). The preliminary results were reported recently at the ESMO annual meeting 2018 (43). The primary endpoints of this study were PFS by blinded independent central review (BICR) and OS in patients with PD-L1+ (≥1 of immune cells). Secondary endpoints included PFS and OS irrespective of PD-L1 expression, objective response (OR), and safety.

A total of 886 patients were randomized; 442 to the combination of avelumab plus axitinib and 444 to the sunitinib arm. Of the 442 patients in the combination arm, 270 were PD-L1+; 290 patients were PD-L1+ in the sunitinib arm. The median PFS in the PD-L1+ tumors was 13.8 months (95% CI, 11.1—not estimable) for the combination vs. 7.2 months (95% CI, 5.7–9.7) in the sunitinib arm (HR = 0.61; 95% CI, 0.475–0.790; $p < 0.0001$). The median PFS irrespective of PD-L1 status was 13.8 months (95% CI, 11.1—not estimable) for the combination vs. 8.4 months (95% CI, 6.9–11.1) in the sunitinib arm (HR = 0.69; 95% CI, 0.563–0.840; $p = 0.0001$). The OS was immature at the time of data cutoff and reporting.

This study met the primary endpoint of improved PFS in treatment-naive patients with PD-L1+ tumors, and supports the combination of avelumab plus axitinib for treatment of patients with aRCC in the first-line setting.

Pembrolizumab Plus Lenvatinib

Lenvatinib is a multi-tyrosine kinase inhibitor with activity against VEGF receptors VEGFR1, VEGFR2, VEGFR3; fibroblast growth factor receptors FGFR1, FGFR2, FGFR3, FGFR4; and platelet-derived growth factor alpha, KIT, and RET. Based on a randomized phase 2 study the combination of lenvatinib plus everolimus was approved for the treatment of aRCC in the second-line setting. A preclinical study with Lenvatinib showed decrease in the macrophage population within the tumor micro-environment that correlated with increased antitumor activity with PD-1 inhibition (46). In a multicenter, open-label phase 1b study, 8 patients with aRCC that had progressed after standard treatment were treated with the combination of lenvatinib plus pembrolizumab. The initial starting dose of lenvatinib was 24 mg/day that was decreased to 20 mg/day due to toxicity. Pembrolizumab was administered at the dose of 200 mg intravenously every 3 weeks. Phase 2 of this study included 22 patients with aRCC who could have received up to 2 prior lines of treatment. The primary endpoint for the phase 2 cohort was ORR at 24 weeks. Secondary endpoints included progression-free survival and duration of response (47, 48).

Of the 30 patients included in the phase 1b/2 study, PD-L1 status was assessed for 26 patients; 12 were PD-L1-positive using 1% cutoff. Eighteen patients had received at least one prior treatment; 12 patients were treatment-naïve. The ORR at 24 weeks was 63.3% (95% CI, 43.9–80.1). The ORR by independent radiographic review using RECIST 1.1 was 66.7% (95% CI, 47.2–82.7). There did not appear to be any impact of PD-L1 status on the outcome; 58% of the PD-L1-positive and 71% of the PD-L1-negative patients had a response.

The most common treatment-related adverse events included diarrhea (83%), fatigue (70%), hypothyroidism (67%), stomatitis (60%), hypertension (57%), and nausea (57%). No new safety signals were observed. Treatment-related adverse events required a dose reduction for lenvatinib in 18 patients.

A phase 3 multicenter, open-label study (49) comparing lenvatinib plus pembrolizumab or lenvatinib plus everolimus vs. sunitinib in treatment-naive patients with aRCC is underway (NCT 02811861).

Nivolumab Plus Cabozantinib

Cabozantinib is a multi-kinase TKI that inhibits VEGF receptors VEGFR1, VEGFR2, VEGFR3, AXL, RET, ROS1, TYRO3, MER, KIT, TRKB, FLT-3, and TIE-2. A phase 1 study to evaluate the tolerability and efficacy of nivolumab plus cabozantinib (NivoCabo) and nivolumab plus cabozantinib plus ipilimumab (NivoCabo+Ipi) in patients with metastatic urothelial carcinoma and other genitourinary malignancies was reported by Nadal et al. (50). Of the 75 patients enrolled, 7/47 patients treated with NivoCabo and 6/28 who recieved NivoCabo+Ipi had advanced renal cell carcinoma. Partial response was elicited in 7 patients with aRCC (50).

CheckMate9ER a phase 3 study (51) assessing the combination of nivolumab plus cabozantinib vs. suntinib in treatment-naive patients with aRCC is underway. Enrollment began in August 2017, and is ongoing (NCT 03141177).

DISCUSSION

The approval of combination immunotherapy with nivolumab plus ipilimumab marks the beginning of a new era in the therapeutic landscape for patients with advanced renal cell carcinoma. In addition, a wave of regulatory approvals with multiple VEGF inhibitor plus ICI combination is expected. While the preliminary data from these newer combinations are encouraging and hold great potential, they will also require new questions and concerns to be addressed as the existing treatment paradigm evolves.

The question of the choice and dose of VEGF inhibitor to use in combination with ICIs is important. The VEGF inhibitor should ideally support immunotherapy with positive immune modulation of the tumor micro-environment and be tolerable. While pazopanib was shown to have adequate VEGF inhibition and immunomodulatory activity, the tolerability has been marginal in combination with ICIs. This was initially observed in the CheckMate 016 phase 1 study that assessed the safety of nivolumab with TKIs, sunitinib or pazopanib or ipilimumab. Early hepatotoxicity noted in the dose-escalation phase of the nivolumab plus pazopanib arm required for the expansion phase to be aborted with this combination. Similar observation with pazopanib was made in the KEYNOTE-018, a phase 1/2 study that assessed the safety and efficacy of pazopanib and

pembrolizumab in patients with aRCC (NCT02014636). Dose-limiting liver toxicity with grade 3–4 events were observed in 80–90% of the patients. It was determined that this combination was not feasible for further testing (52). In the Checkmate 016 study, greater efficacy with both ORR and durability of response was observed with the combination of nivolumab plus sunitinib, yet significant grade 3–4 toxicity precluded further development of the combination at the standard approved dose of sunitinib. Anti-VEGF therapy results in normalization of the vasculature that can reduce suppression in the tumor micro-environment (53). Using higher doses of anti-angiogenic agents may in fact result in hypoxia and decreased pH in the tumor micro-environment that are not conducive for optimal immune activity (54). The dose of VEGF inhibitors to achieve optimal modulation of the tumor micro-environment will need to be tailored (55). Therefore, future combination studies with VEGF inhibitors plus ICIs will need to address the question of optimizing the dose, ideally based on assessment of the TIME.

Increased incidence of higher-grade adverse events has been observed with the combinations of ICIs and VEGF inhibitors compared to monotherapy with the same agents. Toxicities attributed to VEGF inhibitors as well as ICIs have been described previously (56). Given the different underlying mechanisms causing these adverse events, immune-mediated for ICIs vs. direct drug-related for TKIs, the management strategies are very different and warrant clear understanding and education for both the patients and treating physicians. For optimal management appreciation of the above and accurate recognition of which component, ICI, or VEGF inhibitor is causative for a specific toxicity event will become critical, e.g., diarrhea may be the presenting symptom for ICI-induced auto-immune colitis but could also be drug-related to TKI therapy. Urgent treatment with steroids may be required for an auto-immune breakthrough toxicity; alternatively, the drug may simply need to be held for a few days for TKI-related symptoms. While not as critical as it was for high-dose IL-2, patient selection will require greater thought with combination therapy compared to monotherapy.

Interpretation of treatment response is another area of concern that may need reevaluation with newer combination therapy. Conventionally the RECIST criteria have been used for evaluation of response to chemotherapy and targeted therapy; iRECIST criteria were developed for evalution of response to immunotherapy. With the combination of two very different therapeutic modalities, new patterns of response or clinical benefit may emerge. Thought will need to be given to developing criteria that will capture the outcomes appropriately. We suggest this primarily because iRECIST criteria were developed only after new patterns of response were observed, as experience with ICIs accumulated. These considerations will become important for both interpretation of clinical trial data and application to clinical practice.

Another question that will need deliberation is how we should choose between dual-immune checkpoint inhibition of nivolumab/ipilimumab (for intermediate/poor-risk patients), the next wave of VEGF/ICI inhibitors, or sequential monotherapy with ICIs and VEGF inhibitors? Using the inevitable cross-trial comparison, ICI plus VEGF inhibitor combinations have elicited higher response rates (50%) and have a pronounced prolongation in PFS over sunitinib monotherapy, which makes the regulatory approval for many of these combinations very likely. However, none of these phase 3 VEGF/ICI combination trials incorporate a sequential TKI followed by PD-1 monotherapy comparison arm; thus, making it difficult to ascertain the true impact of moving the combination therapy upfront. In fact, only 25–30% of patients on the sunitinib arm of the recent phase 3 trials (Checkmate 214, IMmotion 151, Javelin 101) have had exposure to subsequent PD1 monotherapy. With several combinations to choose from, at present our leaning would be toward favoring dual-immune checkpoint therapy that has demonstrated a survival benefit. It will be important to follow the OS signal as the access to subsequent PD-1/PD-L1 therapy on the TKI control arms of the combination studies matures. At present there is paucity of prospective data as to how ICI-based immunotherapy and VEGF inhibition should be sequenced. Preliminary data from studies have shown activity for axitinib, sunitinib, and cabozantinib following treatment with ICIs (57–59). The notion of priming the tumor micro-environment with VEGF-targeted therapy followed by immunotherapy is intriguing as well (60). In other words, while the high response rates observed with VEGF/ICI combination are promising, it is unclear if we can interpret them as proof of true immunological synergy at this time. More data regarding the durability of responses and the impact on overall survival are critical to establish whether dual-immune checkpoint therapy or ICI plus VEGF inhibitor combinations with higher rate of adverse events, compared to optimally sequenced monotherapy, will become the preferred frontline standard.

Based on the different mechanisms of action for ICIs and TKIs, the choice of first-line therapy and the interpretation of outcomes after discontinuation of treatment due to toxicity will need to be put in appropriate perspective. Discontinuation of therapy secondary to intolerable toxicity with chemotherapy or targeted therapy may not be comparable to discontinuation of immunotherapy for high-grade auto-immune toxicity. Patients who receive ICIs and had their treatment discontinued for immune-mediated adverse events continue to maintain their response without requiring additional treatment, which is generally not the case for targeted therapy. This observation will need to be factored into decision-making for patients who respond to immunotherapy alone and may be spared VEGF pathway inhibition in the first line. These patients can potentially enjoy a significant treatment-free interval after they complete their treatment course or discontinue immunotherapy because of adverse events—some of these patients may never require further therapy. Analysis from the CheckMate 214 study reported by McDermott et al. showed the treatment-free survival after discontinuing nivolumab plus ipilimumab compared to sunitinib was different (42). The quality of response reported by Rini et al. from the same study confirmed that patients who received nivolumab plus ipilimumab continued to maintain responses if their treatment was discontinued for reasons other than progression (61). Additionally, the optimal duration of immunotherapy for patients who respond to

immunotherapy and do not experience adverse events will need to be ascertained. Our current paradigm of continuing treatment until intolerable toxicity or progression of disease is derived from the chemotherapy experience. With immunotherapy-based treatment, the theoretical concerns of inducing resistance by immune-editing, antigenic drift, and irreversible T cell exhaustion after continuous exposure to immune modulation will need to be worked out.

There will certainly be clinical scenarios where eliciting high-response rates becomes critical. Symptomatic patients with high tumor burden, pending visceral crisis, or organ compromise are all scenarios where reliable and rapid cytoreduction, irrespective of mechanism (e.g., driven by VEGF, ICI, or a combined effect), is desirable and should be treated with VEGF/ICI combinations that reach into the 50–70% ORR range. On the other hand, many patients could just be exposed to combined ICI, which not only provides useful information on the responsiveness of different lesions but may also allow for excision and radiation of escape lesions or other future adaptive treatment strategies. This information is obviously lost in patients exposed to concurrent VEGF inhibitors.

Our impression is that the first-line combination treatment of aRCC will dichotomize between nivolumab/ipilimumab on the one hand, and potent VEGF/PD1 inhibitors (e.g., axitinib/pembrolizumab) on the other. The preference of one regimen vs. the other will likely depend on a multitude of factors including the country/health care system, clinical practice setting (academic vs. private practice), familiarity and experience, education, staffing, and patient's choice. Furthermore, most health care systems will have to ask the question of whether they should reimburse all regulatorily approved combinations or just focus on the one or two most promising regimens and try to save cost. This is certainly a question beyond the scope of this review but will undoubtedly have impact moving forward.

Another question in the changing landscape as several combinations are approved will be reaching consensus regarding the appropriate control arms for future studies? Which of these agents/combinations would serve as the most appropriate control? In this context, the optimal sequence for best therapeutic efficacy will need to be ascertained to ensure that the control arm does not compromise care. Over time the long-term follow-up data from dual ICI, VEGF/ICI, and sequential studies will help discern this from patient outcomes.

A multitude of options with potential to become therapeutic reality for patients with aRCC are moving steadily toward fruition. Exciting as this potential is, the new landscape poses new challenges, concerns, and questions that will need to be answered in a rational, thoughtful manner to best move the field forward. Ideally, biomarkers to predict response could help make the most optimal therapeutic choice, but despite intense efforts none have yet been identified. Expression of PD-L1 in the setting of aRCC has displayed mixed data and is not ready for use in clinical decision-making. Several approaches including evaluation of ctDNA and microbiome are under investigation.

AUTHOR CONTRIBUTIONS

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

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Gene Expression Analysis Identifies Novel Targets for Cervical Cancer Therapy

Jason Roszik[1,2†], Kari L. Ring[3†], Khalida M. Wani[4], Alexander J. Lazar[5], Anna V. Yemelyanova[6], Pamela T. Soliman[7], Michael Frumovitz[7] and Amir A. Jazaeri[7]*

[1] Department of Genomic Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [2] Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [3] Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, University of Virginia Health System, Charlottesville, VA, United States, [4] Department of Translational Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [5] Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [6] Department of Pathology, The University of Alabama at Birmingham, Birmingham, AL, United States, [7] Department of Gynecologic Oncology and Reproductive Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, United States

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***Correspondence:**
Amir A. Jazaeri
AAJazaeri@mdanderson.org

† These authors have contributed equally to this work

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Although there has been significant progress in prevention and treatment of cervical cancer, this malignancy is still a leading cause of cancer death for women. Anti-angiogenesis and immunotherapy approaches have been providing survival benefits, however, response rates and durability of response need to be improved. There is a clear need for combination therapies that increase effectiveness of these agents and further improve patient outcome. Previous studies have largely focused on gene expression and molecular pathways in untreated cervix cancer. The goal of this study was to evaluate cancer-specific molecular pathways and their correlation with tumor immune profile in recurrent cervical cancer. Tumor and adjacent normal tissues were used to identify potential combination therapy targets. We found that DNA damage repair pathway genes were significantly overexpressed in the tumor. Based on our results and other recent investigations, we suggest that combination immune checkpoint and PARP inhibitor therapy is a high priority consideration for patients with recurrent, previously treated cervical cancer. We also show that multiple epithelial-mesenchymal transition-related genes, including MAP2K4, ID2, JAK1, FGF2, PIK3R1, AKT3, FGF13, and STAT3 may be potential targets. Interestingly, high-throughput analysis of Cancer Genome Atlas data identified distinct targets, including Fatty acid synthase FASN and Matrix Metallopeptidase 1 MMP1 as novel, promising combination therapy partners.

Keywords: cervical, cancer, retrospective analysis, gene expression profile, immunohistochemistry, combination therapies

INTRODUCTION

Despite effective screening and preventative vaccines, there will be an estimated 13,240 new cases of cervical cancer and 4,170 deaths estimated in 2018 in the United States, with cervical cancer accounting for the second leading cause of cancer death for women age 20 to 39 years (1). Unfortunately, women who present with advanced stage and metastatic disease have a poor

prognosis and limited therapeutic options. Response rates to current therapies range from 35 to 50% with a median survival of less than 2 years (2, 3). Biologic therapy targeting the vascular endothelial growth factor (VEGF) has shown the most recent success in the treatment of cervical cancer and is now used in combination with chemotherapy as the standard of care in the treatment of recurrent and metastatic disease. While anti-angiogenesis therapies have shown an incremental improvement in survival, there remains a high clinical demand for novel treatment strategies in this disease site. At this time, there are no other targeted therapies approved in the treatment of cervical cancer according to clinicaltrials.gov.

Immunotherapy presents an additional rational approach for the treatment of cervical cancer given the molecular underpinnings of this human papilloma virus (HPV) related disease. Impaired local cellular immunity results in persistent infection with high risk HPV and expression of viral oncoproteins E6 and E7. Expression of these oncoproteins in turn leads to downstream genomic instability through interactions with the well described tumor suppressor genes p53 and retinoblastoma (pRb). The loss of cell cycle regulation allows for an increased mutational burden and malignant transformation from cervical intraepithelial neoplasia to invasive carcinoma (4). An increased mutational burden is a source of targetable neoantigens that can be detected in most cervix tumors (5). Cervical cancer, as well as other HPV related diseases, also present a unique viral antigen for T-cells to identify tumor cells from self and serve as ideal candidates for immunotherapy from a biologic standpoint. In contrast, previous studies have also demonstrated that immune checkpoint pathways, including PD-1 /PD-L1 are activated during chronic viral infections to combat T-cell responses to viral antigens. Yang and colleagues evaluated this in cervical intraepithelial neoplasia and showed that upregulation of the PD-1/PD-L1 pathway was associated with HPV positivity and progression of precancerous lesions (6). In addition, diffuse PD-L1 expression has been associated with worse disease specific survival in this patient population (7).

Multiple trials evaluating various immunotherapy strategies are currently underway in both the upfront and recurrent setting, however immune checkpoint blockade has been the most widely studied in cervical cancer to date (clinicaltrials.gov) and the majority of trials have evaluated this strategy in the single agent setting. While initial studies have shown activity of checkpoint blockade in cervical cancer, response rates thus far are disappointing and range from 17 to 27% (8, 9). Despite these low response rates pembrolizumab recently gained FDA approval for patients with recurrent or metastatic cervical cancer with disease progression on or after chemotherapy whose tumors express PD-L1. The current therapeutic landscape highlights the need for: (a) rationale combination therapies with the potential to provide improved responses, and (b) identification of molecularly defined subgroups who may benefit from immunotherapy. Our objective was to identify immune related as well as other potentially targetable cancer pathways in recurrent cervical cancer in an effort to identify rational combination therapies that should be prioritized in developmental therapeutics for cervical cancer (10).

RESULTS

Clinical Characteristics

Twenty-eight patients were treated for recurrent cervical cancer with a pelvic exenteration at MD Anderson Cancer Center from 1994 to 2004. We focused our investigation on this population, because it represents the population with the greatest unmet need in this disease (i.e., patients with recurrent disease following primary treatment with surgery and/or chemoradiation). The majority of patients initially presented with squamous histology ($n = 19$, 67.9%) and locally advanced disease ($n = 17$, 60.7%) Nineteen patients were treated with primary chemoradiation (67.9%), 2 were treated with a radical hysterectomy (7.1%), and 7 (25.0%) were treated with both a hysterectomy and chemoradiation. Seven patients (25.0%) were alive with no

TABLE 1 | Clinical characteristics of patients.

Clinical characteristics ***N* = 28**	**N (%)**
RACE	
White	17 (60.7)
African American	2 (7.1)
Hispanic	8 (28.6)
Asian	1 (3.6)
STAGE	
IA2	1 (3.6)
IB1	8 (28.6)
IB2	5 (17.8)
IIA	0 (0)
IIB	6 (21.4)
IIIA	1 (3.6)
IIIB	4 (14.3)
IVA	1 (3.6)
Unknown	2 (7.1)
GRADE	
1	3 (10.7)
2	11 (39.3)
3	11 (39.3)
Unknown	3 (10.7)
HISTOLOGY	
Squamous cell carcinoma	19 (67.9)
Adenocarcinoma	9 (32.1)
PRIMARY TREATMENT	
Radical hysterectomy	2 (7.1)
Primary chemoradiation	19 (67.9)
Hysterectomy and Chemoradiation	7 (25.0)
DISEASE STATUS AT LAST FOLLOW-UP	
Alive, No evidence of disease	7 (25.0)
Alive with disease	1 (3.6)
Dead with disease	16 (57.1)
Dead of unknown cause	4 (14.3)

disease as of last follow-up and 16 (57.1%) had died as a result of recurrent disease (**Table 1**).

Nanostring Expression Analysis Identifies Immune Alterations in Cervix Tumors

We have performed a NanoString expression analysis using the Cancer Immune genes code set ($n = 730$ genes) to compare tumor and adjacent normal tissue in the patient cohort described in **Table 1**. **Figure 1A** shows the differentially expressed genes, after Bonferroni correction for multiple tests. Interestingly, only cyclin-dependent kinase 1 (CDK1) showed overexpression in the tumor. Genes that were expressed at a lower level in the tumor included IL11RA, NFATC4, MEF2C, MAP2K4, MAP3K7, CD34, STAT5B, ICAM2, TFE3, ATF2, FAS, ITCH, CCL14, IL6ST, and IL6R. Low expression of the ITCH gene was also associated with significantly ($p < 0.01$) shorter progression-free survival (PFS) (**Figure 1B**). We next sought to identify immune related genes expressed in the tumor that correlated with survival. We identified CD58 (lymphocyte function-associated antigen 3 - LFA-3) cell adhesion molecule and macrophage marker, and PSEN1 (presenelin 1), a chemoresistance-associated gene, were significantly associated with improved progression free survival (PFS; **Figures 1C, D**). The p-values from Kaplan-Meier analyses of all genes can be found in **Supplementary Table 1**.

Cancer Pathways Show More Dominant Alterations Compared to Immune Genes

In order to identify cancer-related pathways that may serve as potential targets for therapeutic intervention, we performed a NanoString study using the Cancer Pathways code set which is composed of 730 genes. In this analysis we identified 423 genes that were differentially expressed at $p < 0.05$ level. Out of these, 148 were significant after Bonferroni correction (**Figure 2A**). A notable observation was the significantly higher expression of DNA damage repair pathway genes, in particular those involved in homologous recombination and mismatch repair pathways) in tumor tissues (**Figure 2A**, e.g., BRCA1, BRCA2, BRIP1, FANCA, FANCG, FANCC, RAD51, XRCC4, MSH2, MSH6, MCM7, MCM4, PCNA). Our findings are

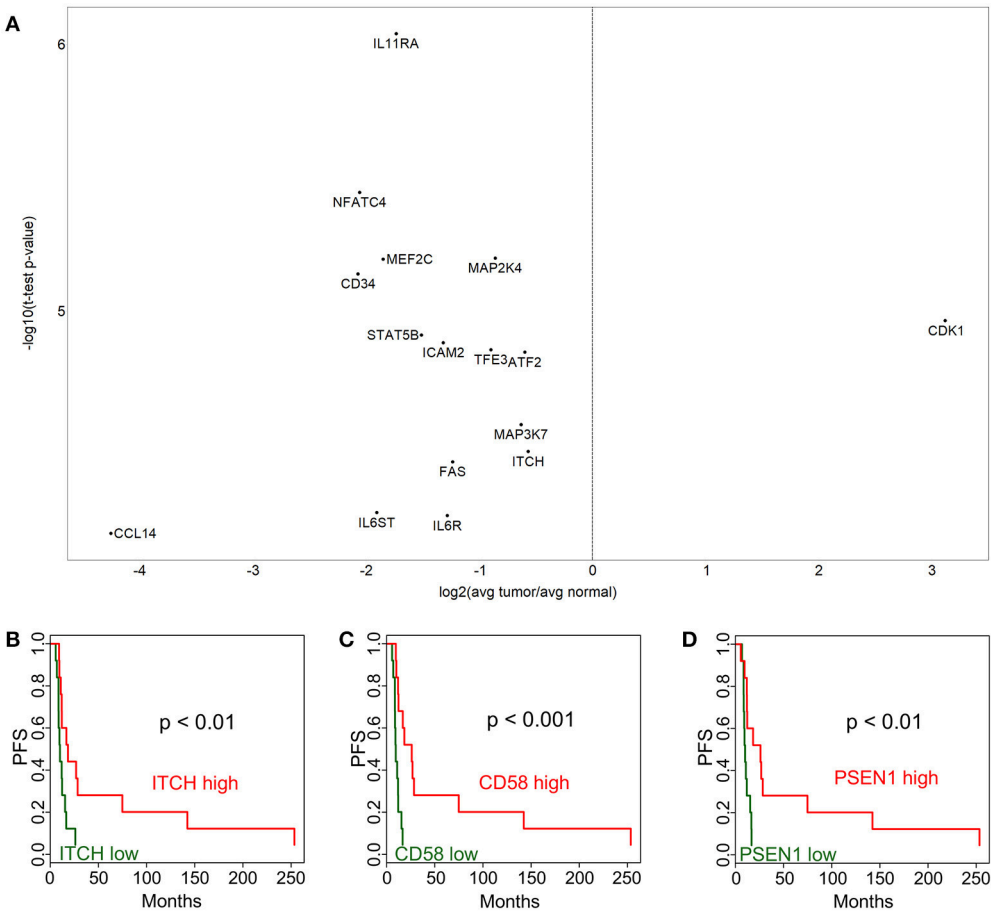

FIGURE 1 | Immune-related gene expression alterations in cervical cancer. Over- and under-expressed immune-related genes in cervix tumors compared to adjacent normal tissue are shown in **(A)**. Association between progression-free survival (PFS) and gene expression is displayed for ITCH **(B)**, CD58 **(C)**, and PSEN1 **(D)**. High expression (above median) is depicted with red color, while low expression (below median) is green.

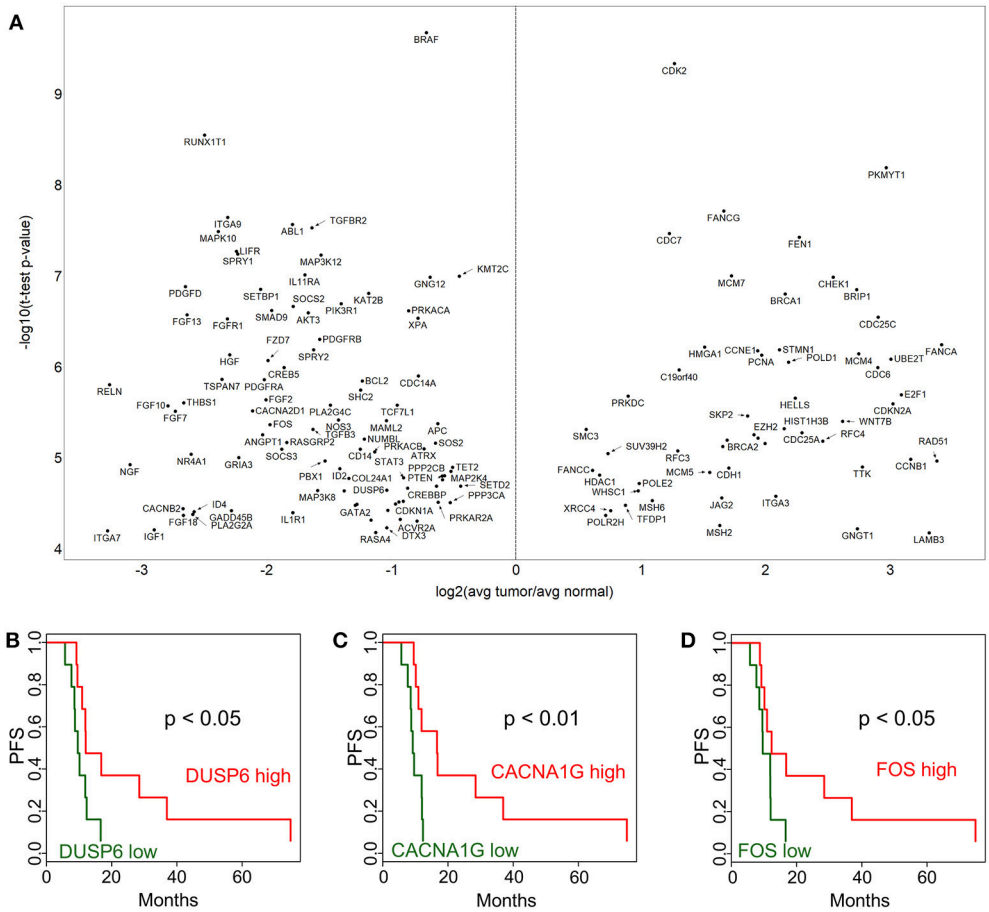

FIGURE 2 | Cancer pathway-associated genes show diverse alterations in cervical cancer. Cervix tumor and adjacent normal tissue expression comparisons are shown in **(A)** for genes in the NanoString Cancer Pathways code set. Progression-free survival (PFS) associations for selected genes in the NanoString panel, DUSP6 **(B)**, CACNA1G **(C)**, and FOS **(D)** are displayed comparing high (above median) and low (below median).

especially intriguing, because it was recently shown that HPV E6 and E7 oncogenes increase the abundance of HR proteins and enhance their ability to form DNA repair foci. However, ironically, E6 and E7 interfere with the ability of the HR pathway to complete double-strand break (DSB) repair, resulting in homologous recombination deficiency (HRD) (11).

In addition, Ingenuity Pathway Analysis revealed that 25 out of these are in the "Regulation of the Epithelial-Mesenchymal Transition Pathway" (MAP2K4, ID2, JAK1, FGF2, PIK3R1, SOS2, FGF13, TGFBR2, BRAF, FGF10, FGF18, HGF, WNT7B, AKT3, FGF7, PDGFRB, JAG2, FGFR1, STAT3, TCF7L1, APC, CDH1, TGFB3, PDGFD, and FZD7). This pathway appeared to be suppressed in tumor cell compared to adjacent normal tissue. A few selected PFS associations are shown for DUSP6 (**Figure 2B**), CACNA1G (**Figure 2C**), and FOS (**Figure 2D**). The *p*-values from Kaplan-Meier analyses are provided for all genes in **Supplementary Table 2.** These expression and survival differences clearly show that multiple oncogenic pathways are active.

Immunohistochemistry Analysis Indicates PFS Associations

In addition to gene expression level comparisons, we also analyzed PFS associations with protein level by immunohistochemistry (IHC). We found that higher CD8+ density and mean membrane PD1 level were associated with better survival ($p < 0.05$) both in adjacent non-tumor and in tumor (**Figure 3**). FoxP3+ phenotype density did not show a statistically significant association with PFS. The PD-L1 expression PFS-association was also not significant, possibly due to the high variation in expression in tumors.

Immunologic Features of Cervical Cancer Are Associated With Targetable Molecular Pathways

We next sought to correlate the immunologic features on cervical tumor microenvironment with gene expression levels to gain additional insight into the potential interactions between the immune and cancer pathways. We performed an unbiased correlation analysis of tumor immune phenotype (as revealed

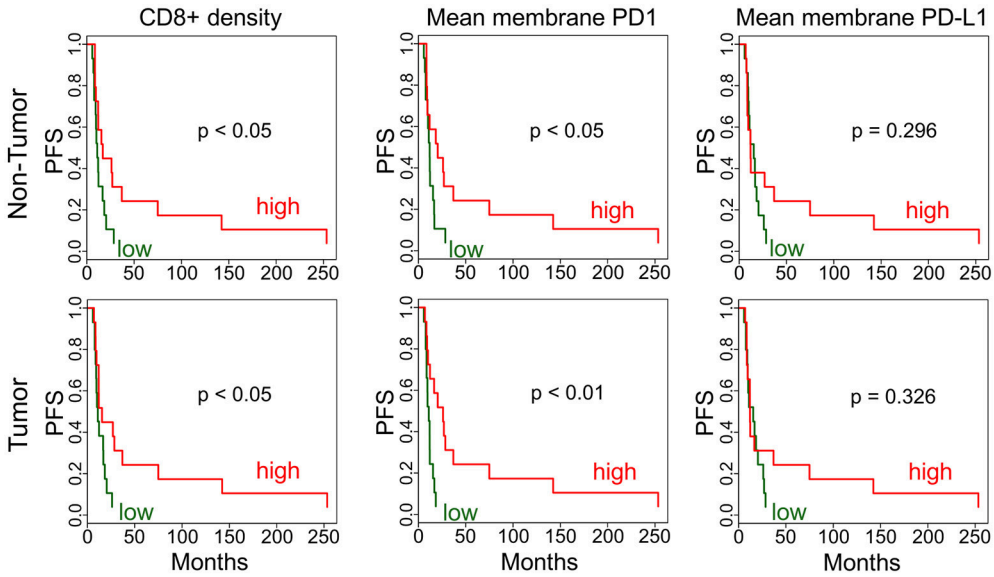

FIGURE 3 | Immunohistochemistry analysis of tumors and adjacent non-tumors. Kaplan-Meier plots are applied to analyze PFS associations in non-tumor and tumor for CD8+ phenotype density (counts/mm^2) and mean membrane PD1 and PD-L1 expression. High (red) is the above median density/expression group, low (green) is below median.

by IHC) and gene expression (using Nanostring) (**Figure 4**). One of the notable observations in this analysis was the negative correlation between STAT3 expression with CD8+ phenotype density. This is important because STAT3 can be over-expressed and constitutively-activated in cervical cancer (12), and decreasing its expression may help to increase CD8+ cell infiltration to the tumor. Another important observation is that macrophage marker CD68+ phenotype density was found to be associated with multiple markers in the tumor including STAT3, ABL1, CDH1, MAK3K1, MAP2K1, MAP2K4, TTK, IKBKB, IL1RAP, NOTCH1, ITGB4, and JAK1.

TCGA Analysis Reveals Potential Combination Therapies

While the main focus of our study was the immune and molecular phenotype of recurrent cervical cancer, the availability of the Cancer Genome Atlas (TCGA) cervix cancer data provided an opportunity to correlate gene expression and survival in untreated cervical cancer samples (used in TCGA). We performed this analysis using the high-throughput architecture which we developed earlier (13). The association of overall survival and gene expression was determined for gene pairs using the following four groups: (1-high-high group): the expression of both genes is above median expression; (2-high-low group): the expression of the first gene is above while the second gene is below median expression; (3-low-high group) the expression of the first gene is below and the expression of the second is above median expression; and (4-low-low group) the expression of both genes is below median expression levels. **Figure 5A** shows the gene pairs where high expression of both genes of a potential combination is associated with shorter or longer median survival than low expression of the same genes. One of the most interesting combination we identified is Fatty acid synthase FASN and Matrix Metallopeptidase 1 MMP1. Low expression of either of these genes is associated with significantly longer overall survival (**Figures 5B,C**), however, when both genes have low expression (**Figure 5D**), median survival is much more improved ($p < 0.0001$). We have also performed a similar analysis focusing on the EMT genes identified above. **Figure 5E** shows the gene pairs with significant overall survival association, and the JAK1 - LOXL2 (**Figure 5F**) and JAK1 - RAB2A (**Figure 5G**) combinations are also shown using Kaplan-Meier plots.

DISCUSSION

Immunotherapy can take many forms including therapeutic vaccines, adoptive transfer of autologous tumor-infiltrating T lymphocytes (TILs), chimeric antigen receptor (CAR)-engineered T-cells, and immune checkpoint blockade. Optimal immunotherapies as well as targeted therapies usually require target overexpression in the tumor compared to normal tissues (14). To identify such targets, we performed gene expression and IHC analyses of tumor and adjacent normal tissues of 28 cervical cancer patients.

Our NanoString Cancer Immune expression analysis identified multiple immune alterations in cervix tumors, but most of the genes with significantly altered expression were under-expressed in the tumor, making them unattractive targets. However, CDK1 was over-expressed, and may be a potential combination therapy target. In addition to this gene, our NanoString Cancer Pathways analysis provided a number of promising target candidates. Several DNA damage repair

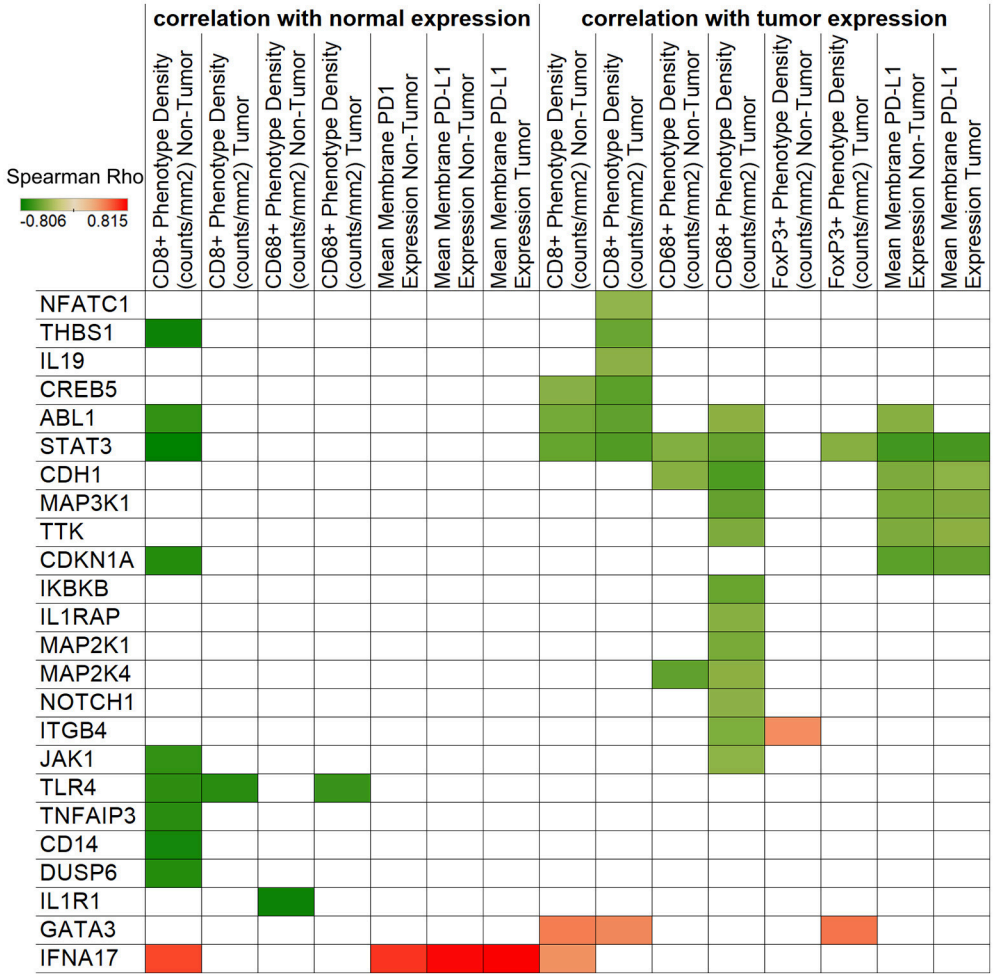

FIGURE 4 | Correlation of IHC and NanoString expressions. Spearman's rank correlation coefficients are represented by color for NanoString genes and IHC densities/expressions. The left side of the figure shows correlations with normal tissue gene expression, while the right side displays tumor expression associations. Only significant ($p < 0.05$) correlations are shown.

pathway genes were overexpressed in the tumor. In particular there was an overabundance of genes in the BRCA-Fanconi Anemia and homologous recombination pathways. These findings along with a previous report that HPV E6 and E7 oncogenes increase the abundance of HR while producing functional homologous recombination deficiency (HRD) (11), support the clinical investigation of poly (ADP-ribose) polymerase inhibitors (PARPi) alone or in combination with other therapies in recurrent cervical cancer. In fact a phase I study of paclitaxel, cisplatin, and the PARPi veliparib in the treatment of persistent or recurrent carcinoma of the cervix found this combination safe and feasible (15). Another study of rucaparib and bevacizumab combination in patients with recurrent cervical cancer is currently ongoing (NCT03476798). Considering both the immune and gene expression profiling data provided by our study we feel that combination immune checkpoint and PARPi therapy is a high priority consideration in patients with recurrent previously treated cervical cancer.

Furthermore, epithelial-mesenchymal transition (EMT) was one of the top pathways associated with these differentially expressed genes. Although EMT has been shown to be targetable in cervical cancer, the regulation of EMT is not well known (16). Out of the 25 genes identified in our study, multiple have been implicated in cancer and may be targetable, including MAP2K4 in prostate cancer (17), ID2 in glioma (18), JAK1 in multiple cancer types (19), FGF2 to address resistance to anti-VEGF therapy (20), PIK3R1 and AKT3 using PI3K/AKT/mTOR inhibitors (21), FGF13 which mediates resistance to platinum therapy in cervical cancer (22), and STAT3 which has also been proposed as a target in cervical cancer (23).

Our IHC analyses clearly indicate immune activity in cervix cancer, however, cytotoxic T cell density was found to be lower in tumors compared to a relatively high density in adjacent normal tissues. FoxP3+ regulatory T cells (Tregs) may be partially responsible for an immune-suppressive tumor microenvironment, however, our results indicate that Tregs

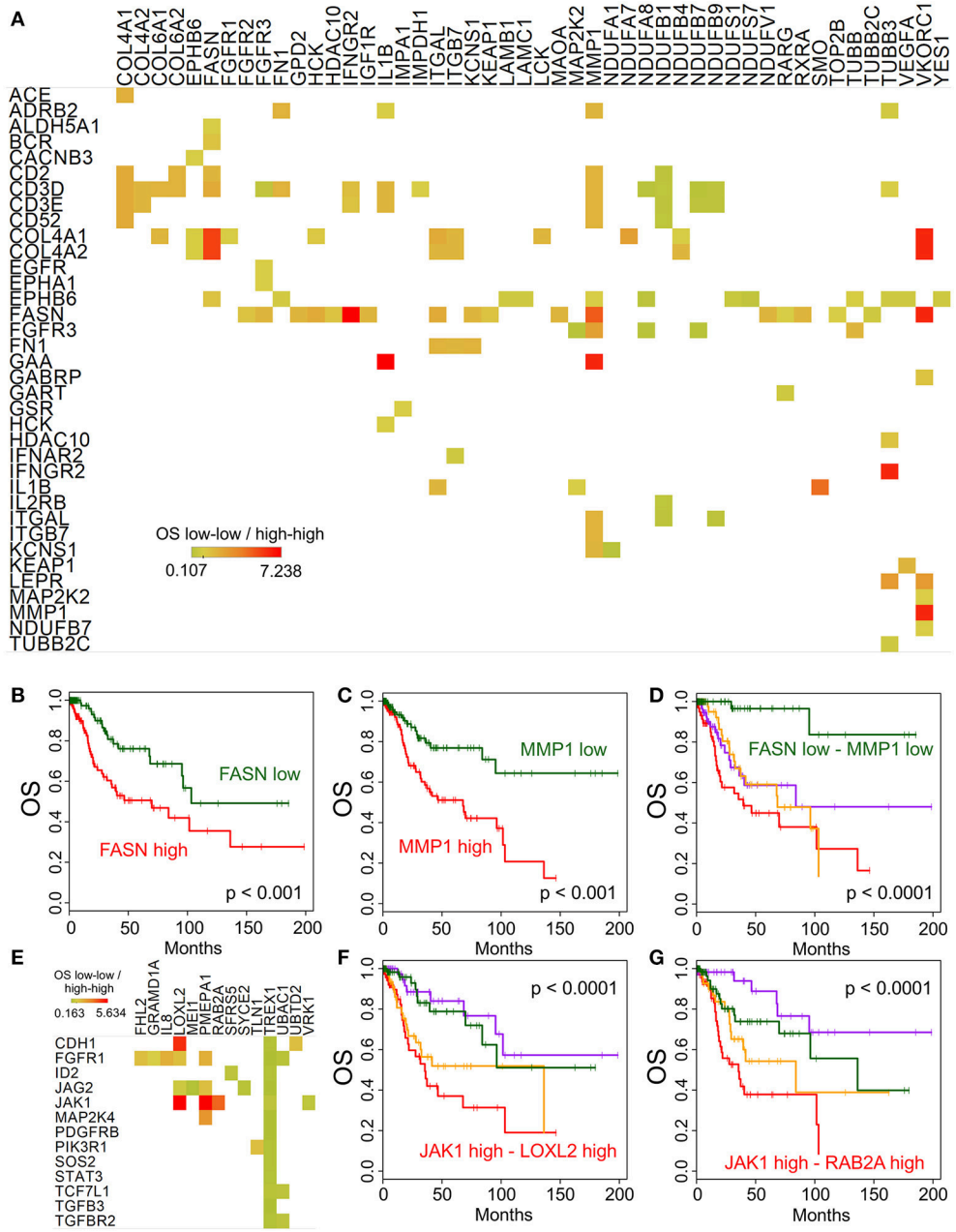

FIGURE 5 | Potential targets for combination therapies. Median overall survival ratio of low-low (both below median) and high-high (both above median) expression of gene pairs is depicted as shown by the color legend **(A)**, only $p < 0.0001$ associations where median expression >5 TPM). Kaplan-Meier plots show the difference between high and low expression for FASN **(B)** and MMP1 **(C)** alone, and in combination **(D)**. **(E)** Displays the overall survival ratio analysis results for the EMT genes (shown in rows) identified by our Ingenuity Pathway Analysis. The JAK1 - LOXL2 **(F)** and JAK1 - RAB2A **(G)** combination survival analyses are shown as examples.

are also excluded from tumors to adjacent areas. CD68+ macrophages were present in high quantities in both tumor and adjacent non-tumor tissues. We also showed that CD68+ phenotype density was inversely associated with NOTCH1 expression. Notch1-activating agents have been proposed earlier (24), and based on our results this may be considered together with targeting macrophages in the tumor. We also identified some PD-L1 protein expression, which may be a useful marker for a subset of patients for immune checkpoint inhibitor therapy (25). Interestingly, PD-L1 gene expression may also be associated with higher neoantigen burden and expression of HPV master regulators (5).

In addition, our TCGA-based high-throughput combination therapy prediction identified Fatty acid synthase (FASN) and Matrix Metallopeptidase 1 (MMP1) as a promising target candidate pair. FASN inhibitors have shown promising results

for therapy of breast cancer (26) and orthotopic tongue oral squamous cell carcinoma (27). MMP1 is overexpressed in cervical cancer, and knockdown of MMP1 reduced the proliferation and migration of cervical cancer cells, while expression of epithelial marker E-cadherin increased and expression of mesenchymal marker Vimentin decreased (28). These and our results suggest that a combined FASN and MMP1 inhibition may be beneficial for cervical cancer patients.

We would like to acknowledge some of the limitations of this study. This study was retrospective and performed on a small but relevant patient population in that all patients had recurrent disease and had received prior radiation representing the at need population. Despite this limitation, these findings present an opportunity to rationally approach future combination immunotherapy trials in the treatment of recurrent cervical cancer.

METHODS

Patient Sample Preparations

Following institutional review board approval (PA15-0286), patients were identified retrospectively through a departmental database (MDA 2008-0095). All women with a diagnosis of squamous cell carcinoma or adenocarcinoma of the cervix who underwent a pelvic exenteration procedure at The University of Texas MD Anderson Cancer Center from 1994 to 2004 were included. Clinical and pathologic data were abstracted from the medical record. Formalin-fixed, paraffin-embedded tumor samples were identified and specimens were reviewed for pathologic diagnosis and dissected if necessary to ensure that $\geq$90% of the sample represented tumor. The normal tissue was directly adjacent to the tumor on the same slide.

Nanostring Analyses

NanoString was performed using the nCounter® PanCancer Immune Profiling panel (XT–CSO-HIP1-12) composed of 770 immune response genes and the nCounter® PanCancer pathways panel (XT–CSO-PATH1-12) comprised of 770 genes from 13 canonical cancer associated pathways (NanoString Technologies). RNA was extracted using the Highpure miRNA isolation kit (Roche) from FFPE blocks, following initial confirmation of tumor presence and content by two pathologists by H&E. For gene expression studies, 1 μg of RNA was used as per manufacturer's instructions (NanoString Technologies). All samples included in this study passed a quality check before the NanoString analysis. Raw NanoString results were normalized using standard NanoString housekeeping genes before comparing tumor-normal pairs. We performed a "Core Analysis" with the significantly differently expressed genes (after Bonferroni correction) in the Cancer Immune and separately with the significant results in the Cancer Pathways panel.

Immunohistochemistry

We performed further survival and expression correlation analyses with our previously published immunohistochemistry data described in (29).

Statistical Analyses

To compare tumors and normals we used two-tailed paired Student's *t*-tests. Statistically significant differences were noted when $p < 0.05$. In expression analyses we used Bonferroni correction to adjust the 0.05 threshold of significance for comparing expression level of 730 genes.

Data Visualization

We used the Tableau Desktop business intelligence tool to prepare the figures. Kaplan-Meier plots were made using the "survival" R package.

ETHICS STATEMENT

This study was carried out in accordance with the approval of MD Anderson Cancer Center IRB with a waiver of informed consent due to the retrospective and minimal risk nature of this study.

AUTHOR CONTRIBUTIONS

AJ: Study concept and design; JR, KR, KW, AL, AY, PS, MF, and AJ: Acquisition, analysis, or interpretation of data; JR, KR, KW, AL, AY, PS, MF, and AJ: Preparation, review, or approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02102/full#supplementary-material

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Ex vivo Hsp70-Activated NK Cells in Combination With PD-1 Inhibition Significantly Increase Overall Survival in Preclinical Models of Glioblastoma and Lung Cancer

*Maxim Shevtsov[1,2,3,4], Emil Pitkin[5], Alexander Ischenko[6], Stefan Stangl[1], William Khachatryan[4], Oleg Galibin[3], Stanley Edmond[1], Dominik Lobinger[1] and Gabriele Multhoff[1]**

[1] Radiation Immuno-Oncology, Center for Translational Cancer Research, TUM (TranslaTUM), Munich, Germany, [2] Institute of Cytology of the Russian Academy of Sciences (RAS), St. Petersburg, Russia, [3] Pavlov First Saint Petersburg State Medical University, St. Petersburg, Russia, [4] Almazov National Medical Research Centre, Polenov Russian Scientific Research Institute of Neurosurgery, St. Petersburg, Russia, [5] Wharton School, University of Pennsylvania, Philadelphia, PA, United States, [6] Research Institute of Highly Pure Biopreparations, St. Petersburg, Russia

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***Correspondence:**
Gabriele Multhoff
gabriele.multhoff@tum.de

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Heat shock protein 70 (Hsp70) which is expressed on the plasma membrane of highly aggressive tumors including non-small cell lung carcinoma and glioblastoma multiforme serves as a target for Hsp70-targeting NK cells. Herein, we aimed to investigate the antitumor effects of a combined therapy consisting of *ex vivo* Hsp70-peptide TKD/IL-2-activated NK cells in combination with mouse/human anti-PD-1 antibody in a syngeneic glioblastoma and a xenograft lung cancer mouse model. Mice with membrane Hsp70 positive syngeneic GL261 glioblastoma or human xenograft A549 lung tumors were sham-treated with PBS or injected with *ex vivo* TKD/IL-2-activated mouse/human NK cells and mouse/human PD-1 antibody either as a single regimen or in combination. Tumor volume was assessed by MR scanning and tumor-infiltrating $CD8^+$ T, NK, and PD-1^+ cells were quantified by immunohistochemistry (IHC). We could show that the adoptive transfer of *ex vivo* TKD/IL-2-activated mouse NK cells or the inhibition of PD-1 resulted in tumor growth delay and an improved overall survival (OS) in a syngeneic glioblastoma mouse model. A combination of both therapies was well-tolerated and significantly more effective with respect to both outcome parameters than either of the single regimens. A combined treatment in a xenograft lung cancer model showed identical effects in immunodeficient mice bearing human lung cancer after adoptive transfer of TKD/IL-2-activated human effector cells and a human PD-1 antibody. Tumor control was associated with a massive infiltration with $CD8^+$ T and NK cells in both tumor models and a decreased in PD-1 expression on immune effector cells. In summary, a combined approach consisting of activated NK cells and anti-PD-1 therapy is safe and results in a long-term tumor control which is accompanied by a massive tumor immune cell infiltration in 2 preclinical tumor models.

Keywords: membrane Hsp70, glioblastoma, lung carcinoma, immunophenotyping, NK cell therapy, anti-PD-1 antibody

INTRODUCTION

Stress-inducible Hsp70 is frequently overexpressed in the cytosol of many tumor entities where it fulfills a large variety of chaperoning functions such as folding/unfolding and transport of other proteins (1). Furthermore, highly aggressive tumors including glioblastoma (2–4) and lung cancers (5) present Hsp70 on their plasma membrane as a tumor-specific biomarker. Membrane Hsp70 positive, viable tumor cells have been found to actively release Hsp70 in exosomes, and therefore elevated exosomal Hsp70 levels in the serum are predictive for viable tumor mass (5). Increased Hsp70 membrane densities are detectable in highly aggressive tumors including primary glioblastoma multiforme (2) and advanced non-small cell lung cancer (NSCLC) (6). Both tumor types are debilitating, life-threatening diseases with poor prognosis. Despite combined treatment regimens consisting of surgery, radiotherapy (RT) and chemotherapy, OS and local progression-free survival (LPFS) in patients with glioblastoma multiforme and NSCLC in stage IIIA/B remains poor with <15 months (7–9). In preclinical tumor models, radio-chemotherapy (RCT) has been found to induce abscopal effects (10–13), however, due to anti-apoptotic pathways and immunosuppressive mechanisms (14) these bonafide immunostimulatory effects are unable to mediate long-term protective anti-tumor immunity (15). A major breakthrough has been achieved by the application of immune checkpoint inhibitor antibodies which provide inhibitory feedback loops for an immune cell mediated tumor rejection (16). Many cancer types including brain and lung tumors use the PD-1 pathway for immune escape (17). Nivolumab, a fully humanized IgG_4 antibody, targets PD-1 and thereby attenuates inhibitory signals in immune cells such as T and NK cells (16, 18), which results in objective tumor responses predominantly in highly immunogenic ("hot") tumors (19, 20). Despite these promising results a relevant proportion of patients, however, does not profit from immune checkpoint inhibitor blockade therapies. Therefore, herein a combined regimen consisting of Hsp70-targeting activated NK cells and anti-PD-1 inhibition was tested in a preclinical syngeneic glioblastoma and a xenograft lung cancer model.

MATERIALS AND METHODS

Cells

The mouse glioblastoma cells line GL261, human lung carcinoma A549 cells (American type culture collection (ATCC #CCL-185) and the NK target cell line K562 (ATCC #CCL-243) were cultured in Roswell park Memorial Institute 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics (100 IU/mL penicillim, 100 μg/mL streptomycin) at 37°C in 95% humidity and 5% (v/v) CO_2. Lewis lung carcinoma (LLC) cells were cultured in DMEM medium supplemented with 10% FCS, 2 mM L-glutamine and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin). All cell lines are positive for membrane-bound Hsp70 as determined by flow cytometry (21, 22).

Animals

C57Bl/6 male 10-week-old mice were purchased from the animal nursery "Rappolovo" of the Russian Academy of Medical Sciences (St. Petersburg, Russia). NMRI nu/nu 8–10-week male mice were obtained from an animal breeding colony (Charles River). All animal experiments were approved by the local ethical committee of Pavlov First St. Petersburg State Medical University (St. Petersburg, Russia) and were in accordance with institutional guidelines for the welfare of animals.

Orthotopic Injection of GL261 Glioblastoma Cells Into C57Bl/6 Mice

Briefly, C57BL/6 mice were anesthetized by ip injection with fentanyl (0.05 mg/kg), midazolam (5 mg/kg) and medetomidine (0.5 mg/kg) mixture before mounting them in a stereotactic frame (David Kopf Instruments, Tujunda, CA, USA). GL261 cells (1×10^5) resuspended in sterile PBS (2 μl) were stereotactically injected into the *nucleus caudatus dexter* of anesthetized mice.

Orthotopic Injection of A549 Lung Cancer Cells Into Immunodeficient Mice

After anesthesia, NMRI nu/nu mice were injected percutaneously in the upper margin of the sixth rib on the right anterior axillary line into the right lung (5 mm depth) with a single cell suspension (100 μl) of A549 cells (5×10^6 cells/ml).

Ex vivo Stimulation of Mouse/Human NK Cells With TKD/IL-2

Peripheral blood lymphocytes (PBLs) were isolated of sacrificed C57BL/6 mice by Ficoll-Paque gradient centrifugation. After separation, PBL were resuspended in RPMI-1640 supplemented with 2 mM L-glutamine, 10% FCS, and antibiotics (100 IU/ml Penicillin G and 100 μg/ml Streptomycin). Previous data have indicated that NK cell activation is superior when, instead of purified NK cells, PBL are stimulated with the 14-mer TKD peptide (TKDNNLLGRFELS, 2 μg/ml, Bachem, Bubendorf, Switzerland) and IL-2 (100 IU/ml) at defined cell densities of 5–10 × 10^6 PBL/ml for 3–4 days (23, 24). Since the human TKD sequence differs only in one amino acid in human and mouse (TKDNNLLGRFELSG and TRDNNLLGRFELSG, respectively), it is possible to stimulate mouse NK cells with the human TKD peptide (4).

Human PBL for NK cell stimulation for the treatment of the A549 xenograft tumor mouse model were obtained from Caucasian healthy volunteers (age range 22–24 year, age mean 23.1 years). All healthy individuals who participated in this study provided written informed consent. The study was approved by the local ethical committee.

Ten ml of peripheral blood was collected into EDTA tubes and PBL were isolated by density gradient centrifugation using Ficoll-Paque, as described earlier. After separation, PBL were resuspended in RPMI-1640 supplemented with 2 mM L-glutamine, 10% FCS, and antibiotics (100 IU/ml Penicillin G and 100 μg/ml Streptomycin). PBL were stimulated either with the 14-mer TKD peptide (TKDNNLLGRFELS, 2 μg/ml, Bachem, Bubendorf, Switzerland) or recombinant, low-endotoxin Hsp70

protein (10 μg/ml) that was obtained and purified from bacteria transformed with a pMSHSP plasmid, as described previously (23), and IL-2 (100 IU/ml) at cell densities of 5–10 × 10^6 PBL/ml for 3−5 days (24, 25). Flow cytometry was performed on day 5 after stimulation with TKD/IL-2 using FITC/PE/PerCP or APC conjugated mouse IgG1 antibodies (BD Biosciences), FITC-conjugated mouse antibody against CD94 (BD Pharmingen), FITC/PE or APC conjugated mouse antibodies against CD56 (BD Biosciences), PerCP conjugated antibody against CD3 (BD Biosciences), FITC conjugated antibody against CD4 (BD Pharmingen), FITC or PE conjugated antibodies against CD8 (BD Pharmingen), PE conjugated antibody against CD19 (BD Pharmingen), PE conjugated antibody against CD16 (BD Pharmingen), PE conjugated monoclonal antibodies against NK cell activatory receptors (NKG2D (R&D Systems), NKp30 (Beckman Coulter), NKp46 (Beckman Coulter), APC-conjugated antibodies against CD45 (Life Technologies) and CD69 (BD biosciences). The percentage of positively stained cells was determined following subtraction of cell stained with an isotype-matched negative control antibody. Only PI (propidium iodide, Sigma) negative, viable cells were gated and analyzed.

Cytotoxicity Assay

GL261, A549, and LLC cells and K562 cells were employed as target cells for analysis of the cytolytic activity of NK cells. The effector cells were isolated from C57/Bl6 mice (for GL261 and LLC cells) and peripheral blood of healthy individuals (for human A549 adenocarcinoma cells). Target cells were treated as follows: (1) control; (2) NK cells following co-incubation with IgG isotype antibody (20 μg/ml); (3) NK cells co-incubated with mouse/human anti-PD-1 immune checkpoint inhibitor antibody (20 μg/ml); (4) NK cells without stimulation; (5) NK cells *ex vivo* TKD/IL-2-stimulated (2 μg/ml for TKD peptide and 100 IU/ml for IL-2); (6) NK cells *ex vivo* TKD/IL-2-stimulated in combination with anti-PD-1 immune checkpoint inhibitor antibody (20 μg/ml). The incubation of the effector and target cells at various ratios (1:12.5, 1:25, and 1:50) lasted 4 h. CytoTox 96® non-radioactive cytotoxicity assay (Promega, USA) was employed to determine the amount of dying target cells according to the manufacture's protocol.

Treatment Protocol

For comparing the efficacy of singular or combined therapies consisting of an adoptive transfer of *ex vivo* TKD/IL-2-stimulated mouse/human NK cells and mouse/human anti-PD-1 immune checkpoint inhibitor antibody (RMP1-30, eBioscience, Frankfurt/Main, Germany) animals with comparable tumor sizes (according to MRI volumometrics) were randomly divided into 5 groups (8 animals per group): Animals of the control groups were injected either with 100 μl PBS (iv) or with 250 μg isotype-matched IgG antibody (ip) on days 6, 9, 12 and 15. Animals of the treatment groups were iv injected either with NK cells (6 × 10^6 in 100 μl PBL) on days 6, 9, and 12 and/or ip injected with anti-PD-1 antibody on days 6 (500 μg), 9 (250 μg), 12 (250 μg), and 15 (250 μg) in a volume of 500 μl PBS.

Magnetic Resonance (MR) Tumor Imaging of Mouse Glioblastoma

Tumor progression was assessed before and after each therapy on days 5, 10, 15, 20, 25, and 30 using a high-field 11.0 T MR scanner (Bruker, Bremen, Germany) with a customized rodent coil. High-resolution anatomical T_2-weighted scans (repetition time [TR]/echo time [TE] 4,200/36 ms, flip angle 180°, slice thickness 1.0 mm, interslice distance 1.2 mm, field of vision (FoV) 3.0 × 3.0 cm, matrix 256 × 256, in total 20 slices) were performed in coronal planes. Additionally T_1-weighted scans (TR/TE 1500/7.5 ms, flip angle 180°, slice thickness 1.0 mm, FoV 3.0 × 3.0 cm, matrix 256 × 256), FLASH scans (TR/TE 350/5.4 ms, flip angle 40°, slice thickness 1.0 mm, 3.0 × 3.0 cm, matrix 256 × 256) in coronal planes were performed. The obtained images were analyzed using adequate software (AnalyzeDirect Inc, Overland Park, KS, USA).

Mouse Tumor Immunohistochemistry (IHC)

Animals were anesthetized by ip injection of 150–200 mg/kg pentobarbital. After perfusion with 100 ml saline/4% paraformaldehyde, whole brains were removed and tumor volumes were assessed. Tissue was fixed in 4% paraformaldehyde/30% sucrose, embedded in Tissue-Tek® and blocks were cut into serial sections (5–7 μm). $CD8^+$ T cells, $NK1.1^+$ cells and $PD\text{-}1^+$ lymphocytes were stained on IHC sections using anti-CD8 (53-6.7, Biolegend, San Diego, CA, USA), anti-NK1.1 (PK136, Biolegend, San Diego, CA, USA) and anti-PD-1 (RMP1-30, eBioscience, Frankfurt/Main, Germany) antibodies according to an established protocol. Tumor-infiltrating $CD8^+$ T cells, NK1.1 cells and $PD\text{-}1^+$ cells were counted in 3 fields of views by two independent researchers.

Human Tumor Immunohistochemistry (IHC)

For IHC formalin-fixed, paraffin-embedded (FFPE) specimens of the A549 lung tumors were cut at 4 μm and transferred onto slides. All staining procedures were automatically performed on a Ventanas Benchmark XT for analysis of tumor-infiltrating $CD8^+$, $PD\text{-}1^+$, and $CD56^+$ cells.

Statistics

The comparative survival of animals was assessed with Kaplan-Meier curves that are based on the Kaplan-Meier estimator. All such estimates were computed and visually presented with corresponding confidence intervals. The Kaplan-Meier estimator is a non-parametric statistic that accommodates right-censoring in the data. When the means of the groups of two continuous variables were compared, the parametric Student's *t*-test was employed. Variances between groups were not considered to be equal, and degrees of freedom for such tests were computed accordingly. The significance level for all tests was alpha = 0.05, and all confidence intervals are reported at the 95% level. All p-values reported for all t-tests are two-sided. When comparing multiple groups, each of which had so few observations that standard parametric assumptions could not be validated, the Kruskal-Wallis test, which is a non-parametric analog to the one-way ANOVA test, was applied. The Krukal-Wallis test analyzes the differences in ranks between groups, rather than

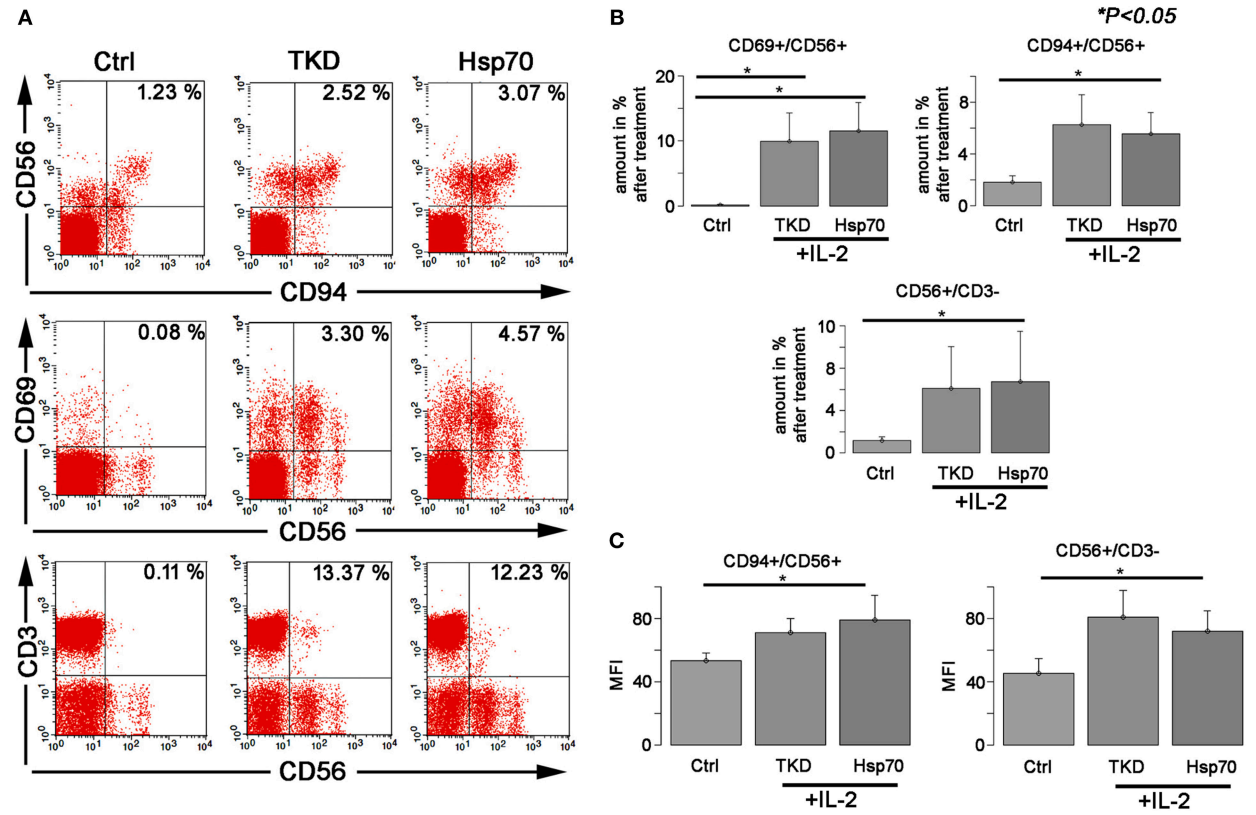

FIGURE 1 | The effect of TKD/IL-2 or Hsp70/IL-2 on the expression of CD56, CD94, and CD69 receptors in peripheral blood lymphocytes (PBLs) of healthy individuals. The expression of the receptors was examined within the fraction of stimulated and unstimulated PBLs derived from 10 healthy Caucasian individuals. **(A)** Representative figure of the healthy individual following stimulation with TKD/IL-2 or Hsp70/IL-2. **(B)** The amount and **(C)** the median fluorescence intensity (MFI) of the receptors expressed on NK cells. Data presented as means ± standard error (M ± SE).

the difference in means. Depending on the test, either Statistica Version 9.2 for Windows or the R programming language was run for all tests. All experiments were conducted once on each animal.

RESULTS

Analysis of the Phenotype of Human NK Cells After Stimulation With TKD/IL-2

Compared to unstimulated cells, a treatment with TKD/IL-2 for 5 days results in a significant upregulation of CD94, CD69, and CD56 on CD3-negative, human NK cells (**Figures 1A,B**). The percentage of $CD94^+$ cells increased from 1.83 ± 0.48 to 6.27 ± 2.31%, that of $CD69^+$ cells from 0.14 ± 0.09 to 9.94 ± 4.35% and that of $CD56^+$ cells from 1.19 ± 0.35 to 6.13 ± 3.9% ($p < 0.05$) (**Figure 1B**). A similar upregulation of the receptors was observed after an incubation of PBL with recombinant Hsp70 protein instead of TKD peptide: ($CD94^+$ cells: 5.55 ± 1.65; $CD69^+$ cells: 11.58 ± 4.38; $CD56^+$ cells: 6.72 ± 4.75) (**Figure 1B**). Concomitantly, the mean fluorescence intensities of CD94, CD56 which serve as surrogate markers for the Hsp70-specificity increased significantly on CD3-negative NK cells compared to unstimulated control cells (**Figure 1C**). No significant changes in activation markers were observed on $CD3^+$ T cell population upon stimulation with TKD/IL-2 or Hsp70/IL-2 (*data not shown*).

Ex vivo TKD/IL-2-Stimulated NK Cells Combined With Anti-PD-1 Antibody Demonstrate Enhanced Cytotoxic Activity Toward Tumor Cells

To assess the effect of a combined application of TKD/IL-2-stimulated NK cells with anti-PD-1 antibody *in vitro,* tumor cells (GL261, A549, and LLC) were co-incubated with activated lymphocytes at various effector:target (E:T) cells ratios ranging from 1:50 to 1:12.5. To prove that NK cell activity is measured in the assay the lysis of the NK target cell line K562 was assessed. The lysis of K562 cells at an E:T ratio of 1:50 was 20, 34, and 55% by unstimulated NK cells, NK cells stimulated with TKD/IL-2, and NK cells stimulated with TKD/IL-2 plus PD-1 antibody, respectively. With respect to the tumor cell lines GL261, A549, and LLC a co-incubation of unstimulated PBL with species-specific PD-1 antibody resulted in a more than two-fold increase in the lysis of all tumor cells (**Figure 2**). This effect was comparable to that of a stimulation of mouse and human PBL with TKD/IL-2. The most prominent anti-tumor cytolytic

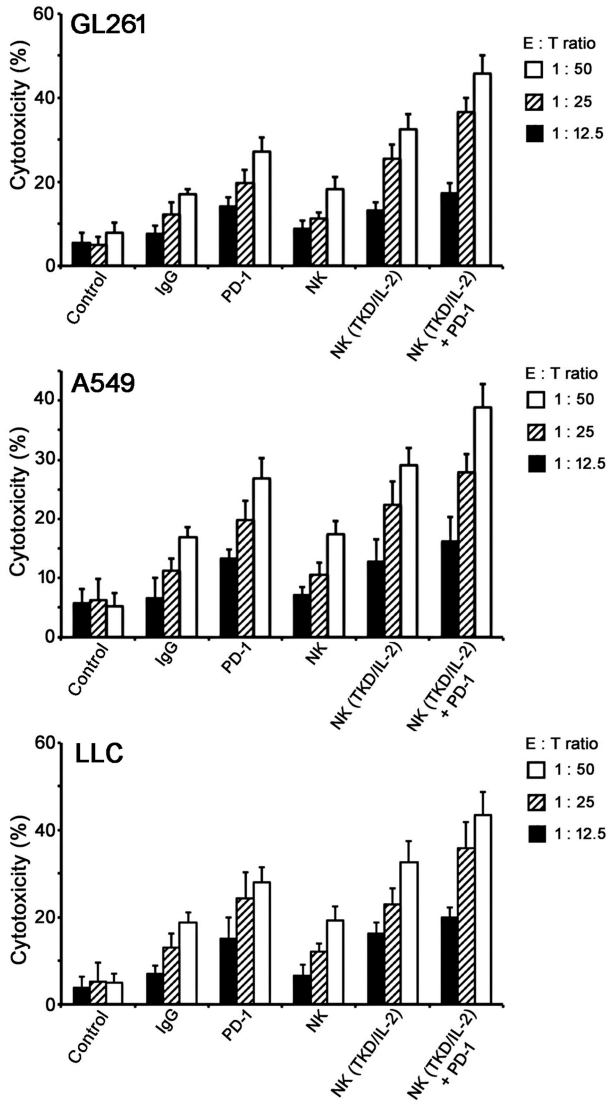

FIGURE 2 | Cytolytic activity of the *ex vivo* stimulated NK cells with anti-PD-1 antibody toward GL261, A549, and LLC tumor cells. Data presented as means ± standard error (M ± SE) for three independent experiments.

activity was achieved when PBL were stimulated with TKD/IL-2 concomitant with anti-PD-1 antibody ($p < 0.001$).

Treatment With *ex vivo* TKD/IL-2-Activated, Mouse NK Cells, and Anti-PD-1 Antibody Significantly Enhances OS and Induces Immune Cell Infiltration in a Syngeneic Glioblastoma Mouse Model

The effects of a singular or combined treatment consisting of *ex vivo* TKD/IL-2-stimulated mouse effector cells (NK) and immune checkpoint inhibitor blockade against mouse PD-1 (PD-1) were determined in mice with membrane Hsp70 positive orthotopic glioblastomas (GL261) (22). The treatment was started when the tumors reached a size of 100 mm^3 approximately on day 6. The most rapid tumor growth was observed in sham-treated (PBS, IgG isotype-matched antibody) control mice, as determined by MRI scanning (**Figure 3A**). On day 10, tumors reached a volume of 179 ± 12 mm^3 (PBS) and 203 ± 12 mm^3 (IgG, **Table 1**), and all mice of the control groups died before day 15 (**Figure 3B**). Three iv injections of *ex vivo* TKD/IL-2-activated NK cells, or 4 ip injections of mouse anti-PD-1 antibody caused a significant tumor growth delay. The maximum tumor volume of 203 ± 33 and 205 ± 24 mm^3 was reached 10 and 15 days later than in the sham-treated control group (**Table 1**). The best therapeutic outcome was achieved after a combined treatment with *ex vivo* mouse NK cells and PD-1 antibody. Even on day 30, the size of the tumors of 4 mice was only 124 ± 22 mm^3, and 4 out of 8 mice treated with the combined therapeutic approach showed complete tumor control (**Table 1**).

As shown by Kaplan-Meier analysis, OS of mice treated either with NK cells (3 injections, iv) or anti-PD-1 antibody (4 injections, ip) was significantly ($p < 0.05$) higher than that of sham-treated mice (PBS, 3 injections, iv; IgG, 4 injections, ip) (**Figure 3B**, **Table 2**). The p-values constituted $p < 0.0001$ for both, NK cell and anti-PD-1-treated groups vs. control. It appeared that 4 treatment cycles with mouse anti-PD-1 antibody were not significantly different regarding the OS as compared to the animals treated with three cycles of pre-activated NK cells ($p = 0.22$). Due to the iv route, the number of NK injections was limited to three cycles. The best therapeutic outcome was observed in mice after a combined treatment. OS of these mice was significantly higher than that of the sham-treated control groups ($p < 0.00001$) and that of NK or PD-1 antibody treated mice ($p < 0.00001$). In line with these findings, the number of tumor-infiltrating $CD8^+$ T and NK1.1 cells in tumor sections of mice, treated with NK cells and PD-1 antibody was significantly higher than in the control group ($p < 0.01$), and in the group of mice treated either with NK cells or PD-1 antibody ($p < 0.05$; **Figure 4**, **Table 3**). Vice versa, the number of tumor-infiltrating effector cells expressing the immune checkpoint inhibitor PD-1 decreased significantly ($p < 0.001$) in the treatment groups (PD-1, NK, NK + PD-1).

Treatment With *ex vivo* TKD/IL-2-Activated, Human NK Cells, and Anti-PD-1 Antibody Significantly Enhances OS in a Xenograft Lung Carcinoma Mouse Model

Following iv injection of *ex vivo* TKD/IL-2-stimulated, human effector cells (38.6 ± 9.7 days) a significant increase in the OS of tumor-bearing animals was observed compared to sham (PBS or IgG control antibody) treated control animals (**Figure 5**, **Table 4**). A combination of the NK cell therapy and the humanized anti-PD-1 antibody showed a 2.3-fold increase in OS as compared to control animals 48.8 ± 12.4 (NK) and 21.2 ± 6.2 (PBS), 22.3 ± 6.3 (IgG) days, respectively ($p < 0.001$) (**Figure 5**). Subsequent IHC analysis of the tumor sections showed an increased infiltration by $CD56^+$ NK cells and $CD8^+$ cells in the treatment groups with a highest infiltration of immune effector cells in the group who received the combined treatment regimen

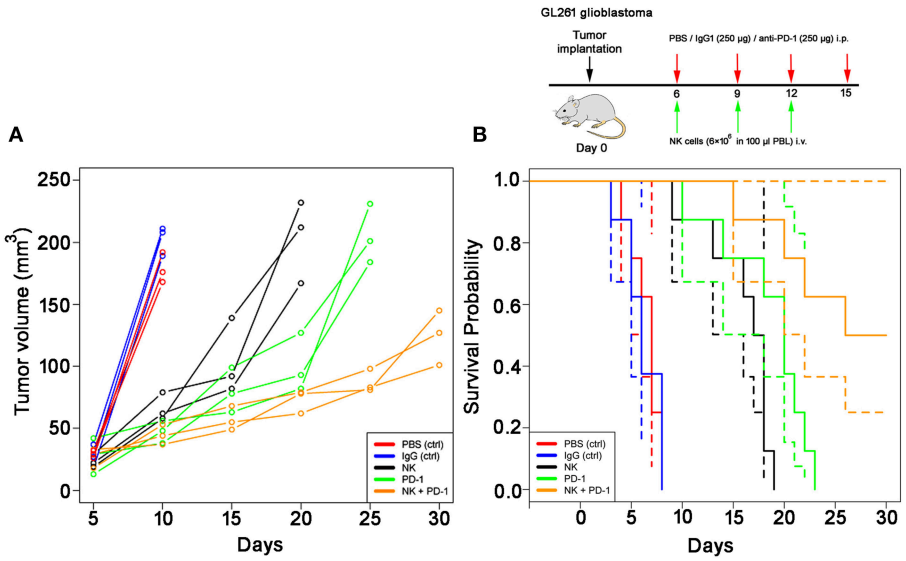

FIGURE 3 | Therapeutic potency of a combined treatment with *ex vivo* TKD/IL-2- stimulated, mouse NK cells with anti-PD-1 antibody in the model of intracranial GL261 glioma. **(A)** Volumetric studies of the GL261 glioma. Tumor volume (mm^3) as determined over time by T_1-weighted and T_2-weighted MRI scans in control (PBS, red lines; IgG control antibody, black lines) and treated (NK cells, green lines; PD-1 antibody, orange lines; NK cells + PD-1 antibody, blue lines) glioblastoma (GL261)-bearing C57/Bl6 mice ($n = 3$ per group). Tumor progression was calculated by measuring the cross-sectional areas on each slice and multiplying their sum as related to the thickness of the sections. **(B)** Kaplan-Meier analysis of the cumulative survival (days after treatment) in control (PBS, red lines; IgG control antibody, blue lines) and treated (NK cells, black lines; PD-1 antibody, green lines; NK cells + PD-1 antibody, orange lines) glioblastoma (GL261)-bearing C57/Bl6 mice ($n = 8$ per group). Solid lines: mean values; dotted lines: SD within 95% confidence interval.

TABLE 1 | Tumor volumes (mm^3) of mice ($n = 8$ per group) of control (ctrl) and treatment groups (NK, PD-1, NK + PD-1).

	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
PBS (ctrl)	29 (3)	178.67 (12.22)				
IgG (ctrl)	27.33 (9.50)	202.67 (11.93)				
NK	23.33 (5.13)	66.33 (11.15)	104.33 (30.43)	203.67 (33.29)		
PD-1	28 (14.52)	47.33 (9.02)	80 (18.08)	100.67 (23.46)	205.33 (23.80)	
NK + PD-1	26.67 (7.77)	44.66 (8.02)	57.33 (9.71)	73 (9.54)	87.33 (9.29)	124.33 (22.12)

Sham treatment: PBS (ctrl, 100 µl, iv), IgG (ctrl, 500 µl, ip) isotype-matched control antibody; treatment: NK, ex vivo TKD/IL-2-activated NK cells ($6x10^6$ cells in 100 µl PBS, iv); PD-1, PD-1 antibody (500 µl, ip); NK + PD-1, ex vivo activated NK cells ($6x10^6$ cells in 100 µl PBS, iv) + PD-1 (500 µl, ip) antibody over 6 time points (days 5, 10, 15, 20, 25, 30). The data represent mean values ± SD.

(**Figure 6**, **Table 5**). Furthermore, a significant decrease in PD-1^+ effector cells was observed inside the tumor ($p < 0.01$), as shown by IHC analysis.

DISCUSSION

Immune checkpoint inhibitors directed against CTLA-4, PD-1 or PD-L1 have recently demonstrated a therapeutic benefit in various solid tumors (e.g., melanoma, head and neck squamous cell carcinoma, gastric cancer, colorectal cancer, NSCLC, etc.) and lymphoid malignancies (26–31). Recently, evidence has accumulated that combined therapeutic strategies that consist of several immune checkpoint inhibitors or immune checkpoint inhibitors and other treatment modalities (32, 33) are beneficial. In the presented study anti-PD-1 immune checkpoint antibodies were combined with a NK cell therapy in a syngeneic and xenograft tumor mouse model. As shown previously, a blockade of immune checkpoints could improve NK cell-based therapies (34). Guo et al. demonstrated that anti-PD-1 antibody significantly increased the cytotoxicity of NK cells (i.e., enhanced expression of NKp30, NKp44 and NKG2D) that resulted in therapeutic effect toward multiple myeloma cells (35). Subsequent studies proved combined effect of Pidilizumab

TABLE 2 | Means and standard deviations (SD) of survival in days for mice with orthotopic GL261 glioblastoma subjected to different treatment and control regimes.

	PBS (Ctrl)	IgG (Ctrl)	NK	PD-1	NK + PD-1
Mean	11.5	11.1	21	23.5	29.9
SD	1.41	1.8	3.4	4.4	5.3

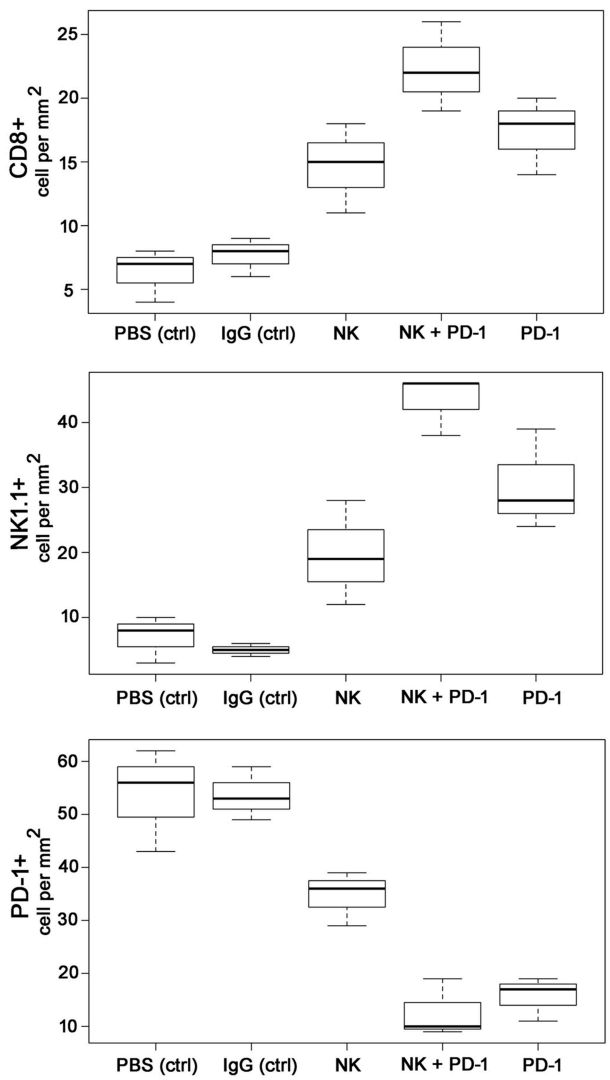

FIGURE 4 | Boxplots of the CD8+, NK1.1+ and PD-1+ cells infiltrating the GL261 tumor in control (PBS and IgG treated animals) and experimental groups. Tumor infiltrating CD8+ cytotoxic T cells, CD3-/CD56+ NK cells and PD1+ effector (T/NK) cells were counted in three representative IHC sections of the tumors of the different treatment groups (PBS ctrl, IgG ctrl, NK, NK + PD-1, PD-1) and infiltrating immune cells per mm^2 were calculated.

TABLE 3 | Number of tumor-infiltrating CD8$^+$ T cells, NK1.1 cells and PD-1$^+$ expressing effector cells in tumor sections of mice of the sham-treated control (ctrl) and treatment groups (NK, PD-1, NK+PD-1).

	NK 1.1 cells	CD8 cells	PD-1$^+$ cells
PBS (ctrl)	7 (3.61)	6.33 (2.08)	53.67 (9.71)
IgG (ctrl)	5 (1)	7.67 (1.53)	53.67 (5.03)
PD-1	30.33 (7.77)	17.33 (3.06)	16.67 (4.16)
NK	19.67 (8.02)	14.67 (3.51)	34.67 (5.13)
NK + PD-1	40.33 (4.62)	22.22 (3.51)	12.67 (5.51)

Sham-treated groups: PBS (ctrl), PBS (100 μl, iv), IgG (ctrl), isotype-matched IgG control antibody (500 μl, ip). Treated groups: NK, ex vivo activated NK cells (6x10^6 cells in 100 μl PBS, iv), PD-1, PD-1 antibody (500 μl, ip), NK+PD-1, ex vivo activated NK cells (6x10^6 cells in 100 μl PBS, iv) + PD-1 antibody (500 μl, ip). The data represent mean values of three fields of view ± SD.

(anti-PD-1) either alone or in combination with Rituximab in facilitation of the cytolytic activity of NK cells in patients with follicular lymphoma, multiple myeloma and renal cell carcinoma (36, 37).

In series of *in vitro* experiments for analysis of NK cells cytolytic activity toward tumor cells (GL261, A549, LLC) we demonstrated the therapeutic potential of a monotherapy when *ex vivo* TKD/IL-2-activated NK cells were applied (**Figure 2**). The effect was significantly higher as compared to non-stimulated lymphocytes. TKD/IL-2 activation of NK cells upregulated expression of CD56, CD69, and CD94 (**Figure 1**) that subsequently resulted in an enhanced cytotoxicity of lymphocytes (38). Previously, Gross et al. demonstrated that an increased expression density of CD94/NKG2C and CD56 initiates the NK cells capacity to kill membrane Hsp70-positive tumor cells (39, 40) and thereby acts as a surrogate marker for Hsp70-reactivity. The observed cytolytic effect of TKD/IL-2-stimulated NK cells was comparable to that of lymphocytes which have been pre-incubated with anti-PD-1 monoclonal antibodies (**Figure 2**). Previously, it was shown that blockade of PD-1 on NK cells could improve the cytotoxicity of the lymphocytes (even of exhausted NK cells in advanced tumor stages) (36, 41). A combination of TKD/IL-2-stimulated NK cells with anti-PD-1 antibodies resulted in 1.5-fold increase of anti-tumor cytotoxicity of lymphocytes (**Figure 2**) that indicates the synergistic effect of both therapeutic concepts.

In our experiments a preclinical proof-of-principle study has shown promising results of a combined therapy consisting of *ex vivo* TKD/IL-2-stimulated NK cells and anti-PD-1 antibody with respect to local tumor control, OS and immune stimulation in immunocompetent and immunodeficient mice with membrane Hsp70-positive tumors (GL261 glioblastoma, A549 lung cancer). The observed therapeutic efficacy was comparable to the effects reported earlier (42–44). Intriguingly, a significantly improved OS was observed when NK cell therapy was combined with anti-PD-1 antibody in a syngeneic GL261 glioblastoma and a xenograft A549 lung cancer model. Previously it was reported that cancer types (including NSCLC and melanoma), which are most responsive to checkpoint inhibitors, have a high mutational load (45, 46). Anti-tumor responses in mice were accompanied by a massive infiltration of the tumors with CD8$^+$ cytotoxic lymphocytes and NK1.1 cells, and a reduction in the amount of PD-1$^+$ immune cells in the tumor. Although NK cells or anti-PD-1 antibody, as a single treatment modality, have been shown to trigger anti-tumor immune responses that increase OS, a combined therapy has been found to be significantly more efficient. Presumably this could be explained by the effect of the anti-PD-1 antibody on NK cells. Programmed death 1 (PD-1) receptor was originally determined as an exhaustion marker on T cells, however, this receptor is also expressed on NK cells. In the recent study by Concha-Benavente et al. it was shown that PD-1 blockade increased Cetuximab-mediated NK cell activation and cytotoxicity in the head and neck patients (47). The anti-tumor effect achieved by monotherapies

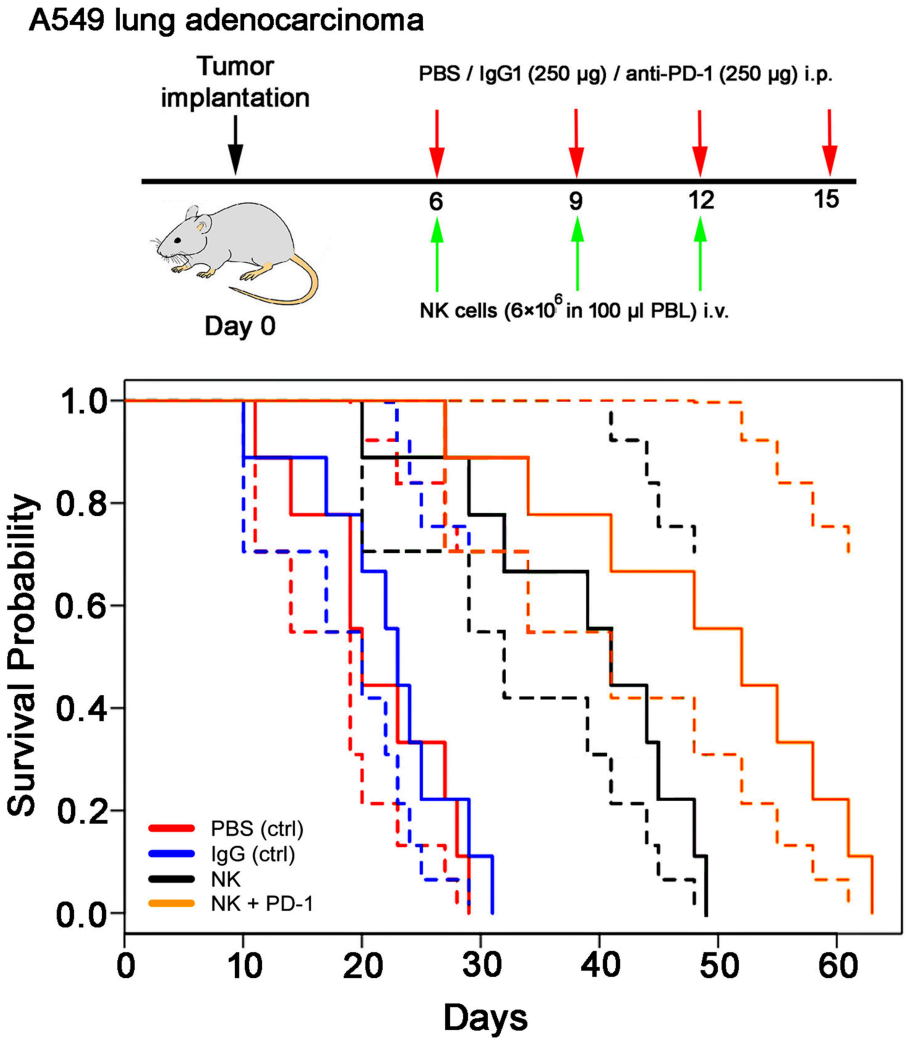

FIGURE 5 | Therapeutic effect of *ex vivo* stimulated, human NK cells with anti-PD-1 antibody in the orthotopic xenograft model of A549 lung carcinoma. Kaplan-Meier analysis of the cumulative survival (days after treatment) in control (PBS, red lines; IgG control antibody, blue lines) and treated (NK cells, black lines; NK cells + PD-1 antibody, orange lines) lung cancer (A549)-bearing mice ($n = 8$ per group). Solid lines: mean values; dotted lines: SD within 95% confidence interval.

(i.e., TKD/IL-2-stimulated NK cells or anti-PD-1 antibodies) that resulted in the delayed tumor progression (**Figure 3A**) was shortly abrogated after the discontinuation of the therapies. However, combined treatment approaches demonstrated the sustainability of the therapeutic effect after the discontinuation. Presumably, to further potentiate the therapeutic benefit a long-term combinatorial immunotherapy should be considered.

In our study we employed the inhibitor of PD-L1/PD-1 axis for the enhancement of NK cell adoptive therapy. Recently other immune checkpoint inhibitors (e.g., anti-CTLA-4 antibodies, anti-NKG2A antibodies) have been reported to restore cytolytic functions of NK cells and thereby enhance their anti-tumor activity (48, 49). Thus, André et al. showed that humanized anti-NKG2A antibodies enhanced NK cell activity against various tumor cells and rescued $CD8^+$ T cell function (49). Presumably, combination of TKD/IL-2-stimulated NK cells with several therapeutic antibodies could improve the anti-tumor activity of the adoptive cell immunotherapies.

TABLE 4 | Means and standard deviations (SD) of survival in days for mice with orthotopic lung A549 adenocarcinoma subjected to different treatment and control regimes.

	PBS (Ctrl)	IgG (Ctrl)	NK	NK + PD-1
Mean	21.2	22.3	38.6	48.8
SD	6.2	6.3	9.7	12.4

Depending on its subcellular or extracellular localization, Hsp70 fulfills different functions (50). On the one hand membrane Hsp70 serves as a tumor-specific target for TKD/IL-2-activated NK cells (4, 51), on the other hand, high

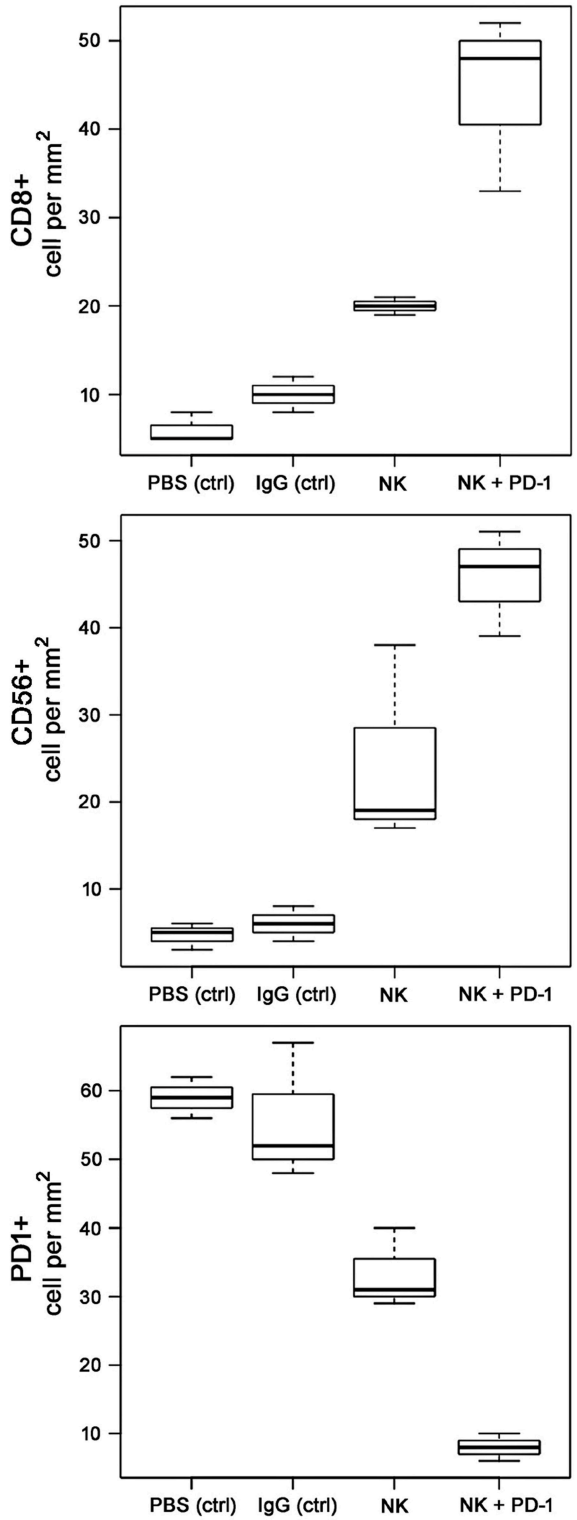

FIGURE 6 | Boxplots of the CD8+, CD56+NK, and PD-1+ cells infiltrating the A549 lung carcinoma in control (PBS and IgG treated animals) and experimental groups. Tumor infiltrating CD8+ cytotoxic T cells, CD3-/CD56+ NK cells, and PD1+ effector (T/NK) cells were counted in three representative IHC sections of the tumors of the different treatment groups (PBS ctrl, IgG ctrl, NK, and NK + PD-1) and infiltrating immune cells per mm^2 were calculated.

TABLE 5 | Number of tumor-infiltrating CD8+ T cells, CD56+ NK cells and PD-1+ expressing effector cells in A549 lung carcinoma sections of mice of the sham-treated control (ctrl) and treatment groups (NK, PD-1, NK+PD-1).

	Cell type		
Treatment	**CD56+ NK cells**	**CD8+ cells**	**PD-1+ cells**
PBS (ctrl)	4.67 (1.53)	6.00 (1.73)	59.00 (3.00)
IgG (ctrl)	6.00 (2.00)	10.00 (2.00)	55.67 (10.02)
NK	24.67 (11.59)	20.00 (1.00)	33.33 (5.86)
NK + PD-1	45.67 (6.11)	44.33 (10.02)	8.00 (2.00)

Sham-treated groups: PBS (ctrl), PBS (100 μl, iv), IgG (ctrl), isotype-matched IgG control antibody (500 μl, ip). Treated groups: NK, ex vivo activated NK cells (6 ×10^6 cells in 100 μl PBS, iv), ex vivo activated NK cells (6 × 10^6 cells in 100 μl PBS, iv) + PD-1 antibody (500 μl, ip). The data represent mean values of three fields of view ± SD.

cytosolic Hsp70 levels can interfere with apoptotic pathways that mediate radio-chemotherapy resistance. However, as was shown previously the upregulation of the membrane-bound Hsp70 following anti-tumor therapies (e.g., ionizing radiation, chemotherapy, etc.) also increases the efficacy of the Hsp70-targeted therapies (52, 53).

In line with the data shown in two preclinical models most recently, we could demonstrate the efficacy of the combined therapeutic concept consisting of radiochemotherapy, TKD/IL-2-activated NK cells and PD-1 inhibition in a patient with membrane Hsp70 positive stage IIIb NSCLC. Identical to the mouse models, the therapy was well tolerated, induced anti-tumor immune responses mediated by T and NK cells and resulted in a long-term OS of more than 35 months (54).

In summary our data indicate that immunotherapeutic approaches with minor monoactivity could be enhanced by the addition of immune checkpoint inhibitors. The efficacy of a combined therapy consisting of *ex vivo* stimulated NK cells and anti-PD-1 blockade which has been shown to be feasible, safe, and effective needs to be validated in randomized clinical trials.

AUTHOR CONTRIBUTIONS

The study was conceived and designed by MS and GM. MS, EP, AI, SS, WK, OG, SE, DL conducted and analyzed the experiments. EP supported statistical analysis. MS and GM wrote the manuscript with valuable comments from DL, SS, WK, OG, and AI.

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OPINION
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How to Increase the Efficacy of Immunotherapy in NSCLC and HNSCC: Role of Radiation Therapy, Chemotherapy, and Other Strategies

*Valerio Nardone[1], Pierpaolo Pastina[1], Rocco Giannicola[2], Rita Agostino[2], Stefania Croci[1], Paolo Tini[1,3], Luigi Pirtoli[4], Antonio Giordano[4,5], Pierosandro Tagliaferri[6,7] and Pierpaolo Correale[2]**

[1] Radiation Oncology Unit, University Hospital of Siena, Siena, Italy, [2] Medical Oncology Unit, Grand Metropolitan Hospital "Bianchi Melacrino Morelli", Reggio Calabria, Italy, [3] Sbarro Health Research Organization, Temple University, Philadelphia, PA, United States, [4] Department of Biology, College of Science and Technology, Temple University, Philadelphia, PA, United States, [5] Department of Medicine, Surgery and Neurosciences University of Siena, Siena, Italy, [6] Department of Experimental and Clinical Medicine, Magna Graecia University, Catanzaro, Italy, [7] Medical Oncology Unit, Azienda Ospedaliero – Universitaria "Mater Domini", Catanzaro, Italy

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Patrik Andersson, Massachusetts General Hospital, Harvard Medical School, United States

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***Correspondence:**
Pierpaolo Correale
correalep@yahoo.it

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An extraordinary large amount of strategies potentially able to elicit and empower an efficient anti-tumor immune-response in cancer patients, has already been described (1). However, a number of hurdles have delayed the translation of these results in efficacious treatments for many years leaving the immunological treatments confined to malignant melanoma and renal cell carcinoma(2, 3). In the latter few years, the discovery of priming (CTLA-4/B7.1) and effector (PD-1/PDL-1) immune-checkpoints and the availability of highly specific blocking mAbs has lead to a terrific clinical development of the immune-oncology approaches. Some of these mAbs, especially those directed to PD-1 (Nivolumab and Pembrolizumab) expressed on activated CTLs, or PDL-1 (Atezolizumab, Durvalumab, and Avelumab) expressed on inflammatory and cancer cells, have in fact, gained a stable role in the treatment of very common malignancies such as non-small cell lung cancer (NSCLC), head and neck squamous cell carcinoma (HNSCC), and urological malignancies, where they are capable of producing significant benefit to many patients and prolonging their survival in about a quarter of the cases (4). Even though this kind of strategy is considered quite successful, it is however, hampered by the fact that its efficacy is unpredictable and is associated to immune-related adverse events (irAEs) and unsustainable costs. At the present, the identification of reliable biomarkers of response to immune-oncology treatments as well as the design of combined strategies to enhance their efficacy and field of action represent one of the mainstream immune-oncology research lines. PD-1/PDL-1 is a peripheral immune-checkpointaimed to attenuate the cytotoxic response of tumor-specific infiltrating lymphocytes. Thus, its blockade by anti PD-1 (Nivolumab and Pembrolizumab) or anti PDL-1 mAbs (Atezolizumab, Durvalumab, and Avelumab) rescues these CTLs and triggers a fast cytolytic effect in the tumor tissue (5). This effect may triggera rapid antitumor effect;neverthelessthis renewed CTL reaction is not sufficient alone to prolong patients' survival. In fact the antitumor activity of these reactivated cells, is more or less rapidly extinguished if a continuous and self-sustained supply of fresh tumor-specific immune-effectors does not occur (immunopriming) (6). Experimental evidence suggests in fact, the achievement of a prolonged patient survival requires a continuous immune-priming, in order to avoid CTL exhaustion in the tumor and to prevent an adaptive response by the tumor cells (7, 8). In this context, CTLA-4/B7.1 immune-check point, acts by attenuating the proliferative activity of antigen specific CTL clones, expressing CTLA-4 and by stimulating the immune-suppressive activity of immune-regulatory

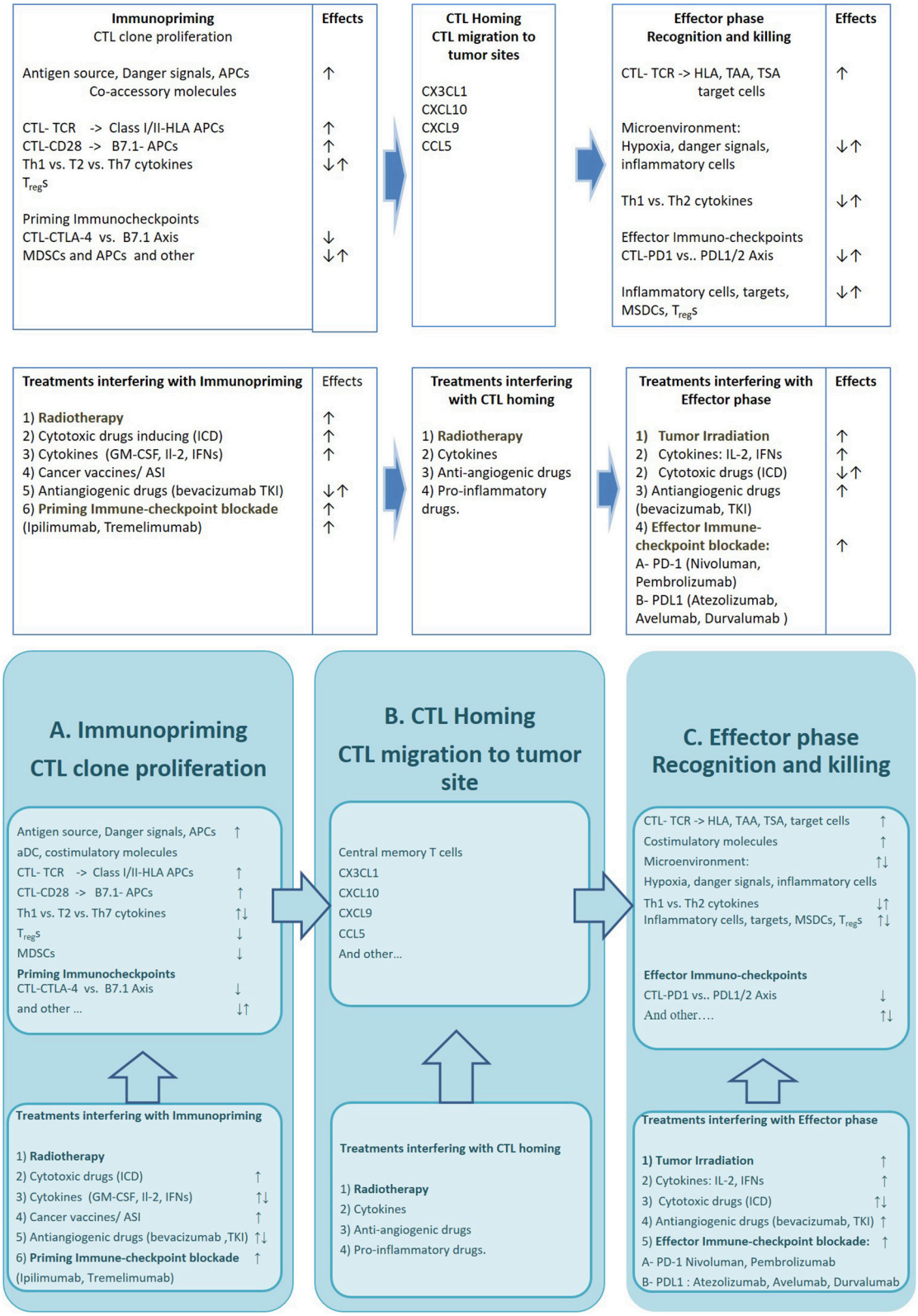

FIGURE 1 | The figure describes the critical mechanisms involved in three phases of the immune-response against cancer and available drugs and strategies which may improve its efficacy **Upper row:** Specific cell lineages, molecular structures and immune-checkpoints involved in immunopriming process (A), T cell Homing (B), and modulation of CTL mediated Tumor cell killing (C). **Bottom row:** Strategies (AKA radiation therapy), cytotoxic Drugs, cytokines and Immunocheckpoint inhibitors

(Continued)

FIGURE 1 | interfering with the immunopriming (A), T cell Homing (B), and T cell mediated killing (C). APCs, antigen presenting cells; CTL-TCR, cytotoxic T lymphocites–T cell Receptor; HLA, Human Leucocyte Antigen; MDSCs, myeloid derived suppressor cells; TAA, Tumor Associated Antigen; TSA, Tumor Specific Antigen; ICD, Immunogenic Cell Death inducers; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IFNs, interferons; ASI, active specific immunotherapy; TKI, tyrosine kinase inhibitor; CTLA-4, Cytotoxic T cell antigen−4; PD-1, Programmed cell death receptor-1; PDL, Programmed cell death ligand.

TABLE 1 | Ongoing trials testing immunotherapy (IT) in combination with radiation therapy (RT) in patients with NSCLC or HNSCC.

NCT number	Study phase	Disease stage	Trial design *(Experimental arm)*	Estimated primary completion date
NCT03391869	Phase 3	Metastatic NSCLC	Local Consolidation Therapy (RT or surgery) after Nivolumab and Ipilimumab (LONESTAR)	December, 2022
NCT03523702	Phase 2	Locally Advanced NSCLC	Selective Personalized Radio-Immunotherapy for Locally Advanced NSCLC Trial (SPRINT)	August, 2020
NCT03176173	Phase 2	Metastatic NSCLC	Radical-Dose Image Guided Radiation Therapy in Treating Patients with Metastatic NSCLC undergoing IT	June, 2020
NCT03110978	Phase 2	Stage I, selected IIa or isolated recurrent NSCLC	Immunotherapy Plus Stereotactic Ablative Radiotherapy (I-SABR) vs. SABR Alone	June, 2022
NCT03164772	Phase 2	Metastatic NSCLC	Safety and preliminary efficacy of the addition of a vaccine therapy to 1 or 2 checkpoint inhibitors for NSCLC.	March, 2021
NCT03313804	Phase 2	HNSCC and NSCLC undergoing IT	Short-course radiation to a single systemic (non-CNS) site within 14 days of the beginning of IT	June, 2018
NCT02999087	Phase 3	Locally advanced HNSCC front-line	Avelumab and Cetuximab plus RT vs Cetuximab-RT and Cisplatin-RT	October, 2019
NCT03085719	Phase 2	Advanced HNSCC after first line therapy	Immunotherapy (Pembrolizumab) in combination with high dose and low dose radiation therapy	October, 2020
NCT03317327	Phase 1/2	Recurrent HNSCC	Re-irradiation and Nivolumab in loco-regionally recurrent HNSCC	November, 2023
NCT03247309	Phase 1	Recurrent HNSCC and NSCLC	TCR-engineered T Cells in Solid Tumors With Emphasis on NSCLC and HNSCC (ACT engine)	December, 2019
NCT02892201	Phase 2	HNSCC with residual disease	Pembrolizumab after RT in patients with residual disease (biopsy proven)	December, 2018
NCT03247712	Phase 1/2	HNSCC before surgery	Test the safety of neoadjuvant immune-radiotherapy to down-staging HNSCC prior to surgical resection.	December, 2025

T cells (T_{reg}s). Its blockade by Ipilimumab and Tremelimumab, two mAbs to CTLA-4, represents a valid therapeutic option for both metastatic malignant melanoma and renal cell carcinoma and is under clinical investigation in combination with effector PD1/PDL-1 immunocheckpoint blockade (9–12). An efficient Immune-priming however, requires the expression of multiple tumor associate (TAAs) and tumor specific antigens (TSAs) by cancer cells, released as consequence of cancer-associated inflammation, necrosis, previous use of cytotoxic drugs or radiation therapy (13). A number of studies have shown that the efficacy of both immune-effectors and antigen cross-priming may be hardened by cancer vaccines, specific anticancer treatments (radiotherapy, chemotherapy, steroid hormones, and immune-adjuvant agents), hypoxic response and/or tumor associated inflammation (14, 15) (**Figure 1**).

Radiation therapy in particular, together with its direct cyto-reductive activity on tumor burden is also capable of eliciting radio-induced DNA damage on target cells and triggering specific immunological effects (16) which are believed to be responsible for the "abscopal effects" observed in those rare cases, where tumor irradiation is paralleled by regression of non-irradiated tumor sites (17, 18). This hypothesis is in line with the results of a large number of studies showing that tumor irradiation may really influence all the phases of the immune-response. Tumor irradiation may in fact, trigger immunogenic cell death, and significant release of TAAs and TSAs in a context of immunological danger signal. The latter is consequent to DNA damage by radiation which is able to activate of Damage-Associated-Molecularbiochemical Patterns (DAMP) which in turn are able of enhancing tumor antigens presentation to CTL precursors and their proliferation in the draining lymph-nodes (19). Furthermore, the irradiated-tumor cells release inflammatory cytokines, chemokines (such as CXCL16) and tumor vessel associated adhesion molecules (VCAM-I and ICAM-I) able to reinforce the presence of activated CTLs in the tumor site (19–21). Finally, strong evidence does exists concerning the ability of radiation therapy to induce up-regulation of class I MHC, multiple death receptors (e.g., FAS, NKG2DL) in the target cells thus enhancing their susceptibility to recognition and killing by tumor specific CTLs (19). Clinical

evidences in line with these preclinical results have also been reported.

IAn abscopal response to radiation was recorded in metastatic NSCLC patients who were receiving immunological treatment with ipilimumab (22). We recently carried out a retrospective analysis in advanced NSCLC patients enrolled in the BEVA2017, who had received an immune-modulating treatment with metronomic chemotherapy (mPE) +/– bevacizumab (mPEBev) reporting that that the use of radiotherapy given on palliative setting, was associated to a prolonged survival and that this effect was indeed correlated to a significant treatment-related increase in activated DCs and effector memory CTLs (23). Similarly, in a retrospective analysis of the KEYNOTE-001 phase I study aimed to investigate Pembrolizumab in a cohort of 495 patients advanced NSCLC patients, it has been detected a much longer PFS and OS in a group of 97 patients who had received radiation therapy prior immunotherapy (24). Finally, a perspective randomized phase III study in un-resectable lung stage III cancer patients aimed to receive chemoradiation followed by Durvalumab or placebo for 12 months (PACIFIC) reported a significant advantage in PFS in the experimental arm, which was unrelated to PDL-1 expression in the tumor (25).

In HNSCC, the immune system is known to have a pivotal role, as high density of tumor-infiltrating lymphocytes (TILs) is associated with improved outcome of patients (26, 27) while tumor tissues and draining lymph-nodes respectively, present a high density of CTLs expressing PD-1 and regulatory T_{reg}s over-expressing CTLA-4; a finding that clearly suggests a high suppressive activity of either peripheral and central immune-checkpoints in these patients (28).

Based on this solid rationale, PD-1 blockade with Nivolumab, Pembrolizumab, and Durvalumab represented a concrete option for the treatment of recurrent or metastatic HNSCC to be investigated. At the present, the results of three large trials in HNSCC patients on or after frontline platinum-based chemotherapy, concur to show an median overall response rate of 11.3–18%, with a median time to progression of 9.7 months and a 32%reduced risk of death at 1 year of (29–33). These encouraging results led to the design of a number of clinical trials which are currently ongoing with the specific aim of combining tumor irradiation with immunological agents and/or immune-check point blockade in patients with advanced HNSCC (see **Table 1**).

On these premises, a rationale use of radiation therapy may be included among the various strategies that could potentially increase the efficacy of immunotherapy at different disease settings. We believe that more successful immune-oncological trials should take in consideration this knowledge to improve their benefit NSCLC and HNSCC patients.

AUTHOR 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
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Combining Radiotherapy With Anti-angiogenic Therapy and Immunotherapy; A Therapeutic Triad for Cancer?

Ruben S. A. Goedegebuure[1†], *Leonie K. de Klerk*[1,2†], *Adam J. Bass*[2,3], *Sarah Derks*[1*†] and *Victor L. J. L. Thijssen*[1,4*†]

[1] Amsterdam UMC, Location VUmc, Medical Oncology, Cancer Center Amsterdam, Amsterdam, Netherlands, [2] Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, United States, [3] Cancer Program, The Broad Institute of MIT and Harvard, Cambridge, MA, United States, [4] Amsterdam UMC, Location VUmc, Radiation Oncology, Cancer Center Amsterdam, Amsterdam, Netherlands

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Edited by:
Patrik Andersson,
Harvard Medical School,
United States

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Andreas Pircher,
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Université de Namur, Belgium

***Correspondence:**
Sarah Derks
s.derks@vumc.nl
Victor L. J. L. Thijssen
v.thijssen@vumc.nl

[†] These authors have contributed equally to this work

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Radiotherapy has been used for the treatment of cancer for over a century. Throughout this period, the therapeutic benefit of radiotherapy has continuously progressed due to technical developments and increased insight in the biological mechanisms underlying the cellular responses to irradiation. In order to further improve radiotherapy efficacy, there is a mounting interest in combining radiotherapy with other forms of therapy such as anti-angiogenic therapy or immunotherapy. These strategies provide different opportunities and challenges, especially with regard to dose scheduling and timing. Addressing these issues requires insight in the interaction between the different treatment modalities. In the current review, we describe the basic principles of the effects of radiotherapy on tumor vascularization and tumor immunity and vice versa. We discuss the main strategies to combine these treatment modalities and the hurdles that have to be overcome in order to maximize therapeutic effectivity. Finally, we evaluate the outstanding questions and present future prospects of a therapeutic triad for cancer.

Keywords: radiation, immune response, angiogenesis, therapy, combination treatment, clinical trials, tumor microenvironment, cancer

INTRODUCTION

Radiotherapy has been an integral part of cancer treatment for over a century. More than half of all cancer patients undergo radiotherapy at some stage during treatment, either with curative intent, or in a palliative setting once the possibility for cure has been lost (1, 2). Radiotherapy was introduced shortly after the discovery of X-rays and gamma-rays in the late nineteenth century. Patients with different types of cancer were treated with radiotherapy, resulting in a paradigm shift in cancer therapy (3, 4). Since then, the clinical benefit of radiotherapy continuously improved, both by technical advancements and by increased insight in the biology behind the radiation response. For example, optimized treatment planning and more precise delivery techniques have made it possible to safely increase the tumor-targeted radiation dose while sparing the surrounding normal tissues. In addition, research into the cellular effects of ionizing radiation has provided detailed understanding of e.g., the cell cycle, apoptosis and DNA repair. This has offered insight in optimal dose-scheduling of radiotherapy (3). For example, the advantages of delivering a high dose of irradiation in multiple smaller fractions was already recognized in the 1930's (5).

Further research has resulted in the definition of "the five Rs of radiobiology" which represent five different cellular aspects that affect the efficacy of fractionated irradiation and that later have been exploited to develop combination therapies (6, 7) (**Box 1**).

Initially, radiobiology research was mainly focused on the cancer cells without appreciating the role of the tumor microenvironment. However, over the past decades it has become clear that components within the tumor microenvironment such as the tumor vascular bed and tumor infiltrating immune cells have a pivotal impact on radiotherapy efficacy (5). For instance, radiotherapy can exert opposing effects on tumor vascularization and perfusion depending on dose-scheduling (8, 9). In addition, the abscopal effect, i.e. the observation that local tumor irradiation can also lead to regression of distant tumor masses, has been linked to the immune system (10). Consequently, both anti-angiogenic therapy and immunotherapy are evaluated in combination with radiotherapy. In the current review, we describe the basic concepts of the interactions between radiotherapy and the tumor vasculature as well as between radiotherapy and the tumor immune microenvironment. In addition, we discuss how both anti-angiogenic therapy and immunotherapy can influence the efficacy of radiotherapy and how a therapeutic triad might emerge as a powerful anti-cancer treatment modality.

RADIOTHERAPY AND THE TUMOR VASCULATURE

The relation between radiotherapy and tumor vascularization has become apparent when it became clear that the effects of ionizing radiation largely depend on the generation of reactive oxygen species (ROS) (11). These highly reactive oxygen radicals can induce irreparable DNA damage that eventually leads to cancer cell death. As the generation of ROS depends on oxygen availability, well-vascularized and perfused tumor tissues are more susceptible to ionizing radiation. Thus, radiation damage is positively correlated with oxygen availability and while lack of oxygen, e.g., in hypoxic tumors, hampers treatment efficiency (11, 12). Indeed, a clinical study in patients with head and neck squamous cell carcinoma (HNSCC) comparing tumors with a median oxygen tension below and above 10 mmHg, reported disease free survival rates after radiotherapy of 22 vs. 78%, respectively (13). Furthermore, the uptake of hypoxia PET tracers has been reported to be of prognostic value for response evaluation (14). In line with this, it has been shown that tumor perfusion is a predictive factor for radiotherapy efficacy. Measuring blood flow and blood volume using either perfusion CT or the apparent diffusion coefficient with diffusion weighted MRI, has been found to predict the response to radiotherapy in patients with HNSCC (15, 16). Similar results were reported in patients with rectal cancer or cervical cancer (17, 18). These findings indicate that monitoring tumor perfusion and/or oxygenation prior to radiotherapy can be of value for setting up a proper treatment plan. This requires robust and reproducible imaging protocols as well as validated imaging biomarkers (14, 19). Modern PET/CT radiotherapy simulators already offer FDG-PET and dynamic contrast-enhanced CT imaging for a combined volumetric assessment of tumor metabolism and perfusion (14). With the current advances of MRI-guided adaptive radiotherapy, real time evaluation of tumor perfusion for predicting and monitoring treatment response might also become available. To what extent the clinical implementation of such techniques is feasible awaits further studies.

Apart from predicting treatment outcome, measuring tumor perfusion and oxygenation might also be of value to monitor the response during radiotherapy. Especially since perfusion not only affects radiotherapy, but radiotherapy also affects perfusion. The latter is related to the effects of radiotherapy on the vasculature, which are complex and appear to be dependent on the dose and scheduling of radiotherapy. Based on a literature review, Park et al. concluded that high dose irradiation, i.e., a dose above 10 Gy, induces acute vascular damage leading to deterioration of the tumor microenvironment and indirect cancer cell death (9). This was recently confirmed in a study showing that irradiation with a dose of 15–30 Gy resulted in dose-dependent secondary cell death. This was not observed after low-dose radiotherapy and most likely caused by vascular damage (20). Possibly, the vascular damage was caused by endothelial cell apoptosis, which can be induced by the upregulation of acid sphingomyelinase production in endothelial cells after high dose irradiation (21, 22).

Interestingly, fractionated low dose radiotherapy, i.e., daily fractions of up to 2 Gy, appears to exert a positive effect on the tumor vasculature and tissue perfusion (9, 23, 24) in multiple tumor models (25–27) as well as in patients (28–33). For example, an increased tumor blood volume during treatment with chemoradiation (27×1.8 Gy) was observed in cervical cancer patients (34). Using dynamic contrast-enhanced MRI and contrast-enhanced ultrasonography, we recently also observed increased tumor perfusion following two weeks of fractionated irradiation in a xenograft mouse tumor model. This was accompanied by reduced intratumoral hypoxia and increased tumor viability (35). Of note, increased tumor oxygenation during radiotherapy has been linked to different mechanisms, such as decreased oxygen consumption and vasorelaxation via increased inflammation (36). In addition, fractionated low dose irradiation can promote the growth of new blood vessels which might also contribute to enhanced perfusion, as discussed in the next section (23, 35, 37).

Collectively, there is clear evidence of a reciprocal relation between radiotherapy and the tumor vasculature in which an adequate tumor vascularization enhances radiotherapy efficacy, while irradiation induces dose-dependent effects on the vasculature (Summarized in **Figure 1A**). Exploiting this relation for combination therapies with angioregulatory strategies appears both feasible and challenging, especially with regard to dose scheduling.

COMBINING RADIOTHERAPY AND VASCULAR TARGETED THERAPY

As described previously, proper tumor oxygenation is an important predictor of radiotherapy efficacy. Therefore,

BOX 1 | The 5 Rs of radiotherapy.

The 5 Rs of radiotherapy represent a conceptual framework that form the rationale behind fractionation of radiotherapy. The 5 Rs are: Repair, Redistribution, Reoxygenation, Repopulation, and Radiosensitivity. **Repair** is the one of the primary reasons to fractionate radiotherapy. By applying fractionated radiotherapy, normal cells have the opportunity to repair sublethal DNA damage between each fraction while cancer cells are unable to sufficiently repair DNA damage due to defective or suppressed repair pathways. **Redistribution** relates to the ability of cells to progress in the cell cycle. Cells in S-phase are typically radioresistant, while cells in late G_2 and M phase are relatively sensitive. Fractionated application of irradiation increases the chance that cells that were in a radioresistant phase at one fraction have 'redistributed' to a radiosensitive phase at the following fraction. **Reoxygenation** is related to the dynamic and changing hypoxic status of tumor tissue. Fractionated radiotherapy increases the chance that all areas of the tumor tissue receive a dose of irradiation when oxygenation is improved. **Repopulation** refers to the increase in cell division that is seen in normal and cancer cells after radiation. Cells that proliferate between fractions increases the number of cells that have to be killed by radiotherapy. Consequently, repopulation is affected by the time between fractions. **Radiosensitivity** refers to the intrinsic radiosensitivity or radioresistance of different cell types. It influences the total dose that is required for a given level of damage.

modification of tumor hypoxia and perfusion in order to enhance the clinical benefit of radiotherapy has been explored using different strategies. A straightforward approach to counteract a hypoxic tumor environment involves the use of hyperbaric oxygen or of hypoxic sensitizers like nitroimidazoles. Both strategies can result in a treatment benefit, as shown in a meta-analysis with HNSCC patients (38). Unfortunately, data on other tumor types is scarce (11). Today, neither hyperbaric oxygen nor nitroimidazoles have been implemented in routine clinical practice due to the small benefit in relation to either practical difficulties or toxicity. Accelerated radiotherapy with carbogen and nicotinamide (ARCON) is a more recent development, in which radiotherapy is combined with inhalation of a hyperoxic gas and a vasoactive agent, thereby decreasing both perfusion-limited hypoxia as well as diffusion-limited hypoxia in the lungs (39). Although promising, results of clinical trials are not conclusive with respect to local tumor control (40, 41). Vasodilating agents, such as nitric oxide, calcium antagonists and hydralazine, have also been studied as an approach to improve tumor perfusion in order to enhance radiotherapy efficacy, as reviewed by Sonveaux (42). However, both variable effects on radiosensitivity as well as the mutual systemic effects preclude their clinical use. To date, the most effective method to improve tumor perfusion in a clinical setting appears to be hyperthermia. While hyperthermia can promote cell death via induction of apoptosis or mitotic catastrophy, it has also been shown to improve the efficacy of radiotherapy by inhibition of DNA damage repair pathways and enhancement of tissue perfusion and oxygenation (43–45).

A somewhat unexpected method that was discovered to improve tumor perfusion and oxygenation is anti-angiogenic therapy. Anti-angiogenic therapy refers to treatment strategies that aim to block or hamper angiogenesis, i.e., the growth of new blood vessels of pre-existing capillaries (**Box 2**). It was proposed as an effective anti-cancer therapy in the early 1970's by prof. J. Folkman after his discovery that the growth of most solid tumors is dependent on angiogenesis (47). Initially, it was anticipated that anti-angiogenic drugs would hamper the effect of radiotherapy due to decreased perfusion and oxygenation. However, multiple preclinical studies observed an enhanced effect of the combinatorial approach (48–50). These findings have been confirmed in multiple conducted clinical trials investigating the combinatorial approach. For example, in a phase I study in patients with locally advanced pancreatic cancer, the vascular endothelial growth factor (VEGF) blocker bevacizumab displayed acceptable toxicity in combination with radiotherapy and capecitabine. Interestingly, only one of the 46 patients had progressive disease and median survival from the start of the protocol was 11.6 months (51). Promising results were also reported when bevacizumab was combined with capecitabine, oxaliplatin and radiotherapy in patients with rectal cancer (52). Thus far, the results from larger and more recent clinical trials are less conclusive, reporting variable efficacy as well as increasing toxicity [extensively reviewed by us previously (53, 54)].

While the clinical observations warrant further investigation regarding therapy optimization, the potential positive interaction between radiotherapy and anti-angiogenic therapy has been attributed to several distinct mechanisms, such as vessel normalization and the vascular rebound effect. The concept of vessel normalization was coined by prof. R. Jain to explain the paradoxical observation that drugs aimed at vessel pruning could in fact enhance the effect of therapies that rely on a functional vasculature, including radiotherapy (55). Based on the premise that the tumor vasculature is abnormally structured and dysfunctional due to a continuous imbalance between pro- and anti-angiogenic signaling, it was suggested that anti-angiogenic therapy restores the angiogenic balance thereby improving vessel function and tissue perfusion (55). Normalization of the tumor vasculature would thus result in enhanced tumor oxygenation and thereby increase the efficacy of radiation therapy. Indeed, transient improvement of hypoxia and pericyte coverage was reported in different tumor models treated with either a VEGF-receptor 2 blocking antibody, or a VEGF-receptor tyrosine kinase inhibitor (56, 57). Dings et al. (58) also studied tumor oxygenation in multiple tumor models during treatment with different anti-angiogenic drugs. Treatment with either bevacizumab or the anti-angiogenic peptide anginex induced elevated oxygenation levels and increased pericyte coverage in the first 4 days (58). Moreover, the anti-tumor effect improved when radiotherapy was applied within the window of increased oxygenation (57, 58).

While the previous findings indicate that vascular normalization could improve tumor perfusion, it has also become clear that vascular normalization occurs only transiently and that continuation of anti-angiogenic treatment eventually

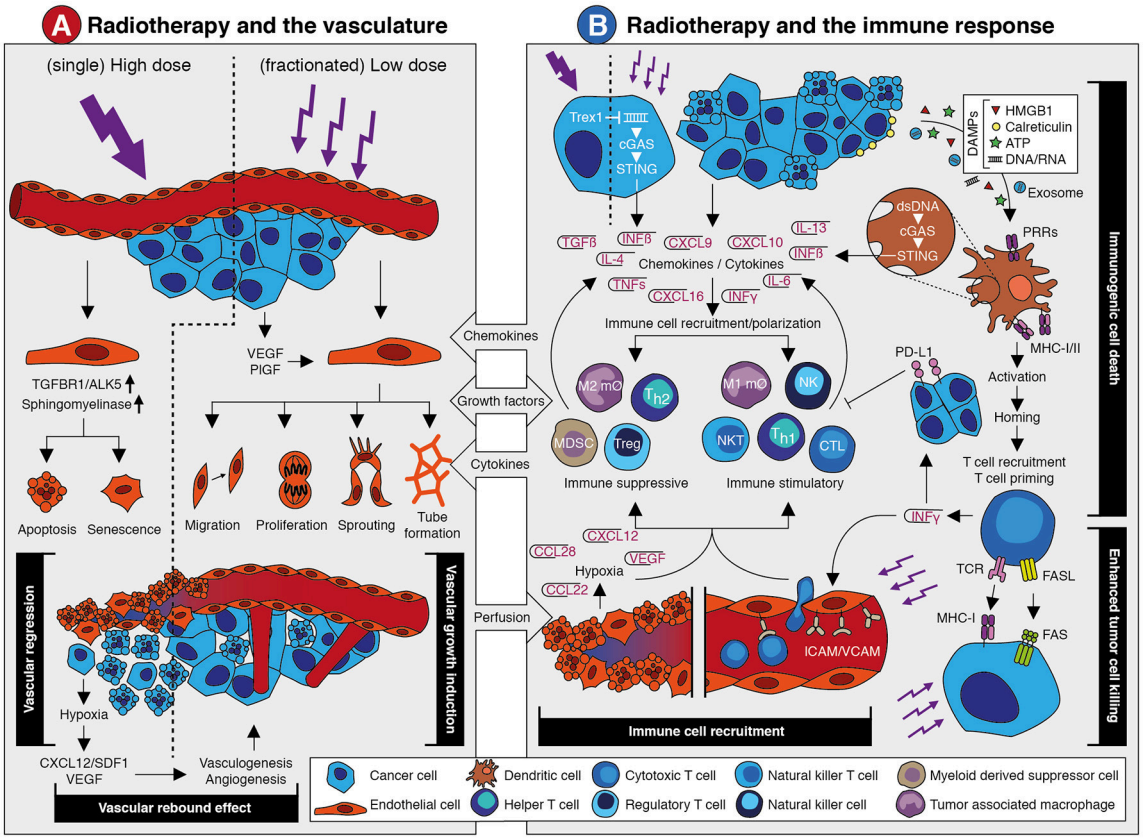

FIGURE 1 | The effects of radiotherapy on the vasculature and the immune response. **(A)** Schematic overview of the main effects that occur in the vasculature in response to radiotherapy. A detailed description is provided in the main text. In brief, single high dose irradiation induces endothelial cell apoptosis and senescence via increased ALK5 and Sphingomyelinase expression. This causes vessel regression and vascular collapse which is accompanied by reduced perfusion. This eventually results in tissue hypoxia which leads to a vascular rebound effect by growth factor-induced vasculogenesis and angiogenesis. Fractionated low dose irradiation also induces an increased expression of angiostimulatory growth factors like VEGF and bFGF. This promotes different endothelial cell functions that results in vascular growth induction and enhanced tissue perfusion. Both the vascular rebound effect and vascular growth induction provide opportunities for therapeutic intervention in combination with radiotherapy. **(B)** Schematic overview of the main effects that occur in the vasculature in response to radiotherapy. A detailed description is provided in the main text. In brief, irradiation of tumor cells can induce expression of interferon beta (IFNβ) through cytosolic dsDNA/cGAS/STING signaling. This is dependent on dosing, as high dose irradiation induces Trex1 which causes clearance of cytosolic dsDNA. Apart from IFNβ, radiotherapy induces the expression and release of several chemokines, cytokines and growth factors that promote the recruitment of immune cells. This includes both suppressive and stimulatory immune cell subsets. At the same time, irradiation promotes an immune response via the induction of immunogenic cell death. The release of damage-associated molecular patterns (DAMPs) upon radiotherapy-induced cell death causes the activation of antigen presenting cells like dendritic cells through pattern recognition receptors (PPR). This eventually results in the recruitment and priming of cytotoxic T cells. This is accompanied by the release of cytokines like interferon gamma (IFNγ) which exerts diverging effects on the immune response. At one hand, IFNγ induces PD-L1 expression on tumor cells which is immunosuppressive. At the other hand, it stimulates the expression of leukocyte adhesion molecules in the vessel wall which contributes to increased immune cell recruitment. Vessel regression induces hypoxia which increases expression of growth factors and chemokines that affect immune cell recruitment and polarization. Finally, radiotherapy induces the expression of molecules on the tumor cell surface like MHC-I and Fas, which increases tumor cell killing by immune cells. Targeting the immune suppressive mechanisms provide opportunities for therapeutic intervention in combination with radiotherapy.

causes vessel regression and reduced tumor oxygenation (57–60). This has important therapeutic consequences, especially since the data on the exact occurrence and timing of the vascular normalization window in patients is limited (61–63). Characteristic features of vessel normalization like reduction of immature vessels and increased pericyte coverage have been observed in patient treated with bevacizumab (64). Furthermore, improved perfusion has been reported in a subset of glioblastoma multiforme (GBM) patients treated with cediranib (a pan-VEGF TKI) or cediranib-containing regimens, and was associated with survival benefit (61, 65). Notwithstanding these latter observations, the temporary character of vessel normalization in mice, i.e., a few days, seems to be in contrast with the beneficial effects for patients receiving anti-angiogenic drugs during several weeks of fractionated irradiation. Moreover, anti-angiogenic therapy is not only beneficial when applied prior to radiotherapy but also when given during or after radiotherapy (54). Thus, although vessel normalization might partially explain the beneficial effects, other mechanisms might be equally relevant for the

BOX 2 | Angiogenesis.

Angiogenesis is the growth of new blood vessels out of pre-existing capillaries. It is one of the hallmarks of cancer since most solid tumor cannot grow beyond a few cubic millimeters if they are unable to induce angiogenesis. The key players in the angiogenic process are endothelial cells. These cells form the inner lining of all blood vessels. Under hypoxic conditions, cancer cells undergo the so-called "angiogenic switch" which results in an elevated expression and secretion of soluble factors like vascular endothelial growth factor (VEGF). Secreted VEGF binds to it receptors on surface of endothelial cells in a nearby capillary vessel. As a result, the endothelial cells become activated and secrete proteases that degrade the capillary basement membrane as well as the underlying extracellular matrix. Subsequently the activated endothelial cells to proliferate and migrate into the direction of the growth factor gradient, thereby forming novel vascular sprouts toward the tumor that will eventually reassemble into a capillary bed. Due to an imbalance between angiostimulatory and angioinhibitory factors, the newly formed vasculature is abnormally structured, dysfunctional and unable to adequately relief tumor hypoxia. As a consequence, the pro-angiogenic stimulus is maintained and endothelial cells lose some of their typical functional features, including the expression of adhesion molecules that regulate the extravasation of leukocyte into the tumor tissue [For an extensive review see (46)].

interaction between both radiotherapy and anti-angiogenic therapy.

Another possible mechanism that could explain the benefit of anti-angiogenic drugs involves the stimulation of angiogenesis by irradiation, referred to as the vascular rebound effect. As described previously, low dose irradiation has been found to increase tumor perfusion and oxygenation. While this was linked to mechanisms such as vasodilation by enhanced inflammation and reduced oxygen consumption (36), we and others have shown that low-dose irradiation can also influence angiogenesis by inducing the expression of pro-angiogenesis growth factors like VEGF by cancer cells or other cells that reside in the tumor microenvironment (35, 66–68). For example, Sofia-Vala et al. (23) showed that low dose irradiation induces VEGF signaling in endothelial cells. Likewise, macrophages in the stromal tissue have been shown to enhance their VEGF expression after irradiation (69). We observed induction of VEGF and PlGF after 2 weeks of fractionated irradiation (daily fractions of 2 Gy) in cultured cancer cells as well as in xenograft tumor tissues (37). The induction of VEGF coincided with increased tumor perfusion, increased tissue viability and reduced hypoxia. In addition, the levels of VEGF were sufficient to stimulate endothelial cell migration and sprouting. Importantly, the anti-angiogenic drug sunitinib, which blocks VEGF-dependent signaling, could hamper these effects (37). These findings suggest that ionizing radiation can enhance tumor perfusion by induction of a pro-angiogenic response which can be counteracted by anti-angiogenesis treatment (35). Interestingly, when exploring the optimal dose-scheduling of fractionated low-dose radiotherapy with sunitinib, a small molecule that inhibits multiple tyrosine kinase receptors including VEGFR, we observed that the beneficial effects of the combination treatment could be obtained with a lower dose of anti-angiogenic drugs than what is currently applied for cancer treatment (35, 54). A similar observation was made by Wachsberger et al. (70) using VEGFtrap, a soluble receptor that "traps" VEGF. These findings are clinically relevant since the implementation of combination therapy is currently restricted due to increased toxicity in tumor types such as rectal cancer, nasopharyngeal cancer and glioblastoma (53). Of note, high dose irradiation can also induce a vascular rebound effect due to the vascular collapse and subsequent tissue hypoxia. In addition, intermediate and high dose irradiation have been suggested to trigger vasculogenesis, i.e., the influx of endothelial progenitor cells from other parts of the body or bone marrow to build vessels (71). This process is mediated via various chemokines including CXCL12/SDF1. Interfering in this process by blocking the CXCL12/SDF1 receptor (CXCR4) could be of interest in relation to radiotherapy (72). Furthermore, recent research on the role of endothelial cell metabolism in cancer have led to new insights and potential targets for anti-angiogenesis therapy. For example, inhibition of PFKFB3, which is a regulator of glycolysis, can promote vessel normalization, albeit that this effect is dose-dependent (73). Whether and to what extend such inhibitors synergize with radiotherapy awaits further investigation.

Collectively, the findings described above point toward the importance of proper dose-scheduling of both treatment modalities to achieve optimal beneficial effects. On one side, the dose-scheduling of anti-angiogenic drugs influences whether and when vessel normalization occurs and whether and when the angiogenic rebound effect is countered. On the other side, the dose-scheduling of radiotherapy influences whether and when tumor perfusion is affected and whether and when an angiogenic (rebound) effect occurs. This complex relation illustrates the challenges that accompany the combination of radiotherapy with anti-angiogenic therapy. It also explains that, while a plethora of pre-clinical evidence suggests a treatment benefit for the combination of radiotherapy with anti-angiogenic therapy, the clinical practice is less conclusive. The radiotherapy efficacy might be strengthened by a pro-angiogenic response, enhancing both tumor perfusion and oxygenation but this could at the same time induce unwanted tumor growth. Thus, optimal dose-scheduling of both treatment modalities is key to achieve beneficial effects and limit toxicity of the combination therapy.

RADIOTHERAPY AND THE IMMUNE SYSTEM

The link between radiotherapy and the immune system was recognized already several decades before the role of the tumor vasculature was uncovered. The first clear observation that the host immune system contributes to radiotherapy efficacy was presented in the late seventies of the previous century. In a preclinical study it was shown that the effect of radiotherapy is compromised in immunodeficient and CD8+ T cell depleted mice (74). Prior to this, radiotherapy was more or

less considered to be immunosuppressive (75, 76). Additional evidence for a role of the immune system during radiotherapy was obtained from preclinical research and multiple case studies that reported on regression of (metastatic) tumor masses that were distant from the irradiated site (77–79). This so-called abscopal effect (**Box 3**) was already described in 1953, but it took about 50 years to link this to a systemic anti-tumor immune response initiated by radiotherapy (80, 81). Still, the exact mechanisms behind the abscopal effect are not entirely elucidated. Nevertheless, the clear link between radiotherapy and the immune response, together with the breakthrough of immunotherapy in recent years, has renewed the interest in combining radiotherapy and immunotherapy. Similar as for anti-angiogenic therapy, preclinical and clinical studies using this combination therapy have made it clear that successful implementation of radiotherapy combined with immunotherapy relies on a proper understanding of the interaction between both treatment modalities. In recent years, several mechanisms have been proposed that explain how radiotherapy affects the tumor immune response (82, 83) (Illustrated in **Figure 1B**).

A well-recognized mechanism by which radiotherapy can enhance the anti-tumor immune response is the induction of immunogenic cell death. Unlike normal cell death, immunogenic cell death makes cancer cells visible to the immune system by the release of damage-associated molecular patterns (DAMPs), such as calreticulin, HMGB1 and ATP, along with the presentation of neoantigens and tumor associated antigens (84–91). DAMPs bind to pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) on antigen presenting cells, including dendritic cells (DCs). This leads to DC activation which subsequently cross-present antigens and migrate to the tumor-draining lymph node (92, 93), where they prime naive T cells and B cells to initiate a systemic immune response (92–99). Recent studies have identified the STING pathway, activated upon recognition of double-stranded DNA (dsDNA) via cytosolic DNA sensors, as an important regulator of this immunogenic cell death response (100–105). Double-stranded DNA can be transferred via exosomes from irradiated cancer cells to DCs. Subsequently, STING-dependent activation of type-I interferons and upregulation of co-stimulatory molecules is triggered (106). Collectively, these findings show that radiotherapy can promote an anti-tumor immune response via immunogenic cell death-mediated activation of antigen presenting cells like DCs leading to increased priming of tumor antigen-specific T cells.

Apart from enhanced T cell priming through immunogenic cell death, radiotherapy can also promote the trafficking of immune cells into the tumor. In fact, multiple mechanisms contribute to this enhanced immune infiltration. Firstly, radiotherapy can improve tumor perfusion (as described above) which will increase the number of leukocytes passing through the tumor tissue. Secondly, irradiation induces the endothelial expression of leukocyte adhesion molecules like ICAM and VCAM (93, 107–109). Consequently, leukocyte extravasation from the circulation into the tumor tissue will be increased. Thirdly, radiotherapy has been shown to increase the expression of pro-inflammatory chemokines such as CXCL9, CXCL10, and CXCL16 by cancer cells. This will help to attract leukocyte populations like cytotoxic CD8+ T cells, Th1 cells, NK cells, and NKT cells (108, 110, 111). Finally, radiation can induce MHC-I expression on cancer cells, either by an accumulation of damaged proteins and their break-down products (89, 97, 112), or in response to a general increase of IFN gamma (IFNγ) within the tumor microenvironment (108). Preclinical studies have also shown that radiotherapy enhances the expression of the death receptor Fas (CD95) on cancer cells, making them more susceptible to Fas ligand mediated cell death (97, 113–116). Altogether, enhanced tumor perfusion, increased leukocyte chemoattraction and extravasation, as well as increased susceptibility to T cell-mediated cell death contribute to an improved immune response during radiotherapy.

Unfortunately, there are some ifs and buts to the immunostimulatory effect of radiotherapy. Similar as with the angioregulatory response, the immunoregulatory response to irradiation appears to be dose and schedule dependent. For example, the induction of MHC-I (97, 112) and immunogenic cell death (89) depend on the dose, and in preclinical models moderate to high doses of radiotherapy seem to have most effect (92, 117, 118). For instance, Filatenkov et al. showed in weakly immunogenic CT26 and MC38 colon tumors that only a single dose of 30 Gy increased intratumoral CD8+ T cells, whereas 10×3 Gy did not (118). On the other hand, radiotherapy doses of $\geq$12 Gy have been shown to attenuate radiotherapy-induced tumor immunogenicity through the induction of DNA exonuclease TREX1 (Three prime repair exonuclease 1), which degrades cytosolic dsDNA, thereby preventing cGAS/STING mediated induction of interferon beta (IFNβ) (119). With regard to the abscopal effect, only a few comparative studies are available, but a systematic review of 46 case reports revealed a broad range in cumulative dose at which the effect was observed (range 0.45–60.75 Gy; median 31 Gy) (77). With regard to scheduling there is also no clear answer yet. It has been reported that a single fraction is better than multiple fractions (93), that there is no difference between single or multiple fractions (92), or that multiple fractions are better (120, 121). From a tumor perfusion perspective there is evidence that fractionated low dose is preferred over single high dose as described previously. At the same time, the induction of leukocyte adhesion molecule expression appears to be dose-dependent (109, 122, 123). So, a major future challenge will be to unravel at what dose-scheduling regime an optimal immunostimulatory effect of radiotherapy will occur.

Most likely, the overall effect of radiotherapy on the immune response is not only dose-scheduling dependent but is also determined by tumor type and the tumor microenvironment. Regarding the latter, it has been shown that the efficacy of radiotherapy is influenced by the composition of the pretreatment tumor immune microenvironment (124). Thus, it would be of interest to explore to what extent the pre-treatment immunogenic profile in the tumor tissue can predict the response to radiotherapy. This is also relevant given the observation that radiotherapy can induce an immunosuppressive microenvironment. After all, apart from the induction of pro-inflammatory chemokines, as described above, radiotherapy can also induce chemokines and cytokines

BOX 3 | The abscopal effect.

The concept and term "abscopal" was proposed in 1953 by dr. R.H. Mole to describe effects of irradiation that occur distant from the site of irradiation, but within the same organism (78). The term originates from the prefix *ab-* (away from) and Latin word *scopus* (mark or target). As such, it can be considered as a systemic response following a local trigger. Today, the abscopal effect has been reported in a wide variety of both solid and hematologic tumor types. While the mechanism is still not fully elucidated, it has been established the abscopal effect involves the immune system [For an extensive review see (80)].

that attract immunosuppressive cell populations such as Tregs (97), myeloid derived suppressor cells (MDSCs) (125), M2 macrophages, and Th2-skewed CD4+ T cells (126) to the tumor immune microenvironment (127). Multiple *in vitro* studies demonstrated that unpolarized macrophages tend to acquire a M1 phenotype after irradiation with 2–5 Gy. Interestingly, Klug et al. (128) showed in an *in vivo* model reprogramming of TAMs to a M1 phenotype after irradiation with 2 Gy. Different dose-effects of radiotherapy on TAMs, as well as mechanisms involved, has been described in detail by Genard et al. (129). Blockade of the macrophage chemoattractant CSF-1 and repolarization of macrophages into a M1 tumor suppressive phenotype by blocking interleukin-4 (IL-4) and IL-13 significantly improved responses to radiotherapy in a mouse breast cancer model (126, 130). In addition, IFN gamma expression within the tumor immune microenvironment is an important driver of PD-L1 expression on tumor and immune cell which leads to impairment of T cell function (131–133). In fact, it were these kind of observations that led to the hypothesis that the combination of immunotherapy with radiotherapy might have clinical benefit.

ENHANCEMENT OF IMMUNOTHERAPY EFFICACY BY RADIOTHERAPY

One of the major breakthroughs in oncology in recent years has been the development of drugs that enhance the potency of the immune system. These drugs are predominantly inhibitors of so-called immune checkpoint proteins (**Box 4**) and they are able to re-activate T cells to attack cancer cells. Although we are only starting to understand the effect of such immune checkpoint inhibitors, it has become clear that these drugs are most effective when the T cells that they activate are already in the tumor microenvironment (134–136). However, many tumors lack a proper lymphocyte infiltration. As described above, radiotherapy can elicit an anti-tumor T cell response, which has spurred the interest to apply radiotherapy in order to augment the local and systemic effect of immunotherapy. Evidence that radiotherapy can reliably and consistently achieve this effect in cancer patients is currently not available but multiple retrospective studies have shown that radiotherapy can increase the response to immunotherapy. Several studies [for overview see (137)] in predominantly melanoma and lung cancer patients have shown that radiotherapy given during the course of immunotherapy increases the median overall survival compared to no radiotherapy (138, 139). Also in lung cancer it has been shown that radiotherapy somewhere in the course of the disease prior to the first cycle of PD-1 inhibitor pembrolizumab significantly increased overall and progression free survival (139). In metastatic non-small cell lung cancer (NSCLC) preliminary results of an ongoing trial (NCT02492568) with pembrolizumab preceded by stereotactic body radiation therapy showed a doubling of the overall response rate (140). However, other studies in melanoma and various solid tumors evaluating the combination of radiotherapy with ipilimumab (98) or pembrolizumab (141) showed disappointing results. The same holds true for a large phase III trial testing radiotherapy followed by ipilimumab or placebo in castration-resistant prostate cancer patients (142).

Interestingly, there is also a variety of case reports describing major systemic antitumor effects of palliative radiotherapy in patients that had progressed on immunotherapy. For instance, Postow et al. (94) showed, in a case report of a metastatic melanoma patient that had progressed under ipilimumab, re-induction of an anti-tumor immune response after palliative radiotherapy. This response was accompanied by the expansion of existing, and appearance of new anti-tumor antibodies (94). Another retrospective analysis of 21 patients with advanced melanoma who received radiotherapy after progression on ipilimumab showed partial systemic response and stable disease in 43% and 10% of cases, respectively (143). A beneficial effect of radiotherapy following progression on checkpoint inhibition has also been reported for a patient with NSCLC (144) and HNSCC (145). Another study of patients with stage IV melanoma treated with ipilimumab followed by palliative radiotherapy within the first 5 days of treatment showed that around 50% of patients experienced clinical benefit (146). Nevertheless, most clinical success of combined radiotherapy with immunotherapy has been shown in the adjuvant use of PD-1 pathway inhibitors. The largest study among those is the PACIFIC study, a multicenter randomized controlled trial comparing the use of PD-L1 inhibitor durvalumab as consolidation therapy following definitive chemoradiation in stage III NSCLC which showed a median progression free survival of 16.8 months compared to 5.6 months with placebo and an acceptable toxicity profile, resulting in prompt FDA approval of the adjuvant use of durvalumab for stage III NSCLC patients (147). Importantly, the combination of radiotherapy and immunotherapy appears to be safe and well tolerated without severe toxicities (138, 146–150). Altogether, these studies suggest a bright future for combined radiotherapy and immunotherapy for certain patients. Of note, the high expectations might be somewhat hampered by clinical studies that explored the concurrent use of immunotherapy and radiotherapy to stimulate an anti-tumor immune response by both modalities at the same time. Although the results of such studies are still in early phase, a recent phase I trial in patients with metastatic or locally advanced bladder cancer was paused early due to intolerable in-field toxicities (151). Trials

BOX 4 | Immune checkpoint proteins.

Immune checkpoints programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) are negative regulators of T cell responses and act as a brake on the immune system. Although CTLA-4 and PD-1 have similar negative effects on T cells activity, the immune checkpoints operate on different stages of an immune response. CTLA-4 expression is confined to T cells and functions mostly during the priming phase of T cell activation in lymph nodes. The PD-1 checkpoint is predominantly at play during the effector phase within peripheral tissues, where it interacts with its ligand PD-L1 which is broadly expressed on both tumor and immune cells. Despite these differences, inhibitors of both PD-1/PD-L1 and CTLA-4 are able to (re-)activate T cells to attack cancer cells and have shown unprecedented durable responses in many cancer types.

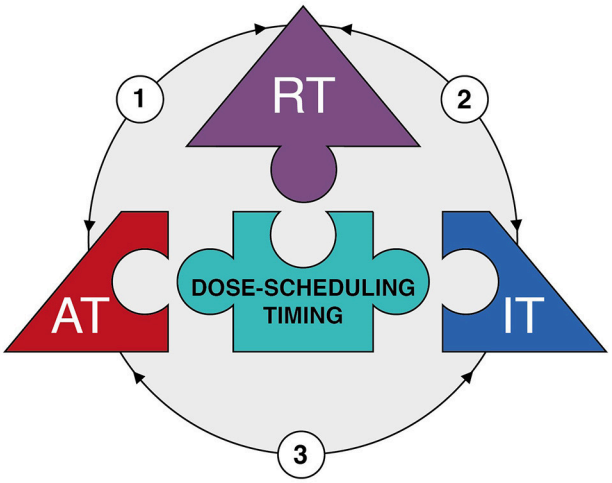

FIGURE 2 | The therapeutic triad. Diagram depicting the main components of the 'therapeutic triad' as pieces of a jigsaw puzzle, i.e. radiotherapy (RT), anti-angiogenic therapy (AT), and immunotherapy (IT). Optimization of dose-scheduling and timing of the three treatment modalities is the center piece of the puzzle, for it is essential to achieve effective combination therapy with minimal toxicities. The arrows reflect the interactions between the different treatment modalities (see main text for more detailed information). In brief: (1) Radiotherapy has dose-dependent effects on tumor vessels resulting a vascular rebound effect due to either vascular collapse or direct induction of angiogenesis. This provides an opportunity for anti-angiogenic therapy. Anti-angiogenic therapy itself induces vessel normalization which improves tumor perfusion and oxygenation; this in turn enhances the efficacy of radiotherapy. (2) Radiotherapy induces immunogenic cell death which enhances specific T cell priming. In addition, radiotherapy can induce the expression of adhesion molecules on endothelial cell and chemokines by cancer cells which both improve the extravasation of immune cells into the tumor tissue. This enhances the efficacy of immunotherapy. In addition, the tumor immune microenvironment itself affects the response to radiotherapy. (3) Anti-angiogenic therapy induces vessel normalization which improves extravasation of immune cells into the tumor tissue. Likewise, immunotherapy might result in recruitment of immune subsets with angioregulatory activity which can be targeted by anti-angiogenic therapy.

to test the safety and feasibility of neoadjuvant immunotherapy with radiotherapy in NSCLC, HNSCC, and gastroesophageal cancer (NCT03245177, NCT03383094, and NCT03044613, respectively) amongst others are currently ongoing. Apparently, and in line with the observations of anti-angiogenic therapy combined with radiotherapy, the timing, dosing and scheduling of both treatments is key in achieving optimal therapeutic effects.

ALTERNATIVE COMBINED RADIOTHERAPY-IMMUNOTHERAPY APPROACHES

While currently most (pre)clinical research is mainly focused on the combination of radiotherapy with immune checkpoint inhibitors, several alternative immunomodulatory approaches are also being explored. For example, the combination of radiotherapy with immunostimulatory factors such as interleukin-2 (IL-2) (152, 153), granulocyte-macrophage colony-stimulation-factor (GM-CSF) (154), and agonists of the T cell co-stimulatory receptor OX40 (155, 156) has yielded promising responses in early phase clinical trials. Also strategies to trigger an anti-tumor immune response by intratumoral injection of TLR9 agonists in combination with concurrent low-dose radiotherapy on the injection site has shown promising results and excellent safety and tolerability in different tumor types, including low-grade B cell lymphomas (157), cutaneous T cell lymphoma (158) and follicular lymphoma (159). A TLR3 agonist in combination with concurrent fractionated radiotherapy was recently tested in a single arm phase II trial in 30 patients with newly diagnosed glioblastoma multiforme and was found to be well tolerated (160). Others have performed studies in which radiotherapy was combined with intratumoral injections of autologous immature DCs after radiotherapy in hepatocellular carcinoma (161) and soft tissue sarcoma (162). This treatment was also well tolerated and based on the observed responses, future phase II and III studies were recommended. Finally, efforts have been made to combine radiotherapy with vaccination against carcinoembryonic antigen (CEA) combined with GM-CSF in colorectal cancer (163), or against prostate specific antigen (PSA) combined with GM-CSF and IL-2 in patients with prostate cancer (164, 165). Despite the clear rationale behind these trials, both studies showed limited effectivity (163–165). On the other hand, a phase I clinical trial in chemo-naïve esophageal squamous cell carcinoma did show vaccine-specific cellular and clinical responses (CT evaluation) after treatment with a peptide vaccine containing five tumor-associated peptides (TTK, URLC10, KOC1, VEGFR1, and VEGFR2) in combination with chemoradiation (60 Gy, cisplatin, 5-FU) (166). All these studies exemplify the current interest and feasibility to combine radiotherapy with immunostimulatory treatments. Still, many questions have to be answered and challenges have to be met, especially with regard to dosing, scheduling and timing of both treatments. Nevertheless, the outlook for radiotherapy in combination with immunotherapy appears promising.

FUTURE PERSPECTIVES – A THERAPEUTIC TRIAD

Based on aforementioned interactions and synergy, a trimodal approach combining radiotherapy with anti-angiogenic therapy and immunotherapy is a promising therapeutic strategy. To our best knowledge, no clinical trials have been published combining all three treatment modalities. Radiotherapy with either anti-angiogenic therapy or immunotherapy appears feasible, but presents both researchers and clinicians with many challenges.

While this review focused on the interaction of radiotherapy with either anti-angiogenic therapy or immunotherapy, there is growing awareness that the latter two treatments are also intrinsically interwoven. Indeed, the combination of immunotherapy and anti-angiogenic therapy has recently emerged as a novel therapeutic strategy (167). This is based on the observation that anti-angiogenic therapy can enhance immune effector cell trafficking to the tumor site. This would strengthen the efficacy of immunotherapy since low immune cell infiltration still represents a major obstacle for cancer immunotherapy (168). A recent review on this subject by Fukumura et al. (169) provides an up-do-date table of pre-clinical and clinical trials. The improved recruitment of immune cells during anti-angiogenic therapy is partly explained by vessel normalization. In the tumor endothelium, the expression of adhesion molecules that facilitate rolling, adhesion and extravasation of immune cells is reduced due to exposure of endothelial cells to tumor-derived angiogenic growth factors (170–172). This phenomenon is referred to as endothelial cell anergy and it makes the underlying tumor tissue invisible or at least less reachable to the immune system (173). In addition, hypoxia due to impaired perfusion results in the expression of several chemokines such as stromal cell–derived factor 1 (SDF1-α), CC-chemokine ligand 22 (CCL22) and CCL28. These chemokines initiate a state of tolerance by recruiting Tregs, MDSCs and M2-type TAMs to induce an immunosuppressive microenvironment (174, 175). Furthermore, hypoxia as well as VEGF can induce the expression of immune checkpoint molecules on cancer cells and immune cells (176, 177). Collectively, the hypoxic and pro-angiogenic tumor microenvironment are generally immunosuppressive. Thus, strategies that normalize the dysfunctional vasculature can not only restore immune cell functions and facilitate their antitumor activities, but also enhance immunotherapy effects (8). As already described, anti-angiogenic therapy can induce vascular normalization and reduce hypoxia. In line with this, anti-angiogenic drugs have been shown to facilitate tumor infiltration of CD8+ T lymphocytes and potentiate cancer immunotherapy (178–181). This effect could thus add up to the previously described induction of adhesion molecule expression in endothelial cells by radiotherapy itself. While anti-angiogenic therapy can influence the immune system, evidence is emerging that immunotherapy also affects the tumor vasculature. Interferon gamma is suggested to play an important role in this process, as it is produced by activated T cells and, upregulates ICAM-1 and induces T cell migration. Interestingly, Th1 cell infiltration is reported to reciprocally promote blood vessel normalization which would further contribute to an immunostimulatory microenvironment, in a process that is also dependent on IFNγ signaling. For example, in mice treated with anti PD-1 antibodies, Th1-mediated vessel normalization was improved (182). Thus, a mutual regulatory feedback loop is identified in which vessel normalization and T lymphocyte infiltration can amplify the positive effects conferred by each individual effect. Possibly, this combinatorial approach could lead to a more pronounced vessel normalization window which could be exploited to enhance the effect of radiotherapy. In this context it is noteworthy to mention that is has been shown in melanoma models that the improved immune response following STING activation actually depends on the production of IFNβ by endothelial cells (183). While this effect was observed after STING activation by intratumoral injection of cyclic dinucleotide GMP-AMP (cGAMP) and not by irradiation, it further indicates that targeting endothelial cells to improve immunotherapy could be of interest during radiotherapy. Thus, combining the three treatment modalities as a "therapeutic triad" offers an innovative and interesting approach to cancer treatment (**Figure 2**), but will even present with additional challenges regarding optimal dose-scheduling, timing and overcoming potential toxicities as compared to the combination of two treatments.

CONCLUDING REMARKS

Although combining radiotherapy with either anti-angiogenic therapy or immunotherapy has been extensively studied the last decade, phase III studies showing a clear benefit of combinatorial approaches are scarce. This not only illustrates the complex relationship between the cancer cells and the tumor microenvironment, but it also emphasizes that many challenges have to be overcome to make these combination therapies effective. In particular, future studies should shed light upon the optimal timing and dosing of the different treatments. In addition, finding predictive and prognostic biomarkers could help determine which cancer types and disease stages are particularly suitable for combinatorial approaches. Interestingly, radiotherapy, anti-angiogenic therapy and immunotherapy all exert effects on both the tumor vasculature and the anti-tumor immune response. Better understanding of their reciprocal interactions in the tumor microenvironment is the main future challenge to allow the development of a therapeutic triad that combines the three treatment modalities for effective cancer therapy.

AUTHOR CONTRIBUTIONS

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

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PRMT5 Associates With the FOXP3 Homomer and When Disabled Enhances Targeted p185$^{erbB2/neu}$ Tumor Immunotherapy

*Yasuhiro Nagai[1], Mei Q. Ji[1], Fuxiang Zhu[2], Yan Xiao[1], Yukinori Tanaka[1], Taku Kambayashi[1], Shigeyoshi Fujimoto[3], Michael M. Goldberg[4], Hongtao Zhang[1], Bin Li[5], Takuya Ohtani[6] and Mark I. Greene[1]**

[1] Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States, [2] Unit of Molecular Immunology, Key Laboratory of Molecular Virology & Immunology, CAS Center for Excellence in Molecular Cell Science, Institute Pasteur of Shanghai, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China, [3] Adviser of Seishin Medical Group, Takara Clinic, Tokyo, Japan, [4] Macrophage Therapeutics, Englewood Cliffs, NJ, United States, [5] The Department of Immunology and Microbiology & Shanghai, Institute of Immunology, Shanghai JiaoTong University School of Medicine, Shanghai, China, [6] Penn Institute for Immunology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States

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Martin Luther University of
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***Correspondence:**
Mark I. Greene
greene@reo.med.upenn.edu

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Regulatory T cells (Tregs) are a subpopulation of T cells that are specialized in suppressing immune responses. Here we show that the arginine methyl transferase protein PRMT5 can complex with FOXP3 transcription factors in Tregs. Mice with conditional knock out (cKO) of PRMT5 expression in Tregs develop severe scurfy-like autoimmunity. In these PRMT5 cKO mice, the spleen has reduced numbers of Tregs, but normal numbers of Tregs are found in the peripheral lymph nodes. These peripheral Tregs that lack PRMT5, however, display a limited suppressive function. Mass spectrometric analysis showed that FOXP3 can be di-methylated at positions R27, R51, and R146. A point mutation of Arginine (R) 51 to Lysine (K) led to defective suppressive functions in human CD4 T cells. Pharmacological inhibition of PRMT5 by DS-437 also reduced human Treg functions and inhibited the methylation of FOXP3. In addition, DS-437 significantly enhanced the anti-tumor effects of anti-erbB2/neu monoclonal antibody targeted therapy in Balb/c mice bearing CT26Her2 tumors by inhibiting Treg function and induction of tumor immunity. Controlling PRMT5 activity is a promising strategy for cancer therapy in situations where host immunity against tumors is attenuated in a FOXP3 dependent manner.

Keywords: PRMT5, FOXP3 regulatory T cells, autoimmunity, scurfy, tumor immunity, breast cancer

INTRODUCTION

Regulatory T cells (Tregs) limit autoimmune processes directed at self-antigens. While the Treg's immunosuppressive functions are beneficial to limit autoimmune processes, studies examining certain syngeneic tumors in animals have confirmed a detrimental role for Tregs in cancer (1). In human tumors, infiltration of tumor sites with Foxp3 Treg cells can be associated with a less favorable prognosis (2, 3). Thus, targeting Tregs in tumor bearing hosts represents a strategy for tumor immunotherapy (4–6) although studies in some human tumor tissues have suggested FOXP3 effects only as a contributory, but not a deterministic role, on tumor malignant growth (7).

FOXP3 is a dimeric and/or tetrameric transcription factor in Tregs and fulfills an important role in both Treg development and function (8, 9). FOXP3 forms an ensemble with several proteins, many of which have been shown to play roles in enhancing or modifying FOXP3 activities. These include several epigenetic modulators for FOXP3, such as the histone acetyltransferases Tip60 (KAT5) and p300 (KAT3b) that acetylate FOXP3 at discrete lysines and enhance Treg function (10–12) through stabilization and limiting degradation caused by FOXP3 ubiquitination (13). In contrast, histone deacetylases (HDAC7) can remove the acetyl groups, and this process promotes degradation of FOXP3. Phosphorylation of FOXP3 at serine 418 is also important for Treg function (14). Phosphorylation of FOXP3 by the Pim1 and Pim2 serine/threonine kinases diminishes FOXP3 functions (15, 16). Thus, post-translational modifications (PTM) of FOXP3 are important for modifying Treg functions and stabilities. In an effort to characterize other PTM proteins of FOXP3, we have identified Protein Arginine Methyl Transferase 5 (PRMT5) as a novel PTM protein that modifies FOXP3 functions by arginine methylation.

MATERIALS AND METHODS

Plasmid and Antibodies

All human PRMT cDNAs were purchased from OpenBiosystem and GE Dharmacon, and subcloned into the mammalian expression vectors *pIRESpuromycin-HA2* with two HA eptitope tags or *pIRESpuromycin-FLAG2* with two FLAG eptitope tags, and HA2-or FLAG2-Foxp3 vector was generated as previously described (10). PRMT5 shRNA vector was obtained from TRC shRNA vector library (GE Dharmacon). The sequence is below: TATTCCAGGGAGTTCTTGAGG (shPRMT5 85); ATAAGGCATCTCAAACTGGGC (shPRMT5 86). For the point mutation of Foxp3, Quick change II site-directed mutagenesis kit (Agilent) was used per manufacturer's instructions.

Mice

To generate the PRMT5$^{fl/fl}$ mouse, PRMT5 conditionally targeted ES cells were obtained from the International Mouse Phenotyping Consortium (Prmt5$^{tm2a(EUCOMM)Wtsi}$). In the targeted cells, Exon 6, which encodes the catalytic domain, is sandwiched by two loxp sites, and lacZ reporter and Neomycin genes are inserted upstream together with two FRT sequences. We injected the ES cells into C57BL/6 blastocysts and obtained chimeric animals. The founder animals were mated with flippase transgenic mice (B6.Cg-Tg (ACTFLPe)$^{9250Dym/J}$, 005703, Jackson Lab) to delete lacZ and Neomycin genes. Foxp3^{Creyfp} (B6.129(Cg)-Foxp3$^{tm4(YFP/Cre)Ayr}$/J, 016959) and CD4^{cre} (Tg(Cd4-cre)1Cwi/BfluJ, 017336) mice were obtained from Jackson Laboratory. All animals were housed and bred in a specific pathogen-free animal facility of the University of Pennsylvania. All the experiments were performed following national, state, and institutional guidelines. Animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Cell Culture and Transfection

293T cells were grown in DMEM supplemented with 10% heat inactivated fetal bovine serum and antibiotics (1% penicillin/streptomycin; Invitrogen) at 37°C in a humidified incubator with 5% CO2 (v/v). Cells were grown to 80% confluency in 6-well plates, and transient transfection was carried out using a mixture of 6 μg DNA and 18 μl FuGENE 6 (Roche) according to manufacturer's instructions. Twenty-four hours after transfection, the cells were lysed with high salt lysis buffer [20 mM Tris-Cl pH 7.5, 420 mM NaCl, 1% TritonX-100, and complete mini protease inhibitor cocktail (Roche)], then prepared for western blot analysis. For the PRMT5 inhibitor treatments cells were transfected with HA-Foxp3 vector and cultured for 24 h. Then inhibitors were added to the cells with indicated concentrations of CMP5 (IC50: unavailable, Millipore), DS-437 (IC50: 5.9 μM, Sigma), HLCL-61 (IC50: 7.21-21.46 μM for acute myeloid leukemia cell line), EPZ004777 [IC50: 50 μM for PRMT5 (17)], and EPZ015666 (IC50: 20 nM, Selleckchem) and incubated for 16 h. For T cell culture, RPMI-1640 medium supplemented with 10% FBS, 1X non-essential amino acids (Invitrogen), 2 mM sodium pyruvate (Invitrogen) and 50 μM β-mercaptoethanol (Sigma) was used.

Mass Spectrometry

293T cells were transfected with FLAG-Foxp3 or empty vectors, lysed with high salt lysis buffer, and then immunoprecipitated with anti-FLAG agarose beads (Sigma) overnight at 4°C. The precipitates were then washed three times with lysis buffer and boiled for 5 min in SDS loading buffer. Samples were analyzed by SDS-PAGE and specific bands were cut and subjected to mass spectrometry by the University of Pennsylvania Proteomics and System Biology Core. For the methylation analysis, 293T cells were transfected with HA-Foxp3 vector and immune precipitated with anti-HA magnetic beads (Thermo FIsher). Proteins were eluted with elution buffer (Thermo Fisher) and concentrated by vivaspin 500 (GE Healthcare). Samples were analyzed by SDS-PAGE and subjected for mass spectrumtry by the CHOP Proteome Core at the University of Pennsylvania.

Immunoprecipitation and Western Blotting

Cells were lysed in lysis buffer and the soluble fractions were collected and incubated with anti-HA angarose, anti-FLAG agarose (Sigma-Aldrich), or anti-symmetric dimethyl arginine antibody Sym10 (Upstate) conjugated with Dynabeads protein G magnetic beads (Invitrogen) for 2 h at 4°C. The precipitates were then washed three times with lysis buffer and boiled for 5 min in SDS loading buffer. Samples were analyzed by SDS-PAGE, transferred to Immobilon-P (Millipore) PVDF membrane, and probed with anti-Flag M2-Peroxidase (Sigma), or anti-HA Peroxidase (3F10; Roche). For the detection of tag proteins, immunocomplexes were detected using Immobilon Western Chemiluminescent horseradish peroxidase (HRP) Substrate (Millipore). For human Tregs, expanded cells were harvested and lysed on ice for 1 h with RIPA buffer (50 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% NonidetP-40, 0.25% NaDOC, 1 mM $MgCl_2$, and 10% (vol/vol) glycerol) containing protease inhibitor (1:100;

P8340; Sigma-Aldrich), NaF (10 mM), and PMSF (1 mM). Cell lysates were cleared by centrifugation, and the supernatants were incubated with anti-FOXP3 (1 μg, eBio7979), anti-PRMT5 (1 μg, Millipore O14744), or IgG (1 μg, 5415S; Cell Signaling) at 4°C overnight, and then immunoprecipated with Protein G-Sepharose beads (P3296; Sigma) for 1 h at 4°C. The immunocomplexes then were washed with RIPA buffer and examined by Western blotting.

Flow Cytometry

Spleen, axillary and inguinal lymph nodes, and thymus of 18–21 day-old male mice were collected, and single-cell suspensions were made. The cells were stained with L/D aqua (Thermo Fisher Scientific) per manufacturer's instruction for eliminating dead cells. Then their membranes were stained with anti-CD4-percp (100432, Biolegend), CD8-APC/R700 (564983, BD), TCRβ-BV605 (109241, Biolegend), CD25-PE (553866, BD Bioscience), GITR-PE (120208, Biolegend), CD134-PE/Cy7 (119415, Biolegend), CD304-PE-Cy7 (145211, Biolegend), and/or ICOS-PE/Cy7 (313519, Biolegend), CD44-APC-Fire750 (103062, Biolegend), and/or CD62L-PE-Cy7 (104418, Biolegend). After 20 min of incubation on ice, the cells were then fixed with a Foxp3 staining buffer set (eBioscience) and intracellularly stained with CTLA4-BV421 (106312, Biolegend), KI67-BV421 (652411, Biolegend), Foxp3-APC (17-5773-82, eBioscience) and/or Helios-PE (137206, Biolegend), and subjected to flow cytometry using the fluorescence-activated cell sorter (FACS) LSR (BD Biosciences). FACS data were analyzed with FlowJo software (Tree Star). For separating $YFP^{+/-}$ cells, the cells were fixed with 1% paraformaldehyde in PBS for 5 min on ice after membrane staining, then re-fixed using the Foxp3 staining kit, followed by intracellular staining as described above.

For the cytokine expression studies, the spleen cells were harvested and stimulated with PMA (50 ng/ml) and ionomycin (10 μM) and anti-CD3 (0.5 μg/ml) for 6 h with Protein Transport Inhibitor Cocktail (× 500, eBioscience). The cells were then membrane stained with L/D aqua followed by anti-CD4-percpCy5.5 (100434, Biolegend) CD8-AF488 (100723, Biolegend) and fixed with 1% paraformaldehyde in PBS for 10 min on ice and then re-fixed using the Foxp3 staining kit, followed by intracellular staining as described above. After fixation, the cell were stained with anti-IL-4-BV711 (504133, Biolegend), IL-17a-BV421 (506926, Biolegend), IFNγ-PE-cy7 (505826, Biolegend), and Foxp3-APC as described above and analyzed with FACS LSR.

For TILs, tumors were dissected and minced in digestion buffer (0.025 mg/ml liberase TM, 0.05 mg/ml DNAse I (Roche) in RPMI) and then incubated at 37°C with rotating for 30 min. Then cells were filtered through a cell strainer (Falcon). Cells were then stained with anti-CD4-BV785 (100453, Biolegend), CD8-APC/R700 (564983, BD), PD-1-BV421 (135221 BIolegend), TCRβ-BV605, PD-L1-PE (124308, Biolegend), Ly6G-Percp (127654, Biolegend), CD206-PE/Cy7 (141720, Biolegend), CD45-AF488 (103122, Biolegend), F4/80-BUV395 (565614, BD), NKp46-BUV737 (565085, BD), and Foxp3-APC as described above and analyzed with FACS LSR.

RNA Sequencing

$TCR\beta^{+}$ $CD4^{+}$ $CD45RB^{low}$ $CD25^{high}$ cells from $CD4^{Cre/+}$ or $CD4^{+/+}$ $PRMT5^{fl/fl}$ mice were sorted using the FACS Aria II. A total of 100,000 cells per sample were used for mRNA extraction using Trizol (Invitrogen) and RNeasy micro kit (Qiagen) according to manufacturer's instructions. The extracted mRNA was subjected for RNA sequencing and statistics were analyzed by the High Throughput Sequencing Core at CHOP at the University of Pennsylvania. Differentially expressed gene (DEG) was defined as fold change ≥ 2 and post-probability of equally expressed (PPEE) <0.05 calculated by EBSeq method.

Human Treg Expansion

$CD4^{+}$ T cells were provided by the Human Immunology Core at the University of Pennsylvania from healthy donors. $CD4^{+}$ $CD25^{high}$ $CD127^{low}$ Tregs were sorted using the FACS Aria II (BD Bioscience) to a purity >98%. For *in vitro* expansion, the cells were cultured in T cell medium supplemented with recombinant human IL-2 (200 IU/ml, Peprotech) and anti-CD3/CD28 beads at a 1:1 ratio. The media was changed every other day.

Lentivirus Production and Transfection to Human Tregs

Lentivirus was produced using the ViraSafe™ lentivirus packaging system (Cell Biolabs) per manufacturer's instructions. Viral supernatants were concentrated by adding 50% PEG8000 and 1.5 M NaCl (Final concentration: 5% PEG8000 and 0.15 M NaCl), rotated overnight at 4°C, then centrifuged for 30 min at 3,300 g. The pellets were suspended in T cell media and used for lentivirus transfection. For lentivirus transfection to human Tregs, expanded human Tregs (3×10^5 cells) were harvested and suspended in lentivirus-containing medium with IL-2 (200 IU/ml) and anti-CD3/CD28 beads in 5 ml round bottom tubes (Fisher) and then cenreifuged for 2.5 h at 450 g at RT. The cells were then re-suspended in T cell medium supplemented with IL-2 (200 IU/ml) and cultured in a 12 well-plate. After 2 days of culture, medium was changed and puromycin (1 μg/ml) was added for selecting transfected cells. After 6 days, live cells were sorted and used for the *in vitro* suppression assay as described below.

Retrovirus Production and Transfection to Human $CD4^{+}$ Tcells

Foxp3-introduced MIGR1 vectors were used for retrovirus production with pMD2. G and pUMVC (Addgene) at 7.5:5:2.5 ratio using 293T cells. Viral supernatants were concentrated as described above. $CD4^{+}$ T cells were transfected with the same method as lentivirus transfection as described above. Two days after transfection, GFP^{+} cells were sorted and expanded for 5 days. Then GFP^{+} cells were re-sorted and subjected to the suppression assay as described below.

Treg Suppression Assays

For the mouse cells, $CD4^{+}$ T cells were enriched from splenocytes using a mouse $CD4^{+}$ T cell isolation kit (Stem Cells). $CD4^{+}$ $CD25^{-}$ $CD45RB^{high}$ Teff cells and $CD4^{+}$ $CD25^{+}$ YFP^{+}

CD45RB^{low} Treg cells were separated from CD4$^+$ cells using the FACSAria II (BD Biosciences). Teff cells were labeled with Cell Trace Violet (CTV, Molecular Probes) and mixed with Tregs and Dynabeads® Mouse T-Activator CD3/CD28 (Life Technologies) in a V-bottom 96-well plate (20,000 cells/well of Teffs, indicated ratio of Tregs, and 0.2 μl/well of CD3/CD28 beads). For the human cells, healthy donor PBMC were provided by the Human Immunology Core of the University of Pennsylvania, and CD4$^+$ T cells were enriched using the MACS CD4$^+$ isolation kit (Miltenyi Biotech). Then CD4$^+$ CD25$^-$ CD127^{high} Teff cells and CD4$^+$ CD25$^+$ CD127^{low} Treg cells were separated from CD4$^+$ cells using the FACSAria II (BD Biosciences). Teff cells were labeled with Cell Trace Violet (Molecular Probes), and mixed with Tregs and irradiated (2000 rad) CD3$^-$ PBMC, and then stimulated with anti-human CD3 OKT3 (eBioscience) in a V-bottom 96-well plate (20,000 cells/well of Teffs, indicated ratio of Tregs, 50,000/well of irradiated PBMC from which CD3$^+$ cells were eliminated by human CD3$^+$ T cell isolation kit (Stem Cells), and 0.1 μg/ml of anti-CD3 antibody). After 3 days of culture, cell proliferation was analyzed by flow cytometry. For the human transfected cells, anti-CD3/CD28 beads (0.02 μl/well, Thermo Fisher) were used instead of anti-CD3 and irradiated PBMC.

iTreg Induction

Mouse CD4$^+$ CD25^{low} CD45RB^{high} naïve T cells were sorted as described above. The cells were labeled with CTV, then seeded in a V-bottom 96-well plate (20,000 cells/well) and incubated with the indicated amount of TGFβ (Peprotec), 20 IU/ml of IL-2 and a 1:1 ratio of anti-CD3/CD28 magnetic beads (0.5 μl/well, Invitrogen) for 3 days. After the incubation, the cells were fixed and stained with anti-Foxp3-APC as described above, then analyzed using a FACS Canto (BD).

IL-2 Promoter Assay

Jurkat cells (1,000,000 cells/ml) were transfected with IL-2 promoter (1.2 μg, Panomics), pRL-TK (0.03 μg, Promega), and indicated vectors (0.8 μg) using Fugene 6 per manufacturer's instructions. Twenty Four hours after transfection, cells were stimulated with PMA (50 ng/ml) and ionomycin (10 μM) for 6 h. A luciferase assay was then performed using Dual luciferase assay kit (Promega). Fold changes were calculated as (P.I. stimulated Firefly Luciferase/Renilla Luciferase)/(Non-stimulated Firefly lusciferase/Renilla Luciferase).

Histology

Tissues were fixed with 10% neutral buffered formalin and embedded in paraffin. Sections were de-paraffinized and stained with H & E by the Cell Imaging Core of the Abramson Family Cancer Research Institute.

Mouse *in vivo* Experiments

Six to Ten weeks old female Balb/c mice ($n = 4$ per group) were used for this set of experiments. Humanaized mAb 4D5 was obtained from Roche. For the *in vivo* treatment model, mice were randomly divided into groups and 5×10^5 CT26Her2 cells were injected subcutaneously into both sides of the back of shaved mice. Treatments began a week later. 4D5 was injected IP (5 mg/kg) twice a week. DS-437 was injected (10 mg/kg) 5 times a week. Tumor size was measured with a digital caliper and calculated using a simple algorithm (3.14 × length × wide × height ÷ 6). For some experiments, 6–10 weeks old female MMTV-neu mice ($n = 4$ per group) were used. mAb 7.16.4 was purified from a hybridoma that was generated in our lab. For the *in vivo* treatment model, mice were randomly divided into groups and a rodent erbB2/neu transformed Balb/c breast tumor cell line, H2N113 (1×10^6 cells) was injected subcutaneously into both sides of the back of mice. Treatments began 2 weeks later. 7.16.4 was injected IP (1.5 mg/kg) twice a week. EPZ004777 was injected (5 mg/kg or 25 mg/kg) 5 times a week. The experiments were not blinded but performed by a technician who wasn't knowledgeable about the expected outcome. The cell lines were tested for mycoplasma using a MycoSensor PCR Assay Kit (Agilent).

Bisulfite Sequencing Analysis

Lymph node Tregs from Foxp3^{Creyfp} and Foxp3^{Creyfp}-PRMT5$^{fl/fl}$ mice (male, 18–21 days old) were collected using a FACSAria II as described above and then subjected to bisulfite sequencing analysis using the EZ-DNA Methylation Kit (Zymo Research) and primers described previously (18–20).

Statistical Consideration

Statistical analysis was performed using the Student's *t*-test with unequal variance test using Microsoft Excel (one tail for *in vivo* TIL, two tail for others) for comparison of two groups. For 3 or more groups, one way ANOVA with Tukey HSD analysis were used for calculating the significance. At a minimum, data with a p-value < 0.05 were deemed significant. All *in vitro* experiments were performed in triplicate for calculating statistical significance. In all cases, experiments shown are representatives that were performed at least twice for *in vivo*, and 3 times for *in vitro* experiments.

RESULTS

Identification of PRMT5 as a FOXP3 Binding Partner

To define the molecular interactions of FOXP3, we transfected and expressed FLAG-FOXP3 in 293T cells and then immunoprecipitated FOXP3 proteins with anti-FLAG agarose beads for identifying FOXP3-interacting proteins. We immunoprecipitated FOXP3 proteins with anti-FLAG agarose beads (**Figure 1A**). Several FOXP3 specific binding proteins were identified from the precipitation. Each specific band was extracted and subjected to mass spectrometry. One protein at ∼70 kDa size was identified as the protein arginine methyltransferase (PRMT5) (**Figure 1B**). To verify the interaction between FOXP3 and PRMT5, we co-transfected HA-FOXP3 with FLAG-PRMT5 into 293T cells. As shown in **Figure 1C**, co-precipitation confirmed that FOXP3 binds to PRMT5. We further investigated whether other PRMT family members bind to FOXP3. We tested PRMT1, 2, 4, and 7 using 293T cells. PRMT1, 2, and 4 are know to be expressed in T cells (21), and PRMT7 is know to methylate arginine symmetrically

(22). We found that PRMT5 preferentially binds to FOXP3 (**Figure 1D**). In addition, immunoprecipitates of anti-PRMT5 with human Treg lysate clearly included FOXP3 protein, and anti-FOXP3 precipitates included PRMT5 proteins (**Figure 1E**). These results indicate that PRMT5 is a binding partner of FOXP3 in Tregs.

Deletion of PRMT5 in Tregs Causes Autoimmunity in Mice

To reveal the role and function of PRMT5 on Tregs, we generated transgenic mice which have conditional PRMT5 deletion in Tregs since PRMT5 constitutive deficiency results in early embryonic lethality (23). We crossed PRMT5$^{fl/fl}$ and Foxp3^{Creyfp} mice (11, 12) to produce PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice. The transgenic mice that possess PRMT5 deficient Tregs displayed severe scurfy-like autoimmunity. Clinical features included weight loss, dermatitis and splenomegaly; and all animals died by 40 days after birth (**Figures 2A–C**). Necropsied livers revealed massive infiltration of lymphocytes as deduced by H&E sections (**Figure 2D**). This pathologic feature resembled those we observed in the liver of scurfy and Tip60$^{fl/fl}$ Foxp3^{Creyfp} mice (12).

To study the Treg populations that still exist in the PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice, we isolated CD4^{+} Foxp3^{+} YFP^{+} cells from spleen, lymph node and thymus (**Figure 2E**). Compared with control mice, PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice possessed fewer Tregs in their spleens. However, the number of Tregs in peripheral lymph nodes was comparable. In the surviving Tregs, PRMT5 expression was dramatically deleted (**Figure S1A**), confirming conditional deletion in those Tregs. We observed increased Foxp3^{+} populations in the PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mouse thymus, which is reminiscent of Tip60$^{fl/fl}$ Foxp3^{Creyfp} mice (12). This may be because massive inflammatory signaling induces Treg differentiation in the thymus.

We next investigated whether T cells in lymph nodes are activated and differentiated into the effector cells as deduced by staining for CD44 and CD62L. In the PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice, the population of CD8^{+} and CD4^{+} Foxp3^{-} cells contained significant amounts of effector cells (the CD44^{high} CD62L^{low} population in **Figure 2F**), which is consistent with the severe scurfy phenotype. Also consistent with this observation, we found increased numbers of CD25^{+} cells in the CD4^{+} Foxp3^{-} and CD8^{+} population in the PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice (**Figure S1B**), suggesting that PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice have significantly increased amounts of activated T cells in their lymph nodes. In those T cell populations, we noted increased expression of IL-4 and IFNγ in the CD4^{+} Foxp3^{-} cells, and significant amounts of IFNγ^{+} cells in CD8^{+} T cells (**Figure S1C**), which is similar with the other scurfy-like phenotype reported previously (24).

We further analyzed the suppressive function of Tregs from lymph nodes by suppression assays. As shown in **Figure 2G**, PRMT5 deficient Tregs from PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice had significantly less suppressive activity than Tregs from Foxp3^{Creyfp} littermates. We investigated if PRMT5 knockdown could also impair human Treg functions. Expanded human Tregs were transfected with lentivirus with or without shRNA targeting

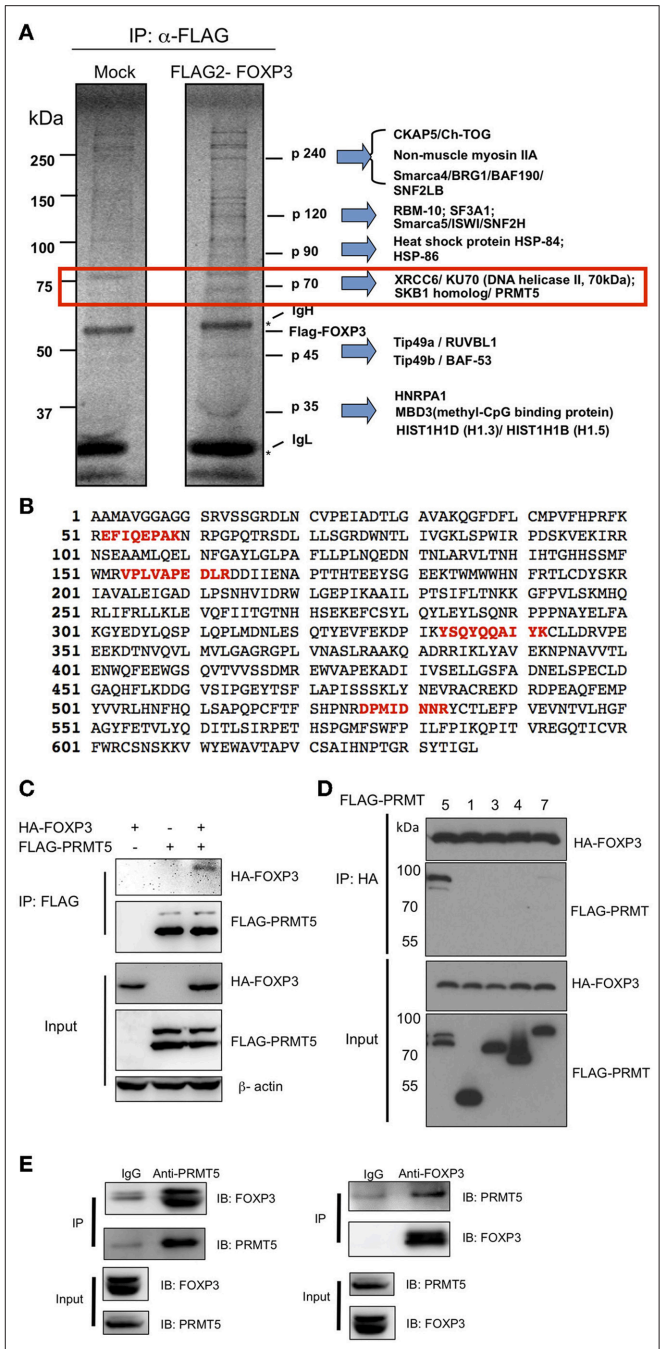

FIGURE 1 | Identification of PRMT5 as a binding partner of FOXP3. **(A)** mass spectrum analysis of FOXP3 binding protein. 293T cells were transfected with pIPFLAG2-FOXP3 vector and immunoprecipitated with anti-FLAG agarose beads and subjected to SDS-PAGE. The proteins were visualized using a SilverQuest silver staining kit, and specific bands in FLAG-FOXP3 samples were analyzed by LC-MS/MS. **(B)** 4 peptide sequences of PRMT5 (labeled in red) were identified by MS sequencing as FOXP3 bound proteins. **(C,D)** specific interaction of PRMT5 with FOXP3 proteins. 293T cells were transfected with HA-FOXP3 and FLAG-PRMT5 **(C)** or HA-FOXP3 and FLAG-PRMT1, 3, 4, and 7 **(D)**. **(E)** endogenous interactions of FOXP3 with PRMT5 in human Tregs. Expanded human Tregs were lysed and subjected to immunoprecipitation with anti-PRMT5 (left panel) or anti-FOXP3 antibody (right panel). The samples were then blotted and protein detected with indicated antibodies.

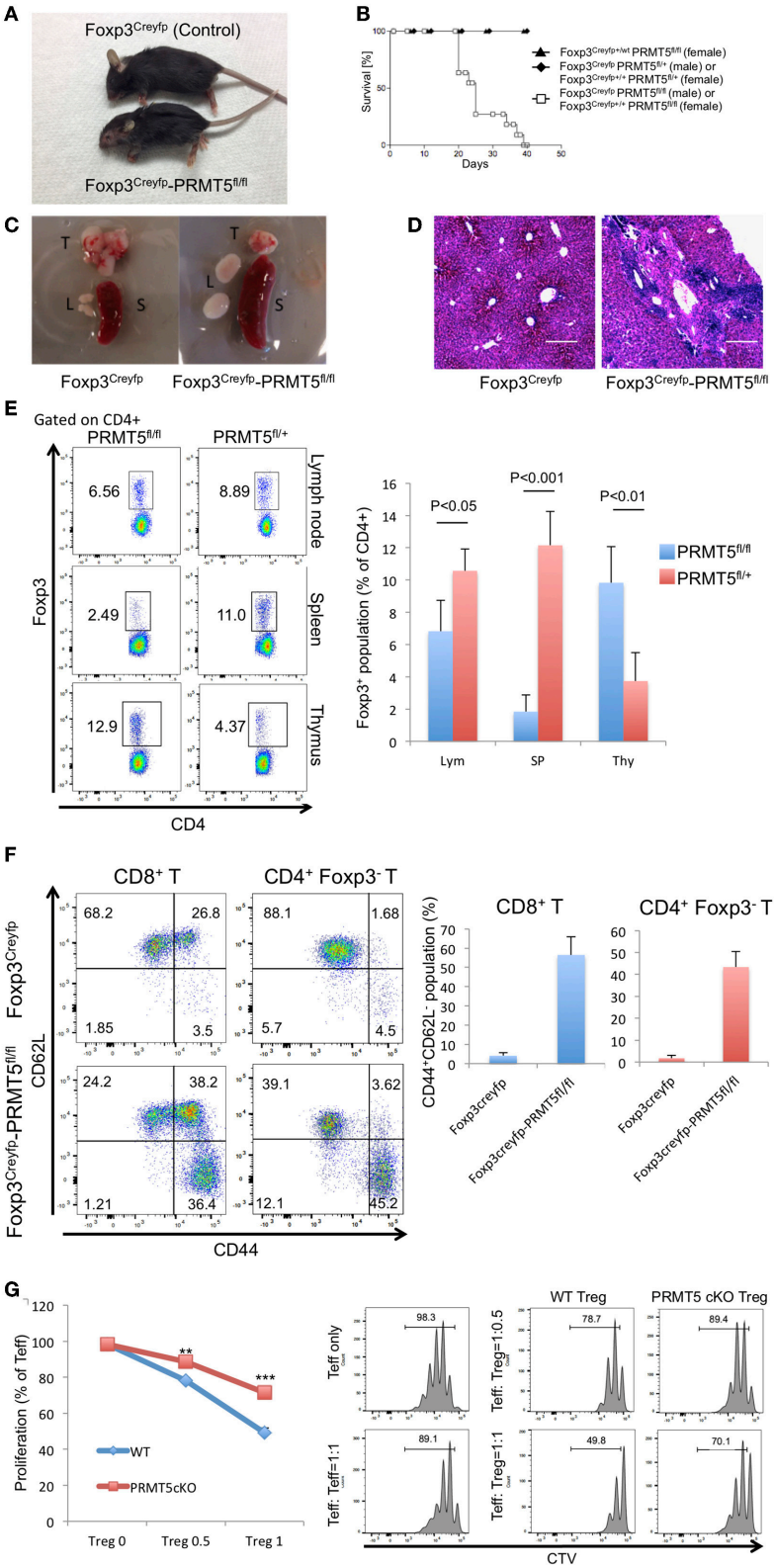

FIGURE 2 | Foxp3^{Creyfp}-PRMT5$^{fl/fl}$ mice display scurfy-like symptoms. **(A)** phenotype of Foxp3^{Creyfp}-PRMT5$^{fl/fl}$ mouse (male, 18–21 days old). **(B)** survival curve of Foxp3^{Creyfp}-PRMT5$^{fl/fl}$ mouse (10 mice). **(C)** spleen (S), thymus (T), and lymph nodes (L) from Foxp3^{Creyfp} and Foxp3^{Creyfp}-PRMT5$^{fl/fl}$ mouse. **(D)** H&E staining of

(Continued)

FIGURE 2 | liver sections from Foxp3^{Creyfp} and Foxp3^{Creyfp}-PRMT5$^{fl/fl}$ mouse. Bar = 200 μm. Data shows a representative of 3 different mice. **(E)** Treg populations of spleen, lymph node and thymus from Foxp3^{Creyfp} and Foxp3^{Creyfp}-PRMT5$^{fl/fl}$ mouse Data shows a representative of 4 different mice. **(F)** activation of T cells in Foxp3^{Creyfp}-PRMT5$^{fl/fl}$ mice. TCRβ^{+} T cells from Foxp3^{Creyfp} and Foxp3^{Creyfp}-PRMT5$^{fl/fl}$ mouse lymph nodes (axillary and inguinal) were stained with anti-CD44 and CD62L antibodies, and analyzed by flow cytometry. Data shows a representative of 4 different mice. **(G)** suppressive functions of lymph node Tregs from Foxp3^{Creyfp} (WT) and Foxp3^{Creyfp}-PRMT5$^{fl/fl}$ (Prmt5 cKO) mouse. The error bars indicate the SD value. $^{**}P < 0.005$, $^{***}P < 0.001$ with Foxp3^{Creyfp} groups calculated by student *t*-test.

PRMT5. PRMT5 was successfully knocked down by shRNA in human Tregs (**Figure S2A**). Those Tregs showed impaired suppressive functions compared with empty vector transfected cells (**Figure S2B**), indicating PRMT5 is also important for human Treg function. These results indicate that a lack of PRMT5 in Tregs causes a reduction in Treg suppressive activity in cells that occupy the peripheral lymph nodes.

Expression of Treg-Associated Molecules in PRMT5 cKO Tregs

We further investigated the molecules that are expressed in Tregs and associated with their function and maturation. In Tregs from PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice, many function related surface markers such as CD25 and GITR were up-regulated (**Figure S3A**). Interestingly, Nrp-1 and Ki67 were dramatically decreased, compared with Foxp3^{Creyfp} mice. When we examined ICOS, the overall expression was increased, but the population of ICOS^{high} cells was decreased in PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice. In addition, most of CD4^{+} Foxp3^{+} cells remained as CD44^{low} CD62L^{high} population compared with Foxp3^{creyfp} mice, suggesting that PRMT5 deletion prevents the maturation of Tregs as the effector/memory phenotype (**Figure S3B**).

To exclude the possibility that scurfy-like autoimmune phenotype affected the expression of these molecules, we used Foxp3^{Creyfp} heterozygous mice (Foxp3^{Creyfphet}). In those heterozygous mice there is no scurfy-like phenotype as half of the Tregs are Foxp3^{Creyfp} positive because of random inactivation of the X chromosome (25). We observed a decreased number of YFP^{+} Tregs in PRMT5$^{fl/fl}$ Foxp3^{Creyfphet} mouse spleen and lymph node (**Figure 3A**). In contrast, there were no notable differences between YFP^{+} and YFP^{-} Treg numbers in PRMT5$^{fl/+}$ Foxp3^{Creyfphet} mice.

Next we compared the expression level of other known Treg-associated molecules. YFP^{+} cells from PRMT5$^{fl/fl}$ Foxp3^{Creyfphet} mice showed decreased expression of Nrp-1, CTLA4, ICOS, and Ki67 (**Figure 3B**). These results indicate that PRMT5 deficient Tregs display both decreased suppressive and proliferative functions.

We also investigated whether PRMT5 deletion affects iTreg generation from naïve T cells by using CD4^{cre}-PRMT5$^{fl/fl}$ mice. PRMT5 deletion reduced the iTreg development compared with WT littermates (**Figure 3C**). In those iTregs, Foxp3 expression level was lower than WT. These results indicate that PRMT5 deletion also affects iTreg generation and Foxp3 expression.

Gene Expression Profiles in PRMT5 Deleted Tregs

To reveal how PRMT5 deletion affects overall mRNA profiles expressed in Tregs, we collected PRMT5 deleted Tregs and subjected them to RNA sequencing analysis. For this experiment, we used the Tregs from CD4$^{+/+}$ and CD4$^{cre/+}$ -PRMT5$^{fl/fl}$ mice because CD4$^{cre/+}$ PRMT5$^{fl/fl}$ mice do not display scurfy-like symptoms, principally because of reduced function of CD8^{+}T and CD4^{+}T cells (Tanaka et al. in preparation), to avoid the effect of endogenous inflammation in their bodies on their Tregs. In addition, CD4^{cre} enables an earlier deletion of PRMT5 expression in the T cells' developmental stage in the thymus that differs from Foxp3^{cre}. This event possibly defines the complete phenotypic changes in generated Tregs. As shown in **Figure 4A**, Tregs from CD4$^{Cre/+}$ showed significantly lower suppressive functions compare with WT Tregs. RNA sequencing analysis showed that 159 genes were down-regulated and 318 genes were up-regulated in the PRMT5 deleted Tregs (**Figure 4B**). Foxp3 induced genes, as reported by Kwon et al. (26), that include *igfr1* and *jun* were significantly decreased, and Foxp3 repressed genes such as *il18rap*, *Eomes*, *Gzmb*, and *il4* were up-regulated by PRMT5 deletion, (**Table S1**). Pathway analysis showed that PRMT5 deletion impacts a number of cell cycle pathway participating genes. Changes in p53, Foxo, and cytokine-cytokine receptor interaction pathways (**Figure S4**) were noted, supporting the notion that PRMT5 deletion causes both cell cycle abnormalities and induces an inflammatory phenotype in Tregs.

Stability of PRMT5 cKO Tregs

Since we found a dramatic reduction of Tregs in Foxp3^{creyfp} PRMT5$^{fl/fl}$ mouse spleen, we next studied the stability of PRMT5 deleted Tregs by analyzing epigenetic modifications of the CNS2 region, which mediates an important role in sustained expression of Foxp3 during Treg activation (27). To elucidate if a lack of PRMT5 can affect the methylation status in the Treg CNS2 region, we collected lymph node Tregs from Foxp3^{Creyfp} and PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice and then analyzed their methylation status by bisulfite sequencing (**Figure S5A**). In the PRMT5-lacking Tregs, we noted that de-methylation of the CNS2 region was not observed, unlike wild type controls. We did not observe major alterations in the methylation status in the CNS1, CNS3, and upstream regions commonly hypomethylated and downstream hypermethylated regions. Consistent with this, proliferation of those Tregs resulted in the loss of Foxp3 expression (**Figure S5B**).

Analysis of Symmetrical Dimethylation Sites on Foxp3

To test whether FOXP3 can be methylated by PRMT5, we first analyzed if PRMT5 knockdown decreases symmetric arginine di-methylation signals by immunoprecipitation with anti-symmetric arginine di-methylation antibody sym10. PRMT5

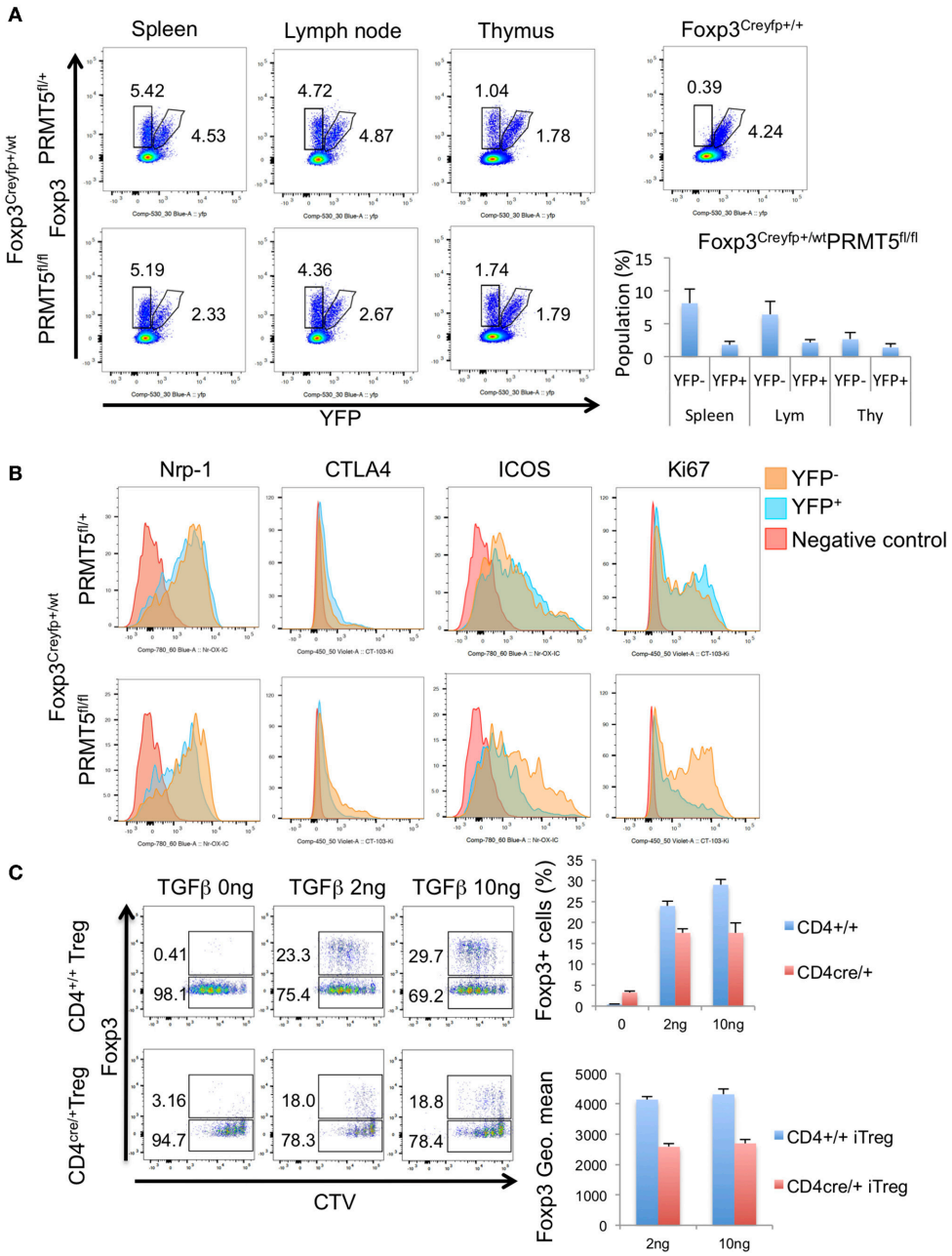

FIGURE 3 | Populations and phenotypes of Tregs in Foxp3$^{Creyfp+/-}$-PRMT5$^{fl/+}$ or -PRMT5$^{fl/fl}$ mice. **(A)** cells were harvested from spleen, lymph node, and thymus. Populations of YFP^{+} or YFP^{-} cells were analyzed. Numbers show the percentage of gated cells in CD4^{+} TCRβ^{+} cells. **(B)** phenotypes of Tregs. Each type of YFP^{+} or YFP^{-} cell was gated as **(A)**. Then expression levels of indicated proteins were analyzed. Data shows a representative of 3 different mice. **(C)** iTreg induction of CD4^{+}T cells from CD4^{cre}-PRMT5$^{fl/fl}$ mice. CTV-labeled CD45RB^{high} CD25^{low} naïve CD4^{+}T cells were treated with indicated concentrations of TGFβ, 20 IU/ml of IL-2 and 1:1 ratio of CD3/CD28 beads; then cultured for 3 days. After the incubation, cells were stained with anti-Foxp3 APC and analyzed by Flow cytometry.

deletion by shRNA (vector 86) could reduce PRMT5 expression, and symmetric arginine di-methylation signals (**Figure 5A**).

Next, we sought to identify the symmetric methylation sites. First we computationally analyzed the methylation sites on FOXP3 using the on- line web tool first reported by Kumar et al. (28). We found several possible methylation sites in Foxp3, including R51 and proximal R48 position. Consistent with this, Geoghegan et al. have suggested that FOXP3 can be di-methylated at the R51 position (21). However, Geoghegan and colleagues did not show whether that methylation is symmetric or asymmetric. We analyzed the effect of point mutation of FOXP3 R51 position to K on its symmetric methylation signals.

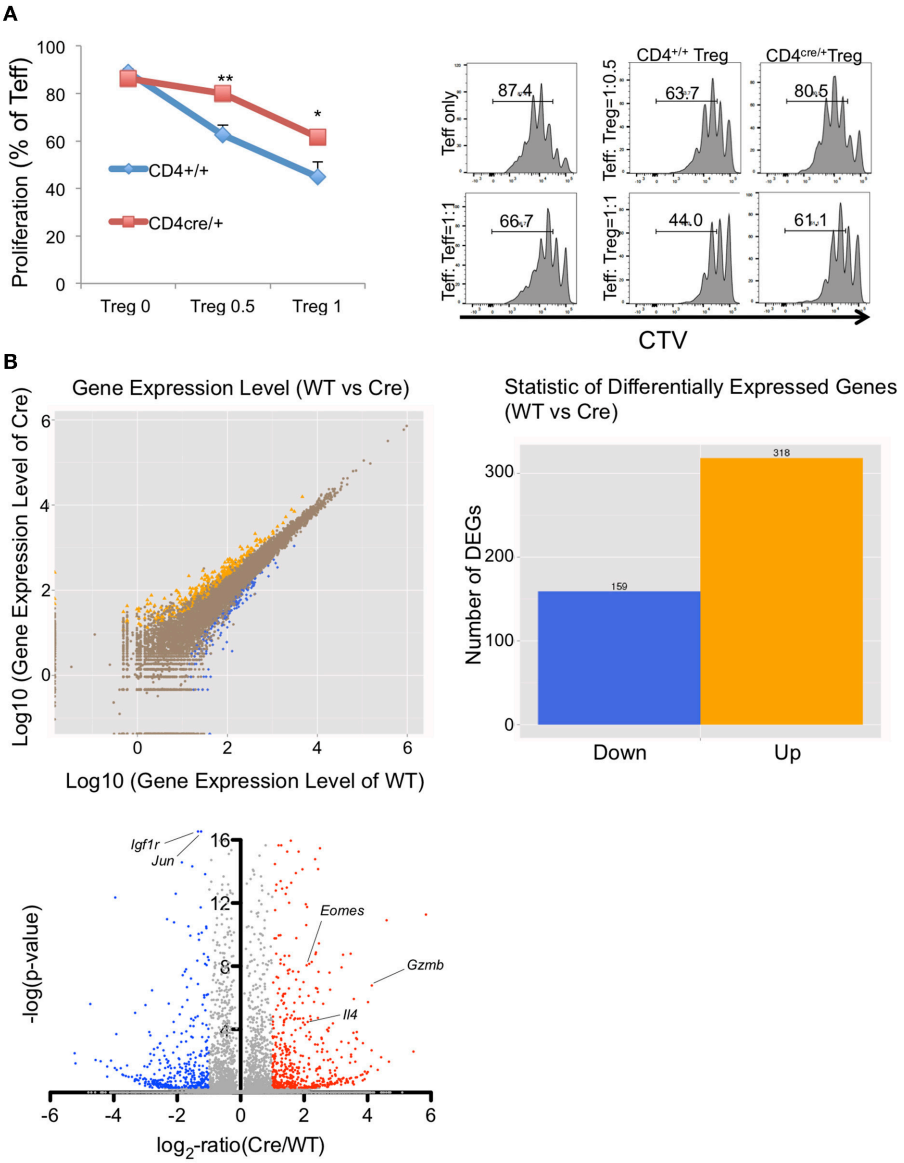

FIGURE 4 | Functional and RNA sequencing analysis of PRMT5-deleted Tregs. **(A)** functional analysis of $CD4^{cre/+}$ -$PRMT5^{fl/fl}$ Tregs. $CD4^{+/+}$ and $CD4^{cre/+}$ -$PRMT5^{fl/fl}$ Tregs were sorted as $CD4^{+}$ $CD25^{high}$ $CD45RB^{low}$ cells and used for the Treg suppression assay as described above. CTV-labeled $CD4^{+}$ $CD25^{low}$ $CD45^{high}$ cells from $CD4^{+/+}$ $PRMT5^{fl/fl}$ cells were used as effector cells. The error bars indicate the SD value. $^{*}P < 0.05$, $^{**}P < 0.01$ with $CD4^{+/+}$ groups calculated by student *t*-test. **(B)** differential gene expression between WT ($CD4^{+/+}$ -$PRMT5^{fl/fl}$) and Cre ($CD4^{cre/+}$-$PRMT5^{fl/fl}$). Two mice per group were subjected for RNA sequencing analysis.

R51K and proximal arginine R48K mutations both reduced endogenous FOXP3 methylation signals in 293T (**Figure 5B**). Over-expression of PRMT5 increased symmetric arginine di-methylation signals, but they were still lower in the FOXP3 R51K mutant, suggesting that position R51 is likely di-methylated by PRMT5.

We also confirmed by mass spectrometry that FOXP3 is di-methylated. Since 293T expresses high levels of PRMT5 proteins and is efficient for gene transfection, we used 293T transfected with HA-FOXP3 and purified the HA-FOXP3 using anti-HA magnetic beads. We found that there are several distinct dimethylation sites on FOXP3: R27 and R146, in addition to R51 (**Figure 5C** and **Table S2**).

We next analyzed the effect of the R51 point mutation on Foxp3 functions. Human $CD4^{+}$ T cells were retrovirally transfected with the empty vector, FOXP3 WT or a mutated FOXP3 R51K gene and subjected to suppression assays. We found that the R51K mutation dramatically decrease its suppressive functions on T effector cells compare with FOXP3 WT (**Figure 5D**). We also analyzed if R51 mutation affects the direct binding of its target gene promoters by IL-2 promoter luciferase assay (**Figure 5E**).

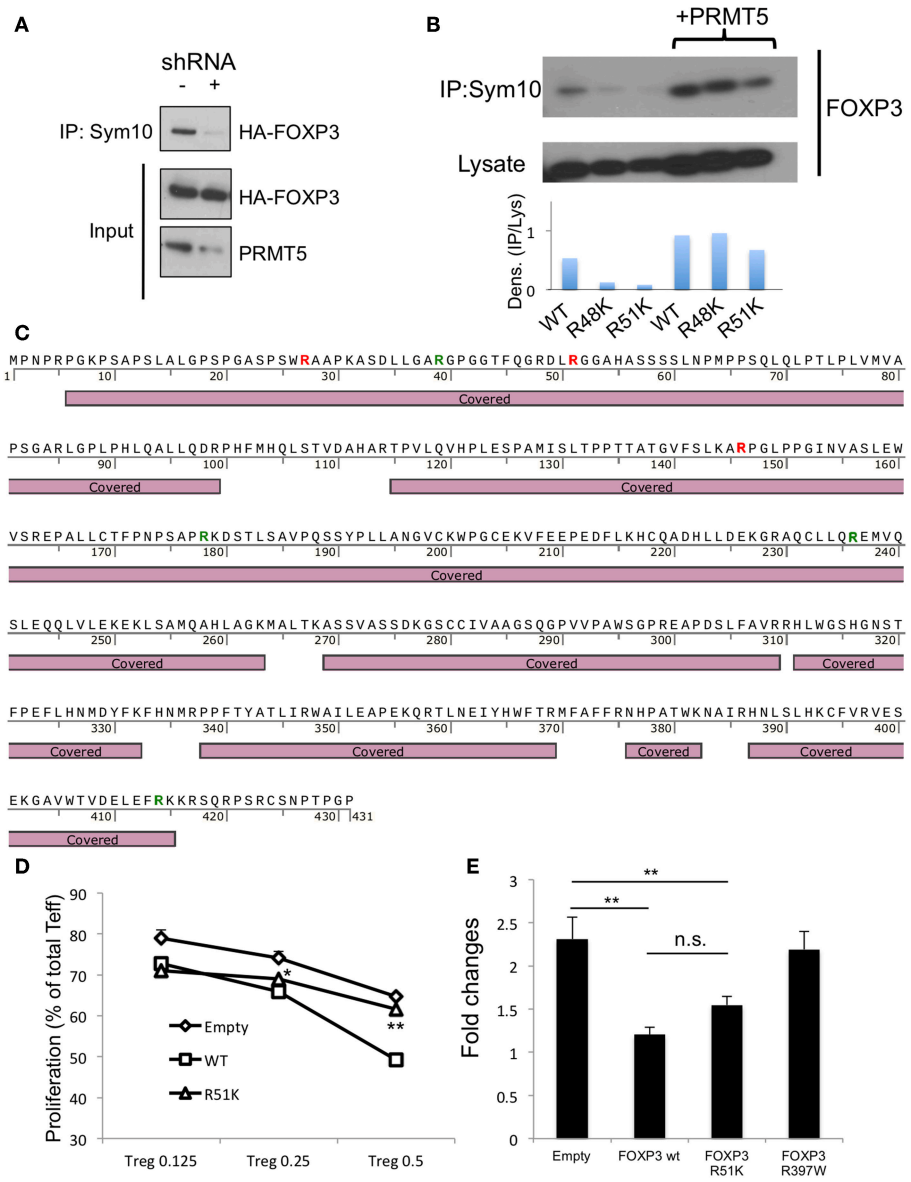

FIGURE 5 | Analysis of methylation sites in human FOXP3. **(A)** symmetrical methylation of FOXP3. 293T cells were transfected with FOXP3 and empty or PRMT5 shRNA86 vector. After 24 h of transfection, the cells were lysed and immunoprecipitated with anti-sym10, which recognizes symmetrical arginine dimethylation, and then subjected to western blotting. **(B)** symmetrical methylation analysis of human FOXP3 wild type (WT), R48K and R51K mutant in 293T systems. **(C)** methylation analysis of FOXP3 by mass spectrometry. HA-FOXP3 transfected 293T cells were lysed and immunoprecipitated with anti-HA beads, then subjected for mass spectrometry. Covered: FOXP3 sequences detected by mass spectrometry. Red letters show the arginine that is di-methylated. Green letters show the arginine that is mono-methylated. **(D)** suppressive functions of FOXP3 R51K. Human $CD4^+$ T cells were transfected with empty, FOXP3 WT and FOXP3 R51K mutant, then used as suppressors in the suppression assay. $^*P < 0.05$, $^{**}P < 0.01$ with FOXP3 WT groups calculated by student *t*-test. **(E)** IL-2 promoter assay with FOXP3-transfected Jurkat cells. Jurkat cells were transfected with the IL-2 promoter vector and additional indicated vectors. Twenty four hours after transfection, cells were transferred to 24 wells (250 μl/well), stimulated with PMA and ionomycin for 6 h, and then subjected to the luciferase assay. The error bars indicate the SD value. $^{**}P < 0.01$ with control vehicle groups calculated by one way ANOVA with Tukey HSD test.

The R51 mutation by itself did not alter FOXP3 suppressive activity on IL-2 promoter activity. On the other hand, the FOXP3 R397 mutation to W, which is known as a natural IPEX mutation and cannot bind to the promoter DNA sequences (29–31), did not display any suppressive function of IL-2 promoter. This result suggests that FOXP3 R51K mutation still has DNA binding ability.

PRMT5 Is Important for Human Treg Functions

Pharmacologic PRMT5 inhibition should also impair Treg functions. There are several reported small molecule PRMT5 inhibitors including EPZ015666, CMP5, HLCL61, EPZ004777, and DS-437. DS-437 and EPZ004777 that are S-adenosylmethionine (SAM) competitive inhibitors (17, 32).

CMP5 and HLCL-61 were computationally developed to bind the PRMT5 catalytic site, but they are not SAM-competitive inhibitors (33–35). EPZ015666 only binds to the complex of PRMT5, MEP50, and SAM (36), and MEP50 is a co-factor of PRMT5 (37). We initially investigated whether those inhibitors successfully inhibit FOXP3 methylation by 293T systems with anti-sym10 antibody. SAM-competitive inhibitor DS-437 and EPZ004777 showed better inhibition activities than other inhibitors, especially at 2.5 μM concentration (**Figure 6A**).

The effect of those inhibitors was examined in assays of Treg suppressive function to Teff cells. Treg suppression assays with mouse T cells revealed inhibition of mouse Treg functions with DS-437 (**Figure 6B**). EPZ015666 by itself led to strong inhibition of Teff proliferation (**Figure 6B**, red and green columns), while other inhibitors had no direct effect on Teff proliferation at the tested concentrations. EPZ004777, when used at 5 μM could inhibit mouse Treg functions (**Figure S6A**). This tendency was also noted in human Treg suppression assays (**Figure 6C**). In addition, DS-437 and EPZ00477 successfully inhibited endogenous FOXP3 methylation in the human expanded Tregs (**Figure 6D** and **Figure S6B**).

SAM-competitive PRMT5 Inhibitors Enhance the Effect of Targeted Antibody Therapy in Murine Tumor Model

Our laboratory previously established many of the features of p185$^{erbB2/neu}$ ectodomain targeted monoclonal antibody therapy. Our laboratory had also identified a contribution of Treg activity in dampening tumor elimination (5, 38), which has relevance to human breast tumor patients (7). To assess whether targeting PRMT5 could boost tumor immunity, we first employed the syngeneic MMTV-neu breast tumor model with EPZ004777 treatment, which is has previously been used in a mouse tumor model (32). Our preliminary experimental study found that EPZ004777 had better activity than EPZ015666 (**Figure S6C**). We next employed the combination of anti-erbB2/neu targeted therapy using the same model. Ten days after inoculation of syngeneic neu-transformed breast tumor cells (H2N113), mice were treated with anti-p185$^{erbB2/neu}$ antibody 7.16.4, EPZ004777, or the combination of both. While we have noted that treatment with high dose EPZ004777 alone had modest beneficial effects on tumor growth inhibition (**Figure S6D**), even more effects were observed when mice were treated with the combination of EPZ004777 and the anti-p185$^{erbB2/neu}$ antibody. Interestingly, the 7.16.4 group alone had the highest Treg levels among TILs (**Figure S6E**). Importantly, EPZ004777 treatment lessened 7.16.4 induced Treg infiltration into tumors.

We next sought to determine whether inhibition of PRMT5 could improve anti-p185$^{erbB2/neu}$ targeted therapy in the resistant tumor model. CT26-Her2 cells were used. CT26 cells were established by engineering the Balb/c syngeneic tumor line CT26 to express human Her2 (39) as a naturally resistant model for anti-p185^{erbB2} antibody therapies as it carries the oncogenic K-RasG12D mutation (40, 41). Although the animals treated with DS-437 alone had some beneficial effects on inhibiting tumor growth (**Figure 7A**), the combination of DS-437 and the anti-p185$^{erbB2/neu}$ antibody 4D5 had even more dramatic effects (**Figure 7A**). Remarkably, only 3/8 tumors continued to grow in the combination group mice (**Figure S7A**). 4D5 treatment by itself did not show a beneficial effect in this model. The mice treated with DS-437 showed significantly lower Treg activities compare with PBS-treated mice (**Figure 7B**). We further analyzed tumor infiltrating lymphocytes (TILs) in the treated mice (**Figure S7B**). In the DS-437 treated groups, there are increased total CD8$^+$ and CD8$^+$ PD-1$^+$ T cells, but more significantly in combination groups compared with controls (**Figure 7C**). Interestingly, the combination groups had significantly increased NKp46$^+$ cells in their tumors. We also noted that there are not significant changes of the expression of CD206 in the F4/80$^+$ macrophage population from combination groups (**Figure S7C**).

DISCUSSION

In this study, we defined a FOXP3 post-translational modifier, PRMT5. As far as we know, this is the first report that Foxp3 interacts with and can be methylated by PRMT5. Acuto et al. have shown that FOXP3 is methylated at R51, however they did not specify which member of the PRMT family methylates FOXP3 (21). Mass spectrometry analysis revealed that there are several di-methylated arginines in FOXP3. We found that the FOXP3 R51 position is di-methylated and appears to be symmetrically di-methylated by PRMT5. The point mutation of FOXP3 at R51 did not alter IL-2 promoter binding, but decreased the suppressive function of transduced CD4$^+$T cells, suggesting FOXP3 methylation does not alter DNA binding by itself, but has a role in FOXP3 suppressive functions, and di-methylation at R51 is important for Treg function.

Post-translational arginine modification of proteins is known to regulate many biological processes. PRMT proteins transfer methyl groups from SAM to arginine residues. There are 3 types of PRMTs; and PRMT5 belongs to the Type 2 PRMT set, which can symmetrically di-methylate arginine residues of target proteins (37). Although there are some reports identifying PRMT7 as a Type 2 methylation protein (22), our data showed that PRMT7 does not interact with FOXP3, supporting our supposition that PRMT5 is the dominant protein that can methylate FOXP3 symmetrically.

PRMT5 proteins were identified as histone methylating enzymes that led to suppression of gene expression. However, more recently, PRMT5 has been found to methylate and alter the activity of transcription factors. Di-methylation of the NF-kB p65 subunit enhances its function (42), while methylation of the tumor suppressor genes p53 and E2F1 attenuates their activity (22). Our results indicate that PRMT5 is indispensable for normal Treg activity, which prevents the severe autoimmunity scurfy-like symptoms. Interestingly, although those PRMT5 cKO Tregs show defective functions and less expression of CTLA4, Nrp-1, and ICOS, expressions of other important molecules for Treg suppressive functions such as GITR, OX40, and Helios are still comparable to normal (data not shown). We also found that PRMT5 deleted Tregs show less activation as demonstrated by

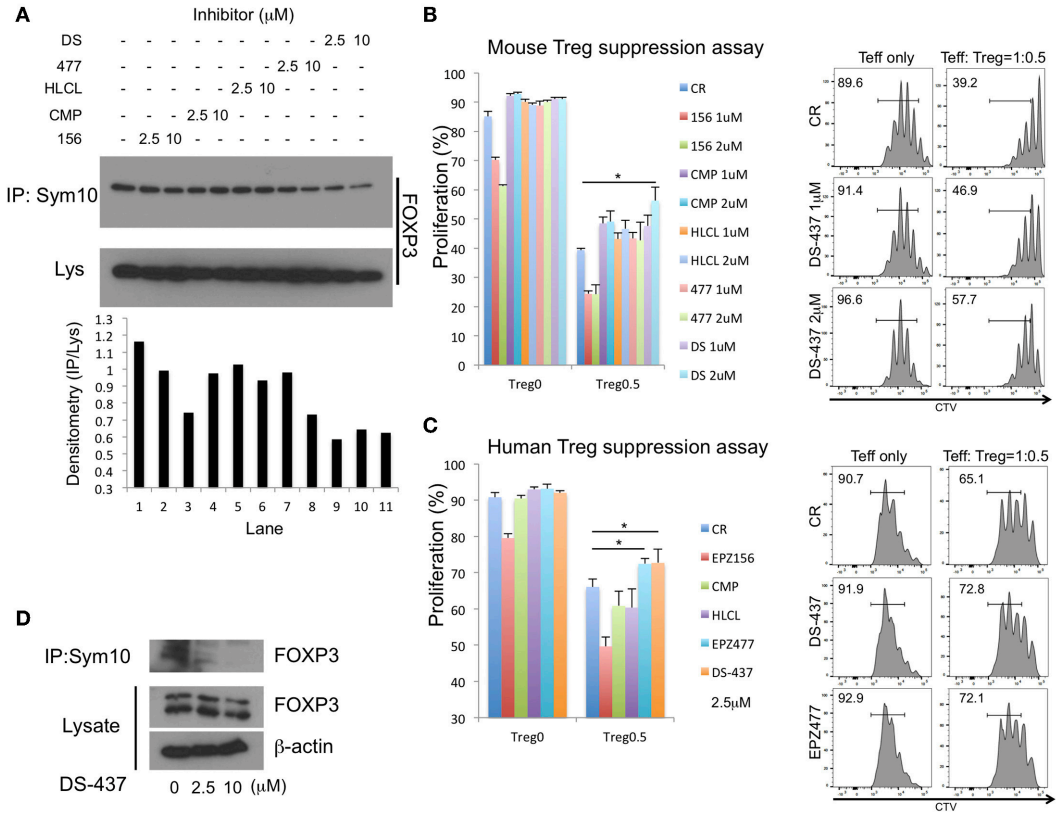

FIGURE 6 | Effect of PRMT5 inhibitors on FOXP3 methylation and Treg function. **(A)** effect of PRMT5 inhibitors on FOXP3 methylation in 293T cells. 293T cells were transfected with FOXP3. After 24 h of transfection, the cells were incubated with indicated inhibitors for 16 h, then subjected to symmetrical dimethylation analysis as described above. 156: EPZ015666. CMP: CMP5. HLCL: HLCL61. 477: EPZ004777. DS: DS-437. **(B,C)** effect on suppressive function of mouse **(B)** and human **(C)** Treg suppression assay. The error bars indicate the SD value. *$P < 0.05$ with control vehicle groups calculated by one way ANOVA with Tukey HSD test. **(D)** effect of PRMT5 inhibitor DS-437 on FOXP3 methylation in expanded human Tregs.

dramatic loss of the CD44^{high} CD62L^{low} population. In addition to CD44 and CD62L, ICOS expression in Tregs is also known to reflect effector and memory phenotype of Tregs and has strong immune suppressive functions (43–46). These results suggest that PRMT5 deletion prevents Treg cell progression to effector/memory phenotypes, and that may have a role in the strong scurfy-like symptoms in Foxp3^{cre}-PRMT5$^{fl/fl}$ mice.

RNA sequencing results and Ki-67 staining clearly indicate that PRMT5 deletion affects Treg proliferation. In addition, those Tregs show increased inflammatory cytokine and cytokine receptor expression. These results also suggest that PRMT5 deletion weakens the Foxp3 suppressive function of expression of those inflammatory cytokines. Indeed, several Foxp3 regulating genes reported by Kwon et al. (26) are dramatically changed in the PRMT5 deleted Tregs. Interestingly, *igfr1*, which is up-regulated by Foxp3 (26) and is important for Treg proliferation and function (47–49), is significantly decreased in the PRMT5 deleted Tregs. Reduction of *igfr1* may also influence the decrease of Treg proliferation in PRMT5$^{fl/fl}$ Foxp3^{Creyfp} mice.

Zhao et al. showed that the symmetrical methylation of histone H4 at R3 position specifically recruits the DNA methyltransferase DNMT3a to suppress the targeted genes (50, 51). Other studies were unable to verify that interaction (52). To identify the effect of PRMT5 deletion on Treg CNS methylation status, we also examined direct DNA methylation. Our study showed that the PRMT5 deletion was associated with hypermethylation in the CNS2 region, suggesting that the Histone H4R3-mediated DNMT3a recruitment by PRMT5 does not affect the methylation status of CNS2 region. While CNS2 hypermethylation may contribute to a less proliferative phenotype, PRMT5 cKO Tregs suffer from less stable Foxp3 expression and expansion in the periphery. Fang et al. have shown that de-methylation of CNS2 sustains Foxp3 expression in mature proliferating Tregs (27). Nevertheless, mice whose CNS2 region has been deleted in a Foxp3-dependent manner do not display severe scurfy-like symptoms as seen with the PRMT5 cKO mice we describe herein.

In our experimental conditions, we have not noted increased expression of Foxp3 by inhibiting PRMT5. Those studies include genetic knock outs, shRNA, and inhibitor experiments in both mice and humans. On the other hand, a recent publication by Zheng et al. found that PRMT5 negatively regulates FOXP3

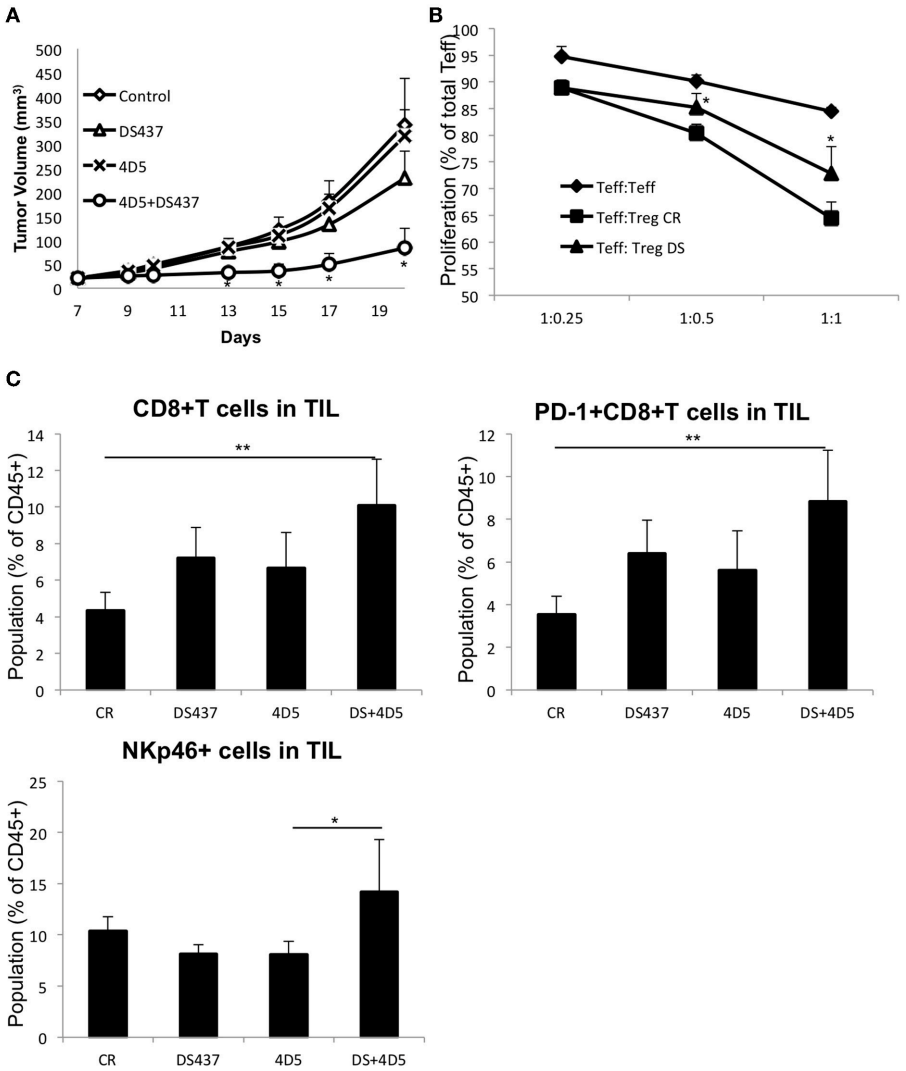

FIGURE 7 | Effect of PRMT5 inhibitor DS-437 on targeted therapy. **(A)** effect of DS-437 combined with anti-erbB2 antibody 4D5 on tumor size in CT26Her2 tumor model. CT26Her2 tumor cells (0.5×10^6) were injected subcutaneously into Balb/c mice that were treated with control PBS, 4D5 (5 mg/kg, twice per week), DS-437 (10 mg/kg, 5 times per week), or both 4D5 and DS-437. Data represent mean + SEM. One way ANOVA with Tukey HSD test was performed to compare the difference in the tumor size of different treatment groups. **(B)** function of Tregs in tumor bearing mice. Teffs and Tregs of PBS (CR) or DS-437 (DS) treated mouse lymph nodes were collected by sorting as described above. Teffs were labeled with CTV and stimulated with dynabeads CD3/CD28 beads (0.2 μl/well) with or without Tregs as indicated ratios. After 3 days of culture, proliferation of Teffs were analyzed by flow cytometry. The error bars indicate the SD value. $^*P < 0.05$ with control groups calculated by student *t*-test. **(C)** TIL were obtained after treatment as in **(A)** and the cell population analyzed by flow cytometry (4 mice per group, one tumor from each mouse). One way ANOVA with Tukey HSD test was performed to compare the difference in the populations of TIL of different groups. $^{**}P < 0.01$ (CR vs. DS+4D5); $^*P < 0.05$ (4D5 vs. DS+4D5).

expression in human Tregs, especially in ulcerative colitis patients (53). However, Zheng et al. used the AMI-1 inhibitor, which is known to inhibit PRMT1, 2, 4, and 6. AMI-1 does not selectively effect PRMT5 (54). In addition, the Zheng studies did not clearly demonstrate shRNA effects. It is possible that PRMT5 inhibition decreases inflammatory disease. The substrate (e.g., histone H3) competitive inhibitor EPZ015666, which acts as an inhibitor of H3 methylation but is not effective for inhibiting Foxp3 methylation, strongly inhibits Teff cell proliferation. The decrease of PRMT5 seen in the shRNA experiment in the Zheng et al. studies, might demonstrate effects on histone methylation.

PRMT5 is known to be expressed universally but is overexpressed in a large number of cancers, including breast cancer (37, 55). Thus, there are efforts to develop pharmaceutical inhibitors for this protein. CMP5, which was developed by computational simulation, showed suppressive function of Th1 cells at high doses (56). In our experiments, EPZ015666 showed significant inhibition of Teff cell proliferation, which we consider

as disadvantageous for tumor therapy. We found that SAM-competitive inhibitors efficiently inhibit Foxp3 methylation and do not inhibit Teff cell proliferation, at least at the concentration we tested. These results indicate that a SAM-competitive PRMT5 inhibitor may be a better candidate because of its activities in both malignant phenotype inhibition and induction of tumor immunity. Consistent with this, we found better activity of EPZ004777 than EPZ015666 on our syngeneic tumor models (data not shown).

Our results suggest that treatment with a PRMT5 inhibitor in a syngeneic mouse erbB2/neu breast tumor model shows only a slight effect on tumor growth by itself, but significantly enhanced anti-erbB2 targeted antibody therapy. This model only demonstrated a small number of tumor-infiltrating Tregs; but, importantly and unexpectedly, the level of Tregs actually increases with targeted antibody treatment. This inhibitor limits antibody-dependent Treg infiltration.

In human patients, increased frequency of Tregs during trastuzumab therapy coincided with disease progression (57, 58). In addition, recent clinical observations indicate that early-stage HER2-positive breast cancers that did not react with neoadjuvant combinations such as docetaxel, carboplatin, and trastuzumab with or without pertuzumab demonstrate an immunosuppressive tumor microenvironment phenotypes, and this phenotypic change involves $CD4^+$ and $FoxP3^+$ cells that represent the Treg population (59). Thus, targeting Tregs during anti-$p185^{erbB2}$ targeted therapy may represent a therapeutic benefit to erbB2 driven breast cancer patients.

We did not see increased Tregs in humanized 4D5-treated tumors in our CT26Her2 tumor model (data not shown). This difference probably arises because CT26Her2 malignant growth is driven by the RAS oncogene and resistant to erbB2/neu targeted therapy. Rather, we observed inhibited Treg functions in DS-437 treated mouse lymph nodes and increased total $CD8^+$ T cells, $CD8^+$ PD-1^+ T cells, and $NKp46^+$ cells in their TIL. $CD8^+$ PD-1^+ T cells are known to be tumor-reactive T cells (60), indicating that DS-437 treatment induced tumor-specific immunity in those mice.

We found that the combination of 4D5 and DS-437 also increased the $NKp46^+$ cell population. NKp46 is a marker of NK cells, suggesting that the combination therapy also induced NK cell activity. We noted DS-437 does not affect the tumor associated macrophage's phenotype, suggesting that PRMT5 inhibition during anti-erbB2 mAb therapy would not be dependent on the macrophage activity.

Our studies identify PRMT5 as a FOXP3 binding partner and epigenetic modulator, and potentially important for Treg-targeting small molecules. PRMT5 deletion in Tregs caused scurfy-like symptoms and severe autoimmunity in the mice. PRMT5 deleted Tregs clearly showed proliferation abnormality and decreased suppressive functions. In this study we used many different inhibitors of PRMT5. Most of the inhibitors appeared to inhibit FOXP3 methylation. However, DS-437 and EPZ004777, which are SAM competitive inhibitors, displayed the most efficient suppression of FOXP3 methylation and greater activity in limiting Treg function.

Our experiments indicate that limiting PRMT5 function may promote tumor immunity by inhibiting Treg function and limiting Treg migration into tumors. Interestingly, PRMT5 levels and activity is reported to be elevated in several tumor cells and abnormal PRMT5 functions may contribute to some aspects of the malignant phenotype (37). Therefore, targeting PRMT5 coupled with targeted therapy represents a rational strategy for both cancer immunotherapy and tumor-targeted therapy.

AUTHOR CONTRIBUTIONS

YN performed *in vivo* and *in vitro* experiments, interpreted the results and wrote manuscript. MJ performed *in vivo* tumor study. FZ performed endogenous interaction of Foxp3 with PRMT5 in human Tregs. YX made Foxp3 constructs. YT and TK generated $CD4^{cre}$-$PRMT5^{fl/fl}$ mice. SF helped in the conceptual organization of the paper. MMG initiated efforts to define the role of tumor localized myeloid cells in the erbB2/neu targeted therapy. BL performed mass spectrometry and discovered Foxp3-PRMT5 interaction. TO generated $Foxp3^{creyfp}$-PRMT5fl/fl mice and performed bisulfite sequencing. HZ helped to design the *in vivo* tumor experiments. MIG wrote manuscript, interpreted the results, designed experiments and organized this project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00174/full#supplementary-material

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Anti-cancer Therapies Employing IL-2 Cytokine Tumor Targeting: Contribution of Innate, Adaptive and Immunosuppressive Cells in the Anti-tumor Efficacy

Lorenzo Mortara[1]*, *Enrica Balza*[2], *Antonino Bruno*[3], *Alessandro Poggi*[4], *Paola Orecchia*[5] *and Barbara Carnemolla*[5]

[1] Immunology and General Pathology Laboratory, Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy, [2] UOC Cell Biology, IRCCS Ospedale Policlinico San Martino, Genoa, Italy, [3] Vascular Biology and Angiogenesis Laboratory, Scientific and Technologic Park, IRCCS MultiMedica, Milan, Italy, [4] UOSD Molecular Oncology and Angiogenesis Unit, IRCCS Ospedale Policlinico San Martino, Genoa, Italy, [5] UOC Immunology Unit, IRCCS Ospedale Policlinico San Martino, Genoa, Italy

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***Correspondence:**
Lorenzo Mortara
lorenzo.mortara@uninsubria.it

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Antibody-cytokine fusion proteins (immunocytokine) exert a potent anti-cancer effect; indeed, they target the immunosuppressive tumor microenvironment (TME) due to a specific anti-tumor antibody linked to immune activating cytokines. Once bound to the target tumor, the interleukin-2 (IL-2) immunocytokines composed of either full antibody or single chain Fv conjugated to IL-2 can promote the *in situ* recruitment and activation of natural killer (NK) cells and cytotoxic $CD8^+$ T lymphocytes (CTL). This recruitment induces a TME switch toward a classical T helper 1 (Th1) anti-tumor immune response, supported by the cross-talk between NK and dendritic cells (DC). Furthermore, some IL-2 immunocytokines have been largely shown to trigger tumor cell killing by antibody dependent cellular cytotoxicity (ADCC), through Fcγ receptors engagement. The modulation of the TME can be also achieved with immunocytokines conjugated with a mutated form of IL-2 that impairs regulatory T (Treg) cell proliferation and activity. Preclinical animal models and more recently phase I/II clinical trials have shown that IL-2 immunocytokines can avoid the severe toxicities of the systemic administration of high doses of soluble IL-2 maintaining the potent anti-tumor effect of this cytokine. Also, very promising results have been reported using IL-2 immunocytokines delivered in combination with other immunocytokines, chemo-, radio-, anti-angiogenic therapies, and blockade of immune checkpoints. Here, we summarize and discuss the most relevant reported studies with a focus on: (a) the effects of IL-2 immunocytokines on innate and adaptive anti-tumor immune cell responses as well as immunosuppressive Treg cells and (b) the approaches to circumvent IL-2-mediated severe toxic side effects.

Keywords: anti-tumor therapy, IL-2, targeting immunotherapy, chemotherapy, T-cell responses, NK cells

INTRODUCTION

Viewing the tumor microenvironment (TME) like a critical orchestrator in tumor biology has been a central paradigm shift of the cancer field during the past two decades. Within this time, the distinct role of tissue-residing cells in promoting or suppressing tumor growth, metastasis and resistance to therapy has been gradually elucidated. Among the host-dependent biological features of the tumor, the hallmarks defined by Hanahan and Weinberg, such as the "evading immune destruction", the "tumor-promoting inflammation", and the "immune orchestration of angiogenesis" point out the key role of the immune system in neoplastic disease (1). Therefore, diverse cells both from innate or adaptive immunity, as a consequence of their plasticity, have been reported to acquire an altered phenotype and functions upon TME interaction; indeed, the cross-talk between TME and immune system leads to (a) attenuation of targeting and killing of tumor cells, (b) generation of tolerogenic/immunosuppressive behavior, and (c) acquisition of pro-angiogenic activities (1–5). As soon as the immune checkpoint inhibitors entered the clinic showing important and long lasting responses, the immune system gained greater attention in cancer biology.

Cytokines are molecular messengers, allowing immune cells to communicate with each other and with the TME compartments. Growing interest has been focused in exploiting the immune system to eradicate cancer using different cytokines. Nonetheless, toxicity and dual ambivalent activities of some cytokines (tumor promoting vs. tumor inhibiting) still remain relevant issues. In this context, immunotherapy approaches, and cytokine therapy, has been a promising strategy for the treatment of cancer (6).

IL-2 cytokine displays multiple immunological effects and acts by binding to the IL-2 receptor (IL-2R). The association of IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132) subunits results in the trimeric high affinity IL-2Rαβγ. CD25 confers high affinity binding to IL-2, whereas the β and γ subunits (expressed on natural killer (NK) cells, monocytes, macrophages and resting $CD4^+$ and $CD8^+$ T cells) mediate signal transduction (7, 8). It appears that the expression of CD25 is essential for the expansion of immunosuppressive regulatory T cells (Treg); on the other hand, cytolytic $CD8^+$ T and NK cells can proliferate and kill target cells responding to IL-2 by the IL-2Rβγ engagement in the absence of CD25 (9). The IL-2 cytokine acts as a master activation factor for helper/regulatory T cell and NK cell proliferation, differentiation and acts as a relevant mediator for pro- and anti-inflammatory immune responses (10). Treatment with IL-2 has been associated with stable and curative regressions in patients with metastatic melanoma, renal cancer and advanced non-Hodgkin's lymphomas, representing the first effective immunotherapeutic agent (11). Generally, IL-2 can evoke some mild common side effects, such as a flu-like syndrome, fever, asthenia, nausea, and vomiting. These side effects are more frequent and more relevant when IL-2 administration is associated with chemotherapy. However, the very rare observation of potential life-threatening clinical toxicities, such as vascular leakage syndrome (VLS), severe flu-like symptoms and coma has recommended that the IL-2 be employed under the supervision of the oncologist in a hospital setting. IL-2 immunocytokines have been developed both to avoid these undesired clinical side effects, and to target cancer cells with specific anti-tumor antibodies fused with IL-2 that can elicit a potent anti-tumor immunological response within the immunosuppressive TME.

Here, according to the literature reviewing, we summarized and discussed: (a) the effects of IL-2 immunocytokines on innate and adaptive anti-tumor immune response and (b) their use in combination with other immunocytokines, chemio- and radio-therapy, immune checkpoint blockade, and immunotherapies.

IL-2-TARGETED ANTI-CANCER THERAPIES

IL-2 was identified in 1976 as a T cell growth factor and later approved for treatment of patients with metastatic melanoma and renal cell carcinoma with beneficial results in a subset of patients (11, 12). Administration of high-dose IL-2 can be associated with relevant adverse effects that include the VLS, fever, chills, malaise, hypotension, organ dysfunction and cytopenia, well-reviewed previously (13–17). Low-doses of IL-2 lead to the preferential expansion of Treg cells; this is an unwanted effect in anti-cancer immunotherapy (18). To overcome the toxicity related to the systemic administration of IL-2 at high-dose, diverse IL-2 immunocytokines composed of IL-2 fused to antibodies directed against tumor-associated antigens (TAAs) have been tested in preclinical models with promising results (10, 19–48). Indeed, some IL-2 immunocytokines are currently in phases I–II of several clinical trials, in combination with other therapeutics (49–56) (https://www.clinicaltrials.gov). These immunocytokines showed beneficial effects for a wide range of tumor types with manageable and reversible side effects and toxicities (49–51, 53, 54, 57–60). IL-2 immunocytokines-targeted proteins are represented by either surface membrane TAAs, such as disialoganglioside 2 (GD2), epithelial cell adhesion molecule (EpCAM), carcinoembryonic antigen (CEA), CD20, and CD30 or proteins belonging to tumor extracellular matrix (ECM) like extra domain A (ED-A) and B (ED-B) of Fibronectin A-FN and B-FN, respectively and tenascin-C. The targeting of proteins expressed on the cell surface of tumor cells presents some limitations, such as the transitory expression of TAAs, the rapid immunocytokine internalization, localization and degradation into the lysosomal compartment, determining failure of the expected therapeutic effect. The tumor ECM proteins have been proposed as good targets due to the over-expression of isoforms absent or barely expressed in the ECM of normal tissues (61, 62). An overview of IL-2-immunocytokines in preclinical and clinical development for treatment of cancer is summarized in **Table 1** and some examples will be discussed here.

Among the IL-2 immunocytokines directed to ECM, the first and most studied was L19-IL-2. L19-IL-2 is specific for the angiogenesis-associated B-FN isoform selectively accumulated on tumor neovasculature; L19-IL-2 showed a good anti-tumor activity in preclinical models both in solid and hematological

TABLE 1 | IL-2-immunocytokines in preclinical and clinical development for treatment of various types of cancer.

IL-2-immunocytokine (target)	Type of study	Indication	Administration	Results	References
L19-IL2 (B-FN)	Clinical (PhI/II)	Met. Melanoma (stage III)	IT	A complete response was achieved in 25% of patients with met. melanoma.	(49)
	Clinical (PhI/II)	Solid tumors and metastatic renal cell carcinoma	IV infusion	The immunocytokine can be safely administered in patients with advanced solid tumors with clinical activity in patients with mRCC.	(50)
L19-IL2	Preclinical	Teratocarcinoma; Orthotopic pancreatic cancer	IV	Tumor volumes were significantly reduced after therapy.	(10, 19)
L19-IL2 plus L19-TNF	Clinical (PhI/II)	Metastatic Melanoma (stage IIIC/IVM1a)	IT	Effective methods for local control of inoperable lesions.	(58)
L19-IL2 plus Dacarbazine	Clinical (PhI/II)	Metastatic Melanoma	IV infusion	More than 60% of patients were still alive 12 months of the start treatment.	(51)
L19-IL2 plus L19-TNF	Preclinical	Fibrosarcoma	IT	Totally eradication of fibrosarcoma and acquired protective immunity (100%).	(21)
	Preclinical	Teratocarcinoma		Totally eradication of smaller teratocarcinoma.	(22)
	Preclinical	Neuroblastoma		Neuroblastoma eradication in 70% of treated mice that aquired a protective immune response.	(23)
	Preclinical	Myeloma	IV	Tumor eradication in 58% of treated mice.	(24)
L19-IL2 plus RT	Preclinical	Colon, Lung and mammary carcinomas	IV	The combination therapy cured 75% of colon carcinoma bearing mice, induced addittive effect for lung carcinoma and no effect in the mammary model.	(25)
	Preclinical	Colon carcinoma	IV	Long-lasting immunological protecting against tumors in colon carcinoma models.	(26, 27)
	Preclinical	Teratocarcinoma	IV	Survival increase when RT was administrated before L19-IL2 in teratocarcinoma model.	(28)
L19-IL2 plus CTLA-4 (mouse analog of Ipilimumab)	Preclinical	Teratocarcinoma	IV/IT	The treatment induced 20–40% survival in teratocacinoma bearing mice.	(22)
	Preclinical	Colon carcinoma	IV/IT	Totally colon carcinoma eradication and acquired protective immunity (100%).	(22)
L19-IL2 plus OC-46F2 (scFv anti CD138)	Preclinical	Metastatic melanoma	IV	Combined therapy led to a complete tumor eradication until day 90 from tumor implantation in 71% of treated mice with significant differences compared to monotherapy.	(29)
L19-IL2 plus Rituximab	Preclinical	Non Hodgkin's lymphoma	IV	Complete remission in 75% or in 28.6% of the treated mice in localized or disseminated MCL, respectively.	(30)
				Complete remission in 80% or in 100% of the treated mice in two different lymphoma models.	(31)
L19-IL2 plus AAZ+-ValCit-MMAE	Preclinical	Renal cell and colon carcinoma	IV	The combination of the two agents induced complete remissions in all mice.	(63)
F8-IL2 (EDA-FN)	Preclinical	Lung adenocarcinoma	IV	Dose dependent therapeutic efficacy and survival increase in the treated mice.	(32)
F8-IL2 plus F8-TNF	Preclinical	Teratocarcinoma, Fibrosarcoma, Lung carcinoma; Melanoma	IT	Eradication of neoplastic lesions (50–75%).	(33)
F8-IL2 plus Paclitaxel or Dacarbazine	Preclinical	Melanoma	IV	Tumor eradication in the 82% of F8-IL2 plus Paclitaxel treated mice.	(21, 34)
F8-IL2 plus Sunitinib	Preclinical	Human renal cell carcinoma	IV	Partially tumor eradication (28%).	(35)
F8-IL2 plus F8-TNF (EDA FN)	Preclinical	Acute myeloid leukemia	IT	No tumor eradication.	(33)
F8-IL2 plus F8-SS-CH2CEM	Preclinical	Acute myeloid leukemia	IV	Complete and long-lasting tumor eradication in 80% of treated mice that acquired protective immunity (100%).	(36)

(Continued)

TABLE 1 | Continued

IL-2-immunocytokine (target)	Type of study	Indication	Administration	Results	References
F8-IL2 plus Cytarabine	Preclinical	Acute myeloid leukemia	IV	Complete and long-lasting tumor eradication in 100% of immunocompetent treated mice.	(37)
F16-IL2 (TNC) plus Temozolomide	Preclinical	Human glioblastoma	IV; IP	Totally tumor eradication.	(38)
F16-IL2 plus Doxorubicin or Paclitaxel	Preclinical	Human breast cancer	IV	Significant therapeutic benefit compared monotherapies.	(39)
F16-IL2 plus Cytarabine (low dose)	Clinical (Phl/II)	Acute myeloid leukemia in patients relapsed after chemotherapies	IV infusion	Stimulation of effector cells at the bone marrow site.	(52)
hu14.18-IL2 (GD-2)	Clinical (Phl/II)	Metastatic Melanoma	IV infusion	One patient out fourteen had a partial response while four had stable disease.	(53)
	Clinical (Phl/II)	Neuroblastoma	IV infusion	Patients with disease evaluable with (123)I-MIBG scintigraphy and/or BM histology had a 21.7% CR.	(54)
	Clinical (Phl/II)	Cutaneous melanoma (stage IV)	IV infusion	There are no major objective tumor responses.	(55)
hu14.18-IL2	Preclinical	Neuroblastoma	IV/IT	Eradication of established bone marrow and liver metastases.	(20, 40)
hu14.18-IL2 plus anti-CTL-4 plus RT	Preclinical	Primary and metastatic melanoma	IT; IV	The triple-combination eradicated large tumors and metastasis, and improved animal survival compared with combinations of any two treatments.	(41)
hu14.18-IL2 plus IL-2	Preclinical	Neuroblastoma	IV	Prolonged tumor eradication of established tumors and acquired protective immunity.	(42)
antiCEA-IL2 (CEA)	Preclinical	Colon carcinoma	IV	Tumor volumes were significantly reduced after therapy.	(43)
antiCEA-IL2v	Preclinical	MC38-CEA and syngeneic pancreatic PanO2-CEA models	IV	Statistically significant increase in survival in both models.	(44)
anti CEA-IL2v Plus anti muPDL1 or Cetuximab or trastuzumab or imgatuzumab	Preclinical	Pancreatic ADK; lung, breast, colon and gastric cancers	IV	Preclinical results support the use of CEA-IL2v for combination immunotherapy with ADCC-competent or -enhanced antibodies of the IgG1 isotype, T cell bispecific antibodies that rely on $CD8^+$ T effector cells and also with PD-L1 checkpoint blockade in immunogenic tumor.	(44)
huKS/IL-2 (EpCAM)	Preclinical	Melanoma and neuroblastoma	IT; IV	Significant anti tumor effect of immunocytokyne when IT administered vs. IV.	(27)
huKS/IL-2 plus Paclitaxel and Cyclophosphamide	Preclinical	Colon, lung and mammary carcinomas	IP; IV	Combined treatments resulted in enhanced anti tumor responses.	(45)
huKS/IL-2 plus Cyclophosphamide	Clinical (Ph II)	Small-cell lung cancer (SCLC)	IV infusion	The combination therapy was well-tolerated in extensive-disease SCLC, but did not show PFS and OS compared with best supportiva care.	(56)
DI-Leu16-IL2 (CD20)	Preclinical	Human lymphoma	IV	The modified anti-CD20 antibody fused IL2 retained full anti-CD20 activity but had enhanced ADCC respect to the unfused antibody or control Rituximab.	(46)
HRS3scFv-IL12-Fc-IL2 (CD30)	Preclinical	Hodgkin's lymphoma	IV	Suppressed tumor growth in immunocompetent mice compared to the control.	(48)
B3-IL2 (PDL1)	Preclinical	Orthotopic pancreatic carcinoma	IP	Reduced tumor growth (50%)	(47)
B3-IL2 plus TA99	Preclinical	Melanoma	IP	Slowed tumor growth and prolonged mice survival	(47)
NHS-IL2/IL2LT (DNA-histone complex)	Preclinical	Experimental lung and liver metastasis	IV	NHS-IL2LT retained anti tumor activity against established neuroblastoma and non–small cell lung cancer metastases in syngeneic mouse tumor models.	(59)
NHS-IL2+Cisplatin+RTX	Preclinical	Murine Lewis lung carcinoma	IP/IV	The combination of cisplatin plus radiotherapy with NHS-IL2 resulted in marked tumor reduction and delayed outgrowth that was statistically significant.	(60)

tumors (**Table 1**). A complete tumor eradication was reported when L19-IL-2 was administered in combination with CTLA-4 blockade in two syngeneic immunocompetent mouse models of teratocarcinoma and colon carcinoma; in the latter model, responder mice to this combination treatment were resistant to tumor re-challenge (22). Complete remissions of established localized lymphomas were induced when L19-IL-2 was co-administered with the anti-CD20 antibody rituximab (31). Recently, similar preclinical results were observed in renal cell carcinoma and colorectal cancer models when L19-IL-2 was co-administered with a small molecule-drug conjugate, capable of selective homing to tumor cells expressing surface carbonic anhydrase IX (63). Rekers et al. have recently reported that radiotherapy (RT) combined to systemic administration of L19-IL-2, resulted in a long-lasting immunological protection against tumors in mouse colon carcinoma. These authors hypothesize that the IL-2 immunocytokine and RT could elicit an immune-mediated abscopal effect with tumor regression far from the irradiated tumor field (25–28). Preclinical data have shown that a combination of L19-IL-2 and L19-TNF-α induced complete remission when administered as a single intratumoral injection in two immunocompetent mouse models of melanoma and sarcoma (21). These promising results led to multicenter phase II trials with the intralesional application of L19-IL-2 as single agent or in combination with L19-TNF-α in stage IIIB/IIIC and IVM1 melanoma patients as recently reviewed by Weide et al. (64).

Cancer immunotherapy holds promising synergistic potential when combined with chemotherapy and anti-angiogenic therapy. However, to our knowledge, the combined therapy of L19-IL-2 with anti-angiogenic drugs, such as bevacizumab, has not been reported until now. It is known the pro-angiogenic role of the cell surface proteoglycan syndecan-1 (CD138) whose ectodomain is a target of ADAM17 sheddase activity. The soluble ectodomain of syndecan-1 binds several matrix effectors (e.g., VEGF, FGF-2, or cytokines) and presents them to the corresponding cell surface receptors promoting angiogenesis and tumor growth (65, 66). We have recently reported that blocking syndecan-1 activity via the specific antibody OC-46F2 leads to an anti-tumor effect by inhibiting vascular maturation and tumor growth in experimental models of human melanoma and ovarian carcinoma (67). Furthermore, we have shown that OC-46F2, in combination with the L19-IL-2 resulted in complete inhibition of melanoma growth until day 90 from tumor implantation in 71% of treated mice with a significant increase of their tumor free survival (29).

The hu14.18-IL-2 immunocytokine containing a humanized anti-GD2 mAb linked to IL-2 was mainly studied as mono-therapy in neuroblastoma and in combination with anti-CTLA-4 plus RT in primary and metastatic melanoma. The triple-combination therapy eradicated large tumors and metastasis improving animal survival (41). A still ongoing phase II clinical trial using hu14.18-IL-2, reported stable disease in four patients and a partial response in one patient out of fourteen in metastatic melanoma (53) while not significant results were shown by the other two phase I/II trials already completed (54, 55).

Recently, it has been described as a novel class of monomeric tumor-targeted immunocytokines in which a single engineered IL-2 variant (IL-2v) with abolished CD25 binding is fused to the C-terminus of an antibody against the CEA or fibroblast activation protein-α (FAP). CEA-IL-2v and FAP-IL-2v demonstrated superior safety, pharmacokinetics and tumor targeting, while lacking preferential induction of Treg cells due to abolished CD25 binding. At the same time, these constructs showed monovalency and high-affinity tumor targeting as compared to classical IL-2 based immunocytokines. They retain the capacity to activate and expand NK and $CD8^+$ effector T cells through IL-2Rγβ in the periphery and in the TME (Klein C.; 1st Immunotherapy of Cancer Conference (ITOC1) Munich, Germany, 2014) (44, 68).

Both in MC38-CEA and syngeneic pancreatic PanO2-CEA models, animals treated with CEA-IL2v monotherapy showed a statistically significant increase in median survival compared to untreated animals. Moreover, CEA-IL2v treatment resulted in a superior efficacy when administered in combination with PD-L1 checkpoint blockade or with ADCC competent antibodies, such as trastuzumab and cetuximab (44). In the syngeneic PancO2 model, similar results were recently described using FAP-IL2v associated with a CD40 agonistic and PD-L1 inhibitory checkpoint antibodies as reported by Nicolini V. et al. at AACR Annual Meeting 2018; Chicago, IL (https://www.clinicaltrials.gov, NCT02627274; NCT03386721).

Although it is not an immunocytokine, it is important to analyze the effects of OMCP-mutIL-2, a mutated form of IL-2 (mutIL-2) linked to a high-affinity NKG2D ligand (OMCP), which is directed to cytotoxic immune effector cells rather than tumor cells. This targeted therapy resulted in preferential binding to and activation of NK cells rather than Treg cells and a significant decrease of tumor growth was obtained after treatment with OMCP-mutIL-2 in mouse models of Lewis lung carcinoma (LLC) (69).

OUTSTANDING RELEVANCE OF TRIGGERING NK CELL ACTIVITY IN THERAPEUTIC EFFECT OF IL-2 IMMUNOCYTOKINES

It is well-established that IL-2 can trigger T lymphocytes to expand and acquire a therapeutic anti-tumor activity (70). Noteworthy, cytotoxic $CD8^+$ T cells can be activated with IL-2 mainly by IL-2Rβγ, whereas regulatory T cells can efficiently respond to IL-2 through the IL-2Rαβγ complex (71–75). These peculiar features of $CD8^+$ T cells have been used to design unique IL-2 molecules and favor the expansion of cytotoxic anti-tumor rather than regulatory T lymphocytes (72–75). Likewise, NK cells can respond efficiently to IL-2 through the IL-2Rβγ in the absence of IL-2Rαβγ heterotrimer (18, 70, 71, 76). Since NK cell can kill their target without prior sensitization or priming, they may represent a good candidate to respond to *in vivo* during administration of immunocytokines composed of IL-2 (20, 38, 70, 77). This is the case for the hu14.18-IL-2 immunocytokine, where depletion of NK cells resulted in

the abrogation of the anti-tumor response detected *in vivo* in preclinical murine model of NXS2 neuroblastoma (20). Furthermore, the effect of hu14.18-IL-2 immunocytokine was strongly enhanced when combined with poly I:C or recombinant mouse IFN-γ which can be considered potent NK cell stimulating factors (20). Impressively, only NK cells, but not $CD8^+$ T cells, isolated from these mice exerted a detectable cytolytic activity against the NK cell target YAC-1. This would indicate that in this murine model system NK cells can cure from neuroblastoma. It is not clear whether this effect is dependent only on IL-2-mediated activation of NK cells, or other cytolytic effector cells, such as NK-like T and/or γδ T cells not expressing CD8. In addition, both poly I:C and IFN-γ can be potent stimulators of antigen presenting cells (APC) as monocytes and monocyte-derived dendritic cells (mDC) (20, 78, 79). More importantly, APC can produce IL-12 (79), a strong inducer of NK cell cytotoxicity, and it is still to be defined whether poly I:C and IFN-γ can exert both direct and indirect effect on NK cell activation. We can speculate that the crosstalk between NK and DC, further reinforced by the triggering with poly I:C and IFN-γ of both NK and DC, could generate a positive loop to produce high IL-12 and amplify NK cell response (80, 81); this could eventually generate a Th1 microenvironment favoring anti-tumor adaptive immune response (**Figure 1A**).

Regarding the therapeutic efficiency of NK cells under administration of IL-2 immunocytokines, it is relevant to analyze the role of major histocompatibility complex antigens (82). It is well-established that NK cells in allogeneic hematopoietic stem cell transplant (HSCT) can show the so called "killer immunoglobulin-like receptor (KIR)/KIR-ligand incompatibility". An improvement of leukemia control is related to a difference in HLA-I between the donor and recipient because the corresponding KIR expressed on NK cell does not recognize the HLA-I antigen (83). Thus, due to the KIR/KIR-ligand mismatch the KIR on NK cell donor does not deliver a signal in NK cell leading to inhibition of NK-cell mediated killing of residual leukemia cells present in the recipient. Importantly, the KIR/KIR-ligand mismatch can happen also in an autologous setting (84, 85). In relapsed/refractory neuroblastoma patients, hu14.18-IL-2 immunocytokine administered to the cohort with the KIR/KIR-ligand mismatch showed a better anti-tumor response to that of the matched cohort patients (82). Thus, the KIR/KIR-ligand mismatch analysis should be associated with the immunocytokine therapy to further improve the NK cell response in anti-neuroblastoma activity (82). The involvement of NK cells in the therapeutic effect of IL-2 immunocytokine has been further confirmed in targeting the tumor stroma with the F16-IL-2 immunocytokine (52). In point of fact, F16-IL-2 treatment of acute myeloid leukemia (AML) relapsed patients after HSCT led to a massive accumulation of lymphocytes in the bone marrow and $CD56^+CD16^+$ NK cells represented the most prominent increment, besides γδT and $CD8^+$ T cells (52). In addition, lymphocytes appeared in contact with clusters of leukemic blasts suggesting that a recognition of tumor cells and formation of immunological synapses have been clearly established in F16-IL-2 treated patients.

ROLE OF ADAPTIVE T-CELL RESPONSES IN THE ANTI-TUMOR THERAPEUTIC EFFICACY IN IL-2 IMMUNOCYTOKINES TREATMENTS

L19-IL-2 has been largely studied in different tumor preclinical mouse models, indicating a powerful action of this compound in the ability to induce a pro-inflammatory reaction and a tumor influx of lymphocytes together with an IFN-γ response and NK and/or T cell responses. Interestingly, in the CT26 colon carcinoma murine model, L19-IL2 as well as anti-CTLA-4 mAb treatments have been shown to be active as single agents; importantly, the combination of the IL-2 immunocytokine and the immune checkpoint blocker determined an enhanced anti-tumor therapeutic effect and a prolonged survival (22). In this tumor model, treated and cured mice have been protected following tumor re-challenging, indicating memory of anti-tumor immunity. But there was no synergy between L19-IL-2 and anti-PD-1 checkpoint blockade with increased survival of CT26-tumor bearing mice; this would suggest that to obtain with this IL-2 immunocytokine a synergistic anti-tumor effect a distinctive immune checkpoint blocker should be targeted. Intratumoral L19-IL-2 in combination with L19-TNF-α immunocytokine led to a complete cure of 100% treated F9 teratocarcinoma-bearing mice. By contrast, in athymic mice, tumor rejection capacity elicited by the combination of these immunocytokines was impaired; only a delayed tumor growth in comparison to the control immunocompetent mice was observed; this points out the relevance of T cell response for the complete eradication of tumors (22). Synergy between L19-IL-2 and L19-TNF-α has been also documented in neuroblastoma models (**Figure 1B**) (23). L19-TNF-α is also considered a crucial element in combined anti-tumor immunotherapeutic strategies, showing encouraging results in both preclinical (86–88) and clinical studies (58).

Intratumoral injections with hu14.18-IL-2 in neuroblastoma NXS2 murine model showed an enhanced inhibition of tumor growth and prolonged survival compared with controls; this therapeutic effect involved both NK and T cells localized *in situ* and peripheral blood (40). Interestingly, after intratumoral injection, an enhanced proportion of both NK and T cells expressing NKG2A/C/E antigens in comparison to control mice and intravenous-treated tumor-bearing mice was detected. Moreover, this therapeutic approach induced a remarkable tumor infiltration of $CD8^+$ CTLs, $CD4^+$ T cells as well as macrophages. In *in vivo* immune cell subsets depletion assays demonstrated a key role for $CD4^+$, $CD8^+$ T and NK cells. Remarkably, the hu14.18-IL-2 immunocytokine has been shown to synergize with local RT and systemic checkpoint blockade (anti-CTLA-4 mAb) to eradicate large tumor and metastases in different tumor murine models (38). An important issue derived from these studies was that the cooperative effect was mediated, at least in part, by NK cells through ADCC and that the use of tumor-specific IL-2 immunocytokine led to memory T-cell responses (38).

The F8-IL-2 immunocytokine when used as monotherapy in a metastatic adenocarcinoma lung mouse model resulted in

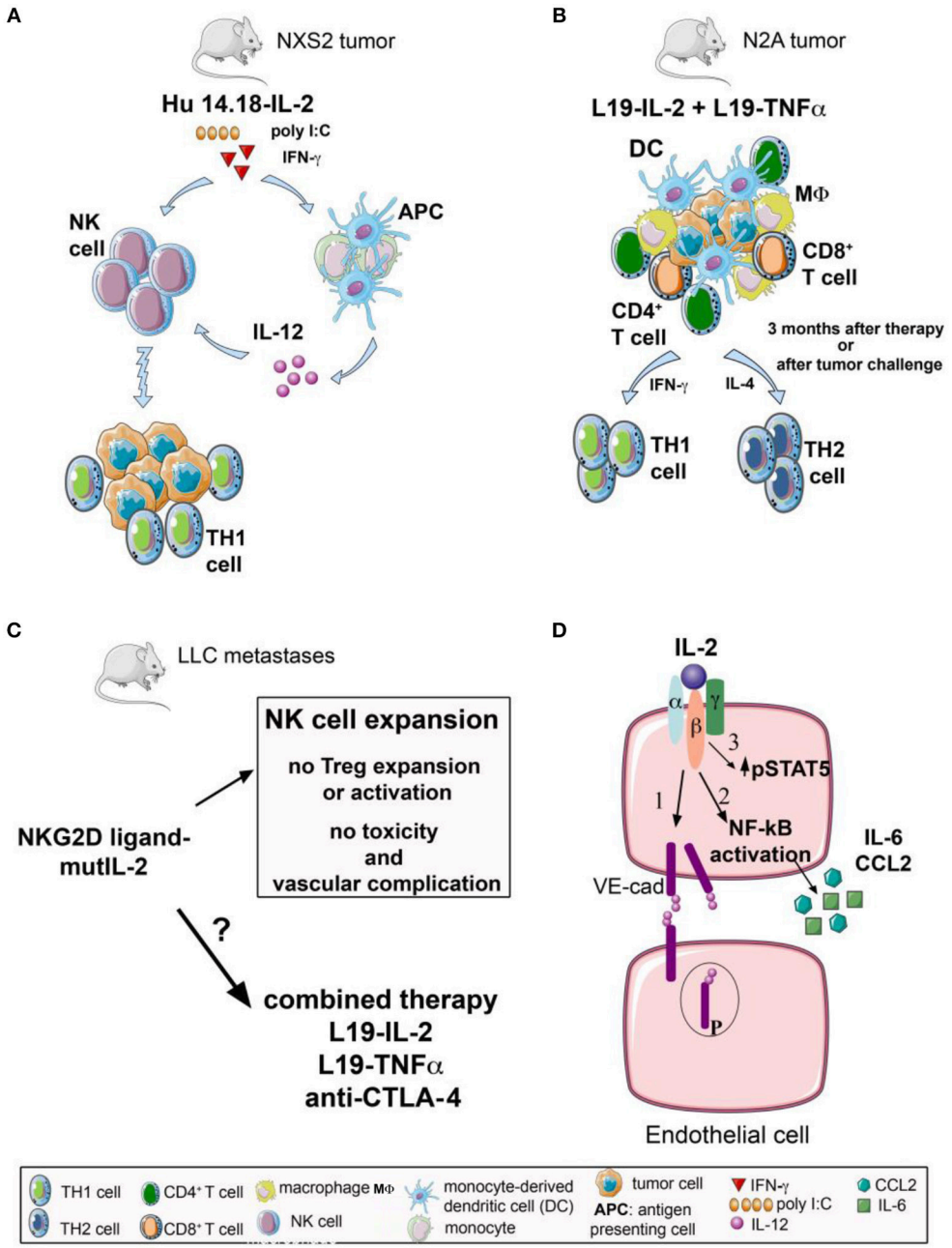

FIGURE 1 | Effects on innate and adaptive immune response of IL-2 immunocytokines and IL-2 fusion protein either alone or in combination with other therapeutic approaches, and IL-2 mediated modulation of endothelial cells. **(A)** The NK cell stimulating effect of hu14.18-IL2 immunocytokine, containing a humanized anti-GD2 mAb linked to IL-2, is strongly enhanced when combined with poly I:C or recombinant mouse IFN-γ. Poly I:C and IFN-γ can be potent stimulators of antigen presenting cells (APC) as monocytes and monocyte-derived dendritic cells (mDC) that can produce IL-12, a strong inducer of NK cell cytotoxicity. This mechanism could eventually generate a Th1 microenvironment favoring anti-tumor adaptive immune response. **(B)** L19-IL-2 in combination with another immunocytokine, L19-TNF-α, shows therapeutic synergistic effects in neuroblastoma N2A murine model. 70% of systemically treated mice result in a specific long-lasting anti-tumor immune memory, with efficient priming of $CD4^+$ T helper cells and $CD8^+$ CTL effectors, massive tumor infiltration of $CD4^+$, $CD8^+$ T cells, macrophages and dendritic cells, accompanied by a mixed Th1/Th2 response. **(C)** The use of a fusion protein consisting in a mutated form of IL-2 targeting NKG2D-positive cells (OMCP-mutIL2) is employed as a monotherapy, in a preclinical model of Lewis lung carcinoma (LLC). This protocol is highly efficient in stimulating anti-tumor NK cells and their cytotoxicity with no involvement of Treg cells and in absence of vascular-related toxicity. It is still to be investigated if OMCP-mutIL2 can display a synergistic effect in those combination therapies which trigger the anti-tumor adaptive T cell response. **(D)** IL-2 is able to interact with IL-2R complex (IL-2Rβ and IL-2Rγ) on brain microvascular endothelial cells (BMEC) inducing: (1) destabilization of adherent junctions through an increase in VE-cadherin (VE-cad) phosphorylation and internalization accompanied by NF-kB activation, and (2) release of pro-inflammatory mediators, such as CCL2 and IL-6, resulting in brain oedema. Moreover, (3) IL-2 binds directly to $CD25^+$ lung endothelial cells with an increase of STAT5 phosphorylation inducing pulmonary oedema.

strong tumor infiltration of both $CD3^+$ T and NK cells but not of Treg cells and $F4/80^+CD11b^+$ macrophages. Of note, in this model, TILs also contained an enhanced percentage of intratumoral proliferating $Ki67^+$ Granzyme B^+ $CD8^+$ T cells (32).

Finally, monotherapy with OMCP-mutIL2, demonstrated a strong NK cell-mediated anti-tumor effect but no involvement of adaptive immune response, at least in the LLC model (66). It is still to be elucidated if OMCP-mutIL2 could have additive or synergistic anti-tumor effects in association with therapies that can trigger T-cell responses (**Figure 1C**).

COULD IL-2 BE DIRECTLY INVOLVED IN THE TUMOR VESSELS DESTRUCTION?

As mentioned above, one of the major and potentially fatal side effects upon administration of high-dose IL-2 is the VLS. This syndrome is characterized by the accumulation of fluid in the extravascular space in multiple organs, such as heart, lung, kidney, and brain. IL-2 can induce VLS acting either indirectly or directly on endothelial cells. Indeed, VLS is caused by the release of pro-inflammatory cytokines, such as TNF-α from IL-2–activated NK cells (89); in turn, this TNF-α alters the vascular permeability. Furthermore, IL-2 is able to induce both pulmonary and brain oedema binding directly CD25 expressed on lung and brain microvascular endothelial cells (BMEC); this engagement leads to disruption of the integrity of lung vascular permeability and blood-brain barrier (BBB) (90, 91).

Moreover, IL-2 interacts on BMEC with intermediate affinity IL-2Rβγ complex inducing destabilization of adherent junctions through an increase in VE-cadherin phosphorylation and internalization accompanied by NF-kB activation; this results in the release of pro-inflammatory mediators, such as CCL2 and IL-6 (**Figure 1D**) (91, 92). Thus, it is essential that the targeted therapy with IL-2 immunocytokines should avoid, or at least reduce, the VLS in healthy organs. Importantly, recent results from tumor-targeting IL-2 immunocytokines composed of variant forms of IL-2 lacking vascular effects and low or absent Treg cell stimulation have shown promising new avenues for IL-2 applications (44, 69, 90, 93). In addition, the molecular and biochemical mechanisms of IL-2-mediated activation of endothelial cells could be investigated *in vitro* and *in vivo* on several types of tumor-associated cells (e.g., tumor-associated endothelial cell and tumor-associated fibroblast) expressing the different chains of IL-2R; this could be potentially exploited for the treatment of tumors.

CONCLUSION

IL-2 therapy can lead to durable responses in cancer patients but it is associated with significant toxicity and even life-threatening syndromes. IL-2 immunocytokines, alone or in combination with other immunocytokines, checkpoint blockade, chemio-, radio- and/or immunotherapies showed cooperative anti-tumor effects without relevant toxicities; indeed, the vast majority of preclinical tumor models have shown a strong therapeutic response to IL-2 immunocytokine. This is the firm starting point to employ IL-2 immunocytokine to improve the patients' survival and to treat metastatic cancers as well.

AUTHOR CONTRIBUTIONS

LM, EB, AB, AP, PO, and BC planned, organized, wrote and revised the manuscript, and prepared the figure and table.

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REVIEW
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Targeting Myeloid Cells in Combination Treatments for Glioma and Other Tumors

*Andy S. Ding†, Denis Routkevitch†, Christina Jackson and Michael Lim**

Department of Neurosurgery, Johns Hopkins School of Medicine, Baltimore, MD, United States

Myeloid cells constitute a significant part of the immune system in the context of cancer, exhibiting both immunostimulatory effects, through their role as antigen presenting cells, and immunosuppressive effects, through their polarization to myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages. While they are rarely sufficient to generate potent anti-tumor effects on their own, myeloid cells have the ability to interact with a variety of immune populations to aid in mounting an appropriate anti-tumor immune response. Therefore, myeloid therapies have gained momentum as a potential adjunct to current therapies such as immune checkpoint inhibitors (ICIs), dendritic cell vaccines, oncolytic viruses, and traditional chemoradiation to enhance therapeutic response. In this review, we outline critical pathways involved in the recruitment of the myeloid population to the tumor microenvironment and in their polarization to immunostimulatory or immunosuppressive phenotypes. We also emphasize existing strategies of modulating myeloid recruitment and polarization to improve anti-tumor immune responses. We then summarize current preclinical and clinical studies that highlight treatment outcomes of combining myeloid targeted therapies with other immune-based and traditional therapies. Despite promising results from reports of limited clinical trials thus far, there remain challenges in optimally harnessing the myeloid compartment as an adjunct to enhancing anti-tumor immune responses. Further large Phase II and ultimately Phase III clinical trials are needed to elucidate the treatment benefit of combination therapies in the fight against cancer.

Keywords: combination immunotherapy, myeloid therapy, glioma, myeloid-derived suppressor cells, checkpoint inhibitors, radiation, chemotherapy, tumor-associated macrophages

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****Correspondence:***
Michael Lim
mlim3@jhmi.edu

†These authors have contributed equally to this work

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INTRODUCTION

The recent rise to prominence of immunotherapy into the forefront of cancer treatment has resulted in an abundance of research aimed at harnessing various components of host immunity in anti-tumor treatments. Immunotherapy efforts have historically focused on boosting the activities of the lymphocyte compartment, specifically $CD8^+$ cytotoxic T lymphocytes (CTLs), with the use of immune checkpoint inhibitors (ICIs), chimeric antigen receptor (CAR) T cells, peptide vaccines, and oncolytic viral therapy. While T cell-based therapies, particularly those involved with immune checkpoint inhibition, have shown improved survival and tumor regression in multiple systemic cancers including non-small cell lung cancer and melanoma, their benefits are not universal. The efficacy of T cell-based therapies is predicated on the presence of tumor

infiltrating lymphocytes (TILs); tumors with fewer TILs are less responsive to these therapies and are considered immunologically "cold tumors." Myeloid cells are a significant, yet sometimes overlooked component of immunotherapy. In normal physiologic states, myeloid cells play an important role in innate immunity while also contributing to the adaptive immune response through antigen presentation. However, in the setting of cancer, they can be induced by a multitude of factors to adopt an immunosuppressive phenotype that can lead to the inhibition of anti-tumor responses by CTLs. These suppressive myeloid cells are particularly abundant in immunologically cold tumors prompting increasing efforts to target these cells to improve the efficacy of immunotherapy. Furthermore, there is increasing evidence that adjuvant therapies such as chemotherapy and radiation can have conflicting effects on the efficacy of immunotherapy, with the potential to be synergistic or antagonistic when reshaping the myeloid population. Therefore, it is critical to understand the interplay between the tumor, immune cells, and adjuvant therapy to fully optimize the efficacy of immunotherapy.

The myeloid compartment is especially relevant in the study of gliomas, including glioblastoma (GBM), which is the most aggressive and most common primary central nervous system (CNS) malignancy in adults with a dismal median overall survival of 12–15 months even with the current standard care of surgery followed by adjuvant chemoradiation (1). A growing body of evidence has highlighted the poor immunogenicity of GBM with a paucity of $CD8^+$ CTLs, relative abundance of $Foxp3^+$ regulatory T cells (Tregs), high infiltration of tumor-associated immunosuppressive macrophages and microglia (TAMs), and presence of myeloid-derived suppressor cells (MDSCs) (2–5). These factors are likely responsible for the minimal efficacy of T cell-based therapies in GBM. MDSCs are divided into two groups: granulocytic/polymorphonuclear (PMN-MDSCs) and monocytic (M-MDSCs) which are phenotypically and morphologically similar to neutrophils and monocytes, respectively. Although similar to typical myeloid cells, M-MDSCs are distinguishable from monocytes by low/absent expression of HLA-DR, while PMN-MDSCs are distinguishable from neutrophils by LOX-1 expression (6). Studies have shown TAMs and MDSCs to constitute a large proportion of tumor infiltrating immune cells in the GBM tumor microenvironment (TME) (7), ranging from 30 to 90% in human GBM samples, with $CD11b^+$ MDSCs comprising the majority of infiltrating inflammatory cells in human gliomas (8, 9).

Unique to gliomas and other brain tumors, a significant portion of the tumor-associated myeloid compartment consist of microglia, the resident macrophages of the CNS. Historically, these tumor-associated macrophages and microglia have been used interchangeably. The advent of genome-wide microarray and single-cell RNA sequencing analyses have allowed for phenotypic and transcriptomic differentiation between these two populations. These studies have demonstrated that microglia are characterized by low expression of CD45 and major histocompatibility complex II (MHCII), absence of C-C motif chemokine receptor 2 (CCR2), and high expression of purinergic receptor P2RY12, C-X3-C motif chemokine receptor 1 (CX3CR1), and transmembrane protein 119 (TMEM119), while blood-derived macrophages demonstrate high expression of CD45, MHCII, and tyrosine-protein kinase Mer (MERTK) (10–12). Single-cell RNA sequencing of gliomas have also shown that microglial TAMs are enriched in the leading edge of the tumor and surrounding white matter, while blood-derived TAMs are more often found within regions of microvascular proliferation and peri-necrotic regions within the core of the tumor. This is correlated with higher expression of pro-inflammatory factors in the periphery and anti-inflammatory factors in the core (13). In fact, TAMs that originate from the blood and migrate to brain tumors, where they adopt a more tissue-specific phenotype, have been shown to have a distinct metabolism as well as increased expression of immunosuppressive markers when compared to microglia (14). Additionally, as glioma grade increases, the ratio of blood-derived TAMs to microglia concurrently increases (15). However, despite the increased tendency in microglia toward a pro-inflammatory phenotype, both cell types have the potential for tumor-based induction toward MDSCs and can thus be targets for myeloid therapy (16).

For tumors that endorse a myeloid-enriched TME, like gliomas, therapies that are able to re-program the immunosuppressive myeloid population back to immunostimulatory phenotypes or limit the function of TAMs and MDSCs may enhance the effectiveness of and reduce resistance to existing therapies. This review aims to highlight potential targets for myeloid therapy, with a specific focus on recent efforts in combining myeloid targeted therapy with other treatment options to optimize the efficacy of immune-based therapies.

PRECLINICAL GLIOMA MODELS FOR MYELOID STUDY

The most commonly used glioma murine models in preclinical studies of myeloid populations and myeloid-based therapies are orthotopic models that are accomplished by intracranial injection of established glioma cell lines such as GL261 and CT2A. However, these models harbor inherent limitations in representing *de novo* tumorigenesis in the host and have variable immunogenic responses due to the necessity of using immunosuppressed or immunodeficient animal hosts for orthotopic implantation (17–20). To address some of these limitations, genetically engineered models that employ overexpression of relevant oncogenic receptors or downstream signaling pathways, such as replication-competent avian sarcoma-leukosis virus (RCAS) engineered with the sleeping beauty (SB) transposon, have been developed and result in *de novo* tumor formation (21–24). These genetically engineered mice (GEMs) have the advantage of having the tumor originate from the host's own cells, as well as the utility of using immunocompetent animals to assess tumor immunogenicity and response to therapy, but are poorly reproducible and are more representative of genetic predispositions to cancer rather than random tumorigenesis by point mutation (25). A combination of the two techniques, in which donor mouse

cells are transfected with the RCAS system and implanted into recipient mice, has also been explored (11, 26), which improves the correlation to human gliomagenesis, but is limited in reproducibility.

TARGETS FOR MYELOID THERAPY

Strategies for targeting the myeloid compartment generally fall into three main categories: (A) modulating the recruitment of MDSCs from peripheral blood; (B) promoting an immunostimulatory phenotype, primarily through maturation of myeloid precursors into inflammatory macrophages and antigen presenting dendritic cells (DCs); and (C) inhibiting the polarization of myeloid cells to MDSCs. The pathways involved in these three methodologies are shown in **Figure 1**, organized in the context of the TME in which each target is involved.

Inhibiting the Recruitment of MDSCs

CCL2/CCR2

C-C motif chemokine ligand 2 (CCL2, MCP1) was first characterized as a cytokine that interacted with its receptor, CCR2, on peripheral blood monocytes to facilitate chemotaxis to active areas of inflammation (27). In a murine K1492 GBM model, Zemp et al. demonstrated that in addition to recruiting peripheral monocytes to sites of infection, inflammation, and other neuropathological conditions, CCR2 also plays a role in recruiting glioma infiltrating monocytes and macrophages to the TME (28). The authors showed that when oncolytic myxoma virus therapy was given to CCR2-null mice, there was impaired monocyte infiltration and clearance of the virus, leading to increased effectiveness of the therapy and increased survival compared to wild-type mice. Concurrently, Lesokhin et al. confirmed in a B16 melanoma-bearing mouse model that chronic secretion of GM-CSF from the tumor led to recruitment of monocytic MDSCs, characterized by CCR2/CD11b co-positivity, which inhibited TIL proliferation and infiltration in the TME (29). The same group found that while CCR2 was not necessary for MDSC activation, knockdown of CCR2 resulted in a 50% reduction in tumor-infiltrating MDSCs. These results were corroborated by Zhu et al. who directly blocked CCL2 with a monoclonal antibody in C57BL/6 mice bearing intracranial either GL261 or U87 glioma cancer cells and found that blockade of CCL2 led to an increase in median survival in both mouse models (30).

Chang et al. further expanded upon the role of the CCL2/CCR2 axis in glioma immune evasion (31). Using a murine GL261 glioma model, they showed that glioma cells are capable of secreting CCL2 to recruit MDSCs to the tumor site, and that tumor-derived CCL2 can further induce TAMs to secrete CCL2 leading to synergistic tumor immune suppression. In addition to recruiting myeloid cells, the group also found that tumor- and TAM-secreted CCL2 can lead to the recruitment of Tregs through CCR4, further dampening the ability of CTLs to exert anti-tumor effect. This positive feedback loop can help explain the efficacy of CCL2 blockade in anti-tumor response as shown by previous groups. In addition to antagonistic anti-CCL2 antibodies, minocycline has also been shown to inhibit the synthesis of CCL2 by TAMs and has the potential to block this immunosuppressive pathway to work synergistically with current glioma therapeutics (32).

Chen et al. confirmed the relevance of CCL2 in glioma patients by querying the TCGA database and showed that high CCL2 expression was associated with worse prognosis and shorter median survival compared to patients who exhibited low expression of CCL2 (11). These findings suggest that the CCL2/CCR2 axis is a primary mechanism by which glioma cells can recruit MDSCs to promote tumor growth and reduce the effectiveness of anti-cancer therapeutics. The aforementioned studies all highlight the importance of the CCR2 axis in recruiting MDSCs to the tumor and suggest the potential for its blockade in combination with other therapies to potentiate an anti-tumor immune response.

VEGF-A/MET/TIE2/VEGFR2

The vascular endothelial growth factor receptor 2 (VEGFR2) pathway has been well-known to stimulate angiogenesis during tumor development. Recent work from Huang et al. has shed new light on VEGFR2, not only as a regulator of angiogenesis in response to hypoxia, but also as an inducer of myeloid differentiation to MDSCs and their subsequent recruitment to the TME (33). The group showed that myeloid-derived hematopoietic cells that express higher levels of VEGFR2 were correlated with higher tumor grade, worse prognosis, and higher rates of tumor progression in glioma patients. In murine glioma models, the same group knocked down VEGFR2 in bone marrow-derived macrophages, which resulted in significantly decreased tumor blood perfusion and tumor volume, as well as a relative absence of tumor-associated MDSCs. These results suggest that VEGFR2 in peripheral myeloid cells aids in MDSC polarization and trafficking. Interestingly, while Piao et al. demonstrated that anti-angiogenic therapy via bevacizumab prolonged survival in murine glioma models, the group also observed an increase in MDSC recruitment and increased expression of transforming growth factor beta 1 (TGFβ1), an immunosuppressive cytokine, in the TME post treatment (34). Similar results of TGFβ1 upregulation were observed by Osterberg et al. in GL261-implanted mice with selective VEGF-A knockout in $CD11b^{+}$ myeloid cells (35). Furthermore, the use of anti-angiogenic agents such as bevacizumab and sorafenib in clinical trials has historically been unsuccessful at improving patient survival in glioma (36, 37). These findings suggest that while conventional anti-angiogenic therapy can lead to decreased tumor blood perfusion and MDSC trafficking, it also triggers the upregulation of compensatory or alternative pathways for angiogenesis, MDSC recruitment, and therapy resistance.

To expand on their initial findings, Piao et al. identified an alternative angiogenic pathway involving MET and TIE2 that was upregulated after bevacizumab treatment (38). This pathway acts on the same effectors as VEGF-A and is therefore also a mechanism through which the TME can recruit MDSCs. In GBM stem cell (GSC) xenograft mouse models, the group found that treatment with altiratinib, a MET/TIE2/VEGFR2 inhibitor, in combination with bevacizumab, significantly prolonged survival compared to either monotherapy (38). Altiratinib alone

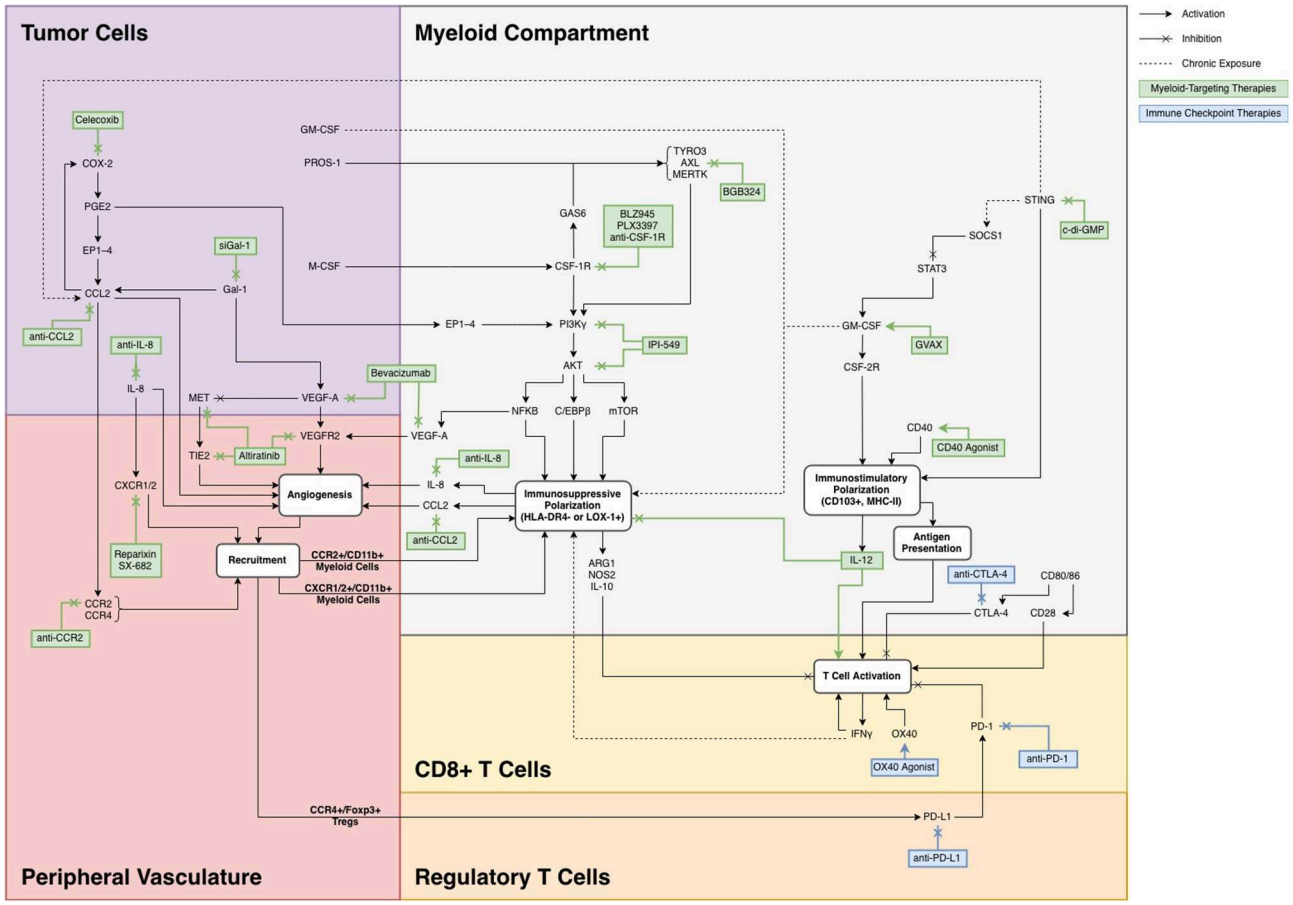

FIGURE 1 | A summary of previously targeted myeloid pathways with potential for combination therapy.

conferred no survival benefit, supporting the argument that the MET pathway is normally suppressed by VEGF-A activity. As VEGF inhibitors like bevacizumab are often considered in cancer treatment, their potential suppressive functions on the immune system through the expression of MET may provide new insight on mechanisms of tumor recurrence and resistance to therapy.

IL-8/CXCR1/2

Interleukin-8 (IL-8, CXCL8) is a chemoattractant cytokine that was originally described to attract and activate peripheral neutrophils and myeloid cells to areas of inflammation by acting on G protein-coupled receptors C-X-C motif chemokine receptor 1 and 2 (CXCR1 and CXCR2) (39). Recent studies have also characterized IL-8 as a tumor-secreted agent that promotes an immunosuppressive TME via MDSC and neutrophil recruitment, as well as tumor angiogenesis (40–42). Importantly, since the rodent genome lacks the IL-8 gene, preclinical studies evaluating the role of IL-8 in MDSC recruitment have been difficult to conduct. To address this obstacle, Asfaha et al. developed a transgenic mouse model (IL-8Tg), in which a bacterial artificial chromosome that encodes for the human IL-8 gene and its regulatory elements is spliced into the mouse genome (43). Using this model, the group found that carcinogen challenge with azoxymethane and dextran sodium sulfate, produced more colorectal tumors in IL-8Tg mice compared to wild-type mice. Furthermore, the group showed that injection of recombinant human IL-8 resulted in increased trafficking of MDSCs to the TME. Alfaro et al. confirmed these findings in BALB/c mice harboring HT29 colorectal adenocarcinoma flank tumors (44), demonstrating that IL-8 induced MDSC migration from the spleen in a dose-dependent fashion. The same group further found that treatment with reparixin, a CXCR1 and CXCR2 inhibitor, abrogated the trafficking of MDSCs in immunocompromised HT29 tumor-bearing mice that underwent IL-8 hydrodynamic gene transfer. In human melanoma-xenografted BALB/c mice, Huang et al. have further shown that IL-8 blockade with the monoclonal antibody ABX-IL8 significantly inhibited tumor growth and decreased angiogenesis, which in turn inhibited MDSC migration (45). By analyzing the expression of biomarkers in human glioma conditioned media, Kumar et al. have shown that IL-8 is a predominant chemokine in the glioma TME, suggesting that glioma-secreted IL-8 helps contribute to MDSC trafficking to the tumor site (46). Pre-clinical studies evaluating the impact of IL-8 blockade on survival have yet to be conducted in glioma models.

Gal-1

Galectin-1 (Gal-1) is the prototypic member of a family of lectins that bind to β-galactosides. Recently, Gal-1 has been described as an important regulator of immune cell trafficking and T cell fate (47). Work from Verschuere et al. has elucidated a link between Gal-1-mediated recruitment of immune cells via CCL2 and VEGF-A (48). The group showed that mice implanted with Gal-1 knockdown GL261 glioma cells not only had prolonged median survival compared to wild-type GL261-implanted mice, but also had decreased levels of MDSCs. Reverse transcription polymerase chain reaction (RT-PCR) revealed that Gal-1 knockdown abrogated CCL2 and VEGF-A mRNA expression in the tumor, resulting in decreased recruitment of MDSCs to the TME and decreased angiogenesis, respectively. Interestingly, Gal-1-knockdown mice implanted with Gal-1-expressing GL261 tumors showed no treatment advantage over wild-type mice, emphasizing the importance of Gal-1 specifically in the TME. By cancer database transcriptomic analysis and immunohistochemistry-based quantifications of GL261, Chen et al. further confirmed that *LGALS1*, the gene encoding Gal-1, was significantly correlated with CCL2 and VEGF-A mRNA expression in the tumor (49). In BV2-bearing mice cells, the same group also knocked down Gal-1 mRNA expression via RNA interference and observed a resulting decrease in MDSCs in the TME. From these results, the expression of Gal-1 is strongly suggested to be an upstream regulator of CCL2 and VEGF-A expression and subsequent inducer of MDSC and Treg recruitment.

There currently exists a variety of Gal-1 inhibitors, including galactoside-derivatives and peptides (50). Of note, Thijssen et al. treated F9 teratocarcinoma-bearing mice with anginex, a polypeptide angiogenesis inhibitor that binds Gal-1, and showed a 70% decrease in tumor growth compared to control mice (51). Shih et al. observed similar findings with LLS2, a small-molecular inhibitor of Gal-1 that decreased tumor growth in a murine ovarian cancer model (52). Finally, in a GL261 glioma mouse model, Van Woensel et al. have targeted Gal-1 in via intranasal administration of nanoparticles loaded with siRNA against Gal-1 (siGal-1), showing a significant reduction of Gal-1 expression in the TME (53). These recent findings have highlighted Gal-1 as a potential target in limiting tumor growth and recruitment of MDSCs via its downstream effectors.

Promoting an Immunostimulatory Phenotype

GM-CSF

Granulocyte macrophage colony stimulating factor (GM-CSF, CSF-2) has a complex role in the regulation of myeloid cells. On one hand, it is commonly used as a method to increase myeloid cell activation and differentiation into DCs (54–63). On the other, it has been shown to promote myeloid immunosuppression through expression of associated markers and inhibition of T cell activation (24, 64, 65). As such, it is important to consider the context of GM-CSF treatment in order to effectively promote immune stimulation.

A common use of GM-CSF for immune stimulation is through vaccination with irradiated tumor cells that have been genetically modified to express GM-CSF (54–57, 66–68), commonly known as GVAX. This technique is based on the rationale that irradiating tumor cells before vaccination causes effective uptake of tumor antigens by macrophages, granulocytes, and DCs without tumor formation, while the expression of GM-CSF allows for activation of the myeloid and dendritic compartments working synergistically to allow successful antigen presentation to T cells (54, 68). Smith et al. demonstrated increased cytotoxic T cell activity with the administration of either GM-CSF vaccine or interferon gamma (IFNγ) in GL261 murine glioma models (55). Interestingly, the administration of GM-CSF alone also showed increase in Tregs and MDSCs. Combination therapy with GM-CSF and IFNγ showed synergistic effects with significantly prolonged survival and long-term immunologic memory at rechallenge. In this case, it is likely that GM-CSF tumor vaccine alone helped enhance antigen presentation by myeloid cells but was not enough to fully activate T cells against the tumor. Combination therapy with other adjuncts is needed to fully harness the immunostimulatory effects of GM-CSF.

As hinted by Smith's study, GM-CSF can also result in immunosuppression with recruitment of Tregs and MDSCs in other contexts. Sielska et al. showed that mice implanted with GL261 tumors knocked down for GM-CSF had significantly improved survival and decreased MDSC infiltration to the TME (64). However, they also found that GM-CSF secreted from the tumor cells resulted in higher expression of immunosuppressive genes, such as arginase 1 (*ARG1*), within the myeloid population. Notably, this occurred at later timepoints, indicating that chronic GM-CSF exposure likely led to myeloid immunosuppression. Kohanbash et al. demonstrated that interleukin-4 (IL-4) and IL-4 receptor alpha (IL-4Rα) are likely responsible for this immunosuppressive effect (24). They showed that GM-CSF is expressed in glioma tissues and can induce IL-4Rα expression *in vitro*. Knockdown of IL-4Rα in BALB/c mice with *de novo* SB transposon-induced gliomas subsequently resulted in downregulation of immunosuppressive pathways involving TGFβ, ARG1, and cyclooxygenase-2 (COX-2). Additionally, Ribechini et al. showed that GM-CSF induces MDSC polarization *in vitro* through simultaneous activation of the protein kinase B (AKT) cascade and the interferon regulatory factor-1 (IRF-1) pathway (65). As GM-CSF has been implicated in immunosuppression, it could be useful to combine GM-CSF treatment with IL-4α or AKT inhibitors to minimize pro-tumor effects.

The opposing immunomodulatory effects of GM-CSF are important to consider when administering this therapy. GM-CSF is associated with immunosuppression when secreted by an active tumor, where there are a host of other suppressive factors, and when chronically secreted through activation of pathways such as PI3K/AKT (65) and via expansion of MDSCs (55). When in the context of irradiated tumor cells without inhibitory signals, as in GVAX, however, GM-CSF causes immunostimulation by instigating the expansion of a subset of antigen presenting, activated myeloid cells (68). By providing GM-CSF in a context

that maximizes the immunostimulatory effects, it can be possible to improve its efficacy alone as well as in combination with other treatments.

STING

Stimulator of IFN genes (STING) is another component in the myeloid compartment that has the ability to both inhibit and stimulate the immune system. STING is activated in the presence of cytosolic DNA, resulting in expression of type I IFNs, with cyclic dinucleotides often used as STING agonists. Ohkuri et al. demonstrated the immunostimulatory effects of STING in SB-induced gliomas by showing that STING knockout resulted in increased infiltration of MDSCs and Tregs and lower infiltration of CTLs. Treatment with the STING agonist cyclic diguanylate (c-di-GMP) resulted in enhanced T cell activity (22). Zhang et al. demonstrated another immunostimulatory function of STING in a nasopharyngeal carcinoma model whereby it inhibited the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in both tumor and myeloid cells through suppressor of cytokine signaling 1 (SOCS1), an intracellular STAT inhibitor (69). This decreased the production of GM-CSF and inhibited the polarization of MDSCs. Foote et al. also demonstrated that STING agonists can promote immunostimulation through increased expression of type I IFNs and increased DC activation (70). However, the group also showed the potential suppressive effects of STING agonists through an increase in myeloid expression of programmed death-ligand 1 (PD-L1), the ligand to programmed cell death protein 1 (PD-1) and an often-targeted immune checkpoint, suggesting the presence of alternative pathways and a potential target for combination therapy. The dual nature of STING was also emphasized by Liang et al. who used an MC38 colon cancer model to show that STING is implicated in myeloid-mediated radioresistance through MDSC recruitment from CCR2 signaling (71).

These results suggest that STING agonist treatment is generally immunostimulatory but can also activate immunosuppressive pathways and interfere with other types of treatment. To optimize the anti-tumor function of this pathway, an agonist could be used in combination with blockade of the suppressive downstream pathways of STING, such as PD-L1 and CCR2. The resulting increase in immune activation could then be used synergistically with other treatment therapies such as ICIs and radiation.

CD40

CD40 is a costimulatory protein found on myeloid cells and DCs. Activation of CD40 with its ligand, CD154, or an agonist antibody promotes antigen presentation in these cells (72, 73). As a result, agonistic CD40 antibodies have been explored as an option to decrease immunosuppression and increase T cell activation. Chonan et al. used an anti-CD40 agonist in in several glioma models and showed a modest improvement in survival as a result (74). Shoji et al. used convection-enhanced delivery of anti-CD40 agonist to the tumor site and showed moderately improved survival (75). Both groups showed increased T cell infiltration with CD40 stimulation, but neither reported long-term survivors, indicating that a CD40 agonist on its own is not enough to sustain a full anti-tumor immune response. Kosaka et al. additionally demonstrated that CD40, as part of a combination treatment with COX-2 inhibition, polarized myeloid cells away from a suppressive phenotype (21). From these findings, we conclude that in a combination treatment, CD40 effectively stimulates antigen presentation by myeloid cells and inhibits myeloid-derived suppression. Although survival is enhanced, the lack of long-term survivors suggests that a full immune response is not mounted. By polarizing the myeloid compartment away from the immunosuppressive phenotype, CD40 agonists could increase the efficacy of other treatments, including T cell-based therapies.

IL-12

Interleukin-12 (IL-12) is secreted by macrophages and promotes an anti-tumor immune response through stimulation of T cells and natural killer cells. Though there is also evidence showing that IL-12 also influences the myeloid compartment. On its own, IL-12 treatment leads to strong systemic toxicity in humans (76). However, localized expression via intratumoral viral transduction or delivery of IL-12 in the tumor has shown promise as a treatment both as a monotherapy (77) and in combination with checkpoint blockade (78, 79). In these cases, the treatment effect was most likely due to anti-tumor T cell stimulation. However, Elzey et al. showed that IL-12 also has the ability to inhibit MDSCs in the TME in a murine breast cancer model by decreasing expression of suppressive genes like *ARG1* (80). In glioma, Thaci et al. found that IL-12 treatment increased myeloid DCs in the TME (81). Work from both groups highlighted the role of IL-12 as both an inhibitor of MDSC function and a promoter of DC maturation, rendering it a promising candidate for combination therapy with T cell-based immunotherapy.

Inhibition of MDSC Formation From Myeloid Precursors

M-CSF/CSF-1R

Macrophage colony stimulating factor (M-CSF, CSF-1) has been well-characterized as a growth factor that binds to colony stimulating factor 1 receptor (CSF-1R) on macrophages and monocytes to stimulate survival and proliferation (82). Coniglio et al. found that microglia surrounding the tumor expressed CSF-1R and responded to CSF-1 via invasion into the TME (83). In a murine GL261 glioma model, the group also found that treatment with PLX3397, a CSF-1R inhibitor that can cross the blood brain barrier, significantly decreased the proportion of microglia in the TME. Concurrently, they observed less tumor invasiveness post-treatment, compared to control groups that experienced extensive tumor cell migration into the brain parenchyma. Their findings suggest that CSF-1R mediates myeloid invasion into the TME and aids in promoting a pro-tumoral environment. As a follow-up, Pyonteck et al. used another CSF-1R inhibitor, BLZ945, on a murine model of RCAS-human platelet-derived growth factor subunit B (hPDGF-B) induced gliomas, which resulted in significantly improved long-term survival rate of 64.3% and no detectable lesions in

55.6% of asymptomatic mice (84). Interestingly, they found that CSF-1R inhibition did not decrease the number of TAMs, but rather abrogated MDSC polarization by downregulating immunosuppressive genes, such as mannose receptor C-type 1 (*MRC1*), adrenomedullin (*ADM*), coagulation factor XIII A chain (*F13A1*), and *ARG1*, in the myeloid compartment. Yan et al. from the same group observed similar results with PLX3397 in the same hPDGF-B-driven glioma model, showing reduced expression of immunosuppressive genes (85). Furthermore, the group found that PLX3397 was significantly more successful at inhibiting tumor growth compared to receptor tyrosine kinase (RTK) inhibitors vatalanib and dovitinib. However, in an another RCAS-hPDGF-B inducible glioma model, Quail et al. found that although CSF-1R inhibition resulted in tumor shrinkage, secretion of IGF-1 in TAMs, and upregulation of IGF-1R in tumor cells resulted in activation of the PI3K pathway in glioma cells, stimulating tumor rebound growth, and recurrence (86). Co-treatment with an IGF-1R or PI3K inhibitor significantly prolonged median survival in mice treated with PLX3397. These findings demonstrate that CSF-1R inhibition has the ability to inhibit MDSC polarization and activity, thereby sensitizing tumor cells to other forms of immunotherapy.

PI3Kγ

The phosphoinositide 3-kinase (PI3K) pathway is important in driving cellular proliferation and differentiation in both tumor and immune cells. PI3K can originally be activated in gliomas as a result of hypoxia, with downstream signaling resulting in recruitment of macrophages that can then be polarized to MDSCs (87). In the myeloid compartment, PI3K is expressed as PI3Kγ, which can be selectively targeted over PI3K expressed in other types of cells (88). Kaneda et al. demonstrated that PI3Kγ is crucial to immune suppression by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and inhibiting CCAAT-enhancer-binding proten beta (C/EBPβ) during macrophage polarization, resulting in an immunosuppressive phenotype (89). As a result, the group found that PI3Kγ knockout resulted in decreased tumor growth in several cancers. De Henau et al. found similar anti-tumor effects when 4T1 breast tumors and B16 melanomas bearing mice were treated with IPI-549, a myeloid-selective PI3Kγ inhibitor (90).

Within glioma cells, the PI3K pathway also has important effects that can potentially interact with the myeloid compartment. For example, PI3K has been implicated in radioresistance *in vitro* by Wang et al. (91), where PI3K inhibition resulted in increased radiation-induced apoptosis. In a later section, we see that the myeloid compartment is also involved in radioresistance, and thus non-selective PI3K targeting has the potential to improve outcomes of radiotherapy on multiple fronts. Quail et al. used non-selective PI3K blockade to overcome CSF-1R inhibition resistance, where the pathway has been implicated in a late resurgence of tumor growth following treatment with a CSF-1R inhibitor (86). In this case, the initial treatment effect was myeloid-based, but the resurgence was caused by signaling within the tumor, and addressing both sides resulted in improved survival. Considering these associations, PI3K inhibition has the potential to play a dual role by inhibiting myeloid immunosuppression and sensitizing the tumor to adjuvant therapies.

TYRO3/AXL/MERTK Receptor Tyrosine Kinases

TYRO3, AXL, and MERTK are a family of RTKs called TAM-RTKs and have been implicated in cell survival (92). Classically, their ligands protein S (PROS1) and growth arrest specific 6 (GAS6) are secreted by macrophages and cancer cells to activate PI3K, extracellular signal-regulated kinase (ERK), and NFκB pathways to promoted tumor proliferation and immune suppression (93). The role of TAM-RTKs in resistance to anticancer therapies have also been well-documented (94). Of note, shRNA knockdown of MERTK and AXL in G12 and A172 astrocytoma cell lines increased tumor apoptosis and autophagy pathways leading to increased chemo sensitivity to temozolomide and carboplatin (95). In mesenchymal GSCs, AXL was also found to be a key regulator of tumorgenicity and clonogenicity (96). Interestingly, AXL activation in head and neck squamous cell carcinoma cell lines resulted in increased expression of PD-L1 and radioresistance (97), providing rationale for potential synergy between AXL inhibition and anti-PD-1 therapy. In the context of the myeloid compartment, Ludwig et al. used a the novel AXL inhibitor BGB324 on a pancreatic cancer murine model and observed a prolonged median survival that was enhanced in combination with gemcitabine (98). The group also found that BGB324 treatment decreased MDSCs in the TME, suggesting that TAM-RTKs create an immunosuppressive environment by enriching the myeloid landscape with MDSCs.

COX-2

In various cancer types, COX-2 has been shown to push myeloid cells toward an immunosuppressive phenotype through prostaglandin E_2 (PGE_2) (99–101). Fujita et al. showed that COX-2 plays an important immunosuppressive role in gliomas as well (102). They found that COX-2 inhibition through acetylsalicylic acid (ASA) or celecoxib contributed to improved survival in C57BL/6J mice with implanted tumors derived from *SB de novo* gliomas. Total knockout of the COX-2 gene within the mice produced a similar result. ASA was further shown to decrease MDSC infiltration, lower the expression of CCL2; and increased influx of CTLs to the tumor site. Interestingly, COX-2 and CCL2 have been described as part of a positive feedback loop via prostaglandin E2 (PGE_2), in which PGE_2-mediated production of CCL2 induces COX-2 activity to produce more PGE_2 (103). In this way, COX-2 promotes a pro-tumor response by immunosuppressive polarization as well as recruitment of MDSCs into the TME.

COX-2 inhibition has been combined with other myeloid targeted treatments to augment their anti-tumor response. For example, Kosaka, Ohkuri, and Okada found that combination treatment of GL261-bearing C57BL/6 mice with anti-CD40 agonist and celecoxib resulted in prolonged survival compared to monotherapies alone (21). Combination therapy resulted in decreased expression of *ARG1* in myeloid cells, a more robust $CD4^+$ T cell activation, and a decrease in Tregs. In another potential combination, Kohanbash et al. showed that a subset of MDSCs in *SB*-induced gliomas express COX-2 (24).

This expression was associated with the expression of other suppressive markers such as ARG1 and TGFβ. Expression of these markers could be induced by GM-CSF, which has been shown to have immunosuppressive properties in the setting of active tumor and chronic exposure but is also commonly used in immunotherapy for myeloid activation. Based on this result, targeting COX-2 could potentially enhance the efficacy of GM-CSF, as COX-2 inhibition can limit the immunosuppressive downstream effects of GM-CSF treatment, while preserving the myeloid-activating pathways. In fact, Eberstal et al. showed that in GL261 tumors, COX inhibitors improved survival in combination with GVAX, when compared to either treatment alone, as a result of greater T cell activation (63).

PRECLINICAL STUDIES OF COMBINATION TREATMENTS INVOLVING MYELOID-TARGETING THERAPY

Although the myeloid compartment is important in modulating the immune system, targeting myeloid cells alone is often not sufficient to elicit an effective immune response. As a result, combination therapies are often necessary to achieve desired treatment outcomes. Additionally, many existing treatment modalities affect and are affected by the myeloid compartment, therefore, emphasizing the need for combination with myeloid targeting to prevent myeloid-mediated therapy resistance. A summary of the following preclinical studies is available in **Table 1**.

T Cell Therapies

Immune checkpoints under normal conditions provide co-stimulatory and co-inhibitory signals to modulate T cell immune responses. In the setting of cancer, tumors can manipulate ICs by expressing inhibitory receptors or upregulating T cell expression of inhibitory ligands to blunt the body's normal anti-tumor immune response. Inhibitory antibodies to PD-1, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and lymphocyte-activation gene 3 (LAG3) have emerged as strategies to facilitate enhanced anti-tumor response and have shown efficacy in systemic cancers such as melanoma and non-small cell lung cancer. Their effects have been less dramatic in cold tumors such as GBM. This is likely due to the presence of alternative tumor-induced inhibitory pathways. Previous work has shown that MDSCs in the TME express immune checkpoint molecules and contribute to the inhibition of CTL function and maintenance of Tregs, which ultimately exert a suppressive effect on TILs (135, 136). The presence of MDSCs therefore acts as an escape mechanism by which the tumor can overcome immune checkpoint blockade. Impeding the recruitment of MDSCs or re-educating the myeloid compartment to a more immunostimulatory phenotype as supplemental therapy to checkpoint blockade has been shown in preclinical models to enhance anti-tumor effects.

The combination of GVAX to prime the myeloid compartment and anti-CTLA-4 checkpoint blockade to disinhibit TILs has been well-characterized in murine melanoma models and patients with metastatic melanoma (60). Agarwalla et al. have conducted the first reported study combining GVAX and anti-CTLA-4 therapy in an intracranial GL261 glioma mouse model and concluded that sequential injection of irradiated GL261 cells expressing GM-CSF followed by anti-CTLA-4 therapy significantly prolonged survival compared to individual monotherapies (57). In a similar fashion, Zhang et al. recently reported therapeutic success in combining anti-PD-1 checkpoint blockade with an anchored GM-CSF vaccination in MB49 bladder cancer (58). The synergistic effect of combining myeloid based therapy and immune checkpoint blockade is likely resulting from activation of TILs with GM-CSF followed by anti-PD-1 treatment negating functional immunosuppression from tumor PD-L1 expression. Ma et al. has recapitulated these findings in murine models of pancreatic ductal adenocarcinoma (PDAC) and breast cancer, two solid-tumor cancers that are known to have limited immunogenicity (59). Triple therapy with agonist anti-CD40, a 3T3 fibroblast analog of GVAX (3T3neuGM), and anti-PD-1 significantly shifted the myeloid compartment from MDSCs to activated DCs and led to increase TIL infiltration. In gliomas, Jahan et al. (118) found that agonist anti-OX40 immunotherapy enhances activity of activated lymphocytes and works synergistically with GVAX against an intracranial glioma model in C57BL/6 mice. Combination therapy resulted in improved survival as well as improved T cell infiltration and anti-tumor function. The same group added to this by using a triple therapy regimen of GVAX, anti-PD-1, and agonist anti-OX40 in GL261 glioma-bearing mice, demonstrating 100% long-term survivorship (137). Dual therapy with GVAX and anti-PD-1 also significantly prolonged median survival, with an observed 50% long-term survival rate. Here again we see that GVAX likely stimulated the myeloid compartment toward an activated, antigen presenting phenotype, while the OX40 agonist promoted activation of T cells (138).

The concept of using colony stimulating factors to stimulate or target the myeloid compartment is multi-faceted. While GM-CSF signaling through GM-CSF receptor 2 (CSF-2R) contributes to macrophage polarization into DCs, M-CSF signaling through CSF-1R shapes the myeloid landscape into an immunosuppressive phenotype (139). Since Antonios et al. found that DC vaccination increases the amount of PD-L1-expressing MDSCs in the TME, the group hypothesized that CSF-1R blockade with anti-PD-1 therapy would enhance anti-tumor effects (111). In a murine GL261 glioma model, triple therapy with CSF-1R blockade, anti-PD-1 therapy, and DC vaccine conferred a 50% long-term survival rate and prolonged median survival compared to double therapies. Their proposed model for triple therapy argues that DC vaccination ultimately stimulates TIL infiltration but does not address TIL inactivation via PD-1/PD-L1 signaling. To this end, CSF-1R inhibition in combination with anti-PD-1 therapy further endorses an immunostimulatory environment by converting MDSCs to pro-inflammatory myeloid cells and by inhibiting PD-1-mediated T cell inhibition. Saung et al. also found that administration of GVAX and anti-PD-1 therapy in a PDAC mouse model upregulated CSF-1 expression in the TME and

TABLE 1 | Preclinical studies of combination treatments targeting myeloid cells.

Myeloid treatment	Treatment effect	Combination therapy	Cancer type and reference	Synergistic effects
Alisertib	MDSC depletion	Oncolytic virus	Malignant peripheral nerve sheath tumor (104)	Oncolytic virus causes tumor lysis and myeloid recruitment, alisertib causes MDSC depletion
All-trans retinoic acid	Decrease level of MDSCs	CAR-T cells	Sarcoma (105)	Decrease in MDSCs lowers inhibition of CAR-T cells
Axitinib	VEGF inhibition, increase myeloid infiltration, reduce suppressive capacity	anti-CTLA-4	MO4 tumors (106, 107)	Axitinib allowed for more stimulatory TME, which allowed ICI to be more effective
CCL2 blockade	Prevention of MDSC polarization	TMZ	Glioma (30)	Both result in decrease in MDSCs
CD40	Activation of myeloid dendritic cells	ICI + GVAX	PDAC (59)	See above for ICI + GVAX effect. CD40 improves efficacy of antigen presentation
		Sunitinib	Melanoma and fibrosarcoma (108)	Sunitinib causes MDSC depletion, while CD40 activates antigen presentation in myeloid DCs
COX-2 inhibition	Prevention of MDSC polarization	Sunitinib	Renal cancer (109)	Sunitinib causes MDSC depletion, while COX inhibition prevents further MDSC polarization
CSF-1R inhibition	Prevention of MDSC polarization	ICI + GVAX	PDAC (110)	See below for ICI + GVAX effect. CSF-1R decreases myeloid suppression
		ICI + DC vaccination	Glioma (111)	CSF-1R decreases myeloid suppression while DC vaccination and ICIs promote targeted T cell response
		Topotecan	Neuroblastoma (112)	Decrease in MDSCs enhances efficacy of chemotherapy
Gemcitabine	MDSC depletion	Oncolytic virus	ID8 (113), colorectal cancer (114)	Oncolytic virus causes tumor lysis and myeloid recruitment, gemcitabine causes MDSC depletion
GM-CSF	Antigen uptake and presentation by myeloid cells	Oncolytic virus	Glioma and melanoma (115), colorectal cancer (116), breast cancer (117)	Oncolytic virus causes tumor lysis and myeloid recruitment, GM-CSF promotes DC differentiation
GVAX/GM-CSF vaccination	Antigen uptake and presentation by myeloid cells	ICI	Glioma (57), bladder cancer (58), PDAC (59)	GVAX allows for a more targeted T cell response while ICI blocks immune checkpoint
		OX40 activation	Glioma (118)	GVAX allows for a more targeted T cell response while OX40 increases T cell activation
		Radiation + Agonist anti-CD137	Glioma (62)	Increased myeloid activation allows for better response to radiation-weakened tumor. CD137 agonist enhances T cell response
IL-12	Myeloid (and T cell) activation	ICI	Glioma (78, 79, 119)	IL-12 inhibits myeloid suppression (and promotes T cell activation) while ICI blocks immune checkpoint
		Oncolytic virus	Sarcoma (120), glioma (79, 119)	Oncolytic virus causes tumor lysis and myeloid recruitment, IL-12 promotes myeloid activation
Macrophage phagocytosis agonist	Activation of macrophages	TMZ	Glioma (121)	Undetermined
Magnetic nanoparticles	Prevention of MDSC polarization	Radiation	Glioma (122)	Decrease in MDSCs results in decreased radioresistance
PGE2 targeting	Prevention of MDSC polarization	Oncolytic virus	Variety of tumors (123)	Oncolytic virus causes tumor lysis and myeloid recruitment, PGE2 targeting prevents MDSC polarization
PI3K inhibition	Prevention of MDSC polarization	ICI	Breast cancer and melanoma (90)	PI3K inhibition decreases myeloid suppression while ICI blocks immune checkpoint
siGal-1	Decrease level of MDSCs	ICI	Glioma (124)	Decrease in MDSCs allows for more effective checkpoint blockade
		DC vaccination	Glioma (48)	Decrease in MDSCs allows for more effective DC vaccination promotes targeted T cell response from DC vaccination
		TMZ	Glioma (124)	Both result in decrease in MDSCs
STING agonist	Increase in myeloid (and other) DCs	ICI + OX40 activation	Breast cancer (70)	STING agonist allows for stronger T cell response resulting from other therapies

(Continued)

TABLE 1 | Continued

Myeloid treatment	Treatment effect	Combination therapy	Cancer type and reference	Synergistic effects
STING agonist + CCR2 blockade	Decrease in MDSCs and increase in myeloid DCs	Radiation	Colon cancer (71)	Myeloid therapy decreases myeloid suppression, thereby decreasing radioresistance
TAM-RTK blockade	Prevention of MDSC polarization	ICI	Glioma (125)	TAM-RTK inhibition decreases myeloid suppression while ICI blocks immune checkpoint
Toca 511/Toca FC	Depletion of MDSCs	Toca 511/Toca FC	EL4 (126), glioma (127, 128), colorectal cancer (129)	Toca causes depletion of MDSCs and both cause tumor cell death
		Chemotherapy (TMZ or lomustine)	Glioma: TMZ (130), lomustine (131)	Toca causes depletion of MDSCs and tumor cell death
		Radiation	Glioma (132)	Toca causes decrease in radioresistance
Tumor peptide vaccination	Antigen uptake and presentation by myeloid cells	Sunitinib	Melanoma (133), TC1 (134)	Depletion of MDSCs allows for more effective vaccination

resulted in increase of MDSCs (110). Of note, treatment with anti-CSF-1R both prior to and after GVAX and anti-PD-1 was necessary for synergistic anti-tumor effects. Using anti-CSF-1R exclusively before or after the other treatments resulted in fewer T cell infiltration. This indicates that persistent inhibition of the CSF-1 axis is needed for myeloid cells to remain in an immunostimulatory state.

The PI3Kγ axis has been recognized as a critical component of tumor recurrence and of polarization of macrophages to the immunosuppressive phenotype. In fact, other myeloid targets, such as COX-2 (140) and CSF-1R (141) exert their pro-tumoral effects through upregulation of expression of PI3Kγ in macrophage and microglia. De Henau et al. used a novel macrophage-targeting PI3Kγ inhibitor IPI-549 in combination with anti-PD-1, and anti-CTLA-4, which resulted in prolonged median survival and long-term survival in breast cancer and melanoma murine models (90). The group also demonstrated that IPI-549 polarized myeloid cells to an anti-tumor phenotype, subsequently increasing the CTL/Treg ratio *in vivo*.

vom Berg et al. utilized a different approach in mice implanted with GL261 glioma cells by inhibiting the suppressive activity of MDSCs through IL-12 (78). Interestingly, IL-12 therapy was found to prolong survival in an IFNγ-independent fashion and resulted in upregulation of CTLA-4 in TILs. Although local delivery of IL-12 via osmotic minipump markedly prolonged median survival, combination with systemic CTLA-4 blockade resulted in full remission in 80% of treated mice. As a follow-up to this study, and to assess the efficacy of IL-12 on GSCs, which are involved in polarization of myeloid cells toward immunosuppressive phenotypes, Saha et al. engineered a G47Δ strain of oncolytic herpes virus (oHSV) to express mouse IL-12 (oHSV G47Δ-mIL-12) (119). Combination of oHSV G47Δ-mIL-12 with either anti-PD-1 or anti-CTLA-4 therapy corroborated with vom Berg et al's findings and conferred a moderate increase in median overall survival. More significantly, triple combination with oHSV G47Δ-mIL-12, anti-PD-1, and anti-CTLA-4 therapy virtually eliminated the GSC tumors in mice and conferred universal long-term survival upon reinoculation with GSCs.

Since TAM-RTKs have been characterized to block IL-12 production (142) and facilitate immunosuppressive polarization (94), Sadahiro et al. have targeted the AXL arm of TAM-RTKs as a potential therapy in mice implanted with mesenchymal GSC-derived tumors (125). The authors used the small molecule AXL inhibitor BGB324 in combination with anti-PD-1 checkpoint blockade and found a moderate increase in median survival compared to the control group with long term survival in about 10% of treated mice. They also found that AXL activation in the TME is mediated through increased expression of PROS1, a known TAM ligand that was originally thought to only bind to TYRO3 and MERTK. Furthermore, anti-PD-1 therapy was observed to increase AXL expression and levels of $CD11b^+$ myeloid cells in the TME, confirming the rationale of using an AXL inhibitor in combination with checkpoint blockade.

To address Gal-1-mediated regulation of MDSCs and subsequent therapy resistance in glioma, Van Woensel et al. treated mice implanted with GL261 glioma cells with anti-Gal-1 siRNA (siGal-1) and anti-PD-1 therapy (124). siGal-1 treatment was shown to decrease the pool of M-MDSCs and Tregs in the TME. Co-staining also revealed an increase in infiltrating T cells in the TME. The group found that siGal-1 works synergistically with anti-PD-1 treatment, almost doubling median survival (30 days in anti-PD-1 arm vs. 51.5 days in the combination arm) with a 20% long-term survival rate. Verschuere et al. (48) showed that Gal-1 knockdown also worked synergistically with DC vaccination to result in long-term survival in 34% of inoculated mice. The immunopermissive niche allowed by the Gal-1 knockout likely prevented suppression of the T cells activated by DC vaccination, enabling them to properly mount an anti-tumor response. Gopinath et al. targeted downstream effectors of Gal-1 by disrupting the CCL2-CCR2 axis in gliomas to target MDSC trafficking to the TME (143). Using the CCR2 antagonist CCX598, the group showed that while monotherapy did not prolong survival in GL261 tumor-bearing mice, the addition of anti-PD-1 or anti-PD-L1 treatment slowed glioma progression. In a similar fashion, Highfill et al. demonstrated that rhabdomyosarcoma (RMS) utilizes the IL-8/CXCR1/2 axis

to recruit MDSCs (144). The group showed that, while CXCR2-knockout mice did not consistently exhibit increased survival compared to wild-type mice when implanted with RMS cell lines, combination therapy with anti-PD-1 checkpoint blockade resulted in markedly increased median survival in all cell lines, as well as long-term survivorship in one cell line.

Combination of multiple myeloid targeted therapies in addition to lymphocyte targeted therapies has also been shown to have synergistic effects on the anti-tumor response. In FVB mice with breast tumors expressing human epidermal growth factor receptor 2 (HER-2), Foote et al. used a STING agonist to improve the efficacy of combination treatment with anti-PD-L1 and OX40 agonist by increasing activation of myeloid DCs, priming tumor-specific CTLs, and abrogating immunosuppressive signals (70). In this treatment, the STING agonist allowed for a more immunopermissive TME with enhanced antigen presentation, while OX40 increased T cell activation, rendering anti-PD-L1 therapy much more effective.

In addition to immune checkpoint blockade, targeting the myeloid compartment has also been shown to boost the anti-tumor effect of CAR T cell therapies. Long et al. showed that treatment of NOD scid gamma (NSG) mice in a sarcoma model with all-trans retinoic acid, which is known to promote differentiation of immature myeloid cells into immunostimulatory phenotypes, resulted in a loss of monocytic MDSCs and loss of suppressive function in granulocytic MDSCs (105). Combination treatment of all-trans retinoic acid with CAR-T cells improved survival. Interestingly, the CAR-T cells expressing OX40 and CD28 receptors, both of which are involved in T cell activation, were more effective than CAR-T cells that did not express both receptors, highlighting the importance of multiple modes of activation.

Oncolytic Viral Therapy

Oncolytic viruses are viruses that preferentially kill tumor cells and also enhance the induction of anti-tumor immunity that accompanies the oncolytic activity. Therefore, a combination of oncolytic viral therapy with other immune based therapies targeting the myeloid compartment can enhance the efficacy of oncolytic viral therapies. Several different types of virus have been used for their specificity in targeting cancer cells. For example, Liu et al. have created an oHSV strain for intratumoral injection, in which a neurovirulence factor has been inactivated, resulting in tumor-specific infection in murine gliomas and melanomas (115). Upon the insertion of the GM-CSF gene into the virus, the group showed an increase in activation of splenocytes *in vitro*, correlated with greater tumor shrinkage *in vivo*. In a clinical trial involving patients with metastatic melanoma treated with GM-CSF-transfected oHSV, Kaufman et al. also showed a decrease in MDSCs in vaccinated lesions (145). Yin et al. demonstrated a similar enhanced effect in colorectal cancer with a different type of GM-CSF-expressing oHSV, which resulted in decreased MDSC infiltration and an increase in local mature DCs (116). oHSV has also been engineered to express IL-12, which was demonstrated by Ring et al. to decrease infiltrating MDSC levels in sarcomas more so than oHSV alone (120). While oncolytic viruses can elicit anti-tumor immune response through antigen release after tumor cell death, they are also subject to targeting by circulating antibodies and host immune response, therefore leading to recruitment of immune cells to the injected tissue. Currier et al. demonstrated that oHSV can induce accumulation of MDSCs in the TME (104). This phenomenon was reversed by alisertib, a serine/threonine-protein kinase 6 inhibitor, in malignant peripheral nerve sheath tumors. Combination of oHSV and alisertib lead to a synergistic decrease in tumor growth. In support of these results, Esaki et al. also showed enhanced anti-tumor response by enhancing oHSV therapy with myeloid depletion therapy gemcitabine in colorectal cancer models (114). These studies highlight the synergistic anti-tumor response of combining oncolytic viral therapy with myeloid targeted therapies.

Vaccinia virus has also been used for its oncolytic properties. Similar to oHSV, vaccinia expressing GM-CSF has been used by de Vries et al. to enhance anti-tumor immunity in breast cancer (117). Vaccinia has also been engineered by Hou et al. to target PGE_2, which, as described previously, is involved in MDSC polarization (123). This resulted in improved survival over treatment with viruses without PGE_2 targeting capability. It has been shown that vaccinia virus can attract both immunostimulatory myeloid cells and MDSCs to the site of injection. Tan et al. showed that treatment with vaccinia viral therapy alone led to infiltration with MDSCs that suppressed DC function (146). On the other hand, Kilinc et al. (147) found an increase in activated myeloid cells upon treatment with vaccinia. Thus, it may be advantageous to deplete the suppressive MDSCs that are induced by vaccinia, while preserving stimulatory myeloid cells, highlighting the need for combination therapies. In fact, Liu et al. circumvented this issue after finding a large amount of PD-L1 expressing MDSCs in the tumor after vaccinia treatment by treating with anti-PD-L1, improving survival and decreasing tumor burden (148).

Reovirus is another commonly used oncolytic virus that has been shown to impact the myeloid population in the TME. Clements et al. demonstrated that in ovarian cancer, reovirus increases intratumoral MDSCs and expression of immunosuppressive genes (149). Katayama et al. found that reovirus inhibited the T cell suppressive function of MDSCs through toll-like receptor 3 (TLR3), which was abrogated in a TLR3 knockout model (150). Furthermore, Gujar et al. found that the anti-tumor effect of reovirus therapy was enhanced by MDSC depletion with gemcitabine (113).

Other viruses have also been used for their oncolytic properties. In colon carcinoma, Scherwitzl et al. showed that treatment with Sindbis virus expressing tumor-specific antigen in colon carcinoma lead to anti-tumor immune responses (151). Combination therapy of Sindbis virus with PD-1 blockade resulted in significantly improved overall survival and reduced MDSC infiltration in the TME. Another oncolytic strain is Newcastle disease virus, which Koks et al. found that treatment with another oncolytic virus, Newcastle disease virus, resulted in increased T cell activation, MDSC depletion, and improved survival in gliomas (152). Finally, in a creative treatment, Eisenstein et al. (153) found that MDSCs can be used as a vehicle for oncolytic rhabdovirus. MDSCs infected with the virus and

transferred to the host not only lead to a localized infection at the tumor, but also assumed a more immunostimulatory phenotype.

In summary, oncolytic viruses appear to recruit many types of myeloid cells into the tumor, both immunosuppressive and immunostimulatory. Combination treatments are most effective upon inhibition of the suppressive myeloid cells while preserving those that enhance anti-tumoral immune responses.

Combination Myeloid-Targeting Therapies

In addition to the success of combining myeloid targeted therapy with immunotherapy aimed at boosting other aspects of the immune system, combination therapies targeting multiple immunosuppressive myeloid pathways have also shown some promise. However, it is important to consider that altering only the myeloid compartment, even through multiple pathways, may not suffice for a full anti-tumor response. It has been shown previously that while GM-CSF can activate the immune system, it can also upregulate suppressive factors in myeloid cells such as COX-2 (24). As previously mentioned, Eberstal et al. combined GVAX with COX-2 inhibition via systemic administration of parecoxib and intratumoral administration of valdecoxib, demonstrating that inhibition of COX-2 enhances the efficacy of GVAX and improves survival (63). Kosaka et al. used the COX-2 inhibitor celecoxib in concert with CD40 to inhibit MDSC polarization, promote DC differentiation, and increase T cell activation (21). Finally, Chen et al. used triple therapy with intratumoral delivery of GM-CSF, IL-12, and irradiated tumor vaccine in the treatment of gliomas (154). These therapies acted on different components of the myeloid cells, with irradiated tumor vaccines promoting antigen presentation, IL-12 inhibiting MDSC polarization and promoting DC maturation, and GM-CSF promoting growth of the antigen-presenting myeloid cells. The authors found that each dual therapy combination enhanced survival in gliomas with triple therapy leading to the longest survival benefit. However, it should be noted that IL-12 also has significant effects on the CTL populations, which would be necessary for an effective immune response.

Molecular Therapies

Molecular targeted therapies have also been used in combination with myeloid targeted therapies to modulate the myeloid compartment in anti-cancer treatments. RTK inhibitors, for example, can inhibit VEGF, which can lead to MDSC differentiation and recruitment to the TME. Sunitinib, a RTK inhibitor, has been shown to reduce MDSC levels in human renal cell carcinoma (155). Several groups have taken advantage of this, thereby combining sunitinib treatment with myeloid targeting therapies. van Hooren et al. demonstrated that sunitinib synergistically enhanced treatment of B16 melanomas and T24 fibrosarcomas with anti-CD40 agonist antibodies (108). Sunitinib resulted in a decrease in MDSCs while anti-CD40 increased DC activation. Zhao et al. (109) used sunitinib in conjunction with celecoxib to drastically inhibit tumor growth. In this case, both modalities worked to decrease the level of MDSCs. Bose et al. was able to enhance the efficacy of tumor-specific peptide-pulsed DC vaccine with sunitinib, showing that combination treatment had a lower level of MDSCs (133). This effect was mirrored when Draghiciu et al. showed lower levels of MDSCs and increase in CTLs when combining sunitinib with a viral vaccine for HPV-induced oncoproteins (134).

Another RTK inhibitor, sorafenib, has shown promise as well in promoting anti-tumor immune responses. Heine et al. has shown that sorafenib leads to decreased MDSC immunosuppressive capacity *in vitro*. However, the effect of sorafenib appears to be dependent on MDSC levels (156). Chang et al. showed that the treatment efficacy of sorafenib was decreased in tumors with high levels of MDSCs, but that upon antibody-mediated depletion of MDSCs, the efficacy of sorafenib was restored (157). This suggests that combining sorafenib with an MDSC-depleting therapy could result in a synergistic effect. Axitinib, a VEFGR inhibitor, has also been shown to increase myeloid infiltration while simultaneously reducing the suppressive capacity of MDSCs (106). This could prime the TME into an immunostimulatory state, allowing for improved efficacy of additional immune based therapies. In fact, Du Four et al. combined axitinib with CTLA-4 blockade and found that combination treatment synergistically reduced tumor growth and improved survival (107).

Cetuximab is an antibody against EGFR that has been shown to impact myeloid function and phenotypes. EGFR is a commonly mutated gene in multiple cancers has served as a common molecular target. Li et al. hypothesized that the Fc portions of cetuximab may interact with the Fcγ receptor on myeloid cells to alter their phenotype (158). This hypothesis was further supported by a recent clinical trial (NCT01218048) that analyzed blood from head and neck squamous cell carcinoma patients treated with cetuximab and showed polarization of myeloid cells toward an immunostimulatory phenotype. Jia et al. then showed in mice that EGFR molecular inhibitors also cause an increase in immunostimulatory myeloid cells (159). However, this effect was transient, lasting only the length of treatment, and was counteracted by alterative immunosuppressive pathways involving CCL2, ultimately leading to a persistent increase in MDSCs. The authors suggested that a combination of CCL2 and EGFR inhibition could increase anti-tumor effects compared to EGFR inhibitors alone.

Radiation

While immunotherapy has been integrated into the treatment regime of several systemic cancers, radiation, and chemotherapy remains the main stay of treatment for various tumors, especially gliomas. Therefore, it is crucial to understand the effects of standard adjuvant therapies on the anti-tumor immune response and vice-versa to optimally integrate these novel therapies into current standard of care. The effect of radiation on the myeloid population is 2-fold. It has been shown to increase tumor infiltration of both immunosuppressive and immunostimulatory populations of myeloid cells (160). Furthermore, radiation-induced necrosis and apoptosis of tumor cells also lead to release of tumor antigens and antigenic spread leading to enhanced tumor-specific immune responses (161–163). In an MC38 colon cancer model, Liang et al. showed that radiation treatment resulted in increased levels of MDSCs, which subsequently contributed to radiation resistance

developed by the tumors (71). Interestingly, this radioresistance was abrogated by CCR2 blockade. The group further showed that combination therapy with radiation and CCR2 blockade resulted in improved treatment outcomes when compared to radiation alone. Similarly, combination therapy with a STING agonist, cGAMP, CCR2 blockade, and radiotherapy resulted in lower MDSC infiltration and decreased tumor volume.

In the setting of gliomas, Newcomb et al. showed in a GL261 glioma models that treatment with radiotherapy in combination with GVAX resulted in improved survival over either treatment alone (62). The group theorized that GVAX was able to prime the myeloid population toward a more immunostimulatory state, rendering the tumors more sensitive to radiotherapy. The same group also showed that radiation enhances the antitumor effect of agonist anti-CD137 antibody therapy. Combination-treated mice observed increased TILs in the TME, as well as prolonged survival compared to control and monotherapy groups. CD137 (4-1BB) has been implicated in differentiation of monocytes into DCs (164) and activation of $CD4^+$ and $CD8^+$ T cells leading to increased anti-tumor responses (165).

Nanoparticles that target MDSCs to promote polarization of myeloid cells to an anti-tumor phenotype have also been used in combination with radiotherapy with promising effects. In CT2A and U87 gliomas, Wu et al. treated mice with magnetic nanoparticles, aimed at targeting both MDSCs and tumor cells directly, along with radiation therapy (2 Gy/day for 4 days) 7 days post-tumor implantation (122). They showed increased median survival with the combination therapy when compared to radiation alone. The combination therapy pushed the myeloid compartment into an anti-tumor phenotype and increased the expression of immunostimulatory genes such as tumor necrosis factor alpha (TNFα) and inducible nitric oxide synthase (iNOS). In addition to radiosensitizing tumor cells and modulating the TME, nanoparticles can also be readily uptaken by myeloid cells such as macrophages. Myeloid cells can then traffic the phagocytosed nanoparticles to the TME where they can lead to anti-tumor response and MDSC repolarization (166). Better understanding of the interplay between nanoparticles, MDSCs, and radiotherapy will be necessary to optimally combine these therapies to boost anti-tumor response. With continued evolution of radiotherapy aimed at increasing targeted radiation dose to the tumor while minimizing collateral damage to surrounding normal tissue, other types of radiation such as carbon irradiation (CIR) and proton irradiation (PIR) have gained popularity over traditional photon irradiation. It has been shown that different types of radiation can affect the myeloid compartment differently. Chiblak et al. showed CIR to be more beneficial than standard PIR in immunotherapy in several key areas (167). They showed that treatment with CIR led to decreased MDSC infiltration and an increase in pro-inflammatory myeloid cells compared to PIR. *In vitro*, microglial migration was reduced with CIR and increased with PIR. Monzen et al. showed that CIR can inhibit the growth of MDSCs and their progenitors over PIR, which could partially explain the treatment advantage of CIR over PIR in the context of immunotherapy (168).

Chemotherapy

Currently, the most commonly used chemotherapeutic in GBM is temozolomide (TMZ), an alkylating antineoplastic drug that causes cytotoxicity through guanine and adenine methylation (169). Mathios et al. demonstrated that synergistic effects can exist between locally-delivered TMZ and immunotherapy (170). Unfortunately, systemic TMZ, which is the current standard of care, results in immunodepletion and thus can limit the efficacy of immune-based therapies. In the case of MDSCs, immunodepletion could be advantageous, as several of the following groups have shown.

In GL261 glioma and human U87 glioma xenograft mouse models, Zhu et al. showed that CCL2 antibody blockade in combination with TMZ resulted in improved survival over either monotherapy alone (30). As discussed previously, CCL2 binds to CCR2 and results in the recruitment of TAMs and polarization to MDSCs (171).

Van Woensel et al. demonstrated that treatment with siGal-1 can lower the presence of MDSCs and Tregs in GL261 tumors (124). Knockdown of Gal-1 also resulted in more normalized vasculature that allowed for greater penetration by TMZ leading to more effective tumor killing. In BALB/c nude mice and C57BL/6 mice subcutaneously injected with U87 and GL261 cells, respectively, Zhang et al. (172) showed that combination treatment with an agonist for macrophage-mediated phagocytosis (SIRPα-Fc), an autophagy inhibitor (chloroquine), and TMZ resulted in significantly prolonged survival compared to control, monotherapy, and double therapy groups.

In another study, Webb et al. treated a patient-derived neuroblastoma xenograft in T cell deficient mice with a combination of the CSF-1R inhibitor BLZ945 and the chemotherapeutic topotecan (112). They showed a decrease in myeloid cells with BLZ945 alone, but no effect on survival. However, upon addition of chemotherapy, there was an increase in survival with combination therapy over chemotherapy alone. It is clear from these results that myeloid cells can have an inhibitory effect on chemotherapy independent of T cell function and inhibition of myeloid immunosuppression can improve chemotherapy outcomes.

Another creative chemotherapy with effects on the myeloid compartment utilizes the retroviral vector Toca 511. With this technique, Toca 511 selectively delivers a cytosine deaminase gene to cancer cells. Cytosine deaminase is then expressed by the infected cells, causing them to convert the pro-drug 5-fluorocytosine (5-FC), commonly delivered in the oral extended-release form of Toca FC, into the potent chemotherapeutic 5-fluorouracil (5-FU), which causes death of the tumor cells (173). Besides tumor cell death, 5-FU has also been shown by Vincent et al. to selectively kill MDSCs in tumor cells while preserving other immune populations and resulting in greater T cell IFNγ production (126). Mitchell et al. then confirmed this effect in the context of Toca by pretreating tumor cells before flank implantation with Toca 511, followed by treatment with Toca FC (127). Intratumoral injection of Toca 511 in gliomas by Hiraoka et al. (128) and colorectal

cancer by Yagiz et al. (129) also resulted in immunological benefit. The immunological effects were correlated with survival and long-term resistance to tumor rechallenge. Survival effects were preserved on combination therapy with both TMZ (130) and lomustine, another chemotherapeutic (131). Additionally, Takahashi et al. demonstrated a decrease in radioresistance caused by treatment of gliomas, although this was done in athymic mice, and thus the possible immunological contribution is unclear (132). The success of Toca supports the findings of Mathios et al. where localized chemotherapy was beneficial for cultivating an immune response (170). The exact mechanism of the synergistic anti-tumor effects of combining myeloid targeted therapies with chemotherapy is unclear. It has been postulated that chemotherapy, similar to radiation therapy, generates new antigenic targets and boosts antigenic uptake and presentation thereby priming the TME for adaptive anti-tumor immune responses. Combination with myeloid targeted therapies can further abrogate alternative immunosuppressive pathways to enhance anti-tumor effects of chemotherapy.

Corticosteroids

An adjunct treatment that is unique to the treatment of gliomas and other brain tumors is corticosteroids. Steroids such as dexamethasone are used to decrease cerebral edema caused by the tumor and has been shown to lead to significant alterations to the immune compartments. Maxwell et al. previously showed that administration of steroids led to decreased peripheral $CD4^+$ and $CD8^+$ T cells and led to decreased efficacy of anti-PD-1 treatment for peripheral flank tumors compared to intracranial tumors (174). While historically, steroids were thought to affect the lymphocyte population, studies now have shown similar effects of steroids on the myeloid compartment as well. Moyes et al. has demonstrated that treatment with dexamethasone resulted in an increase in peripheral circulator myeloid populations (175). As we continue to elucidate the mechanism of immunosuppression caused by steroids in the context of cancer immunotherapy, it is important to account for their potential effect on the myeloid compartment in addition to lymphocyte populations.

MDSCS AS PREDICTIVE BIOMARKERS

In addition to identifying the therapeutic benefits of myeloid-based therapies, characterizing the myeloid compartment offers the potential for stratifying patient prognosis and response to immunotherapy by measuring myeloid-specific biomarkers. Other biomarkers such as tumor mutational burden, checkpoint expression, and T cell receptor diversity have been used to predict response to immunotherapies, where increases in each are correlated with improved patient outcomes (176). In gastric cancer, higher levels of TAMs have been associated with increased tumor progression (177), which could be inferred from the immunosuppressive functions of tumoral myeloid cells. Circulating MDSC levels in patients have also been used as a biomarker for response to immunotherapies in patients with melanoma, colorectal, kidney, prostate, and breast cancer, with increases in blood MDSCs correlating with worse patient outcomes (178–180). Alban et al. found a similar correlation in GBM, with higher circulating MDSCs associated with worse prognosis and survival (181). Additionally, in melanoma patients, Huber et al. found that microRNAs (miRNAs) through which tumors induce MDSC formation can be used as a predictive biomarker of response to immunotherapy (182). The authors found that clustering patients based on the quantity of intratumoral MDSC miRNA stratified patients' response to ICI therapy. These studies indicate that myeloid-based characterizations have the potential to serve as biomarkers of outcome and treatment response to identify patients who are most likely to respond to a particular therapy.

CLINICAL TRIALS

Although the majority of preclinical evidence for combination treatments involving various immune based and cytotoxic cancer therapies along with myeloid targeted therapies has shown promise, targeting myeloid cells for immunotherapy is a fairly recent endeavor. As a result, many clinical trials using combination treatment with myeloid therapies are still ongoing, with results yet to be reported. Some encouraging results have emerged from a few published trials. In a Phase I trial (NCT02526017) of combination therapy of cabiralizumab, a CSF-1R blocking antibody, with nivolumab (anti-PD-1) in a variety of solid tumors showed significant depletion of TAMs. They further demonstrated a tolerable safety profile and durable clinical benefit, with response in 5 of 31 advanced pancreatic cancer patients (183). Based on this trial, a Phase II trial in of cabiralizumab and nivolumab in combination with chemotherapy is underway for advanced pancreatic cancer (NCT03336216).

GM-CSF has also been investigated in the clinical setting as a combination therapy. A Phase II trial in GBM (NCT01498328) utilizing GM-CSF with an EGFRvIII peptide vaccine and bevacizumab has recently completed. The combination treatment showed efficacy over bevacizumab alone, as measured by overall response rate (ORR) and progression free survival (PFS), confirming its efficacy (184). Similar preliminary results have been reported in an ongoing GBM Phase II trial of ERC1671, or Gliovac, which consists of inactivated tumor cells and tumor lysate, in combination with GM-CSF, cyclophosphamide, and bevacizumab, with vaccinated patients surviving longer than non-vaccinated, bevacizumab treated counterparts (185). GVAX has also been evaluated in combination with pembrolizumab in a Phase II trial in metastatic colorectal cancer. While the treatment was well-tolerated, treatment outcomes were comparable to historical control (186).

PI3Kγ is also being targeted via the aforementioned selective inhibitor, IPI-549. In a Phase I trial in several tumor types (NCT02637531), treatment with IPI-549 in combination with nivolumab has so far been shown to be tolerable. Preliminary results have shown some immunological effects, including a decrease in immunosuppression and increased T cell proliferation (187).

TABLE 2 | Clinical Trials targeting myeloid cells in combination with other anti-cancer therapies.

Myeloid target	ClinicalTrials.gov identifier	Phase	Tumor types	Additional treatment	Results?
CCR2	NCT03767582	1/2	Locally advanced pancreatic ductal adenocarcinoma (PDAC), Pancreatic ductal adenocarcinoma	Nivolumab + Stereotactic body radiation ± GVAX	–
	NCT03778879	1/2	Adenocarcinoma of the pancreas, Pancreas cancer	Stereotactic body radiation	–
CD40	NCT03597282	1	Metastatic melanoma	NEO-PV-01 (peptide vaccine) + Nivolumab (anti-PD-1)	–
Chemodepletion	NCT03302247	2	Non-small cell lung cancer stage IIIB	Nivolumab	–
COX-2	NCT03638297	2	Colorectal cancer	BAT1306 (anti-PD-1)	–
	NCT03864575	2	Metastatic cancer	Nivolumab	–
CSF-1R	NCT02452424	1/2a	Melanoma, Non-small cell lung cancer, Squamous cell carcinoma of the head and neck, Gastrointestinal stromal tumor (GIST), Ovarian cancer	Pembrolizumab (anti-PD-1)	–
	NCT02777710	1	Colorectal cancer, Pancreatic cancer, Metastatic cancer, Advanced cancer	Durvalumab (anti-PD-L1)	–
	NCT03238027	1	Solid tumor, Metastatic tumor, Locally advanced malignant neoplasm, Unresectable malignant neoplasm	Durvalumab	–
	NCT02880371	1/2	Advanced solid tumors	Pembrolizumab	–
	NCT02829723	1/2	Advanced solid tumors	PDR001 (anti-PD-1)	–
	NCT03336216	2	Advanced pancreatic cancer	Nivolumab + Chemotherapy	–
	NCT02526017	1a/1b	Advanced solid tumors, Including but not limited to lung cancer, Head and neck cancer, Pancreatic cancer, Ovarian cancer, Renal cell carcinoma, Malignant glioma	Nivolumab	Some preliminary efficacy observed along with macrophage depletion (183)
CSF-1R + CD40	NCT03502330	1	Advanced melanoma non-small cell lung cancer renal cell carcinoma	Nivolumab	–
CSF-1R + chemodepletion	NCT03697564	2	Pancreatic cancer stage IV	Nivolumab	–
CSF-1R + GM-CSF	NCT03153410	1	Pancreatic cancer	Pembrolizumab	–
GM-CSF	NCT00254592	2	Breast cancer	Bevacizumab OR Trastuzumab + chemotherapy	–
	NCT01498328	2	Glioblastoma, gliosarcoma	Rindopepimut (EGFRvIII peptide vaccine) + Bevacizumab	Treatment demonstrated improved ORR and PFS at 6 months compared to control (184)
GM-CSF vaccine	NCT01551745	2	Stage III, IV ovarian cancer	Bevacizumab	Study terminated due to no patients being analyzed for primary outcomes
	NCT01903330	2	Glioblastoma, Gliosarcoma	Bevacizumab + Cyclophosphamide (CY)	Increased survival in combination treatment over bevacizumab alone
GVAX	NCT01896869	2	Metastatic pancreatic adenocarcinoma	Ipilimumab (anti-CTLA-4)	–
	NCT02451982	1/2	Pancreatic cancer	Nivolumab ± Urelumab (anti-41BB)	–
	NCT03161379	2	Pancreatic cancer	CY + Nivolumab + Stereotactic body radiation	–
	NCT03190265	2	Pancreatic cancer	CY + Nivolumab + Ipilimumab + CRS-207 (listeria)	–
	NCT02243371	2	Pancreatic cancer	CY + CRS-207 + Nivolumab	–
	NCT03006302	2	Metastatic pancreatic adenocarcinoma	Epacadostat + Pembrolizumab + CRS-207	–

(Continued)

TABLE 2 | Continued

Myeloid target	ClinicalTrials.gov identifier	Phase	Tumor types	Additional treatment	Results?
	NCT02648282	2	Pancreatic cancer	CY + Pembrolizumab + Stereotactic body radiation	–
	NCT01510288	1	Prostate cancer	Ipilimumab	Terminated due to company action
	NCT02981524	2	Metastatic colorectal cancer	CY + Pembrolizumab	Treatment is well-tolerated, results comparable to historical controls (186)
Pexa-Vec (GM-CSF)	NCT03294083	1	Renal cell carcinoma	REGN2810 (anti-PD-1)	
	NCT02562755	3	Hepatocellular carcinoma (HCC)	Sorafenib	
	NCT03206073	1	Refractory colorectal cancer	Durvalumab ± Tremelimumab (anti-CTLA-4)	Durvalumab safety established (193)
	NCT02977156	1	Metastatic/advanced solid tumors	Ipilimumab	
	NCT02630368	1/2	Solid tumors soft-tissue sarcoma breast cancer	CY	Safety established (194)
	NCT03071094	1/2a	Hepatocellular carcinoma (HCC)	Nivolumab	
PI3Kγ	NCT02637531	1	Advanced solid tumors non-small cell lung cancer melanoma squamous cell cancer of the head and neck triple negative breast cancer adrenocortical carcinoma mesothelioma high-circulating myeloid-derived suppressor cells	Nivolumab	Treatment is tolerable and results in some preliminary immunological activity, with reduced immunosuppression by macrophages (187)
Reparixin (CXCR1)	NCT02001974	1b	Breast cancer	Paclitaxel	–
STING	NCT03172936	1	Solid tumors and lymphomas	PDR001 (anti-PD-1)	–
	NCT03010176	1	Solid tumors and lymphomas	Pembrolizumab	Dose escalation ongoing, some signs of immunological activity (189)
	NCT02675439	1	Advanced/metastatic solid tumors or lymphomas	Ipilimumab	In STING agonist monotherapy arm, no dose-limiting toxicities yet, most patients dropped out due to disease progression or death, some immunological activity seen (190)
SX-682 (CXCR1/2)	NCT03161431	1	Melanoma	Pembrolizumab	–
Toca	NCT02576665	1	Colorectal cancer, triple negative breast cancer, Pancreatic cancer, non-small cell lung cancer, Head and neck cancer, Ovarian cancer, Lymphoma, sarcoma, Bladder cancer, Melanoma, IDH1 Mutated solid tumors, IDH1 Mutated or MGMT methylated recurrent HGG (Not Recruiting)	None	–
	NCT01985256	1	Glioblastoma multiforme, Anaplastic astrocytoma, Anaplastic oligodendroglioma, Anaplastic oligoastrocytoma	None	–
	NCT01156584	1	Glioblastoma multiforme, Anaplastic astrocytoma, Anaplastic oligodendroglioma, Anaplastic oligoastrocytoma	None	–
	NCT02598011	1	Newly diagnosed high grade glioma (HGG)	None	–
	NCT02414165	2/3	Glioblastoma multiforme, Anaplastic astrocytoma	None	–

(Continued)

TABLE 2 | Continued

Myeloid target	ClinicalTrials.gov identifier	Phase	Tumor types	Additional treatment	Results?
	NCT01470794	1	Glioblastoma multiforme, Anaplastic astrocytoma, Anaplastic oligodendroglioma, Anaplastic oligoastrocytoma	None	Safe dose established, survival improved from external control, all responders remain alive as of August 25, 2017 (192, 195)
TVEC (GM-CSF)	NCT01161498	3	Squamous cell carcinoma head and neck cancer	Cisplatin	Terminated to permit redesign (196)
	NCT03597009	1b/2	Malignant pleural effusion stage IV metastatic cancer lung cancer	Nivolumab	
	NCT01740297	1b/2	Melanoma	Ipilimumab + TVEC	Approximate doubling of ORR in combination (191)
	NCT03802604	1	Breast cancer	Atezolizumab (anti-PD-L1)	
	NCT03069378	2	Sarcoma	Pembrolizumab	
	NCT02819843	2	Melanoma merkel cell carcinoma other solid tumors	Radiotherapy	
	NCT02978625	2	Non-melanoma skin cancer	Nivolumab	
	NCT03747744	1	Melanoma	Myeloid DCs	
	NCT03886311	2	Sarcoma	Nivolumab + Trabectedin	
	NCT02779855	1/2	Breast cancer	Chemotherapy	
	NCT02923778	2	Soft tissue sarcoma	Radiotherapy	
	NCT03256344	1	Metastatic triple negative breast cancer metastatic colorectal cancer	Atezolizumab	
	NCT02965716	2	Stage III-IV melanoma	Pembrolizumab	
	NCT03300544	1	Locally advanced or metastatic rectal cancer	Chemotherapy + Radiation therapy	
	NCT02626000	1	Carcinoma of the head and neck	Pembrolizumab	Combination safety established, showed some clinical effect (197)
	NCT03554044	1	Metastatic, unresectable, or Recurrent HER2- negative breast cancer	Chemotherapy OR Endocrine therapy	
	NCT02509507	1	Liver tumors	Pembrolizumab	
	NCT03088176	1	BRAF mutated advanced melanoma	BRAF/MEK inhibitors	

Acknowledging the role of IL-8 in MDSC recruitment, Collins et al. recently conducted a Phase I clinical trial with HuMax-IL8, an anti-IL-8 monoclonal antibody, to assess the safety profile and efficacy of this therapy in reducing serum IL-8 levels in patients with solid tumors (NCT02536469). Their trial concluded that IL-8 blockade was well-tolerated and successfully decreased serum IL-8 levels in subjects across all doses tested (188). Given the results of this clinical trial, combination with ICI may be underway.

STING agonist MK-1454 is being used intratumorally in combination with systemic pembrolizumab in treatment of solid, glioma tumors and lymphomas in an ongoing Phase I trial (NCT03010176) with reports of partial response in a number of patients (189). In two other Phase I trials with similar tumors, the STING agonist ADU-S100 is being tested in combination with ipilimumab (anti-CTLA-4, NCT02675439) and PDR-001 (anti-PD-1, NCT03172936). A majority of the patients have dropped out of the STING monotherapy arm due to disease progression. However, lesion biopsies have shown an increase in tumor-infiltrating $CD8^+$ T cells, indicating some immunological effect with combination therapy (190).

T-VEC, an oncolytic herpes simplex virus that expresses GM-CSF, has been FDA approved for treatment of melanoma. Since approval, a number of trials have attempted to use this therapy in combination with other immune-based therapies. Notably, a Phase Ib/II trial of T-VEC combined with ipilimumab (NCT01740297) has shown promising results, with a doubling of ORR when compared to ipilimumab alone (191). Pexa-vec, a vaccinia virus also engineered to express GM-CSF, is also the subject of a number of clinical trials of combination treatments.

Finally, the results from a completed Phase I trial of Toca 511 and Toca FC trial in high grade gliomas (NCT01470794) have been released. A safe dose has been established and as of the last report (August 25, 2017), several long-term survivors are still being followed (192).

The remaining clinical trials are listed in **Table 2** and have not released any results to our knowledge.

CONCLUSION

A growing body of work has highlighted the importance of the myeloid compartment in glioma and other cancers. The

composition of myeloid cells in the TME contributes to the success of immunotherapy as well as adjuvant treatments such as radiation and chemotherapy. Often, it is necessary to modulate the myeloid compartment to a phenotype that is pro-inflammatory to exert enhanced anti-tumor effects. Unfortunately, these effects are not often considered when designing new therapies. Additionally, as discussed above, the effect of certain myeloid modulators such as GM-CSF and tyrosine kinase inhibitors can change depending on the context or timing of treatment. To produce the desired treatment outcomes, it is necessary to thoroughly evaluate the therapeutic mechanism of myeloid targets. In this review, we have listed examples of combination therapies that have attempted to modulate the myeloid compartment in ways that improve the efficacy of the other treatments. These treatments introduce further challenges in ensuring that the separate treatments do not interfere, and instead synergize by addressing their respective deficits. Finally, we discussed the clinical trials attempting to target myeloid cells in combination with other therapies. Still a largely unanswered question regarding the myeloid compartment revolves around which patient populations and tumor types will effectively respond to myeloid-targeting therapy. While tumors with MDSC-enriched TMEs would presumably benefit from these strategies, other influencing factors have yet to be elucidated. Factors that may impact therapeutic efficacy may include levels of myeloid cells in the periphery that are eligible for recruitment, density of myeloid cells and their distance from the tumor core, the composition of immunomodulatory factors secreted by the TME, and the mutational landscape of the tumor itself. Although there is some promise of long-term survivors and responses from limited published trial results and as we eagerly await the results of various ongoing trials, it is important to continue to consider the above factors in the design of new combinations. We hope that with improved understanding of the complex interplay between various immune compartments in the TME and continued consideration of the role of myeloid cells in glioma and other tumor types, the efficacy of immune-based therapies will continue to improve with optimally designed combination treatment regimens.

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Several authors were instrumental in making this manuscript. DR, AD, CJ, and ML contributed to the original conceptualization, writing, and editing of the manuscript. DR and AD contributed to the generation of visualizations associated with the manuscript.

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REVIEW
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Targeting Innate Immunity to Enhance the Efficacy of Radiation Therapy

Tahir B. Dar[1], *Regina M. Henson*[1] *and Stephen L. Shiao*[1,2]*

[1] Department of Radiation Oncology, Cedars-Sinai Medical Center, Los Angeles, CA, United States, [2] Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA, United States

Radiation continues to play a major role in the treatment of almost every cancer type. Traditional radiation studies focused on its ability to damage DNA, but recent evidence has demonstrated that a key mechanism driving the efficacy of radiation *in vivo* is the immune response triggered in irradiated tissue. Innate immune cells including macrophages, dendritic cells, and natural killer cells are key mediators of the radiation-induced immune response. They regulate the sensing of radiation-mediated damage and subsequent radiation-induced inflammation. Given the importance of innate immune cells as determinants of the post-radiation anti-tumor immune response, much research has been devoted to identify ways to both enhance the innate immune response and prevent their ability to suppress ongoing immune responses. In this review, we will discuss how the innate immune system shapes anti-tumor immunity following radiation and highlight key strategies directed at the innate immune response to enhance the efficacy of radiation.

Keywords: radiation therapy, innate and adaptive immune response, immunotherapy, macrophages, dendritic cells, NK cells

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Correspondence:
Stephen L. Shiao
stephen.shiao@cshs.org

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INTRODUCTION

Radiation (RT) continues to play a major role in the treatment of cancer with more than 50% of all cancer patients receiving RT sometime during their treatment course (1). Traditionally, the primary mechanism of action for RT's effect on tumors was thought to be RT-induced DNA damage to malignant cells. However, recent evidence demonstrating the critical role of the immune system in regulating the response to cytotoxic therapies such as RT has challenged this long-standing assumption about how RT mediates its anti-tumor activity.

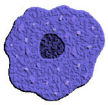
Early work from Stone et al. demonstrated that mice lacking T and B cells required more RT to control the same size tumor compared to immune intact animals (2). Other groups have since gone on to show the importance of IFN-γ producing cytotoxic CD8+ T cells (3, 4) as critical effectors in the tumor response to RT. Thus, it has become clear that a T cell response is required for RT to attain its maximal efficacy. However, T cell responses are the culmination of a multi-step inflammatory response that begins with RT-mediated damage to a tumor and its microenvironment. The sensing of this damage and transmission of the signals to generate a productive immune response is the responsibility of the most ancient form of immunity, the innate immune system. The innate immune system that includes natural killer (NK) cells, macrophages and dendritic cells (DCs) serves as the early warning system of body and the gatekeeper to T cell responses. By virtue of its early role in inflammation, innate immunity has the ability to shape the magnitude and character

of the RT-induced immune response (summarized in **Table 1**). We review here how the innate immune system regulates the response to RT and highlight potential therapeutic approaches that target innate immunity in combination with RT to enhance the RT-mediated anti-tumor immune response.

Innate Immunity

The immune system is often separated into two categories: innate and adaptive immunity. The key distinction between the categories is antigen specificity, i.e., the ability of each cell to uniquely recognize and respond to a single specific molecular entity. Adaptive immunity consisting primarily of B and T cells provide the diverse specificity of the immune system through the essentially infinitely rearrangeable B and T cell receptors. Innate immunity largely composed of dendritic cells, myeloid/macrophages and natural killer (NK) cells provide the *context* for an immune response through a specialized set of receptors designed to distinguish when a given target poses a danger and should be eliminated by the immune system (22). Upon recognition of a common array of molecular patterns called pathogen associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) which signal the presence of pathogens or tissue-damage ("danger"), the innate immune system initiates an immune response (23). Cells of the innate immune system serve not only as early responders to contain the source of inflammation, but also as the gateway to a full and robust immune response by transmitting critical signals to activate the adaptive immune system. Once the combination of the earlier innate immune response and the later adaptive response have eliminated or contained the source of antigen, the innate immune system, particularly the myeloid cells/macrophages, helps restore tissue homeostasis by clearing dead cells, restoring the vasculature and reconstituting the normal tissue structure (24). Thus, given the innate immune system's critical role in the initiation, maintenance, and resolution of an immune response, it is no surprise that the innate immune system plays an important role in regulating the immunobiology of tumors affecting everything from the progression of tumors to their response to therapy.

TABLE 1 | Summary of immune cells in the tumor microenvironment.

Innate immune cells	Interaction with radiation therapy	References
Dendritic cells	• Batf3-dependent DCs induced by RT promote anti-tumor immune responses by activating CD8 + T cells	(5, 6)
	• DCs upregulate MHC-I after RT, promoting efficient tumor antigen presentation for better anti-tumor immune responses	(6–8)
	• RT enhances tumor antigen presenting capacity of infiltrating DC through type I IFN production	(9)
Macrophages 	• RT induces TGF-ß and IL-4 leading to production of alternatively activated/M2 macrophages which inhibit anti-tumor immune responses	(4, 10–13)
	• Macrophages promote matrix remodeling, vasculogenesis which support tumor regrowth post-RT	(14, 15)
	• Inhibiting macrophages via CSF-1R, Axl, Cd11b results in better anti-tumor responses post-RT	(4, 16–18)
NK cells	• RT enhances cytotoxic activity of NK cells against various solid tumors including pancreatic cancer and sarcoma	(19–21)

Innate and adaptive immunity play many roles in the context of tumor biology. Key functions of each of the immune cells is listed.

RADIATION THERAPY AND INNATE IMMUNITY

Among cancer therapies, RT possesses unique biology as a result of its ubiquity in the environment. Given the omnipresent nature of radiation from natural sources such as naturally occurring isotopes and cosmic radiation, all organisms from bacteria to humans have had to develop methods to deal with cells damaged by irradiation. Activation of the innate immune system is one of those methods and likely serves as one of the main mechanisms driving the extraordinary efficacy of RT. Evidence of the importance of innate immunity in the response to RT come from studies that demonstrate reduced efficacy for RT in preclinical models of cancer which are deficient in innate immune cells including NK cells (25), macrophages (4, 16), and DCs (26). These findings are further supported by numerous observations from patients; one study in hepatocellular carcinoma, for example, showed that increased numbers of circulating myeloid cells following RT correlated with poorer responses (27). Thus, given that innate immunity has such an important role in determining the response to RT, multiple groups have explored the mechanisms by which RT interacts with the innate immune system. We discuss the findings from these studies below in the context of the different functions of the innate immune system: initiation of inflammation, activation of the adaptive immune response and resolution of an immune response (**Figure 1**).

Role of Radiation in the Initiation of an Anti-tumor Immune Response

As previously mentioned, one of the primary functions of the innate immune system is to regulate the initiation of an immune response. In the sterile environment of most tumors, innate immune cells initiate an immune response following detection of signals that indicate the presence of cell damage or danger. Radiation activates the innate immune system by inducing both tumor and normal cells to release specific danger signals that leads to activation of multiple inflammatory pathways in innate immune cells. These danger signals include high-mobility group B-1 (HMGB1), calreticulin, complement, and cytosolic DNA all of which act upon receptors on innate immune cells and lead to

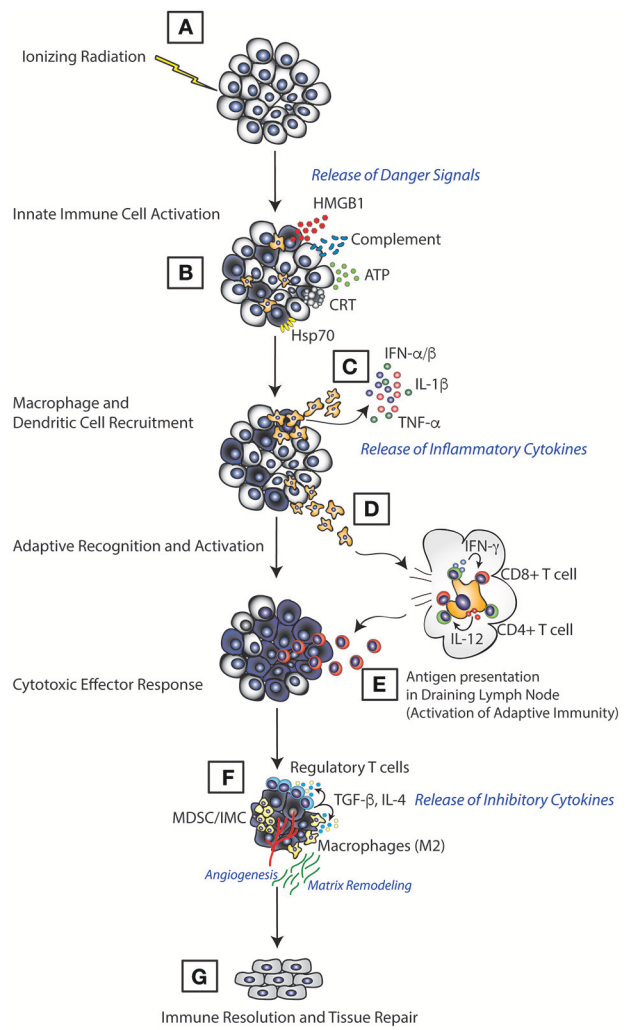


FIGURE 1 | Model of immune activation following RT. RT induces direct tumor cell death which leads to release of various immunological mediators in the form of ATP, HMGB1, calreticulin, and complement **(A)**. This leads to innate immune cell priming, where innate immune cells, such as dendritic cells and macrophages, recognize these mediators through various receptors, migrate to the tumor **(B)** and induce the production of inflammatory molecules such as TNF-α, IL-1β, and type I IFN **(C)**. Innate cells then migrate to the lymphoid tissue **(D)** carrying antigens acquired from the tumor cell for presentation **(E)** resulting in activation of the adaptive immune response and elimination of tumors. Once tumors are eradicated, the RT-induced inflammation is suppressed **(F)**, and tissue damage associated with tumors and the immune response is repaired **(G)**.

release of mediators such as cytokine and chemokines that trigger an immune response (28–30) (**Figure 2**).

The HMGB1 protein is a nuclear protein that is released by damaged cells and binds to toll-like receptor 4 (TLR4), the main receptor for lipopolysaccharide (LPS). Thus, HMGB1, like its bacterial counterpart LPS, can stimulate macrophages and dendritic cells which express high levels of TLR4 leading to cytokine production and upregulation of molecules (MHC, B7.1, B7.2) that lead to activation of T cells. It was one of the first inflammatory molecules identified in the setting of RT. Apetoh et al. demonstrated that RT releases HMGB1 and that depletion of HMGB1 or loss of TLR4 reduced the efficacy of RT (29). Interestingly, they also identified a variant in the TLR4 gene that leads to less efficient binding and patients with the variant seemed to do worse with standard of care therapy which in many instances included a course of RT (28). In addition to HMGB1, calreticulin (CRC) has also been shown to be expressed on the surface of cells following RT leading to better anti-tumor immunity (30). Calreticulin serves as a phagocytic signal for macrophages which engulf the dying cells and subsequently can present tumor antigens (31). TLR4 is highly expressed on innate immune cells, thus the primary responders to RT-associated HMGB1 are likely macrophages and DCs in the tumor microenvironment (28, 30). Further, macrophages as the primary cells responsible for the clearance of damaged cells are responsible for recognizing calreticulin. Thus, for the extracellular inflammatory signals produced by RT, the innate immune system serves as the main conduit to conduct danger signals to the rest of the immune system.

Recent studies have also identified cytosolic DNA as a critical inflammatory signal induced by RT (32, 33). Of the various cancer therapies, RT, in particular, damages DNA both directly and indirectly within the nucleus and mitochondria and in doing so generates DNA fragments both with the nucleus and cytosol. Cytosolic DNA is recognized by an intracellular protein called cGAS (cyclic GAMP synthase) which leads to production of cGAMP (2′-5′ GMP-AMP). cGAMP activates the endoplasmic reticulum (ER)-bound STING (stimulator of interferon genes) pathway which further recruits and phosphorylates TBK1 (TANK-binding kinase 1), leading to phosphorylation and activation of IRF3 (IFN-regulatory factor 3) and subsequent production of Type I interferons like IFN-β (**Figure 1**) (34, 35). cGAS and STING are highly expressed by a variety of innate immune cells such as macrophages, dendritic cells, and others and required for optimal production of type I interferons (36–38). Recent evidence from several groups have shown that the cGAS-STING pathway is responsible for detecting cytosolic tumor–derived DNA after RT-induced damage to the DNA (32, 34, 39, 40). Subsequent production of type I interferons post-RT are critical for generating the anti-tumor cytotoxic CD8+ T cell response. Studies in murine B16 melanoma model revealed that the surge of IFNβ production in irradiated tumors is associated with enhanced RT-induced anti-tumor effects in IFN receptor intact mice which is lost in mice lacking the IFN receptor (IFNAR-1$^{-/-}$) (9, 41). Other DNA damaging agents such as anthracyclines have also been shown to signal through the cGAS-STING-IFN pathway to produce anti-tumor immune responses (42).

Recent observations have shown that a DNA exonuclease called 3′ repair exonuclease 1 (Trex1) regulates RT-induced activation of the cGAS-STING-IFN pathway. Using paired RT-sensitive and resistant orthotopic breast cancers it was revealed that RT-sensitivity depends in part on Trex1 levels. Mechanistically, Trex1 cleaves the DNA that accumulates in the cytosol following RT thereby abrogating IFN-β production through the STING-cGAS pathway. Thus, high

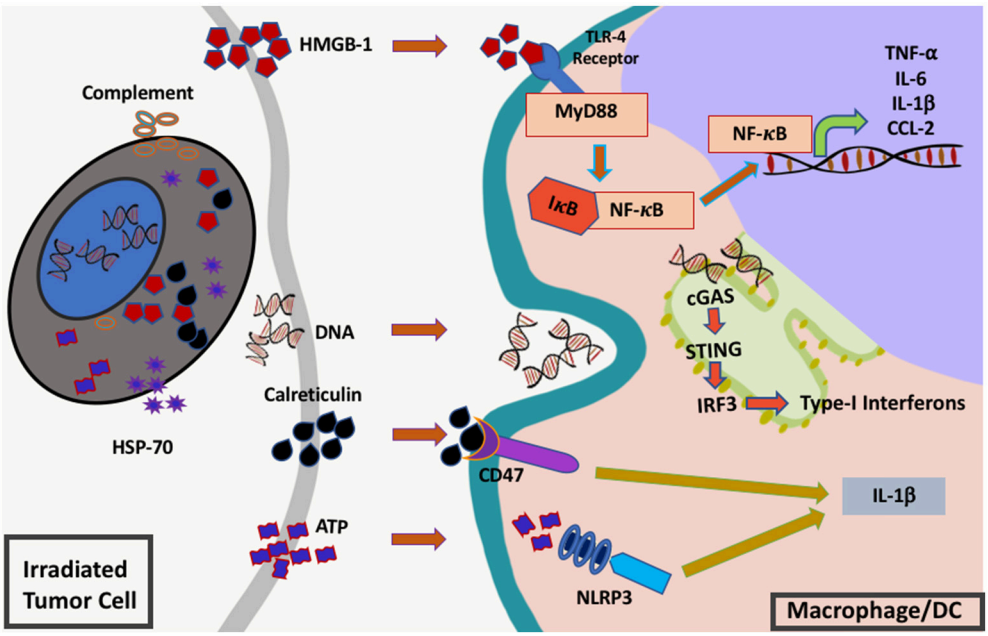


FIGURE 2 | Innate immune signals ("danger signals") triggered by RT. RT induces the release and activation of multiple different inflammatory mediators from injured cells including complement, heat shock protein 70 (hsp70), high-mobility group box protein 1 (HMGB1), cytosolic DNA, calreticulin, and adenosine triphosphate (ATP). These molecules are sensed by innate immune cells such as macrophages or dendritic cells via toll-like receptor 4 (TLR-4), cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING), CD47 and NLR family pyrin domain containing protein 3 (NLRP3). Once sensed these receptors send signals via nuclear factor kappa B (NF-κB) and interferon regulatory factor 3 (IRF3) leading to downstream cytokine production and subsequent inflammation.

levels of Trex1 prevent radiation-induced Type I interferon induced inflammation thereby reducing the efficacy of RT (5). Interestingly, multiple smaller fractions of radiation (8 Gy∗3) did not induce higher levels of Trex1, rather it induced more IFN-β production and activation of Batf3-dependent DCs, leading to enhanced anti-tumor T cells responses. The induction of Trex1 by a single fraction of high-dose radiation dose but not with a short-course of fractionated radiation suggests that it may be essential to fractionate the radiation doses to improve the immunogenicity of RT and its synergy with immunotherapy. Preclinical studies and a recently reported clinical trial support this notion demonstrating synergy between fractionated RT and anti-CTLA (43). In the checkpoint-resistant breast TSA model (mouse), it was observed that single high dose (20 or 30 Gy) of RT did not induce abscopal effects when used along with either anti-CTLA-4 or anti-PD-1 while a short fractionated course (8 Gy∗3) induced an abscopal systemic immune response when given in conjunction with anti-CTLA-4 leading to prolonged/sustained tumor regression. Fractionated lower doses (8 Gy∗3) induced the production of IFN-I stimulated genes in mice followed by enhanced number of CD8α+ tumor infiltrating DCs (with high CD70) within the tumors and IFN-β in TSA cells *in vitro* but interestingly 20 Gy did not in part through in the induction of Trex1 by high-dose single fraction RT. Trex1 knockdown in TSA cells restored Type I interferon production with high doses of radiation (20 Gy∗2) suggesting that induction of Trex1 is a key mediator of RT-induced inflammation. Thus, to ensure an optimal anti-tumor responses, short-course fractionated RT may need to be employed in part to prevent Trex1 induction leading to optimal sensing of the cytosolic DNA produced by RT.

By sensing an array of danger signals produced by irradiated cells, innate immune cells serve as the primary sentinels of the body to identify cells that have been damaged by radiation (**Figure 1**). As such, the innate immune system plays an outsized role in determining the response to radiation damage. Macrophages and dendritic cells integrate the danger signals they received from irradiated cells and their response to these signals shapes the ensuing immune response. Thus, many strategies are currently being explored to augment the response of the innate immune system following RT to help create better anti-tumor immunity.

Targeting Innate Immune Initiation of an Anti-tumor Immune Response

Most of the strategies directed at augmenting the innate immune response have focused on increasing signals that mimic the danger signal sensed by the innate immune system. In preclinical models, these strategies have shown much promise and are beginning to be tested clinically. The oldest and most common strategy that has been utilized to enhance the early innate immune response typically targets toll signaling. While RT naturally leads to release of HMGB1 which binds TLR4, other toll agonists have also been utilized to augment the inflammatory response triggered by RT. For example, addition of CpG, a TLR9 agonist, showed synergy when combined with RT (44) in a murine and canine models of melanoma and a murine

model of breast cancer (45). Imiquimod, a TLR7 agonist, also showed increased activity in conjunction with RT in several different murine models of cancer (46, 47) with enhanced immune activation noted in a trial of human breast cancer skin metastases (48). In one preclinical study, topical application of imiquimod to lesions in a murine breast cancer model in combination with RT and low-dose cyclophosphamide led to tumor regression for both the irradiated and distant lesions and was further associated with an upregulation of IFN-α and IFN-γ signaling and CD8+ T cell homing to the tumor site (49). Similarly, systemic administration of another TLR7 agonist, DSR-6434 resulted in enhanced radiation efficacy with prolonged tumor regression in murine models of colorectal carcinoma and fibrosarcoma with increased type I interferon production (47).

Other strategies to improve innate immunity have focused on the calreticulin pathway. As mentioned previously, calreticulin expression is induced by RT and is an important signal for phagocytosis by macrophages (31). This process is regulated in part by a molecule known as CD47 (integrin associated protein) which interacts with signal regulatory protein-alpha (SIRPα) expressed on myeloid cells. This interaction causes phosphorylation of the SIRPα cytoplasmic immunoreceptor tyrosine-based inhibition motifs and recruitment of Src homology 2 domain-containing tyrosine phosphatases to ultimately result in delivering an anti-phagocytic signal to myeloid cells preventing a cell from being consumed (50). While, not acting directly in concert, the phagocytic stimulation provided by RT-induced calreticulin can be enhanced by blocking the anti-phagocytic signal CD47 which leads to increased dendritic cell and macrophage activation and improved anti-tumor immunity (51, 52). Trials are currently underway testing this pathway in combination with RT (ClinicalTrials.gov, NCT02890368).

One of the most promising newer strategies to augment the RT-mediated activation of innate immunity has been to target type I interferon production through the use of STING agonists. Mostly structured as cyclic dinucleotides, multiple groups have shown the efficacy of STING agonists in combination with chemotherapy and various immunotherapies (53–55). STING agonists in combination with RT have also been examined (56, 57) and in these murine models of pancreatic cancer and prostate cancer, STING agonists in combination with RT showed significant synergy. In this study using a murine model of pancreatic cancer, Baird et al. found that RT along with STING agonist-CDN displayed strong synergy significantly enhancing tumor regression through augmented CD8+ T cell responses (57). Similar synergy was also observed in murine models of lung cancer (LLC) and colorectal cancer (MC38) (56). Like the other agents targeting cancer by augmenting innate immune activation, STING agonists are currently in early phase clinical trials for multiple different cancer types (ClinicalTrials.gov, NCT03172936).

While limited clinical information exists, there is substantial preclinical data suggesting that augmenting the innate immune activation triggered by RT can significantly enhance the anti-tumor immunity produced following RT (**Figure 1**). However, given the significant release of these innate immune activating molecules following RT at baseline there may be other aspects of how innate immune cells interact with tumors that can serve as additional targets.

RT and Regulation of the Anti-tumor Adaptive Immune Response

When cells of the innate immune system detect that there is a problem, e.g., an infection or tissue damage, they activate a program of inflammation that leads to activation of the adaptive response (T and B cells). Activation of the adaptive immune system requires maturation of dendritic cells or macrophages into antigen-presenting cells (APC) which requires appropriate expression of MHC molecules and co-stimulatory signals. Interestingly, RT has been shown to upregulate MHC class I and stimulate presentation of unique antigens (7, 8, 58) as well as costimulatory molecules (58, 59) by dendritic cells. The importance of DC in mediating the efficacy of RT was shown Dewan et al., where fractionated radiotherapy along with anti-CTLA4 had significant abscopal effects in part through the generation of increased numbers of Batf3 DCs (43). Batf3 dependent DC cells are an important subset of dendritic cells with their ability to efficiently cross-present antigens and regulate tumor growth by enhancing CD8+ T cell migration to the tumor microenvironment and fostering effective T cell response (6, 60). Abscopal effects were abolished in the Batf3$^{-/-}$ mice consistent with other observations demonstrating the critical role of Batf3 DC in regulating RT-induced anti-tumor immune responses (60–62). In addition to its effects on DC, RT further contributes to the adaptive immune response by encouraging innate immune cells to establish an inflammatory milieu in irradiated tissue in part through stimulating the release of complement and pro-inflammatory cytokines and chemokines by innate immune cells (63, 64).

Following innate recognition, one of the first proinflammatory molecules activated by RT is complement, soluble effector proteins that are produced by and regulate innate immune cell function (65, 66). Surace et al. demonstrated that components of the complement system are important for RT-induced anti-tumor immunity both in murine and human tumors (66). They showed higher levels of activated C3a and C5a (inflammatory anaphylatoxins) in tumors within 24 h of RT and that these mediate the response to RT-induced damage to tumor cells (66). They went on to demonstrate that in a mouse melanoma model, DC activation post-RT was dependent on these anaphylatoxins. In their model, DC activation post-RT was only observed in wild-type mice but not in mice lacking C3, the C3a receptor or the C5a receptor. Previous studies have shown that anaphylatoxins can bind on their own receptors (67, 68), thus, following RT it was observed that DC increased the expression of some of the complement factors including C3 and the C5a receptor within 24 h following RT and that expression of these complement factors were critical for controlling DC activation and subsequent T-cell responses following RT. As would be expected from a complement response (69), RT-mediated complement activation increased NK1.1+ (natural killer cells) but not NKp46+

(invariant NK-T cell) cell populations which likely served to enhance anti-tumor response of CD8+ T cells.

In addition to expression of complement, RT has been shown to increase the expression of a number of cytokines and chemokines. Aside from the previously mentioned type I interferons, RT has been shown to induce immune cells within the tumor including TAMs and CD8+ T cells and NK cells and others to produce inflammatory cytokines including tumor necrosis factor alpha (TNF-α) (70), interleukin-1 (71), interleukin-6 (72, 73), interferon-gamma (IFN-γ) (3, 74), macrophage colony stimulating factor 1 (CSF-1, M-CSF) (75), and granulocyte macrophage colony stimulating factor (GM-CSF) (76, 77). These cytokines are critical for establishing inflammation at the irradiated site as well as induction of a cytotoxic CD8+ T cell response. Genetic ablation or use of agents that deplete or block the actions of theses cytokines significantly reduced the response to RT across a number of histologies including melanoma, sarcoma and breast in murine models. These cytokines not only serve to attract circulating immune cells, but also help establish inflammation by altering the vasculature (78) and increasing the release of chemokines including CXCL16 (79, 80), CCL2 (81), and CCL5 (82). These chemokines serve to attract CD8+ T cells (CXCL16) and myeloid cells (CSF-1, CCL2, CCL5) to irradiated tumors.

Through the expression of various inflammatory molecules, the innate immune system translates the danger signals they sense in irradiated tissue into an anti-tumor immune response. Multiple strategies have been employed combining RT with various agents in attempt to enhance the innate immune response to RT as we discuss below.

Enhancing Innate Regulation of the Anti-tumor Adaptive Immune Response

In addition to targeting the danger signaling induced by immunogenic cell death, multiple groups have sought to make the downstream responses of the innate immune cells more productive. Strategies to enhance the magnitude and efficiency of antigen presentation and inflammation induced by RT are currently being explored.

The primary target of the strategies to augment the innate immune response following RT has been focused on dendritic cells as they are the primary APC within tumors. Several groups have shown that they can improve the response to RT in murine models and early human trials by increasing the growth and differentiation of dendritic cells. One way to increase the number of DC is the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) which has been shown to be a crucial pathway for the growth, maturation and migration of DC (83, 84). Several human trials of GM-CSF in melanoma and breast cancer have demonstrated the efficacy of GM-CSF administration alone with improved survival compared to historical controls (85) (86) and an increase in circulating DC (87). Based on these successful early studies, trials of GM-CSF and RT were initiated. In one trial of metastatic patients of various histologies, exogenous administration of GM-CSF with a course of fractionated RT (35 Gy in 10 fractions) found evidence of an abscopal, and hence systemic, anti-tumor immune response in 27% of the patients (84). Currently, multiple trials are underway to test the efficacy both locally and systemically of combining GM-CSF with high-dose, short-course radiation (stereotactic body radiation therapy, SBRT) in hepatocellular carcinoma (ClinicalTrials.gov, NCT02946138) and lung cancer (ClinicalTrials.gov, NCT02976740, NCT02623595, NCT03113851) and standard fractionated RT in glioblastoma (ClinicalTrials.gov, NCT02663440).

Another cytokine for DC-specific growth similar to GM-CSF that has been shown to enhance the response to RT is the FS-like tyrosine kinase 3 ligand (FLT3L) (88–90). FLT3L binds and activates FLT3 on hematopoetic progenitors and serves a critical role in steady-state maintenance of DC (91) and increased levels of FLT3L during inflammation mobilizes DC (92). Two studies using preclinical models of non-small cell lung cancer demonstrated reduced tumor growth, metastases, and improved survival with administration of RT and FLT3L in a T-cell dependent manner (89, 90). Based on the success of the preclinical data, FLT3L is currently being tested in a phase II trial in non-small cell lung cancer in combination with SBRT (ClinicalTrials.gov, NCT02839265). Preclinical data in a murine model of hepatocellular carcinoma has also shown that the efficacy of RT can be enhanced by augmenting DC function through the use of exogenous IL-12 to help DCs better generate cytotoxic T cells (93).

Instead of encouraging the creation of more or better DCs, others have taken a more direct approach and have tested combining dendritic cell vaccines with RT (94, 95). In two trials for glioblastoma, DC loaded with tumor lysates were administered either concurrently with chemoradiation (96) or immediately following (97) demonstrated increased numbers of tumor specific T cells, however neither showed correlation between immune response and survival, though they were not powered enough to determine such a correlation. Currently, an open trial in brainstem glioma combines both of the above strategies employing both DC vaccination, GM-CSF, and standard RT for patients (ClinicalTrials.gov, NCT03396575). Though previous trials have not been able to show significant survival impact using the combination of DC targeting with RT, as understanding of the underlying immune mechanisms increases more combinations with various immune therapies as well as different doses and timing of RT may further enhance the response to DC vaccines and RT.

While none of these strategies have had tremendous clinical responses to date, the advent of newer immunotherapy approaches particularly those targeting tumor immunosuppression such as checkpoint inhibitors have generated renewed interest in RT and DC vaccination combinations.

RT and Innate Immunity-Mediated Immunosuppression

The recent success of agents known as checkpoint inhibitors that block immunosuppressive pathways within tumors highlight the importance of targeting the immunosuppressive tumor

microenvironment to foster anti-tumor immunity. Cells of the innate immune system particularly macrophages in conjunction with tumor cells participate in establishing the suppressive environment of tumors. Macrophages play a complex dual role in the context of tumor immunobiology. They have pro-inflammatory roles as outlined above, but more often exhibit a pro-tumor phenotype that suppresses anti-tumor immunity and supports tumor growth (98, 99). In the context of radiation therapy, multiple groups have shown that macrophages play a negative role in regulating the anti-tumor response after RT thus reducing the efficacy of RT. Several groups have reported increased numbers of myeloid-macrophages migration following RT in models of head and neck cancer, glioma, pancreatic, and breast cancer (4, 16, 81, 100, 101). Further, many of these macrophages have been shown to have an immunosuppressive pro-tumor phenotype, also known as the M2 or alternatively activated phenotype, which limits the response to RT (4, 102, 103). Further, macrophages are one of the key cells within tumors that express both PD-1 (104) and PD-L1 (105). Thus, given the role of innate immune cells like macrophages as sources of tumor immunosuppression, it is not surprising that many groups have explored targeting the suppressive capacity of innate immune cells to improve the efficacy of RT.

Targeting Innate Immunity-Mediated Immunosuppression in Combination With RT

With the recent recognition of the need to alleviate the intrinsic tumor immunosuppression to allow anti-tumor immunity to progress, much activity has been devoted to targeting the pathways and cells that mediate immunosuppression. Interestingly, many of the cellular targets are innate immune cells such as macrophages. Since RT generates both an anti-tumor immune response and the corresponding suppressive immune control mechanisms, combinations of RT with agents that target intratumoral immune suppression are thought to allow for an enhanced anti-tumor immune response following RT. Preclinical models strongly support this notion and clinical data is just emerging that suggests that this strategy may also be efficacious in the clinical setting.

One of the most successful regimens targeting intratumoral immunosuppression has been targeting immune suppression with checkpoint inhibitors which are agents that target the PD-1/PD-L1 and CTLA-4 pathways. Innate immune cells are one of the key sources of signal for the PD-1/PD-L1 pathway with dendritic cells and macrophage serving as one of the primary, non-tumor sources of PD-L1 in the tumor microenvironment. Thus, the underlying mechanism of checkpoint blockade likely involves disrupting the effects of innate immune cells on immune response in tumors. To date, an increasingly large amount of data has demonstrated the efficacy of using checkpoint inhibitors in the preclinical and clinical setting in combination with RT. As several excellent recent reviews have examined the role of combining checkpoint blockade with RT in detail, we will not discuss combinations with checkpoint blockade further here though it should be recognized that including one of these agents as a part of any immune-directed therapeutic regimen will be an important consideration for the foreseeable future (106, 107).

Beyond checkpoint blockade, macrophages serve as the main source of immunosuppression within the tumor microenvironment following RT. As evidence of the importance of macrophages, various studies have revealed a strong negative correlation between the presence of macrophages and survival in various solid tumors including breast, colon, bladder, and lung cancer (10–12, 108). As we described above, macrophages are often associated with resistance to radiotherapy and chemotherapy by providing both pro-survival signals and tissue repair functions that protect and/or repair the damage done by these therapies. Various studies have shown that macrophages, the most abundant cells of the tumor microenvironment, are altered by RT to support tumor growth after being damaged and sensing damage resulting from irradiation. For example, Leblond et al. found an increase in density of pro-tumor M2 macrophages in the tumor microenvironment post-RT in glioblastoma (109). Kioi et al. showed that the RT-recruited macrophages help rectify the damage done by RT by promoting vasculogenesis (14). Given, the pro-tumor role of macrophages following RT multiple groups have shown that blocking macrophage recruitment via targeting CD11b (16), CCL2 (81), or CSF-1R (4, 75, 100), enhance the efficacy of RT in preclinical murine models. For example, in a squamous cell carcinoma model Ahn et al. found that administration of a CD11b antibody enhanced the efficacy of RT by blocking myeloid cell recruitment to the tumor site after RT leading to delayed regrowth in part through impaired angiogenesis (16). Other studies have revelead that inhibition of macrophages following RT increases both the anti-tumor immune response (4) and prevents pro-tumor repair mechanisms such as angiogenesis and matrix remodeling (14, 16). Thus, these studies all demonstrate that targeting macrophages can synergize with RT, however, given the potentially positive role of macrophages in producing cytotoxic anti-tumor immune responses, other groups have sought to preserve the pro-inflammatory activation capacity of macrophages while preventing their suppressive differentiation to even further synergize with RT.

Given the successful preclinical models showing enhanced responses to RT in combination with agents that target macrophages including CSF-1R inhibitors and CD11b, several trials are currently underway to test the validity of this observation in human trials. Based on the work of Stafford et al. a trial using the small molecule inhibitor of CSF-1R (Pexidartinib, PLX3397, Plexxikon) in newly diagnosed glioblastoma in combination with standard chemotherapy and RT was opened and accruing (ClinicalTrials.gov, NCT01790503). Another group is also testing the CSF-1R inhibitor in combination with concurrent standard dose RT and androgen deprivation for localized unfavorable risk prostate cancer (ClinicalTrials.gov, NCT02472275). Interestingly, agents targeting tumor-associated macrophages such as the CCL2 inhibitor carlumab have had limited effect as single-agents (110) and in fact may only have efficacy when combined with other agents such as RT that perturb the tumor immune microenvironment (4, 111).

In order to preserve the macrophage capacity to activate anti-tumor immunity while preventing their differentiation into pro-tumor, immunosuppressive phenotypes, several groups including our own have examined the potential of targeting the pathways that lead to pro-tumor phenotypes in macrophages including IL-4 (4), arginase 1 (102), TGF-β (15), and Tyro3/Axl/Mer (TAM) tyrosine kinases (18, 101) in combination with RT. Targeting macrophage differentiation led to improved anti-tumor immunity, particularly cytotoxic CD8+ T cells, resulting in dramatically enhanced responses to RT. Though each of these strategies targets a distinct pathway found in myeloid-macrophages, they result in reduction but likely not elimination of immunosuppressive differentiation suggesting that even modest reductions in tumor-associated immunosuppression can have profound effects on therapeutic responsiveness to RT.

The findings from these preclinical studies targeting macrophage phenotype in combination with radiation are just beginning to be explored in the clinical trial setting. One promising target is TGF-β a cytokine for which several inhibitors have been developed. Though TGF-β has pleiotropic effects, its upregulation post-RT is one of the primary drivers of immunosuppression in the irradiated tumor microenvironment particular effects on the development of regulatory macrophages and T cells. Using an agent that binds all isoforms of TGF-β (fresolimumab, Sanofi-Aventis) in combination with SBRT for patients with metastatic breast cancer (ClinicalTrials.gov, NCT02538471), Formenti et al. found that the highest dose combination led to improved survival and systemic immune responses compared to lower doses (112). Other clinical trials testing TGF-β inhibition with RT and/or chemotherapy are currently underway in non-small cell lung cancer (ClinicalTrials.gov, NCT02581787), glioblastoma (ClinicalTrials.gov, NCT01220271), and hepatocellular carcinoma (ClinicalTrials.gov, NCT02906397). Other pathways targeting macrophage phenotypes have not yet been explored clinically, but the experience with TGF-β suggests that strategies that help create favorable macrophage phenotypes may mirror the preclinical data in improving the efficacy of RT.

CONCLUSIONS

The innate immune system plays a critical role in regulating the response to RT from the recognition of RT-mediated tissue damage to shaping of the RT-mediated anti-tumor immune response. Strategies to augment the innate immune response have met with varying success clinically, however promising new strategies based on our improved understanding of innate immune biology such as STING agonists, adjuvants to enhance DC activity and anti-macrophage agents will undoubtedly shape future therapeutic approaches to combination therapies with RT.

AUTHOR CONTRIBUTIONS

TD and SS conceived this article. TD, RH, and SS wrote the article and designed the figures.

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REVIEW
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Role of Radiation Therapy in Modulation of the Tumor Stroma and Microenvironment

*Hari Menon[1], Rishab Ramapriyan[1], Taylor R. Cushman[1], Vivek Verma[2], Hans H. Kim[3], Jonathan E. Schoenhals[4], Cemre Atalar[1], Ugur Selek[5], Stephen G. Chun[1], Joe Y. Chang[1], Hampartsoum B. Barsoumian[1], Quynh-Nhu Nguyen[1], Mehmet Altan[6], Maria A. Cortez[7], Stephen M. Hahn[1] and James W. Welsh[1]**

[1] Departments of Radiation Oncology, University of Texas MD Anderson Cancer Center, Houston, TX, United States, [2] Department of Radiation Oncology, Allegheny General Hospital, Pittsburgh, PA, United States, [3] Department of Radiation Medicine, School of Medicine, Oregon Health and Sciences University, Portland, OR, United States, [4] Medical School, University of Texas Southwestern, Dallas, TX, United States, [5] Department of Radiation Oncology, School of Medicine, Koç University, Istanbul, Turkey, [6] Thoracic/Head and Neck Medical Oncology, Houston, TX, United States, [7] Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, United States

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Edited by:
Patrik Andersson,
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***Correspondence:**
James W. Welsh
jwelsh@mdanderson.org

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In recent decades, there has been substantial growth in our understanding of the immune system and its role in tumor growth and overall survival. A central finding has been the cross-talk between tumor cells and the surrounding environment or stroma. This tumor stroma, comprised of various cells, and extracellular matrix (ECM), has been shown to aid in suppressing host immune responses against tumor cells. Through immunosuppressive cytokine secretion, metabolic alterations, and other mechanisms, the tumor stroma provides a complex network of safeguards for tumor proliferation. With recent advances in more effective, localized treatment, radiation therapy (XRT) has allowed for strategies that can effectively alter and ablate tumor stromal tissue. This includes promoting immunogenic cell death through tumor antigen release to increasing immune cell trafficking, XRT has a unique advantage against the tumoral immune evasion mechanisms that are orchestrated by stromal cells. Current studies are underway to elucidate pathways within the tumor stroma as potential targets for immunotherapy and chemoradiation. This review summarizes the effects of tumor stroma in tumor immune evasion, explains how XRT may help overcome these effects, with potential combinatorial approaches for future treatment modalities.

Keywords: radiation therapy (radiotherapy), immunotherapy, stroma, cancer, tumor microenvironment

INTRODUCTION

Cancer therapy has advanced greatly over the past several decades, and recent advances in immunotherapy have led to marked improvement in outcomes and quality of life in patients with cancers previously thought to be incurable (1, 2). However, responses to immunotherapy are not as robust as previously hoped. This has led to increased interest in the mechanisms of tumor immune evasion. Increasing observations strongly suggest the tumor microenvironment (TME) and stroma are sources for tumor evasion of the immune system and related immunotherapies.

The stromal microenvironment of a tumor presents an underlying challenge to the efficacy of cancer immunotherapy. In their seminal review, Hanahan and Weinberg named evading immune

destruction as an emerging hallmark of cancer among other related activities, such as metabolic reprogramming and induction of angiogenesis within the TME (3). For cytotoxic T cells and other immune cells to kill cancer cells, physical cell-to-cell contact is necessary (4). However, stromal cells actively orchestrate resistance to antitumor immunity by restricting T cells from making physical contact with cancer cells (5). The stroma surrounding tumor islets of solid malignancies consists of a myriad of molecular and cellular components: immune cells, including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs); fibroblasts; epithelial cells; extracellular matrix (ECM) proteins; blood and lymphatic vessels; and various metabolites, chemokines, and cytokines.

Leveraging the components of the stromal microenvironment, tumors employ a variety of strategies for immune evasion. These strategies can be broadly grouped thematically into the following categories: immune cell regulation, metabolic reprogramming, and hypoxia. These immune evasion strategies collectively synergize to blunt the efficacy of tumor-infiltrating lymphocytes (TILs) with regard to both activation and infiltration. Clinically, this may significantly limit significant limitation of cancer immunotherapy. Indeed, evidence suggests that baseline infiltration of both T cells and natural killer cells as well as expression of various chemokines involved in immune cell recruitment to the TME are strongly associated with prognosis for a variety of histological types of cancer (6). Therefore, we believe that the stroma is an underexplored target for immunotherapies that can also synergize with other therapeutic modalities, such as radiation therapy (XRT). Overcoming the immune-suppressive stroma may prove to be integral to unleashing the full potential of immunotherapy and bolstering its antitumor effects.

Radiation therapy is a gold standard of cancer treatment, with more than 50% of cancer patients needing local therapy with XRT (7). With increasing knowledge of the TME's role in immune evasion, interest in the effect of XRT on the TME is growing. From increasing tumor antigen presentation to facilitating trafficking of T cells, XRT plays an important immunogenic role in treatment of cancer and its microenvironment. In this review, we describe how the stroma affects antitumor immunity, XRT's role in disrupting the tumor stroma and TME, and future role of XRT combined with immunotherapy to enhance antitumor immunity.

TUMOR STROMA: EVADING THE ANTITUMOR IMMUNE RESPONSE

Exclusion of Effector Immune Cells From the Tumor Microenvironment

The most observable effect of the tumor stroma in the context of cancer immunotherapy is the exclusion of T cells from tumor beds, resulting in a "cold" phenotype. Inflammatory chemokines are the primary factors involved in trafficking and homing of T cells to the TME. Gene expression profiling performed with a series of melanoma metastases identified six chemokines—CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10—that are associated with $CD8^+$ T-cell recruitment and demonstrated that chemokine blockade inhibited migration of $CD8^+$ effector T cells *in vivo* (8). To induce rapid chemotaxis toward inflammatory chemokines, activated T cells have increased expression of surface chemokine receptors, including CXCR3, which, along with its interferon (IFN)-γ-inducible ligands, has been associated with a Th1 immune response and accumulation of both T and natural killer cells in the tumor bed (9–11).

However, tumors commonly dysregulate normal chemokine pathways and express different chemokines, such as nitrosylated CCL2 and CCL28, which result in the recruitment and accumulation of Tregs, TAMs, immature dendritic cells (DCs), and MDSCs and form an immune-suppressive TME (12). TME conditions are partly responsible for such changes in chemokine networks. Nitrosylation of CCL2, which normally supports tumor-infiltrating lymphocyte trafficking into the tumor core, occurs through the production of reactive nitrogen species in the TME (13). CCL28 is produced as a result of tumor hypoxia and the release of damage-associated pattern molecules (14). In addition, tumors often specifically target chemokines that are responsible for cytotoxic T lymphocyte (CTL) infiltration. One such chemokine is CXCL11, which specifically attracts $CXCR3^+$ $CD8^+$ cells and undergoes proteolytic alterations induced by the tumor, resulting in failure to attract TILs (15). In addition, preclinical and clinical evidence has demonstrated that expression of CCL27, which also plays a role in T-cell homing under inflammatory conditions, is downregulated by hyper-activation of the epidermal growth factor receptor (EGFR)/Ras/mitogen-activated protein kinase (MAPK) signaling pathway in melanoma (16). Overall, manipulation of chemokine networks in the TME results in an abundance of M2 TAMs and other regulatory components that blunt the antitumor activity of CTLs.

In the stroma, both tumor cells and these abundant M2 TAMs secrete various molecules, such as vascular endothelial growth factor (VEGF), interleukin (IL)-10, transforming growth factor (TGF)-β, adenosine, and prostaglandin E_2, that inhibit DC activation and maturation and suppress the activity of CTLs and natural killer-mediated immunity (17). For example, the production of VEGF, which is a well-known mediator of angiogenesis, can play a strong role in preventing DC precursors from maturing into DCs (18). Likewise, prostaglandin E_2 secretion modulates chemokine production in favor of Tregs and MDSCs differentiation while inhibiting CTLs and natural killer cell populations and decreases production of IL-2 and IL-12 (19). M2 TAMs have immune-suppressive roles that extend beyond the production of soluble factors. The "immune-excluded" phenotype can physically occur via long-lasting interactions between CTLs and TAMs. Peranzoni and colleagues showed that stromal macrophages impede $CD8^+$ T cells from reaching tumor islets by making long-lasting contacts that reduce T-cell motility (20). Upon pharmacological depletion of TAMs, T-cell infiltration and migration into the tumor islets were no longer impeded, and this enhanced the efficacy of anti-programmed cell death protein 1 (PD-1) immunotherapy (20). Clinically, the same

study found that lung squamous cell carcinoma patients with high tumor: stroma ratios, which reflected increased $CD8^+$ T-cell infiltration into tumor islets, had better overall survival than did patients with low ratios (20).

Tumor vasculature may play a strong role in the stromal mechanisms of immune exclusion. The migration of T cells through the endothelium, which is often dysregulated as a result of vasculature remodeling, is another challenge to antitumor immunity. For T cells to migrate to the tumor bed, they must adhere to the endothelium (21). However, expression of various endothelial adhesion molecules, such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion protein (VCAM)-1, is downregulated in endothelial cells surrounding solid tumors (22). Recently, Motz and colleagues have described a mechanism by which the tumor endothelial barrier regulates T cell migration into tumors (23). In both human and mouse tumor vasculature, the expression of Fas ligand (FasL), which induces apoptosis, was detected, but it was not detected in normal vasculature (23). Additionally, the expression of FasL on endothelium was associated with decreased $CD8^+$ infiltration and accumulation of Tregs, which were resistant to FasL due to higher c-FLIP expression. However, this blunting of $CD8^+$ T cell infiltration was reversed by pharmacologic inhibition of prostaglandin E_2 and VEGF, which were shown to cooperatively induce FasL expression on this tumor endothelial "death barrier" (23). The dense stroma matrix architecture also presents a unique challenge to T cell infiltration, and matrix reduction with collagenase has been shown to improve T cell infiltration (24, 25). Finally, cancer-associated fibroblasts (CAFs) in the stroma have pleiotropic roles in secretion of chemokines, cytokines, and metabolites that alter antitumor immunity (26). Molecular strategies to normalize tumor vasculature and induce tertiary lymphoid structures have shown much promise in orchestrating effective T cell immunotherapy preclinically (27–30). Overall, tumor cells employ a combination of these above mechanisms in excluding cytotoxic T-cells from the tumor microenvironment, blunting anti-tumor immunity (**Figure 1**).

Metabolic Reprogramming

Metabolic competition between tumor cells and immune cells is known to cause T-cell anergy and immune resistance. Tumor cells, as well as stromal endothelial cells and CAFs, are characterized by the Warburg effect (31). The Warburg effect is traditionally recognized as a unique type of cancer metabolism described as the switch from oxidative phosphorylation to anaerobic glycolysis in the presence of oxygen (32). Warburg found that cancer cells mainly depend on anaerobic glycolysis survival even in the presence of oxygen, which leads to the substantial depletion of glucose from the TME, causing pleiotropic immuno-suppressive effects (33). Excessive depletion of glucose and essential amino acids such as glutamine, tryptophan, and arginine in the TME, coupled with production of metabolites such as lactate, adenosine, and kynurenine, blunts cytotoxic T-cell function while promoting accumulation of regulatory immune cells, such as Tregs, TAMs, and MDSCs (34). Therefore, altered cancer metabolism of tumor stromal cells is a significant factor that mediates resistance of cancer to immunotherapy.

Many metabolic alterations are driven by the need for NADPH, a unique high-energy molecule that is required for lipid synthesis, a building block for the plasma membrane in rapidly growing tumor cells. In almost all types of cancer, both cancer cells and stromal cells like CAFs overexpress transketolase, an enzyme in the pentose phosphate pathway, which importantly produces NADPH and ribose (35). Transketolase is now considered one of the most universally overexpressed genes in cancer metabolism. Additionally, investigators recently found that patients with isocitrate dehydrogenase 1 (IDH1)-mutant glioblastoma had a better prognosis than IDH1 wild type glioblastoma (36). Interestingly, mutation leads to depletion of elevated NADPH pools in cancer cells. Unlike wild-type isocitrate dehydrogenase 1, the mutant form found in glioma patients depletes NADPH pools by converting NADPH to NADP+ (37). Multiple studies have implicated the well-known tumor suppressor p53 in regulating metabolic reprogramming. When Ahmad and colleagues induced overexpression of p53 in human prostate cancer cells and combined it with treatment with 2-deoxy-D-glucose, they showed that the cancer cells overexpressing p53 died of oxidative stress by disrupting glucose influx using 2-deoxy-D-glucose, demonstrating a major role for p53 in glucose metabolism major metabolic switch (38). Their work supports the recently identified role of p53 as a metabolic suppressor of NADPH production (39). One of the enzymes controlled by p53 is malic enzyme, a major NADPH producer in cells (40). Another enzyme whose activity is inhibited by p53 is glucose-6-phosphate dehydrogenase, which is the first step in the pentose phosphate pathway (39). Targeting of cancer metabolism is a crucial consideration in any therapeutic approach and we will later discuss the role XRT plays in this context.

XRT: CHALLENGING THE TUMOR STROMA AND TME

The Evolving Role of XRT in Antitumor Immunity

The field of radiation biology has historically focused on the effects of radiation in killing cancer cells in isolation. Although the earliest cellular radiobiology experiments yielded significant advances in the understanding of DNA damage and repair, they did not account for the impact that local and systemic factors may have on radiation responses. *In vitro* clonogenic and colony formation assays, in which radiation log kill curves were first generated, but did not include an understanding of stromal microenvironment and immunity (41). Moreover, *in vivo* tumor xenograft experiments have historically relied on immune-deficient animal models (42). As such, these classic models that radiation biologists relied on for decades were insufficient to elucidate phenomena such as the abscopal response (43, 44) and the efficacy of PD-1–directed therapies (45).

Increasing evidence has implicated the stromal microenvironment as being a critical mediator of radiation responses both locally and systemically. For example, the

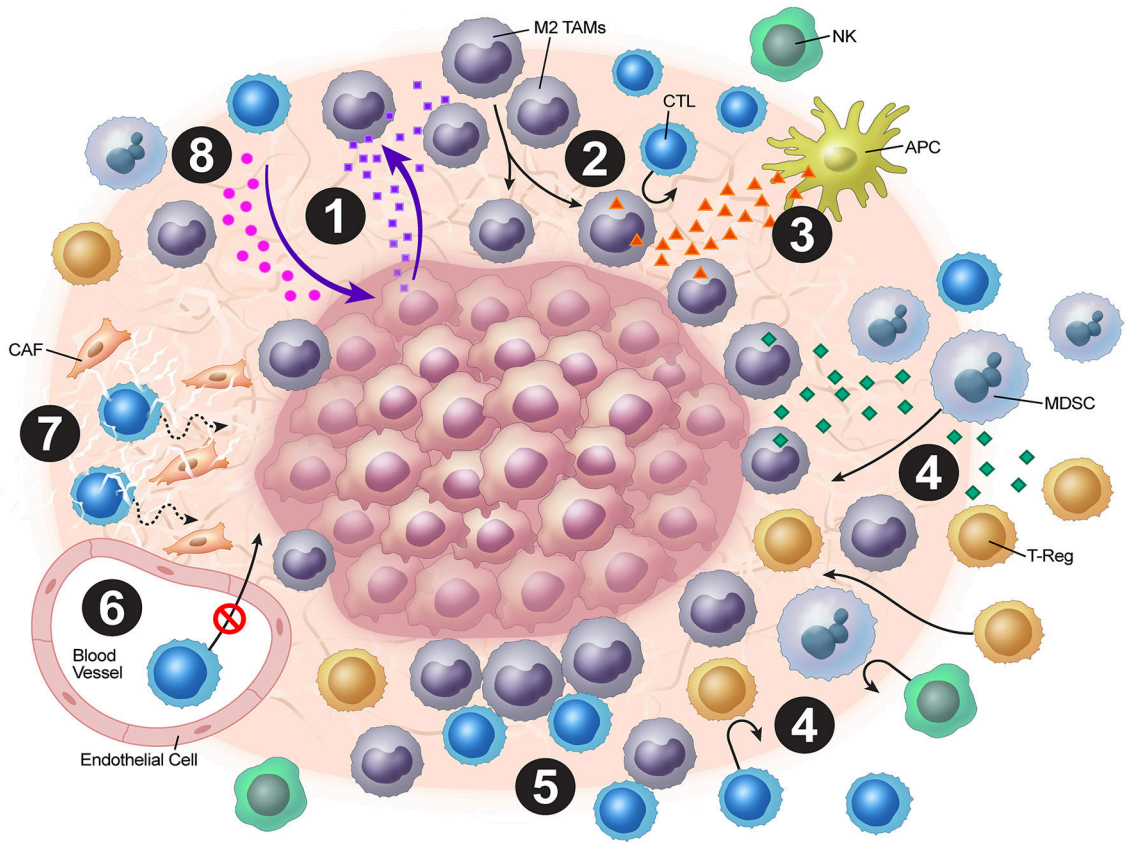

FIGURE 1 | Overview of tumor stromal mechanisms of immune evasion. (1) The tumor stroma disrupts normal chemokine pathways. (2) Chemokine dysregulation leads to increased M2 TAM populations. (3) M2 TAMs release VEGF, which inhibits DC maturation. (4) M2 TAMs also release chemokines and cytokines (e.g. TGF-β), which attract Tregs and MDSCs. (5) Stromal macrophages limit $CD8^+$ T-cell infiltration and migration. (6) ICAM and VCAM downregulation lead to decreased CTL penetration. (7) CAFs and the stromal matrix inhibit CTL mobility. (8) Depletion of resources and accumulation of tumor metabolic byproducts leads to blunting of CTL functionality.

observation that COMMA-D cells demonstrate enhanced tumorigenicity when implanted into pre-irradiated fat from murine mammary stroma *in vivo* underlined the hypothesis that radiation can have differential effects on tumors and the surrounding microenvironment (46). Radiation is a potent inducer of vascular injury, inflammation, and fibrosis. Also, hypoxia and activation of hypoxia-inducible factor-1α/VEGF signaling as a result of radiation-induced vascular dysfunction can promote radioresistance (47). Furthermore, irradiation sets in motion a robust inflammatory and fibrotic response in stroma mediated by cytokines such as IL-1, IL-6, IL-10, and TGF-β that can modify tumor responses to both XRT and chemotherapy (48). Indeed, radiation has a myriad of pleiotropic effects in tumors and their stroma that are only starting to be understood (49, 50). With increasing recognition of the fundamental role played by stromal immune signaling in tumor maintenance and radioresistance, pursuing mechanism-based strategies to overcome XRT resistance based on a comprehensive understanding of not only tumor biology but also local stromal and systemic immunobiology is crucial.

Historically, XRT was thought to be primarily immunosuppressive. However, the discovery of the abscopal effect in multiple tumor types (although rare) has significantly altered our understanding of XRT's role in the immune system. This new paradigm demonstrates XRT to be an immunomodulatory tool that facilitates for recruitment and activation of the immune system to fight tumors. The main underpinning of XRT's effect on antitumoral immunity is increasing the release of tumor antigens and their availability for antigen-presenting cells (APCs) to take up and prime T cells. However, XRT also has direct effects on the surrounding stroma that enables the immune system to increase antitumoral responses.

In addition, a potential strategy involves XRT to eradicate all gross disease followed by immunotherapy to eliminate remaining microscopic disease in cancer patients. Researchers demonstrated the benefit of this strategy in the recent PACIFIC trial (NCT02125461) examining sequential XRT and immunotherapy (51). Antonio et al. recently reported results from this randomized phase 3 study of 713 patients with stage 3 locally advanced, unresectable non-small cell lung

cancer who received anti-programmed death-ligand 1 antibody, durvalumab, or a placebo after completion of two or more cycles of platinum-based chemoradiation. Recent updated results have demonstrated a markedly longer median progression-free survival (PFS) duration with durvalumab than with the placebo (17.2 vs. 5.6 months) following chemoradiation (52).

We can suggest that XRT and immunotherapy worked synergistically in the PACIFIC trial, in which XRT first ablated all gross disease, leaving behind only microscopic metastases, which immunotherapy controlled. The lack of recurrences in that study, resulting in extended PFS in patients receiving immunotherapy, stems from the enhanced ability of immune cells to infiltrate and eliminate microscopic metastases, which lack a stromal microenvironment but can still seed the growth of larger metastases. Indeed, a study by Zhang and colleagues demonstrated poor prognoses and increased rates of recurrence in non-small cell lung cancer patients with stroma-rich tumors, in whom the tumor: stroma ratio was quantified using hematoxylin-stained tissue specimens (53). Therefore, XRT can ablate all gross disease and its stroma to enhance the effects of immunotherapy on remaining microscopic disease with a less dense stromal microenvironment.

Immunogenic Mechanisms of XRT

The landmark PACIFIC trial suggests significant improvements in patient outcome by utilizing XRT combined with immunotherapy. This will impact many future trial designs for multiple solid tumors with the goal of improving the patient outcomes. As described previously, XRT was initially used for its direct induction of DNA damage, leading to tumor cell death (54). Historically, this DNA death mechanism was seen as immunosuppressive due to the radiosensitivity of lymphocytes (55). However, with recent advances in technology including stereotactic body radiation therapy (SBRT), which allows for tighter dose distributions and higher doses given, there has been increasing evidence that XRT can serve to help activate T cells and destroy much of the immune inhibitory stroma.

A direct immune-related result of XRT is the release of tumor antigens, which allows for APC presentation and subsequent $CD8^+$ cell activation. This modality of cell death is termed immunogenic cell death (ICD). Traditionally, apoptosis is considered a tolerogenic process, which limits the ability of the immune system to develop a full response. However, with ICD, an external stress source facilitates the release of danger-associated molecular patterns (DAMPs), which elicit a signal to APCs and instigate cell death (56). Several DAMPs have been implicated in the ICD pathway, such as CRT, HMGB1, and secreted ATP (57, 58).

Even with increased antigen release due to XRT leading to increased ICD, the TME does not allow for proper activation of the immune response. For example, tumors have demonstrated downregulated major histocompatibility complex class I (MHC-I) expression, which leads to decreased recognition of tumor cells by effector T cells (59, 60). Clinically, increased MHC-I expression has been associated with improved survival of multiple cancer types (61, 62). Biologically, this makes sense, as an increase in the number of T cell-mediated reactions can occur, conferring stronger immune responses. The reduced expression of MHC-1, found biologically is found in the tumor stroma can be overcome by XRT. *In vitro* studies have demonstrated that XRT can upregulate MHC-I expression at sublethal doses (63–66). One underlying mechanism promoting this phenotype occurs through increased peptide availability following XRT and subsequent mammalian target of rapamycin (mTOR) activation, leading to an increase in MHC-I protein subunits in a dose-dependent fashion (67). Ultimately, this leads to an increase in effector activity by facilitating proper effector signaling, thereby increasing the overall T-cell repertoire.

Having antigens and the necessary cell-surface receptors alone is not sufficient to overcome all of the negative effects of tumor stroma on the immune system. Activation of pro-inflammatory signals to overcome the immunosuppressive population of Tregs, M2 TAMs, and MDSCs is imperative. XRT has been shown to facilitate this process through several chemokine/cytokine modulations within the TME. Type I IFNs play a role in this process, as they are required for proper DC maturation, increasing MHC-I expression and T-cell priming (68). IFN expression is upregulated by XRT through the cGAS-STING pathway. In this process, cGAS is activated by the DNA damage caused by XRT, with downstream effects leading to production of nuclear factor-κB and other transcription factors for IFN (69). Indeed, in a recent *in vivo* study using an anti-PD-1 therapy-resistant mouse lung cancer cell line, suppression of type I IFN expression was associated with anti-PD-1 therapy resistance due to reduced MHC-I expression, but tumors became responsive to anti PD-1 therapy after XRT (66).

XRT has also been shown to orchestrate T cell immunotherapy by promoting T-cell homing into the tumor bed through a variety of mechanisms including chemokine expression, macrophage polarization, and expression of adhesion molecules on tumor vasculature. As described previously in this review, the stroma provides signals that prevent trafficking and homing to a tumor using several chemokines. With XRT, these chemokine signals are altered and allow for better lymphocyte "pulling" into the TME. Expression of CXCL16, a chemokine that assists in T-cell infiltration, has been upregulated in breast cancer cells after XRT at 2 fractions at 12 Gy. This allows for increased $CD8^+$ activation of T cells expressing CXCR6 *in vivo*. Subsequently, loss of CXCR6 results in loss of this phenotype and poor outcomes *in vivo* (70). Also, immune cell infiltration has occurred with low-dose XRT (2 Gy). Klug et al. demonstrated polarization of M2 TAMs to NOS^+ M1 TAMs after low-dose XRT (71). Moreover, low-dose XRT can increase T-cell recruitment to pancreatic tumors *in vivo*.

Adhesion molecules are also altered after XRT. Studies of K562 cells have demonstrated upregulation of VCAM-1 expression *in vitro* after exposure to 16–20 Gy within 24 h (72). This upregulation of VCAM-1 has been further observed in other cancer types *in vivo* after low-dose XRT (73). Upregulation of adhesion molecule expression is not limited to tumor cells, as lymphatic endothelial cells have also demonstrated this change after single doses of XRT (74). Furthermore, ICAM-1 expression is increased in several tumor cell lines after XRT (75, 76). Overall, upregulation of these adhesion molecules allows for increased

infiltration of lymphocytes to tumor cells, increased affinity binding to $CD3^+$ cells, and ultimately increased immunogenicity.

In a nutshell, XRT leads to neo-antigens and DAMPs release, upregulation of MHC-I, expansion of T-cell repertoire, activation of the STING pathway and production of Type-I interferons, and upregulation of adhesion molecules such as VCAM/ICAM. Additionally, low dose XRT could polarize the M2 macrophages to M1 and reduce the levels of tumor-induced Tregs. Further work is needed to make conclusions regarding the optimal combinations and timings of XRT with immunotherapy and other targeted treatments to overcome immune resistance that is orchestrated by tumor stroma. The tumor stroma is complex and intricately dynamic with multiples layers of cytokine signaling and XRT provides a much-needed tool to combat such a clinical challenge. We believe the above-mentioned mechanisms of XRT work in concert to elicit systemic anti-tumor responses. In summary, XRT has multiple effects on the tumor stroma to increase anti-tumor immunity (**Figure 2**).

Targeting Cancer Metabolism

Based on recent discoveries in the field of cancer metabolism that we discussed previously, researchers have proposed new rationales behind cancer metabolism, providing insight into why XRT and immune therapy are perhaps the best clinically available weapons we have to fight cancer. Very little effort has been directed toward tackling the metabolic aspect of cancer using radiation and addressing targeting of immune metabolism to improve cancer therapies (77). XRT is the only effective established clinical tool that takes advantage of the metabolic aspect of cancer. In their 2005 article, Spitz et al. described that when glucose was deprived from cell media culture, cancer cells died of oxidative stress (78). What they showed was that by shutting off the glucose influx into cancer cells, they were unable to manipulate the metabolic environment to fight oxidative stress, which can be induced by XRT. Later, Coller et al. discussed the importance of protection against reactive oxygen species manifested in patients with abnormal cancer metabolism (79).

As of now, the only available clinical tool to induce oxidative stress is XRT, which works by increasing the amount of reactive oxygen species, such as hydroxyl radical, which causes DNA damage and depletes NADPH pools needed for the proliferation of cancer cells. XRT causes oxidative stress to kill cancer cells by effectively depleting the pool of NADPH, which is rapidly consumed by proliferating cancer cells to support their growth, reduce their levels of oxidized glutathione, or neutralize any oxidative damage they go through. Of note, both endothelial cells and fibroblasts demonstrate upregulation of the glycolytic pathway and pentose phosphate pathways, so these stromal cells would be affected by XRT as well.

XRT LIMITATIONS: STRATIGIES TO OVERCOME RESISTANCE

Radiation provides strong antitumor immunogenic responses to help overcome the anti-tumor immune evading mechanisms that the TME provides. However, the TME also has mechanisms that help tumors evade the full effects of initial and subsequent rounds of XRT. One important mechanism of this evasion comes from fibrosis after XRT. Fibrosis, which is initiated by the activation of inflammatory pathways, allows for further radioprotection and decreased vascular permeability of tumors which lead to increased resistance to subsequent therapies (50). CAFs are one of the largest cell populations within the tumor stroma that are drivers of stromal proliferation (80). After XRT, these CAFs are further activated. This additional activation enables CAFs to produce several cytokines, proteins, and enzymes to promote stromal expansion (80, 81). *In vivo* studies demonstrated that CAFs can mediate autophagy and irradiated tumor cell recovery through insulin-like growth factor 1-mediated mechanisms (82).

CAFs also produce important proteins such as collagen, fibronectin and integrins. Studies have demonstrated integrins to be of particular interest following XRT. A modest post-XRT increase in expressions of both α and β integrins within the stroma occurred *in vivo* (83, 84). These integrins help anchor tumors in place as well as initiate integrin-specific signal transduction. These effects lead to and promote chemoradiation resistance of tumors and induce tumor growth for multiple cancer types (85). Mantoni et al. demonstrated this association in pancreatic cancer cases, as cancer cells co-cultured with irradiated fibroblasts demonstrated greater radioresistance and integrin concentration than did their non-irradiated counterparts (86). Integrins are also implicated to have roles in tumor invasion and metastasis (87). Clinically, integrin expression is strongly associated with radioprotection and increased proliferation of breast cancer (88). Mechanistically, how integrins enforce this phenotype has yet to be determined. One *in vivo* study demonstrated that β_1-integrins produced inhibitory signals in an insulin-like growth factor 1 receptor-dependent manner in irradiated prostate tumors (84). Another study demonstrated that radioresistance develops in small-cell lung cancer cells through β_1-integrin–mediated phosphatidyl inositol 3-kinase activation (89).

Inhibition of these CAFs is an area of active investigation. Tirosh et al. sought to elucidate genotypic and phenotypic states of melanomas using single-cell RNA sequencing of tumor samples from patients with metastatic disease (90). They found that the enzyme NADPH oxidate 4 (NOX4), is an integral component of fibroblast differentiation and may be a viable target for inhibition of CAF-associated tumor immune evasion. Although multiple phase 1/2 trials have demonstrated CAF inhibitors to be safe, they did not demonstrate improved tumor control or survival in patients with metastatic colorectal or pancreatic cancer (91–93). Notably, none of these trials included patients receiving XRT. In theory, combination of CAF inhibitors with XRT will minimize immunosuppression and maximize anti-tumorigenicity.

Another important aspect of the TME is the presence of Tregs, which suppress immunity through a variety of mechanisms, including TGF-β and IL-10 production, IL-2 depletion, and ATP degradation into immunosuppressive adenosine via the ectoenzymes CD39 and CD73 (94). Importantly, Tregs are known to correlate with poor prognosis for various cancer subtypes (95). When a tumor is irradiated, various changes in

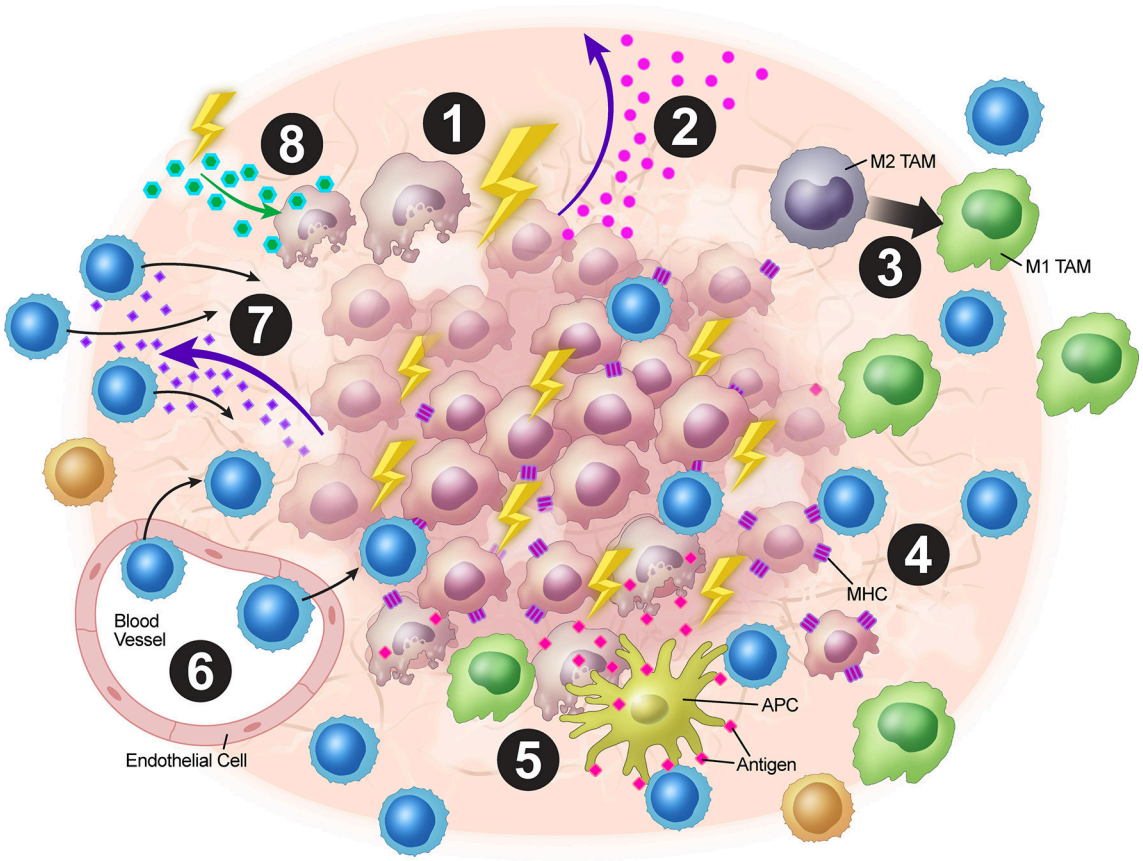

FIGURE 2 | Overview of XRT's effects on the tumor stroma. (1) XRT ablates and reprograms the stroma. (2) Increased STING pathway activation leads to upregulation of type I IFNs. (3) TAMs are polarized from the M2 to the M1 phenotype. (4) Radiation increases MHC-I expression on tumor cells. (5) Tumor destruction leads to increased antigen presentation via ICD. (6) Upregulation of VCAM-1 and ICAM-1 expression leads to increased T-cell adhesion within the stroma. (7) Upregulation of expression of chemokines such as CXCL16 leads to T-cell trafficking into the TME. (8) Radiation alters stromal cell metabolism leading to increased reactive oxygen species and subsequent surrounding cell death due to changes in oxygen requirement.

its Treg population occur. Researchers showed that Tregs appear to be more radioresistant than other subsets of T cells, thus increasing the prevalence of Tregs at a tumor site (96, 97). Muroyama et al. further demonstrated Treg proliferation with increased Ki-67 staining for Tregs after XRT when compared to control (98). In addition, the authors blocked T-cell migration into tumors using fingolimod and saw similar results, suggesting that the Tregs at tumors proliferate. In a different study, 8.5 Gy given five times decreased the population of Tregs and their suppressive capabilities (99). These studies suggest that different doses of radiation can have different effects on the Treg population, with hypofractionation perhaps having more anti-Treg effects than single doses.

Thus, depletion of Tregs in combination with XRT is a logical antitumor strategy. Schoenhals et al. investigated the effects of an IgG2a (depleting isotype) anti-glucocorticoid-induced tumor necrosis factor-related protein (GITR) antibody in an anti-PD-1–resistant murine lung adenocarcinoma model (100). They found that the protein was highly expressed at the tumor site, that anti-GITR therapy preferentially depleted Tregs at the tumor site, and that combining this therapy with XRT and anti-PD-1 therapy generated a systemic and durable antitumor response. These results highlight the potential of XRT to overcome treatment resistance of cancer, an area of intense interest in the field of cancer immunotherapy.

MDSCs are also are also believed to be among the main drivers of TME immunosuppression, and their presence has been correlated with poor prognosis and response rates for many types of human tumors (101). Studies demonstrated that the frequency of circulating MDSCs was higher with increased tumor burden for multiple solid tumors (101–105). Also, XRT has been shown to inhibit MDSC infiltration into the TME via CCR2 blockade (106). Studies have shown the administration of low-dose gemcitabine depletes MDSCs at low doses in murine models (107). Clinical studies of the safety and efficacy of combined low-dose gemcitabine and anti-PD-1 therapy (NCT03302247) are currently underway. Given the synergy between anti-PD-1 therapy and XRT described above, the addition of XRT to this dual therapy may further improve outcomes.

In addition to these immunosuppressive cells, XRT can impact the fitness of CD8+ effector T cells through cytokines. Interferons have been found to play a role in signaling for T cell exhaustion through programmed-death ligand 1 (PD-L1), which is a member of the B7 superfamily (68, 108). One study found IFNγ is produced after hypofractionated XRT doses with a subsequent increase in PD-L1 expression *in vivo* (109). They found that when combined with anti-PD-L1 immunotherapies, T cells can be rescued from this exhaustive phenotype. Clinically, anti-PD-L1 therapies have recently shown promise, with the ES-SCLC patients demonstrating significant survival benefit with atezolizumab in addition to standard-of-care chemotherapy (110). Given this information together, clinical trials with anti-PD-L1 immunotherapy and XRT may bear even more pronounced results.

Hypoxia also poses a challenge for XRT and its ability to ablate tumors and recruit effector immune cells within the TME. MDSCs and TAMs are heavily recruited to these hypoxic environments through various mechanisms, including colony-stimulating factor 1, VEGF, endothelin, and several other proteins (111). Within the tumor stroma, TAMs have plasticity in their phenotype. As noted above, M1 TAMs are characterized as pro-inflammatory and encourage anti-tumoral responses, whereas M2 TAMs are anti-inflammatory and encourage tumor growth. The distribution of these macrophages within the tumor stroma mirrors their stimuli. Specifically, hypoxic conditions promote the tumoral production of IL-4, IL-10, and TGF-β, which promote M2 polarity and attenuate proper anti-tumoral immune responses (112). *In vivo* studies of prostate cancer demonstrated that M2 TAMs which express arginase-1 and COX-2 are recruited to these hypoxic centers after irradiation and promote tumor growth (113, 114). At higher populations and stronger signaling compared to M1 TAMs and other immunoproliferative cells, these immunosuppressive cells dampen the effector anti-tumor immunity within the stroma after XRT.

Overall, the evidence that XRT modulates the TME and the balance between pro-tumoral and antitumoral signaling is substantial. Investigators have placed an emphasis on how fractionation and dosing play a role in these changes (115, 116). However, from a broader perspective, even with XRT's greater immunogenic capabilities through increased antigen release and ICD, the overall number of cases in which true abscopal effects are seen has been limited (44). Further studies are warranted to evaluate the impact of dosing on these immunogenic characteristics of XRT.

Additionally, the practicality of XRT in the setting of systemic disease is uncertain given increased time demands. For example, the time on the therapy table for each patient per isocenter would increase dramatically. Also, the precision required to target multiple isocenters is not yet possible even with the currently available SBRT technology. Given these practical and mechanistic limitations, our current understanding of XRT as monotherapy for systemic disease is limited. Further studies are warranted to evaluate the timing, dosing, and tolerance of multisite irradiation.

Meanwhile, given the current landscape of multi-agent immunotherapy, such as the combination of anti-PD-1 and anti-CTLA-4 therapy, the body of literature on the synergy of XRT and immunotherapies is rapidly growing (117–119). Targeting immunosuppressive cell populations upregulated by XRT, such as CAFs, Tregs, MDSCs, and TAMs, may further enhance systemic responses to combined XRT-systemic treatment strategies. Future studies must build upon our translational knowledge of these critical relationships to incorporate into clinical trials.

FUTURE CONSIDERATIONS AND GOALS

The rationale for combining XRT and immunotherapy is clearly apparent based on the aforementioned synergistic mechanisms. This is exemplified by the rapidly increasing number of ongoing prospective trials of combined-modality therapy for cancer (120, 121). Although a few of these are phase 3 studies, most are phase 1/2 trials given little, low-quality evidence of the safety and efficacy of combined immunotherapy and XRT for cancer at various sites (122–125).

The construction of these prospective investigations has several implications for the design of future studies. Although many of the trials in a previous systematic review evaluated concurrent therapy, few specifically evaluated the risks and benefits of this approach with sequential therapy (120). Mechanistically, as described above, delivering XRT prior to immunotherapy has several theoretical benefits, namely regarding antigen presentation, lack of T-cell depletion from concurrent therapy, and modulation of the TME. However, although some data points to a benefit of XRT delivered prior to immunotherapy (126), other data demonstrates better outcomes if both are given concurrently (127) or immunotherapy is followed by XRT (128). Thus, because most of the aforementioned ongoing trials are phase 1 studies, future phase 2/3 work will be dependent on the paradigm put forth by phase 1 data, researchers sincerely hope that future randomized studies directly evaluate the timing of XRT and immunotherapy (e.g., NCT02525757). However, the effect of their timing is likely dependent on the clinical setting, neoplasm, and/or immunotherapeutic agent.

Future studies must also evaluate combinatorial therapy consisting of XRT and multi-agent immunotherapy as well as chemoimmunotherapy. Although a clear concern is that multi-agent immunotherapy may be more toxic than a single agent alone, multi-agent treatment yields better outcomes than do some single agents as noted in the CheckMate 067 metastatic melanoma trial (129). However, whether additional XRT creates unacceptable toxicity with the use of multiple immunotherapeutic compounds is unknown. Likewise, use of chemoimmunotherapy may increase in the future based on the findings of the KEYNOTE-189 trial, which compared chemoimmunotherapy with chemotherapy alone (130). For most disease sites, although delivering concurrent chemoradiation increases the toxicity over that of a single modality alone, the effect of XRT with chemoimmunotherapy remains unknown and must be addressed.

Just as candidate radiosensitizers have been and continue to be developed for XRT, another goal is to explore candidate immunosensitizers that are not immunotherapeutic compounds but rather promote and stimulate the immune system in ways that allow for enhanced immunotherapy effects while

minimizing complication risks to normal tissues, thereby improving the therapeutic ratio. This is important because excessive immune system "drive" may result in potentially lethal toxic effects. Nevertheless, because the response rate for most immunotherapeutic compounds in seminal clinical trials is about 20%, novel biomolecules are needed to increase this rate (131, 132).

Furthermore, the synergy between XRT and immunotherapy may be exemplified by using XRT as a "pseudo-systemic agent" in patients with oligometastatic disease or even widely metastatic disease with good initial responses to chemotherapy and/or immunotherapy (133). Because these patients are expected to survive longer than those with widely disseminated disease, aggressive therapy in them is becoming more reasonable.

Recently, we have seen an increasing trend in the number of positive trials in which XRT is used to treat up to three metastatic sites. Patients with a greater number of metastases would also benefit, but such an approach would be logistically arduous due to the need for multiple isocenters. The development of technologies that make multi-site XRT easier, together with technologies that automate target delineation and treatment planning, such as deep and machine learning, may make XRT more pseudo-systemic in the future, especially when integrating it with other synergistic treatments, such as immunotherapy (134).

CONCLUSIONS

The stroma is an important component of the TME to study because it has significant implications for limiting antitumor immunity. XRT has long been considered to damage cancer cell DNA, but its effects on the stroma have received little consideration. Given reported evidence, one of the greatest benefits of XRT is its ability to eradicate and reprogram the stroma, the same stroma that too often limits the delivery of systemic treatments such as chemotherapy, targeted therapy, immunotherapy, and even cellular therapy. Radiation's ability to eradicate areas of gross disease provides a strong rationale for its use with systemic agents, which would eradicate remaining circulating microscopic disease. Systemic agents are much more effective against the microscopic disease for many reasons, as they no longer face the hypoxic and metabolic changes associated with gross tumor deposits, providing much greater access to target tissues and improving T-cell functionality.

Going forward, we must rationally combine radiation with other stroma-modifying agents, such as colony-stimulating factor 1 receptor, indoleamine 2, 3-dioxygenase 1, and TGF-β inhibitors, to further exploit these advantages. XRT already provides substantial benefits to patients with localized or oligometastatic disease. Combining XRT with immunotherapy will potentially benefits to patients with more advanced metastatic disease and continue to improve survival.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the manuscript. HM and RR compiled the first draft of the manuscript in preparation for submission. All authors contributed to revisions and subsequent edits.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Previous Radiotherapy Increases the Efficacy of IL-2 in Malignant Pleural Effusion: Potential Evidence of a Radio-Memory Effect?

Dawei Chen[1], Xinyu Song[2,3], Haiyong Wang[2], Zhenwu Gao[4], Wenjuan Meng[5], Shuquan Chen[6], Yunfeng Ma[7], Youda Wang[8], Kong Li[1], Jinming Yu[1] and Jinbo Yue[1]**

[1] Department of Radiation Oncology, Shandong Cancer Hospital affiliated to Shandong University, Jinan, China, [2] Department of Internal Medicine-Oncology, Shandong Cancer Hospital affiliated to Shandong University, Jinan, China, [3] School of Medicine and Life Sciences, University of Jinan-Shandong Academy of Medical Sciences, Jinan, China, [4] Department of oncology, Affiliated Hospital of Weifang Medical University, Weifang, China, [5] Weifang People's Hospital, Weifang, China, [6] Laiwu Hospital of Traditional Chinese Medicine, Laiwu, China, [7] Laiwu People's Hospital, Laiwu, China, [8] Linyi City People's Hospital, Linyi, China

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Germany

***Correspondence:**
Jinming Yu
sdyujinming@163.com
Jinbo Yue
yuejinbo@hotmail.com

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Preclinical and clinical studies have shown that prior receipt of radiotherapy enhances antitumor immune responses, a phenomenon we call the "radio-memory effect." However, all of the evidence regarding this effect to date comes from work with PD1/PDL1 inhibitors. Here we explored whether this effect also occurs with other forms of immune therapy, specifically interleukin-2 (IL-2). We retrospectively assessed outcomes in patients with malignant pleural effusion (MPE) who had previously received radiotherapy for non-small-cell lung cancer (NSCLC) within 18 months before the intrapleural infusion of IL-2 or cisplatin. Radiotherapy sites included lungs, thoracic lymph nodes, and intracranial. All patients received intrapleural infusion of IL-2 or cisplatin, and most had had several cycles of standard chemotherapy for NSCLC. We identified 3,747 patients with MPE (median age 64 years [range 29–88]) treated at one of several institutions from August 2009 through February 2015; 642 patients had been treated with IL-2 and 1102 with cisplatin and had survived for at least 6 months afterward. Among those who received IL-2, 288 had no radiotherapy, 324 had extracranial (i.e., thoracic) radiotherapy, and 36 had intracranial radiotherapy. The median follow-up time for surviving patients was 38 months. Patients who had received extracranial radiotherapy followed by IL-2 had significantly longer PFS than patients who had not received extracranial radiotherapy (i.e., either no radiotherapy or intracranial radiotherapy). Patients who had received intracranial or extracranial radiotherapy followed by IL-2 had significantly longer OS than did other patients. No survival advantage was noted for prior radiotherapy among patients who received intrapleural cisplatin. We speculate that previous radiotherapy could enhance the efficacy of subsequent intrapleural infusion of IL-2, a "radio-memory" effect that could be beneficial in future studies.

Keywords: non-small-cell lung cancer (NSCLC), radiotherapy, radio-memory effect, immunotherapy, interleukin-2 (IL-2), malignant pleural effusion (MPE)

INTRODUCTION

Immune checkpoint inhibitors have recently gained popularity owing to their efficacy and low toxicity (1). Immunotherapy, in combination with conventional oncology treatment, can activate the body's autoimmune response to recognize tumor cells throughout the body (2). Radiotherapy has classically been considered a form of local treatment, causing direct damage to DNA in tumor cells. Because some immune cells are inherently sensitive to radiotherapy, radiotherapy also has both immunosuppressive and immunostimulatory activities (3). Combinations of local radiotherapy and systemic immunotherapy have been shown recently to have significant advantages in preclinical and clinical studies (4). Some studies have also confirmed that radiotherapy combined with immunotherapy can produce abscopal effects and perhaps what we term a "radio-memory" effect such that the addition of one to the other has synergistic effects (5, 6). Although radiation-induced abscopal effects have been reported in many studies (7–10), little is known about the radio-memory effect.

In terms of immunostimulatory effects, localized radiotherapy can stimulate systemic immune responses (3) by promoting the expression of tumor-associated antigens and the production of new tumor antigens to activate antitumor immune responses, counteracting the tumor's ability to inhibit antigen presentation. For instance, MHC-1, a key antigen recognized by CD8+ T cells, is significantly reduced in tumor cells (11). Radiotherapy can effectively promote the expression of MHC-1, promote the maturation of dendritic cells, and infiltrate tumors (12); reduce the numbers of Tregulatory cells (Tregs) in tumors; expand T-cell lineages; and enhance T cell migration. Radiotherapy has also been shown to partially or in some cases completely convert non-immunogenic tumors into immunogenic tumors (13). Radiotherapy in combination with anti-PD1/PDL1 antibodies, anti-CTLA4 antibodies, immune cytokines, dendritic cell vaccines, and Toll-like receptor antagonists can control local tumor progression, thereby improving overall survival (OS) and inducing specific immune responses to cancer (14).

The KEYNOTE-001 and PACIFIC studies confirmed that having had radiotherapy followed by treatment with a PD1/PDL1 inhibitor could produce a memory-specific immune anti-cancer effect (14, 15). We postulate that this result results from radiotherapy acting as a catalyst or ignition agent that may change a patient's overall immune microenvironment; radiotherapy may enhance the production and storage of immune memory cells, which when followed by immunotherapy would amplify the efficacy of the immunotherapy. With the above hypothesis, we evaluated the effects of intrapleural infusion of IL-2 for the treatment of malignant pleural effusions (MPE) caused by lung cancer, particularly whether these effects varied in patients who had received radiotherapy vs. in those who had not. Specifically, we sought to determine whether the radio-memory effect could occur after radiotherapy combined with other immunotherapy agents, or whether this effect is restricted to anti-PD1/PDL1 drugs.

Abbreviations: NSCLC, non-small cell lung cancer; IL-2, interleukin-2; MPE, malignant pleural effusion; ECOG, Eastern Cooperative Oncology Group; SBRT, stereotactic ablative radiotherapy or radiosurgery; CR, complete response; PR, partial response; SD, stable disease; DCR, disease control rate; ORR, objective response rate.

METHODS

Patients

We retrospectively identified and reviewed records of patients with MPE to identify patients with non-small-cell lung cancer (NSCLC) who had received intrapleural infusion of IL-2 or cisplatin, with or without prior radiotherapy. Patients were aged 18 years or older, had adequate organ function, with no history of pneumonitis, systemic immunosuppressive therapy, or other autoallergic diseases. Efficacy evalution and disease progression were determined with the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. All patients provided written informed consent, and this study was approved by the appropriate institutional review boards. All patients enrolled in our study were treated at one of the following institutions: Shandong Cancer Hospital and Institute; Affiliated Hospital of Weifang Medical University; Weifang People's Hospital; Laiwu Chinese Medicine Hospital; and Linyi People's Hospital, all in Shandong, China.

Procedures

Patients received intrapleural infusion of one cycle of either IL-2 (20,000 U) or cisplatin alone (40–60 mg) until the effusion progressed. Before this infusion, all patients underwent more than one ultrasound-guided pleural catheterization for drainage to remove as much of the pleural effusion as possible. To ensure the uniform distribution of the agents in the pleural cavity, patients were advised to turn over smoothly every 15 min.

Patients were considered to have a history of radiotherapy if they had received any radiotherapy for NSCLC at any time during the 18 months before the intrapleural treatment. Radiotherapy sites included the lungs or metastases in the thoracic lymph nodes, brain, and other extracranial metastatic sites. Radiotherapy schedules before MPE were categorized as (1) chemotherapy followed by concurrent chemoradiotherapy, (2) upfront concurrent chemoradiotherapy followed by adjuvant chemotherapy, (3) induction chemotherapy followed by sequential radiotherapy, concurrent (4) chemoradiotherapy only, or (5) intracranial radiotherapy. Radiotherapy type was categorized as conventional (i.e., 2-dimensional), three-dimensional conformal radiotherapy, or intensity-modulated radiation therapy.

Data Collection and Evaluation Criteria

Clinicopathologic data collected for all patients included sex, age, Eastern Cooperative Oncology Group (ECOG) performance status score, smoking history. Hematologic indicator including total lymphocytic counts, neutrophils and neutrophil–lymphocyte Ratio (NLR) were also collected. Response to treatment (by RECIST v1.1) were evaluated as described elsewhere (7, 15, 17). Short-term efficacy was classified as complete response (CR; MPE and symptoms disappeared and

the patient's condition was stable for >8 weeks), partial response (PR; MPE size reduced by 50%, symptoms improved, and no subsequent growth in the MPE over 8 weeks), stable disease (SD; MPE size reduced by <50% or unchanged), or progressive disease (PD; MPE size increased). The objective response rate (ORR) included both CR and PR, and the disease (MPE) control rate (DCR) included CR, PR, and SD.

Outcomes

The primary objective was to determine whether prior receipt of radiotherapy affected PFS or OS after intrapleural IL-2 or cisplatin treatment. The secondary objective was to determine the effect of previous extracranial radiotherapy on PFS and OS, to account for potential blood-brain barrier effects. Additional objectives included evaluating the effect of previous radiotherapy on treatment efficacy and pulmonary toxicity. Progression-free survival (PFS) was defined as the interval between the initiation of intrapleural IL-2 (or cisplatin) and the time to either effusion progression or death. Overall survival (OS) was measured from the date of the initiation of intrapleural IL-2 to the date of death from any cause or the last known follow-up date.

Statistical Analysis

All statistical analyses were done with SPSS (Statistical Package for the Social Sciences) version 17.0 and Prism GraphPad 6.0. Clinicopathologic characteristics and short-term efficacy were analyzed by using χ^2 tests, Fisher's exact tests, or Student's *t* tests. Independent predictors associated with PFS and OS were identified by using a Cox regression model. PFS and OS were analyzed with the Kaplan-Meier method, with differences between groups evaluated with log-rank tests. Two-sided *P*-values of <0.05 were considered statistically significant.

RESULTS

Patient Characteristics

A total of 3,747 patients with MPE were treated at the participating hospitals between August 2009 and February 2015. Of these patients, 1,506 received intrapleural IL-2 and the other 2,241 received intrapleural cisplatin. Among the 1,506 patients given IL-2, 1,098 had NSCLC; after exclusion of 456 patients who survived for <6 months after treatment, the study group consisted of 642 patients (**Figure 1**). The control group (i.e., those given intrapleural cisplatin but not IL-2) consisted of 1,102 patients with NSCLC who survived for more than 6 months after treatment. The enrollment flowchart is shown in **Figure 1**, and baseline patient characteristics are shown in **Table 1**. The median age of the 642 patients in the study group was 62 years (range 29–87). Most patients had received intrapleural chemotherapy and several lines of systemic therapy before receiving the intrapleural IL-2. Slightly more than half of the 642 patients given IL-2 (324, or 50.5%) had previously received radiotherapy, which was extracranial in 288 (44.8%). The median follow-up for patients alive at this analysis was 38 months. Radiotherapy was delivered for a median period of 6.4 months (range 0.8–85.0, IQR 3.0–15.5), before the first cycle of intrapleural IL-2.

Regardless of whether patients had received prior radiotherapy or not, significant differences were found between groups with regard to sex, age, ECOG status, histopathologic classification, smoking history, diagnosis

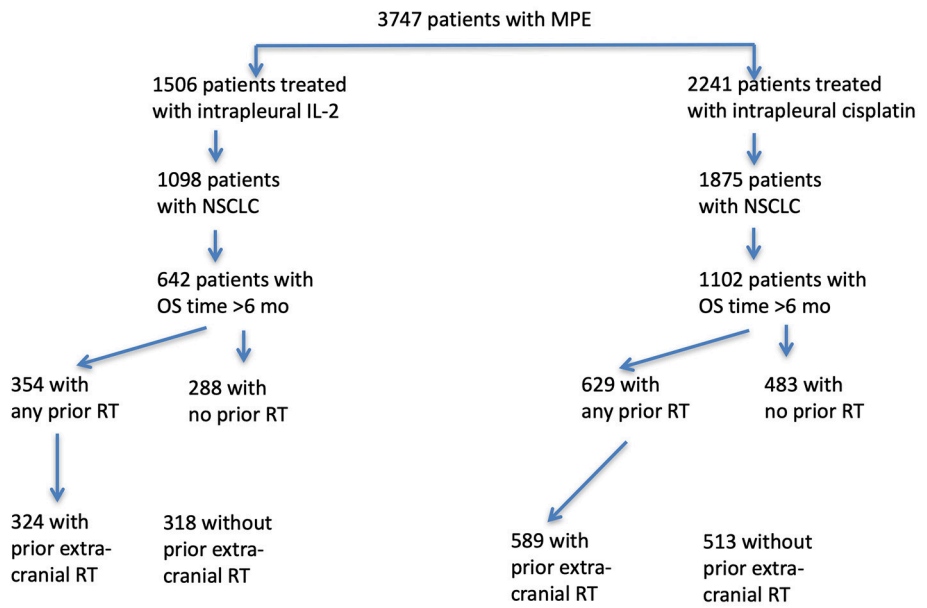

FIGURE 1 | Flowchart of eligible patients enrolled in this study. From a total of 3,747 patients with malignant pleural effusion (MPE), we identified 1,506 who had been treated with interpleural interleukin-2 (IL-2) and 2,241 who had been treated with intrapleural cisplatin. Of the 1,098 patients given IL-2 (and the 1,875 patients given cisplatin) who had non-small cell lung cancer, 642 who had received IL-2 survived for more than 6 months, and 1,102 who had received cisplatin survived for more than 6 months. Patients in each group were subdivided according to whether they had any vs. no radiotherapy (RT), or extracranial vs. no extracranial RT.

TABLE 1 | Baseline characteristics of 642 patients with non-small cell lung cancer who received IL-2.

Variable	No. of patients	Any previous RT			Previous extracranial RT		
		No (n = 318)	Yes (n = 324)	P-Value	No (n = 354)	Yes (n = 288)	P-Value
SEX							
Male	360	168 (53%)	192 (59%)	0.501	198 (56%)	162 (56%)	0.974
Female	282	150 (47%)	132 (41%)		156 (44%)	126 (44%)	
AGE, YEARS							
≥55	312	144 (45%)	168 (52%)	0.497	174 (49%)	138 (48%)	0.899
<55	330	174 (55%)	156 (48%)		180 (51%)	150 (52%)	
ECOG PS SCORE							
0	122	60 (19%)	62 (19%)	0.836	69 (19%)	53 (18%)	0.827
1	412	204 (64%)	208 (64%)		221 (62%)	191 (66%)	
2	108	54 (17%)	54 (17%)		60 (18%)	48 (16%)	
HISTOPATHOLOGIC CLASSIFICATION							
Squamous cell	198	90 (28%)	108 (33%)	0.573	120 (34%)	78 (27%)	0.448
Adenocarcinoma or other	444	228 (72%)	216 (67%)		234 (66%)	210 (73%)	
SMOKING HISTORY							
Never-smoker	402	210 (66%)	192 (59%)	0.469	234 (66%)	168 (58%)	0.409
Former/current smoker	240	108 (34%)	132 (41%)		120 (34%)	120 (42%)	
DIAGNOSIS METHOD FOR MPE							
CT-guided biopsy	264	126 (40%)	138 (43%)	0.438	138 (39%)	126 (44%)	0.220
Pleural effusion cytology	144	78 (25%)	66 (20%)		90 (25%)	54 (19%)	
Thoracotomy	174	72 (23%)	102 (31%)		78 (22%)	96 (33%)	
Neck lymph node biopsy	60	42 (12%)	18 (6%)		48 (14%)	12 (4%)	
COLOR OF PLEURAL EFFUSION							
Bloody	426	222(70%)	204 (63%)	0.453	246 (69%)	180 (63%)	0.447
Light yellow	216	96(30%)	120 (37%)		108 (31%)	108 (37%)	
HEMATOLOGIC FINDINGS							
Neutrophil count, mean ± IQR, × $10^3/\mu L$		6.12 ± 1.78	4.48 ± 1.34	0.032	6.09 ± 1.81	4.4 ± 1.32	0.034
Total lymphocyte count, mean ± IQR, × $10^3/\mu L$		1.34 ± 0.35	2.21 ± 0.70	0.021	1.35 ± 0.36	2.19 ± 0.69	0.022
Neutrophil-to-lymphocyte ratio		4.56 ± 1.36	2.06 ± 0.70	<0.01	4.52 ± 1.41	2.02 ± 0.75	<0.01
INTRAPLEURAL CHEMOTHERAPY BEFORE IL-2							
Yes	528	270 (85%)	258 (80%)	0.766	300 (85%)	228 (79%)	0.739
No	114	54 (15%)	60 (20%)		54 (15%)	60 (21%)	
History of brain metastases		0	68 (21%)	<0.01	36 (10%)	32 (11%)	0.699
No. of previous systemic therapies, mean (range)		2 (0–5)	2 (0–5)	0.017	2 (0–5)	3 (0–6)	0.017
PREVIOUS SYSTEMIC THERAPIES BEFORE IL-2							
No		236 (74%)	250 (77%)	0.18	259 (73%)	227 (79%)	0.096
Yes		82 (26%)	74 (23%)		95 (27%)	61 (21%)	
RADIOTHERAPY SCHEDULE BEFORE DISTANT METASTASIS							
ChT → CCRT			140 (43%)			140 (49%)	
CCRT → ChT			61 (19%)			61 (21%)	
ChT → RT			66 (20%)			66 (23%)	
CCRT alone			21 (6%)			21 (7%)	
Intracranial radiotherapy			36 (12%) 0			0	
RADIOTHERAPY TYPE							
Conventional (2D) radiotherapy			164 (51%)			148 (52%)	
3D-CRT/MRT			160 (49%)			140 (48%)	
PREVIOUS SABR							
Yes	20		20 (6%)			20 (7%)	
No	622		304 (94%)			268 (93%)	

ECOG, Eastern Cooperative Oncology Group; MPE, malignant pleural effusion; ChT → CCRT, chemotherapy followed by concurrent chemoradiotherapy; CCRT → ChT, upfront concurrent chemoradiotherapy followed adjuvant chemotherapy; ChT → RT, induction chemotherapy followed by sequential radiotherapy; CCRT alone, concurrent chemoradiotherapy alone; IMRT, intensity modulated radiotherapy; 3D-CRT, three dimensional conformal radiotherapy; SBRT, stereotactic body radiotherapy or stereotactic radiosurgery.

method, color of MPE, and history of concurrent intrapleural chemotherapy (**Table 1**). Patients who had prior radiotherapy had significantly higher frequency of brain metastases and had received more systemic therapies than patients who had not had prior radiotherapy (**Table 1**). Frequency of brain metastases was similar among patients who had received extracranial radiotherapy and those who had not had prior extracranial radiotherapy.

Survival Outcomes

PFS time was similar among patients given IL-2 regardless of whether they had had prior radiotherapy or not (median PFS time 4.0 months prior vs. 3.67 months no prior, $p = 0.18$; **Figure 2A**). PFS time for patients who received IL-2 preceded by extracranial radiotherapy was longer than that for patients who had not had extracranial radiotherapy (median PFS time 4.06 months extra vs. 3.64 months no extra, $p = 0.046$; **Figure 2B**). Otherwise, previous radiotherapy or previous extracranial radiotherapy did not confer an advantage in PFS for patients with cisplatin (**Supplementary Figure 1**). In univariate analysis of the 642 patients who received intrapleural IL-2, ECOG score ($p = 0.036$), previous systemic therapy ($p = 0.041$), previous intrapleural chemotherapy ($P = 0.049$), having had any prior radiotherapy ($p = 0.019$) and having had extracranial radiotherapy ($p = 0.32$) were associated with longer PFS. Multivariate analysis revealed that ECOG score, having had any radiotherapy, and having had extracranial radiotherapy were independent predictors of PFS (**Table 2**).

As for OS time, among the patients given IL-2, having received any radiotherapy was associated with longer OS time than no radiotherapy (median OS time 8.8 months vs. 7.34 months, $p = 0.0116$; **Figure 2C**), as was having received extracranial radiotherapy compared with no extracranial radiotherapy (median OS time 8.93 months vs. 6.92 months, $p = 0.0003$; **Figure 2D**). Otherwise, previous radiotherapy or previous extracranial radiotherapy did not confer any advantage in OS for patients with cisplatin (**Supplementary Figure 1**). In univariate analysis of the 642 patients who received intrapleural IL-2, age ($p = 0.041$), ECOG score ($p = 0.04$), smoking history ($p = 0.016$), previous intrapleural chemotherapy ($p = 0.046$), having received any radiotherapy ($p = 0.042$), and having received extracranial radiotherapy ($p = 0.013$) were associated with longer OS times. Multivariate analysis confirmed that smoking history, receipt of any radiotherapy, and receipt of extracranial radiotherapy were independent predictors of OS (**Table 3**).

Hematologic Outcomes

Patients who had had any previous radiotherapy had higher TLC than patients with no prior radiotherapy (1.34 ± 0.35 vs. 2.21 ± 0.70, $p = 0.021$); had lower neutrophil counts than patients with no prior radiotherapy (6.12 ± 1.78 vs. 4.48 ± 1.34, $p = 0.032$); and had lower NLR than patients with no prior radiotherapy (4.56 ± 1.36 vs. 2.06 ± 0.70, $p < 0.01$). These patterns also held for patients who had had extracranial radiotherapy vs. no extracranial radiotherapy (TLC: 6.09 ± 1.81 vs. 4.4 ± 1.32,

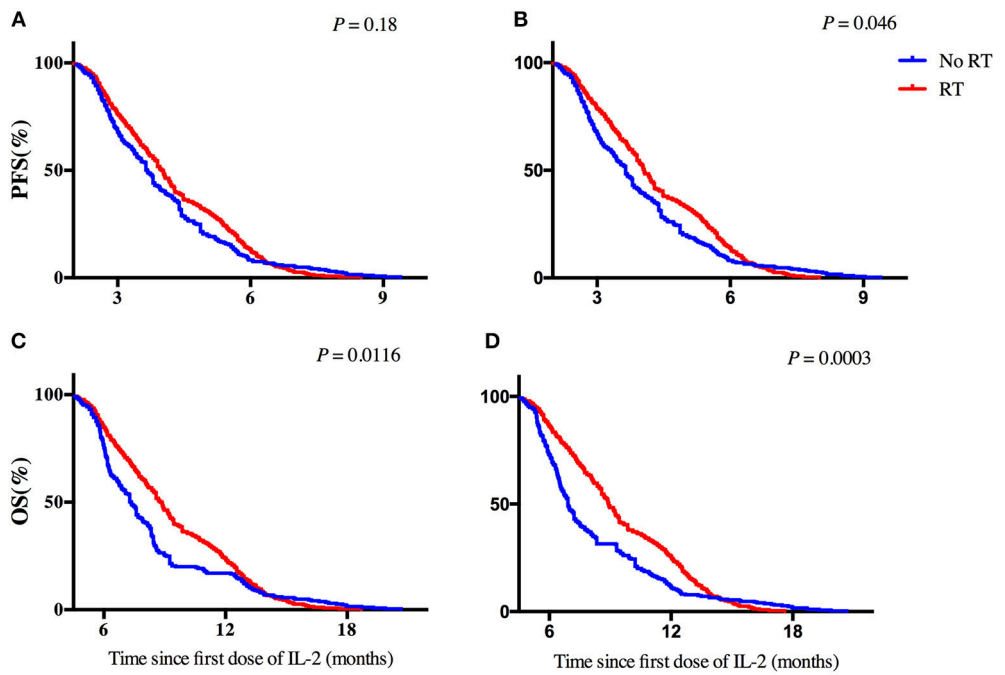

FIGURE 2 | Effect of previous radiotherapy on progression-free survival and overall survival for patients with IL-2. **(A,B)** Progression-free survival in patients according to a history of **(A)** any radiotherapy or **(B)** extracranial radiotherapy. **(C,D)** Overall survival in patients according to a history of **(C)** any radiotherapy or **(D)** extracranial radiotherapy. Hazard ratios [HRs] are shown.

TABLE 2 | Factors associated with progression-free survival.

	PFS (Univariate analysis)		Any previous RT and PFS (Multivariate analysis)		Previous Extracranial RT and PFS (Multivariate analysis)	
	HR (95% CI)	***P*-Value**	**HR (95% CI)**	***P*-Value**	**HR (95% CI)**	***P*-Value**
Sex (Female vs. male)	1.69 (0.82–2.94)	0.229				
Age (≥55 y vs. <55 y)	0.75 (0.48–1.41)	0.312				
ECOG PS score (0 vs. 1 vs. 2)	0.71 (0.45–0.82)	0.036	0.57 (0.23–0.92)	0.032	0.59 (0.25–0.89)	0.033
Adenocarcinoma and other (vs. squamous)	1.63 (0.75–2.32)	0.52				
SMOKING HISTORY (Never vs. former/current)	1.43 (1.00–2.14)	0.051				
Color of pleural effusions (Bloody vs. yellow)	1.12 (0.66–2.43)	0.23				
Previous systematic therapy	0.56 (0.32–0.91)	0.041	0.62 (0.25–1.06)	0.071	0.64 (0.23–1.12)	0.069
Previous intrapleural chemotherapy (Yes vs. no)	1.65 (1.12–2.32)	0.049	1.75 (0.87–2.32)	0.062	1.77 (0.85–2.34)	0.066
Any previous radiotherapy (Yes vs. no)	0.68 (0.34–0.89)	0.019	0.65 (0.31–0.87)	0.024		
Previous extracranial radiotherapy (Yes vs. no)	0.87 (0.64–0.94)	0.032			0.84 (0.62–0.91)	0.024

Progression-free survival was defined as the time from the first dose of intrapleural interkeukin-2 until disease progression or death.
HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status.

$p = 0.034$; neutrophils: 1.35 ± 0.36 vs. 2.19 ± 0.69, $p = 0.022$); and NLR: 4.52 ± 1.41 vs. 2.02 ± 0.75, $p < 0.01$).

Treatment Efficacy

Short-term treatment efficacy did not differ substantially between patients who had had any radiotherapy and those who had not had any radiotherapy. Treatment outcomes were also no different between patients who had extracranial radiotherapy vs. no extracranial radiotherapy (**Table 4**).

DISCUSSION

Preclinical and clinical studies have shown that radiotherapy can enhance antitumor immune responses. A secondary analysis of the KEYNOTE-001 phase 1 trial, in particular, suggested that previous radiotherapy may improve the outcomes of patients given pembrolizumab relative to those for patients not given radiotherapy. Here we sought to determine whether radiotherapy combined with another well-known immune agent would produce similar radio-memory effect. In our retrospective analysis of outcomes among patients given intrapleural IL-2 or cisplatin for MPE in light of prior receipt of radiotherapy for NSCLC, we found that having received radiotherapy before IL-2 led to longer PFS and OS times compared with patients who had not had prior radiotherapy. No such results were found for patients given cisplatin but not IL-2. We further found that patients who had had any prior radiotherapy or prior extracranial radiotherapy had higher TLC, lower neutrophil counts, and lower NLR than those who had no prior radiotherapy which generally are associated with better treatment efficacy and longer survival (16, 17). To our knowledge, this was the largest such analysis undertaken to date, and its results strongly suggest that having had radiotherapy may improve the efficacy of immune therapy other than checkpoint inhibitors. These findings are consistent with the results of several preclinical studies indicating significant, synergistic antitumor effects achieved by combining radiotherapy with immunotherapy. Our findings thus seem to demonstrate that combining radiotherapy with immunotherapy other than the previously studied anti-PD1/PDL1 can indeed induce a "radio-memory" effect.

IL-2, also known as T-cell growth factor, includes a range of bioactive cytokines produced primarily by activated CD4+ T cells and CD8+ T cells, acts as a growth factor for all T-cell subsets and can also promote the activation of B-cell proliferation (18, 19). IL-2 is also important in the regulation of the immune response, including antibody responses, hematopoiesis, and tumor surveillance. Numerous animal models have shown that combining radiotherapy with IL-2 has a synergistic antitumor effect (20–23) and has been shown to be useful clinically in oral cancer (24), renal cell carcinoma (25), and prostate cancer (26). Our results are consistent with these studies. In a study published in 2011, Koji suggested that radiotherapy combined with IL-2 may have radiopharmaceutical effects (27); however, to our knowledge, no studies to date have demonstrated a radio-memory effect.

In our study, patients given intrapleural IL-2 who had previously received radiotherapy for NSCLC had better OS than those who had not had radiotherapy ($p = 0.0116$, **Figure 2C**), but no differences were found in short-term efficacy or PFS between these to groups ($p = 0.18$, **Figure 2A**). We further found better PFS and OS among patients who had received extracranial irradiation than among those who had not received extracranial irradiation ($p = 0.046$, **Figure 2B**; $p = 0.0003$, **Figure 2D**). These findings may reflect the following. First, compared to extracranial

TABLE 3 | Factors associated with overall survival.

	OS (Univariate analysis)		Any previous RT and OS (Multivariate analysis)		Previous extracranial RT and OS (Multivariate analysis)	
	HR (95% CI)	***P* Value**	**HR (95% CI)**	***P* Value**	**HR (95% CI)**	***P* Value**
Sex (Female vs. Male)	1.34 (0.74–2.81)	0.381				
Age (≥55 y vs. <55 y)	0.87 (0.61–0.98)	0.041	0.9 (0.82–1.00)	0.056	0.93 (0.84–1.00)	0.063
ECOG PS score (0 vs. 1 vs. 2)	0.61 (0.36–0.92)	0.04	0.51 (0.32–0.94)	0.073	0.52 (0.3–0.91)	0.072
Adenocarcinoma and other (vs. squamous)	1.85 (0.86–2.93)	0.23				
Smoking history (Never vs. former/current)	1.76 (1.09–2.42)	0.016	1.79 (1.09–2.45)	0.015	1.78 (1.09–2.44)	0.015
Color of Pleural effusions (Bloody vs. yellow)	1.26 (0.64–2.56)	0.49				
Previous systemic therapy	0.74 (0.36–0.92)	0.15				
Previous intrapleural chemotherapy (Yes vs. no)	1.81 (1.03–2.54)	0.046	1.92 (0.91–2.35)	0.074	1.96 (0.83–2.49)	0.062
Any previous radiotherapy (Yes vs. no)	0.52 (0.31–0.93)	0.042	0.56 (0.30–0.85)	0.045		
Previous extracranial radiotherapy (Yes vs. no)	0.44 (0.24–0.86)	0.013			0.36 (0.16–0.82)	0.022

Overall survival was defined as the time from the first dose of intrapleural interkeukin-2 until disease progression or death. OS, overall survival; HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status.

TABLE 4 | Short-term treatment efficacy for patients with intrapleural IL-2.

	Previous radiotherapy			Previous extracranial radiotherapy		
	No (*n* = 318)	**Yes (*n* = 324)**	***P* Value**	**No (*n* = 354)**	**Yes (*n* = 288)**	***P* Value**
Complete response, no.	16	20	0.529	18	18	0.585
Partial response, no.	140	139	0.317	145	134	0.157
Stable disease, no.	60	52	0.347	63	49	0.797
Disease control rate	67.92	65.12	0.452	63.84	69.79	0.112
Objective response rate, %	49.06	49.07	0.669	46.05	52.78	0.0897

radiotherapy, cranial radiotherapy will damage more lymphocyte cells and some believe that cranial irradiation cannot change the immune microenvironment in the brain (28). Second, the immune microenvironment within the brain may not change because of the presence of the blood-brain barrier. Even if such changes in the immune system did occur within the brain, it is unlikely that this effect would extend to the immune microenvironment throughout the body, as the blood-brain barrier serves as an obstacle.

Typically the choice of radiation dose, distribution, sequence, and target area is made to ensure thorough destruction of tumor cells while minimizing damage to surrounding normal tissues. When radiotherapy is used as an intervention for immunization, however, this classical approach may require adjustment. For example, it may not be necessary to include the entire tumor area to high-dose radiation, because exposing only a part of the tumor may be required to trigger the immune system. Radiation dose and segmentation schemes that trigger an immune response probably depend on many unknown factors, but the required dose is likely to be much lower than the dose required for definitive radiotherapy, which would in turn reduce the likelihood of adverse reactions. Finally, proton therapy may be more suitable than photon therapy for combining with immune therapy, as proton therapy doses are usually lower and protons may have an as-yet unrecognized role in immunotherapy. However, more comprehensive research in this area is required before recommendations can be made.

Our study of patients with NSCLC who also developed MPE involved a large number of patients with which to analyze the overall clinical effect of previous radiotherapy when those patients were given IL-2. Nevertheless, our study had several limitations, chief among them its retrospective nature, with the attendant unclear inclusion criteria and somewhat inaccurate statistical analyses. Second, the effects on the clinical endpoints studied (OS and PFS) were relatively modest, and the statistical significance levels were marginal. Third, our data could not shed light on the mechanism underlying the radio-memory effect of previous radiotherapy. Also, it is possible that bias

may have been introduced by differences in standards of living among patients, perhaps including distance from and access to radiotherapy facilities. Overall, although this study could be considered hypothesis-generating; its conclusions should be validated through prospective controlled trials. From surgery to medicine, and from radiotherapy to immunotherapy, many approaches have been attempted to eradicate cancer. However, single treatments have had relatively modest success at best, and hence the greatest challenge faced by modern-day oncologists is to identify the optimal combinations of existing forms of cancer therapy.

CONCLUSIONS

Our findings suggest that previous receipt of radiotherapy for NSCLC may enhance the efficacy of IL-2 for treating MPE and improve outcomes for such patients. They further suggest that the radio-memory effect is not restricted to combining radiotherapy with PD1/PDL1 inhibitors but may extend to other immunotherapy agents. Future clinical trials involving radiotherapy and immunotherapy should consider this observation in designing more rational regimens.

AUTHOR CONTRIBUTIONS

JmY and JbY designed the study. DC, XS, HW, WM, YW, YM, ZG, and SC participated in the collection and analysis the data. DC and XS wrote the manuscript. KL and JbY were responsible of the critical review and revision of this manuscript. All authors provided the approval of the final manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02916/full#supplementary-material

Supplementary Figure 1 | Effect of previous radiotherapy on progression-free survival and overall survival for patients with Cisplatin. **(A,B)** Progression-free survival in patients according to a history of **(A)** any radiotherapy or **(B)** extracranial radiotherapy. **(C,D)** Overall survival in patients according to a history of **(C)** any radiotherapy or **(D)** extracranial radiotherapy. Hazard ratios [HRs] are shown.

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REVIEW
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Overcoming Resistance to Combination Radiation-Immunotherapy: A Focus on Contributing Pathways Within the Tumor Microenvironment

*Laurel B. Darragh, Ayman J. Oweida and Sana D. Karam**

Department of Radiation Oncology, School of Medicine, University of Colorado, Aurora, CO, United States

Radiation therapy has been used for many years to treat tumors based on its DNA-damage-mediated ability to kill cells. More recently, RT has been shown to exert beneficial modulatory effects on immune responses, such as triggering immunogenic cell death, enhancing antigen presentation, and activating cytotoxic T cells. Consequently, combining radiation therapy with immunotherapy represents an important area of research. Thus far, immune-checkpoint inhibitors targeting programmed death-ligand 1 (PD-L1), programmed cell death protein 1 (PD-1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have been the focus of many research studies and clinical trials. The available data suggest that such immunotherapies are enhanced when combined with radiation therapy. However, treatment resistance, intrinsic or acquired, is still prevalent. Various theories as to how to enhance these combination therapies to overcome treatment resistance have been proposed. In this review, we focus on the principles surrounding radiation therapy's positive and negative effects on the tumor microenvironment. We explore mechanisms underlying radiation therapy's synergistic and antagonistic effects on immune responses and provide a base of knowledge for radio-immunology combination therapies to overcome treatment resistance. We provide evidence for targeting regulatory T cells, tumor-associated macrophages, and cancer-associated fibroblasts in combination radio-immunotherapies to improve cancer treatment.

Keywords: immunotherapy, radiation therapy (RT), myeloid derived suppressor cell (MDSC), regulatory T (Treg) cell, tumor microenvironment (TME), immunotherapy resistance, cancer associated fibroblast (CAF)

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***Correspondence:**
Sana D. Karam
sana.karam@ucdenver.edu

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INTRODUCTION

Radiation therapy (RT) represents standard-of-care treatment for more than half of all cancer patients (1). RT was originally used for its ability to induce double-stranded DNA damage resulting in cell death via apoptosis, necrosis, autophagy, mitotic catastrophe, or replicative senescence (2, 3). But RT can also modulate the immune system and the tumor microenvironment (TME) in a dose-dependent manner (4–6). Our increased knowledge of the positive immune-modulating effects of RT has led

to the development of novel combination therapies. Several preclinical studies have shown that combining RT with immunotherapy (IT) can result in better local and systemic tumor control (5). Combining RT with anti-CTLA-4 therapy (7–10), anti-PD-1 (11–13), or anti-PD-L1 therapy (14–16), with RT doses ranging from 2 to 20 Gy in single and fractionated regimens, has resulted in prolonged survival and reduced tumor growth in preclinical tumor models (17). Emerging data from clinical trials combining RT and IT have also shown promise (18–21). Most recently, a Phase II clinical trial in which patients with locally advanced non-small cell lung cancer (NSCLC) or metastatic disease were treated with RT followed by pembrolizumab (anti-PD-1) found that this combination prolonged overall survival by 19.8 weeks (NCT02407171). Administration of nivolumab (anti-PD-1) before RT in another Phase II clinical trial looking at advanced NSCLC was shown to increase the 18 months survival of patients by 29% (22). Similarly, RT increased the effectiveness of PD-L1 inhibition in a retrospective study of recurrent/metastatic nasopharyngeal carcinoma (23). Although combining IT with RT has shown promising improvements in survival in these clinical trials, patients eventually relapse, and durable responses are rare (24). Several parameters can influence the response to IT and RT combinations, including RT dose, sequencing, and tumor oncogenic and immune composition. This variable success rate is thought to be caused by resistance—regrowth of the tumor—and is still common in most patients treated with radio-immunotherapy as some cancers like head and neck squamous cell carcinoma have a low response rate of 13% (25). By considering the cancer tumor microenvironment (TME) and its components, and how to specifically modulate them with RT and IT, we can potentially determine how to override resistance to radio-immunotherapy and improve outcomes.

Various elements of the TME can prevent effective lymphocyte priming, reduce immune cell infiltration, and suppress effector cell function that can lead to a failure of the host to reject tumors (26). These elements identify several potential mechanisms that could affect the efficacy of radio-immunotherapy: suppressive immune cells including regulatory T cells (Tregs), macrophages, or myeloid derived suppressor cells (MDSC); lack of antigen stimulation/co-stimulation for dendritic cells (DCs) leading to inadequate T cell priming; physical barriers such as a thick extracellular matrix (ECM) produced by fibroblasts around tumor tissues preventing immune cell entry into tumors; and exhausted or short-lived activation of antigen-specific cytotoxic $CD8^+$ T cells through activation of immune checkpoints like PD-1. Although tumor-intrinsic factors also play an important role in mediating growth and survival of the primary tumor (27), the focus of this review is on how elements of the TME can impact treatment outcomes, how RT modulates the immune TME, and potential immunotherapies that could improve RT's effects (as shown in **Figure 1**). This will provide a foundation for developing rational targeted ITs aimed at reducing the development of resistance when combined with RT. Further, it presents a rationale for shifting from broad targeting of immune checkpoint receptors to targeting of regulatory T cells, tumor-associated macrophages, and cancer-associated fibroblasts as specific targets for combination radio-immunotherapies. We conclude by suggesting that a thorough understanding of the biological pathways underlying known interactions between RT and various immune targets is and will continue to be invaluable for informing design of combination radio-immunotherapies to improve cancer treatment.

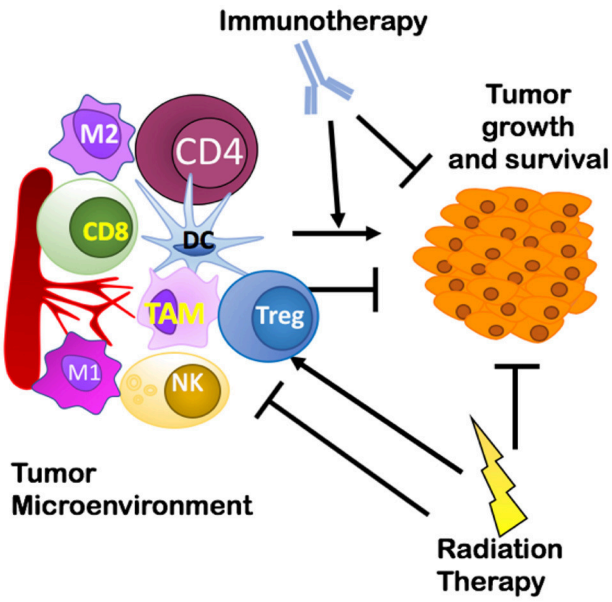

FIGURE 1 | Overview of the interplay between a tumor and its microenvironment and potential targets of immunotherapy and radiation therapy covered in this review. Treg, Regulatory T cells; TAM, Tumor Associated Macrophages.

HIGHLIGHTING RT'S DELICATE BALANCE BETWEEN PROMOTING IMMUNOSUPPRESSION AND TUMOR CYTOTOXICITY

To maximize the therapeutic ratio, it is important to establish a combination of ITs that activate pathways to promote anti-tumor immunity and effector T cell function while limiting pathways that mediate an immunosuppressive TME. Several mechanisms are involved in immune regulation and response to stress stimuli, including RT.

RT INCREASES TYPE I IFN SECRETION VIA STING ACTIVATION: A DICHOTOMY BETWEEN DENDRITIC CELL AND MDSC RECRUITMENT DETERMINES THE THERAPEUTIC RESPONSE TO RADIATION

When RT induces tumor cell death, DNA from dying tumor cells is delivered to antigen presenting cells (APCs), most notably

CD11c+ dendritic cells (DCs). In this process DCs are stimulated to present antigens, and express costimulatory molecules (28). A critical mediator of DC function is the stimulator of interferon genes (STING). STING pathway activation (**Figure 2**) occurs when DNA from tumor cells taken up by APCs is sensed by cyclic-GMP-AMP (cGAMP) synthase, which interacts directly with STING to induce a conformational change leading to translocation of STING from the endoplasmic reticulum to perinuclear vesicles (29). Inside the nucleus, STING recruits and phosphorylates TANK-binding kinase 1 (TBK1), which activates interferon regulatry factor 3 (IRF3). Finally, IRF3 induces expression of Type I IFNs (30). Type I IFN release from APCs facilitates the ability of Batf3 DCs to take up antigen (31). This stimulates maturation of DCs and cross-presentation of tumor associated antigens (TAA) to $CD8^+$ T cells, which mediate antitumor immunity after proliferation and infiltration into the tumor microenvironment.

Type I IFNs induced by RT include IFN -α, -β and the less studied IFN-τ, -ε, -κ, and -ω. Expression of Type I IFN and Type I-inducible genes is associated with T cell-infiltrated tumors (32, 33). In addition, Type I IFN expression can be induced by RT. Burnette et al. showed increased Type I IFNs in a melanoma cancer model (B16-SYI) after 20 Gy of local RT (34). Knockdown of IFN-β receptor (IFN-α receptor 1) in B6/IFNAR1 KO mice abolished RT's ability to reduce tumor growth in this model. Lim et al. showed similar findings with a dose of 15 Gy (35). These data suggest that Type I IFNs, specifically IFN-β, may be key targets by which RT modulates the TME.

Deng et al showed that innate immune sensing following RT is predominantly mediated by a STING-dependent mechanism (31). The study demonstrated that cGAS- and STING-dependent cytosolic DNA sensing in DCs is required for type I IFN induction after RT and that adding the STING agonist cGAMP reduces radioresistance and enhances antitumor immune responses. However, the paradox of RT-mediated STING activation is that it can also recruit MDSCs (34, 36). While this could be an RT dose-dependent phenomenon, the recruitment of MDSCs by RT can inhibit $CD8^+$ T cells and DC activity, thus negating any benefit from activation of the Type I IFN pathway. This has been demonstrated in MC38 colon tumors where irradiation was shown to primarily increase monocytic MDSCs ($Ly6c^{hi}$ CD11b+ cells) (36). In support of this being mediated via the STING pathway, tumor irradiation in STING KO mice led to a significant decrease in MDSC recruitment (36). This evidence supports STING as an initiating factor in MDSC recruitment. It is possible that STING-mediated RT effects are tumor-specific. Tumors that are poorly MDSC infiltrated and/or do not induce MDSC chemoattractants in response to RT may benefit from a STING agonist in combination with RT. In contrast, tumors that are MDSC rich and/or activate MDSC recruitment in response to RT may require strategies for targeting MDSCs. Combining MDSC targeting therapies with RT may not only enhance STING activation, but also increase Type I IFN production and recruitment of $CD8^+$ T cells (37, 38).

A potential target through which RT increases MDSC recruitment is the monocyte chemoattractant CCL2. In the MC38 colon tumor model above, genetic knockdown of CCL2 yielded complete tumor eradication in 60% of irradiated mice further supporting MDSCs as a major driver of immunosuppression (36). Similarly, monoclonal antibodies against CCL2 led to tumor rejection in 40% of mice, but only when combined with RT (36). Anti-CCL2 antibody therapy combined with RT also resulted in an increase of $CD8^+$ T cell activity, measured by INF-γ by Elispot assay (36). Antitumor immune-mediated effects of CCL2 genetic knockdown or anti-CCL2 antibody treatment were abolished when both $CD8^+$ and $CD4^+$ T cells were depleted (36). This evidence indicates that MDSCs block RT-induced T-cell anti-tumor activity via CCL2 and suggests this is a therapeutic target that could be manipulated to tip the balance in favor of dendritic cell recruitment.

Combining MDSC targeting therapies with RT may not only enhance STING activation, but also increase Type I IFN production and recruitment of $CD8^+$ T cells (37, 38). For tumors where MDSCs play a prominent role, using RT with STING immunotherapies may not be sufficient. MDSCs may be able to block the positive effects of these therapies by inhibiting $CD8^+$ T cell activity. For these tumors, adding anti-CCL2 antibodies to the treatment may be prudent.

In addition to MDSCs, M2 macrophages play a similar role in mediating immune suppression and resistance to RT (39). The IL-6/JAK/STAT3 pathway has been shown to polarize macrophages toward the pro-tumoral M2 phenotype through activation of STAT3, and anti-IL-6 immunotherapy increased the number of M1 polarized macrophages in a hepatocellular carcinoma mouse model (40). A review focused on the IL-6/JAK/STAT3 pathway, its role in cancer, and possible inhibitors of the pathway was recently published by Johnson et al. (41). The effects of targeting IL-6 with RT in a murine model of prostate cancer resulted in attenuation of angiogenesis, MDSC recruitment and decreased tumor growth (39).

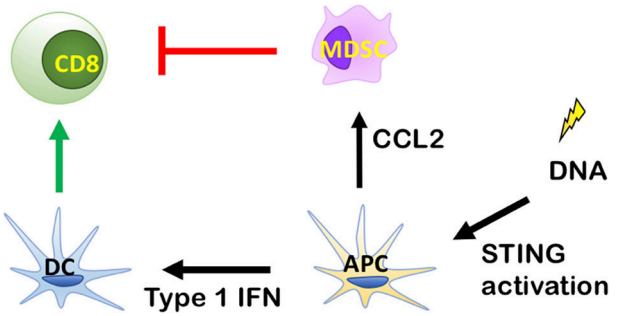

FIGURE 2 | Role of the stimulator of interferon genes (STING) signaling pathway in antitumor immunity. By inducing Type I IFN release from antigen presenting cells (APCs), radiation therapy (RT) can enhance antigen uptake by specialized dendritic cells (DCs) known as Batf3 DCs. This stimulates maturation of DCs and the cross-presentation of tumor associated antigens (TAA) to $CD8^+$ T cells, which exhibit antitumor immunity after proliferation and infiltration into tumor microenvironment. DNA from tumor cells is recognized by cytosolic DNA sensor cGAS to produce cGAMP for STING activation and cytokine production, which stimulate the maturation of DCs and stimulate the cross-presentation of TAA to $CD8^+$ T cells, which exhibit antitumor immunity after proliferation and infiltration into the tumor microenvironment.

Collectively, these studies highlight that it may be prudent to combine RT with immunotherapies that target MDSC and/or M2 macrophage recruitment and polarization to enhance anti-tumor immune responses. Some initial successes in targeting macrophages have been achieved. Anti-CSF1 immunotherapy, when used in combination with RT, prolonged survival in a glioblastoma (GBM) mouse model and significantly reduced RT-mediated macrophage recruitment to the tumor (42). Chloroquine, a common drug used to treat malaria, has also been shown to have anti-tumor effects via its ability to convert M2s into an M1 phenotype. Alone, chloroquine was able to reduce tumor burden in a murine melanoma and a hepatocarcinoma model (43). Since chloroquine was shown to be dependent on T cells for its effects (43), it might induce an even larger reduction in tumor burden when combined with RT given RT's potent effect on increasing T cell infiltration. Finally, myeloid cells' activation status can be targeted for therapeutic development. One such example is CD40 a surface protein present on most APCs (44). When CD40 is activated on APCs by binding to CD40L, APCs are able to present antigens to T cells (45). Increasing antigen presentation with anti-CD40 therapy in combination with RT was shown to increase survival of a B-cell lymphoma mouse model to 100% when the study ended at 100 days post-tumor-inoculation (46).

PD-L1-DEPENDENT RESISTANCE AND PD-L1-INDEPENDENT RESISTANCE: HOW CD8$^+$ T CELLS NEGATIVELY REGULATE THEIR OWN ACTIVATION BY IFN-γ AND CCL22 SECRETION

RT's ability to recruit and activate CD8$^+$ T cells by inducing secretion of chemoattractant molecules CXCL9, CXCL10, CXCL16, and CCL5 as a response to tissue damage is well-known (47–52). Despite this, resistance to RT still occurs. This is in part explained by CD8$^+$ T cell exhaustion, which is characterized by increased expression of immune checkpoint receptors such as PD-1, resulting in PD-L1-dependent resistance (53). Tumor-intrinsic factors can determine the extent of PD-L1 expression in tumors treated with RT and chemotherapeutic agents (27), but it also increases in response to IFN-γ (53). Gajewski et al. found evidence that activated CD8$^+$ T cells and their secretion of IFN-γ are responsible for promoting PD-L1 expression in the TME in a negative feedback loop *in vivo* (36). IFN-γ has been known for supporting an anti-tumor TME by promoting Th1 polarization, cytotoxic T cell activation, DC maturation (54), and increased CXCL9 secretion (55). But evidence now suggests that IFN-γ can also upregulate PD-L1 in the TME (53) (**Figure 3**).

IFN-γ's upregulation of PD-L1 has been shown in both murine and human tumor cell lines (56). The presence of both high CD8$^+$ T cell infiltration and IFN-γ is required for PD-L1's increase in tumors. This has been demonstrated by comparing levels of PD-L1 and IFN-γ in WT mice and CD8 KO mice in multiple murine melanoma models (53). It has been postulated that IFN-γ upregulates PD-L1 expression through activation

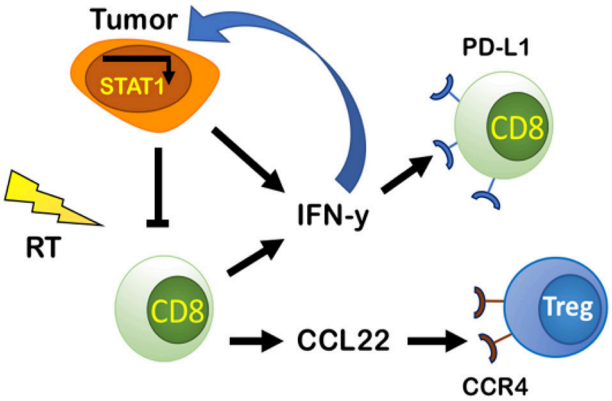

FIGURE 3 | PD-L1-dependent and independent resistance by CD8 effector cells and tumor cells. Tumor cells secrete IFN-y and IFN-I that can bind to IFNGR and IFNAR on tumor cells and promote PD-L1-independent resistance through constitutive activation of STAT1. Tumor cells and CD8 effector cells produce and secrete IFN-y that increases PD-L1 in the TME and causes exhaustion of CD8 cells promoting PD-L1-dependent resistance. CD8 effector cells increase production of CCL22, a chemoattractant that binds to CCR4 on Tregs increasing their presence in the TME, thus decreasing CD8 effector cell activity.

of IRF-1, an interferon regulatory factor with a binding site on the promotor of the gene coding for PD-L1 (57). IFN-γ's upregulation of PD-L1 supports the rationale for anti-PD-L1/PD-1 axis therapies in cancer therapy, but it also highlights why these therapies are only useful for a small portion of patients with high baseline levels of PD-L1 expression. Many tumors are devoid of T cells at baseline, and thus lack PD-L1 expression or effector T cells (Teff cells) that can be activated by anti-PD1/PD-L1 therapies (58). Combining such therapies with RT could be beneficial as RT increases PD-L1 expression and enhances infiltration of Teff cells (59).

Although combining RT and PD-L1 therapy has improved outcomes in more patients than anti-PD-L1 treatment alone, emerging data suggest that resistance still develops (24). In preclinical models, Benci et al. identified a novel role for INF-γ and Type I IFNs in PD-L1-independent resistance and showed that targeting IFN-γ/Type I IFNs resulted in decreasing T cell exhaustion (60). To determine if IFN-γ was responsible for resistance independent of PD-L1 expression, PD-L1 was deleted in tumor cells using CRISPR and PD-L1 was deleted in tumor associated macrophages (TAMs) or globally deleted with anti-PD-L1 therapy. The authors reported that IFN-γ expression was still able to induce resistance when PD-L1 was deleted, but when IFN-γ's receptor IFNGR and the receptor for Type I IFNs IFNAR were knocked out on tumor cells, exhausted T cells were significantly reduced and response to RT and anti-CTLA4 was enhanced (60). These data demonstrate that IFN-γ and Type I IFNs are responsible for promoting resistance to combined RT and anti-CTLA-4 treatment in a PD-L1-independent manner (60). Benci et al. further showed that this resistance is mediated by constitutive activation of STAT1 expression in tumor cells through genomic studies and effect studies involving STAT1 KOs combined with anti-PD-L1 treatment (60). Based on these

results and the finding that IFN-stimulated genes are increased in patients who develop resistance to anti-PD-L1 therapy (60), screening patients for IFN-stimulated genes may determine if patients qualify for therapeutic combinations of RT, anti-PD-L1, or anti-IFN therapy.

$CD8^+$ T cells can also regulate their own activity by recruiting Tregs through the CCL22-CCR4 axis (**Figure 3**). Gajewski et al. demonstrated that an increase in CCR4-expressing Tregs as a percentage of total immune cells was observed only when $CD8^+$ T cells were present (53). In CD8 KO mice, Tregs represented a lower percentage of total immune cells (53). Through a series of experiments, they showed that secretion of CCL22 by $CD8^+$ T cells recruits T cells and supports their proliferation without inducing T cell differentiation (53). Additionally, inhibition of CCR4 using the antagonist C021 prevented Treg accumulation in tumors (53). Targeting CCR4 could be a promising therapeutic target, especially in Treg enriched tumors. Such a therapy may have enhanced efficacy when combined with RT to induce Teff cell infiltration.

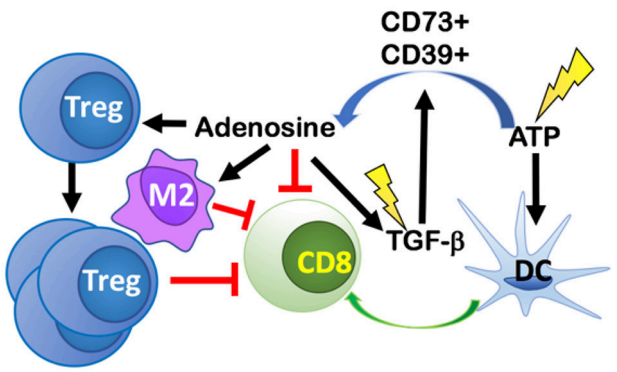

FIGURE 4 | RT-induced cancer cell death leads to release of ATP that both recruits and activates dendritic cells (DCs) thus initiating antitumor immune responses. ATP is rapidly catabolized into adenosine in the TME by CD39 and CD73 expressed on tumor cells, stromal cells, and immune cells. Local accumulation of extracellular adenosine suppresses DCs and CD8 T cells, while promoting proliferation of Tregs, M2 polarization, and increasing the release of TGF-β into the TME. RT, can also directly activate TGF-β via activation of reactive oxygen species (ROS). The increase in TGF-β promotes more adenosine formation in a positive feedback loop.

RT-INDUCED ADENOSINE: SHIFTING THE TME FROM DENDRITIC CELL RECRUITMENT TOWARD TREG- AND M2-MEDIATED IMMUNE SUPPRESSION

Immunogenic cell death resulting from tumor irradiation alerts the immune system to a potential threat via upregulation or release of DAMPs, including adenosine triphosphate (ATP). The dose-dependent release of ATP as a result of RT-induced cancer cell death (61), can recruit and activate DCs to uptake tumor antigens and cross-present them to naïve T cells, thus initiating antitumor immune responses (62) (**Figure 4**). However, ATP is rapidly catabolized into adenosine in the TME by the ectoenzymes CD39 and CD73 expressed on tumor cells, stromal cells, and immune cells, primarily, Tregs and Th17 cells. CD39 hydrolyzes ATP to ADP, and ADP into AMP, and then CD73 converts AMP into adenosine (63). Local accumulation of extracellular adenosine suppresses DCs and Teff cells while promoting proliferation of Tregs, increases the expression of CTLA-4 and adenosine receptor 2A (A2AR) on Tregs, and enhanced the polarization of tumor-associated macrophages (TAMs) into an M2 suppressive phenotype (64, 65).

Conversion of ATP to adenosine can be induced directly by RT. One mechanism for this conversion is mediated via the induction of reactive oxygen species (ROS) by RT, which then converts pro-TGF-β into its activated form (66). TGF-β promotes TAM polarization into M2s and upon glucocorticoid induction, TGF-β modifies gene expression in M2 macrophages to express additional immune-suppressive genes like the one coding for IL-17 receptor (IL17RB) that promotes development of Th17 cells. TGF-β is also able to increase the expression of ectonucleotidases CD73 and CD39 on Th17 cells by downregulating zinc finger protein growth factor independent-1 (Gfi-1) and by inducing Stat3 expression, respectively (67). Taken together, TGF-β increases the number of Th17 cells and the expression of genes responsible for converting ATP into adenosine in Th17 cells.

Therapeutic targeting of A2AR, CD73, and TGF-β may shift the TME to a pro-ATP environment and reduce resistance to immunotherapy in the setting of RT. In preclinical animal models, targeting A2AR, the receptor for adenosine, with a pharmacological inhibitor SCH58261 led to a significant decrease in tumor growth and reduced Tregs while enhancing Teff cell activity in a spontaneous Cre/lox HNSCC model (68). Targeting A2AR alone with CPI-444 led to a significant reduction in tumor burden for mice implanted with MC38 tumors (69). Further tumor regression was achieved by the addition of anti-PD-L1 and anti-CTLA-4 treatment in both MC38 and CT26 tumors. The combination of CPI-444 and anti-PD-L1 in MC38 implanted mice led to a 50% eradication of the tumors (5/10) (69). Another way to reduce the effects of adenosine is to limit its production in the first place by targeting CD73. Targeting CD73 with an anti-CD73 monoclonal antibody (mAb), anti-CD73 decreased the tumor burden and increased the survival of mice with MC38-OVA tumor cells (70). This effect was even greater when combined with anti-PD-L1 and anti-CTLA-4 (70). Another group found that CD73 knockout mice had greater homing of Teff cells and that this effect was primarily driven by CD73 expression on Tregs (71). Although blocking production and direct action of adenosine has been shown to be effective, therapeutic strategies aimed at targeting TGF-β can be of more significant benefit in combination with RT. TGF-β increases the expression of both CD73 and CD39 and is responsible for promoting a variety of pro-tumor effects. There has been some hesitation in targeting TGF-β in the past because of the potential for cardiac toxicities, but new-generation small-molecule inhibitors have been shown to have limited side effects in clinical trials (72–74). The newly developed bifunctional fusion protein, M7824, TGF-β Trap (75) is another potential therapeutic

target to combine with RT, as it simultaneously blocks the PD-L1 and TGF-β pathways and might yield increased response compared to monotherapy alone.

CLASSIC RT ANTI-TUMOR EFFECTS MEDIATED THROUGH ROS HAVE A DARK SIDE: INCREASING ADENOSINE THROUGH TREG APOPTOSIS AND CREATING A HYPOXIC IMMUNOSUPPRESSIVE TME

RT is classically known to act on cancer cells by inducing apoptosis, senescence, and mitotic catastrophe through the production of reactive oxygen species (ROS) that, at high enough concentrations, can damage cells and cause double-stranded DNA damage (2, 3). It was thought that these effects would primarily affect tumor cells, causing tumor cell death, and—based on current understanding—increase tumor associated antigens for immune cell recognition. Recently, Zou et al. showed that within the immune TME, ROS resulting from RT induced apoptosis of Tregs driving increased immunosuppression. Their data support a hypothesis that apoptotic, but not proliferating, Tregs release high levels of ATP and subsequently metabolize ATP into adenosine because CD73 and CD39 are still metabolically active (76) (**Figure 5**). This fundamentally changes the current dogma of targeting all Tregs with immunotherapies. If Treg apoptosis is driving immunosuppression, an ideal immunotherapy would decrease Treg activity and proliferation, without inducing their apoptosis.

A more hypoxic environment will be less sensitive to the effects of RT (77), and many solid tumors are known to be more radioresistant in hypoxic regions. Although there is intrinsic hypoxia due to the nature of solid tumors, RT can worsen hypoxic conditions by increasing hypoxia-inducible factor-1α (HIF-1α). HIF-1α has been shown to cause radioresistance of endothelial cells (78), angiogenesis through expression of vascular endothelial growth factor A (VEGF-A) (79), malignant progression (79), and poor survival outcomes after RT treatment (80, 81). Upregulation of HIF-1α by RT can be a direct result of stabilizing HIF-1α in cancer cells (78, 79), or it can occur indirectly as RT increases TAMs, which also stabilize HIF-1α (82).

Within the TME, HIF-1α mediates immunosuppression by modulating specific immune cell functions (**Figure 6**). It modulates gene expression and cytokine production in MDSCs, thereby increasing their role in T cell suppression. HIF-1α inhibits myeloid cell differentiation through a VEGF-A mediated mechanism leading to accumulation of MDSCs (83, 84). Induced by RT, VEGF-A can also increase inhibitory receptors on $CD8^+$ T cells (e.g., Tim-3, CTLA-4, PD-1, Lag-3) (85) as well as PDL-1 expression on tumor cells and MDSCs (86), thereby promoting T cell exhaustion and inactivity (85). Another mechanism by which RT-induced VEGF-A secretion can enhance a pro-tumor environment is through its influence on endothelial cells by inducing expression of CD95L (or FasL), the ligand for FAS (87, 88). In response to RT, expression of Fas can be induced by tumor cells secreting IL-10, prostaglandin E2, and VEGF-A (89). Fas can induce apoptosis of Teff cells, while sparing Tregs to support an anti-tumor environment (90).

HIF-1α represents an ideal target for reducing the immunosuppression driven by a hypoxic environment, but

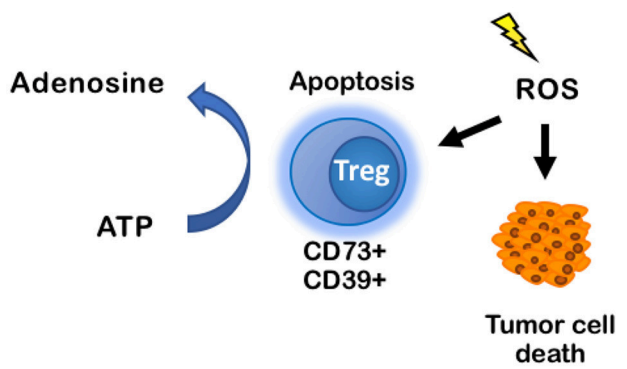

FIGURE 5 | ROS induces apoptosis of CD73+CD39+ Tregs, increasing production of adenosine in the TME. ROS classically induces tumor cell death through DNA-mediated cell damage, but it also induces apoptosis in immune cells. When CD73+CD39+ Tregs undergo apoptosis, they produce high levels of ATP that are rapidly converted into adenosine by CD73 and CD39 on the Tregs cell membrane. Accumulation of adenosine promotes an immuno suppressive environment. ROS, reactive oxygen species.

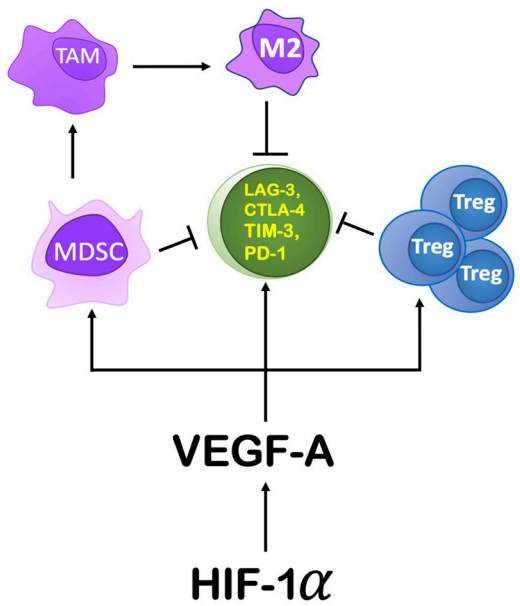

FIGURE 6 | The role HIF-1α and its downstream components play in producing an immunosuppressive environment. HIF-1α's action on the TME is primarily through its induction of VEGF-A. VEGF-A drives immunosuppression by recruiting MDSCs, promoting proliferation of Tregs, and by increasing the expression of immune checkpoint inhibitor genes on $CD8^+$ T cells. Increasing MDSCs in the TME leads to their conversion to TAMs, specifically an M2 polarization.

currently no drugs are approved for clinical trials in humans. Drugs designed in an attempt to target HIF-1α have had many off-target effects, including but not limited to inhibiting mRNA expression, protein synthesis, protein degradation, and DNA binding (91). In the future, more effective and specific inhibitors of HIF-1α will be developed. In the meantime, targeting VEGF-A may have some potential. There are several FDA-approved drugs that target VEGF-A, including the monoclonal antibody bevacizumab (66). Pre-clinical and clinical applications of these drugs have been well-described by others recently (92–94). Briefly, inhibiting VEGF-A appears to produce only a modest increase in survival for patients with a wide range of tumor types (95–99). These modest effects could be the result of indiscriminate administration of the drugs and/or parallel pathways of resistance. Combination approaches targeting both VEGF-A and HIF-1α axes or with cox-1 inhibitors as described in the next section could prove to be more beneficial than any single approach.

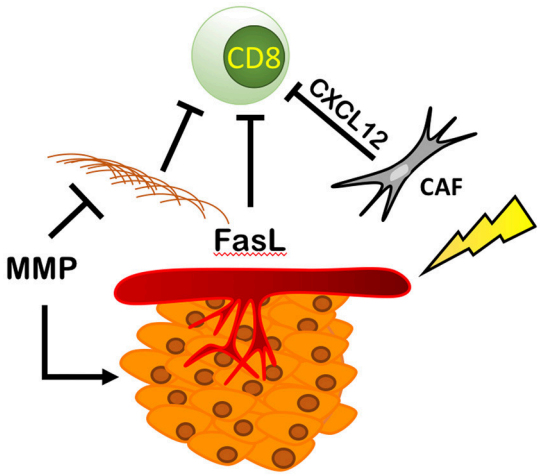

FIGURE 7 | RT modulates fibroblasts, ECM, and endothelial cells resulting in an immunosuppressive environment. RT increases the number and activity of cancer associated fibroblasts (CAFs) increasing the production of CXCL12, blocking $CD8^+$ T cell recruitment and increasing the amount of ECM proteins produced by fibroblasts physically blocking immune cells from entering the TME. This is countered by RT's ability to increase the expression of MMPs that break down the ECM, increasing cancer spread and metastasis. Finally, RT is able to increase the expression of Delta, Jagged, Notch, and FasL, thus reducing $CD8^+$ T cell recruitment and promoting tumor growth and survival.

RT'S REMODELING OF THE EXTRACELLULAR MATRIX AND ENDOTHELIAL CELLS: PROMOTING FIBROSIS, MMP ACTIVITY, AND FASL EXPRESSION

By increasing the number and activity of fibroblasts and MMPs, and increasing pro-tumoral endothelial cell function, RT can directly modulate the extra-cellular matrix (ECM) component of the TME (**Figure 7**). RT-mediated TGF-β signaling increases the number of cancer-associated fibroblasts (CAFs) or myofibroblasts in the ECM. These cells deposit type I, type II, type III, and type V collagen, fibronectin, and matrix metalloproteinases (MMPs) that regulate ECM homeostasis (100–102). CAFs also express fibroblast activation protein (FAP) (103) enhancing immunosuppression within the TME via CXCL12 (104), a chemokine that reportedly coats tumor cells and inhibits recruitment of T cells in the area (104) and reduces ECM-associated fibrosis (105).

RT can directly modulate endothelial cell function to inhibit Teff cell immune function and create a pro-tumoral TME. Upregulation of FasL, on endothelial cells has been shown to be a critical mediator of Teff cell inhibition in a variety of cancers (87, 88, 90). Fas can induce preferential apoptosis in Teff, while sparing CD25-expressing Tregs, favoring an immunosuppressive TME (90). Tumor-derived IL-10 and prostaglandin E2 can independently increase endothelial cell expression of Fas, and tumor derived VEGF-A is dependent on the presence of IL10 or prostaglandin E2 to further increase Fas expression (90). This explains why a blockade of FasL expression in different ovarian cancer cell lines by targeting VEGF-A was shown to be drastically enhanced when combined with COX1 inhibitors (90). VEGF-A's effects were dependent on the amount of COX 1 expression, implying that VEGF-A is necessary but not always sufficient to produce FasL (90). These results have been corroborated by findings in four distinct murine cancer models: ovarian, skin, colon, and renal cancer (90). Treatment of these tumors with anti-VEGF-A combined with a COX 1 inhibitor, salicylic acid, resulted in depletion of FasL expression on tumor endothelial cells, an increase in $CD8^+$ T cells infiltrating the TME, and a reduction in tumor growth (90). Targeting VEGF-A alone has had modest effects on overall survival in clinical trials (92–94). Of note, aspirin has also been associated with prevention of colorectal cancer and a reduction in colorectal cancer mortality (106). Combining VEGF-A inhibitors with daily aspirin use may present a potential therapeutic combination to improve upon these modest anti-VEGF-A effects.

RT can also modulate the vascular TME to enhance tumor metastasis, which is in part mediated by upregulation of various genes involved in migration/metastasis. Tumor cell dissemination via blood vessels requires tumor cells to undergo transendothelial migration. This occurs at sites where leukocytes and macrophages are in direct contact with tumor cells and endothelial cells. The best studied proteins at these sites are Jagged, Delta, and Notch (107). Activated Notch1 has been shown to inhibit apoptosis and enhance radioresistance (108), while downregulation of Notch1 expression can induce radiosensitization and alleviate radiation-induced epithelial-mesenchymal transition (EMT) (109–111). Activation of the Notch signaling pathway upregulates E-selectin expression on endothelial cells that shields tumor cells in the blood stream from anoikis (112). In breast cancer models, inhibition of the Notch signaling pathway blocked macrophage-induced intravasation *in vitro* and the dissemination of tumor cells from the primary tumor *in vivo* (113). The association between EMT and radioresistance and the prominent role of Notch signaling as a

driving force in the EMT process, suggest that Notch inhibition will result in radiosensitization of tumors that underwent EMT.

Targeting the fibrotic TME can be challenging. Kalluri et al. found that when myofibroblasts were eliminated from the TME using transgenic mice, cancer progression and outcome were worse (113). Deletion of myofibroblasts was also associated with a reduced Teff/Treg ratio and elevated CTLA-4 expression (113). This could be because certain structure is needed to allow for normal functioning of immune cells and to keep the tumor in place. However, some tumor types are known to be highly fibrotic, hence reducing, but not eliminating, fibrosis may be important for enhancing anti-tumor immunity. As treatment with RT can result in a significant increase in fibrosis in these tumors (114–118), it may also be important to use anti-fibrotic agents to reduce fibrosis to pre-RT levels. Some available drugs are being tried pre-clinically to reduce fibrosis. For example, Pirfenidone, which inhibits TGF-induced fibrosis by targeting the TGF-β1/Smad/CTGF pathway (119) has been shown to reduce RT-mediated fibrosis in a murine lung carcinoma model (120) and increase survival (121). Another avenue to increase T cell infiltration is targeting CXCL12 or its receptor, CXCR4. Targeting both has been shown to reduce RT-associated lung fibrosis (122), and anti-CXCL12 therapy alone increases T cell infiltration into tumors (104). Although directly targeting FAP+ cells represents an attractive therapeutic strategy, thus far targeting FAP alone has shown no benefit in clinical trials (115).

CONCLUSION

There is now considerable evidence that single-agent immune therapies have limited response in various cancer sites (123–126). Radiation therapy has been shown to synergize with immune modulating therapy through several mechanisms including exposure of neo-antigens, STING activation and PD-L1 upregulation (31, 59, 127). In addition to synergy where each component contributes to tumor response, radiation therapy can transform tumors and sensitize them to immune therapies (7, 9, 12, 17). However, in both cases the response to combination RT and immune therapy can be transient. The challenge ahead is to determine why the combination of RT and immune therapy provides a durable response in some patients and a limited response in others. Specifically, future studies should focus on identification of how RT's paradoxical effects manifest in responders and non-responders. The response to RT and immune modulating therapy can be suppressed through additional mechanisms of immune-evasion and immune-suppression including chronic IFN-γ activation, conversion of ATP to adenosine, ECM remodeling and secretion of immunosuppressive factors that promote infiltration of Tregs, MDSCs and macrophages. These mechanisms are likely activated by tumor-intrinsic factors that should be identified and targeted to develop effective therapies. It is conceivable that such factors will affect RT response differently between patients with the same cancer type and across different cancer types. Therefore, identifying diagnostic biomarkers for these factors is an important next step. Tumor staining for PD-L1 expression has been successfully implemented in NSCLC and melanoma patients to identify candidates who will benefit from anti-PD-1/anti-PD-L1 therapy. Additional markers are warranted to identify candidates such as immune checkpoint receptors such as TIM-3, LAG-3, CTLA-4, as well as assessment of intratumoral Tregs, MSDCs and macrophages are warranted. Furthermore, assessment of secreted factors will be important for identifying patients who can benefit from therapies that target recruitment and homing of immune suppressive cell populations. Such factors include TGF-β, ATP, CCL2, CCL20, and CCL22. Another challenge of integrating RT with immunotherapy is identifying the RT dose and fractionation resulting in optimal synergy. Most evidence suggests that hypofractionated RT is better suited for integration with immunotherapy, but there is also evidence that conventional fractionation can achieve similar results. Consideration for when certain immune cell populations are more abundant may be beneficial in determining an optimal dosing schedule (128, 129). It is important to design clinical trials that address RT's effects on the TME, as well as dosing and fractionation when combined with immunotherapy. Selecting rational combinations of therapies based on both forward and reverse translation, rigorous preclinical studies, and careful analysis of trial specimens is needed to generate a mechanistic understanding of the effects of treatment on the tumor and the associated microenvironment.

AUTHOR CONTRIBUTIONS

LD, AO, and SDK contributed to the writing and editing of the manuscript. SDK is responsible for supervision of this work.

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Targeting the Immunomodulatory CD73/Adenosine System to Improve the Therapeutic Gain of Radiotherapy

*Simone de Leve, Florian Wirsdörfer and Verena Jendrossek**

Institute of Cell Biology (Cancer Research), University Hospital Essen, University of Duisburg-Essen, Essen, Germany

Extracellular adenosine is a potent endogenous immunosuppressive mediator critical to the maintenance of homeostasis in various normal tissues including the lung. Adenosine is either released from stressed or injured cells or generated from extracellular adenine nucleotides by the concerted action of the ectoenzymes ectoapyrase (CD39) and 5′ ectonucleotidase (CD73) that catabolize ATP to adenosine. An acute CD73-dependent increase of adenosine in normal tissues mostly exerts tissue protective functions whereas chronically increased adenosine-levels in tissues exposed to DNA damaging chemotherapy or radiotherapy promote pathologic remodeling processes and fibrosis for example in the skin and the lung. Importantly, cancer cells also express CD73 and high CD73 expression in the tumor tissue has been linked to poor overall survival and recurrence free survival in patients suffering from breast and ovarian cancer. CD73 and adenosine support growth-promoting neovascularization, metastasis, and survival in cancer cells. In addition, adenosine can promote tumor intrinsic or therapy-induced immune escape by various mechanisms that dampen the immune system. Consequently, modulating CD73 or cancer-derived adenosine in the tumor microenvironment emerges as an attractive novel therapeutic strategy to limit tumor progression, improve antitumor immune responses, avoid therapy-induced immune deviation, and potentially limit normal tissue toxicity. However, the role of CD73/adenosine signaling in the tumor and normal tissue responses to radiotherapy and its use as therapeutic target to improve the outcome of radiotherapy approaches is less understood. The present review will highlight the dual role of CD73 and adenosine in tumor and tissue responses to radiotherapy with a special focus to the lung. It will also discuss the potential benefits and risks of pharmacologic modulation of the CD73/adenosine system to increase the therapeutic gain of radiotherapy or combined radioimmunotherapy in cancer treatment.

Keywords: CD73, adenosine, radiotherapy, therapeutic window, normal tissue toxicity, T_{reg}, macrophages, tumor microenvironment

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Reviewed by:
Frederique Vegran, INSERM U1231 Lipides, Nutrition, Cancer (LNC), France
Zoltan Vereb, University of Szeged, Hungary

***Correspondence:**
Verena Jendrossek
verena.jendrossek@uni-due.de

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INTRODUCTION

Radiotherapy is a mainstay in the treatment of cancer patients. About 60% of all cancer patients receive radiotherapy during the course of their disease alone or in multimodal combinations of surgery, radiotherapy, and chemotherapy, with beneficial effects of these highly effective treatments on long-term survival and tumor cure (1–5). Moreover, much progress has been made with

technical improvements in treatment planning that increase accuracy of dose delivery, as well as by the development of particle therapy approaches (6). Nevertheless, cure rates still need to be improved for prevalent cancer types with high loco-regional failure-rates or a high risk for invasive growth or metastatic spread. For example, patients suffering from locally advanced non-small cell lung cancer (NSCLC) are typically treated with fractionated radiotherapy to the thoracic region, or concurrent platinum-based radiochemotherapy (RCT) yielding local control rates of 40–66% with doses of 60–66 Gray (Gy) (7–9). But loco-regional failures upon definitive RCT or disease progression by distant metastases are common and it is thought that improving local control rates will directly improve survival rates (9, 10).

Herein biological factors such as the high intrinsic tumor cell radio resistance, a pronounced tumor heterogeneity, diversity in radiation responses, and a resistance-promoting microenvironment reduce the efficacy of radiotherapy and thus contribute to failures. Otherwise, the high radio sensitivity of the normal lung tissue limits the application of curative radiation doses to the thoracic region and therapy intensification efforts of RCT (9, 11). Technical advances in image guidance and modern radiation techniques have significantly increased the safety profile of thoracic radiotherapy (12–14); but radiation-induced lung disease (RILD) still represents a serious normal tissue complication associated with radio(chemo)therapy of thoracic neoplasms or total body irradiation in conditioning regimens for hematopoietic stem cell transplantation (15–17). Moreover, toxicity rates can increase or new toxicities can be observed when using molecularly targeted drugs in combination with radiotherapy (18–21). Thus, there is a high need for further innovations in radiotherapy practice that improve the tumor response without increasing toxicity.

The progress in cancer immunotherapy and the discovery that radiotherapy activates T-cell-mediated antitumor immune responses under certain conditions, particularly when combined with established immune checkpoint blockade, expedited interest, and research in exploiting a potential benefit of combining radiotherapy with immunotherapy in pre-clinical and clinical cancer research (22–30). However, there are still major challenges in defining optimal dosing and treatment schedules and understanding the dual face of radiation-induced immune changes with potential impact on immune-related adverse effects. Moreover, only a fraction of patients responds to the treatment with immune checkpoint blockade alone or in combination with radiotherapy as tumors may not be immunogenic, dispose of efficient strategies to escape from tumor immune surveillance, or responses may not be durable (31–34).

In this context, accumulation of extracellular adenosine through activation of 5′ectonucleotidase (CD73) and subsequent signaling through adenosine receptors is a common mechanism how tumors escape from tumor immune surveillance. This makes CD73/adenosine signaling an attractive target in immuno-oncology and the related studies and underlying principles are well covered in various reviews (35–39).

But the role of CD73/adenosine signaling in the response of tumors and normal tissues to radiotherapy and its potential impact on the outcome of radiotherapy and combined radioimmunotherapy are less well described. Herein it is important to consider that the effects of CD73/adenosine activation on the immune system and reconstitution of tissue homeostasis might well differ among tissues of different origins as well as between acute and chronic activation stages. Therefore, we will first introduce the contribution of radiotherapy-induced changes in the innate and adaptive immune cell compartments to acute and chronic tumor and normal tissue responses and point to beneficial and adverse roles to the outcome of radiotherapy. We will then summarize current knowledge about the role of CD73 and adenosine in tumor and normal tissue responses to radiotherapy, and highlight the potential of targeting CD73/adenosine for improving the therapeutic gain of radio (immuno)therapy in thorax-associated tumors with high risk of adverse late effects in the highly radiosensitive normal lung tissue.

PARADIGM CHANGE: RADIATION ACTIVATES LOCAL AND SYSTEMIC IMMUNE EFFECTS

The broad use of radiotherapy as standard treatment option in the therapy of solid human tumors is based on its ability to damage cellular macromolecules, particularly the DNA, thereby effectively inducing growth arrest and cell death locally in irradiated tumor cells. But bystander effects such as the transmission of lethal signals between cells via gap junctions or the production of diffusible cytotoxic mediators can also contribute to local antineoplastic action of radiotherapy. However, despite reported transient immunosuppressive effects by local induction of immune cell death (40) and or immune impairment (41, 42), multiple reports highlight the ability of radiotherapy to induce systemic effects that involve activation of the innate and adaptive immune systems (22, 23, 43, 44).

In the context of tumor therapy, exposure to ionizing radiation can modulate immunosuppressive barriers in the tumor microenvironment, trigger the recruitment of immune effector cells to the local tumor, render tumors accessible to

Abbreviations: ADA, adenosine deaminase; ADOR, adenosine receptor; α-SMA, alpha smooth muscle actin; BLM, Bleomycin; B_{reg}, regulatory B cells; CD39, ectoapyrase, ectonucleoside triphosphate diphosphohydrolase 1; CD73, 5′ ectonucleotidase; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; cGAS/STING, cyclic GMP-AMP Synthase/Stimulator of Interferon Genes; CSF-1, colony-stimulating factor 1; CTGF, connective tissue growth factor; CTLA-4, cytotoxic T-lymphocyte-associated Protein 4; DAMPs, damage associated molecular patterns; ENPP1, ectonucleotidepyrophosphatase (phosphodiesterase 1); ENT, equilibrative nucleoside transporter; Gy, Gray; HMGB1, high-mobility-group-protein B1; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon gamma; IL, Interleukin; mAb, monoclonal antibody; MDSC, myeloid-derived suppressor cells; MMR, macrophage mannose receptor; MSC, mesenchymal stem cell; NSCLC, non-small cell lung cancer; PD-1, programmed cell death 1; PD-L1, programmed death-ligand 1; PEG-ADA, pegylated ADA; PDGF, platelet-Derived Growth Factor; RILD, radiation-induced lung disease; ROS, reactive oxygen species; RT, Radiotherapy; SCID, severe combined immunodeficiency; TAM, tumor-associated macrophages; TGF-β, transforming growth factor beta; TLR, toll-like receptor; T_{reg}, regulatory T cells; VEGF, vascular Endothelial Growth Factor; WTI, whole thorax irradiation.

infiltration of immune effector cells by modulating restrictive tumor vessels, and even elicit tumor-specific immune responses leading to the regression of tumor lesions locally and at tumor sites outside the radiation field (abscopal effects) (22, 45–50). Elegant pre-clinical investigations helped to reveal the importance of T-cell responses to the local and abscopal antitumor effects in response to radio(immuno)therapy and to uncover the underlying mechanisms (47, 51–57).

Since abscopal effects seem to be rare in the clinical situation (49, 58–61), current clinical trials focus on combining radiotherapy with different immunotherapies (30, 47, 48, 62–64). Notably, there is hope from first clinical studies that blockade of the programmed cell death 1/programmed death-ligand 1 (PD-1/PD-L1) immune checkpoint might improve progression-free survival in lung patients with an acceptable safety profile, when given after radiotherapy or platinum-based RCT (65, 66). But further studies are needed to explore the efficacy and the safety profile of combined therapy of cancer patients suffering from thorax-associated neoplasms with radiotherapy and immunotherapies, to define biomarkers for patient selection and potential compensatory immune-tolerance mechanisms in malignant tumors (27, 67), and to define optimal treatment schedules.

It appears that the local induction of damage to highly radiosensitive resident cells in the lung with subsequent activation of non-targeted immune effector mechanisms might also contribute to the adverse effects of ionizing radiation in normal tissues such as the development of pneumonitis and pulmonary fibrosis (68–74). Similar to other models of sterile inflammation radiation-induced damage to resident normal lung tissue cells triggers a multifaceted damage-signaling cascade including a multifactorial secretory program in order to stimulate repair and recovery (74). However, radiation induces chronic changes in irradiated tissues that presumably result from a persistent damage signaling. These chronic environmental changes impact not only the phenotype of resident cells but also the recruitment and polarization of immune cells infiltrating the previously irradiated lung tissue, thereby disturbing the balance between inflammatory and repair processes and promoting chronic fibrosis progression (73).

DUAL FACE OF RADIATION-INDUCED IMMUNE CHANGES: BALANCE BETWEEN IMMUNOACTIVATING AND IMMUNOSUPPRESSIVE EFFECTS

As outlined above, exposure to ionizing radiation has the capacity to induce immune responses in normal and tumor tissues. These changes involve a complex interplay between cells of the irradiated malignant or healthy normal tissues and cells of the innate and adaptive immune systems. But, depending on the type (tumor vs. normal) and origin of the irradiated tissue, the temporal appearance (acute vs. chronic), and the basal immune status of the tissue before exposure to ionizing radiation (pro- vs. anti-inflammatory), the response of the immune system can either adopt immunostimulatory or immunosuppressive effects and have either a positive impact (anti-tumor; normal tissue protection) or a negative impact (pro-tumor; normal tissue toxicity) on treatment outcome.

In the following paragraphs we will highlight the dual roles of the immune system in the response of tumor and normal tissues after irradiation that are mostly derived from pre-clinical studies.

Tumor Tissue

Radiation-induced immune changes in the tumor involve the direct activation of innate and adaptive immune responses influencing tumor growth; but radiation-induced immune responses also include indirect responses such as radiation-induced changes in the tumor vasculature or tumor microenvironment that impact the recruitment and activation state of cells from the innate and adaptive immune system [for a review see (64, 75–79)].

Tumor irradiation induces damage and death of cancer cells resulting in the surface exposure of immunogenic molecules as well as the release of damage associated molecular patterns (DAMPs) such as ATP or High-Mobility-Group-Protein B1 (HMGB1), and potentially tumor antigens, to activate innate and adaptive immune responses (80). Nuclear release and cytoplasmic sensing of altered nuclear acids via Toll-like receptor (TLR)9 or cyclic GMP-AMP Synthase/Stimulator of Interferon Genes (cGAS/STING) is intimately connected to the secretion of cytokines that support innate and adaptive antitumor immunity. Priming of tumor-specific T cell responses requires uptake of tumor antigens by antigen presenting cells e.g., dendritic cells. Furthermore, priming of tumor-specific T cells depends on sensing of cancer-cell derived cytoplasmic DNA. e.g., by the cGAS/STING pathway that is connected to the activation of the interferon (IFN) I response to support antitumor immunity. The initiated migration and antigen presentation of dendritic cells then triggers the activation of B and T cells in secondary lymphoid organs. Activated T and B cells subsequently exert anti-tumor effects by several mechanisms like $CD8^+$ T cell mediated cytotoxicity, antibody-dependent cell-mediated cytotoxicity, and antibody-induced complement-mediated lysis. These processes have been excellently described in more detail elsewhere (27, 43, 47, 57, 75, 80–82).

Thus, the direct induction of anti-tumor immunity in response to ionizing radiation requires a complex interplay between the innate and adaptive immune system and the tumor microenvironment. Moreover, the recruitment and activation of dendritic cells in irradiated tumors that are required for the priming of tumor-specific T cell responses largely depends on the dose and fractionation of radiation in a tumor-dependent manner. Finally, tumor cells dispose of multiple mechanisms to evade this response so that the direct induction of anti-tumor immunity by radiotherapy is a rare event (41, 57, 83).

Besides these beneficial radiation-induced anti-tumor immune responses, local irradiation can also induce subacute or chronic immune changes that mostly exert tumor-promoting effects. Pro-inflammatory cytokines released in tissues as a damage response after radiotherapy as well as the humoral immune response from activated B cells can activate cells of the innate immunity, such as granulocytes, macrophages, and

mast cells (84, 85). These cells release molecules that modulate gene expression programs in favor of pro-survival signaling and cell cycle progression in neoplastic cells thereby supporting malignant tissue expansion (86, 87). Moreover, cells from the innate immunity have the capacity to induce repair, regeneration, and tissue remodeling. By releasing various mediators these cells influence and initiate fibroblast activation, angiogenesis and matrix metabolism thereby indirectly fostering tumor growth (84, 88–90).

Finally, the tumor itself responds to radiation-induced stress or damage through a panel of phenotypic changes. By releasing several cytokines, chemokines, or growth factors as well as up-regulating specific surface receptors e.g., immunosuppressive PD-L1, cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), and others, tumor cells become proficient in dampening immune responses and to escape the immune system (91–96). Detailed reviews from Sharma et al. as well as Wennerberg et al. recently summarized the role of these tumor cell-extrinsic factors for primary and adaptive resistance so that these mechanisms will not be further addressed here (34, 41).

Normal Tissue

Despite technological improvements ionizing radiation still directly hits to some extent tumor-surrounding healthy tissue during treatment, leading to local damage, stress, or cell death in normal resident cells. Moreover, the damage response initiated in malignant tissues and healthy tissues residing in the radiation field not only contributes to the local effects of radiotherapy but can also exert strong systemic effects promoting normal tissue complications (22, 97–99).

Radiation-induced immune changes in normal tissues also include acute and chronic immune effects that will be discussed below. While the effects of radiation-induced normal tissue inflammation are well described (100–106), the contribution of the complex immune mechanisms that support chronic, pathological changes e.g., fibrosis, are less investigated and still not yet completely understood.

Similar to the situation in tumors, radiation-induced acute damage and cell death in normal tissues also results in the release of DAMPs as well as pro-inflammatory cytokines and chemokines which have the capacity to modulate immune responses (105, 107). These "danger signals" trigger the activation and influx of innate and adaptive immune cells at the site of damage resulting in normal tissue inflammation. An excessive inflammation during the acute phase after radiation as a result of an overwhelming secretion of pro-inflammatory cytokines and the release of reactive oxygen species (ROS) supports normal tissue toxicity and severe side effects in treated patients (108).

In addition DAMPs can activate tissue regeneration in normal tissues as well as in tumor tissues. It is known that the extracellular DAMPs HMGB1 and ATP can activate and recruit cells, thus stimulating tissue repair (109). Of these, innate immune cells invade into the damaged tissue to clear dead cells and cellular debris (110). Moreover, stem cells and tissue-associated cells, e.g., fibroblasts, keratinocytes, endothelial cells, and vascular smooth muscle cells, are stimulated by these DAMPs to support angiogenesis and tissue regeneration (111–117). In addition, several DAMPs (e.g., HMGB1, S100A4, uric acid) can also enhance the expression of immunosuppressive mediators like interleukin (IL)-10 and indoleamine 2,3-dioxygenase (IDO) in stem cells, thereby inhibiting lymphocyte responses and contributing to tumor promotion (118). Excellent detailed reviews about the role of DAMPs in mediating regenerative pathways can be found elsewhere (109, 119).

Radiation-induced damage to normal tissues furthermore triggers chronic environmental changes e.g., hypoxia and senescence, that are reminiscent of the changes observed in the tumor microenvironment. These changes support chronic inflammation and repair processes, promote alternative polarization of recruited immune cells, pathologic immune cell interactions and excessive tissue remodeling, and thereby trigger not only the development of tissue scaring and fibrosis but also the development of secondary tumors (120, 121). For more detailed reviews about the acute and chronic events during radiation-induced normal tissue toxicity please refer to Wirsdorfer and Jendrossek (73), McKelvey et al. (79), Schaue et al. (105), Stone et al. (122), Barnett et al. (123), Kim et al. (124), and Ruhle and Huber (125).

The dual face of radiotherapy-induced immune changes in normal tissues can be nicely demonstrated in murine models of RILD. The acute phase of pneumonitis and the chronic event of fibrosis dramatically reveal how complex the radiation-induced alterations of the tissue micromilieu and the immune system impact disease pathogenesis (73).

Own studies in the murine C57BL/6 model of RILD revealed that whole thorax irradiation (WTI) with 15 Gy triggered an acute early immune suppression characterized by a pronounced reduction in the number of lymphocytes and myeloid cells that was followed by an influx of $CD3^+$ T cell lymphocytes into the lung tissue during the pneumonitic phase Interestingly, WTI also enhanced numbers of regulatory T cells (T_{reg}) in the lung tissue of irradiated animals both, during the early inflammatory state as well as at the time of fibrosis development. Of note, radiation-induced pulmonary fibrosis was more severe in recombination-activating gene 2 (RAG2)-deficient mice that lack mature T- and B-lymphocytes suggesting that lymphocytes may have beneficial effects (126). Instead, depletion of $CD4^+$ T cells during the pneumonitic phase decreased radiation-induced lung fibrosis in rats pointing to a contribution of $CD4^+$ T cells to disease pathogenesis (68). These data suggest a causal link between the recruitment or local expansion of specific T-lymphocyte populations and the course of RILD that are also observed in other fibrosis models (127). But further work is required to define the beneficial and adverse effects of recruited and induced T cell subsets during the course of RILD (128).

Thoracic irradiation induces not only changes in the T cell compartment but also in the myeloid compartment and the lung environment. For example, others and we detected a significant reduction in the levels of total pulmonary macrophages and an almost complete eradication of alveolar macrophages early after irradiation as well as time-dependent changes in the macrophage phenotype with increased expression of markers for alternative macrophage activation [e.g., macrophage mannose receptor and

Arginase-1] particularly during the fibrotic phase (102, 126, 129–132). Further, macrophages accumulated in organized clusters and expressed pro-fibrotic mediators such as alpha smooth muscle actin (α-SMA) and transforming growth factor beta (TGF-β) at ≥25 weeks post-irradiation (131). Importantly, a recent report confirmed the formation of organized clusters of $CD163^+$ macrophages also in lung tissue of irradiated patients. Intriguingly, pharmacologic inhibition of colony stimulating factor-1 (CSF-1) inhibited macrophage influx and attenuated RT-induced lung fibrosis in mice supporting a pathologic relevance of macrophages in RT-induced lung fibrosis (132). Similarly, pharmacological treatment with as connective tissue growth factor (CTGF) antibody before or after 20 Gy thoracic irradiation reduced acute and chronic radiation toxicity in mice and abrogated M2-like macrophage infiltration (133). The combined inhibition of TGF-β and Platelet-Derived Growth Factor (PDGF) blockade in a pre-clinical murine model attenuated radiation-induced pneumonitis and lung fibrosis and was accompanied by reduced osteopontin expression and leukocyte infiltration (134). Instead strategies using anti- Vascular Endothelial Growth Factor (VEGF) to target the tumor vasculature in combination with radiotherapy turned out to be highly toxic to normal lung tissue in pre-clinical murine models (21).

THE IMMUNOMODULATORY CD73/ADENOSINE SYSTEM AS THERAPEUTIC TARGET FOR IMPROVING RADIOTHERAPY OUTCOME

Various observations from pre-clinical and clinical studies summarized in the former paragraphs suggest that targeting tumor-induced or radiation-induced immune deviation may offer novel and attractive opportunities for improving the outcome of radiotherapy by modulating the tumor radiation response, radiation-induced adverse late effects, or both. But the complexity of the tumor-induced and radiation-induced changes in the microenvironment as well as the time- and tissue-dependent "dual face" of radiotherapy-induced immune changes highlight the importance to identify strategies suited to balance adverse pro-inflammatory and immunosuppressive effects of radiotherapy and outweigh the beneficial effects of radioimmunotherapy with optimal tumor control and normal tissue protection. In this context, the purinergic CD73/adenosine system recently moved into the focus of research as it is an important endogenous regulator of the innate and adaptive immune systems with a documented role in tumor immune escape but also in adverse late effects of radiotherapy (36, 38, 131, 135–138).

We therefore hypothesized that the purinergic system might offer novel opportunities to interfere with normal tissue and tumor responses to radiotherapy and radiation-induced immune deviation. Extracellular ATP is a danger signal released by dying and damaged cells, and belongs to the earlier mentioned DAMPs, that function as immunostimulatory pro-inflammatory signals (139). In contrast, extracellular adenosine mostly exerts anti-inflammatory, immunosuppressive or regulatory functions and is a critical mediator for the maintenance of tissue homeostasis in various tissues including the lung and to avoid overwhelming inflammation for example in response to infection (140–143). But balancing pro-inflammatory ATP and anti-inflammatory adenosine might also to be crucial for maintaining or re-establishing immune homeostasis and to orchestrate tissue inflammation and repair under conditions of damage-induced sterile inflammation (73, 144).

CD73 and Adenosine Have Physiological Roles in Maintaining and Restoring Tissue Homeostasis

The purinergic signaling pathway is an evolutionary conserved mechanism that regulates immune homeostasis by the conversion of extracellular ATP to extracellular adenosine by using the sequential degradation via the ectoenzymes ectoapyrase (CD39, ectonucleoside triphosphate diphosphohydrolase 1) and CD73. Adenosine is either released from stressed or injured cells, or generated from extracellular adenine nucleotides by the concerted action of CD39 and CD73. While CD39 catalyzes the breakdown of ATP and ADP to AMP, CD73 converts AMP to adenosine. But the action of CD39 in degrading ATP can alternatively be executed by ectonucleotide pyrophosphatase (ENPP1, phosphodiesterase 1) (145).

CD39 and CD73 are expressed on the surface of specific lymphocyte subpopulations such as T_{reg} and regulatory B cells (B_{reg}) and endothelial cells and are important to their regulatory functions (143, 146–149). But CD73 is also expressed on stromal cells, mesenchymal stem cells (MSCs), and tumor-associated stem cells (150–153). Pre-clinical studies demonstrated that CD73 on stromal cells and tumor cells participates in the suppression of immune-mediated responses (152) as well as in homing and stemness of cancer stem cells (151, 154, 155). Furthermore, CD73 on MSCs promoted their immunosuppressive function and MSC were even able to upregulate CD73 expression on T cells (150). Inhibition of CD73 in a pre-clinical model of pancreatic neuroendocrine tumors led to reduced tumor growth and metastatic potential of cancer stem cells (151). Thus, stem cell-mediated immunosuppressive or regenerative processes might help cancer cells to escape natural anti-tumor immune responses, anti-cancer immunotherapies, or both. **Table 1** shows detailed information on the expression of CD73 on multiple cell types in various tissues and their reported prognostic findings. Adenosine suppresses inflammatory functions of cells from innate and adaptive immune system and triggers expansion or differentiation of myeloid-derived suppressor cells (MDSC), M2-like macrophages as well as T_{reg} and B_{reg} and thereby participates in the creation of regulatory environments (144, 146, 149, 180–184). In addition, CD39/CD73-dependent generation of adenosine may also affect other processes in T-cell biology such as naive T-cell homeostasis, memory cell survival, and potentially T cell differentiation (168).

Extracellular adenosine can either be removed by enzymatic inactivation or cellular uptake or exert its actions through

TABLE 1 | CD73 expression on various cell types and tissues and its prognostic finding.

Cell type	Tissue or cell type	Origin	Reported prognostic finding	References
Monocyte	Peripheral blood post-infarcted myocardium	Human Swine	Mesenchymal Stem Cells Induce Expression of CD73 in Human Monocytes *in vitro* and in a Swine Model of myocardial Infarction *in vivo*. Positive ADO loop leads to attenuation of inflammation and promotes the regeneration of the damaged myocardial tissue	(156)
	Monocytes in the inflamed joint	Murine	CD73 expression is associated with the suppression of inflammation in rheumatoid arthritis	(157)
Neutrophil	Neutrophils in the inflamed joint	Murine	CD73 expression is associated with the suppression of inflammation in rheumatoid arthritis	(157)
Dendritic cell	Skin	Murine	Production of Extracellular Adenosine by $CD73^+$ Dendritic Cells Is Crucial for Induction of T cell Anergy and Tolerance in Contact Hypersensitivity Reactions	(158)
MDSC	MDSCs generated from mouse hematopoietic progenitor cells (*in vitro*)	Murine	Generation of ADO by CD73 may promote MDSC expansion and facilitate their immunosuppressive activity	(159)
	Peripheral Blood from advanced melanoma patients	Human	High baseline levels of CD73 on MDSCs negatively correlate with Overall Survival and Progression Free Survival	(160)
Macrophage	Alveolar macrophages	Murine	CD73 expression in the lung tissue contributes to radiation-induced lung fibrosis	(138)
	Peritoneal macrophages	Murine	CD73 regulates anti-inflammatory signaling between apoptotic cells and endotoxin-conditioned tissue macrophages and is required to limit neutrophil influx in a peritonitis model	(161)
NK cells	Peripheral blood 2–5% $CD73^+$ NK cells	Human	ADO induces T cell suppression	(162–164)
	Upregulation of CD73 upon exposure to MSC (*in vitro*)	Human	$CD73^+$ NK cells have the potential to regulate NK cell activation in an autocrine or paracrine manner	(165)
B cells	Subpopulations of murine memory B cells, germinal center B cells	Murine	ADO signaling is prominent in the mature germinal center and required for establishment of the long-lived plasma cell compartment	(166)
	Peripheral blood and tonsil	Human	Dependence of Immunoglobulin Class Switch Recombination in B Cells on Vesicular Release of ATP and CD73 Ectonucleotidase Activity Common variable immunodeficiency (CVID) patients with impaired class-switched antibody responses are selectively deficient in CD73	(167)
	Colon B cells	Murine	B cell CD73/CD39/adenosine mediates immunosuppression in DSS-induced colitis	(149)
T cells	Th1, Th2, Th17, Treg in normal, and tumor tissues	Murine human	CD73 may favor cell homeostasis, memory survival, and differentiation	(168–170)
		Human	$CD73^+$ T cells infiltrate into breast and ovarian tumor tissue	(165)
		Human murine	ADO induces immunosuppression	(146, 171–174)
		Murine	CD73 expression on extracellular vesicles derived from Treg contributes to their regulatory function	(175)
		Murine	CD73 expression in the lung tissue contributes to radiation-induced lung fibrosis	(138)
Fibroblasts	Cancer-associated fibroblasts in High-grade serous ovarian cancer (HGSC)	Human	High CD73 expression on CAFs is associated with worse prognosis	(176)
	Cancer-associated fibroblasts in bladder cancer	Human	High CD73 expression on CAFs is associated with worse prognosis	(177)
Epithelial cells	Retinal pigment epithelial cells	Murine	CD73 expression is associated with the suppression of conventional CD4 cell proliferation	(178)

(Continued)

TABLE 1 | Continued

Cell type	Tissue or cell type	Species	Reported prognostic finding	References
	Renal epithelial cells	Murine	CD73 expression on proximal tubular epithelial cells Is critical in renal ischemia-reperfusion injury protection	(179)
Endothelial cells	Bladder cancer	Human	High CD73 expression is associated with better survival in non-muscle-invasive BC (NMIBC) and muscle-invasive BC (MIBC) tumors	(177)
Mesenchymal stem cells (MSC)	Experimental autoimmune uveitis (EAU)	Murine	Inhibition of T-cell proliferation	(150)
Stem cells (Hematopoitic stem cells; cancer stem cells)		Murine human	CD73/Ado induce stemness, homing	(151, 154)

receptor binding. Adenosine deaminase (ADA) is responsible for the conversion of adenosine to inosine, a process that can happen either extracellularly or intracellularly (144). Adenosine may also be transported into its target cells via four different adenosine transporters, the so-called equilibrative nucleoside transporters (ENT) 1-4. Instead, adenosine exerts its actions by binding to one of four different G-protein-coupled adenosine receptors (ADORA1, ADORA2A, ADORA2B, and ADORA3) that are widely expressed on immune cells and resident tissue cells (185). ADORA1 and ADORA2A are high-affinity receptors responding to low concentrations of extracellular adenosine, while ADORA2B and ADORA3 are low affinity receptors and are mainly activated if the extracellular adenosine concentration rises above physiological levels (186). The adenosine receptors have various biological functions aimed at maintaining or restoring tissue homeostasis by triggering context-dependent pro- or anti-inflammatory effects (187–189).

Role of CD73 and Adenosine in Radiation-Induced Adverse Late Effects in the Lung

There is evidence from pre-clinical studies in models of injury-induced sterile inflammation that an acute CD73-dependent increase in adenosine mostly exerts tissue protective functions (142, 181, 190, 191). Herein, the role of purinergic signaling to self-terminate TLR-responses in macrophages might contribute to the observed effects (187, 192).

In contrast, chronically increased adenosine-levels induced for example by genetic deficiency of the adenosine-degrading enzyme ADA or chronic treatment with the chemotherapeutic drug Bleomycin (BLM) can promote pathologic remodeling processes in various tissues leading to fibrosis development (136, 193–200). The pathologic effects of BLM-induced chronic adenosine-accumulation in the lung have been attributed to alternatively activated myeloid cells (201, 202).

So far the role of purinergic signaling for radiation-induced adverse late effects in the lung has only been addressed in own investigations (131, 138) while others investigated its role the skin (136). Our work demonstrated a pathologic role of chronically increased CD73/adenosine signaling in irradiated lungs of C57BL/6 mice, presumably by promoting or amplifying profibrotic signaling cascades. Pathologic signaling involved a time-dependent increase in the expression and activity of the CD73 in the lung tissue that could be confined to resident $CD45^-$ cells as well as $CD45^+$ immune cells ($CD4^+$ T cells including T_{reg}, alveolar macrophages) and was associated with a progressive increase in adenosine levels in the bronchioalveolar lavage fluid C57BL/6 mice with a knockout of CD73 ($CD73^{-/-}$) failed to accumulate high levels of adenosine in response to WTI resulting in decreased levels of fibrosis-associated proteins and mediators, reduced recruitment/formation of T_{reg}, and attenuated pulmonary fibrosis with absence of clusters with alternatively activated macrophages. A similar protective effect was obtained by treatment of irradiated C57BL/6 mice either with pegylated ADA (PEG-ADA) to catabolize adenosine, or with the CD73 monoclonal antibody (mAb) TY/23 as of week 16 post-irradiation (131, 138).

Taken together, the progressive up-regulation of CD73/adenosine signaling in the irradiated lung environment promotes the accumulation of immunosuppressive cell types of the innate and adaptive immune system, e.g., T_{reg} and M2-like macrophages and supports a pro-fibrotic cross-talk between damaged resident cells and infiltrating immune cells. Thereby, CD73/adenosine signaling helps to amplify radiation-induced lung fibrosis as a late normal tissue complication (**Figure 1**). In support of our findings, adenosine also promoted radiation-induced skin fibrosis; but here the pro-fibrotic effects had mainly been attributed to T-cell infiltrates and signaling via ADORA2A, without a role for alternatively activated macrophages (136).

Although radiation-induced intestinal injury models exist, the role of CD73/adenosine has only been studied so far in acute inflammatory disease models where CD73/adenosine executes tissue protective functions (203–208). Moreover, while CD73/adenosine had protective effects in acute renal disease models, chronic kidney injury in patients and murine studies was again linked to up-regulation of CD73 and ADORA2B (179, 198, 209, 210).

In summary, these studies point to disease-promoting effects of chronic CD73/adenosine signaling with tissue-specific and damage-specific mediators and immune changes.

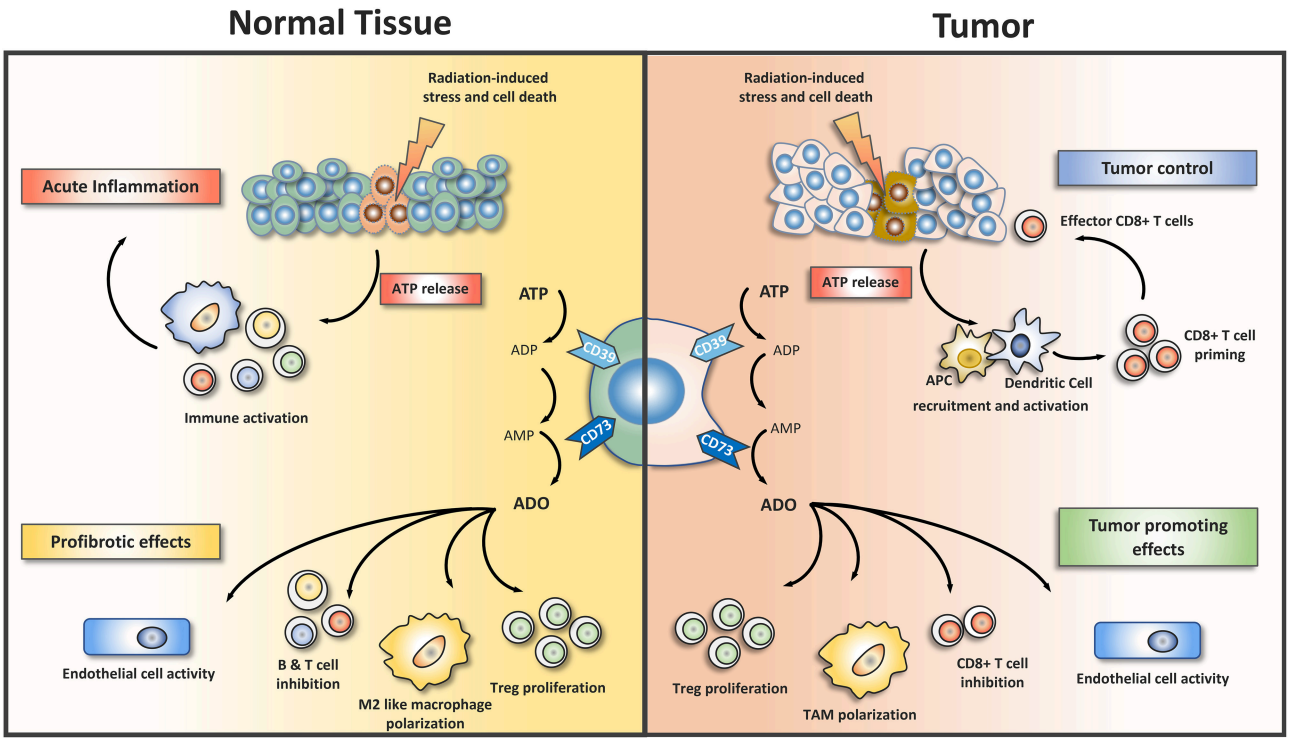

FIGURE 1 | Purinergic signaling shapes the microenvironment in irradiated normal and tumor tissues. Exposure of normal tissues to ionizing radiation induces damage to tissue resident cells, e.g., endothelial cells and epithelial lung cells, as well as in resident immune cells. Equally exposure of tumor tissue results in radiation-induced damage to tumor cells and stromal cells. The resulting cell damage initiates stress responses and/or cell death with subsequent release of damage associated molecular patterns (DAMP). Release of ATP from dying cells is one component of radiation-induced tissue damage. Extracellular ATP acts as a potent inflammatory mediator that promotes inflammation and subsequent further damage to normal tissues. In tumor tissues extracellular ATP is an important mediator of anti-tumor $CD8^+$ T cell responses as it participates in activation of dendritic antigen presenting cells (APC). To avoid excessive inflammation in normal tissues pro-inflammatory ATP is rapidly removed from the extracellular room by a two-step enzymatic conversion into adenosine, involving CD39 (or alternatively ectonucleotide pyrophosphatase) and CD73. Extracellular adenosine is an important endogenous regulator of inflammatory and repair processes as well as vascular functions. Adenosine exerts its pleiotropic actions in a tissue- and context-dependent manner through 4 different adenosine receptors that are expressed on various resident cells and immune cells (not shown). The immunosuppressive actions of adenosine involve the polarization of recruited immune cells toward regulatory or alternatively activated phenotypes, e.g., regulatory T cells (T_{reg}), or M2-like macrophages. Moreover, adenosine mediates the inhibitory action of T_{reg} and other regulatory cell types on proliferation and activation of cytotoxic T cells. By regulating endothelial cell activity CD73 and adenosine impact not only endothelial cell proliferation/angiogenesis but also vascular barrier function and the transmigration of leukocytes into damaged tissues. The expression of CD39 and CD73 thus balances the levels of pro-inflammatory ATP and immunosuppressive adenosine in normal tissues and tumors. The chronic activation of adenosine-driven processes observed in irradiated normal tissues promotes pathologic tissue remodeling and fibrosis development. Tumors coopt the CD73/adenosine system as a mechanism for promoting tumor growth and progression, angiogenesis, and immune escape. ADO, adenosine; CD39, ectonucleoside triphosphate diphosphohydrolase 1; CD73, 5′ ectonucleotidase; TAM, tumor associated macrophages.

Role of CD73 and Adenosine in the Control of Tumor Growth and Response to Therapy

Analyses of patient biopsies have shown that immune cell infiltrates in human tumors exhibit pronounced differences in cell types and numbers, not only intratumorally but also between patients and different tumor entities (211, 212). Interestingly, distribution and type of infiltrating immune cells turned out to have prognostic relevance; for example, the presence of infiltrating T cells was mostly linked to a favorable clinical outcome (213–218). Further pre-clinical and clinical studies showing that tumors can be strongly or poorly immunogenic supported these findings (31, 219). Moreover, the degree of immunogenicity positively correlated with reduced tumor growth and increased survival of tumor-bearing mice in response to immunotherapy indicating that the immune status can be seen as a predictive factor for therapy outcome (220).

High numbers of tumor-infiltrating cytotoxic lymphocytes were also predictive for the response of head and neck cancer patients to treatments involving radiotherapy whereas relapse after chemoradiotherapy and early recurrence correlated to infiltration with myeloid cells (217, 221–224). Local or systemic increases in T_{reg}, high numbers of tumor associated macrophages, or recruitment of $CD11b^+$ myeloid cells have also been associated with poor tumor response to radiotherapy and tumor relapse in pre-clinical models (88, 225). As a proof of concept for the synergistic interaction of radiotherapy and immunotherapy it has been shown that the combination with cancer vaccines, immune checkpoint blockade or inhibition

of CD11b$^+$ cell recruitment can improve the outcome of radiotherapy (52, 62, 89, 226–228).

Of note, tumors coopt the activities of the purinergic CD39/CD73/adenosine system to shape the immune landscape in the tumor microenvironment at multiple levels (**Figure 1**): For example, tumor cells and tumor-associated T_{reg} use CD73-dependent adenosine generation to dampen intratumoral immune responses, particularly in hypoxic tumors (229, 230). The re-direction of the immune response involved suppression of T cell effector functions through CD73-dependent production of extracellular adenosine by CD39$^+$/CD73$^+$ T_{reg} and signaling via stimulation of the ADORA2A on effector T cells (229). Adenosine and ADORA2A thus participate in shaping an immunosuppressive tumor microenvironment by negatively regulating CD8$^+$ T cells (231–233). An adenosine-dependent suppression of immunosurveillance via IFN-γ, NK cells, and CD8$^+$ T cells had also been demonstrated in other pre-clinical models (35, 162). Finally, the creation of an immunosuppressive tumor microenvironment involved the expansion of immunosuppressive myeloid cells, e.g., myeloid-derived suppressor cells, M2-like macrophages, and potentially N2-like neutrophils (234–236). More details about the various effects of CD73 and adenosine on cells from the innate and adaptive immune systems in the tumor microenvironment and the involved ADOR receptors can be found in the following reviews: (137, 143).

In addition, the CD73/adenosine system also supports tumor growth-promoting neovascularization, tumor metastasis, and chemotherapy resistance though part of these actions could also be attributed to the CD73/adenosine-induced modulation of immune cell types in the tumor microenvironment (36, 143, 229, 237–244).

For example, CD73$^{-/-}$ mice were strongly resistant to growth of subcutaneous MC38-ova colon and EG7 lymphoma tumors as well as carcinogen-induced or *de novo* growth of endogenous prostate tumors in transgenic TRAMP mice (162, 245, 246). These interesting observations pointed to a role of CD73$^+$ host cells in tumor growth. However, CD73$^{-/-}$ mice were less resistant to growth of AT-3 mammary and B16F10 melanoma tumors revealing that the effect of host CD73 on the growth of experimental tumors also depends on the tumor type (245, 246). Of note, treatment with an anti-CD73 mAb reduced the growth of experimental 4T1.2 and E0771 breast tumors in wild-type mice, but not in severe combined immunodeficient (SCID) mice, suggesting a role of the adaptive immune system (245, 246). Anti-CD73 treatment also inhibited growth of carcinogen-induced fibrosarcoma tumors and of transgenic prostate tumors in transgenic TRAMP mice (162). The authors could further attribute the efficient tumor rejection to the action of CD8$^+$ T cells whereas CD4$^+$ T cells and NK cells were not involved (162, 246). These data highlight immunosuppressive CD73$^+$ T_{reg} as an important component of the tumor growth-promoting effects of CD73 and adenosine (162, 246).

Interestingly, CD73$^{-/-}$ mice also developed less lung metastases after intravenous injection of B16F10 or TRAMP-C1 cells (162, 246) suggesting that host CD73 also supports metastasis. In line with these observations treatment with an anti-CD73 mAb (TY/23) strongly reduced the lung metastases after injection of 4T1.2 or TRAMP-C1 tumor cells (162, 245). However, the suppression of metastasis formation was observed in both, immunocompetent and in SCID mice, and turned out to be independent of CD8$^+$ T cells and NK cells (162, 245). Thereby the authors revealed a role of CD73$^+$ non-hematopoietic host cells in metastasis formation, potentially endothelial cells, they could further link the pro-metastatic effect to signaling of tumor-derived extracellular adenosine via ADORA2B activation, at least in the 4T1.2 model (245, 246).

In further studies, tumor-derived adenosine attracted myeloid cells and promoted their differentiation into adenosine-generating tumor-associated macrophages (TAM) to amplify adenosine-dependent tumor-immune escape (247). In support of these findings, *in vitro* exposure to adenosine promoted alternative activation of macrophages and enhanced the immunosuppressive responses of macrophages to danger signals, particularly if stimulated in the presence of TLR ligands (141, 187). Interestingly, tumor-derived CD73-dependent adenosine promoted growth, neovascularization, and metastasis of subcutaneous B16F10 melanoma tumors and this was linked to infiltration and polarization of macrophages: genetic or pharmacologic inhibition of CD73 on the B16F10 melanoma cells significantly reduced the number of tumor-infiltrating macrophages recruited to subcutaneous B16F10 melanoma tumors on CD73$^{-/-}$ mice when compared to untreated B16F10 wildtype tumors on CD73$^{-/-}$ mice. Cytokine measurements in CD73$^+$ B16F10 wildtype tumor lysates grown on CD73$^{-/-}$ mice revealed a down-regulation of pro-inflammatory cytokines [Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN-γ] and enhanced expression of anti-inflammatory/pro-angiogenic cytokines (IL-4, IL-10, IL-13, M-CSF) (248). Although the number of infiltrating macrophages did not change in CD73$^+$ B16F10 WT tumors on CD73$^{-/-}$ mice, less MMR$^+$ macrophages were found inside the tumor. Only a pharmacological CD73 inhibition or knockdown of CD73 in the tumor host reduced the amount of infiltrating macrophages (248, 249). The results indicate a role for CD73 in activation and polarization of macrophages that promote tumor progression. Furthermore, it was shown, that the recruitment and activation of tumor-infiltrating macrophages was dependent on ADORA1, ADORA2A, and ADORA3 (250).

Taken together, CD73-dependent adenosine from host cells and tumor cells participates in the support of tumor growth amongst others by promoting tumor immune escape whereas loss of CD73/adenosine signaling enhances tumor immunity. As nicely summarized in a recent review from Allard et al. CD73/adenosine has become an attractive therapeutic target in (immuno)-oncology (38). Several early-phase clinical trials currently evaluate the therapeutic potential of CD73/adenosine inhibitors to inhibit tumor growth and increase tumor immunity. Besides the direct inhibition of CD73 the identification of the respective ADOR involved in promoting tumor immune escape will offer additional opportunities for therapeutic intervention (38, 251, 252).

Intriguingly, co-inhibition of adenosine signaling via CD73 and ADORA2A achieved better anti-tumor immune

responses compared to single treatments, at least in pre-clinical models of breast and colon cancer (253). These effects were associated with improved immune cell infiltration, DC-priming and $CD8^+$ T cell expansion. In line with these findings, Young et al. also observed increased tumor growth delay in CD73/ADORA2A double knockout mice (254). Furthermore, investigations with the human monoclonal anti-CD73 antibody MEDI9447 that is currently in Phase I clinical trials, showed high efficacy in inhibiting CD73 *in vitro* and potent inhibition of pre-clinical syngeneic tumor models *in vivo* as well as additive activity in combination with immune checkpoint inhibitors. Interestingly, MEDI9447 efficiently modulated the tumor microenvironment with significant alterations in the number of both, $CD8^+$ effector T cells and activated macrophages (255).

The immunosuppressive actions of CD73 and adenosine in the microenvironment of established tumors also attract major attention as an interesting target for combined treatment approaches, particularly with immunotherapy. In this context, inhibition of CD73 enhanced efficacy of immunotherapy with α-PD-1 or α-CTLA4 in pre-clinical models (251, 256). The synergistic effect of the combined treatment involved improved T cell effector function as well as reduced CD73 expression on tumor-infiltrating lymphocytes and was dependent on interferon gamma (IFN-γ) and perforin (253, 256). Therapeutic inhibition of ADORA2A was also able to modulate expression of T cell co-inhibitory receptors and to improve effector function for enhanced efficacy of immune checkpoint blockade and adoptive cell therapy in murine cancer models (251, 252).

Since the focus of this review is to highlight the therapeutic potential of CD73 and adenosine inhibition to improve the therapeutic gain in radiotherapy we will not discuss such approaches in more detail here. For further information please refer to reviews discussing the therapeutic potential of the purinergic pathway in immunotherapy in more detail (38, 251, 252).

Instead the therapeutic potential of combining radiotherapy or radioimmunotherapy with CD73/adenosine-inhibition in cancer has been highlighted as an attractive approach but sound data are missing so far (41, 137). Pre-clinical studies are now underway to test such approaches including investigations in our own laboratory (257).

Several pre-clinical studies addressing the role of CD39 in cancer revealed that genetic deficiency of CD39 in mice promotes resistance to metastasis of melanoma and colorectal cancer models (258). Similarly, inhibition of angiogenesis in a CD39-deficient background resulted in reduced growth and pulmonary metastasis of LLC and B16F10 tumors (259). Expression of CD39 was important for angiogenesis and the suppression of NK cell-mediated antitumor activity (260, 261). In line with these findings, overexpression of CD39 enhanced the metastatic potential in pre-clinical models whereas the pharmacological inhibition of CD39 reduced metastasis and enhanced antitumor immunity (261). Of note, clinical data also support a correlation of high CD39 expression with poor prognosis indicating that CD39 might be another promising target for cancer therapy (262–264). But CD39 is much less investigated as a cancer target compared to CD73 underlining the need for further pre-clinical studies.

Taken together various pre-clinical studies highlight the potential of CD39/CD73/adenosine-signaling as promising therapeutic target in immuno-oncology. So far, the observed effects have been associated in multiple studies with activation of T-cell dependent tumor immunity. However, it is important to consider further immunoregulatory actions of CD73 and adenosine in the tumor microenvironment, particularly their influence on the biology of myeloid cells and macrophages, respectively.

Targeting CD73 in Lung Cancer

Only limited data are available so far of the role of CD73 and adenosine in lung cancer. Herein, CD73 was found to be expressed in tumor tissue from NSCLC patients on tumor cells, tumor-promoting mesenchymal stromal cells and myeloid-derived suppressor cells, respectively (265–267). Tumor-derived TGF-β stimulated CD39 and CD73 expression in $CD11b^+CD33^+$ MDSC in tumor tissues and peripheral blood of NSCLC patients, thereby inhibiting activity of T cells and NK cells and protecting tumor cells from the cytotoxic effect of chemotherapy (267).

Moreover, the prognostic value of high CD73 expression for the survival in lung cancer patients remains controversial: Although one study reported a correlation of high CD73 gene expression and improved overall survival of NSCLC patients (268) another study identified high CD73 protein expression as an independent prognostic marker for poor overall survival and shorter recurrence free survival in NSCLC (269). Interestingly, in the same study, high ADORA2A gene expression was an independent predictor of favorable prognosis for overall survival (269). Own *in silico* analyses of publicly available datasets for gene expression of CD73 in lung cancer confirmed the positive correlation between high CD73 gene expression and better overall survival of NSCLC patients. Of note, if radiotherapy-treated patients were excluded from the analysis the correlation to an improved overall survival was abrogated. In addition, the *in silico* analyses revealed poorer overall survival in lung cancer patients with high gene expression of ADORA1, ADORA2A, and ADORA2B (**Figure 2**). Again, the results about the prognostic value of ADORA2A using immunohistochemical data revealed opposite results (269, 270). The discrepancy in the above findings may be due to the use of gene expression analyses vs. immunohistochemical data as CD73 expression in tumor samples turned out to be highly heterogenous (269). We speculate that the heterogeneity in CD73 protein expression in distinct tumor areas might be linked to heterogeneous tumor oxygenation and make the acquisition of representative gene expression data challenging. So far, CD39 inhibitors are not yet involved in clinical trials for cancer patients but such studies are underway (259).

Taken together, adenosine released in an inflammatory milieu or generated by the CD39/CD73 axis will impact the

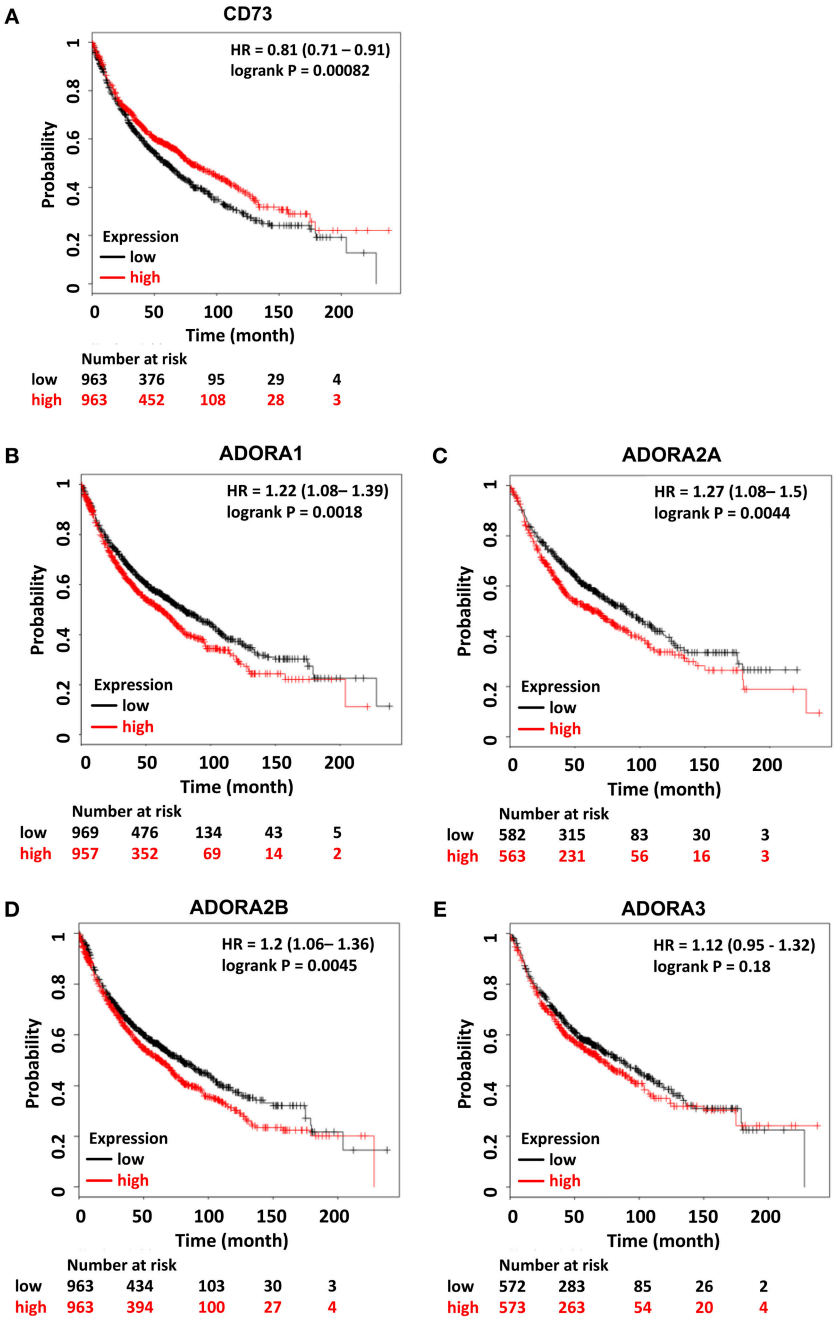

FIGURE 2 | Prognostic relevance of components of the CD73/adenosine signaling system in lung cancer. Kaplan–Meier survival curves relative to **(A)** NT5E, **(B)** ADORA1, **(C)** ADORA2A, **(D)** ADORA2B, and **(E)** ADORA3 expression from publically available datasets for lung cancer. Data were analyzed using the KM-plotter tool (270). Red and black lines indicate patients with higher and lower gene expression, respectively. The total number of patients in the two categories are shown below the graph. Hazard ratios (HR) and *p*-values (log rank p) are shown inside the graph. Patient data is not restricted and includes all datasets.

immune landscape of lung tumors presumably by limiting T cell immunity and promoting immunosuppressive and tumor-promoting lymphoid and myeloid immune cell phenotypes (**Figure 1**). We thus speculate that modulating CD73/adenosine signaling in the lung tumor microenvironment is an attractive strategy to limit tumor progression, improve antitumor immune responses, and avoid escape from therapy in combination with radiotherapy and potentially radioimmunotherapy. On the other hand, the pathologic role of the radiation-induced increase in CD73/adenosine signaling in promoting chronic inflammation and fibrosis in the normal lung tissue strongly suggest that pharmacologic inhibition of CD73/adenosine offers the opportunity for widening the therapeutic window by reducing radiation-induced lung toxicity, particularly in

CD73-rich thoracic tumors with a high risk for CD73-dependent normal tissue toxicity.

Targeting the CD73/adenosine pathway or the involved receptors may thus provide a clear therapeutic gain in the treatment of lung cancer and other CD73/adenosine-rich thorax-associated neoplasms: we expect that inhibition of CD73/adenosine signaling will limit lung toxicity during thoracic irradiation without protecting the tumor or even reinstall anti-tumor immunity when applied during therapeutic irradiation of adenosine-rich tumors with high radioresistance such as NSCLC (138). However, a tight regulation of pro- and anti-inflammatory actions of resident and immune cells is necessary to protect the lung from inflammation-induced loss in its vital function (271–273). For example, immunosuppressive T_{reg} are also to be part of a protective response limiting inflammation-induced collateral normal tissue damage after radiotherapy (44, 274). Therefore, pharmacologic strategies targeting the CD73/adenosine pathway in combination with radiotherapy or combined radioimmunotherapy will require careful validation of potential normal tissue complications. Such complications might include excessive inflammation or autoimmunity by abrogating protective signals mediated by various ADORAs, particularly during acute disease stages. Moreover, the dual effects of acute and chronic CD73 activation as well as spatiotemporal heterogeneity of CD73 and ADOR expression in normal and tumor tissues need to be considered when designing combination treatments for therapeutic intervention.

CURRENT RESEARCH AND FUTURE PERSPECTIVES

So far work from our group identified CD73/adenosine signaling as a novel mechanism promoting RILD through local and systemic actions. Consequences of pathologic CD73/adenosine signaling involved amongst others the accumulation and/or alternative activation of macrophages in organized clusters, their expression of pro-fibrotic mediators, or both. We speculate that the radiation-induced increase in CD73/adenosine is necessary to amplify pro-fibrotic signaling in the irradiated lung environment by fueling the multifaceted cross-talk between damaged resident cells, local and infiltrating immune cells, immunosuppressive T_{reg} and other pro-fibrotic mediators such as hyaluronic acid and TGF-β.

Though immunomodulatory effects of adenosine had been linked to CD73/adenosine-induced adverse effects in other injury models (136, 202) the tissue specific effector and target cells of CD73/adenosine-signaling in response to genotoxic treatment (BLM, radiotherapy) are still controversial and need to be further investigated (73, 200). In this context radiation-induced normal tissue toxicity had also been linked to endothelial cell damage and dysfunction as well as endothelial cell loss as long-term complication (275, 276). As a direct consequence of impaired vascular function, WTI increased numbers of total $CD45^+$ leukocytes, particularly profibrotic $CD11b^+$ myeloid cells and $Ly6C^+$ inflammatory monocytes, in lungs of irradiated mice. However, on the long term, persistence of an activated pro-coagulant endothelial cell type, thickening of the basement membrane, endothelial loss, and collapse of microvessels will contribute to the creation of a hypoxic, pro-inflammatory disease-promoting environment. We assume that the pathologic environment involves a hypoxia-induced up-regulation of CD73 and pathology-associated ADOR on resident cells and immune cells. It is tempting to speculate that therapeutic inhibition of CD73 might also impact adverse late effects in the lung by reducing radiation-induced vascular impairment, but this remains to be determined. Interestingly, further work demonstrates that locally irradiated MSC play a role in the pathogenesis of radiotherapy-induced pulmonary fibrosis by acquiring a pro-fibrotic myofibroblast-like phenotype that promotes extracellular matrix deposition, tissue remodeling, and the development of pulmonary fibrosis upon WTI (276). Since CD73 is expressed on endothelial cells and on MSC of healthy lungs (153) future studies should explore whether the expression of CD73 on the surface of endothelial cells or resident MSC impacts the development of RILD. The same holds true for the expression of CD39 and CD73 on cancer exosomes, which have also been shown to suppress T cells through adenosine production (239).

Adenosine released in an inflammatory milieu or generated by the CD39/CD73 axis impacts the tumor microenvironment and limits tumor immunity at multiple levels. Thus, modulating cancer-derived adenosine in the tumor microenvironment emerges as an attractive strategy to limit tumor progression and improve antitumor immune responses and our own studies suggest that this might be possible without excessively increasing late normal tissue complications (36, 187, 242, 243, 277). Fortunately, multiple approaches for pharmacologic modulation of adenosine levels exist or are being developed and multiple clinical studies have been initiated to evaluate the use of novel inhibitors of CD73 or ADORA2A signaling in cancer therapy alone and in combination with immune checkpoint blockade (38, 39, 143, 188). These studies will give insight into efficacy, compatibility, and potential side effects.

Herein, major attention in oncology has so far been attributed to adenosine signaling via ADORA2A as it is known to effectively dampen immune responses in tumors and normal tissues. However, it has to be taken into account that depending on the tissue of origin and the molecular and immune signature of the tumor, other ADOR may be more important. Moreover, the role of purinergic signaling in the radiation response of malignant tumors and the potential of CD73 or ADOR inhibitors to enhance the efficacy of RT alone and in combination with immunotherapy is still largely unknown. Finally, no reliable biomarkers for the prediction or diagnosis for the individual risk of RILD upon treatment are available to date. Thus, further studies are needed that correlate the gene and protein expression of CD73 and the ADORAs to the outcome after radio(chemo)therapy or immunotherapy. Moreover, as mentioned before, the receptors differ in their affinity for adenosine and extracellular adenosine levels will vary depending on the tissue, the treatment modality and intensity in a spatiotemporal manner. It would therefore be highly beneficial to perform an immunoscore of tissues from pre-clinical studies and test association of high or low expression of CD73 and the ADORAs with the presence of immunosuppressive

lymphoid and myeloid cell subsets, and potentially tissue hypoxia. Such knowledge could later be translated into patient samples. Here, it was an intriguing observation that a high expression of CD73 in normal tissues was indicative for a poor infiltration of prostate tumors with $CD8^+$ T cells whereas high CD73 expression in the tumor stroma was indicative for a longer recurrence-free survival (278). This highlights that CD73 expression in both, normal and tumor tissue should be evaluated.

FINAL REMARKS

Nowadays it is increasingly recognized that strategies for a biology-based optimization and individualization of radiotherapy should include not only the available knowledge about tumor promoting mutations, tumor heterogeneity, tumor cell plasticity, and unfavorable gene expression profiles indicative of the individual radiosensitivity of tumor and normal tissues, but also consider knowledge about the modulation of the radiation response by the immune system and vice-versa. Such a comprehensive view shall allow to harness the combined potential of high precision local radiotherapy, cytotoxic chemotherapy, molecularly targeted small molecule signal transduction inhibitors, and immunotherapy approaches for biologically optimized therapeutic strategies with acceptable safety profile and durable responses in the future (22, 30, 41, 279–284).

The observation that radiotherapy can help to reactivate anti-tumor immunity in immunogenic tumors or increase the potential of immunotherapy has attracted major attention to the use of radiotherapy in combination with various immunotherapies, particularly immune checkpoint blockade immunostimulatory antibodies, and cancer vaccines (24–26, 28–30, 62, 67). However, tumors have evolved effective strategies to escape from immune surveillance and therapy-induced enhancement of tumor immunity is balanced by feed-back inhibition of immune activation in residual tumors, the mobilization of tissue regeneration mechanisms with tumor promoting actions, or both (41, 88, 89, 285–287).

We believe that the identification of mediators driving both, adverse immune changes in irradiated normal tissues and tumor immune escape, will allow us to uncover attractive new therapeutic targets for improving the outcome of radiotherapy. The CD73/adenosine pathway is such a signaling system that regulates adverse immune responses in tumors and normal tissues to microenvironmental stress (e.g., tumor hypoxia) and radiotherapy. So far, CD73/adenosine is mostly considered as a metabolic immune checkpoint that supports immunosuppressive signaling of T_{reg} via ADORA2A. However, there is evidence that CD39, CD73 and adenosine are involved in further immunosuppressive and tumor-promoting signals in the tumor microenvironment beyond modulating T_{reg} function. Intriguingly, radiochemotherapy was also shown to trigger up-regulation of CD73 and CD39 in circulating immune cells of cancer patients (288). This suggests that a radiotherapy-induced systemic upregulation of CD73/adenosine signaling may additionally dampen systemic anti-tumor immune responses during standard fractionated radiotherapy.

Thus, pharmacologic inhibition of CD73/adenosine signaling is an attractive approach to increase the therapeutic ratio in the RT of thoracic tumors with high risk of adverse late effects in the highly radiosensitive normal lung tissue by (i) dampening growth and metastasis of lung tumors, (ii) enhancing the radiation-induced activation of the antitumor immune response, (iii) by restricting the immunosuppressive action of CD39/CD73 on circulating immune cells, and (iv) attenuating adverse late effects in the lung. Moreover, pharmacologic modulation of CD73, adenosine or the four adenosine receptors might offer opportunities to enhance the potential of combined radioimmunotherapy to mount efficient and durable responses with acceptable safety profile.

But the complexity of the tumor-induced and radiation-induced changes in the microenvironment and the multifaceted interactions between damaged resident cells and recruited immune cells outlined above underline the necessity of further work suited to identify strategies that achieve the required balance between pro-immunogenic and immunosuppressive effects of radiotherapy and outweigh the beneficial effects of radioimmunotherapy with optimal tumor control and normal tissue protection. Moreover, further work is required to gain a better mechanistic understanding of the tissue-, injury-, and disease stage-dependent beneficial or adverse effects of CD73/adenosine as well as the identification of involved ADORAs and effector cells for a successful restriction of lung damage during therapeutic lung irradiation by targeting CD73, adenosine or specific ADORAs (73).

Finally, it remains to be determined which approach for targeting the CD73/adenosine axis might be best suited to be used in combination with RT. Above all, the immune effects of RT also depend on physical parameters such as total dose, fractionation schemes (43, 57, 225, 289) and potentially the quality of radiation (290). Thus, attention has also to be given to the best sequence of application, as well as appropriate radiation doses and fractionation-schemes as they may largely impact the effects of radiotherapy on microvessels, immunogenic cell death, immune cell infiltration, the production of immune modulatory mediators, and the activation of CD73/adenosine signaling in both, normal and tumor tissues. Here, the major challenge will be to therapeutically redirect the immune response toward anti-tumor action and avoid tumor recurrence without enhancing collateral normal tissue damage.

AUTHOR 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
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Potential Role of CXCR4 Targeting in the Context of Radiotherapy and Immunotherapy of Cancer

Franziska Eckert[1], Karin Schilbach[2], Lukas Klumpp[1,3], Lilia Bardoscia[1,4], Efe Cumhur Sezgin[1], Matthias Schwab[3,5], Daniel Zips[1] and Stephan M. Huber[1]*

[1] Department of Radiation Oncology, University Hospital Tuebingen, Tuebingen, Germany, [2] Department of General Pediatrics/Pediatric Oncology, University Hospital Tuebingen, Tuebingen, Germany, [3] Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany, [4] Department of Radiation Oncology, University of Brescia, Brescia, Italy, [5] Departments of Clinical Pharmacology, Pharmacy and Biochemistry, University Hospital and University Tuebingen, Tuebingen, Germany

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Viktor Umansky, German Cancer Research Center (DKFZ), Germany

***Correspondence:**
Franziska Eckert
franziska.eckert@med.uni-tuebingen.de

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Cancer immunotherapy has been established as standard of care in different tumor entities. After the first reports on synergistic effects with radiotherapy and the induction of abscopal effects—tumor shrinkage outside the irradiated volume attributed to immunological effects of radiotherapy—several treatment combinations have been evaluated. Different immunotherapy strategies (e.g., immune checkpoint inhibition, vaccination, cytokine based therapies) have been combined with local tumor irradiation in preclinical models. Clinical trials are ongoing in different cancer entities with a broad range of immunotherapeutics and radiation schedules. SDF-1 (CXCL12)/CXCR4 signaling has been described to play a major role in tumor biology, especially in hypoxia adaptation, metastasis and migration. Local tumor irradiation is a known inducer of SDF-1 expression and release. CXCR4 also plays a major role in immunological processes. CXCR4 antagonists have been approved for the use of hematopoietic stem cell mobilization from the bone marrow. In addition, several groups reported an influence of the SDF-1/CXCR4 axis on intratumoral immune cell subsets and anti-tumor immune response. The aim of this review is to merge the knowledge on the role of SDF-1/CXCR4 in tumor biology, radiotherapy and immunotherapy of cancer and in combinatorial approaches.

Keywords: immunotherapy, cancer radiotherapy, CXCR4, SDF-1 (CXCL12), T cells, dendritic cells, NK cells, regulatory T cells

INTRODUCTION

In radiation oncology, chemokine receptor CXCR4 and its ligand SDF-1 (stromal cell derived factor-1, CXCL12) have been described as prognostic factor for head and neck squamous cell carcinoma [e.g., (1)]. Functional data in glioblastoma models point to a role in migration and invasion of cancer cells (2). These and other observations strongly suggest SDF-1/CXCR4 signaling as promising target in anti-cancer therapy, in particular, in combination with radiation therapy (3). However, clinical development of CXCR4 antagonists has mainly focussed on mobilization of hematopoietic stem cells from the bone marrow to peripheral blood (4).

Radiation therapy has proven to elicit both pro-inflammatory, immunostimulatory activities, and anti-inflammatory, immunosuppressive mechanisms. These effects are dependent on radiation dose, tumor biology and the host predisposition (5). As immunotherapy for cancer has been established as standard of care for several cancer entities, such as melanoma (6) and lung cancer (7), the immunologic effects of standard anti-cancer treatment, such as radiation therapy and targeted therapies are of major interest. Radiation-induced immune modulation has been described as direct effects on irradiated tumor cells ("on-target" immunogenic effects) as well as indirect effects in the tumor immune microenvironment ("off-target" effects) (8). Remarkably, recent data also link CXCR4 blockade with antitumor immunity in the tumor immune microenvironment suggesting SDF-1/CXCR4-targeting as a therapeutic tool to interfere with the immune system.

The present article, therefore, aims to give an overview about the plethora of functions of SDF-1/CXCR4 signaling in tumor biology and immune responses in the context of combined radiotherapy and immunotherapy. The knowledge about these functions is indispensable for developing new concepts of anti-cancer therapy that comprise radiotherapy, immunomodulation and SDF-1/CXCR4 targeting.

INTERFERENCE OF IONIZING RADIATION WITH IMMUNE RESPONSES

Radiotherapy effects on cancer had been mostly attributed to direct cytotoxic effects on cancer cells (especially DNA damage) (9). With the advance of cancer immunotherapies preclinical and clinical observations pointed toward synergistic effects. The so-called "abscopal effect" describes responses to radiotherapy (mostly in combination with immunotherapy) outside the irradiated volume and has been linked to immune mechanisms (10). The combination of immune checkpoint inhibitors with local tumor irradiation seems to induce synergistic effects and is currently tested in multiple clinical trials (11, 12). In addition, theoretical rationales and preclinical data provide the basis for also combining radiotherapy with other immunotherapy strategies, such as anticancer-vaccines and cytokine-based therapies (13).

Immune Effects of Tumor Irradiation

The mechanisms of radiation-induced immune effects have been summarized as immune-stimulating and immunosuppressive either directly in tumor cells or in the microenvironment (8). Radiation triggers anti-tumor immune responses directly in the cancer cells by upregulation of MHC-I molecules (14, 15) and possible induction of new tumor associated antigens (14). Cell death mechanisms induced by tumor irradiation lead to "immunogenic cell death" (ICD) (16, 17) characterized by the ability to stimulate the innate immune system and thus indirectly also adaptive immune responses (18, 19). ICD is characterized by the release of danger associated molecular patterns (DAMPs), such as calreticulin (20), high-mobility-group-box 1 (HMGB1) (21) and extracellular adenosine-tri-phosphate (ATP) (22). Additional mechanisms include cytokine release, such as CXCL10 (23), and type-1 interferon (24). Indirect immune stimulation has been attributed to increase and activation of tumor-infiltrating lymphocytes (25, 26), as well as maturation of dendritic cells (DCs) (27). The fact that clinically relevant anti-tumor responses (e.g., abscopal effects after palliative radiotherapy in metastatic cancer patients) are rare despite of these strong immune-stimulating effects is most probably due to simultaneously induced immunosuppression by tumor irradiation. Irradiated tumor cells upregulate immune checkpoint molecules, such as PD-L1 (28, 29) and release immunosuppressive cytokines, such as TGFβ (30, 31). Immunosuppressive cells increased in the tumor immune microenvironment upon local irradiation include regulatory T cells (32, 33) and myeloid-derived suppressor cells (MDSC) (34–36).

Combination Therapies of Irradiation and Immunotherapy for Cancer

These mechanisms have become the basis for combining radiotherapy and immunotherapy in order to exploit pro-immunogenic properties of irradiation and block immunosuppressive actions. Clinical trials ongoing with combinatorial approaches include immune checkpoint inhibition, cytokine based therapies and vaccination strategies (37, 38). Combination of radiotherapy with immune checkpoint inhibition has been recently summarized (39). Besides its use in metastatic cancer patients, durvalumab as adjuvant treatment after definitive radiochemotherapy for non-small cell lung cancer has shown significantly improved disease free survival (40). Vaccination strategies used in combination with radiotherapy include peptide and mRNA based approaches (41–43) which showed promising results in syngeneic mouse xenograft models. IL2 and IL12 as tumor targeted immunocytokines have been tested in combination with tumor irradiation in *in vivo* models showing promising results (44–47).

In conclusion, the strong rationale and promising results led to an increasing use of immunotherapeutics in combination with local tumor irradiation in standard of care treatment of palliative cancer patients as well as in numerous clinical trials with high expectations of the oncological field to improve survival and prognosis of cancer patients.

SDF-1/CXCR4 FUNCTION IN TUMOR BIOLOGY

SDF-1/CXCR4 signaling has been shown to contribute to virtually all processes in tumor biology. As described in this section, SDF-1/CXCR4 signaling reportedly contributes to neoplastic transformation, malignant tumor progression, infiltration, metastasis, angiogenesis and vasculogenesis, and consequently therapy resistance of many different tumor entities.

CXCR4, a Marker of Cancer Stem(-Like) Cells or Tumor-Initiating Cells

CXCR4 chemokine receptors are expressed by hematopoietic stem cells and are required for the trapping of these cells within the stem cell niches of the bone marrow. CXCR4 antagonists, such as AMD3100 (Plerixafor), therefore, can be used to mobilize stem cells into the peripheral blood for hematopoietic stem cell donation (see below). Beyond that, SDF-1/CXCR4 signaling has been shown to be functional in neural progenitor cells and to direct neural cell migration during embryogenesis (48). Notably, CXCR4 expression is further upregulated when neural progenitor cells differentiate into neuronal precursors whereas SDF-1 is upregulated during maturation of neural progenitor cells into astrocytes. While CXCR4 is localized in the cell body of neuronal precursors, expression is primarily restricted to axons and dendrites in mature neurons (49). In addition, SDF-1/CXCR4 signaling has been reported to contribute to chemotaxis and differentiation into oligodendrocytes of engrafted neural stem cells resulting in axonal remyelination in a mouse model of multiple sclerosis (50). Together this suggests that neurogenesis requires functional SDF-1/CXCR4 signaling and CXCR4 as marker of especially the neuronal lineage of neural stem cells.

Primary glioblastoma multiforme (GBM) develops directly by neoplastic transformation of neural stem cells and not by malignant progression from astrocytic gliomas or oligodendroglomas (the latter two are characterized by mutations in the IDH genes). Not unexpectedly, stem(-like) subpopulations of GBM functionally express SDF-1/CXCR4 signaling (51–56). Notably, auto-/paracrine SDF-1/CXCR4 signaling is required for maintenance of stemness and self-renewal capacity (57–59) since SDF-1/CXCR4 targeting leads to loss of stem cell markers and differentiation of stem(-like) cells into "differentiated" tumor bulk.

Besides glioblastoma, SDF-1/CXCR4 signaling has been shown to be functional in stem(-like) subpopulations of retinoblastoma (60), melanoma (61), pancreatic ductal adenocarcinoma (62), non-small cell lung cancer (63), cervical carcinoma (64), prostate cancer (65), head and neck squamous cell carcinoma (66), rhabdomyosarcoma (67, 68), synovial sarcoma (56), and leukemia (69). In summary, these data might hint to an ontogenetically early onset of SDF-1/CXCR4 signaling in mesenchymal and epithelial primordia of the different organs which might be the reason for SDF-1/CXCR4 expression in stem(-like) subpopulations of many different tumor entities.

Transition of stem(-like) cells and differentiated tumor bulk and *vice versa* seems to be highly dynamic and regulated by the reciprocal crosstalk with untransformed stroma cells of the tumor microenvironment (70–72). Beyond that, this crosstalk seems to induce phenotypical changes of cancer stem(-like) cells as deduced from the following observation. Sorted $CD133^+$ stem(-like) cells and $CD133^-$ differentiated bulk cells of GBM did not differ in repair of radiation-induced DNA double strand breaks *in vitro*. Upon orthotopic transplantation in immunocompromized mice, however, $CD133^+$ cells repaired more efficiently than $CD133^-$ cells indicating tumor-microenvironment-mediated upregulation of DNA repair selectively in $CD133^+$ GBM cells (73). The next paragraph introduces the impact of SDF-1/CXCR4 signaling on the crosstalk of tumor cells with non-transformed stroma cells and its function for the cancer stem(-like) cell phenotype.

SDF-1/CXCR4 Signaling in the Crosstalk of Cancer Stem(-Like) Cells With Non-transformed Stroma Cells

The functional significance of SDF-1/CXR4 signaling between tumor cells and the tumor stroma is suggested by a retrospective analysis of genetic SDF-1 variants in patients with colorectal cancer where a certain SDF-1 polymorphism in fibroblasts is associated with higher stromal SDF-1 expression and increased risk for lymph node metastases in stage T3 colorectal cancer (74). Moreover, diffuse-type gastric cancer probably develops from quiescent $Mist1^+$ stem cells upon Kras and APC mutation and loss of E-cadherin. Most importantly, this seems to be dependent on inflammatory processes triggered by SDF-1-expressing endothelial cells and CXR4-expressing gastric innate lymphoid cells that form the perivascular gastric stem cell niche (75).

Likewise, GBM cells co-opt vessels and home to perivascular stem cell niches. Reciprocal signaling between endothelial and GBM cells within these niches has been shown to induce and maintain a stem(-like) cell phenotype of GBM cells on the one hand and to promote angiogenesis on the other [for review see (76)]. Moreover, trans-differentiation of GBM stem-like cells into endothelial cells (77, 78) and pericytes (79) contributes to the adaptation of the tumor microvasculature to the needs of the GBM cells. SDF-1/CXCR4 signaling reportedly exerts pivotal functions in these processes. In particular, CXCR4 on GBM cells and SDF-1 produced by endothelial cells direct perivascular invasion as demonstrated *in vitro* and in orthotopic glioma mouse models (79–81). Accordingly, SDF-1-degradation by the cysteine protease cathepsin K facilitates evasion of GBM cells out of the niches (82). In addition to chemotaxis, CXCR4 stimulation by SDF-1 induces the production of vascular endothelial growth factor (VEGF) in GBM (83) and especially in $CD133^+$ GBM stem-like cells (84). VEGF, in turn, stimulates beyond angiogenesis upregulation of CXCR4 (85) and SDF-1 (86) in microvascular endothelial cells. Moreover, VEGF is required for trans-differentiation of GBM-derived progenitor cells into endothelial cells (77). The significance of targeting VEGF and SDF-1/CXCR4 signaling for stem cell niche formation can be deduced from the observation that targeting of both, VEGF and CXCR4, decreases the number of perivascular GBM cells expressing stem cell markers in an orthotopic glioma mouse model, which was associated with improved survival of the tumor-bearing mice (87).

A further example of up-regulation of a stem(-like) cell phenotype mediated by SDF-1 signaling was reported for breast cancer cells where SDF-1 release from tumor-associated fibroblasts is required for the maintenance of tumor initiation capability (88). Finally, leukemia cells have been demonstrated to be trapped in stem cell niches of the bone marrow (89–91), and follicular lymphoma stem(-like) cells to follicular DCs in the germinal center of lymph nodes (92) by SDF-1/CXCR4

signaling. Combined, these data suggest that SDF-1 directed chemotaxis to certain microenvironmental stem cell niches is a general phenomenon of CXCR4-expressing hematopoietic and non-hematopoietic cancer cells. Of utmost importance, interactions with stromal cells within these niches contribute to a malignant and therapy-resistant phenotype of niche-residing cancer cells as outlined in the next paragraph.

SDF-1/CXCR4 Signaling in Tumor Microenvironment-Induced Therapy Resistance of Cancer Stem(-Like) Cells

Subventricular zones (SVZs) of the brain accommodate neural stem cells and have been shown to attract human GBM stem(-like) cells through SDF-1/CXCR4 signaling in an orthotopic glioma mouse model (93). Importantly, SVZ residence induces radioresistance of GBM stem(-like) cells in direct dependence on SDF-1 release by the SVZ stromal cells (94). Evidence for radioresistance conferred by SDF-1/CXCR4-dependent residency in perivascular niches was further provided by the observation that CXCR4 knockdown in mouse GBM cells resulted in both, diminished perivascular invasion and increased radiosensitivity (81).

Likewise, in mouse models of acute myeloid leukemia CXCR4 antagonism mobilized leukemia cells out of the bone marrow niches and, at the same time, enhanced chemosensitivity (90, 91). Mechanistically, bone marrow mesenchymal cells have been demonstrated to upregulate a signaling complex in the leukemia cells comprising CXCR4 and activating pro-survival signals via extracellular signal-related kinase 1/2 (ERK1/2) and the phosphoinositide 3-kinase (PI3K)/Akt pathway (95). Moreover, bone marrow disseminated *xeno*grafted head and neck squamous cell carcinoma (HNSCC) exhibits a higher cisplatin resistance than lung metastases *ex vivo*. This difference critically depends on TGF-β-triggered SDF-1/CXCR4 signaling (96). In summary, these preclinical *in vivo* and *ex vivo* data strongly suggest that SDF-1/CXCR4-mediated residency of tumor cells in stem cell niches induces resistance to chemo- and/or radiation therapy probably by inducing expression of a therapy-resistant cancer stem(-like) cell phenotype. The maintenance of the latter—as discussed above and impressively demonstrated by the *ex vivo* comparison of bone marrow and lung disseminated HNSCC—itself crucially depends on SDF-1/CXCR4. Beyond cancer stem(-like) cell induction, SDF-1/CXCR4 signaling has been demonstrated to trigger tumor invasion and metastasis as discussed in the next chapter.

SDF-1/CXCR4 Signaling in Triggering Tumor Invasion and Distant Metastasis

Associations between SDF-1/CXCR4 polymorphisms or SDF-1/CXCR4 abundance in tumor specimens and clinical outcome in several but not all studies might suggest a role of SDF-1/CXCR4 signaling in metastatic progression in a variety of tumor entities, such as renal cell carcinoma (97), prostate cancer (98), HNSCC (99–102), esophagogastric cancer (103), colorectal cancer (74), hepatocellular carcinoma (104), or osteosarcoma (105). In preclinical studies, overexpression of CXCR4 has been demonstrated to dramatically increase lung and liver metastases of murine pancreatic cancer in tail vein metastasis assays in nude mice (106). Consistently, antagonizing CXCR4 inhibited lung metastasis of human tongue squamous cell carcinoma (107), esophageal cancer (108), breast cancer (109) in immunocompromized mice. Intra-arterially injected circulating CXCR4-expressing melanoma cells require SDF-1 signaling by mesenchymal stem cells that act as pericytes for extravasation to bone and liver and perivascular niche formation as demonstrated by humanized heterotopic bone formation assay (110). Combined, these examples suggest that CXCR4 expression by cancer cells contribute to their tropism to metastatic niches.

Along those lines, CXCR4 downregulation by overexpression of miR-613 reportedly inhibits lung metastasis of osteosarcoma orthotopically *xeno*grafted in nude mice (105). Notably, epigenetic downregulation of SDF-1 has been demonstrated to boost metastases of CXCR4-expressing sarcoma in mouse models (111). Likewise, a SDF-1 polymorphism with low SDF-1 expression in breast cancer has been proposed to associate with susceptibility to metastases (112). It is, therefore, tempting to speculate that SDF-1^{-}/CXCR4^{+} tumor cells are particularly prone to metastasize. As a matter of fact, high CXCR4 and low SDF-1 expression by the tumor has been associated with poor overall survival in osteosarcoma (111) and metastasis-free survival in head and neck squamous cell carcinoma (113). The latter association, however, was not confirmed by a recent study (114). Nevertheless, these reports strongly suggest a pro-metastatic function of SDF-1/CXCR4 signaling in several cancer entities.

In GBM which usually does not metastasize outside the central nervous system, SDF-1/CXCR4 signaling has been demonstrated *in vitro* to exert pivotal function in cell migration and chemotaxis (115–117). Most probably, SDF-1/CXCR4-dependent migration/chemotaxis does not only contribute to the above discussed homing of GBM cells to protective perivascular stem cell niches (see above) but also to deep infiltration of the brain parenchyma by highly migratory GBM stem(-like) cells. One driver of glioblastoma dissemination might be hypoxia through HIF-1α mediated up-regulation of SDF-1 and CXCR4 in GBM cells (85, 86). Unexpectedly, VEGF- or VEGF-R-targeting has been demonstrated *in vitro* to directly up-regulate CXCR4 expression and chemotaxis toward SDF-1 in VEGF-R-expressing GBM cells in a TGFβ-dependent manner (118). In accordance with these observations, anti-angiogenic therapy of orthotopic mouse glioma promotes GBM invasion by CXCR4 upregulation. Additional CXCR4-targeting blunts this effect (119). Consistently, combined VEGF- or VEGF-R- and CXCR4 antagonism prolongs survival of mice bearing orthotopically *xeno*grafted GBM as compared to only VEGF/VEGF-R-targeted mice (87, 118, 120). Also along those lines, anti-angiogenic therapy, such as Bevacizumab which has been demonstrated in large clinical trials not to improve overall survival of GBM patients is under suspicion to foster distant spread of the tumor at recurrence (121). Even if the tumor spread-promoting effect of Bevacizumab is under debate (122), nevertheless, these data bear witness to a close interaction between tumor hypoxia and

SDF-1/CXCR4 signaling as introduced in more detail in the next chapter.

SDF-1/CXCR4-Signaling and its Function for Vasculogenesis

Hypoxia-induced up-regulation of SDF-1 secretion in tumors reportedly stimulates homing and engraftment of bone marrow-derived myeloid cells, as well as mesenchymal stem cell-derived endothelial and pericyte progenitor cells. This recruitment promotes neovascularization of the hypoxic tumor by transition of the myeloid and progenitor cells into endothelium and pericytes. Such SDF-1/CXCR4-dependent vasculogenesis has been demonstrated in mouse models of several tumor entities, such as GBM (123–127), HNSCC (128), lung adenocarcinoma (129), hepatocellular carcinoma (130) or breast cancer (131). Importantly, irradiation has been shown to induce SDF-1 expression and thus may boost vasculogenesis and tumor re-growth after end of therapy (125, 131–133) suggesting a radioresistance-conferring action of SDF-1/CXCR4 signaling as discussed in the next paragraph.

SDF-1/CXCR4-Signaling and Radioresistance

In many tumor entities radiation therapy is part of standard of care. Ionizing radiation has been demonstrated *in vitro* as well as *in vivo* to stimulate SDF-1/CXCR4 signaling in different human and mouse tumor entities either directly by S-nitrosylation and stabilization of HIF-1α (134) or indirectly via radiation-induced endothelial cell killing and resulting hypoxia (135) or HIF-1α-independent mechanisms (136). Radiation-induced modifications in SDF-1/CXCR4 signaling, in turn, have been reported in gliomas (116, 125, 127, 137), mesotheliomas (138), prostate (139), cervical (140), lung (131, 141) and breast cancer (131). Aside from the direct effect on cancer cells, radiation-induced SDF-1 secretion is also observed in different normal tissues/cells (94, 136, 142–147) or cancer-associated fibroblasts (144).

Importantly, radiation-modulated SDF-1/CXCR4 signaling has been shown to stimulate tumor re-growth (142, 148), EMT (144), migration (116), invasiveness (81, 127, 138, 141, 144) and metastases (138, 145), as well as homing of hematopoietic progenitor cells and accelerated vasculogenesis (125, 127, 131–133, 136, 137, 142). Thus, radiation-induced SDF-1/CXCR4 signaling may foster radioresistance, malignant progression and recurrence of tumors (94, 125, 139, 149–151). Preclinical evidence shows reduced metastases in orthotopic murine models of cervical cancer with Cisplatin-based radiochemotherapy and AMD3100 (152).

Thus, as CXCR4 is a prognostic marker for local control after curative radiotherapy and irradiation interferes with SDF-1/CXCR4 signaling, there is a strong rationale to develop translational and clinical interventional studies combining CXCR4 targeting with curative radio(chemo)therapy. The roles of SDF-1/CXCR4 signaling in tumor biology are summarized in **Table 1**.

SDF-1/CXCR4 Signaling as Druggable Target in Anti-cancer Therapy

As already touched upon, retrospective clinical data might hint to associations between SDF-1 polymorphisms or SDF-1/CXCR4 expression levels with susceptibility to neoplastic transformation, malignant progression or therapy response in a variety of tumor entities, such as renal cell carcinoma (97), prostate cancer (98), HNSCC (1, 100, 102, 113, 114), esophagogastric cancer (103), hepatocellular carcinoma (104), colorectal cancer (74), breast cancer (153), osteosarcoma (111), low grade glioma (154, 155), or GBM (156, 157). Beyond cancer SDF-1 genetics has been associated with e.g., the pathogenesis of multiple sclerosis (158) or prognosis in patients with cardiovascular disease (159).

Apart from genetic variants, SDF-1 as well as CXCR4 were shown to be regulated epigenetically by DNA methylation. DNA methylation status of the genes was suggested as prognostic biomarkers for e.g., breast or pancreatic cancer and GBM (160–162). Such prognostic or predictive value of SDF-1/CXCR4 might be expected from the plethora of SDF-1/CXCR4 functions in tumor biology mentioned above. These functions contribute to malignancy, progression and therapy resistance of the tumors and render SDF-1/CXCR4 signal to an ideal target in anti-cancer therapy. In particular, a combination of SDF-1/CXCR4-targeting and radiotherapy seems to be promising given the above mentioned radioprotective functions of SDF-1/CXCR4 signaling (**Figure 1**). Moreover, combinatorial treatment of conventional chemotherapy with CXCR4 inhibitors might be an approach to overcome cancer therapy resistance (163).

In fact, several SDF-1- or CXCR4-targeting drugs have been applied in preclinical models [e.g., Ulocuplumab (164), ALT-1188 (165), POL5551 (166), PRX177561 (167)], were well-tolerated in clinical trials [e.g., AMD070 (168), Balixafortide (POL6326, Polyphor) (169)] or are FDA-approved [AMD3100, Plerixafor (170)] indicating that SDF-1/CXCR4 targeting is clinically feasible. Overall, Plerixafor used for stem cell mobilization does not induce severe side effects (171, 172). A randomized phase 3 trial comparing G-CSF with plerixafor vs. placebo reported mainly fatigue, gastrointestinal side effects like nausea and diarrhea and injection site reactions (173).

CXCR4, however, is expressed on immune cells suggesting that SDF-1/CXCR4-targeted anti-cancer therapy at the same time interferes with the immune response to cancers and cancer cells in e.g., circulation or micrometastases. In order to explore these functions and develop a rationale if trimodal therapy combining CXCR4 targeting with immunotherapy and radiotherapy might be of benefit, it is crucial to understand the function of SDF-1/CXCR4 signaling in immune cells and the effects of CXCR4 inhibition on the immune response to cancer.

PHYSIOLOGIC ROLE OF CXCR4 IN THE IMMUNE SYSTEM

In addition to its function in tumor biology, SDF-1/CXCR4 signaling controls multiple physiological processes in

TABLE 1 | (Patho)physiological role of SDF1/CXCR4 signaling and targeting in cancer.

Cell type	(Patho)physiological role of SDF-1/CXCR4 signaling	Effects of CXCR4 targeting	References
Cancer cells	VEGF production in GBM		(83)
		Mobilization of leukemia cells from BM, enhanced chemosensitivity	(90, 91)
	Association with decreased patient survival	Decreased metastasis formation	(97–105, 111)
	Cell migration in GBM		(115–117)
		Inhibition of VEGF-mediated migration in GBM, prolonged survival of mice	(119)
	Vasculogenesis		(123–133)
	Radiation-induced EMT		(143)
	Radiation-induced invasiveness		(81, 127, 138, 141, 144)
Cancer stem(like) cells (CSC)	Maintenance of stemness, self renewal capacity		(51–56)
		Loss of stem cell markers, differentiation to "bulk" cells	(57–69)
	VEGF production in GBM CSCs		(84)
	Attraction to subventricular stem cell niches		(93, 94)
Stroma cell/cancer cell crosstalk	SDF-1 in fibroblasts increases lymph node metastases in CRC		(74)
	SDF-1 in endothelial cells contributes to gastric cancer development		(75)
	Perivascular invasion of GBM	Reduction of perivascular GBM cells, increased radiosensitivity	(79–81, 87)
	SDF-1 in fibroblasts required for tumor initiation in BC		(88)

BC, breast cancer; BM, bone marrow; CRC, colorectal cancer; CSC, cancer stem(like) cell; EMT, epithelial mesenchymal transition; GBM, glioblastoma multiforme; VEGF, vascular endothelial growth factor.

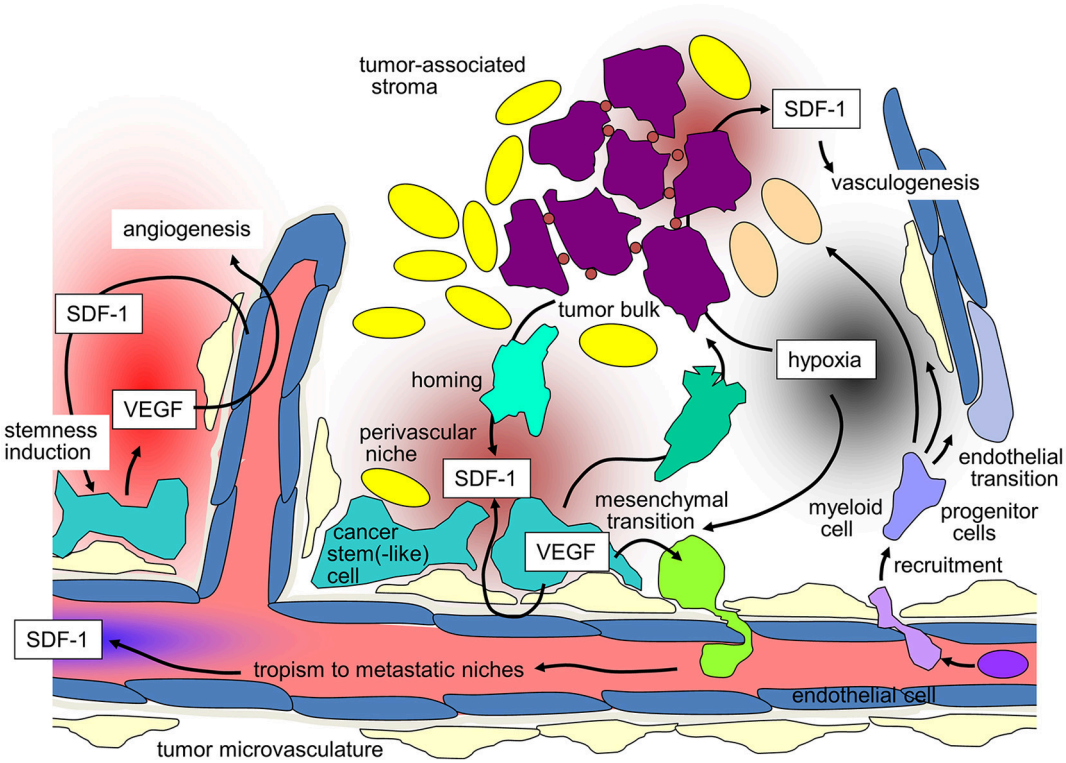

FIGURE 1 | SDF-1/CXCR-4 signaling in tumors and its contribution to maintenance of tumor stemness, recruiting of distant stroma cells, angio- and vasculogenesis, and metastasis (for details see text).

hematopoiesis, T, B and NK cell development and the organization of the immune system. Ablation of either of the components of the SDF-1/CXCR4 axis generates a similar phenotype of deficient B lymphopoiesis and myelopoiesis, disturbed immune responses leading to cancers, autoimmunity and inflammatory diseases (174–176). Recently a 16 amino acid fragment of serum albumin (EPI-X4) was identified as an effective and highly specific endogenous CXCR4 antagonist (177). Peptide EPI-X4 is evolutionarily conserved and generated from the highly abundant albumin precursor by pH-regulated proteases. It antagonizes SDF-1-induced tumor cell migration and suppresses inflammatory responses in mice. In human the peptide is abundant in the urine of patients with inflammatory diseases. EPI-X4 mobilizes stem cells, which explains in part why stem cells can directly respond to inflammation with their migration into the periphery.

Hematopoietic Stem Cell Niche

HSCs (Hematopoietic stem cells) are a rare cell population that can give rise to all lineages of the immune system. HSCs reside in the undifferentiated state in the bone marrow, where the binding of their CXCR4 receptor to its ligand SDF-1—constitutively provided by the bone marrow (BM) niche—promotes their survival (178, 179) while negatively regulating their proliferation (180–182). In addition to direct effects on HSCs, SDF-1/CXCR4 signaling also promotes survival and growth of human bone marrow stromal stem cells (183). Inhibiting the interaction between CXCR4 receptor and SDF-1 leads to the migration of hematopoietic stem and progenitor cells (HSPC) into the periphery, a process termed mobilization, which is required for harvesting stem cells for transplantation [either autologous from the patient (184) or in healthy donors (185)]. A dramatic increase in mobilization efficiency and yields of progenitor cells compared to standard G-CSF is achieved when using CXCR4 antagonists, such as AMD3100, Mozobil® (184, 186, 187). CXCR4 antagonist BL-8040 in a recent phase I clinical study (NCT02073019) besides highly efficient mobilization of pluripotent hematopoietic progenitors showed also superior yields of $CD4^+$ and $CD8^+$ T cells, NKT, NK, and DCs, suggesting increased engraftment ability of CXCR4 mobilized populations, a higher anti-tumor effect and faster immune reconstitution potential. Moreover, mobilization as a 1-day procedure is less wearing for the donor and allows faster access to the stem cells (188). Since HSCs maintain hematopoiesis throughout life, qualitative and quantitative effects through prolonged pharmacologic blockade of the SDF-1/CXCR4 axis need to be investigated. Concerns that the HSC pool in the bone marrow would decrease were not confirmed, as CXCR4-blockade led to higher repopulating capacity and exceptional mobilization along with an expansion of the BM HSC pool, which unexpectedly suggests reversible inhibition of the SDF-1/CXCR4 axis also as a novel strategy to restore damaged BM (189). BM HSCs during reversible long-term CXCR4/SDF1 long term blockade increase their cycling activity 2- to 3-fold [only 10–20% of Lin-Sca1+Kit- (LSK) and 30–40% of LSK SLAM cells being quiescent (G0 phase)] compared to 50–60% of LSK and 70% of LSK SLAM under homeostatic conditions (190, 191). Thus, these findings together with mounting evidence for direct cytolytic and specific anti-leukemic effects of CXCR4 inhibition (192–194) suggests prolonged CXCR4 blockade as a novel safe therapeutic scheme for treatment of (hematologic) malignancies either alone or in conjunction with chemotherapy.

Dendritic Cells

The priming of naïve T cells is dependent on efficient antigen presentation and a strong costimulatory signal both provided by dendritic cells during Th1 polarized immune responses. Th1 polarization is thought to be critical for immune rejection of tumors, while activated T cells polarized to Th2 cytokine and cell profiles might induce even tumor immune evasion (195). Plasmacytoid DCs (pDCs) as type I interferon (IFN)-producing cells play a central role in antiviral and anti-tumor immunity. pDCs are produced from hematopoietic stem cells in the bone marrow where they nestle down with reticular cells in the intersinal space which abundantly provides SDF-1. Concordantly, the number of pDCs and their earliest progenitors is severely reduced in the absence of CXCR4 *in vitro* and *in vivo*, underlining the function of SDF-1/CXCR4 axis as a key regulator of pDC development and the importance of provision of SDF-1 by cellular niches (196, 197). Upon activation, CXCR4 expressing DCs migrate into SDF-1 expressing lymphatic vessels where they initiate immune responses, a process that is severely blocked by systemic CXCR4 antagonist application (198). Since the dysregulated expression of SDF-1/CXCR4 is associated with the pathology of various autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis, targeting SDF-1/CXCR4 axis with 4-F-Benzoyl-TN14003 may be beneficial for prevention of autoimmune disease (198–201). It is not clear, whether these effects on DCs might decrease the efficacy of anti-cancer immune responses upon CXCR4 inhibition.

Myeloid Derived Suppressor Cells

As reviewed in (202), MDSCs are highly immunosuppressive cells in the tumor microenvironment and mainly suppress intratumoral T cells. SDF-1 secreted by tumor associated fibroblasts induces MDSCs and impairs anti-tumor immune responses as shown in a hepatic carcinoma model (203). Another liver cancer model (metastases of colorectal carcinoma) showed less MDSC infiltrates in the metastases after treatment with AMD3100, accompanied by reduced metastases growth (204). Patient samples of ascites also showed that CXCR4 signaling is involved in MDSC recruitment. SDF-1 release of cancer cells as well as CXCR4 signaling in MDSCs could be abrogated by COX2 inhibition.

Regulatory T Cells (nTreg and iTreg)

Regulatory T cells (Tregs) constitute 5–10% of peripheral $CD4^+$ T cells in humans (205, 206). Tregs maintain immune homeostasis, peripheral tolerance and prevent autoimmunity by suppressing and terminating immune responses. They constitute a major barrier for an effective antitumor immunity, and the number of peripheral and intratumoral Treg cells is an independent prognostic factor in malignancies (207). Cancer

cell- and M2 macrophages derived SDF-1 attracts Treg cells into the tumor lesion where they robustly induce FOXP3 and other Treg signature molecules in human naïve $CD4^+$ T cells which display enhanced FOXP3 stability and low expression of pro-inflammatory cytokines (208). Treg cells limit immune effector cell function via cytokines (209–212), direct lysis (213), inhibitory receptors on their cell surface (214–217), via metabolic disruption (218), by starving effector cells via depletion of local IL-2 (219) or indirectly by turning secondary cell types into suppressive ones i.e., IDO (220) and tolerogenic cytokine producing DCs with low costimulatory potential (221). Treg depletion dramatically reduced tumor growth or induced full remission (222–224). In contrast to conventional chemotherapeutic agents which also deplete T effector cells and may induce autoimmunity due to the systemic elimination of T-regs (225), CXCR4 targeting allows the specific targeting of Tregs, since intratumoral Tregs consistently express higher CXCR4 levels than $CD4^+CD25^-$ and $CD8^+$ cells (226). In intraperitoneal papillary epithelial ovarian cancer, CXCR4 antagonism increased tumor cell apoptosis and necrosis, reduced intraperitoneal dissemination, and selectively reduced intratumoral FoxP3 Tregs (226). Superior immune responses as shown for CXCR4 antagonist BL-8040 is not solely owing to a selectively reduced recruitment of Treg cells into the tumor, and an increase in the number of immune and progenitor cells (227). CXCR4 antagonists have shown to also reverse Tregs' suppressive activity. Plerixafor and the antagonistic CXCR4 peptide R29 (228) inhibited Treg-suppressive activity significantly (by 10-fold) in Tregs from primary renal cancer specimens in which high numbers of activated Tregs (expressing CTLA-4/CXCR-4/PD-1/ICOS) were detected. A possible mechanistic explanation involves the demethylation of Treg-FOXP3 promoter (229). Thus, inhibition of Tregs by blocking SDF-1/CXCR4 is one of the major rationales for a better anti-tumor immune response via CXCR4 inhibition.

Effector Cells

T effector (Teff) cells also constitutively express the chemokine receptor CXCR4. Besides T cell migration along SDF-1 gradients, CXCR4 after T cell receptor crosslinking is recruited to and accumulates at the immunological synapse, resulting in stronger T cell-APC interaction, shutdown of T cell responsiveness to chemotactic gradients, and in higher levels of T cell proliferation and IFN-γ production (230). *Vice versa*, the presence of competing external chemokine signals has been shown to disrupt the stability of T-APC conjugates as a result of impaired recruitment of the receptor to the immunologic synapse (231). CXCR4 confers the homing of antiviral T cell responses to bone marrow. Ablation of CXCR4 thus impairs memory cell maintenance due to defective homeostatic proliferation in the bone marrow niche, yet allows fully functional asymmetric cell fates after antigenic rechallenge in $CD8^+$ T cells (232). Antitumoral activity was shown for CXCR4 antagonist BL-8040 in tumor bearing mice, where it induced robust mobilization of $CD4^+$ and $CD8^+$ T lymphocytes and DC in numbers that were significantly higher compared to tumor free naïve counterparts. The authors did not discriminate the lymphocytic population with respect of Teff/Treg ratio or $CD8^+$ content though (233), yet showed in pre-clinical *in vivo* pancreatic cancer models, immune cells mobilized from the bone marrow into the circulation accumulate within the tumor lesion where they inhibit tumor growth. Such a dramatic effect on the

TABLE 2 | (Patho)physiological role of SDF1/CXCR4 signaling and targeting in immune processes.

Cell type	(Patho)physiological role of SDF-1/CXCR4 signaling	Effects of CXCR4 targeting	References
Hematopoietic stem cells	Survival in BM		(178, 179)
	Decreased proliferation in BM		(180–182)
	Survival and growth of bone marrow stromal stem cells		(183)
		Mobilization	(184–187)
Dendritic cells	Dendritic cell development		(196, 197)
		Impaired immune response	(198)
Effector T cells	T cell proliferation and IFN-γ production		(230)
		Increased tumor infiltration	(233)
		Increased cytotoxicity	(234, 235)
Natural killer cells		Migration to periphery, maturation	(238)
		Increased NK cell mediated antitumor immunity	(239)
Regulatory T cells (Tregs)	Attraction to tumor lesions		(208)
		Reduced intratumoral Tregs	(226)
		Reduced Treg suppressive activity	(228)
Myeloid derived suppressor cells (MDSCs)	Induction and hampered immune response	Decreased metastases formation via reduced MDSCs	(203, 204)
	MDSC recruitment to tumors		(203, 204, 240)

APC, antigen presenting cell; BM, bone marrow; MDSC, myeloid derived suppressor cells.

intratumoral T cell compartment function is reflected in a study by Elda Righi (226) where CXCR4 antagonist AMD3100 by favorably modulating the intratumoral Teff/Treg ratio 6-fold, created a phenotype reminiscent of two studies that—although in different contexts—depleted intratumoral T-regs which highly significantly improved cytotoxic T-cell function in the tumor tissue and prolonged survival (234, 235). Along those lines, epigenetic down-regulation of SDF-1 expression in osteosarcoma has been demonstrated to impair cytotoxic T-cell homing to the tumor site (111). In contrast, SDF-1 overexpression by melanoma cells in the B16-ova melanoma model has been shown to chemo-repel antigen-specific cytotoxic T cells (236) suggesting a complex and fine-tuned control of Teff infiltration by SDF-1/CXCR4 signaling.

Chemokines control also the trafficking of developing and mature natural killer cells (NK) in the bone marrow (237). While several CCRs are expressed during progression from precursor to immature and mature NK cells CXCR4 was only detected on immature NK cells. Administration of the CXCR4 antagonist, AMD3100, induced strong reduction of mature NK and immature NK cells in the BM in a murine model and increased their number in blood and spleen, which suggests that this chemokine axis also regulates NK cell subsets localization in the bone marrow niche and their migration to the periphery for their maturation (238). Notably, genetic deletion of CXCR4 in myeloid cells in a melanoma mouse model fostered NK cell-mediated antitumor immunity suggesting indirect suppression of NK cell activity by CXCR4 signaling (239).

In summary, SDF-1/CXCR4 signaling affects most subsets of immune cells, the most prominent and clinically applied effect being the mobilization of HSCs by blocking CXCR4 as summarized in **Table 2**.

Combined Immunotherapy and CXCR4 Targeting

The promotion of antitumor immunity by CXCR4-antagonists was reported for a mouse model of ovarian cancer (226) and in an orthotopic preclinical hepatocellular carcinoma (HCC) model where a CXCR4 antagonist was combined with a checkpoint inhibitor. In this HCC model multi-kinase-inhibitor sorafenib treatment-induced hypoxia fostered SDF-1 production, leading to the recruitment of immunosuppressive tumor-associated macrophages, myeloid-derived suppressive cells, and Tregs all with increased PD-L1 expression. CXCR4 antagonist plerixafor combined with anti-PD-1 therapy showed the most pronounced tumor growth delay, and was associated with increased intratumoral penetration and activation of $CD8^+$ T lymphocytes (241). A novel strategy for the treatment

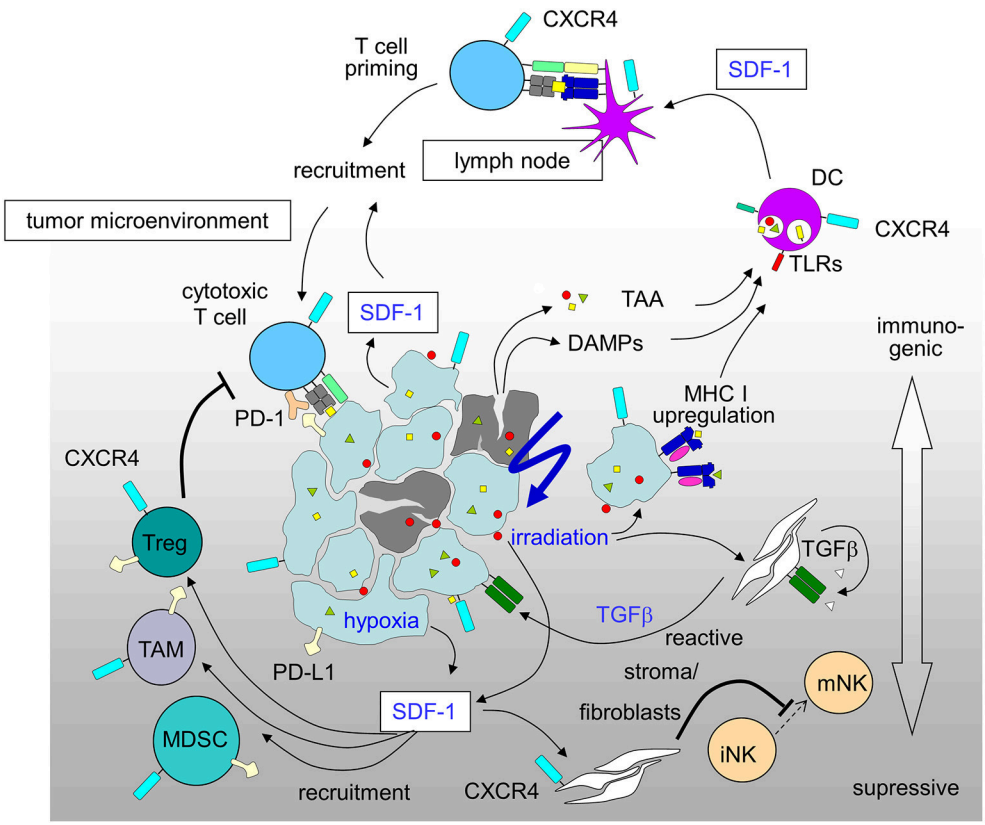

FIGURE 2 | Immunosuppressive and immunostimulatory action of SDF-1/CXCR4 signaling in tumors induced by radiation-therapy and hypoxia (for details see text; DAMPs, danger-associated molecular patterns; DC, dendritic cells; MDSC, myeloid derived suppressor cell; PD-1, programmed cell death protein-1; PD-L1, PD-1 ligand; TAA, tumor-associated antigens; TAM, tumor-associated macrophage; Treg, regulatory T-cell).

of drug-resistant ovarian cancer combines chemotherapy to increase immunogenic cell death and virally delivered CXCR4 to reverse the immunosuppressive tumor microenvironment (242). Ovarian cancer of murine and human ovarian tumor variants resistant to paclitaxel and carboplatin were infected with oncolytic vaccinia virus expressing a CXCR4 antagonist and were +/− treated in combination with doxorubicin. The chemo-resistant variants' augmented expression of CXCR4 was associated with an increased susceptibility to viral infection and doxorubicin-mediated killing compared to parental counterparts *in vitro* and in tumor-challenged mice. Antitumor immune responses in this model culminated in the control of metastatic tumor growth and tumor-free survival. Mechanistically, the authors showed combination treatment increased apoptosis and phagocytosis of tumor material by DCs which efficiently induced adaptive antitumor immunity, reflected by increased intratumoral infiltration of antitumor $CD8^+$ T cells and reduced immunosuppressive Tregs (242). Based on these results (**Figure 2**), the MORPHEUS clinical trials were started including treatment arms combining immune-checkpoint inhibitors with CXCR4 inhibition (NCT03193190, NCT03281369 and NCT03337698 for pancreatic cancer, gastric cancer and non-small cell lung cancer, respectively).

CONCLUDING REMARKS

With combinatorial approaches of radiotherapy and immunotherapy on the rise, it is important to evaluate novel treatment strategies in radiation oncology with respect to tumor and radiation biology as well as immunologic effects. For SDF-1/CXCR4 targeting both perspectives provide a strong rationale for combination therapies. The SDF-1/CXCR4 axis plays pivotal roles in various aspects of tumor biology, and in particular in the stress response of tumors to ionizing radiation. In preclinical *in vivo* models CXCR4 targeting increases the efficacy of radiation therapy and blunts adverse effects, such as radiation-stimulated metastases and vasculogenesis. Mobilization of HSCs, a significant increase of immune and progenitor cells in the periphery that are able to migrate into the tumor and the selective targeting of Treg cells in the tumor lesion provide the rationale for an increased anti-tumor immune response upon CXCR4 inhibition. Preclinical mechanistic studies as well as translational and clinical evaluation of the role of the SDF-1/CXCR4 axis in the context of cancer radiotherapy and immunotherapy might lead to novel treatment strategies implementing SDF-1/CXCR4 targeting in this context using the small molecule inhibitors already approved for the use in patients and healthy donors for HSC mobilization.

AUTHOR CONTRIBUTIONS

FE and SH designed the concept and wrote the manuscript. LK wrote chapter SDF-1/CXCR4-Signaling and Radioresistance. MS wrote chapter SDF-1/CXCR4 Signaling as Druggable Target in Anti-cancer Therapy. LB wrote chapter Interference of Ionizing Radiation With Immune Responses. ES contributed to chapter SDF-1/CXCR4 Function in Tumor Biology. KS wrote chapter Physiologic Role of CXCR4 in the Immune System. DZ and all authors read and approved the manuscript.

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Antitumor Potential of Extracellular Vesicles Released by Genetically Modified Murine Colon Carcinoma Cells With Overexpression of Interleukin-12 and shRNA for TGF-β1

Joanna Rossowska, Natalia Anger, Katarzyna Wegierek, Agnieszka Szczygieł, Jagoda Mierzejewska, Magdalena Milczarek, Bożena Szermer-Olearnik and Elżbieta Pajtasz-Piasecka*

Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland

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Patrik Andersson, Massachusetts General Hospital, Harvard Medical School, United States

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Robin Parihar, Baylor College of Medicine, United States
Roberto Bei, University of Rome Tor Vergata, Italy
Tomer Cooks, Ben-Gurion University of the Negev, Israel

***Correspondence:**
Joanna Rossowska
joanna.rossowska@hirszfeld.pl

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Recent developments demonstrate that tumor-derived extracellular vesicles (EVs) could become a highly effective tool for delivery of antitumor factors. The main objective of the study was to determine whether EVs secreted by MC38 colon carcinoma cells genetically engineered for overproduction of interleukin (IL-)12 and/or shRNA targeting TGF-β1 are effectively loaded with these molecules and whether the obtained EVs could be an efficient tool for antitumor therapy. Fractions of EVs released by genetically modified MC38 cells [both modified tumor-derived exosomes (mTEx) and modified microvesicles (mTMv)] and those released by unmodified, wild-type MC38 cells were characterized in terms of loading efficacy, using real-time PCR and ELISA, as well as their antitumor potential. In order to examine the therapeutic potential of mTEx, they were applied in the form of sole treatment as well as in combination with dendritic cell (DC)-based vaccines stimulated with mTMv in the therapy of mice with subcutaneously growing MC38 tumors. The results demonstrated that genetic modification of wild-type MC38 tumor cells is an effective method of loading the molecules of interest into extracellular vesicles secreted by the cells (both TEx and TMv). The results also showed that mTEx secreted by cells engineered for overproduction of IL-12 and/or shRNA for TGF-β1 are able to induce tumor growth inhibition as opposed to TEx from unmodified MC38 cells. Additionally, antitumor therapy composed of mTEx (especially those deprived of TGF-β1) and DC-based vaccines allowed for regeneration of antitumor immunity and induction of the systemic Th1 response responsible for the sustained effect of the therapy. In conclusion, tumor-derived exosomes loaded with IL-12 and/or deprived of TGF-β1 could become an efficient adjuvant supporting induction of a specific antitumor response in both immuno- and chemotherapeutic schemes of treatment.

Keywords: tumor-derived exosomes, tumor-derived microvesicles, dendritic cells, interleukin 12, TGF-β1 silencing, lentivectors, colon carcinoma, immunotherapy

INTRODUCTION

Tumor development is dependent on reliable communication and interaction between tumor cells and other cellular components of the tumor microenvironment (TME) (1). Recent scientific reports indicate that both tumor and tumor-infiltrating cells secrete large amounts of extracellular vesicles (EVs), which were proved to be an important element of intercellular communication within and beyond the tumor (2, 3). Scientific literature distinguishes at least two major classes of EVs: exosomes and microvesicles. Exosomes, which have an approximate size of 30–200 nm, are formed from the membrane of late endosomes [multivesicular bodies (MVBs)] by its inward budding. They are released from the endosome to the extracellular space in the process of MVB fusion with the plasma membrane. By contrast, microvesicles (200–1,000 nm) are formed through direct budding of the plasma membrane (1, 4). Both of them are potent vectors capable of transporting biologically active molecules (proteins, lipids, RNA, DNA) to target cells. Depending on the final cargo they can induce a wide range of processes that support cancer development, including immune suppression, cell proliferation, angiogenesis, epithelial-to-mesenchymal transition (EMT) and metastasis (5–7). However, given that tumor-derived exosomes (TEx) as well as microvesicles (TMv) are an easily accessible source of tumor antigens, express tumor-specific integrins which direct their migration toward the tumor site or predicted metastatic sites (8), and act as efficient carriers of different biological structures, they seem to be ideal vehicles for delivery of a broad range of therapeutic agents including non-coding RNAs, mRNAs, proteins, and synthetic drugs. The passive loading through the physical mixing of EVs with drugs (i.e., paclitaxel, cisplatin, curcumin), as well as active loading of molecules such as small interfering RNA by electroporation or sonication, are the most common methods to obtain EVs with desired cargo (9–12). EVs with modified content can also be obtained from genetically engineered cells with overexpression of desired molecules (13–15).

Disorders of immune cells caused by highly immunosuppressive TME are the most common reasons for poor results of cancer immunotherapy (16–18). Hence, therapeutic strategies allowing for efficient reprogramming of the hostile TME abundant in suppressive cytokines such as TGF-β, IL-10, or VEGF are intensively studied (19–21). TME can be modulated both by delivery of inflammatory cytokines, i.e., IL-12 or IL-2 and by elimination of suppressing factors. In our work we focused attention on TGF-β1 and IL-12, which play opposite functions in a tumor development. TGF-β1 is an anti-inflammatory cytokine associated with tumor progression and metastasis, often correlated with poor prognosis in patients (22). By contrast, IL-12 is a proinflammatory cytokine with high antitumor potential but also with high toxicity when applied in the recombinant form (23). The main purpose of our study was to develop and analyze TGF-β1-deprived EVs with IL-12 cargo. The TEx and TMv with modified content, referred to here generally as mTEx and mTMv, were obtained from genetically engineered murine colon carcinoma MC38 cells with overexpression of IL-12 and/or shRNA for TGF-β1 (MC38/IL12, MC38/shTGFβ1, or MC38/IL12shTGFβ1). The particles released by unmodified, wild-type MC38 cells, referred to here as TEx MC38 or TMv MC38, were applied as a control. Since EVs are released by cells in response to stress conditions such as hypoxia, acidosis, or oxidation stress (24), we decided to induce EVs secretion by culture of MC38 cells in hypoxic conditions. TEx and TMv fractions were isolated from the wild-type or genetically modified MC38 culture supernatant and separated on the basis of size using sequential centrifugation. The gathered data indicate that genetic modification of tumor cells is an efficient method to change the tumor-derived EVs' cargo to a therapeutic one. Moreover, immunotherapy composed of mTEx, especially those obtained from MC38/shTGFβ1 or MC38/IL12shTGFβ1, and dendritic cells (DCs) stimulated with mTMv from MC38/shTGFβ1 or MC38/IL12shTGFβ1 cells, applied in the treatment of mice with a subcutaneously growing MC38 tumor, induced tumor growth inhibition accompanied by a reduced number of MDSCs in the tumor and enhanced local and systemic Th1-type antitumor activity.

Abbreviations: EVs, extracellular vesicles; mTEx, tumor-derived exosomes secreted by genetically modified cells; mTMv, tumor-derived microvesicles secreted by genetically modified cells; DCs, dendritic cells; MC38, murine colon carcinoma; MDSCs, myeloid-derived suppressor cells.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice were obtained from the Center for Experimental Medicine of the Medical University of Bialystok (Bialystok, Poland). All experiments were performed in accordance with EU Directive 2010/63/EU for animal experiments and were approved by the 1st Local Ethics Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland (authorization number 33/2018). After the experiments, the mice were sacrificed by cervical dislocation.

Cell Culture

The *in vivo* growing cell line of MC38 murine colon carcinoma from the Tumor Bank of the TNO Radiobiology Institute, Rijswijk, Holland, was adapted to *in vitro* conditions as described by Pajtasz-Piasecka et al. (25). The cell culture was maintained in RPMI 1640 (Gibco) supplemented with 100 U/ml penicillin (Polfa), 100 mg/ml streptomycin (Polfa), 1 mM sodium pyruvate (Sigma-Aldrich), 2-mercaptoethanol (Sigma-Aldrich) here called complete medium (CM), and 5% fetal bovine serum (FBS, Sigma-Aldrich). The genetically modified, stable MC38 cell lines with overexpression of murine IL-12 (MC38/IL12) and/or shRNA targeting mRNA for TGF-β1 (MC38/IL12shTGFβ1, MC38/shTGFβ1) were obtained after transduction of the wild-type MC38 cell line with lentiviral vectors encoding murine interleukin 12 (*mil12*) genes (VectorBuilder) or shRNA for TGF-β1 (EzBiolab). Transduced MC38/IL12 or MC38/shTGFβ1 cells were maintained in standard culture medium for MC38 cells supplemented with Geneticin 418 (Gibco, 1 mg/ml) or puromycin (Gibco, 10 μg/ml), respectively. The double

transduced MC38/IL12shTGFβ1 cells were selected using two antibiotics simultaneously. The efficacy of overexpression of IL-12 and silencing of TGF-β1 in MC38 cells cultured in normoxic or hypoxic conditions was estimated by real-time PCR. Total RNA was isolated using a NucleoSpin RNA kit (Macherey-Nagel) and reverse-transcribed with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). Real-time PCR was performed using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay primers for IL-12 and TGF-β1 (Applied Biosystems) in reference to the HPRT gene expression. Production of IL-12 or TGF-β1 was measured by ELISA (eBioscience) in supernatant harvested from MC38 cells cultured for 24 or 48 h (0.5×10^6 cells/ml). DCs were differentiated from bone marrow of C57BL/6 mice according to the protocol described in our previous publication (26). The cells were cultured in CM supplemented with 10% FBS in the presence of rmGM-CSF (ImmunoTools, 40 ng/ml) and rmIL-4 (ImmunoTools, 10 ng/ml). After 6 days the loosely attached immature dendritic cells were stimulated with exosomes or microvesicles isolated from wild-type or transduced MC38 cell lines and used for further tests.

Isolation and Characteristics of MC38 Tumor-Derived Particles

The production of TEx and TMv by MC38 cells was induced by culture of the cells in hypoxic conditions. Wild-type and genetically modified MC38 cells were seeded on multilayer flasks (Merck-Millipore) at the final density of 250×10^3 cells/ml and cultured in the CM supplemented with 5% exosome-deprived FBS (Sigma-Aldrich) in hypoxic conditions (1% O_2) for 48 h. Then the culture supernatants were harvested. TEx and TMv were isolated from the supernatants according to the procedure described by Felicetti (27) using sequential centrifugation at 2,000, 10,000 and 100,000 × *g* (**Figure 2A**). The TMv fraction was collected after centrifugation at 10 000 × g, while TEx fraction was collected after ultracentrifugation. Both fractions were then washed in PBS (IIET) filtered through 0.2 μm filters (Merck Millipore). To determine the number of TEx and TMv in the final suspension we used the flow cytometry method under the control of Absolute Counting Beads (Thermo Fisher) and 1 μm beads (Polysciences INC). After isolation particles were re-suspended in PBS (IIET) filtered through 0.2 μm filters (Merck Millipore). During the analysis the TEx and TMv were separated from flow cytometer- and PBS-derived debris using CFSE staining (Thermo Scientific, 2.5 μM). The quality of the obtained fractions of TEx and TMv was evaluated using transmission electron microscopy (TEM), dynamic light scattering (DLS), flow cytometry (FC), and western blotting (WB).

The Dynamic Light Scattering Method

The dynamic light scattering method was used for measurement of the particle size distribution and the purity of obtained fractions. Isolated particles were resuspended in filtered PBS and then the suspension was evaluated using a DLS Zetasizer (Malvern).

TEM

TEx and TMv fractions were fixed in 2% paraformaldehyde (Serva) and allowed to adsorb onto formvar carbon-coated grids for 20 min. The grids were then washed in PBS (IIET), fixed in 1% glutaraldehyde (Sigma-Aldrich) for 5 min and washed with water (7 × 2 min). Then each grid was transferred to a drop of uranyl-oxalate (4% uranyl acetate and 0.15 M oxalic acid in 1:1 v:v ratio; Sigma-Aldrich) at pH 7 for 5 min. At this stage, samples were counterstained using two protocols: 1. with uranyl acetate or 2. with methylcellulose. 1: The grid was transferred to a drop of 2% uranyl acetate (Chemapol) for 5 min and washed with a drop of water 3 times. Then the grids were allowed to air dry for 10 min. 2: The grids were then embedded in 2% methylcellulose (Sigma-Aldrich) with uranyl acetate (9:1 v:v ratio) for 10 min on ice. The excess of methylcellulose was removed from grids by filter paper and grids were allowed to air dry for 20 min. Preparations were visualized using a JEOL JEM-1200 EX 80 kV TEM.

Western Blotting

MC38 cells, TEx and TMv were washed twice in PBS (IIET) and lysed in RIPA buffer supplemented with protease inhibitor cocktail (both Sigma-Aldrich). Lysates were purified by centrifugation for 10 min at 4°C and 10,000 x *g* followed by supernatant transfer to new tubes. Total protein concentration in lysates was analyzed using the modified Lowry method (Bio-Rad) according to the manufacturer's protocol. Samples containing 20 μg (cell lysates), 100 μg (TEx lysates), or 10 μg (TMv lysates) of proteins were denatured in Laemmli Sample Buffer (Bio-Rad) supplemented with β-mercaptoethanol (Sigma-Aldrich) for 5 min at 100°C and then separated in 4–20% mini-protean TGX gels (Bio-Rad, USA). After the electrophoresis, proteins were transferred from gels to polyvinylidene difluoride (PVDF) membranes (0.45 μm; Merck Millipore). After blocking in 5% non-fat dry milk in 0.1% TBS/Tween-20 (TBST) at room temperature for 1 h, membranes were washed three times for 5 min in 0.1% TBST and then incubated overnight at 4°C with the following primary rabbit antibodies: monoclonal anti-CD81, monoclonal anti-TSG101, monoclonal anti-calnexin, monoclonal anti-CD9 (all from Abcam) or polyclonal anti-GM130 (Proteintech). After incubation with a primary antibody, the membrane was washed four times for 5 min in 0.1% TBST and then incubated at room temperature for 1 h with horseradish peroxidase conjugated secondary anti-rabbit antibody (DAKO). After incubation, the membranes were washed four times for 5 min in 0.1% TBST and protein bands were detected using luminol-based enhanced chemiluminescent substrate according to the manufacturer's protocol (Thermo Scientific). Images were acquired with G:BOX iChemi XR (Syngene).

Flow Cytometry

The flow cytometry method was applied to determine the expression of CD63, CD9, and CD81 on the surface of isolated particles. Isolated TEx or TMv were resuspended in PBS filtered through 0.2 μm filters (Merck Millipore) and then labeled with monoclonal antibodies conjugated with fluorochromes: anti-CD63 APC, anti-CD9 APC, anti-CD81 APC, rat IgG2aκ APC and Armenian hamster IgG APC isotype

controls (all from BioLegend). The expression of cell surface markers was analyzed using FACS Fortessa with FACSDiva software (Becton Dickinson).

Determination of TGF-β1 and IL-12 Levels in Isolated Particles

Levels of mRNA for IL-12 and TGF-β1 in particles isolated from MC38 cells were measured by real-time PCR. Total RNA was isolated using the NucleoSpin RNA XS kit (Macherey-Nagel) and reverse-transcribed with the SuperScript III First-Strand Synthesis System (Thermo Fisher). Real-time PCR was performed using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay primers for IL-12 and TGF-β1 (Applied Biosystems) in reference to the HPRT gene expression. The IL-12 and TGF-β1 proteins inside the TEx and TMv were evaluated by measurement of the cytokine concentration by ELISA (eBioscience) in lysate prepared from isolated particles using Lysis Buffer for ELISA kits (RayBiotech).

Characteristics of Dendritic Cells Stimulated With MC38-Derived Particles

Immature DCs were stimulated with TEx and TMv obtained from wild-type or genetically modified MC38 tumor cells (5×10^6 particles/1×10^6 cells) in the presence of GM-CSF (40 ng/ml) for 24 h. After stimulation dendritic cells were harvested and labeled with monoclonal antibodies conjugated with fluorochromes: anti-CD40 PE (BD Biosciences), anti-CD80 PerCP-Cy5.5, anti-CD86 PE-Cy7, anti-MHC II FITC, anti-PD-L1 APC, and anti-CD11c BV650 (all from BioLegend). The expression of cell surface markers was analyzed using FACS Fortessa with FACSDiva software (Becton Dickinson). Moreover, the ability of TEx or TMv-stimulated DCs to activate naïve lymphocytes was evaluated. Stimulated DCs were co-cultured with splenocytes obtained from healthy mice in the final ratio of 1:10 for 5 days in CM supplemented with 10% FBS and 200 U/ml of recombinant human IL-2. After 5 days, the cells and supernatants were collected. Cytotoxic activity of primary stimulated splc toward DiO-labeled MC38 target cells, as well as the ability of the effector cells to secrete lytic granules, was measured as previously described by Rossowska et al. (28). The cytotoxic effector cells were identified by flow cytometry (FACS Fortessa) using the following monoclonal antibodies: anti-CD49b PE-CF594 (BD Biosciences), anti-CD8 PE-Cy7, and anti-CD107a APC (both from BioLegend). Production of IFN-γ by primed spleen cells was evaluated using commercially available ELISA kits (eBioscience) according to the manufacturer's instructions. A direct effect of EVs on splenocyte activity was evaluated as a concentration of IFN-γ in supernatants from 5-day culture of splenocytes in the presence of TEx or TMv.

Therapeutic Treatment Schedule

Eight- to ten-week old female C57BL/6 mice were subcutaneously inoculated in the right flank with MC38 cells (1.1×10^6/0.2 ml/mouse). The mice were treated according to the scheme presented in **Figure 4A**. Tumor-derived exosomes obtained from the wild-type MC38 cell line (TEx MC38) or genetically modified MC38/IL12 (TEx MC38/IL12), MC38/shTGFβ1 (TEx MC38/shTGFβ1), or MC38/IL12shTGFβ1 (TEx MC38/IL12shTGFβ1) cell lines were inoculated peritumorally (p.t.), twice per week for three consecutive weeks in a dose of 2×10^6 particles/100 μl NaCl/mouse. On the 16th, 23rd, and 30th days, dendritic cell-based vaccines stimulated with wild-type or genetically modified MC38-derived TMv (DC/TMv MC38, DC/TMv MC38/IL12, DC/TMv MC38/shTGFβ1, or DC/TMv MC38/IL12shTGFβ1) were applied p.t. in the final number of 0.7×10^6/0.2 ml/mouse. On the 35th day, mice were sacrificed, and their spleens and tumor nodules were dissected, homogenized and stored in liquid nitrogen for further analyses. The procedure of tumor growth monitoring was presented by Rossowska et al. (29). The therapeutic effect of the treatment was evaluated using tumor growth inhibition (TGI). Statistically significant differences were calculated using Friedman and Dunn's multiple comparison tests.

Analysis of MC38 Tumor-Infiltrating Immune Cells

Tumor cells isolated from mice were thawed and stained for identification of myeloid or lymphoid cell subpopulations according to the procedure described by Rossowska et al. (30). Briefly, tumor-derived cells were stained with LIVE/DEAD Fixable Violet Dead Staining Kit (Thermo Fisher) and then stained with cocktails of fluorochrome-conjugated monoclonal antibodies: anti-CD3 PE-CF594, anti-CD19 PE-CF594, anti-CD49b PE-CF594 (all from BD Biosciences), anti-CD45 BV605, anti-CD11b PerCP-Cy5.5, anti-CD11c BV650, anti-F4/80 AlexaFluor 700, anti-Ly6C PE, anti-Ly6G APC-Cy7, anti-MHC II FITC, anti-CD86 PE-Cy7 (all from BioLegend) for myeloid cell identification, and anti-CD45 BV605, anti-CD3 BV650, anti-CD4 FITC, anti-CD8 APC/Fire 750, anti-CD25 PE, anti-CD44 PE-Cy7, anti-CD62L PerCP-Cy5.5 (all from BioLegend) for lymphocytes identification. Then, the cells were fixed using the FoxP3 Fixation Permeabilization Staining Kit (eBioscience). Tumor cells stained with myeloid or lymphocyte cocktail were additionally incubated with anti-CD206 APC (BioLegend) or anti-FoxP3 APC (eBioscience) antibodies, respectively. The analysis was performed using a FACS Fortessa flow cytometer with Diva software (Becton Dickinson).

Evaluation of Systemic Antitumor Response

In order to determine the polarization of the systemic immune response followed by applied treatment, Tbet and FoxP3 expression, and IFN-γ production by T cells was measured. Spleen cells, obtained from treated and control mice, were stimulated with ConA (0.5 μg/ml; Sigma-Aldrich) and IL-2 (200 U/ml) for 48 h. Then cells were harvested and after staining with the fluorochrome-conjugated antibodies anti-CD4 FITC and anti-CD8 APC/Fire 750 (BioLegend) were fixed and permeabilized for intracellular staining of Tbet, FoxP3 and IFN-γ with the following antibodies: anti-Tbet PE-Cy7, anti-FoxP3 APC, anti-IFN-γ PE (eBioscience). Flow cytometry analyses

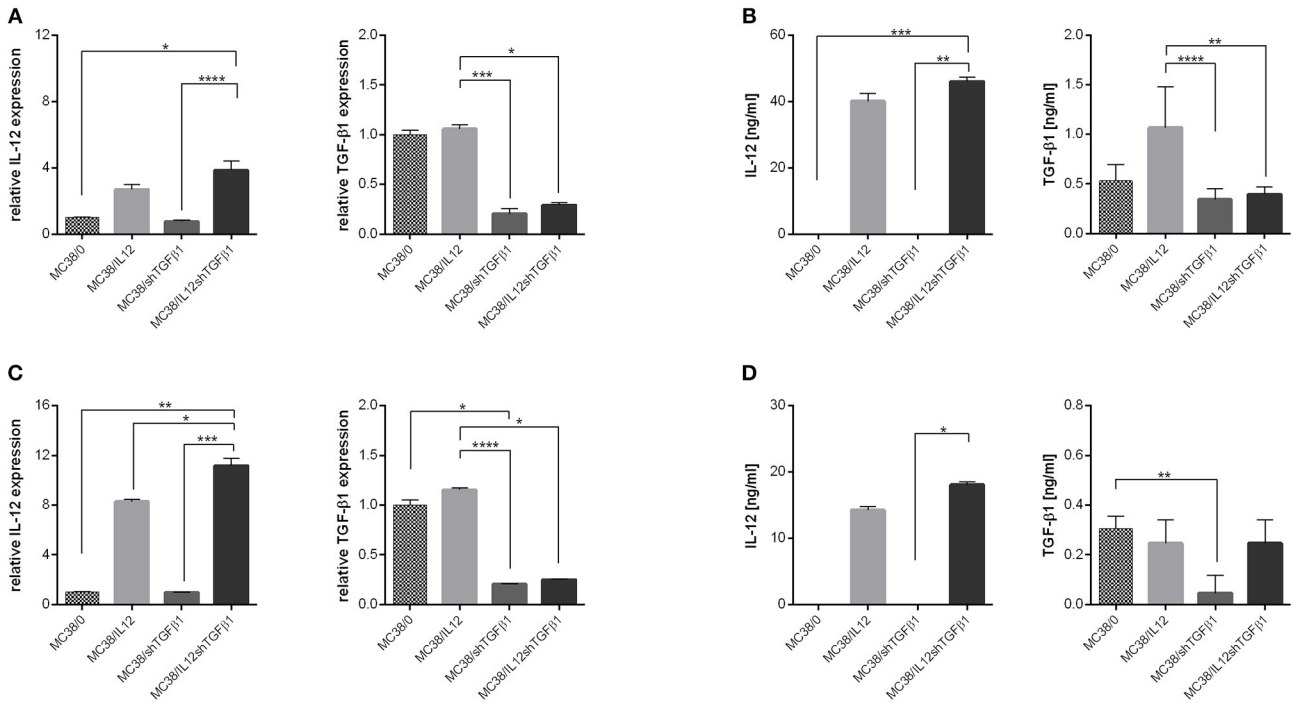


FIGURE 1 | Effectiveness of IL-12 overexpression and TGF-β1 silencing in genetically modified MC38 cell lines. **(A,C)** Relative expression of IL-12 and TGF-β1 measured by real-time PCR in MC38 cells cultured in normoxic **(A)** or hypoxic **(C)** conditions. **(B,D)** Concentration of IL-12 and TGF-β1 in supernatants from MC38 cells cultured in normoxic **(B)** or hypoxic **(D)** conditions. The results are given as the mean ± SD calculated for at least two repeats in two independent experiments. The differences between the groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

were performed using FACS Fortessa with FACSDiva software (Becton Dickinson).

Statistics

All the data were analyzed using GraphPad Prism 6 software. The cytometric data presentations were prepared using NovoExpress software. The statistical significance in kinetics of tumor growth was calculated using the Friedman test followed by Dunn's multiple comparison *post-hoc* test. In all remaining analyses the statistical differences were calculated using the nonparametric Kruskal-Wallis test for multiple independent groups followed by Dunn's multiple comparison *post-hoc* test. Differences with a p-value <0.05 were regarded as significant.

RESULTS

Isolation and Characteristics of TEx and TMv Released by Genetically Modified MC38 Cells

Tumor-derived EVs with modified content (mTEx and mTMv) were obtained from the murine colon carcinoma MC38 cell line with silenced expression of murine TGF-β1 and/or with overexpression of murine IL-12. The MC38/IL12 and MC38/shTGFβ1 cell lines were established by transduction with lentiviral vectors encoding *il12* genes or shRNA for TGF-β1 followed by geneticin or puromycin antibiotic selection, respectively. The MC38/IL12shTGFβ1 cell line was obtained by double transduction followed by selection in the presence of both antibiotics. The quality of the transduction process was monitored using real-time PCR and ELISA (**Figures 1A,B**). In order to stimulate TEx and TMv production by genetically modified or wild-type MC38 cell lines, the cells were cultured in hypoxic conditions for 48 h. To confirm that genetically engineered cells retain their properties in hypoxia we also monitored the IL-12 and TGF-β1 expression after culture of the cells in hypoxic conditions for 48 h (**Figures 1C,D**). Isolation of TEx and TMv from the culture supernatant was carried out according to the scheme presented in **Figure 2A**. After the final washing in PBS the sample from each isolated fraction was collected for further qualitative and quantitative analyses. The quantitative analysis was performed using the flow cytometric method. For this purpose, the samples from TEx and TMv fractions were stained with CFSE dye, fluorescence of which depends on the esterase activity inside the cells or, as in this case, inside particles. This method makes it possible to distinguish the TEx and TMv from flow cytometer and PBS-derived debris during cytometric analysis. The analysis was performed in the presence of counting beads to count the total number of CFSE-labeled TEx or TMv in suspension and reference 1 μm beads to show the approximate size of isolated particles. Representative density plots showing the example of cytometric analysis of TEx and TMv isolated from wild-type MC38 cells are presented in

FIGURE 2 | The method of isolation and characterization of TEx and TMv released by wild-type or genetically modified MC38. **(A)** Scheme of TEx and TMv isolation. **(B)** Representative density plots showing the method of evaluation and counting of CFSE stained TEx and TMv using the LSR Fortessa flow cytometer. The data are

(Continued)

FIGURE 2 | presented for the example of particles isolated from unmodified MC38 cells. TEM analysis of TEx **(C,E)** and TMv **(D,F)** counterstained with uranyl acetate **(C,D)** or with methylcellulose **(E,F)**. Magnification 100,000x. **(G,H)** Representative histograms showing the measurement of MC38-derived TEx and TMv particle size distribution using the DLS Zetasizer (Malvern). **(I)** WB analysis of CD81, CD9, TSG101, GM130, and calnexin in lysates from MC38 cell lines, TEx and TMv fractions. **(J,K,N,O)** Relative expression of mRNA for IL-12 or TGF-β1 in TEx and TMv isolated from wild-type or genetically modified MC38 cell lines. **(L,M,P,Q)** Concentration of IL-12 and TGF-β1 in lysates prepared from TEx and TMv isolated from wild-type or genetically modified MC38 cell lines measured using the ELISA. The results are given as the mean ± SD calculated for at least two repeats in two independent experiments. The differences between the groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*$p < 0.05$, **$p < 0.01$).

Figure 2B. All presented plots represent the result of acquiring 1,000 count beads per sample. Thus, the relation between control plots showing beads and debris and plots showing TEx or TMv is actual. The differences in the number of EVs secreted by the wild-type and genetically modified MC38 cells are presented in **Supplementary Figure 1**. However, it should be stressed that the final number of TEx in preparation may depend on differences in the TEx release as well as differences between proliferation rate of particular cell lines. The quality and size of particles obtained after isolation were measured using TEM and the DLS Zetasizer. The visualization of the EVs with TEM was performed using two protocols of counterstaining: the first with uranyl acetate (**Figures 2C,D**) and second with methylcellulose (**Figures 2E,F**). Both of them showed that the TMv fractions are contaminated by TEx, while the TEx fractions seems to be pure. The TEM analysis showed that the approximate size of TEx is 100 nm, whereas the size of TMv is >200 nm. Histograms presented in **Figures 2G,H** show differences in a size distribution, measured by DLS, between the TMv fraction obtained after centrifugation at 10,000 × *g* for 30 min and the TEx fraction achieved after ultracentrifugation at 100,000 × *g* for 60 min. Using this method we confirmed that the TMv fraction (mean size: 399 nm) was partially contaminated with small TEx particles (mean size: 123 nm), while the TEx fraction was more homogeneous and its mean size was 186 nm. We noted that the size of particles differs when measured by TEM and DLS. The TEM analysis revealed that there are a lot of aggregates in TEx or TMv suspensions. Thus, it seems probable that DLS measurements, which show the mean size of particles, are overstated due to the presence of aggregates in the suspension. There were no significant differences in size distribution between fractions from wild-type and genetically modified cells, thus only representative histograms for TEx and TMv isolated from the wild-type MC38 cell line were presented. In the next step we evaluated the expression of specific markers for exosomes by western blotting and flow cytometry. The western blotting method was used for analysis of both proteins specific for exosomes (CD9, CD81, and TSG101) and cell organelle proteins that exosomes should not contain (GM130 and calnexin). The comparative analysis of lysates from MC38 cell lines, TEx and TMv fractions revealed that GM130 and calnexin were not visible in the TEx-derived lysates, while TSG101, CD81, and CD9 were enriched in the TEx and TMv fractions (**Figure 2I**). The results indicate that TEx fractions were not contaminated with TMv or cell debris. Moreover, expression of CD81 and CD9 in the TMv fractions confirmed our previous observations showing contamination of TMv with TEx. Unfortunately, the comparative analysis of the TEx marker expression between particular cell lines were not possible, because we were not able to determine the precise concentration of total protein in the TEx fractions. We observed that TEx fractions were considerably contaminated with FBS-derived proteins like albumin and the final protein level in a sample was very high, although the concentration of TEx-derived proteins was certainly much lower. For this reason, we decided to perform additional analysis of expression of TEx specific markers using flow cytometry. The analysis showed that the expression of CD63, CD9, and CD81 on the TEx is higher than in TMv. We also noted the difference in expression of these molecules between TEx isolated from genetically modified and wild-type MC38 cells (**Supplementary Figure 2**).

Further studies demonstrated the changes in the content of TEx and TMv following genetic modifications of the MC38 cell line. We observed increased expression of IL-12, measured as mRNA and protein level, in TEx and TMv isolated from cells transduced with lentivectors encoding murine *il12* genes (**Figures 2J,L,N,P**), as well as diminished expression of mRNA and protein for TGF-β1 both in TEx and TMv isolated from MC38 cells with silenced expression of TGF-β1 (**Figures 2K,M,O,Q**). The obtained results indicate that the content of particles produced by tumor cells may be effectively changed following genetic modification of wild-type tumor cells.

The Influence of mTEx and mTMv on Bone Marrow-Derived Dendritic Cell Activity

After 24 h stimulation with mTEx or mTMv the changes in the phenotype of dendritic cells as well as their effectiveness in primary activation of naïve T cells isolated from spleen were evaluated. The results were related to the phenotype and activity of unstimulated DCs, DCs stimulated with lysate prepared by repeated freezing and thawing of wild-type MC38 cells (DC/TAg) or DCs stimulated with TEx or TMv isolated from wild-type MC38 cells (DC/TEx MC38 or DC/TMv MC38). The phenotype analysis showed that all types of TEx strongly affected the maturation of DCs (**Figures 3A,B**). Although after stimulation with TAg, TEx MC38 cells, and mTEx we observed a significant decrease in MHC II expression, the CD40, CD80, and CD86 co-stimulatory molecule expression on the DCs stimulated with TEx MC38 or mTEx considerably increased compared to DC or DC/TAg. The highest maturity stage was shown by DCs stimulated with TEx MC38/shTGFβ1 or with TEx MC38/IL12. Interestingly, TEx isolated from wild-type cells and TEx MC38/IL12shTGFβ1 induced similar phenotypical changes in DCs. Further analyses showed that DCs stimulated with TEx from unmodified MC38 cells or mTEx were more potent to activate cytotoxicity of T lymphocytes and NK cells

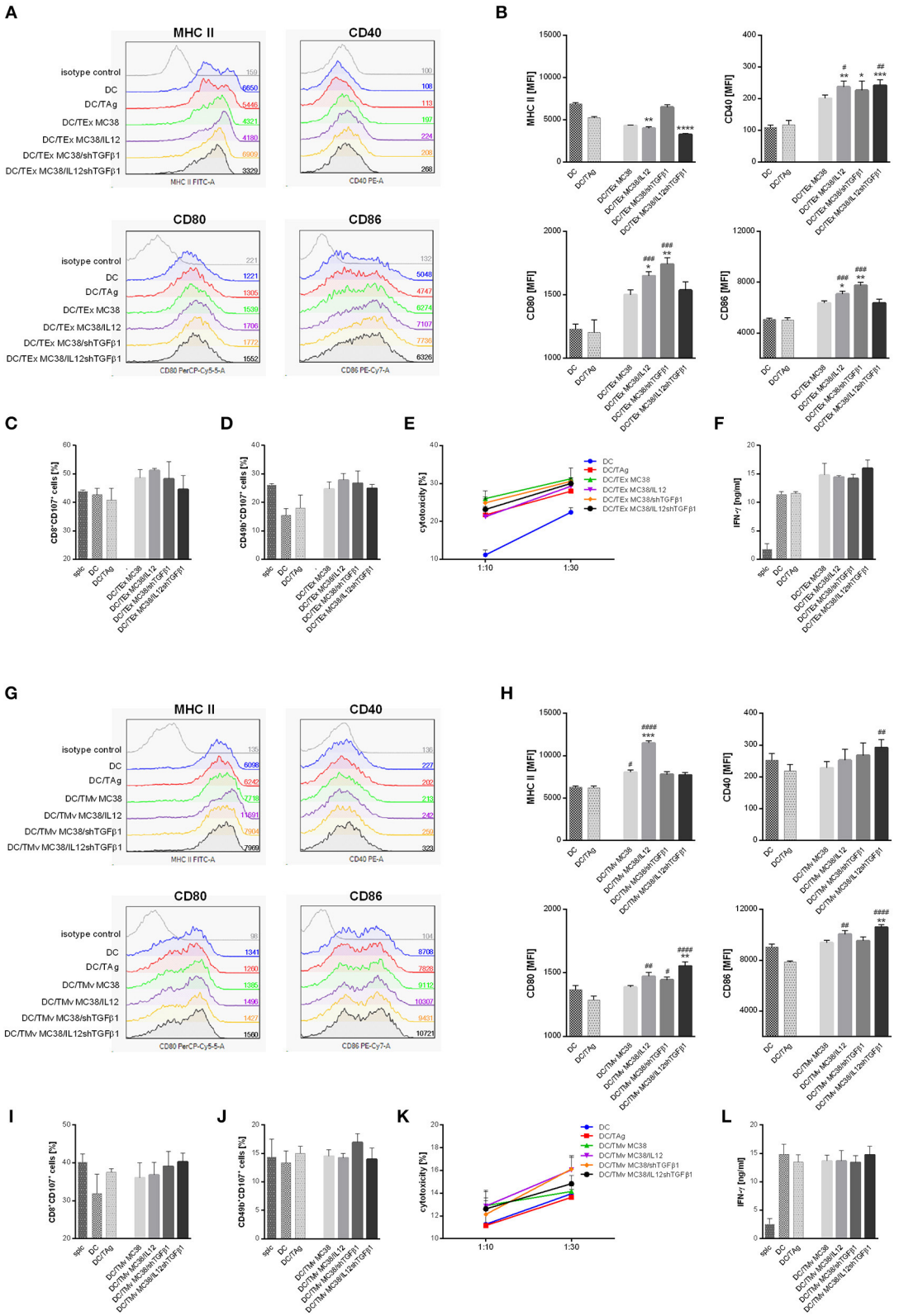


FIGURE 3 | The influence of TEx or TMv isolated from wild-type MC38 cells or MC38 cells with overexpression of IL-12 and/or shTGFβ1 on dendritic cell (DC) activity *in vitro*. **(A,B)** Representative histograms and bar plots showing phenotypic changes of DCs after 24-h stimulation with TEx. **(C–F)** Splenocyte activity after primary

(Continued)

FIGURE 3 | stimulation with TEx-treated dendritic cells. The data show the percentage of $CD8^+CD107a^+$ CTLs **(C)** and $CD49b^+CD107a^+$ NK cells **(D)** among splenocytes obtained after 5-day co-culture with TEx-treated DCs, their cytotoxic activity toward MC38 tumor cells **(E)** and production of IFN-γ during co-culture of splc and DCs **(F)**. **(G,H)** Representative histograms and bar plots showing phenotypic changes of DCs after 24-h stimulation with TMv. **(I–L)** Splenocyte activity after primary stimulation with TMv-treated dendritic cells. The data show the percentage of $CD8^+CD107a^+$ CTLs **(I)** and $CD49b^+CD107a^+$ NK cells **(J)** among splenocytes obtained after 5-day co-culture with mTMv-treated DCs, their cytotoxic activity toward MC38 tumor cells **(K)** and production of IFN-γ during co-culture of splc and DCs **(L)**. The results are given as the mean ± SD calculated for three repeats in three independent experiments. The differences between the groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ in reference to DC; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$ in reference to DC/TAg).

toward wild-type MC38 cells than unstimulated DCs or DC/TAg (**Figures 3C–E**). Moreover, splenocytes produced more IFN-γ during co-culture with DC/TEx MC38 or DC/mTEx than those activated by DC/TAg (**Figure 3F**). Nevertheless, there were no significant differences between groups stimulated with TEx from unmodified MC38 cells and mTEx.

Dendritic cells stimulated with TMv from unmodified MC38 cells or mTMv also showed higher maturity than DC/TAg. All groups stimulated with microvesicles revealed higher expression of co-stimulatory and MHC class II molecules than control DCs and DC/TAg cells (**Figures 3G,H**). However, as in the case of stimulation with TEx, there were no significant changes between groups stimulated with TMv from wild-type MC38 cells or genetically modified MC38 cells. Although we observed similar antitumor activity of DC/TMv MC38 and DC/TAg, the DC/TMv MC38/IL12shTGFβ1 seems to be more effective in activation of cytotoxic CTLs ($CD8^+CD107a^+$), whereas DC/TMv MC38/shTGFβ1 was more potent in activation of cytotoxic NK cells ($CD49b^+CD107a^+$) than control DCs. Additionally, lymphocytes stimulated with these cells showed higher cytotoxic activity toward MC38 cells than in control groups (**Figures 3I–K**). In contrast to TEx, DC/TMv were not able to induce IFN-γ production at a higher level than control DCs and DC/TAg cells (**Figure 3L**). In summary, both mTEx and mTMv induced maturation of dendritic cells, but DCs stimulated with TEx, from both wild-type and genetically modified MC38 cells, seemed to be better activators of antitumor activity of lymphoid cells.

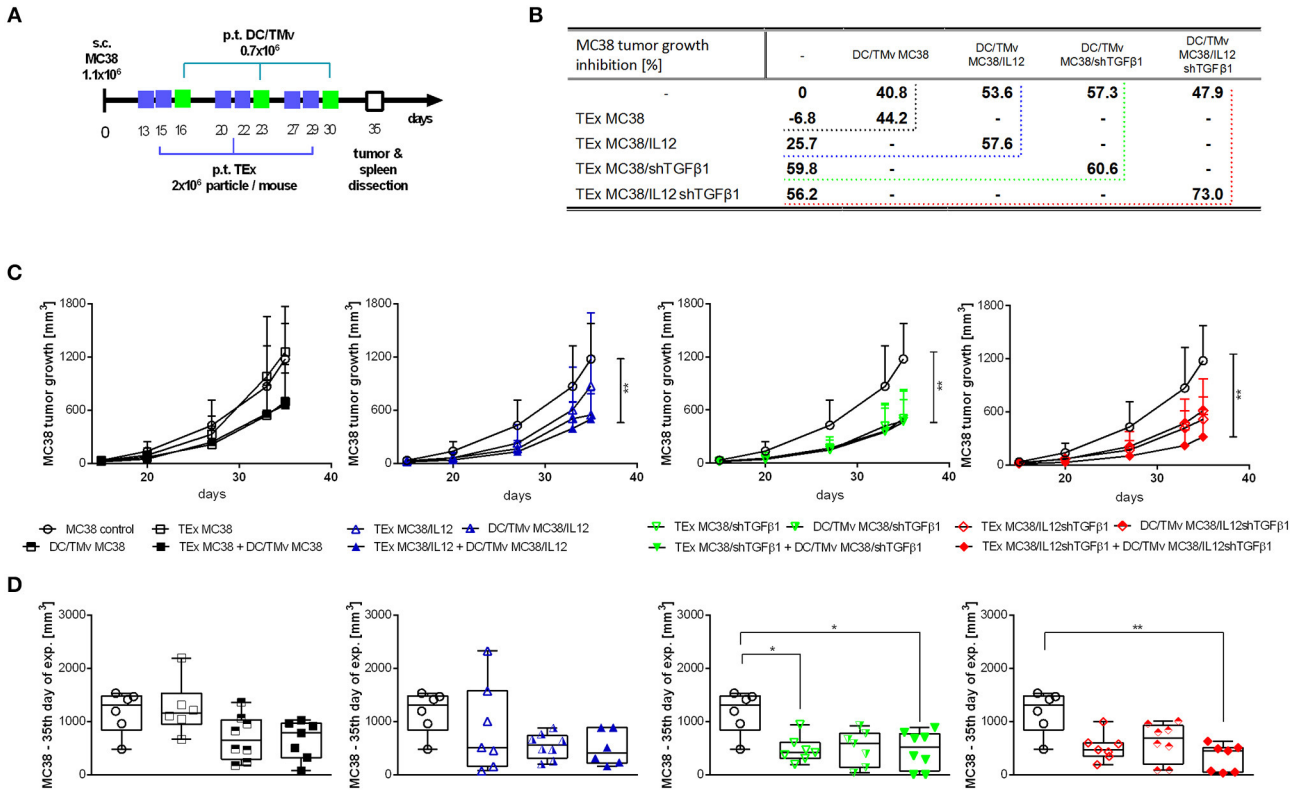
We also performed an experiment which aimed to determine a direct influence of TEx and TMv on splenocyte ability to produce IFN-γ. It was observed that splenocytes stimulated with mTEx or mTMv, especially these isolated from MC38 cells with overexpression of IL-12 produced IFN-γ on significantly higher level than unstimulated cells or stimulated with particles from wild-type MC38 cells (**Supplementary Figure 3**).

Antitumor Activity of mTEx and DCs Stimulated With mTMv

Based on the results obtained in *in vitro* assays, we decided to apply both mTEx and mTMv in the form of immunotherapy of mice with subcutaneously growing MC38 tumors. However, due to the higher activity, mTEx were applied in the form of a vaccine directly inoculated to the host, whereas mTMv were used mainly as the tumor antigen source to stimulate dendritic cell-based vaccines. The scheme of the combined treatment with TEx from unmodified MC38 cells or mTEx (TEx MC38/IL12, TEx MC38/shTGFβ1, TEx MC38/IL12shTGFβ1) and TMv from unmodified MC38 cells or mTMv (TMv MC38/IL12, TMv MC38/shTGFβ1, TMv MC38/IL12shTGFβ1)-stimulated DCs is presented in **Figure 4A**. The kinetics of MC38 tumor growth during therapy as well as the tumor growth inhibition calculated on the 35th day of therapy, are presented in **Figures 4B–D**. The obtained data showed that TEx MC38 accelerated tumor growth and in this case TGI (tumor growth inhibition calculated on the 35th day of the experiment in relation to the untreated MC38 control) reached −6.8%. For comparison, TEx MC38/IL12, TEx MC38/shTGFβ1 or TEx MC38/IL12shTGFβ1 induced TGI at the level of 25.7, 59.8, or 56.2%, respectively. Although TEx MC38/IL12shTGFβ1 did not induce the highest TGI, we observed that application of these exosomes in combination with DC/TMv MC38/IL12shTGFβ1 resulted in the best therapeutic effect and TGI reached 73%. In other groups, which received combinations of mTEx and DC/mTMv, the therapeutic effect did not differ significantly from that obtained for groups which obtained DC/TMv MC38/IL12 or DC/TMv MC38/shTGFβ1 alone. It should also be emphasized that the effect of application of DCs stimulated with mTMv was better than that observed for DCs stimulated with TMv from unmodified MC38 cells. However, the differences between TGI calculated for particular groups were not considerable (**Figure 4B**). Taking into account the particular genetic modifications of wild-type tumor cells, it seems that particles isolated from MC38 cells with silenced expression of TGF-β1 are the most important in induction of a potent therapeutic effect.

The Influence of Therapy Composed of mTEx and DC/mTMv on the Local and Systemic Antitumor Immune Response

We determined the influence of immunotherapy on changes in the immune composition of the MC38 tumor microenvironment applying multicolor flow-cytometric analyses. The lymphoid cell panel (**Figure 5A**) allowed for simultaneous identification of CTLs, Th, Treg, B, NK, and NKT cell subpopulations, as well as determination of their activity. Although all subpopulations were analyzed, we decided to present data for cells which underwent significant changes during applied therapy. The analysis of tumor nodules dissected on the 35th day of immunotherapy revealed very high influx of leukocytes ($CD45^+$ cells; **Figure 5B**) after treatment with a combination of exosomes and DCs stimulated with microvesicles (both TMv and mTMv) as well as after DC/TMv MC38/IL12shTGFβ1. Excluding the group which received unmodified particles, the effect was accompanied by high infiltration of effector CTLs and NK cells (**Figures 5C,E**). We also observed a reduced population



MC38 tumor growth inhibition [%]	-	DC/TMv MC38	DC/TMv MC38/IL12	DC/TMv MC38/shTGFβ1	DC/TMv MC38/IL12 shTGFβ1
-	0	40.8	53.6	57.3	47.9
TEx MC38	-6.8	44.2	-	-	-
TEx MC38/IL12	25.7	-	57.6	-	-
TEx MC38/shTGFβ1	59.8	-		60.6	-
TEx MC38/IL12 shTGFβ1	56.2	-	-	-	73.0

FIGURE 4 | MC38 tumor growth after immunotherapy with mTEx and/or dendritic cells stimulated with mTMv. **(A)** Scheme of treatment. **(B)** Table presenting MC38 tumor growth inhibition (TGI) calculated on the 35th day of the therapeutic experiment in relation to the untreated group. **(C)** Curves presenting the mean tumor volume after immunotherapy. The differences between groups were estimated using the Friedman test (**$p < 0.01$). **(D)** The box graphs present the median tumor volume, calculated on the 35th day. To calculate the mean ± SD, 6–8 mice per group were analyzed. The differences between the groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*$p < 0.05$, **$p < 0.01$).

of Treg cells after combined therapy. However, a statistically lower percentage of the cells compared to the untreated control was noted only after application of monotherapy with TMv-stimulated DCs, especially after DC/TMv MC38/IL12 (**Figure 5D**). The changes occuring in the lymphoid populations were accompanied by modifications in the percentage of myeloid cells infiltrating tumor nodules. The applied myeloid cell panel (**Figure 5F**) allowed for simultaneous identification of TAM, DCs, resident macrophages (Mf), M-MDSCs and PMN-MDSCs as well as identification of the macrophage polarization stage through the evaluation of CD206 expression. We noted that percentages of M-MDSCs and PMN-MDSCs were significantly lower than in the untreated group when combinations of modified exosomes together with DC/mTMv were applied (**Figures 5G,H**). The obtained data also showed a considerably decreased percentage of resident macrophages in TEx MC38/shTGFβ1 + DC/TMv MC38/shTGFβ1 and TEx MC38/IL12shTGFβ1 + DC/TMv MC38/IL12shTGFβ1 groups (**Figure 5J**). We did not observe any changes in the percentage of TAM—the main subpopulation of macrophages in MC38 tumor nodules. However, the M1/M2 rate, which shows the influence of the applied therapy on macrophage polarization, indicates that treatment with combinations of mTEx and DCs stimulated with mTMv (especially with TEx MC38/IL12 + DC/TMv MC38/IL12 and TEx MC38/shTGFβ1 + DC/TMv MC38/shTGFβ1) caused the change of polarization of tumor-infiltrating macrophages (both TAM and Mf) toward M1 (**Figure 5I**). To confirm the influence of the applied immunotherapy on tumor-derived macrophage polarization we performed functional assays, which demonstrated the induction of the Th1 response, corroborating the shift of macrophage polarization toward M1 type. As presented in **Figure 6A**, statistically significant changes in the percentage of spleen-derived $Tbet^{+}IFN\text{-}\gamma^{+}$ Th lymphocytes, corresponding to the shift of the immune response toward Th1 type, were visible only in the group treated with TEx MC38/shTGFβ1 + DC/TMv MC38/shTGFβ1. In the remaining groups treated with mTEx and DC/mTMv the percentage of Th1 lymphocytes was also considerably higher than in the untreated control group. However, it was similar to values obtained for groups which received only DC/mTMv. Taking into account the decreased number of Treg cells among splenic $CD4^{+}$ lymphocytes (**Figure 6B**), we also noted that treatment with TEx MC38/IL12 + DC/TMv MC38/IL12 or TEx MC38/shTGFβ1 + DC/TMv MC38/shTGFβ1 contributed to a significant increase in the proportion of Th1 to Treg cells (**Figure 6C**). The obtained data

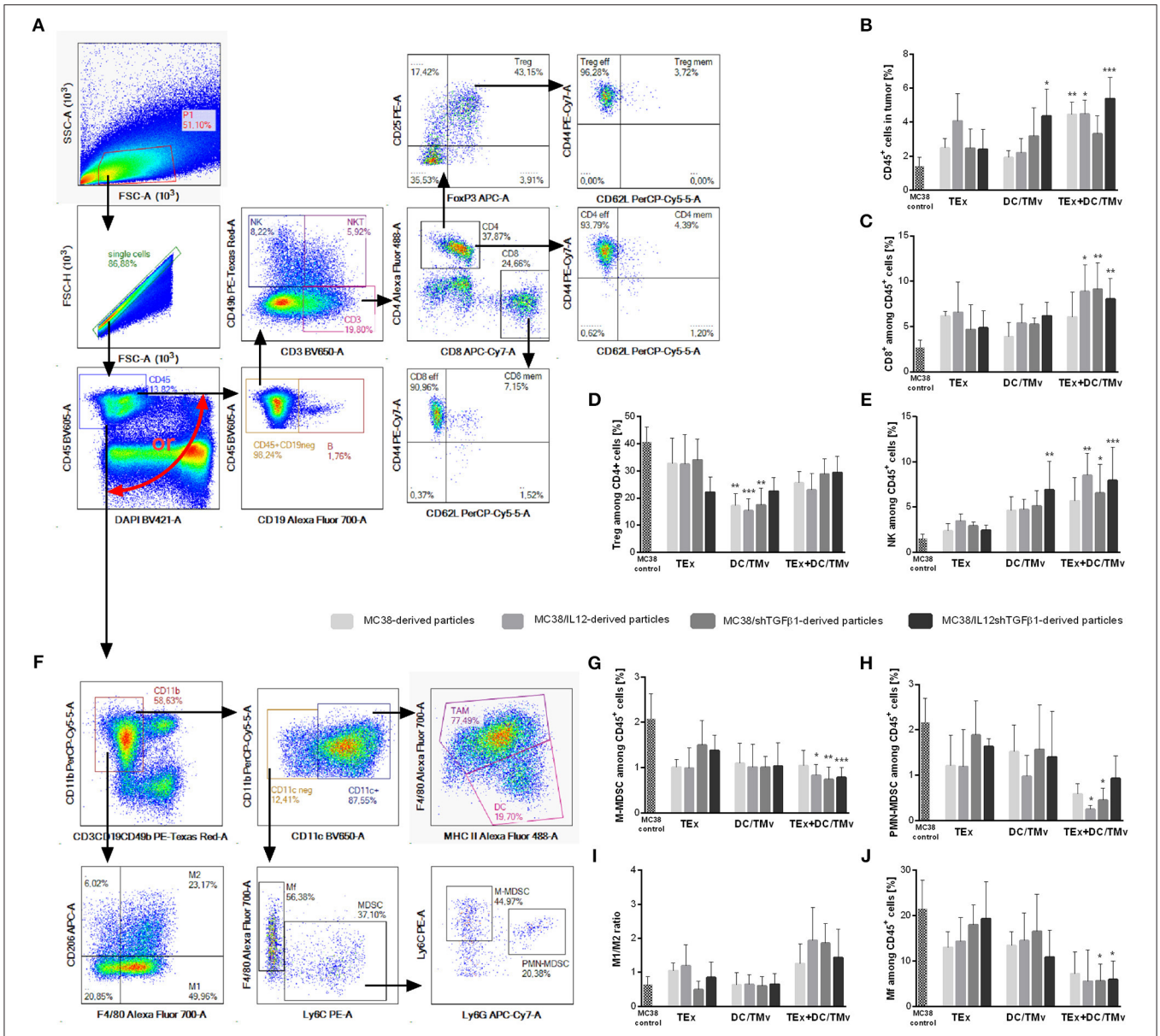


FIGURE 5 | The influence of immunotherapy on the immune landscape in MC38 tumor nodules. **(A,F)** Schemes of multiparameter flow cytometric analyses showing the way of distinguishing lymphoid **(A)** or myeloid **(F)** cell subpopulations. **(B)** The percentage of leukocytes in tumor nodules. **(C–E,G,H,J)** Percentages of effector or suppressor cell subpopulations which underwent changes during therapy. **(I)** The M1/M2 ratio showing changes in polarization of tumor-infiltrating macrophages occurring during therapy. To calculate the mean ± SD, 6–8 mice per group were analyzed. The differences between the groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ in reference to MC38 control).

indicate that combined therapy with mTEx and DC/mTMv, especially application of TEx MC38/shTGFβ1 + DC/TMv MC38/shTGFβ1, is efficient in activation of a potent systemic Th1 response.

DISCUSSION

Scientific reports provide information confirming that cargo-loaded extracellular vesicles (EVs) have shown promising therapeutic effects in a variety of disease models, including cancer (11, 15, 31, 32). Recent research also demonstrated that tumor-derived exosomes, despite their high protumor activity, could become a very effective tool for delivery of chemotherapeutic drugs. Tang and co-workers, as well as Silva and co-workers, reported that chemotherapeutic drugs (methotrexate, cisplatin) and photosensitizers (m-THPC), respectively, can be effectively packed into tumor-derived EVs and used to inhibit tumor growth in murine cancer models (9, 33). It was also discovered that paclitaxel loaded into TEx showed 50 times higher

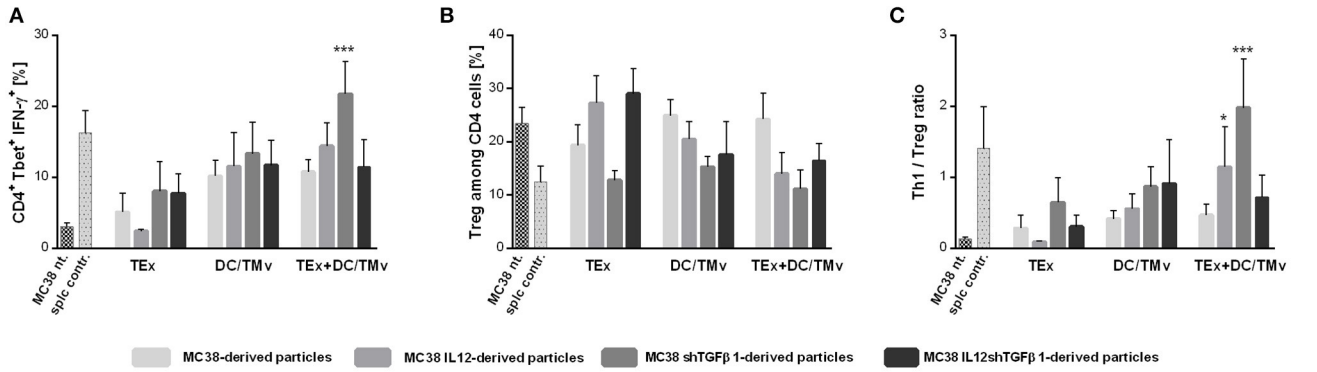


FIGURE 6 | Polarization of immune response following mTEx and DC/mTMv treatment. **(A)** Bar plots presenting changes in Tbet expression and IFN-γ production in $CD4^+$ cells from spleens of treated mice. **(B)** The percentage of Treg cells among $CD4^+$ cells from spleens of treated mice. **(C)** The Th1/Treg ratio. To calculate the mean ± SD, 6–8 mice per group were analyzed. The differences between the groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*$p < 0.05$, ***$p < 0.001$ in reference to MC38 control).

cytotoxic activity than free paclitaxel and caused significant inhibition of lung metastasis growth (34). Besides drug delivery, tumor-derived EVs, due to their biocompatibility and directed migration, are also considered as prime carriers of other types of cargo, such as immunomodulating factors (in the form of non-coding RNA, mRNA, and protein).

The main objective of the study was to determine whether EVs released by tumor cells genetically engineered for overexpression of proinflammatory IL-12 and/or shRNA targeting suppressor TGF-β1 may lose protumor activity and acquire antitumor potential. According to a hypothesis, genetically induced overproduction of antitumor molecules could create a situation where tumor cells would produce protumor molecules at a reduced rate and start loading of the overexpressed molecules into EVs, thereby contributing toward raising the antitumor activity of the latter. Taking that into consideration, in the first stage of the study we confirmed the effectiveness of loading EVs with IL-12 and presence of TGF-β1 in the lumen of isolated particles. The results indicated that genetic modification of wild-type tumor cells is an effective method of loading the molecules of interest into extracellular vesicles secreted by the cells (both TEx and TMv). We observed that EVs released by MC38/IL12 and MC38/IL12shTGFβ1 contained IL-12 in the form of mRNA and active protein, while EVs isolated from MC38/shTGFβ1 or MC38/shIL12shTGFβ1 were characterized by significantly diminished level of TGF-β1 (both in the form of mRNA and protein). The effectiveness of genetic engineering of donor cells to modify the content of exosomes has also been confirmed by other research groups (13, 14, 35). Since, EVs are a source of tumor antigens, our further analyses focused on the influence of mTEx and mTMv as well as TEx and TMv obtained from wild-type MC38 cells on the activity of bone marrow-derived dendritic cells. As expected, modified EVs (both mTEx and mTMv) supported differentiation of DCs toward mature cells capable of presenting tumor antigens to naïve T lymphocytes. However, there were no significant differences between activity of DCs stimulated with EVs isolated from modified and wild-type MC38 cells. Additionally, we observed that DCs stimulated with TEx from wild-type MC38 cells and mTEx seemed to be better activators of antitumor activity of lymphoid cells than TMv MC38 and mTMv. It may be connected with different size of the applied particles. TEx with approximate size of 100 nm can be recognized and taken up by DCs as virus particles, whereas TMv with size >200 nm are recognized as bacteria. The different ways of processing EVs may result in differences in the stimulation efficacy of DCs, although TEx and TMv do not vary especially in a content. It can also be reflected in a diverse expression of MHC II on the surface of DCs stimulated with TEx or TMv. Furthermore, when we compared the effect of TEx MC38 stimulation with tumor lysate (TAg) stimulation—the most frequent method of tumor antigen delivery to DC-based vaccines—we noted that TEx MC38 were better stimulators than TAg. It was observable not only at the level of phenotypic changes occurring in DCs but also at the level of their functional activity. Similar results were demonstrated by Bu et al. (36). They found that T cells primed by DCs stimulated with TEx showed significantly higher cytotoxicity toward glioma cells than those stimulated with TAg. Our research also revealed the direct influence of modified EVs on the activity of spleen cells. We noted that splenocytes stimulated with mTEx or mTMv, especially EVs containing IL-12, produced high amounts of IFN-γ, while splenocytes stimulated with unmodified TEx or TMv produced IFN-γ at the same level as unstimulated cells.

The aim of our subsequent experiments was to confirm the antitumor potential of mTEx in *in vivo* conditions. Taking into consideration the high potential of TEx MC38 and mTEx to activate DCs, we decided to apply them in the form of sole treatment or in combination with DC-based vaccines. The obtained data showed that TEx from unmodified MC38 cells accelerated tumor growth, whereas mTEx, especially those deprived of TGF-β1, caused tumor growth inhibition. Although the combination of TEx MC38/shTGFβ1 with DC/TMv MC38/shTGFβ1 did not further improve the TEx MC38/shTGFβ1 therapeutic effect, we noted that application of

TEx MC38/IL12shTGFβ1 with DC/TMv MC38/IL12TGFβ1 was able to induce over 70% tumor growth inhibition. By contrast, TEx MC38/IL-12 induced minor tumor growth inhibition at the level of 25%.

It also needs to be highlighted that only in groups of mice receiving a combination of mTEx and DCs/mTMv could we observe a statistically significant increase in the percentage of tumor-infiltrating CTLs and NK cells, which was accompanied by a reduction of suppressor MDSCs and Treg cells as well as favorable changes in the polarization of macrophages infiltrating tumor nodules. The obtained data confirm the important role of DC-based vaccines in regeneration of the immune response and induction of a systemic reaction. However, it also shows the necessity of supporting their action by other factors capable of reprogramming the hostile tumor microenvironment. Both selected cytokines play an important role in functioning of DCs. IL-12 induces differentiation of DCs and plays an essential role as the third signal during formation of a fully functional immunological synapse and activation of a Th1 type response (37, 38). On the other hand, TGF-β1 is a strong inhibitor of DCs. Moreover, it induces differentiation of DCs toward regulatory cells characterized by high protumor activity (39). Tumor-derived exosomes isolated from cells with overexpression of both IL-12 and shTGFβ1 can play a dual role in TME. Firstly, they can deliver IL-12, without the "negative cargo" in the form of suppressor TGF-β1, and they support differentiation of myeloid cells, effective presentation of tumor antigens by DCs and induction of a specific antitumor response. Secondly, siRNA against TGF-β1 constitutively produced in large amounts by modified tumor cells can also be loaded by the cells into EVs and may play a significant role in silencing of the TGF-β1 expression in target cells, thus hindering their protumor activity (35). Although both of mentioned factors (IL-12 and shRNA for TGF-β1) are very important for effective reactivation of immune response to fight cancer, our research revealed that the influence of TEx MC38/shTGFβ1 on the activation of specific antitumor response as well as reduction of immunosuppression in TME is significantly higher than the effect of treatment with TEx MC38/IL12. We suppose that the effect of the IL-12 delivered by TEx may be limited due to high activity of suppressor cells such as MDSCs or Treg in the TME. By contrast, TEx MC38/shTGFβ1 deliver tumor antigens without the "negative cargo" in the form of TGF-β1, thereby the response of immune cells for tumor antigens is more efficient. Additionally, we suppose that TEx MC38/shTGFβ1 may support the reactivation of antitumor response by delivery of siRNA for TGF-β1, which reduce the immunosuppression inside a tumor. In our previous study we used lentivectors encoding shTGFβ1 to reduce the concentration of the cytokine in TME and enhance the antitumor activity of DC-based immunotherapy. Although the final effect of the treatment was spectacular (97% TGI), we noted high immunogenicity of the applied lentivectors (20). Compared to lentivectors, TEx seems to be significantly better delivery vectors due to their biocompatibility, targeted migration, and versatility. Moreover, it seems probable that modified exosomes, in contrast to other delivery vectors, can have a the wider spectrum of action. Certainly, peritumorally injected exosomes carrying immunomodulatory factors such as IL-12 and shRNA for TGF-β1 can take part in the reprogramming of suppressive TME and supporting the antitumor activity of peritumorally injected DC-based vaccines as well as reactivating endogenous immune cells arrested in a tumor nodule. In addition, due to their high stability and biocompatibility, modified tumor-derived exosomes could also migrate toward draining lymph nodes, where they could directly support dendritic cells in effective activation of naïve T cells, or to presumable metastatic sites specific for a tumor, where they could prevent formation of a premetastatic niche. However, the hypothesis should be confirmed in further research.

Taken together, the presented results indicate that tumor-derived exosomes secreted by cells engineered for overproduction of IL-12 and shRNA for TGF-β1 are able to induce tumor growth inhibition as opposed to TEx from unmodified MC38 cells. Moreover, their application (especially those deprived of TGF-β1) with DC-based vaccines allowed for regeneration of antitumor immunity and induction of systemic the Th1 response responsible for the sustained effect of the therapy. Nevertheless, further research is required to provide better knowledge of the changes occurring in mTEx following genetic modifications and to determine possible side effects of their application.

ETHICS STATEMENT

This study was carried out in accordance with EU Directive 2010/63/EU for animal experiments and were approved by the 1st Local Ethics Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland (authorization number 33/2018).

AUTHOR CONTRIBUTIONS

JR: substantial contribution to the conception and design of the research, planning, and performing experiments, data analysis, interpretation, and drafting the manuscript; NA: planning and performing *in vitro* and *ex vivo* experiments and data analysis; KW, AS, and JM: planning and performing *ex vivo* experiments; MM: performing western blotting analyses; BS-O: EV visualization by TEM—optimization of the method and sample preparation and analysis; EP-P: planning and performing *in vivo* experiments. All authors reviewed the manuscript and approved its final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00211/full#supplementary-material

Supplementary Figure 1 | Evaluation of the number of TEx and TMv secreted by the wild-type and genetically modified MC38 cells per isolation performed by flow cytometry using Absolute Counting Beads. Bar graphs present the mean ± SD calculated for three repeats. The differences between the groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*$p < 0.05$, ***$p < 0.001$).

Supplementary Figure 2 | Flow cytometry analysis of TEx and TMv isolated from wild-type or genetically modified MC38 cell lines. Histograms represent the expression of CD63, CD9, CD81 on the surface of TEx and TMv. Bar graphs present the mean ± SD calculated for three repeats. The differences between the groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*$p < 0.05$).

Supplementary Figure 3 | Concentration of IFN-γ in supernatants from spleen cells stimulated with TEx or TMv, isolated from wild-type or genetically modified MC38 cell lines. Bar graphs present the mean ± SD calculated for three repeats. The differences between the groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*$p < 0.05$, ****$p < 0.0001$).

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REVIEW
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Cancer Cell-Intrinsic PD-1 and Implications in Combinatorial Immunotherapy

*Han Yao, Huanbin Wang, Chushu Li, Jing-Yuan Fang and Jie Xu**

State Key Laboratory for Oncogenes and Related Genes, Division of Gastroenterology and Hepatology, MOH Key Laboratory of Gastroenterology and Hepatology, Renji Hospital, School of Medicine, Shanghai JiaoTong University, Shanghai, China

Programmed death 1 (PD-1) and its two natural ligands PD-L1 and PD-L2 are responsible for delivering inhibitory signals that regulate the balance between T cell activation, tolerance, and immunopathology. In previous studies, PD-1 was found only expressed on the surface of immune cells, such as T cells and B cells while PD-1's ligands PD-L1 and PD-L2 were found expressed in some tumor cells. However, recent studies revealed intrinsic expression of PD-1 in melanoma and some other cancers. In melanoma cells, PD-1 can be activated by its ligand PD-L1 expressed by tumor cells, modulating downstream mammalian target of rapamycin signaling and promoting tumor growth independent of adaptive immunity. In addition to melanoma, PD-1 was also detected in liver cancer cells as well as in non-small lung cancer cells. Unlike its oncogenic functions in melanoma and hepatic carcinoma cells, PD-1 seemed to play a distinct role in lung cancer, as blockade of PD-1 instead promoted tumor cells proliferation. Tumor-intrinsic PD-1 expression seems to be widespread in many tumor types, according to our reanalysis on cancer transcriptomic and proteomic data. The multifaceted roles of PD-1 in tumor cells beyond immune checkpoint signaling may explain the differential therapeutic effects of anti-PD-1 and anti-PD-L1 drugs and provide crucial information when developing combinatorial approaches to enhance antitumor immunity.

Keywords: tumor cell-intrinsic programmed death 1, combinatorial immunotherapy, mammalian target of rapamycin, tumor growth, blockade

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***Correspondence:**
Jie Xu
jiexu@sjtu.edu.cn

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INTRODUCTION

As the second generation clinical target of immune checkpoint, programmed death 1 (PD-1) is protein of the CD28 superfamily and a kind of cell membrane protein with 288 amino acids (1). PD-1 is expressed on surface of activated T cells as an inhibitory receptor (2), while its ligands PD-L1 and PD-L2 are mainly expressed in antigen-presenting cells and tumor cells (3, 4). After binding to its ligand, PD-1 suppresses the tumor-killing activity of T cells and downregulates T cells responses. The functions of PD-1 in immune cells include the induction and maintenance of peripheral immune tolerance, protecting tissue from immune attack and dampening infectious immunity and tumor immunity (5). Anti-PD-1 and anti-PD-L1 can relieving the immunosuppressive state of T cells through competitively combining with PD-1 or PD-L1 (6). Because of the relatively satisfactory therapeutic effects, anti-PD-1 drugs, such as nivolumab and pembrolizumab, have been approved by FDA for the treatment of patients with advanced melanoma. However, most patients do not show durable remission, and some tumors have been completely refractory to response with checkpoint

blockade, highlighting the requirement of understanding the role of PD-1/PD-L1 axis during the oncogenic and metastatic processes (7–9). In recent studies, the expression of PD-L1 and PD-L2 has been studied in different cancers and stages (10–13). However, most studies on PD-1 expression have focused on immune cells, rendering its potential expression and functions in tumor cells remaining largely unclear. This review summarizes our recent understanding on the multifaceted roles of tumor cell-intrinsic PD-1, aiming to present this interesting research topic to the attentions of researchers in the field of immunotherapy.

SKIN AND LIVER CANCER CELL-INTRINSIC PD-1 PROMOTES TUMORIGENESIS

Malignant melanoma is characterized by early metastasis, rapid progression, poor prognosis, and high mortality, and a large number of antibody drugs for malignant melanoma have entered the clinical research stage (14, 15). Especially in 2014, new antibody drugs targeting the PD-L1:PD-1 interaction (pembrolizumab and nivolumab) were approved by FDA for the treatment of metastatic melanoma (7, 9). As in many other cancers, the therapeutic effects of anti-PD-1 drugs in melanoma were thought as a result of enhanced immunity (16, 17). Most previous research of PD-1 were based on its T-cell specific expression, but there have been emerging studies showing its expression and functions in tumor cells including melanoma and hepatoma carcinoma, even in the above tumor-bearing mice lacking adaptive immunity (18–20). Unlike the reported expression of PD-1 in immune-competent cells of the hematopoietic lineage, two independent studies demonstrated that melanoma and liver cancer cell lines and tissue specimens may express the PD-1 protein.

The biological functions of PD-1 have been intensively studied in T cell, mainly characterized by the binding on cell surface to its ligands PD-L1/PD-L2 and downstream signaling involved in the suppression of T cell proliferation, cytokine production, and cytotoxic functions (schematics in **Figure 1**) (21). Nevertheless, melanoma or hepatoma carcinoma-intrinsic PD-L1 was found to promote tumor growth even in the absence of functional adaptive immune system. In melanoma cells, PD-1 increased phosphorylation of ribosomal protein S6 (RPS6) as an effector of mammalian target of rapamycin (mTOR) signaling (18). Accordingly, S6 phosphorylation dependent of melanoma-PD-1 could be reversed *via* specific inhibitor of mTOR but not PI3K, demonstrating that PD-1 receptor on surface of melanoma activates downstream mTOR signaling independent of PI3K to promote tumor proliferation (18).

In addition to melanoma, PD-1 expression was also found in hepatoma carcinoma cells (20). In xenografted and genetically engineered orthotopic HCC models, antibody blockade of PD-1 displayed therapeutic effects by enhancing tumor-killing ability of T cells. Mechanistically, anti-PD-1 reduced the expression of PD-1 on T cells and the binding to its ligands PD-L1 or PD-L2 on hepatoma carcinoma cell, promoting the activation of T cells and cytokine production. However, a recent research showed that both HCC cell lines and clinical HCC tissues may contain subpopulations that express PD-1, and HCC cell-intrinsic PD-1 promotes tumor progression even in the absence of an immunological environment [20]. By contrast, PD-1 blockade and knockdown (KD) *in vitro* and *in vivo* inhibited tumor growth independently of adaptive immunity. In these tumor cells, the cytosolic domains of PD-1 was found to interact with the eukaryotic initiation factor 4E and RPS6, promoting the phosphorylation of these mTOR effector proteins (**Figure 1**) (20). The authors also proposed that anti-PD-1 drug may function by both stimulating antitumor immunity and blocking the pro-tumorigenic functions of tumor-intrinsic PD-1 (schematics in **Figure 2**). In support to this proposed model, combination of mTOR inhibitors and PD-1 antibody provided more durable and synergistic tumor regression than either single agent alone, each of which presented only modest efficacy.

BLOCKADE OF PD-1 IN NON-SMALL CELL LUNG CANCER PROMOTED TUMOR GROWTH

Given the significant therapeutic effects displayed by checkpoint blockade therapy, FDA approved several anti-PD-L1 or anti-PD-1 drugs in the treatment of advanced cancers including non-small cell lung cancer (NSCLC) (22, 23). Although many NSCLCs displayed durable response to anti-PD-1 or anti-PD-L1 drugs (7), a recent study by Du et al. reported the expression of PD-1 in NSCLC cells and its potential adverse effects to checkpoint blockade therapy (19). In an NSCLC patient expressing tumor-intrinsic PD-1, the tumor rapidly progressed upon anti-PD-1 therapy for 2 months. In the M109 murine NSCLC cell line, PD-1 overexpression significantly decreased cell viability while PD-1 KD increased cell viability (19). The results suggested that blockade of NSCLC-intrinsic PD-1 released PD-1 from tumor-suppression effects under its interaction with the ligands, promoting the growth of NSCLC (described in **Figure 3**). Although the exact mechanisms remain unclear, the author speculated that it may be due to the complex consequences by the phosphatases that interact with activated PD-1 (19). It suggested the possibility that anti-PD-1 immunotherapy will be rendered less efficacious or even deleterious in some patient and it is very necessary to elucidate the mechanism how cell-intrinsic PD-1 regulates tumor growth and development in different tumors.

IMPLICATIONS IN REFINING COMBINATORIAL IMMUNOTHERAPIES

Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is the clinical target of the first generation of immune checkpoint (24). In March 2011, ipilimumab (anti-CTLA-4) was approved by FDA for treatment of advanced melanoma (25). PD-1 is the clinical target of the second generation of immune checkpoint (26), and then both nivolumab and pembrolizumab have been approved by FDA for treatment of malignant melanoma (7, 9). The existence of tumor cell-intrinsic PD-1 and its tumor-regulatory effects

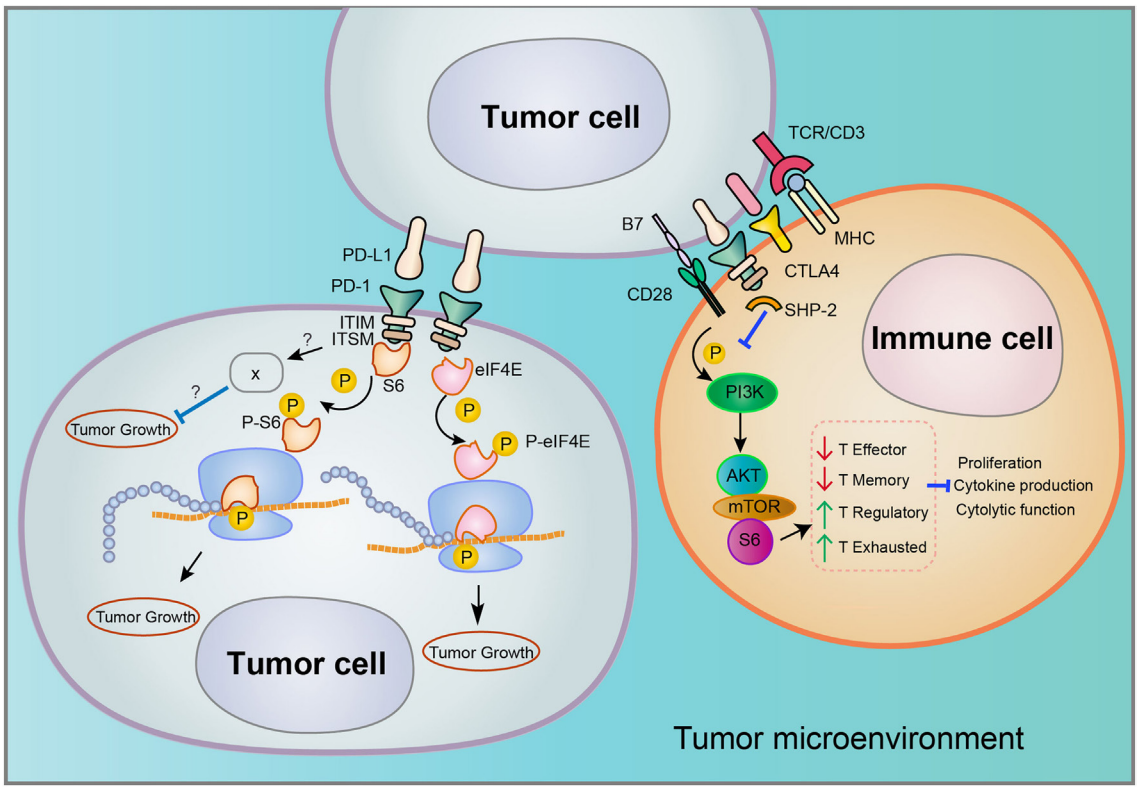

FIGURE 1 | Distinct signaling pathways involved in programmed death 1 (PD-1)/PD-L1 interaction in the tumor microenvironment. Tumor cells may express both PD-L1 and PD-1, but the downstream signaling of PD-1/PD-L1 interaction that occurs in tumor–tumor or tumor–immune cell interfaces may vary considerably. Both the cell type and tumor type may determine the associated signaling pathways.

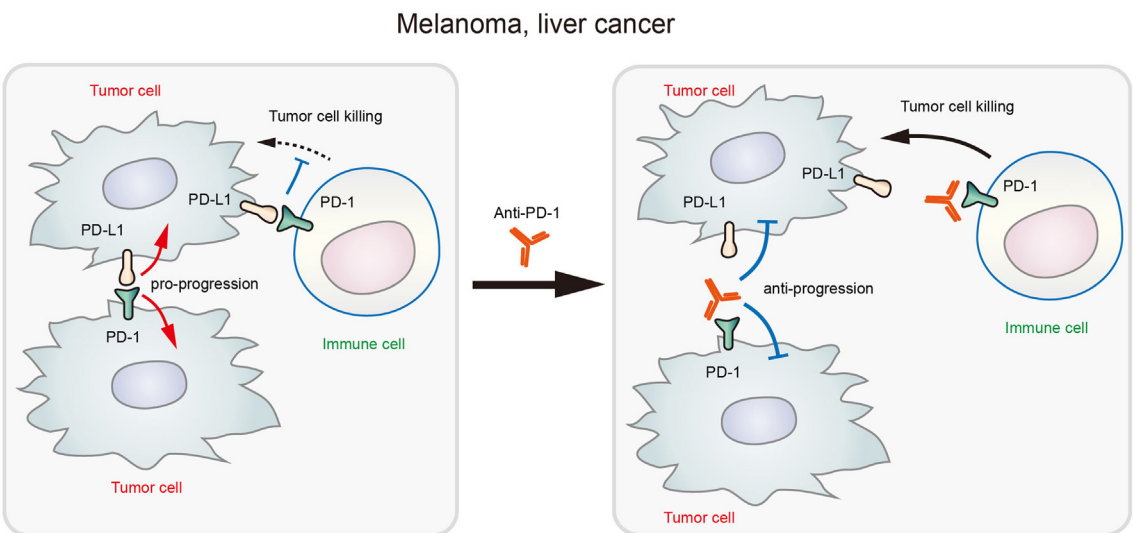

FIGURE 2 | Schematic diagram showing that blockade of tumor-intrinsic programmed death 1 (PD-1) suppresses tumor growth in melanoma and liver cancer.

may explain some baffling question as follows. In addition to tumors with high immunogenicity, PD-1 blockade has also displayed clinical activity in patients with less immunogenic cancers, which have no obvious response to immunotherapy targeting other immune checkpoints (27–30). For instance, patients with advanced melanoma refractory to treatment with

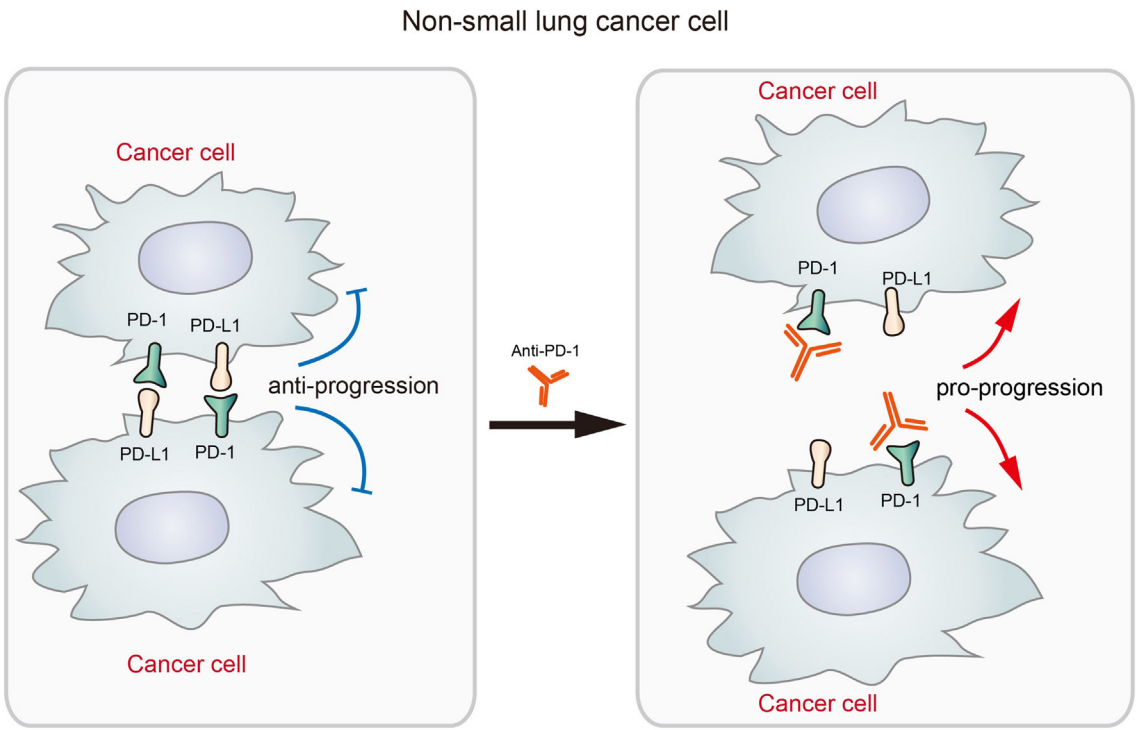

FIGURE 3 | The diagram shows that the blockade of tumor-intrinsic programmed death 1 (PD-1) promotes tumor growth in lung cancer. The interaction between tumor-intrinsic PD-1 and PD-L1 inhibits tumor progression, but the treatment by anti-PD-1 disrupts this inhibitory signaling and promotes tumor progression. This process represents an adverse effect of anti-PD-1 therapy for activating antitumor immunity.

ipilimumab, which is targeting CTLA-4, showed good clinical response to anti-PD-1 therapy (27, 30). It should be noted that the presence of neoantigens and immune-active microenvironment of patients with treatment of PD-1 blockade are similar with patients with CTLA-4-directed checkpoint blockade (31, 32). Moreover, Postow et al. found that PD-1 inhibitors produced greater anticancer activity and fewer immune-related adverse events compared with anti-CTLA-4, ipilimumab (7). These observations collectively suggested that PD-1 antibody may target not only T-cell-specific immune checkpoint functions but also some pro-tumorigenic mechanisms. Since PD-1 is expressed not only on immune cells but also on tumor cells, the superior clinical activity and safety profile of anti-PD-1 compared with anti-CTLA-4 therapy may be due to the additional effects of anti-PD-1 on targeting tumor-intrinsic signaling (7, 9, 28). However, in this context, the principle of precision medicine seems to be crucial, because the roles of tumor-intrinsic PD-1 seem to vary considerably in different tumor types. As PD-1 blockade may promote tumor growth in NSCLC and possibly other cancers, it may be plausible to apply anti-PD-L1 instead of anti-PD-1 for checkpoint blockade, or to combine antiproliferative drugs with anti-PD-1 therapy. In summary, further clarifying the roles of tumor-intrinsic PD-1 may create vast opportunity for refining combinatorial immunotherapy, and extensive efforts are required to precisely define the roles of PD-1 in different cancer types, cell types, and individual cancer cases.

EXPRESSION OF PD-1 IN DIFFERENT CANCER TISSUES AND CELLS

Recent advances in cancer genomic studies have enabled the reanalysis of PD-1 expression in different cancer types. By obtaining and preprocessing the mRNA expression data from The Cancer Genomic Atlas (TCGA) project (33), we have summarized the expression of PD-1 in the tissues of 17 cancer types (**Figure 4A**). Interestingly, most tumor types contain a number of cases expressing higher levels of PD-1 mRNA (above the SD as shown in the box plot). However, considering that cancer tissues may contain infiltrating immune cells, the microarray data from TCGA may not accurately reflect the PD-1 mRNA level in tumor cells. Thus, we reanalyzed the expression of PD-1 mRNA in Cancer Cell Line Encyclopedia (CCLE) dataset, which was based on cultured cancer cell lines and thus without the suspect of immune cell contamination. This analysis including 22 tumor types and 617 cell lines revealed unified low expression of PD-1 in only a few cancer types such as glioma (central nervous system), thyroid cancer, and prostate cancer (**Figure 4B**). But comparing to these tumors, most tumor types exhibited higher expression levels and greater variability in PD-1 expression ($P < 0.001$, *t*-test, highlighted in red font in **Figure 4**). To further analyze the PD-1 protein expression in different cancers, we also summarized the immunohistochemical (IHC) staining results in the Human Protein Atlas (34). Although the IHC results may be affected by

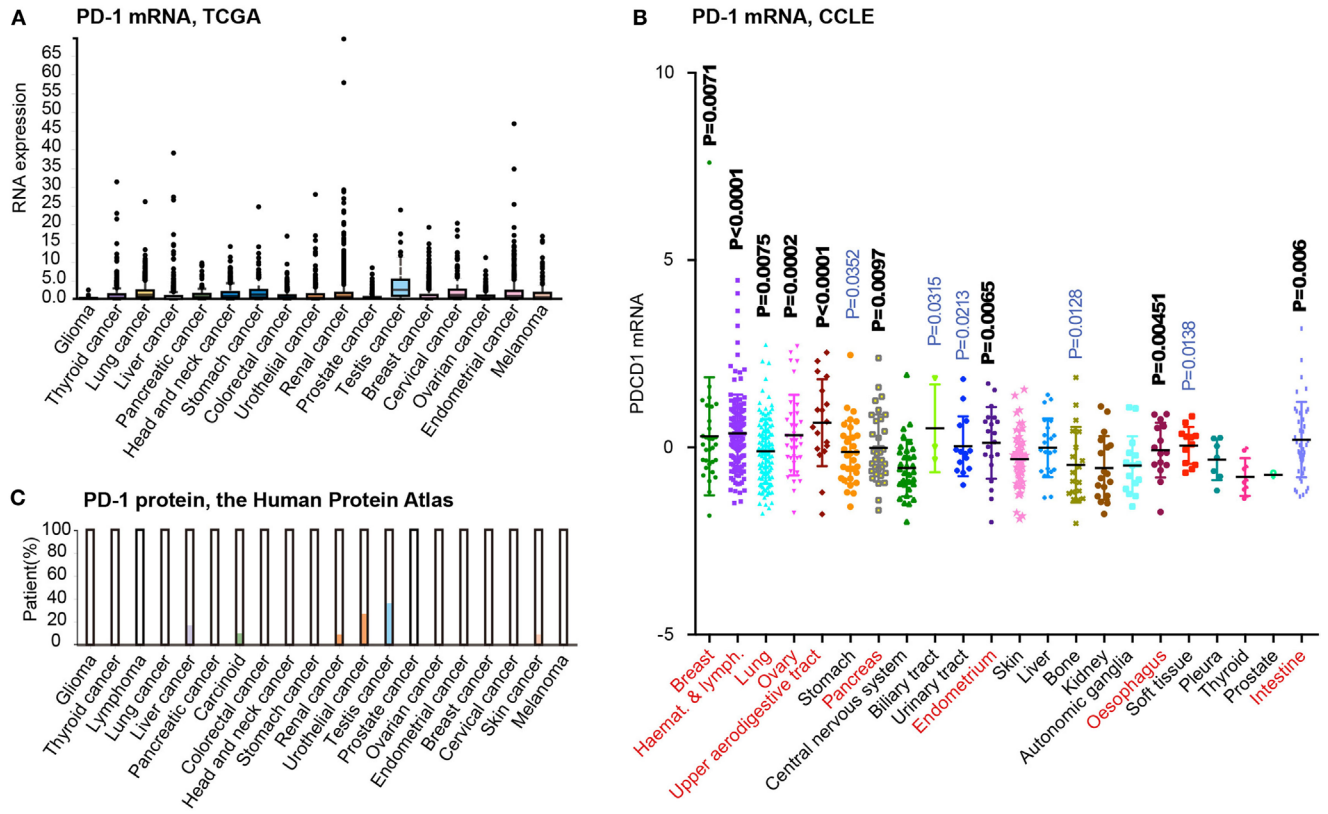

FIGURE 4 | Tumor-intrinsic programmed death 1 (PD-1) expression is widespread in many tumor types. **(A)** PD-1 mRNA expression level analysis in different cancer tissues from the human protein atlas. **(B)** PD-1 mRNA expression level analysis in various kinds of cancer cells based on related data from Cancer Cell Line Encyclopedia (CCLE). **(C)**. PD-1 protein expression level analysis in different cancer tissues using immunohistochemical staining from the human protein atlas. All cancer types were compared to "Central nervous system" tumors by t-test (P-values indicated). Tumors displaying highly significant differences ($P<0.01$) are marked by red font.

more factors (e.g., the specificity/sensitivity of the antibody, the view field selected for analysis, and the quantification approaches, etc.), this approach enabled selective analysis on the expression of PD-1 protein tumor cells. The results suggested that liver cancer, carcinoid, renal cancer, urothelial cancer, testis cancer, and melanoma (skin cancer) have subgroups of tumor cells with positive PD-1 staining (**Figure 4C**). The driving force of PD-1 expression in tumor cells is unknown, but we speculate that multiple factors may be involved, such as gene copy number alterations, epigenetic alterations, and microenvironment, etc. According to the CCLE dataset, PD-1 is amplified in some tumor cell lines, with potential effects on PD-1 mRNA expression. In addition, the cytokines and immune cells in the tumor microenvironment may also participate in the induction of tumor PD-1 expression. Although there is yet no evidence that PD-1 may be transferred from leukocytes to tumor cells, it certain deserves in-depth studies in the future.

Taken together, the analysis of PD-1 expression at both mRNA and protein levels prompted us that PD-1 expression may be more prevalent than the reported three tumor types. Therefore, understanding the roles of cancer cell-intrinsic PD-1 may have broad implications in the refinement of combinatorial immunotherapies.

CONCLUSION

Although the roles of PD-1 in leukocytes have been well established, the expression of PD-1 in tumor cells has been less characterized, with the potential functions largely unclear. When the expression of PD-1 on melanoma cells was reported in 2015, it was not considered as a widespread mechanism. But after the recent studies on the expression and roles of PD-1 in liver cancer and lung cancer, the prevalence and functional importance of tumor-intrinsic PD-1 has attracted the attentions of more researchers. Although accumulating evidence suggests that mTOR signaling may play a role in this scenario, our understanding on the biological roles and therapeutic implications of tumor-intrinsic PD-1 remain very limited. Future in-depth investigations on tumor-intrinsic PD-1 may provide additional insights into the unexpected effects of checkpoint blockade therapies and benefit the development of more effective combinatory immunotherapies.

AUTHOR CONTRIBUTIONS

HY and JX wrote the manuscript, generated the schematics and analyzed data. HW, CL and JYF contributed to the revision of the manuscript.

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frontiers in Immunology

REVIEW
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Combining Immune Checkpoint Inhibitors: Established and Emerging Targets and Strategies to Improve Outcomes in Melanoma

*Duaa O. Khair[1], Heather J. Bax[1,2], Silvia Mele[1], Silvia Crescioli[1], Giulia Pellizzari[1], Atousa Khiabany[1], Mano Nakamura[1], Robert J. Harris, Elise French[1], Ricarda M. Hoffmann[1,2], Iwan P. Williams[1], Anthony Cheung[1,3], Benjamin Thair[1], Charlie T. Beales[1], Emma Touizer[1], Adrian W. Signell[1], Nahrin L. Tasnova[1], James F. Spicer[2], Debra H. Josephs[1,2], Jenny L. Geh[4], Alastair MacKenzie Ross[4], Ciaran Healy[4], Sophie Papa[2], Katie E. Lacy[1] and Sophia N. Karagiannis[1]**

[1] St. John's Institute of Dermatology, School of Basic & Medical Biosciences, Guy's Hospital, King's College London, London, United Kingdom, [2] School of Cancer & Pharmaceutical Sciences, Guy's Hospital, King's College London, London, United Kingdom, [3] Breast Cancer Now Research Unit, School of Cancer & Pharmaceutical Sciences, Guy's Cancer Centre, King's College London, London, United Kingdom, [4] Department of Plastic Surgery at Guy's, King's, and St. Thomas' Hospitals, London, United Kingdom

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Edited by:
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Martin Luther University of Halle-Wittenberg, Germany

Reviewed by:
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Daniel Olive,
Aix Marseille Université, France

***Correspondence:**
Sophia N. Karagiannis
sophia.karagiannis@kcl.ac.uk

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The immune system employs several checkpoint pathways to regulate responses, maintain homeostasis and prevent self-reactivity and autoimmunity. Tumor cells can hijack these protective mechanisms to enable immune escape, cancer survival and proliferation. Blocking antibodies, designed to interfere with checkpoint molecules CTLA-4 and PD-1/PD-L1 and counteract these immune suppressive mechanisms, have shown significant success in promoting immune responses against cancer and can result in tumor regression in many patients. While inhibitors to CTLA-4 and the PD-1/PD-L1 axis are well-established for the clinical management of melanoma, many patients do not respond or develop resistance to these interventions. Concerted efforts have focused on combinations of approved therapies aiming to further augment positive outcomes and survival. While CTLA-4 and PD-1 are the most-extensively researched targets, results from pre-clinical studies and clinical trials indicate that novel agents, specific for checkpoints such as A2AR, LAG-3, IDO and others, may further contribute to the improvement of patient outcomes, most likely in combinations with anti-CTLA-4 or anti-PD-1 blockade. This review discusses the rationale for, and results to date of, the development of inhibitory immune checkpoint blockade combination therapies in melanoma. The clinical potential of new pipeline therapeutics, and possible future therapy design and directions that hold promise to significantly improve clinical prognosis compared with monotherapy, are discussed.

Keywords: checkpoint inhibitors, combination immunotherapy, immunooncology therapeutics, melanoma, CTLA-4, PD-1, PD-L1, antibody engineering

INTRODUCTION

Immune-mediated destruction of tumors has long been considered a potential route of therapeutic intervention. Partial spontaneous regression of melanoma lesions has previously been associated with the presence of endogenous tumor infiltrating lymphocytes (TILs) and the presence of TILs in patient samples has been shown to correlate with improved clinical outcomes and better prognosis (1). Infusion with CD8+ TILs has been reported to induce some responses in patients when combined with other treatments including IL-2 (2). Immunotherapy via cytokine infusion has also been extensively trialed, with IL-2, IL-12, and IFNα2b to activate T cells, showing anti-tumor effects in pre-clinical models and clinical trials, with IL-2 and IFNα2b approved for clinical use (3, 4). Cytokine treatments have however been associated with severe adverse effects resembling severe systemic infections and sometimes resulting in toxic shock or capillary leak syndrome as reported in randomized clinical trials (5, 6). Though not without challenges, these trials confirmed the possibility of reigniting components of the immune system as a cancer therapy.

Increased understanding of tumor evolution and the complex interactions in the tumor microenvironment (TME) has revealed numerous mechanisms by which tumors may escape immune destruction and actively suppress immune activity (7). Immunosuppression by tumor cells may partially be mediated through FoxP3+ regulatory T cell (T-reg) recruitment via tumor-secreted chemokines as shown in an *ex vivo* study (8, 9). Critically, tumor resident T-reg can highly express cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), an important checkpoint that acts as a negative regulator of effector T cell (T-eff) activity *in vivo*, studied in different models including CTLA-4-deficient mice (10) (**Figure 1**). Suppression may also be mediated by tumor expression of the Programmed-death ligand 1 (PD-L1; B7-H1; CD274), known to trigger T cell apoptosis *in vivo* in mouse tumors (11) and to promote formation of FoxP3+ T-regs upon interaction with the T cell-associated checkpoint receptor Programmed-death 1 (PD-1, also known as CD279) (12) (**Figure 1**). These checkpoints, have become therapeutic targets in immune checkpoint blockade therapy, with the aim of overcoming TME-mediated immunosuppression and restoring anti-tumor immune activity (13). Monoclonal antibodies targeting CTLA-4 and PD-1 have now been approved for the treatment of melanoma. These new therapeutic modalities were developed in parallel with targeted MAPK pathway inhibitor therapies, such as vemurafenib and dabrafenib, approved for a subset of melanomas bearing point mutations in the kinase BRAF (e.g., $BRAF^{V600E}$), and the MEK inhibitors trametinib and cobimetinib, all designed to cause cancer cell death via interruption of the MAPK pathway (**Table 1**). Together, these agents have led to an increase in medial survival for advanced melanoma from 9 months in 2010 to over 3.5 years.

While CTLA-4 and PD-1 blockade has proved successful in improving survival rates, many patients do not respond or develop resistance to these interventions. Alongside combinations of checkpoint inhibitors already in clinical use, research into new checkpoints as therapeutic targets has shown promise in pre-clinical and clinical studies, either alone or combined with established agents. Focusing on malignant melanoma as the tumor type for which the first pivotal immunotherapy breakthroughs were demonstrated, in this review, we discuss current and future checkpoint blockade and other immunooncology combination therapies, and the rationale for potential synergistic effects (**Table 2**).

THERAPIES TARGETING CTLA-4 AND PD-1

Anti-CTLA-4 Monotherapy

CTLA-4 is a CD28 homolog expressed constitutively on the surface of both T-reg cells and activated T cells (14). CTLA-4 binds to CD28 co-receptors CD80/60 with a higher affinity and avidity than CD28, thus superseding positive CD28 signaling and thus allowing for inhibition of T cell activation (15, 16). In order to function effectively as an immune checkpoint via endocytosis CTLA-4 is not only able to competitively inhibit T cell co-stimulation but can also clear CD28 ligands CD80/CD86 from the surrounding cells including APCs by trans-endocytosis *in vivo* (17). Physiologically, CTLA-4 has been shown *in vitro* and in mouse models *in vivo,* to suppress T cell responses including activation, proliferation, and pro-inflammatory cytokine production (IFN-γ and IL-2) by antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages (18) (**Figure 1**).

Studies on cells expressing human CTLA-4 in murine models of melanoma, that investigated antibodies aimed at blocking CTLA-4 checkpoints, have documented effects such as enhanced T-eff function, inhibition of T-reg activity and selective depletion of T-reg cells via antibody Fc binding of Fcγ-receptors on atypical macrophages in tumor lesions (19, 20). Hypotheses that CTLA-4 blockade could enhance anti-tumor response were tested by a pre- clinical study using transplantable murine melanoma cell lines, demonstrating that CTLA-4 inhibitors induced rejection of melanoma (21). *Ex vivo* studies of peripheral blood mononuclear cells (PBMCs) and matched melanoma metastases from patients with melanoma treated with ipilimumab have shown evidence that ipilimumab also works by depleting T-reg cell populations by antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by CD16 (FcγRIIIA)-expressing, nonclassical monocytes. In the same study, patients who responded to ipilimumab treatment had higher ratios of intratumoral CD68-expressing vs. CD163-expressing macrophages before treatment and lower T-reg infiltration after treatment (22). Clinical trials involving ipilimumab have demonstrated a dose-dependent response to the antibody in late-stage melanoma patients, with pooled analysis consistently showing improved survival in patients with metastatic disease above historical controls (23, 24). By blocking this key immune escape mechanism, overall survival rates for ipilimumab were significantly improved, alone or in combination with a glycoprotein 100 peptide (GP-100) vaccine when compared to vaccine alone (15, 25). Ipilimumab, a fully humanized IgG1 antibody, was the first anti-CTLA-4 treatment approved by FDA in 2011 (**Table 1**).

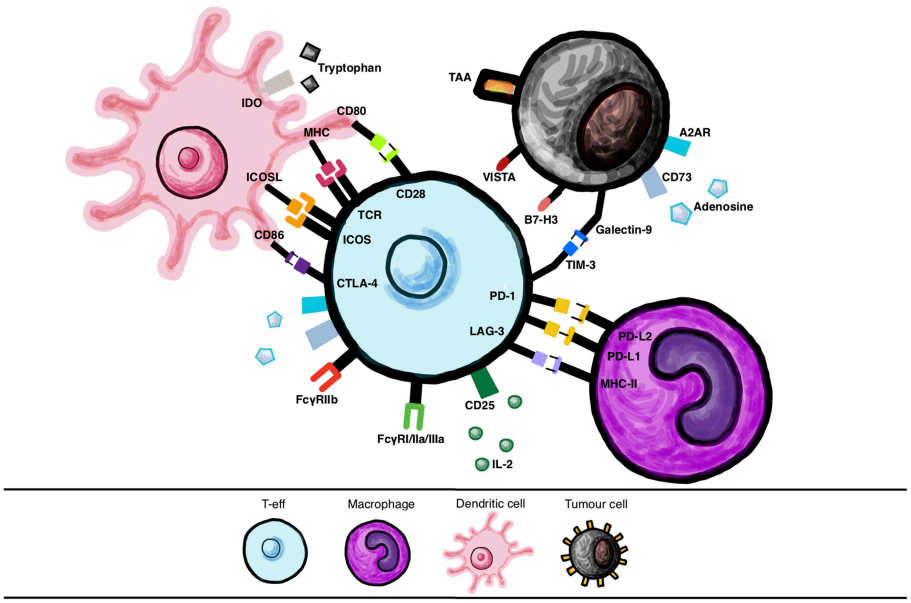

FIGURE 1 | Immune cell interactions via checkpoint molecules and their ligands. Various interactions between checkpoint molecules and their ligands expressed by different cells, such as immune cells (dendritic cells (DC)s, T-effector cells (T-eff), macrophages) and between T-eff and tumor cells, that may be targeted with therapy.

Anti-PD-1 Monotherapy

Another immune checkpoint, the programmed death 1 (PD-1) immunoglobulin-based receptor predominantly expressed on activated, antigen-educated T cells can recognize two ligands, PD-L1 and PD–L2 (B7-DC; CD273). PD-L1 is expressed broadly across many cell types, including leukocytes and tissue cells, whereas PD-L2 expression is limited and specific to expression on immune cells: antigen presenting and stromal cells. Ligation of PD-1 to PD-L1 causes phosphorylation and activation of SHP-2, a phosphatase that can inactivate many downstream molecules in TCR signaling (26). *In vitro* and *in vivo* studies in mouse models of cancer showed that PD-L1 can also enhance the generation of peripherally induced T-regs, (iT-reg), increasing Foxp3 expression and sustaining their immunoregulatory actions such as suppression of $CD4^{+}$ T-eff cells (27). The co-stimulatory molecule CD28 of which CTLA-4 is a homolog, is also preferentially targeted by PD-1-mediated dephosphorylation (28). By this mechanism, PD-1 mediates two immune checkpoints, by reducing immune hyperstimulation via PD-L1 and maintaining tolerance in lymphoid tissues via PD-L2. Both ligands PD-L1 and PD-L2 can also be induced by cytokine signaling during inflammation (29).

PD-L1 expression on tumor cells is often upregulated, resulting in inhibition of T cell responses (15). In melanoma, the expression of PD-L1 may be prognostic, and could correlate with Breslow thickness (30). Mouse melanoma metastasis to the liver was shown to be impaired in PD-1-deficient mice and anti-PD-1 monoclonal antibody administration could inhibit the spread of tumor cells via recruitment of T-eff (31) by blocking the interaction of PD-1 with its ligands (14). Anti-PD-1 and anti-PD-L1 blocking strategies produce different immunologic effects as anti-PD-L1 has effects on more than one pathway. PD-L1 signals negatively to T cells by interacting with both CD80/CD86 and PD-1 (32) preventing both pathways, without interacting with PD-L2, which activates T cell response by producing co-stimulatory signals (32). Anti-PD-L1 studies demonstrated temporary arrest of the growth of melanoma cells in mouse models (33). Following the approval of ipilimumab, the anti-PD-1 monoclonal antibodies nivolumab and pembrolizumab gained FDA approval in 2014 (34) and EMA approval in 2015, following trials showing significantly improved patient outcomes (**Table 1**) (35). Nivolumab is a fully human IgG4 monoclonal antibody which was shown to improve median overall survival to 8.9 months compared to 6.8 months in patients treated with dacarbazine in a phase III study involving patients with previously untreated melanoma (36). Studies into metastatic melanoma have shown superior overall survival of 1 year (72.9 to 42.1%) and better objective response rate (40 to 13.9%) with nivolumab plus dacarbazine compared to dacarbazine plus placebo (36). Pembrolizumab, a humanized IgG4 monoclonal antibody has also shown similar efficacy to nivolumab, with one phase III study reporting increased long-term survival rates when compared to ipilimumab in patients with unresectable melanoma (37).

Comparing Anti-CTLA-4 and Anti-PD-1 Therapies and Toxicity in the Clinic

Both anti-CTLA-4 and anti-PD-1 therapies aim to restore T cell effector function in the TME and establish immune dominance over tumors. Overall, these immune checkpoint blockade monotherapies have generated significant improvements in patient outcomes against traditional dacarbazine therapy, with anti-PD-1 blockade seemingly more effective.

TABLE 1 | Approved targeted, antibody and other immunotherapies and combination treatments for malignant melanoma.

Drug	Target	Mechanism	Indication	Approval
MONOCLONAL ANTIBODY MONOTHERAPIES				
Ipilimumab	CTLA-4	Human IgG1 monoclonal antibody blockade	Advanced unresectable metastatic melanoma	2011
Nivolumab	PD-1	Human IgG4 monoclonal antibody blockade	Advanced metastatic melanoma ± refractory to ipilimumab	2014* *2017: Approved as adjuvant treatment for melanoma with involvement of lymph nodes or for patients with metastatic disease who have undergone complete resection
Pembrolizumab	PD-1	Humanized IgG4 monoclonal antibody blockade	Unresectable melanoma—stage III/IV	2014
COMBINED MONOCLONAL ANTIBODY THERAPIES				
Ipilimumab + nivolumab	CTLA-4 + PD-1	Monoclonal antibody blockade	Unresectable melanoma—stage III/IV PD-L1 negative	2015
TYROSINE KINASE INHIBITOR MONOTHERAPIES				
Vemurafenib	BRAF	BRAF inhibitor causing programmed cell death via interruption of MAPK pathway	Unresectable $BRAF^{V600}$ mutant melanoma	2011
Dabrafenib	BRAF inhibitor	BRAF inhibitor causing programmed cell death via interruption of MAPK pathway	Unresectable $BRAF^{V600}$ mutant melanoma (not wild-type)	2013
Trametinib	MEK inhibitor	MEK1 and 2 inhibitor causing cell death via interruption of MAPK pathway	Unresectable $BRAF^{V600E/K}$ mutant melanoma (not to be used post BRAF inhibitor)	2013
COMBINED TYROSINE KINASE INHIBITOR THERAPIES				
Dabrafenib + trametinib	BRAF + MEK	BRAF+MEK inhibition	Unresectable $BRAF^{V600E/K}$ mutant melanoma	2013
Vemurafenib + cobimetinib	BRAF + MEK	BRAF+MEK inhibition	$BRAF^{V600}$ mutant melanoma	2015** **2018: Approved as adjuvant treatment for patients with nodal involvement and following complete resection
OTHER TARGETED AND IMMUNE THERAPIES				
Interferon	IFNα2b	Systemic IFNα2b administration results in immunostimulatory effects including an increase in tumor-infiltration, decrease in circulating T-regs and modulation of STAT1/STAT3 balance	Adjuvant therapy for stage III melanoma (cancer free but at high risk of recurrence) Adjuvant therapy for stage IIB or IIC melanoma with primary lesions >4mm thickness	1995
Aldesleukin	IL-2	Systemic IL-2 administration promotes T cell proliferation and stimulates CD8 and NK cell cytotoxicity	Metastatic melanoma	1998
T-VEC	Oncolytic herpes simplex virus	Local and direct infection and killing of tumor cells	Unresectable stage IIIB, IIIC or IV melanoma	2015

In 173 patients with advanced melanoma unresponsive to ipilimumab treatment, the overall response rate (ORR) to pembrolizumab was 26%, with the most severe adverse event (AE) reported as grade 3 fatigue in 5 patients (38). In comparison, CTLA-4-blockade treatment-associated toxicity has not been insignificant; 60% of patients treated with ipilimumab experienced adverse immune effects, which were severe (grade 3 or 4) in 10–15% of cases (25). Head-to-head trials of pembrolizumab vs. ipilimumab have shown 47.3 and 26.5% 6-month progression-free-survival (PFS) rates respectively, with AEs grade 3 or higher at rates of 19.9 and 13.3% (39). Nivolumab was shown to be effective in a range of cancers, producing a 28% ORR in melanoma and grade 3 or 4 drug- related AEs occurred in 14% of 296 patients across all groups (including three deaths from pulmonary toxicity) (40). Research in one study demonstrated 1-year and 2-year survival rates of 62 and 43%,

TABLE 2 | Proposed mechanisms of action of selected immune checkpoint blocking agents.

Agent	Specificity	Mechanism
Ipilimumab	CTLA-4	• Inhibits coinhibitory checkpoint molecule CTLA-4 on T cells by preventing CD80/CD86 binding on APCs • Enhances T-eff activation and T-reg inhibition
Pembrolizumab/ nivolumab	PD-1	• Inhibit coinhibitory checkpoint molecule PD-1 on antigen-educated T cells, preventing PD-L1-APC binding • Enhances T-eff activation • Reduces T-eff anergy
BMS-956559/PDR001	PD-L1	• Inhibits coinhibitory checkpoint molecule PD-L1 on APCs and melanoma cells preventing its binding to PD-1 and CD80/CD86 on T cells • Enhances T-eff activation • Reduces T-eff anergy
LAG525	LAG-3	• Inhibits coinhibitory checkpoint molecule LAG-2 on activated T cells preventing binding to MHC-II on DCs, pDC and melanoma cells • Enhances T-eff and myeloid cell responses (DCs, macrophages and NK cells
CP1-444	A2AR	• Inhibits coinhibitory checkpoint molecule A2AR molecule on myeloid cells preventing binding to extracellular adenosine released by CD73 on T-reg and melanoma cells • Enhances myeloid cell response • Reduces T-reg cell response
MBG453	TIM-3	• Inhibits coinhibitory checkpoint molecule TIM-3 on T cells preventing to binding on galactin-9 on immune and melanoma cells and HMG-B on immune cells • Prevents T cell exhaustion • Enhances nucleic acid recognition within endosomes
MGA271	B7-H3	• Inhibits coinhibitory checkpoint molecule B7-H3 on APCs and melanoma cells preventing ligand binding on T cells • Enhances T-eff activation and T-reg inhibition
CA-170	VISTA	• Inhibits coinhibitory checkpoint molecule VISTA on myeloid cells and naïve T cells preventing binding to VSIG-3 • Enhances T-eff activation and T-reg inhibition
Epacadostat	IDO	• Inhibits coinhibitory checkpoint molecule IDO on alternatively activated macrophages, T-regs and melanoma cells preventing the conversion of tryptophan to kynurenines on T cells • Enhances NK/ T-eff activation and T-reg inhibition
N/A	PKC-η	• Inhibits coinhibitory signaling molecule PKC-η on T-regs preventing its induction of anti-inflammatory cytokine transcription • Enhances T-eff activation and T-reg inhibition

respectively (35). Although both therapies have been successful in treating many cases, anti-PD-1 have thus far been more efficacious than anti-CTLA-4 monoclonal antibodies and have fewer adverse drug reactions. This difference may be because PD-1 is expressed on mature T cells; PD-L1 is expressed on antigen-presenting cells such as DCs and macrophages, and other immune cells as well as on tumor cells, while CTLA-4 is widely expressed on T cells across the body including those circulating in lymph nodes and skin. CTLA-4 inhibitor-mediated anti-tumor activity may therefore extend to secondary lymphoid organs rather than only within the TME (13). This wide expression distribution may potentially result in the disruption of other immune-regulating mechanisms and triggering of autoimmune-like events, consistent with toxicities observed in the clinic with anti-CTLA-4 antibody treatment.

Rationale and Pre-clinical Evidence for Checkpoint Blockade Combination Therapy

While checkpoint inhibitor monotherapy provides significant benefits, this is generally only the case in subsets of patients. CTLA-4 and PD-1 are not functionally redundant, acting at different locations and times in the generation of T-eff (41). This may mean that combination therapies may act in a complementary or even synergistic fashion.

Checkpoint blockade therapies are also known to be subject to various forms of resistance mechanisms. Anti-CTLA-4 blockade primary resistance has been shown to correlate with a loss of IFN-γ signaling genes *in vitro* and clinically in patients who had poor clinical responses to ipilimumab therapy (42). Furthermore, in patients, PD-L1 expression on circulating CD4+ T CD8+ T cells may be predictive of resistance to anti-CTLA-4 treatment, providing a potential rationale for combination with PD-1/PD-L1 blockade therapy (43). Studies in murine models and patients receiving anti-PD-1 treatment also point to key roles of infiltrating myeloid cells and their signaling pathways, as well as upregulation of alternative immune checkpoints such as T cell immunoglobulin mucin-3 (TIM-3), all associated with resistance to checkpoint inhibition (44, 45). Checkpoint inhibitor monotherapy has been shown to trigger activation of compensatory T cell-associated checkpoints. Pre-clinical evidence supports a rationale for combination therapy as, by blocking more than one of these pathways, including PD-1, LAG-3, and CTLA-4, may reduce tumor growth (46, 47). Murine studies demonstrated that B16 melanoma cell rejection in mice was improved by combined anti-CTLA-4 and anti-PD-1 antibody therapies (47). The results indicated that combination therapy was more than twice as effective as monotherapy in terms of B16 melanoma rejection by increasing T cell infiltration and the presence of T-eff in the TME; IFN-γ and other pro-inflammatory cytokines were observed to be upregulated, producing an inflammatory rather than immunosuppressive TME (47). Further pre-clinical studies suggested that anti-CTLA-4 and anti-PD-1 therapies may have synergistic effects, increasing the numbers of TILs, reducing T-reg and retarding tumor growth (48). Certain subgroups of patients, such as elderly patients, tend to respond better to anti-PD-1 agents, a phenomenon which may be attributed to a depletion of the number of T-regs in older patients (49). These findings, taken together with studies suggesting that anti-CTLA-4 treatment can reduce CTLA-4-expressing T-regs, may lend

further merit to stratifying appropriate patient groups to receive combination therapies (20, 22).

Therefore, since CTLA-4 and PD-1 inhibitors exert anti-tumor effects through different mechanisms of action, combining these agents could potentially lead to more efficacious treatments. Furthermore, blockade of one pathway resulted in increased activity and an upregulation of other inhibitory pathways, an effect that could perhaps be mitigated by combined therapy. Emerging evidence provided a rationale for the development of combined blockade regimens as well as acceleration of research into further blockade targets. Although toxicity profiles especially those associated with CTLA-4 blockade use may be an important concern in combined therapies, pre-clinical and clinical findings into the efficacy of combining checkpoint blockade antibodies showed encouraging results in melanoma.

Combined CTLA-4 and PD-1 mAb Therapies in the Clinic

In the first clinical trial investigating the efficacy and safety of combined checkpoint blockade antibodies published in 2013 (50), 53 patients with melanoma were treated concurrently with nivolumab and ipilimumab, while 33 patients received only ipilimumab. In the double therapy group the ORR was 40% for combination treatment and the ORR was 20% in the monotherapy group. However, drug-related toxicity was higher in the combined therapy group; 53% of patients experienced grade 3 or 4 AEs compared to 18% in the monotherapy group. These drug related reactions were managed with medications.

Prolonged PFS was also reported in a phase II dose-escalation study of combined nivolumab and ipilimumab in 142 patients (51). ORRs in the combination and ipilimumab alone groups were 61 and 11%, respectively with drug-related AEs of grade 3 or 4 were exhibited by 54 and 24% of the patients, respectively (51). These drug-related AEs were also managed by immune modulation drug intervention. Follow up on these patients (median 24.5 months) showed a 63.8% 2-year overall survival for the combination group compared to 53.6% for ipilimumab alone (51). This clearly indicated the benefit of combined therapy and its longevity.

The registration phase III Checkmate 067 trial randomized 945 previously-untreated patients to nivolumab, ipilimumab and the two combined drugs, showing a median PFS of 6.9 months, 2.9 months and 11.5 months and 3-year overall survival (OS) rates of 52, 34, and 58%, respectively (52). This large well powered trial confirmed the superior efficacy of combination therapy and nivolumab monotherapy when compared to ipilimumab monotherapy as PFS was consistently longer for patients taking preparations that included nivolumab; this included subgroups categorized by PD-L1 or BRAF mutation status and metastasis stage (53). For patients with BRAF mutations the PFS was 11.7 months (11.2 months for those with wild-type BRAF). Positive PD-L1 status patients fared better with a median PFS of 14 months in both the nivolumab mono and dual therapy groups compared to only 3.9 months for patients taking ipilimumab alone. Checkmate 067 also reported higher rates of complete response in patients on a combined regimen (11.5% compared with 8.9% for nivolumab alone and only 2.2% for ipilimumab alone). Tumor burden change (a parameter which can be used for predicting treatment response) was also significantly higher in the combination group;−51.9% compared with−34.5% and 5.9% for nivolumab alone and ipilimumab alone, respectively.

Furthermore, treatment-related adverse events, as reported in the Checkmate 067 trial comparing ipilimumab, nivolumab and the two combined, revealed higher rates of toxicity associated with the combination therapy (96% compared with 86% in both monotherapy regimens) and this also more frequently led to discontinuation of treatment in the combined group than either monotherapy group. Grade 3 or 4 AEs occurred in 59% of patients given dual therapy compared with 21 and 28% of patients on nivolumab or ipilimumab alone, respectively, and these grade 3 or 4 adverse reactions were most frequently gastrointestinal in nature. Side effects were managed with established safety guidelines and usually resolved within 3–4 weeks and the 3-year survival rate for patients who discontinued treatment was 67% (52). These indicate that combined treatment elicited higher rates of toxicity than either monotherapy and that benefit from dual therapy was conferred despite discontinuation of treatment. Median survival in patients with PD-L1-positive tumors was the same in both the combination and nivolumab alone groups. This may reflect T cell infiltration enhanced by ipilimumab, thus favoring a TME that may be amenable to anti-PD-1 agent action (54).

Previous studies of anti-PD-1 monotherapies have suggested that efficacy was higher in patients whose tumors expressed PD-L1 at levels $\geq$5%, compared with those whose tumors showed lower expression (54). Where the patients were PD-L1-negative however, another study demonstrated that PFS was longer in the combination group (11.2 months) than in the nivolumab alone group (5.3 months) (53). Overall studies to-date support these combination therapies, which appear to benefit patients with low PD-L1 tumor expression.

Studies have also successfully treated melanoma with combined checkpoint blockade regimens where the two antibodies were administered sequentially. A phase III study of two groups of patients with unresectable stage III or IV melanoma investigated patients treated with nivolumab then ipilimumab ($n = 68$), or vice versa ($n = 70$); with nivolumab used as maintenance therapy for both groups until toxicity or disease progression (55). Toxicity was comparable between the groups, however, the nivolumab/ipilimumab exhibited a greater 12-month survival (76%) compared with its counterpart cohort of patients who received the treatments in reverse.

Reducing the dose of ipilimumab in combination with PD-1 directed therapy has resulted in lower combined therapy toxicity rates. Nivolumab with low-dose ipilimumab has been approved in some jurisdictions for the treatment of renal cell carcinoma where toxicity rates have reportedly been reduced compared with combined therapies with higher doses of ipilimumab (56). Research has suggested pembrolizumab as an alternative to nivolumab in a combined regimen with ipilimumab (57). A phase I study, investigating the safety of combining standard-dose (2 mg/kg) pembrolizumab with low-dose (1 mg/kg reduced from 3

mg/kg) ipilimumab, was not powered to examine efficacy (although it suggested comparable level of efficacy to a nivolumab/ipilimumab combination) (57). However, the study demonstrated a more manageable toxicity profile with the pembrolizumab preparation (57).

In summary, the first phase I trials demonstrated higher efficacy than previous monotherapy regimens in small patient cohorts, with a concurrent increase in drug-related toxicity (58). Phase III trials have confirmed these findings showing improved outcomes for advanced-stage melanoma patients when treated with both anti-CTLA-4 and anti-PD-1 therapeutics, which showed benefits for patients with PD-L1 negative tumors (53). Significant toxicity associated with dual therapy limits its usage in patients with comorbidities. However, improved clinical outcomes led to the FDA approval of the combination of nivolumab and ipilimumab for the treatment of unresectable stage III/IV PD-L1 negative melanoma (**Table 1**). In 2018 the EMA adopted a positive opinion recommending that nivolumab in combination with ipilimumab is indicated for the treatment of unresectable or metastatic melanoma in adults.

OTHER POTENTIAL IMMUNE CHECKPOINT THERAPY TARGETS AND COMBINATIONS WITH ESTABLISHED CHECKPOINT INHIBITORS

As research into therapies utilizing CTLA-4 and PD-1/PD-L1 blockade to treat melanoma has advanced, further targets have been sought out in an effort to overcome issues such as incomplete tumor regression or relapse following treatment. A concerted effort is underway focusing on inhibitory molecules whose mechanisms may operate within the TME and could have complementary functions to those of approved immunotherapies. Studies in the circulation and tumors of patients with melanoma reveal that the TME promotes T cell, exhaustion demonstrated by upregulation of markers of immunosenescence, thereby allowing T cell impairment and immune escape (59). These markers represent targets for immunotherapy to counteract the immune escape of cancer cells. Targeted treatments combining anti-CTLA-4 or anti-PD-1/PD-L1 alongside blockade of novel checkpoints have the potential to produce comparable effects, perhaps with fewer adverse drug reactions than those of the dual anti-CTLA-4/anti-PD-1 regimens.

LAG-3/PD-L1 Blockade

The lymphocyte-activation gene 3 (LAG-3) is a co-inhibitory receptor known primarily to be expressed on exhausted TILs which have less potent effector functions (60, 61). LAG-3 may downregulate T cell responses via interaction with major histocompatibility complex class-II (MHC-II) on DCs (61) (**Figure 1**). Preclinical studies have shown that, as a result of persistent melanoma antigen expression, LAG-3 expression on TILs is increased, thereby inhibiting T cell action and reducing IFN-γ production within the TME under the influence of PD-1 co-stimulation (61). It has been hypothesized that LAG-3 blockade might produce milder side effects than those observed with checkpoint inhibitors currently in clinical use. Autoimmunity developed by *Lag3*$^{-/-}$*Pdcd1*$^{-/-}$ mice (deficient in LAG-3 and PD-1) was slower: approximately 10 weeks compared with 3–4 weeks in CTLA-4 deficient mice, and less penetrant (80% vs. 100%) than the phenotype observed in *Ctla4*$^{-/-}$ mice (60). Indeed, in one phase I trial of LAG3/PD-1 targeting combined therapy, similar safety profiles to nivolumab monotherapy were reported (62). Moreover, *in vivo* studies in murine cancer models have shown that when expressed at high levels, concomitant LAG-3/PD-1 expression is mostly restricted to infiltrating TILs (60). This may signify that a combination immunotherapy targeting these two molecules may encourage tumor-specific responses, avoiding non-specific or self-antigen specific immune responses, perhaps rendering such treatment less toxic than CTLA-4 blockade. Indeed, preclinical evidence that LAG-3 is synergistically efficacious in combination with anti-CTLA or anti-PD-1 therapies is driving clinical development (46).

In addition, PD-L1 overexpression in melanoma tumors has been associated with increased LAG-3 expression. This may point to potential synergistic treatment effects. Pre-clinical studies demonstrated that combination blockade of PD-1 and LAG-3 can induce immune activation and associated tumor rejection in fibrosarcoma and colorectal cancer models in mice (60). LAG-3 has been shown to be expressed on a subset of alternatively-activated human plasmacytoid DCs (pDC). These cells may be enriched in human melanoma tumor sites and produce anti-inflammatory cytokines in response to interactions with MHC class II (61, 63). This may indicate that LAG-3 blockade may promote innate immunity host defense mechanisms. Additionally, melanoma resistance to FAS-mediated apoptosis has been proposed as a mechanism of immune escape mediated by tumor cells expressing MHC class II through engagement with LAG-3 (CD223) expressed on TILs (64). Murine studies have indicated that combined use of anti-LAG-3 and anti-PD-L1 in melanoma treatment overcame the requirement for tumor specific T-reg depletion (65). Because LAG-3 engages with DCs, it is possible that LAG-3 blockade can promote innate immunity, a first host defense mechanism, hence stopping tumor growth at an early stage.

The human IgG4 monoclonal LAG-3 antibody relatlimab is in late phase clinical trials in combination with nivolumab versus nivolumab monotherapy for first line advanced melanoma treatment (**Table 3**). This regimen holds promise of both efficacy and de-escalation of toxicity.

TIM-3/PD-1 Blockade

T cell immunoglobulin and mucin 3 (TIM-3), a co-inhibitory receptor expressed on T cells, has both inhibitory and activating properties (**Figure 1**). It has been shown to induce T cell apoptosis, anergy and exhaustion via interaction with galectin-9 on immune cells (66). TIM- 3 expressed on TILs has also been found to bind to galectin-9 expressed on tumor cells *in vitro*, promoting immune escape (66). In addition, TIM-3 interactions with the high mobility group box 1 (HMGB1) protein, which is involved in the recruitment of nucleic acids

TABLE 3 | Selection of current anti-LAG-3 (relatlimab) clinical trials. Information sourced from ClinicalTrials.gov.

Trial	Aim	Target molecules	Condition	Phase
NCT02658981	To evaluate the safety and most effective dose of relatlimab or urelumab (CD137) alone and in combination with nivolumab	Relatlimab: anti-LAG-3 mAb Urelumab: anti-CD137 mAb	Recurrent glioblastoma	I
NCT03335540	Evaluate the treatment of solid tumors with various immunotherapy combinations (nivolumab, relatlimab, cabiralizumab, ipilimumab, anti-GITR, IDO1 inhibitor, lirilumab and radiation therapy	Nivolumab: anti-PD-1 mAb Relatimab: anti-LAG-3 mAb Cabiralizumab: anti-CTF1R mAb Ipilimumab: anti-CTLA mAb Lirilumab: anti-KIR mAb Anti-GITR IDO1 inhibitor	Broad biomarker assessment	I
NCT01968109	To assess the safety, tolerability and efficacy of relatlimab alone and in combination with nivolumab in patients with unresectable/metastatic cancer	Nivolumab: anti-PD-1 mAb Relatlimab: anti-LAG-3 mAb	Not previously treated with immunotherapy: • NSCLC • Gastric cancer • Hepatocellular carcinoma • Renal cell carcinoma • Bladder cancer • Squamous cell carcinoma of the head and neck • Melanoma Previously treated with immunotherapy • NSCLC • Melanoma	I/II
NCT02488759	To investigate the safety and effectiveness of nivolumab, and nivolumab combination therapy (relatlimab, ipilimumab and daratumumab)	Relatlimab: anti-LAG-3 mAb Ipilimumab: anti-CTLA mAb Daratumumab: anti-CD38 mAb	Virus associated cancers: • Anal canal cancer • Cervical cancer • Epstein Barr Virus (EBV) positive gastric cancer • HPV positive and negative squamous cell cancer of the head and neck (SCCHN) • Merkel Cell Cancer • Nasopharyngeal cancer (NPC) • Penile cancer • Vaginal and vulvar cancer	I/II
NCT02061761	To evaluate the safety, tolerability and maximum tolerated dose of relatlimab administered alone or in combination with nivolumab to subjects with relapsed hematologic malignancies	Nivolumab: anti-PD-1 mAb Relatimab: anti-LAG-3 mAb	• Relapsed or refractory Hodgkin lymphoma (HL), • Relapsed or refractory Diffuse Large B Cell lymphoma (DLBCL)	I/II
NCT03459222	To investigate safety and anti-tumor activity of relatlimab combination therapy in metastatic/unresectable solid cancers	Relatimab: anti-LAG-3 mAb	Incurable metastatic/unresectable solid tumor excluding CNS metastases	I/II
NCT02750514	To evaluate the efficacy of nivolumab in combination with other agents (dasatinib, relatlimab, IDO1 inhibitor)	Nivolumab: anti-PD-1 mAb Relatimab: anti-LAG-3 mAb Dasatinib: tyrosine kinase inhibitor IDO1 inhibitor	NSCLC	II
NCT02996110	To compare the efficacy and safety of nivolumab combination therapies (relatlimab/IDO1 inhibitor) with nivolumab and ipilimumab	Nivolumab: anti-PD-1 mAb Relatimab: anti-LAG-3 mAb Ipilimumab: anti-CTLA mAb IDO inhibitor	Renal cell carcinoma	II
NCT02935634	To compare the efficacy and safety of nivolumab combination therapies (relatlimab/IDO1 inhibitor) with nivolumab and ipilimumab	Nivolumab: anti-PD-1 mAb Relatimab: anti-LAG-3 mAb Ipilimumab: anti-CTLA mAb IDO inhibitor	Gastric cancer	II
NCT03470922	To compare the efficacy of nivolumab in combination with relatlimab and nivolumab alone	Nivolumab: anti-PD-1 mAb Relatimab: anti-LAG-3 mAb	Unresectable/metastatic melanoma	II/III

into endosomes to be sensed by the innate immunity, impairs this mechanism promoting tumor escape (67). Since TIM-3 has been established as an exhaustion marker in cancer, it is an attractive immunotherapy target (68). Compared with single agent PD-1 blockade in murine cancer models, it has been shown that combined TIM-3/PD-1 blockade led to superior

tumor regression (68). *In vivo* and *ex vivo* research into the properties of TIM-3 has shown that a melanoma peptide vaccine induced CD8+ T cells to upregulate PD-1 and to an extent TIM-3 in immunized patients. Simultaneous TIM-3 and PD-1 blockade enhanced the proliferation of CD8+ T cells (69). Dual anti-TIM-3 (MBG453) plus anti-PD-1 (PDR001) blockade is currently being analyzed in phase I/II trials (NCT02817633, NCT02608268, NCT03099109, NCT03066648).

B7-H3/CTLA-4 Blockade

B7-H3 (CD276) is a receptor of the CD28 (a co-stimulatory molecule) and B7 (a co-inhibitory molecule) family molecules found on APCs (**Figure 1**). B7-H3 has been found to be over-expressed in melanoma, favoring tumor growth and conferring anti-apoptotic processes (70). When targeted by an Fc-optimized anti-B7-H3 (MGA271) humanized IgG1 antibody, potent antibody-dependent cellular cytotoxicity (ADCC) against melanoma *in vitro* and *in vivo* was observed (71). Studies have suggested than B7-H3 blockade could suppress its co-stimulatory properties. Anti-sense oligonucleotides shown to inhibit B7-H3 expression on DCs resulted in inhibition of IFN-γ production by DC-activated T cells and the proliferation of T cells *in vitro* (72). Subsequently, ipilimumab plus enoblituzumab, a first in class mAb targeting B7-H3, have been the subject of a phase I trial (NCT02381314), and also enoblituzumab in combination with pembrolizumab is being tested in refractory cancers (NCT02475213) (73).

VISTA/PD-1 Blockade

V-domain Ig suppressor of T cell activation (VISTA) is a PD-L1 homolog and co-inhibitory receptor of the B7 family predominantly expressed on various hematopoietic cells (myeloid derived suppressor cells (MDSCs), tumor associated macrophages (TAMS) and DCs) (74) and on leukocytes such as naïve T cells (**Figure 1**). Particularly high levels of VISTA were found on tumor infiltrating myeloid cells in murine models (75). VISTA may participate in the suppression of T-eff responses and T-reg induction via interaction with its putative ligand VSIG-3 (75, 76). VSIG-3 is thought to inhibit T cell function and, in the presence of TCR signaling, it may impair T cell proliferation via the VSIG-3/VISTA pathway (77). As well as expression on T cells, it has also been noted to be upregulated in tumors such as colorectal cancer or hepatocellular carcinoma (77). A murine study using B16-OVA melanoma cell lines demonstrated that VISTA blockade with a monoclonal antibody (13F3) enhanced T-eff response within the TME (78). Therefore, blockade of VISTA could enhance innate immunity since it is expressed on myeloid cells thereby promoting early melanoma eradication. VISTA is also expressed on naïve T cells, and inhibition of VISTA could promote early T cell reaction in response to tumor cells (79). VISTA can control T cell activation through nonredundant functions distinct from the PD-1/PD-L1 pathway in controlling T cell activation and antibodies targeting both checkpoints have shown efficacy in preclinical models (80). Therefore, concurrent targeting VISTA and PD-1 pathways has been proposed as a therapeutic approach. The small molecule antagonist CA-170 electively targets PD-L1/2 and VISTA and is presently tested in a phase I trial (NCT02812875) in advanced solid tumors and lymphomas.

A2AR/PD-L1 Blockade

The adenosine-adenosine A2A receptor (A2AR) pathway is of interest as a target for immunotherapy, since the extracellular adenosine, often found in TME due to hypoxia, can inhibit T cell proliferation and cytotoxicity via the A2AR receptor (CD73) on myeloid cells such as macrophages (81) (**Figure 1**). CD73 is a checkpoint molecule expressed on T-reg which converts AMP to adenosine in this pathway and this checkpoint is also expressed on melanoma cells. Studies of patient samples *ex vivo* have shown that via adenosine interaction, tumor cells are able to inhibit immune responses and to simultaneously enhance neovascularization and cancer cell growth via vascular endothelial growth factor (VEGF) and IL-6 expression (82, 83). Synergistic effects of combined CTLA-4 and CD73 as well as combinations of PD-1 and CD73 blockade immunotherapies in breast cancer and colon carcinoma pre-clinical models (84, 85) have been observed. Furthermore, murine studies have demonstrated suppressed melanoma growth and enhanced lymphocytic infiltration in the TME in A2AR-deficient mice. Early phase trials investigating the merits of a combined anti-CD73/anti-PD-L1/anti-PD1 therapies in patients with solid tumors are underway, to ascertain whether or not targeting multiple sites in the pathway can show enhanced anti-tumor effects (e.g. NCT02655822, NCT02503774) (**Table 4**).

IDO/CTLA-4, PD-1 and PD-L1 Blockade

Indoleamine 2'3' dioxygenase (IDO, IDO1 and IDO2) is a catabolic enzyme produced by macrophages and T-regs to convert tryptophan, which is needed for T cell effector function, to kynurines (86) (**Figure 1**). Immune tolerance is promoted by IDO-mediated tryptophan deficiency, with naïve T cells observed to differentiate into T-reg (87). This protein can also be expressed by tumors alongside prostaglandin E2, thereby aiding in immune escape of these cancers via NK cell inhibition (88) and T-reg recruitment resulting in IL-10 mediated induction of myeloid-derived suppressor cells (MDSC) (89). *Ex vivo*-derived and tumor-associated MDSC have been shown to express PD-L1 and MHC-II, and correlated with expression of their receptors, PD-1 and LAG- 3, on T cells, known to be associated with immunosuppression of T cell functions. PD-L1-expressing MDSCs could trigger immunosuppressive effects via IDO (90). Importantly, a pre-clinical study in a melanoma model in mice demonstrated IDO overexpression post-treatment with anti-CTLA-4 and anti-PD-1 (14). This conferred resistance and tumor growth, a property found to be reversible by combination treatment with anti-CTLA-4 and IDO inhibitors (14). Studies using the murine B16.SIY melanoma mouse model have shown that combinations of CTLA-4 or PD-1/PD-L1 with IDO blockade restored both IL-2 production and CD8+ T cell proliferation within the TME (48), pointing to the potential merits of a combinational targeting approach.

IDO and galectin-3 expression are known to promote T-reg upregulation, while suppressing T-eff production (91, 92). Blockade of these two molecules reversed these effects (91),

TABLE 4 | Selection of current A2AR/CD73 inhibitor clinical trials. Information sourced from ClinicalTrials.gov.

Trial	Aim	Target molecules	Condition	Phase
NCT03454451	To evaluate the safety, tolerability, and anti-tumor activity of CPI-006 as a single agent, in combination with A2AR inhibitor CPI-444 and in combination with pembrolizumab	CPI-006: CD23 and adenosine inhibitor CPI-444: A2AR inhibitor Pembrolizumab: humanized anti-PD-1 mAb	• Non-small cell lung Cancer (NSCLC) • Renal cell cancer • Colorectal cancer • Triple negative breast cancer • Cervical cancer • Ovarian cancer • Pancreatic cancer • Endometrial cancer • Sarcoma • Squamous cell Carcinoma of the head and neck • Bladder cancer • Metastatic castration Resistant prostate cancer	I
NCT02503774	To evaluate the safety, tolerability, pharmacokinetics, immunogenicity, and antitumor activity of MEDI9447 (oleclumab) alone and in combination with MEDI4736 (Durvalumab) in adult subjects with select advanced solid tumors	Oleclumab: human anti-CD73 mAb Durvalumab: human anti-PD-1 mAb	• Advanced solid malignancies • NSCLC	I
NCT02655822	To study the safety, tolerability, and anti-tumor activity of A2Ar inhibitor CPI-444 alone and in combination with atezolizumab	CPI-444: A2AR inhibitor Atezolizumab: humanized anti-PD-1 mAb	• Non-small cell lung cancer • Malignant melanoma • Renal cell cancer • Triple negative breast cancer • Colorectal cancer • Bladder cancer • Metastatic • Castration resistant Prostate cancer	I
NCT02740985	To determine the maximum tolerated dose of A2AR receptor antagonist AZD4635 in combination with durvalumab	Durvalumab: human anti-PD-1 mAb AZD4635: A2AR receptor antagonist	• Advanced solid malignancies • NSCLC • Metastatic castrate-resistant prostate carcinoma • Colorectal carcinoma	I
NCT03549000	To assess the safety, tolerability, and anti-tumor activity of anti-CD73 NZV930 alone and when combined with anti-PD1 and/or A2AR inhibitor NIR178	NZV930: anti-CD73 NIR178: A2AR inhibitor PDR001: experimental anti-PD-1	• NSCLC • Triple negative breast cancer • Pancreatic ductal adenocarcinoma • Colorectal cancer microsatellite stable • Ovarian cancer • Renal cell carcinoma	I
NCT02403193	To determine the safety, tolerability, feasibility and efficacy of A2AR inhibitor PBF-509 alone and in combination with anti-PD1	PBF-509: A2AR inhibitor PDR001: experimental anti-PD-1	NSCLC	I/II
NCT03381274	To evaluate the safety, tolerability and antitumor activity of novel combination therapies (oleclumab, A2AR inhibitor AZD4635 and osimertinib)	Oleclumab: human anti-CD73 mAb AZD4635: A2AR inhibitor Osimertinib: tyrosine kinase inhibitor	NSCLC	I/II
NCT03207867	To evaluate the efficacy and safety of A2AR antagonist (NIR178) in combination with anti-PD1	NIR178: A2AR inhibitor PDR001: experimental anti-PD-1	• NSCLC • Renal cell cancer • Pancreatic cancer • Urothelial cancer • Head and neck cancer • Diffused large B cell • Lymphoma • Microsatellite stable colon cancer • Triple negative breast cancer • Melanoma	II

and although further investigations into combined anti-PD-1/IDO (epacadostat) inhibitors underway in a phase I and II trial (NCT02318277) were promising, a phase III trial studying an epacodostat-pembrolizumab combination was abandoned due to a lack of significant improvement in both primary and secondary endpoints—PFS and overall survival (OS) (93).

Epacodostat has had to be discontinued in several trials assessing its efficacy as a monotherapy including KEYNOTE-006, KEYNOTE-010, KEYNOTE-087 and KEYNOTE-052, due to adverse reactions (94). These disappointing results may influence the design of future studies aiming to assess IDO as a potential immunotherapy target. However, different approaches to direct IDO inhibition, including an IDO peptide vaccine, have been promising and have shown significant delay in tumor growth and prolonged survival in a B16 murine model (95). Early phase trials of combinations such as a PD-L1/IDO peptide vaccine with nivolumab (NCT03047928) in advanced melanoma are underway.

PKC-η/PD-1 Blockade

A protein which may synergize with CTLA-4 to mediate immune tolerance is PKC-η, an intrinsic downstream signaling checkpoint molecule (96) (**Figure 1**). PKC-η induces anti-inflammatory cytokine transcription by activating NFkB downstream of the cascade (97). Downregulation of PKC-η in murine models reduced the tumor-suppressive activity of T-reg but did not enhance autoimmune colitis (96). It is possible that inhibition of PKC-η could potentially produce effective T-reg suppression and with fewer adverse drug reactions if used in combination with anti-CTLA-4 blockade. Pre-clinical studies on Rag1 mice have shown that a PKC-η deficiency reduced tumor growth (98) and there are currently studies investigating the efficacy of midostaurin, a tyrosine kinase inhibitor, for the treatment of leukemia as well as studies on the pan-PKC inhibitor AEB071 sotrastaurin which is being tested in uveal melanoma (NCT02273219, NCT02601378, and NCT01430416).

ANTIBODY ENGINEERING AND NOVEL COMBINATIONS TO IMPROVE CHECKPOINT BLOCKADE

Combining checkpoint blockade antibodies may be an effective strategy for treating melanoma (**Figure 1**). However, there remain numerous avenues available for the refinement of existing therapies. With advances in protein engineering, antibodies can be manipulated to introduce novel functionality (99). For combined checkpoint blockade therapies, there is evidence that such engineering could further improve clinical efficacy. Anti-CTLA-4 antibodies have been observed in mouse models of cancer to deplete T-reg cells by ADCC, a function that relies on specific antibody isotypes engaging with FcγR on effector cells (monocytes, macrophages, NK cells) that are cytotoxic to T-regs within the TME (20, 100) (**Figure 2A**).

Until recently, there has been an assumption that Fc receptors would not contribute to the anti-tumor activity of antibodies recognizing checkpoint molecules (101). FcγRs expressed mainly on hematopoietic cells such as NK cells, DCs and macrophages (102) can activate or inhibit; in particular, FcγRI, FcγRIIa, and FcγRIIIa are activating receptors while FcγRIIb is inhibitory (101) (**Figure 2A**). Importantly, FcγRs are co-expressed on the same cells such as monocytes and macrophages, allowing thresholds for activation/inhibition to be fine-tuned (101). Ipilimumab, for instance, exhibits T-reg depletion properties specifically in the presence of FcγR-expressing monocytes and natural killer (NK) cells, consistent with clinical studies that have demonstrated correlations between specific checkpoint inhibitor antibody subtypes of activating hFcγRs and clinical responses (100) (**Figure 2A**). An example of this includes variants in the gene *FCGR (FCGR2A* and *FCGR3A*) which bind more avidly to human IgG1 and IgG2 subtypes, thereby increasing ADCC-mediated cell death and thus have been associated with improved outcomes (100). Whether these single nucleotide polymorphisms (SNPs) can affect the response of other immunomodulatory monoclonal antibodies requires further investigation.

In vitro studies of anti-CTLA-4 monoclonal antibodies with the same IgG1 and IgG2 Fc variants such as ipilimumab and tremelimumab exhibited superior tumor killing with antibodies able to maximize T-reg depletion by ADCC (20, 100) (**Figure 2A**). Additionally, preclinical studies have shown that targeting CTLA-4 on T-reg cells conferred minimal tumor protection in comparison to when antibody bound to CTLA-4 on both T-eff and T-reg compartments *in vivo* (101). Preclinical studies demonstrated a requirement for enriching the TME with effector cells such as myeloid cells expressing high levels of FcγRs (103). One phase II trial showed that granulocyte–macrophage colony-stimulating factor (GM-CSF) plus ipilimumab improved overall survival in patients with metastatic melanoma compared with ipilimumab alone, possibly as a result of a macrophage enriched TME. These findings suggest that macrophages, may be key effector cells in mediating ADCC (101, 103) (**Figure 2A**).

One molecule of note is CD25, highly expressed on T-reg cells in the TME in mouse models and in human tumors, but with minimal expression in the effector cell compartment (104). Anti-CD25 antibody-induced ADCC can be enhanced by optimizing the antibody isotype to engage activating FcγRs in mouse models of cancer (104). Experiments on mouse models have also shown that anti-CD25 therapies can work concurrently with other immunomodulatory drugs such as anti-PD-1 monoclonal antibodies in the TME. Fc-optimized anti-CD25 drug combinations may prove promising through improving the drug therapeutic window (104). However, it is important to note that CD25 is also expressed at lower levels on activated memory T cell populations (105) potentially highlighting a risk for unwelcome T-eff depletion with anti-CD25 treatment.

Bispecific T cell engaging antibodies (BiTE) constitute an extensive class of agents able to recognize a range of tumor antigens expressed on cancer cells on one arm, while simultaneously engaging a T cell-specific molecule such as CD3 through the other arm (**Figure 2B**). BiTEs are able to link the two, promoting cytolysis of tumor cells. A bispecific T cell engaging (BiTE) antibody which recognizes the tumor antigen carcinoembryonic antigen (CEA) with one arm and CD3 with the other has been suggested for improving checkpoint blockade therapies (106) (**Figure 2B**). Following BiTE-mediated cytolysis, upregulation of PD-1 impaired T cell functions (107). If the BiTEs are delivered in conjunction with PD-1 checkpoint

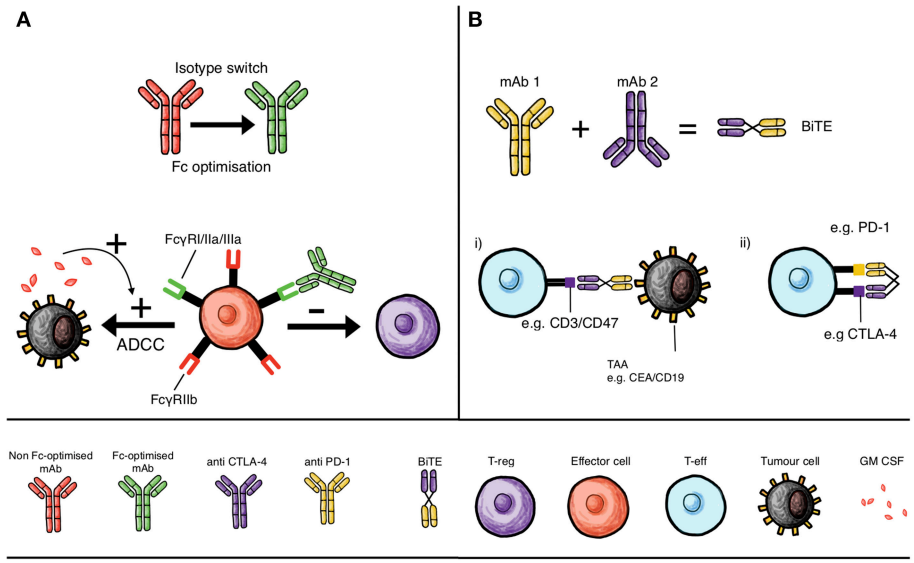

FIGURE 2 | Monoclonal antibody mechanisms of action and interactions with immune cells that can influence checkpoint inhibition: isotype switching, Fc domain optimization and BiTEs. **(A)** Isotype switching and Fc optimization of mAbs to increase binding to activatory Fc receptors on effector cells, such as monocytes, macrophages and NK cells, to enhance T-reg depletion by ADCC, and in the presence of GM-CSF, to enhance ADCC of tumor cells. **(B)** Development of bispecific T cell engaging antibody structures (BiTE) that can either simultaneously engage tumor-associated antigen (TAA, e.g., CEA or CD19) and T cell specific molecules (e.g., CD3 or CD47) to promote cytolysis of tumor cells (**B**,i) or simultaneously inhibit two T cell checkpoint molecules; such as CTLA-4 and PD-1, to circumvent mechanisms of resistance (**B**,ii).

blockers, these T cell modulating effects may be reversed (107). It is therefore possible that checkpoint blockers could synergize with such cancer immunotherapies when used in combination.

Bispecific antibodies have also been generated which target checkpoint molecules directly. CD47 is an immune-regulatory molecule expressed on the surface of many cells and all human cancers. It can prevent phagocytosis of cancer cells by macrophages and dendritic cells by binding to SIRPα on their surface (108). However, the ubiquity of CD47 makes it difficult to specifically target with antibodies. Considering this, a bispecific antibody recognizing both CD47 and a cancer-specific antigen, in this case CD19 for B cell lymphoma, has been generated. *In vitro*, this antibody could promote effective phagocytosis of the cancerous cells (109). It is possible to imagine that this targeted checkpoint blockade approach may be exploited for use melanoma, provided an appropriate targeting antigen can be identified. Furthermore, a BiTE designed to simultaneously target two immune checkpoints on T-eff cells, such as CTLA-4 and PD-1, may improve tumor killing and increase efficacy since dual therapies of anti-PD-1 and anti-CTLA-4 have proven effective (**Figure 2B**). This could circumvent mechanisms of resistance and eschew the need for two mAbs to be administered concurrently.

T cell activation upregulates ICOS expression and targeting the ICOS/ICOSL pathway improved the potency of anti-CTLA-4 therapy in preclinical models (110, 111). The cellular vaccine IVAX (irradiated ICOSL-positive tumor cells) has been shown to function synergistically in the context of CTLA-4 blockade. The combination has been shown to lead to effector cell migration to and survival in the TME, and consequent enhanced tumor elimination in murine models (110) (**Figure 3A**).

Beyond Antibody Engineering: Oncolytic Viruses as Immunotherapies

Oncolytic viruses (OVs) are being developed as cancer immunotherapies, with the modified herpes simplex virus type 1 talimogene laherparepvec (T-VEC, or OncoVEX^{GM-CSF}) being approved by the FDA for malignant melanoma (112), and the ClinicalTrials.gov database currently listing 22 trials studying oncolytic herpes simplex virus following the success of T-VEC for metastatic melanoma (113) (**Figure 3B**). Many tumor cells cannot adequately protect themselves against viral infection, making the development of oncolytic agents an attractive therapy option that may selectively infect tumor cells (113–115). Among the properties of OVs is their ability to improve the immune system response to tumors, and in combination with checkpoint inhibitors they also enhance checkpoint blockade (116–118). By lysing tumor cells, OV treatment can lead to the release of tumor-associated antigens by the dying cells and efficient cross-presentation of antigens to DCs, thus enhancing anti-tumor responses (113, 116) (**Figure 3B**).

In clinical practice, OVs are made cancer specific by attenuating the virus so that preferential infection of tumor cells occurs (119). In addition to this, cytokine expression of OVs may increase the anti-cancer properties of these therapies avoiding systemic toxicity due to the preferential action of OVs on tumor cells (119). IL-12 in particular is an important cancer immune response modulator and oncolytic herpes viruses

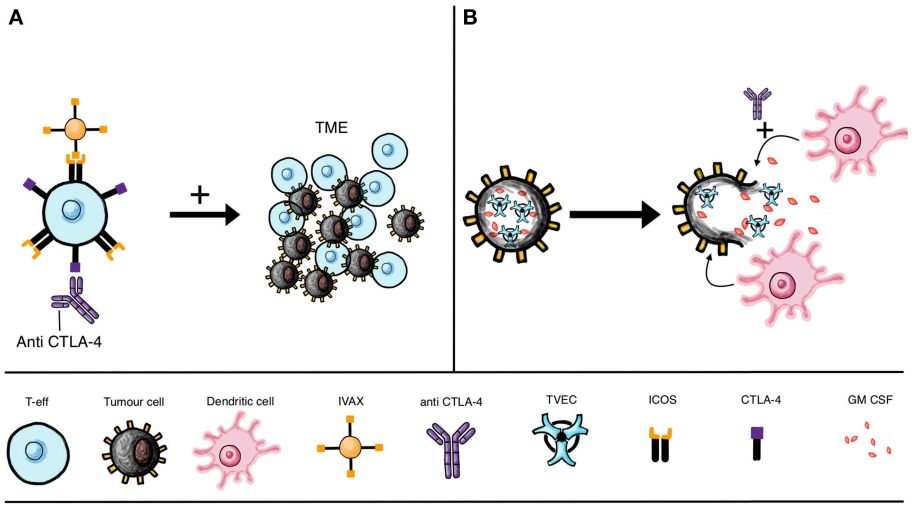

FIGURE 3 | IVAX vaccine and oncolytic viruses combined with checkpoint blockade. **(A)** Combinatorial therapy may include CTLA-4 blockade with anti-CTLA-4 mAb (leading to T cell activation and subsequent ICOS upregulation) together with the IVAX vaccine [which engages ICOS to increase T-eff migration to the tumor microenvironment (TME)]. **(B)** Approaches beyond antibody engineering include attenuated oncolytic virus, such as T-VEC unable to replicate in healthy cells, instead preferentially invading cancer cells. The replicating virus lyses the tumor cells and is armed, for instance with GM-CSF, which when released can recruit DCs to prime T cells to identify and destroy tumor cells. Efficacy of oncolytic virus may be improved by combination with checkpoint inhibitors, such as anti-CTLA-4 treatment to boost T cell activation.

(oHSVs) expressing IL-12 have shown efficacy in preclinical studies on glioblastoma multiforme (120). 'Arming' these OVs with an expression of a particular cytokine may be key for achieving anti-tumor efficacy. The clinically-licensed T-VEC for instance is armed with GM-CSF, an APC activator (121). A murine study also found that an IL-12 armed oHSVs delayed tumor proliferation and reduced tumor growth more effectively than its unarmed counterpart (119) (**Figure 3B**).

Although viral-mediated tumor lysis can be enhanced by tropism targeting and viral arming with immune stimulatory cytokines, clinical efficacy has not been consistent across all patient groups (122). For instance, the cytidine deaminase Apolipoprotein B Editing Complex 3 (APOBEC3), normally involved in the host response to retrovirus infection, is also reported to be associated with virus-resistant tumors. Expression of APOBEC3 been shown to be upregulated in B16 murine melanoma cells infected with an oncolytic vesicular stomatitis virus (VSV) (122). Tumor cell resistance to VSV both *in vivo* and *in vitro* was demonstrated where APOBEC3 was upregulated and knockdown of this enzyme reduced VSV escape from immune clearance (122). This highlights the need for further research to clarify methods for mitigating and preventing tumor resistance to immunotherapies and other novel therapies that often limit efficacy.

Studies have suggested the potential merit of combining OVs with checkpoint inhibitors. In a Phase II study, CD8-expressing T cells, immune activation markers and PD-L1 expression were increased with intralesional administration of coxsackievirus A21 (CVA21), which has a propensity for tumor cells expressing ICAM-1 (123). Phase Ib trials have shown that this combination has minimal additional toxicity or adverse drug reactions, with one study recording an ORR of 73% and a disease control rate (DCR) of 91% of patients with advanced melanoma (124, 125). In the case of T-VEC, long term protection and significant effects on untreated tumors was seen only when the OV was combined with anti-CTLA-4 (121). Finally, several studies are exploring novel OV combinations. One is an oncolytic vaccinia virus armed with a superagonist IL-15 (IL-15-IL-15Rα fusion) given alone or in combination with anti-PD-1: combined treatment demonstrated T cell-driven anti-tumor efficacy in *in vivo* mouse models of cancer (126). Furthermore, TNFα armed viruses have seen success in inducing tumor regression and vascular collapse in solid masses when administered in tumor-bearing mice alongside therapies that inhibit anti-apoptosis proteins *in vitro* (127).

The administration of OVs has often been limited to intratumoral delivery in patients; T-VEC is currently only licensed as a locally-administered treatment (128). This is due to concerns regarding neutralizing antibodies (NAbs) which are often present in human populations in response to common viruses such as HSV and reovirus and may impair the efficacy of systemic delivery of OVs (128). This may be a limitation since systemic delivery of OV therapy would in theory be more effective for targeting metastatic lesions. However, one report (128) found that by loading neutralized reovirus with antibodies in immune complexes onto human monocytes, resulted in the transfer of the virus to melanoma cells, cancer cell lysis and in restriction of cancer growth in a murine model of melanoma (128). This indicates that monocytes and antibodies recognizing viral proteins may be important components in maintaining the potency of OV therapy and may lead to new strategies for OV treatment.

In summary, oncolytic viruses may provide an alternative avenue for cancer immunotherapy due to their properties in targeting cancer cells with some specificity and due to some promising results in combination with immune checkpoint blockade. It appears that these therapies are most efficient when armed with specific cytokine expression and attenuation of the virus is important for both reducing toxicity and allowing preferential infection of cancer cells.

CHALLENGES AND FUTURE DIRECTIONS

Anti-CTLA-1 and anti-PD-1 therapies have been the most extensively researched due to documented benefits for melanoma treatment as evidenced by higher rates of tumor clearance and, crucially, long term disease eradication in some patients compared with traditional treatments (129). Importantly, further research has emphasized improved response and survival rates when combining anti-CTLA-4 and anti-PD-1 therapy (130), leading to the approval of ipilimumab plus nivolumab in BRAF wild-type melanoma in 2015 and regardless of BRAF tumor subtype in 2016 (131). As more and more immune checkpoints are identified, and their mechanisms of action elucidated, new options for single and combined therapy regimens can be derived (**Table 5**). Combining different checkpoint blockade agents may produce potent effects, due in part to targeting more than one immune pathways. The benefits of combination therapies are not limited to the use of multiple checkpoint inhibitors but can extend to combining anti-CTLA or anti-PD-1 drugs with other targeted agents such as MEK and BRAF inhibitors. One such example is a Phase III trial combining PD-L1 and MEK inhibition (atezolizumab and cobimetinib) vs. pembrolizumab in advanced $BRAF^{V600}$ wild-type melanoma (NCT03273153). Combinations of checkpoint inhibitors with chemotherapy, radiotherapy and other immunotherapies such as oncolytic viruses and cell-based therapy approaches are also being investigated. Many challenges, including selecting optimal combinations, predicting and managing toxic effects for different combinations and development of clinically-useful biomarkers to help support the use of such treatments are and will continue to be the subject of intense study.

Toxicities of immunotherapies remains an important challenge. Adverse effects (50, 62), can be managed according to information from resources such as the European Society for Medical Oncology (ESMO) Clinical Practice Guidelines (132, 133). It has been shown that recurrence of immune-related adverse events (irAEs) in patients in whom it was necessary to discontinue treatment as a result of irAEs can occur upon re-challenge with immunotherapies such as anti-PD-1. For instance, a retrospective analysis showed that colitis was less likely to recur in comparison to hepatitis, pancreatitis, pneumonitis and nephritis, and re-challenge with PD-1 blockade has been reported to be tolerated better than other agents (134). Studies have also suggested various biomarkers such as IL-17 or eosinophilia to help predict toxicity in patients, something that could allow early recognition of pathology and thus prompt intervention (133, 135). Investigation into new immunotherapies and combinations may also reveal treatment algorithms with more favorable toxicity profiles than the current regimens.

Improved efficacy has often been associated with increased irAEs, as reported in trials such as Checkmate 067, where the ipilimumab/nivolumab combination had a 10% higher rate of toxicity than either drug administered alone (52). However, immune-related toxic effects may potentially be abrogated in future therapeutic regimens by various approaches. These may include the increasing specificity of targeted immune therapies, to help reduce systemic effects on non-target healthy cells, as well as the implementation of combinatorial therapies with non-overlapping targets. For instance LAG-3 may promote more tumor-specific responses than currently licensed immunotherapies: concomitant LAG-3/PD-1 expression is mostly restricted to infiltrating TILs, expressed at high levels in murine models (60), and mouse studies into other combinations such as a PKC-η/PD-1 blockade therapy has shown a reduction in T-reg immunosuppressive activity without the concomitant increase in autoimmunity (96). Thus, although data from current licensed therapies have suggested a correlation between combinatorial therapies and increased toxicity, this may not necessarily be the case with different checkpoint blockade combinations. In the future, ideally patients would be able to access fully personalized medicine in which treatment such as immunotherapies would be specific to the patient immune response as well as the specific genetic characteristics of their cancers—this would reduce unwanted side effects and maximize the antitumor effect in each individual patient.

The mechanisms behind the lack of clinical responses to single or combination treatments in many patients, how tumors develop resistance to novel immunotherapies and the best criteria to select patients for maximum treatment effect are insufficiently elucidated. A focus on functional evaluations in the context of patient immunity for novel regimens alongside clinical testing may shed some light on the most effective combinations and the best strategies to reduce adverse effects. This may reveal immune or genomic biomarkers linked with better treatment outcomes. For instance, cancer development and therapeutic response may relate to host factors such as the gut microbiome. Approximately 20% of malignancies are linked with microorganism infection and a patient's gastrointestinal microbiota can both positively and negatively influence cancer susceptibility (136). The effects of the microbiome in cancer susceptibility, progression and response to treatment are far reaching insofar as the microbiome is known to guide the immune system's response, host metabolism of medication and endogenously produced chemicals, and can also influence the balance of cell growth and death (136). A recent study of patients with melanoma exploring the role of the microbiome in influencing clinical response to anti-PD-1 therapy found that "favorable" gut microbiota (characterized by higher gut microbe diversity and levels of Ruminococcaceae/Faecalibacterium) mediated higher levels of antigen presentation and T-eff function both in the periphery and within the TME. This promoted better systemic and anti-tumor immune responses compared with patients with "unfavorable"

TABLE 5 | Examples of combination phase III monoclonal antibody immunotherapy trials in melanoma. Information sourced from ClinicalTrials.gov.

Indication	Trial number	Combination therapy	Study design	Target	Status
DUAL MONOCLONAL ANTIBODY THERAPIES					
Previously untreated, unresectable or metastatic melanoma	NCT02905266	Nivolumab + ipilimumab	Arm A: nivolumab and ipilimumab concomitant administration followed by nivolumab monotherapy Arm B: nivolumab and ipilimumab sequential administration followed by nivolumab monotherapy	Anti-PD-1 + anti-CTLA-4	Active, not recruiting
Complete resection of stage IIIB/C/D or stage IV melanoma	NCT03068455 (CheckMate 915)	Nivolumab + ipilimumab	Arm A: nivolumab + ipilimumab Arm B: nivolumab	Anti-PD-1 + anti-CTLA-4	Active, not recruiting
Previously untreated, unresectable or metastatic melanoma	NCT02714218	Nivolumab + ipilimumab	Arm A: nivolumab 3 mg/kg IV + ipilimumab 1 mg/kg IV Arm B: ipilimumab 3 mg/kg IV + nivolumab 1 mg/kg IV Arm C: nivolumab 6 mg/kg IV + ipilimumab 1 mg/kg	Anti-PD-1 + anti-CTLA-4	Active, not recruiting
First-line for advanced melanoma	NCT02599402 (CheckMate 401)	Nivolumab + ipilimumab	Arm A: nivolumab + ipilimumab Arm B: nivolumab	Anti-PD-1 + anti-CTLA-4	Active, not recruiting
Unresectable or metastatic melanoma	NCT03470922	Nivolumab + relatlimab	Arm A: relatlimab + nivolumab Arm B: nivolumab	Anti-PD-1 + LAG-3 inhibitor	Recruiting
COMBINED MONOCLONAL ANTIBODY AND TYROSINE KINASE INHIBITORS					
Stage III-IV $BRAF^{V600}$ melanoma	NCT02224781	Ipilimumab and nivolumab + dabrafenib and trametinib	Arm A: ipilimumab and nivolumab then dabrafenib and trametinib Arm B: dabrafenib and trametinib then ipilimumab and nivolumab	Anti-CTLA-4 and anti-PD-1 + BRAF inhibitor and MEK inhibitor	Recruiting
Previously untreated $BRAF^{V600}$ mutation-positive patients with metastatic or unresectable locally advanced melanoma	NCT02908672	Atezolizumab + cobimetinib + vemurafenib	Arm A: atezolizumab + cobimetinib + vemurafenib + vemurafenib placebo Arm B: atezolizumab placebo + cobimetinib + vemurafenib	Anti-PD-1 + MEK inhibitor + BRAF inhibitor	Active, not recruiting
Previously untreated advanced $BRAF^{V600}$ wild-type melanoma	NCT03273153	Atezolizumab + cobimetinib Pembrolizumab	Arm A: atezolizumab + cobimetinib Arm B: pembrolizumab	Anti-PD-L1 + MEK inhibitors Anti-PD-1	Recruiting
MONOCLONAL ANTIBODY THERAPIES COMBINED WITH OTHER AGENTS					
Anti-PD-1 refractory melanoma	NCT03445533 (ILLUMINATE-301)	Ipilimumab + IMO-2125	Arm A: ipilimumab Arm B: ipilimumab + IMO-2125	Anti-CTLA-4 + TLR9 agonist	Recruiting
Unresectable or metastatic melanoma	NCT02752074 (Keynote-252 / ECHO-301)	Pembrolizumab + epacadostat	Arm A: pembrolizumab + epacadostat Arm B pembrolizumab + placebo	Anti-PD-1 + IDO1 inhibitor	Active, not recruiting
Untreated unresectable stage III or IV melanoma	NCT00324155	Dacarbazine + ipilimumab	Arm A: dacarbazine + ipilimumab Arm B: dacarbazine + placebo	Chemotherapy alkylating agent + anti-CTLA-4	Completed
Unresected melanoma	NCT02263508 (KEYNOTE-034)	Pembrolizumab + T-Vec	Arm A: pembrolizumab + talimogene laherparepvec Arm B: pembrolizumab + placebo	Anti-PD-1 + oncolytic herpes virus	Active, not recruiting
Unresectable or metastatic melanoma	NCT03301636 (NLG2107)	Pembrolizumab/ nivolumab + indoximod	Arm A: pembrolizumab + indoximiod Arm B: pembrolizumab + placebo Arm C: nivolumab + indoximiod Arm D: nivolumab + placebo	Anti-PD-1 + IDO inhibitor	Recruiting

microbiomes (137). Bacteroides has been linked to a poorer anti-tumor response in patients treated with ipilimumab and patient microbiomes enriched with Faecalibacterium and Firmicutes have also been correlated with more efficacious clinical response to ipilimumab (138).

Research exploring predictive markers for checkpoint treatment response has pointed to mutational burden, PD-1 ligand (PD-L1) expression, circulating tumor cells (CTCs) and miRNA signatures. The challenge of identifying biomarkers should also be explored in combination therapies. For melanoma, mutational burden and PD-L1 studied in pretreatment biopsies have been evaluated as predictive markers for guiding therapy. However, these do not always correlate with response (139). A study exploring a noninvasive blood-based monitoring

method of tumor burden, aimed at improving prediction of response for melanoma patients undergoing immune checkpoint inhibition therapy, found longitudinal digital measurements of circulating tumor cells (CTCs) to be predictive of clinical outcomes in patients with metastatic melanoma (139). Other research studies have reported falling levels of circulating free DNA (ctDNA) for tumor survival-promoting mutant kinase BRAF, NRAS, and KIT alleles in melanoma patients who are undergoing immunotherapy (140, 141). Alongside their use as biomarkers of response, perhaps an optimal immune and mutant kinase targeted therapy combination could be derived to further improve clinical outcomes. Tumor lymphocyte and NK cell infiltration and IFNγ upregulation, have been proposed as potential predictors of response alongside mutational burden, however these need to be standardized and widely evaluated in clinical practice (142–144). Tumor mutational load and PD-L1 expression are often clinically available to physicians. PD-L1 expression has been reported to have a good negative predictive value in lung cancer; however, the same results have not been shown in melanoma (142). Finally, new technologies such as analyzing T cell clonality are promising but require specialist equipment not yet widely available as clinical tools (142). Reliable predictive markers of long term outcome are also particularly important for combined therapies with their high toxicity profiles. Identifying signatures to guide selection of patients who do not need to be exposed to the increased risk of toxicity currently inherent in combination strategies would greatly aid clinical decision making.

CONCLUSION

Combinations of existing and novel immune checkpoint inhibitors and discovering predictive biomarkers promise to further build on the success of immunotherapy. A comprehensive approach will be required to produce the most efficacious combination immunotherapies able to circumvent mechanisms of resistance. Antibody engineering may help capitalize on tumor killing effector mechanisms through knowledge of SNPs and FcR-expressing immune effector cells known to influence patient responses to other immunomodulatory monoclonal antibodies. These in addition to considering host factors such as the microbiome, which can affect medication metabolism and uptake, and tumor-associated molecular and immunological characteristics such as mutational burden, expression of checkpoint molecules and immune cell infiltration in the TME, may ultimately determine patient response to treatment and help optimize immunotherapy for melanoma.

AUTHOR CONTRIBUTIONS

DK, SK, KL, and SP conceived and designed the study; DK, HB, SK, BT, CB, ET, AS, NT, KL, and SP searched and studied the literature; DK, SK, BT, CB, ET, AS, NT, KL, and SP wrote the manuscript; DK, SK, HB, KL, and SP discussed and interpreted the literature findings; SM, SC, GP, AK, MN, RJH, EF, RMH, AC, IW, JS, DJ, JG, CH, and AM commented and helped to edit the manuscript; SK supervised the study.

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