IMMUNE CHECKPOINT MOLECULES AND CANCER IMMUNOTHERAPY

EDITED BY: Alexandr V. Bazhin, Amedeo Amedei and Svetlana Karakhanova
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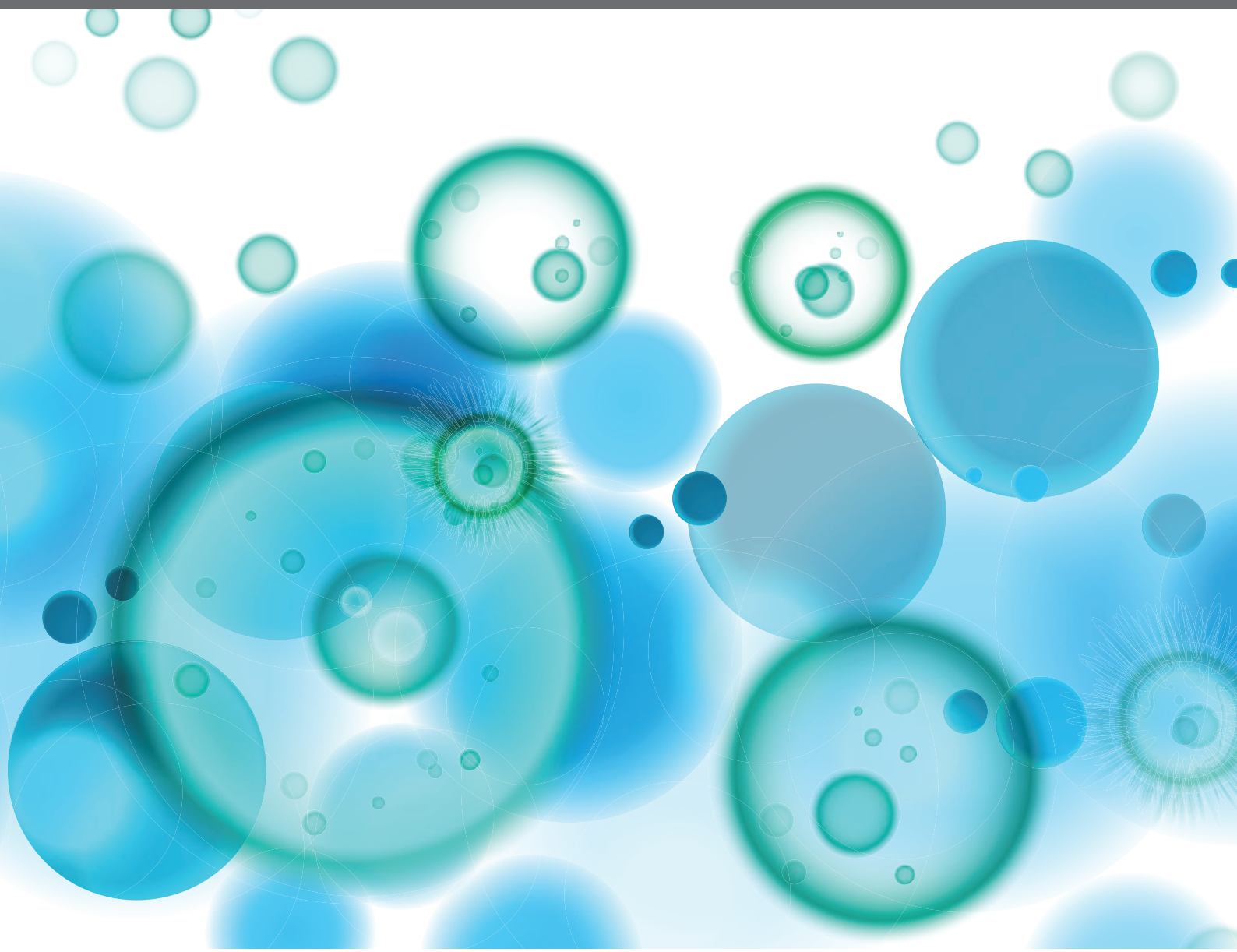

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IMMUNE CHECKPOINT MOLECULES AND CANCER IMMUNOTHERAPY

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EDITORIAL
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Editorial: Immune Checkpoint Molecules and Cancer Immunotherapy

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Editorial on the Research Topic

Immune Checkpoint Molecules and Cancer Immunotherapy

On October 2nd, we experienced with great enthusiasm that the 2018 Nobel Prize in the Medicine and Physiology goes to the fathers of check-point molecules CTLA-4 and PD-1 James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation"(1, 2). This Nobel Prize assignment was a great note for us as guest editors of the special issue "Immune Checkpoint Molecules and Cancer Immunotherapy." Hence, this topic is not just in vogue but represents an enormous important field of translational cancer immunology. The discovery of the checkpoint molecules CTLA-4 and PD-1 stimulated pharma industry to develop specific inhibitors for cancer treatments, encouraging many scientists and clinicians to further explore this field (3, 4). We have witnessed a real success! Especially about the malignant melanoma, where during more than 30 years we saw no progress in the treatment of this tremendous disease (5–7). Meanwhile, inhibitors of checkpoint molecules and their receptors are approved for the treatment of different malignancies and there are impressive case reports of patients (8).

But the way is still stony. During the experience with immunotherapeutic drugs based on targeting of immune checkpoint molecules, many questions, and problems arose. First, not all cancer types as well as not all patients, tested in immunotherapeutic clinical trials, were sensitive to the treatment. Second, it is still not clear how we can monitor the therapy successes. Are there some biomarkers to predict the response to therapy? How can the expression of these immune checkpoint molecules be modulated or influenced? What is the impact of combination of the immune checkpoint molecule therapy with a conventional cancer treatment? Undoubtedly, these and other questions require further intensive research. Therefore, we announced last year this special issue by Frontiers Immunology. The main aim was to collect novel findings from scientists and clinicians involved in basic research on immune checkpoints as well as in translational studies investigating the use of checkpoint inhibitors in immunotherapy in experimental settings.

As mentioned before, not all cancer types tested in immunotherapeutic trials with checkpoint molecule inhibitors had a benefit from such a therapy. One of these exceptions is the pancreatic adenocarcinoma (PDAC). This extremely severe cancer did not respond to the immune checkpoint inhibition treatment. Kabacaoglu et al. attempted to elucidate this problem in the review *"Immune Checkpoint Inhibition for Pancreatic Ductal Adenocarcinoma: Current Limitations and Future Options."* The authors shed light on the immune escape mechanisms allowing PDAC to avoid the effect of immune checkpoint inhibition. Further more they discussed possibilities to potentially

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improve outcome of such immunotherapy in PDAC. In this line, we are pleased to introduce results of the original research of Rataj et al. *"PD1-CD28 Fusion Protein Enables CD4+ T Cell Help for Adoptive T Cell Therapy in Models of Pancreatic Cancer and Non-hodgkin Lymphoma,"* where intriguing data showing a potential for such fusion to overcome the immune suppression due to PD1-PD-L1 axe. Since the authors used two very different cancer models, the results of this study represent a generalized significance.

Importance of crosstalk between immune checkpoint molecules and cellular immunosuppression gets as well increasingly more consideration. About that, the review of Weber et al. *"Myeloid-Derived Suppressor Cells Hinder the Anti-Cancer Activity of Immune Checkpoint Inhibitors"* shed light on a specific immunosuppressive cell population—myeloid-derived suppressor cells, which appear to affect the suppressive potential of immune checkpoint molecules. The authors profoundly discussed the possibility to combine targeting of both immunosuppressive components. This could be valid not only for the malignant melanoma, but also in other cancers, especially in the prostate adenocarcinoma which is highlighted in the review of Elia et al. *"Immune Checkpoint-Mediated Interactions Between Cancer and Immune Cells in Prostate Adenocarcinoma and Melanoma."*

What is new about biomarkers of response to therapy with inhibitors of the immune checkpoint molecules? Kristina Buder-Bakhaya and Jessica Hassel from Heidelberg dealt very intensely with this topic in the review *"Biomarkers for Clinical Benefit of Immune Checkpoint Inhibitor Treatment – A Review From the Melanoma Perspective and Beyond."* The authors thoroughly aggregated the current status of prognostic and predictive biomarkers, used in immune checkpoint inhibition for melanoma and other malignances. They concluded that several possible biomarkers for response to such therapy are now available and should be validated in large clinical trials. Some unexpected relations to the regulatory molecules came as well out in this issue. De Assis et al. could trace a link between circadian clock genes (!) and the aforesaid molecules in their report *"Expression of the Circadian Clock gene BMAL1 Positively Correlates With Antitumor Immunity and Patient Survival in Metastatic Melanoma."* Besides, the gene BMAL1 was found to be associated with an increase in the antitumor immune response but also with the clinical benefit for melanoma patients treated with checkpoint molecule inhibitors; in other words it could be a potential treatment biomarker. What is beyond? In this article collection we are pleased to welcome three original studies, which enlarge the circle of potential biomarkers also apart from malignant melanoma. An interesting report *"Indoleamine 2,3-Dioxygenase Expression Pattern in the Tumor Microenvironment Predicts Clinical Outcome in early Stage cervical Cancer"* was received from the group of Heeren et al. from Amsterdam. The authors claimed that indoleamine dioxygenase can be recognized as a real immune checkpoint molecule. Moreover, they conveniencely demonstrated that a marginal expression of this enzyme predicts a favorable outcome for patients with cervical cancer, making this protein a potential inhibitory target as well as a prognostic biomarker. Another impressive study came from Manjarrez-Orduño et al. *"Circulating T Cell Subpopulation Correlate With Immune Responses at the Tumor Site and Clinical Response to PD-1 Inhibition in Non-Small Cell Lung Cancer."* The title of the paper is self-describing and this work opens new avenues in the field of potential blood biomarkers. In addition to that, head and neck cancer is represented in this special issue. A case report from Qatar by Merhi et al. "*Squamous Cell Carcinomas of the Head and Neck Cancer Response to Programmed Cell Death Protein-1 Targeting and Differential Markers*" introduces a patient suffering from this cancer, who was treated with Nivolumab. The authors indicated that in this case a defined cytokine/chemokine profile might be useful for identifying a response to PD-1 inhibition.

The next series of papers is devoted to the matter of regulation of checkpoint molecule expression. An amiable perspective on the post-transcriptional regulation of CD73/NT5E is delineated by the group of Kordaß et al. from DKFZ Heidelberg in the paper *"Controlling the Immune Suppression: Transcription Factors and MicroRNAs Regulating CD73/NT5E."* They reviewed all contemporary literature concerning this point and highlighted the significance of miRNA involved in the regulation of this checkpoint molecule expression. With respect to transcription factors, Bhat et al. from India showed in their paper *"Checkpoint Blockade Rescues the Repressive Effect of Histone Deacetylases Inhibitors on $\gamma\delta$ T cell Function"* that Eomes and T-bet could be potential regulators of PD-1 expression. The regulation of PD-L1 expression was assessed in the work of Bazhin et al. *"Interferon-α Up-Regulates the Expression of PD-L1 Molecules on Immune Cells Through STAT3 and p38 Signaling."* The authors observed that the type I interferon is indeed involved in the regulation of PD-L1 expression through the above-mentioned transcription factors. A very unorthodox point of view presented by Wang et al. devoted to the gut microbiota in context of immune checkpoint molecules. In their review *"Modulation of Gut Microbiota: A Novel Paradigm of Enhancing the Efficacy of Programmed death-1 and Programmed death Ligand-1 Blockade Therapy"* the authors thoughtfully discussed the influence of gut microbiota on blocking of PD1-PD-L1 axis.

The authors leaded by Yan et al. were concerned with the problems of combining immune checkpoint inhibition with conventional tumor therapy in the manuscript *"Combining Immune Checkpoint Inhibitors With Conventional Cancer Therapy."* They reviewed current literature analyzing the impact of chemo-, radio-, and target therapies on therapeutic effects of immune checkpoint inhibition and discussed the current and the future clinical applications of such combination. Finally, Shevchenko and Bazhin pointed in their work to an importance of discovery of new potential checkpoints molecules. They payed attention in their mini-review "*Metabolic Checkpoints: Novel Avenues for Immunotherapy of Cancer*" to so-called metabolic checkpoint molecules which could be the "new era" of the cancer immunotherapy with checkpoint inhibition.

Summarizing, the wide spectrum of reviews and original papers from this issue provides an insight into new research directions linked to an extremely important topic-immune

checkpoint molecules in context of cancer immunotherapy. We wish all readers of this special issue to have an exciting time to take a close look into a subject of this compendium.

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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Immune Checkpoint Inhibition for Pancreatic Ductal Adenocarcinoma: Current Limitations and Future Options

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Pancreatic ductal adenocarcinoma (PDAC), as the most frequent form of pancreatic malignancy, still is associated with a dismal prognosis. Due to its late detection, most patients are ineligible for surgery, and chemotherapeutic options are limited. Tumor heterogeneity and a characteristic structure with crosstalk between the cancer/malignant cells and an abundant tumor microenvironment (TME) make PDAC a very challenging puzzle to solve. Thus far, targeted therapies have failed to substantially improve the overall survival of PDAC patients. Immune checkpoint inhibition, as an emerging therapeutic option in cancer treatment, shows promising results in different solid tumor types and hematological malignancies. However, PDAC does not respond well to immune checkpoint inhibitors anti-programmed cell death protein 1 (PD-1) or anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) alone or in combination. PDAC with its immune-privileged nature, starting from the early pre-neoplastic state, appears to escape from the antitumor immune response unlike other neoplastic entities. Different mechanisms how cancer cells achieve immune-privileged status have been hypothesized. Among them are decreased antigenicity and impaired immunogenicity *via* both cancer cell-intrinsic mechanisms and an augmented immunosuppressive TME. Here, we seek to shed light on the recent advances in both bench and bedside investigation of immunotherapeutic options for PDAC. Furthermore, we aim to compile recent data about how PDAC adopts immune escape mechanisms, and how these mechanisms might be exploited therapeutically in combination with immune checkpoint inhibitors, such as PD-1 or CTLA-4 antibodies.

Keywords: pancreatic ductal adenocarcinoma, immune checkpoint inhibitors, triple E, antigenicity, immunogenicity, tumor microenvironment

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC), as one of the most fatal malignancies in the world, is the fourth leading cause of cancer-related deaths among both men and women in developed countries (1). Its mortality almost equals its incidence: for 2018 alone, 55,440 diagnoses of pancreatic cancer are projected for the United States with 44,330 associated deaths in the same year (2). At the time of diagnosis, only a minority of patients have localized, resectable tumors (10%); while most patients display locally advanced disease (29%) and/or distant metastasis (52%), and the remainder are not

even staged (2). The 5-year survival rate of pancreatic cancer is only around 7–8% in the United States, which is likely due to late stage diagnosis (2, 3). The high number of estimated pancreatic cancer-related deaths can be hypothesized to be due to several factors: first, late and unspecific symptoms, as well as a lack of PDAC-specific markers or screening resources result in late diagnosis at an advanced stage. Second, delayed diagnosis leads to low resection rates, since most of the tumor patients present with locally advanced or metastatic disease. And third, PDAC is characterized by a low response to radiotherapy (RT) and chemotherapy, which, at least in part, is due to its dense desmoplastic stroma impairing drug delivery (4–6). Also, targeted therapies including small drug inhibitors of key molecular signaling pathways associated with PDAC progression showed almost none (i.e., MEK and PI3K) or only mild benefits (e.g., EGFR) with a moderate increase in overall survival (7–12). Recent advances in chemotherapeutic options for PDAC appear to provide a survival benefit that will likely not be sufficient to decrease mortality rates (13–15). Hence, in developed countries, PDAC is expected to be the second leading cause of cancer-related deaths by 2030 (1, 16). Impaired efficacy of chemotherapy or targeted therapies in cancer was associated with innate and acquired resistance through genetic and epigenetic instability of cancer cells (17, 18). Immunotherapy offers great potential for the treatment of tumors displaying such resistance. Especially T cells with their ability to generate receptors recognizing the heterogeneous and specific repertoire of tumor-related antigens provide great promise in cancer therapeutics. The adaptive immune response might even form an immunological memory providing long-term tumor control. Studies revealing how T cells function under pathophysiological conditions led to development of immune checkpoint inhibitors, which have been successfully translated into the clinic. Thus far, immune checkpoint inhibition (ICI) has shown promising results for the treatment of solid tumors, including melanomas (19–22), as well as lung (23–27), renal (28, 29), bladder (30–32), and head and neck cancers (33, 34), as well as in hematological malignancies, such as Hodgkin's disease (35, 36) and follicular or diffuse-large B-cell lymphoma (37). Although single-agent treatment with immune checkpoint inhibitors showed great promise with many solid tumors, their effect on PDAC has been quite disappointing (38, 39).

Here, we want to discuss the unique characteristics of PDAC immune evasion and why PDAC is unresponsive toward checkpoint inhibition. First, we will provide details concerning immune checkpoint inhibitors and their mechanism of action. Second, the immune-privileged nature of PDAC will be examined. Then, the antigenic and immunogenic attributes of PDAC and how tumor cell-intrinsic and -extrinsic factors within the tumor microenvironment (TME) regulate immunogenicity will be comprehensively discussed, including options for pharmacological modulation of these mechanisms to increase ICI therapy response in the clinic.

IMMUNE CHECKPOINT INHIBITORS

T cells with their various subsets are involved in the regulation of immune responses in autoimmune diseases, but also against infections and cancer. In TME or in tumor-resident lymph nodes, professional antigen-presenting cells (APCs) such as dendritic cells (DCs) display tumor-specific antigens to naïve T cells through major histocompatibility complexes (MHCs) in a process called priming (40). Antigen presentation through MHC-class II acts on naïve $CD4^+$ T cells, giving rise to Th_1, Th_2, and $Foxp3^+$ regulatory T cell (T_{reg}) subtypes, which are all important for immune response orchestration (41): Th_1 polarization induces cytokines (characterized mainly by IFNγ production) further augmenting MHC expression in APCs (42) and antitumor T cell and macrophage cytotoxic activity (43). Th_2 polarized cells are characterized by IL-4 and IL-13 production, leading to exhaustion of T cells and enhancement of other tumor-promoting responses (44, 45). T_{regs} get activated once the effector T cell activation reaches a threshold. With the release of immunosuppressive cytokines (TGF_β and IL-10) T_{regs} negatively regulate T cell effector function (46). On the other hand, antigen presentation through MHC-class I leads to differentiation of naïve $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs), which are directly able to kill antigen-expressing cancer cells (41). Upon MHC:antigen engagement, activated T cells clonally expand in secondary lymphoid organs, and traffic into the inflammatory sites to execute their functions and release intermediary cytokines and ligands to provoke helper immune cells for further support (40).

T cell-mediated immune response is tightly regulated *via* both the repertoire of immunosuppressive cells in the microenvironment and cell-intrinsic regulation of anergy and exhaustion (47). T cell anergy is the state of T cells in which they are hyporesponsive to triggers of naïve T cell differentiation (47). And T cell exhaustion describes a process by which effector T cells become resistant to persistent reactivation (47). Under physiological conditions, T cell activation upon MHC engagement is balanced *via* co-regulation of both stimulatory and inhibitory signals, referred to as immune checkpoints. The balance between stimulatory and inhibitory signals is crucial to generate self-tolerance and to maintain the ability to fight with non-self. However, tumor cells shift this balance toward their benefit by abrogating co-activatory signals and augmenting co-inhibitory signals ultimately heightening anergy and exhaustion (48).

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152) and programmed cell death protein 1 (PD-1 or CD279) are the most studied co-inhibitory receptors of T cell receptor (TCR) signaling (40). The first antibody against CTLA-4, ipilimumab, was approved in 2011 (19), while pembrolizumab and nivolumab, antibodies that both target PD-1, were approved in 2014 for the treatment of melanoma (20, 21, 38). The clinical success of antibodies targeting CTLA-4 and PD-1 marks a breakthrough as these agents established immunotherapy as a new pillar of cancer treatment strategies next to surgery, chemotherapy, and radiation therapy (49).

After TCR engagement with cognate peptide presented by a MHC molecule, costimulatory receptor CD28 binding with CD80 (B7.1) or CD86 (B7.2) amplifies TCR signaling (50). CTLA-4, on the other hand, has higher affinity for CD80 and CD86, outcompeting CD28 binding (50, 51), and subsequently sequestering CD80 and CD86 from the APC surface (52). Initial TCR activation with CD28 co-activation increases IL-2

release, which induces metabolism, proliferation, and survival in a paracrine manner. However, gradual CTLA-4 accumulation on the T cell membrane replaces the activation signal of CD28, blocking IL-2 accumulation (53). Since B7 proteins are expressed on APCs but not on solid tumor cells, the action of CTLA-4 inhibition is thought to take place in secondary lymphoid organs where early T cell activation occurs. CTLA-4 action on $CD8^+$ CTLs is inhibitory, as shown in several studies (54, 55). Still, the overall inhibitory action of CTLA-4 is thought to mainly show itself through its action on $CD4^+$ $Foxp3^+$ T_{regs}, indirectly modulating $CD8^+$ CTL action (48). T_{regs} produce CTLA-4 constitutively through the action of their subset defining transcription factor Foxp3 (56–58). Deletion of CTLA-4 in T_{regs} reduces their activity, blocking their immune-suppressive action (59, 60). Still, use of CTLA4 antibodies in preclinical mouse models of PDAC did not affect T_{reg} infiltration in tumors while enhancing total $CD4^+$ T cell presence (61). T_{regs} might also mediate effector T cell activation through APCs, impairing their B7 ligand expression, and thereby decreasing the CD28 co-activation signal on effector T cells (52). Overall, CTLA-4 engagement downregulates effector T cell activity, while enhancing T_{reg} immunosuppressive activity (59, 62). Inhibiting CTLA-4 action might enhance immunosurveillance through both its action on effector and T_{regs}.

Programmed cell death protein 1 belongs to the family of CD28 proteins, initiating co-inhibitory signaling upon TCR engagement (63, 64). Ligands of PD-1 receptor PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC or CD273) belong to the B7 family of proteins (64–67). PD-1 is expressed mostly on late effector phase $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells in peripheral tissues (63, 68). Especially chronically activated, then exhausted $CD8^+$ cytotoxic T cells show constitutive PD-1 production (69–72). Therefore, PD-1 action is mostly associated with the late phase of immune response, which counterbalances cytotoxic T cell activity. PD-1 is also expressed on T_{regs} and PD-1 blockage leads T_{reg} apoptosis (73). Also, PD-L1 stimulation of naïve T cells can skew differentiation toward the T_{reg} subset (74). Therefore, anti-PD-1 treatment might show an indirect effect on antitumor T cells through its inhibitory actions on T_{regs} (75).

Programmed cell death protein 1 knock out mice show reduced peripheral tolerance and display autoimmunity (76, 77), with a milder phenotype compared with CTLA-4 knock out mice (78, 79). There is a prominent difference between CTLA-4 and PD-1 effects. Anti-CTLA-4 action mostly results in changes in secondary lymphoid organs during the initial phase of naïve T cell activation, while anti-PD-1 treatment targets the effector phase of T cell activation in the periphery where the activated T cells attack the target (40, 48, 80). In addition, CTLA-4 is mobilized to the cell membrane upon TCR engagement in naïve T cells directly from the protein stores, implicating its importance for initial T cell activation (81). By contrast, PD-1 transport requires an initial transcriptional production causing a 6–12 h delay in response upon TCR engagement (48). Considering the differences in mode of action between CTLA-4 and PD-1, PD-1 blockage is thought to be effective in TME (80). Tumor-infiltrating lymphocytes (TILs) in the TME are frequently exhausted due to chronic exposure to the tumor antigens and PD-L1 directly produced by the tumor cells or anti-inflammatory cells of the TME (82). Anti-PD-1 or anti-PD-L1 therapy aims to reduce this exhausted state of TILs in the TME. Of note, PD-1 blockage (e.g., nivolumab) shows milder autoimmunity-related side effects than anti-CTLA-4 treatment (e.g., ipilimumab) in melanoma patients (19, 83). Considering anti-CTLA-4 and PD-1 therapy has implications in different phases of immune response, combination therapy with nivolumab and ipilimumab showed prolonged progression free survival and a higher objective response rate than ipilimumab alone, albeit with concomitant higher toxicity (84).

THE IMMUNE-PRIVILEGED NATURE OF PDAC: IMMUNOSURVEILLANCE AND IMMUNOEDITING

The immunosurveillance hypothesis was proposed by Paul Ehrlich (85) in the early 1900s and later developed further by Thomas and Burnet (86, 87). As a very important concept for cancer immunotherapy, immunosurveillance states that immune cells continually survey somatic cells for any malignant transformation to then destroy them (88). The concept of cancer immunoediting is a byproduct of the immunosurveillance process, in which cancer cells undergo a Darwinian-like selection for their capacity to evade an attack by the immune system (88). The concept of tumor immunoediting proposed by Schreiber and colleagues in 2002 states three different phases of tumor immunoediting: elimination, equilibrium, and escape (i.e., triple E hypothesis) (**Figure 1**) (88, 89). As being more comprehensive than immunosurveillance, immunoediting proposes that not only innate immunity but also adaptive immunity is involved in the elimination process of tumor cells. During the equilibrium phase, tumor cell variants surviving the dynamic but relentless pressure of adaptive and innate immunity undergo a Darwinian-like selection. At the end of the equilibrium phase, many of the tumor cells are dead, whereas new clones generated, likely through genetic instability with better resistance to the immune response, remain. In the escape phase, survivors of the equilibrium phase start to expand in numbers, maintaining an immune-privileged state (89).

Before the wide use of genetically engineered mouse models (GEMMs) of PDAC, human or mouse tumor transplantation into mice had been the main model for preclinical studies of therapeutic response (90). To eliminate simple tissue rejection of tumor xenografts, mostly immune-incompetent mouse models had been utilized. However, these models are unsuitable for studies of the immune response toward tumors. Furthermore, syngeneic murine transplantation models do not provide information regarding the tumorigenesis process. GEMMs for PDAC harboring pancreas-specific expression of mutant Kras recapitulate carcinogenesis of human PDAC, as pre-neoplastic lesions (PanIN) reliably progress to invasive and metastatic cancer (91). In this mouse model, $CD45^+$ leukocytes were shown to accumulate in time as the disease progresses. However, $CD4^+$ T cells observed in PanIN lesions were mostly of the $Foxp3^+$ T_{reg} subtype, accompanied by an abundance of myeloid-derived suppressor cells (MDSCs) and M2 macrophages (92). Strikingly, infiltration by $CD8^+$ antitumor

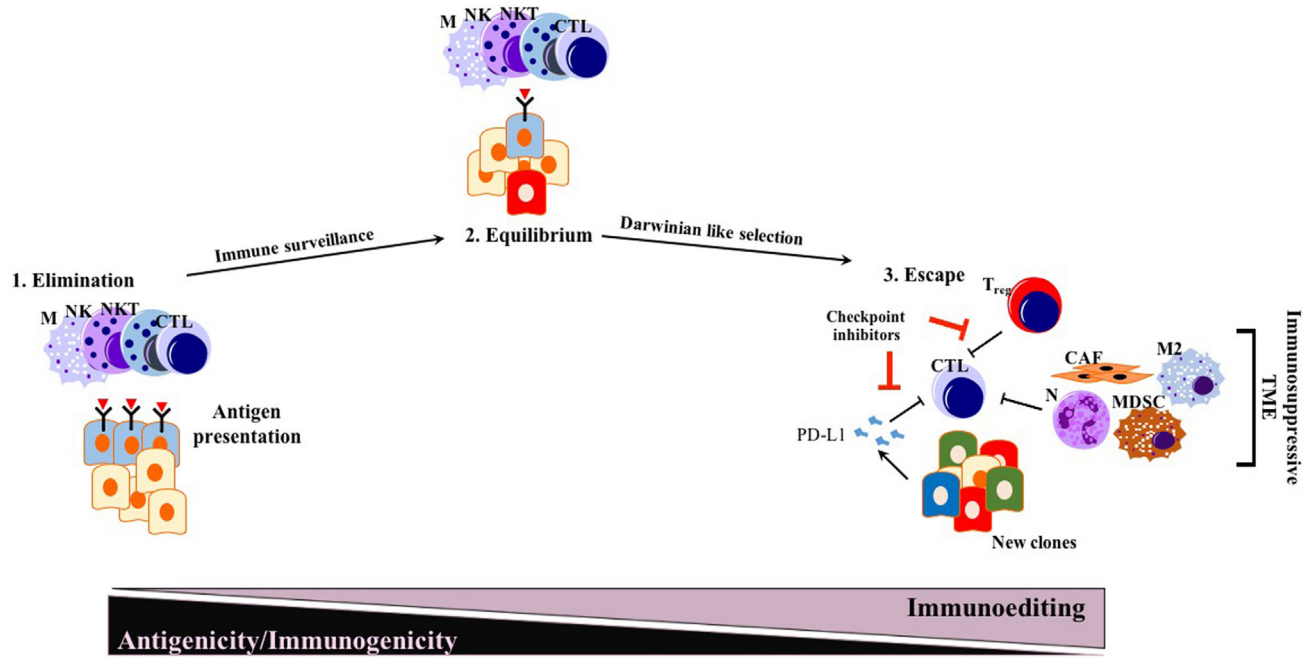

FIGURE 1 | Conventional triple E hypothesis: elimination, equilibrium, and escape. While many solid tumors responding to ICI therapy follow triple E of immunoediting, PDAC is mostly an exception. Generated new clones due to Darwinian-like selection reduce their antigenicity and immunogenicity, escaping from immunosurveillance. Abbreviations: M, macrophages; NK, natural killer; NKT, natural killer T cells; CTLs, cytotoxic T lymphocytes; N, neutrophils; M2, M2 phenotype macrophages; MDSCs, myeloid-derived suppressor cells; T_{reg}, regulatory T cells; CAFs, cancer-associated fibroblasts; ICI, immune checkpoint inhibition.

cytotoxic T cells was very scarce in early PanIN lesions, and only a small portion of advanced tumors actually showed presence of active $CD8^+$ CTLs (92). This spontaneous carcinogenesis model of PDAC highlights the immune-privileged status of PDAC even in the early neoplastic state (92). Unlike for many other solid tumors, the elimination phase of the triple E hypothesis is almost absent or substantially impaired during murine carcinogenesis due to the scarcity of cytotoxic immune cells and the abundant presence of immunosuppressive cells (92). Thus, ablation of T cells did not affect the spontaneous formation of cancer in KPC models (LSL—$Kras^{G12D/+}$; LSL—$Trp53^{R172H/+}$; Pdx—1Cre) (93). However, ectopic expression of a strong neoantigen (e.g., ovalbumin) in cancer cells boosted T cell-mediated immunity, rescuing the elimination phase of the immunoediting sequence. Expression of a single, yet strong, neoantigen thus allowed tumor control *via* CTL infiltration and "Triple E" (immune active) immunoediting. This implies that the scarcity of neoantigens in PDAC is not a result of the elimination step of immunoediting, but rather due to an alternative mechanism more like immune quiescence (**Figure 2**). Because of immune quiescence in tumors with low basal adaptive immune activation, CTLs cannot invade into the TME to initiate conventional immunoediting during carcinogenesis, which is true for the KPC model (93). This model represents human PDAC fairly well, showing an "immune quiescence like" phenotype rather than an "immune active" one (94). Reduced $CD8^+$ CTL and increased $CD4^+$ $Foxp3^+$ T_{reg} infiltration in progressive PDAC has also been validated in human patient samples (95).

In summary, PDAC frequently does not undergo a Darwinian-like selection with respect to the adaptive immune response. Thus, it retains vulnerability toward the natural T cell repertoire. Thus, strategies boosting T cell priming, activation levels, and attraction are promising for the treatment of this cancer (96).

FACTORS DETERMINING THE EFFICACY OF ICI AND FAILURE IN PDAC

Two important factors determine the prospects of immunotherapy of cancer in general, and checkpoint inhibition, in particular, antigenicity and immunogenicity, the latter being modulated by both intrinsic properties of tumor cells and TME (97) (**Figure 3**). Considering the, to date, low efficacy of immunotherapy, and especially checkpoint inhibition in PDAC, a better understanding of the immune escape mechanisms present in PDAC will pave the way for combination factors of checkpoint inhibition for the treatment of this generally intractable disease. A list of selected preclinical mouse model studies focusing on ICI combination therapies in PDAC can be found in **Table 1**.

Antigenicity

Antigenicity refers to the ability of tumor cells to produce and present tumor-specific antigens (TSA) and tumor-associated antigens (TAAs) to the adaptive immune system (97). The bottlenecks of antigenicity include the range of TAA and TSA production, and their ability to be presented to the immune system

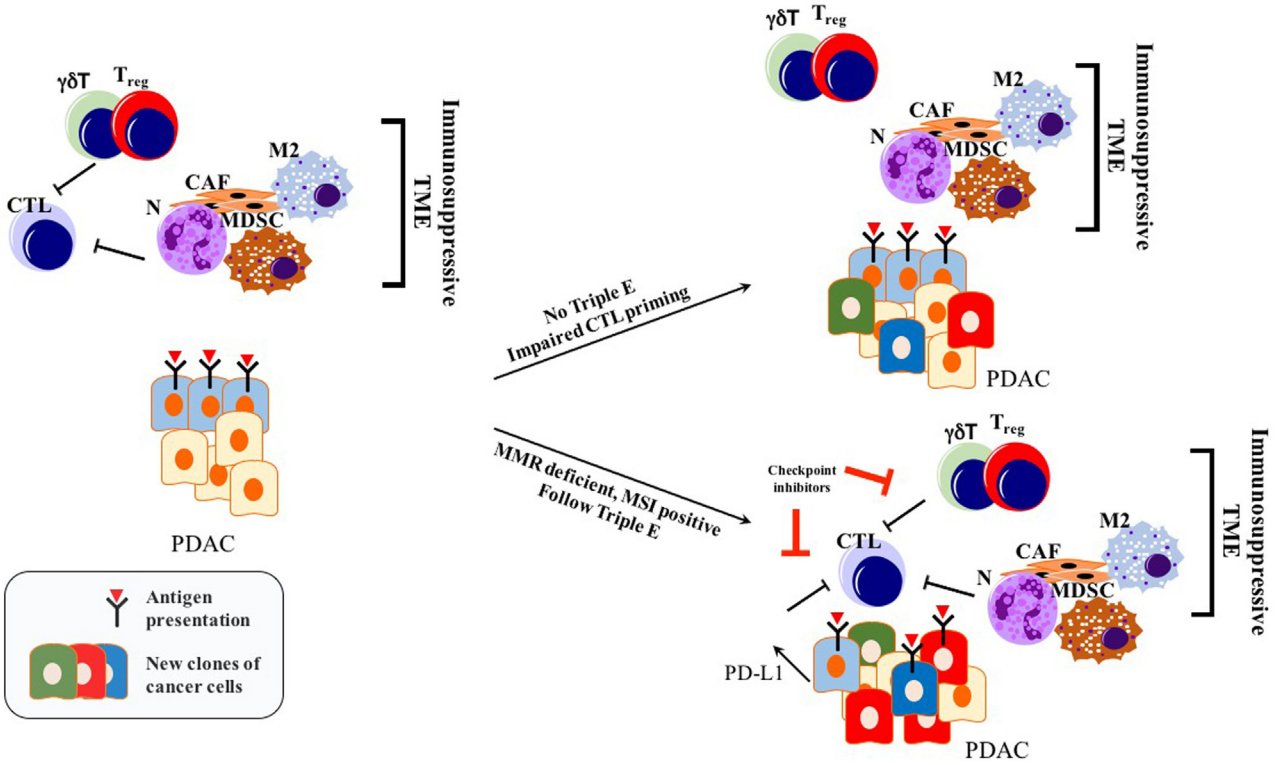

FIGURE 2 | Immunoediting in PDAC: only tumors with genetic instability follow Triple E, while others cannot. Immunosuppressive TME blocks initial CTL priming. Therefore, cancer cells are not forced to undergo Darwinian-like selection. PDAC can still retain its antigenic capacity while impairing immunogenicity making it unresponsive to checkpoint inhibitors. Abbreviations: CTLs, cytotoxic T lymphocytes; N, neutrophils; M2, M2 phenotype macrophages; MDSCs, myeloid-derived suppressor cells; T_{regs}, regulatory T cells; CAFs, cancer-associated fibroblasts; γδT, γδT cells; MMR, mismatch repair; MSI, microsatellite instability; TME, tumor microenvironment.

through MHC complexes (human leukocyte antigen—HLA—in humans) (97, 98). TAAs are overexpressed in cancer cells while their expression is low in normal cells, whereas TSA subtype neoantigens are produced *de novo* upon mutational changes of tumor cells (98). These mutations can favor neoantigen tethering to MHCs, produce a new residue on neoantigens increasing TCR recognition, or generate a proteolytic cleavage site, providing better processing for antigen presentation (99). Since TSAs are expressed only in malignant cells, they provide great specificity for T cell cytotoxicity (98). Epigenetic regulation of TAAs in tumor cells can also represent an important target for T cell action (99). In melanoma patients, even tumors with a low mutational burden, but with a high expression of TAAs, which is likely mediated by epigenetic mechanisms, showed considerable response to immunotherapy (100).

Cancers with high mutation rates such as melanoma, bladder cancer, and lung cancer show better response to check point inhibition compared with other types with a lower mutational burden, for instance, PDAC (101–105). Especially tumors with mismatch repair (MMR) deficiency or with more microsatellite instability (MSI) are shown to respond better to immunotherapy (106). As a matter of fact, impairing MMR through genetic inactivation of mutL homolog 1 gene (MLH1) in PDAC mouse models provoked hypermutation, triggering more neoantigen production. This, in turn, prolonged immunosurveillance with better therapeutic response to immune check point inhibitors (**Figure 2**) (107). Humphris et al. reported that among the 385 resected patient samples only 1% of showed MSI with inactivation of MLH1 and MSH2 (mutS protein homolog 2). This may provide a possible explanation for low response rate to immunotherapy in PDAC (108). Pembrolizumab, a PD-1 antibody, was approved by the Food and Drug Administration in 2017 for solid tumors with MMR defects or MSI, including PDAC (106). Use of DNA damage response (DDR) inhibitors may also enhance the genetic instability of the cancer cells upon exposure to DNA damaging agents, increasing the production of neoantigens. On the other hand, DDR inhibition may show a tumorigenic effect by acting on antitumor immune cells (109). Still, mutational load is not a reliable biomarker for the prediction of response to immunotherapy, considering the patients who were not responding to immunotherapy even if they had a high mutation burden. Likewise, tumors with a low mutational load, such as renal cell carcinomas, responded well to immunotherapy (28, 110).

Recently, Balachandran et al. described a neoantigen quality fitness model identifying long-term survivors of PDAC *via* selecting neoantigens with great resemblance to disease derived peptides (111). On the other hand, a neoantigen quantity model showing more immunosurveillance in response to increasing

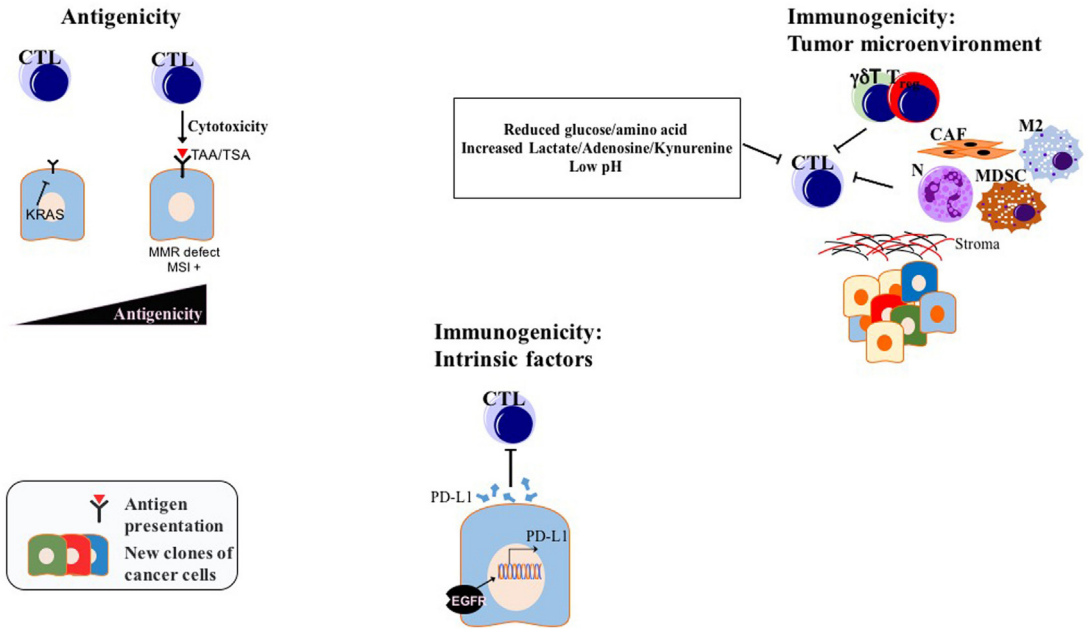

FIGURE 3 | Factors determining ICI efficiency in PDAC: while modulation of antigenicity, intrinsic immunogenicity, and extrinsic immunogenicity *via* TME might be valid for many tumors, drawn examples above are experimentally shown for PDAC. Abbreviations: TAAs, tumor-associated antigens; TSAs, tumor-specific antigens; CTLs, cytotoxic T lymphocytes; N, neutrophils; M2, M2 phenotype macrophages; MDSCs, myeloid-derived suppressor cells; T_{regs}, regulatory T cells; CAFs, cancer-associated fibroblasts; γδT, γδT cells; MMR, mismatch repair; MSI, microsatellite instability; TME, tumor microenvironment; ICI, immune checkpoint inhibition.

neoantigen numbers revealed no long-term survivors by itself. Only tumors showing both, high neoantigen numbers and abundant $CD8^+$ cytotoxic T cell infiltration, were associated with a significant survival benefit for the patients. Supporting the immune quiescence-like phenotype of PDAC, a modest decrease in high-quality neoantigen transcript levels was seen. More strikingly, they identified a loss of high-quality neoantigen expression in metastatic tumors compared with their primary counterparts. In conclusion, identification of hotspot neoantigens and methods to exploit or target them may increase the response to checkpoint inhibition, not only regarding the primary tumor but also regarding metastatic lesions.

Another mode of reduced antigenicity is the loss of antigen presentation, which can reduce immunosurveillance either by blocking priming of naïve T cells, or by making cancer cells invisible to effector T cell function (97, 112). In other cancer types, reduced antigen presentation was achieved by downregulation of MHC class proteins or impaired antigen processing and shuttling (113–116). Oncogenic RAS signaling was shown to reduce antigen presentation in different cancer types, including PDAC (113, 117, 118). Also, HLA-1 and transporter for antigen presentation production was demonstrated to be reduced in human PDAC specimens (113, 117–119). Manipulating cancer cells for enhanced antigen presentation can reinforce the checkpoint inhibition response.

As a matter of fact, Pommier et al. recently showed that disseminated cancer cells (DCCs, metastatic, quiescent single cancer cells) are undergoing a Darwinian-like selection during immune surveillance of metastasis (120). These investigators elegantly showed that only the metastatic cancer cells, negative for MHC-I and CK-19 expression on the surface, could form DCCs, avoiding T cell-mediated killing in pre-immunized mice. ER stress was the barrier for DCCs to maintain a quiescent state, and also to escape from T-cell-mediated immunity. Therefore, to form macrometastasis, in addition to ER stress relieve, a systemic immunity depletion was required. All these results show how important it is for cancer cells (both primary and metastatic tumor cells) to have good quality neoantigens, and a competency to present neoantigens through MHC complexes to immune cells.

The correlation between total neoantigen load and checkpoint inhibition response is absent in PDAC unlike in other, immunogenic tumors, such as melanoma or lung cancer (104, 121–125). This implies that other factors, determined by the immunogenic properties of PDAC, play an important role in the response to immunotherapy of this malignancy.

Immunogenicity

Immunogenicity of cancer refers to its ability to induce an adaptive immune response. Based on comprehensive integrated genomic analysis, PDAC was classified into different subgroups by several studies (126–130). RNA expression analysis identified an immunogenic subtype of PDAC in 25 among 96 PDAC patient specimens. This subtype is associated with an increased immune cell infiltration, and enriched signatures such as $CD4^+$ and $CD8^+$ T cell signaling, antigen presentation, B cell signaling, and most notably CTLA-4 and PD-1 signaling. Signatures enriched in immunogenic subtype might represent predictive biomarkers for immunotherapeutic response in PDAC (130).

TABLE 1 | Selection of studies focusing on immune checkpoint inhibition (ICI) combination therapies in preclinical mouse PDAC model.

Combination approach	Method	Preclinical mouse model	Control group/treatment	Experimental group/treatment	Results	Reference
Oncogenic signaling	MEK inhibition	Subcutaneous transplantation of $KP^{lox/+}C$ mouse cell line	Either MEKi (GSK1120212) or mPD-1-Ab	MEKi and mPD-1-Ab	Reduced tumor growth and possible regression	(140)
Stromal remodeling	FAP^+ cell depletion	$KP^{R172H}C$ transgenic mouse model with modified *fap* gene driving diphtheria toxin receptor expression in FAP^+ cell	Only diphtheria toxin (DTx)	DTx with mPD-L1-Ab	Reduced tumor volume	(158)
			Only diphtheria toxin (DTx)	DTx with cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)-Ab	Deceleration of tumor growth	
	CXCR4 inhibition	$KP^{R172H}C$ autochthonous mouse model	CXCR4i (AMD3100) with isotype control	CXCR4i and CTLA-4-Ab	No effect	
			CXCR4i (AMD3100) with isotype control	CXCR4i and mPD-1-Ab	Reduced tumor growth	
	Focal adhesion kinase (FAK) inhibition	Syngeneic and orthotopic tumor transplantation of mouse PDAC cell lines isolated from $KP^{lox/+}C$ mice	Low dose gemcitabine with either FAKi (VS4718) or mPD-1-Ab	Low dose gemcitabine with FAKi and mPD-1-Ab	Reduced tumor burden, improved overall survival	(160)
			Low dose gemcitabine with either FAKi or anti-CTLA4	Low dose gemcitabine with FAKi, and CTLA-4-Ab	No benefit	
			Low dose gemcitabine with FAKi and mPD-1-Ab	Low dose gemcitabine with FAKi and mPD-1-Ab and CTLA-4-Ab	Reduced tumor burden	
		$KP^{lox/lox}C$ autochthonous mouse model	Low dose gemcitabine with mPD-1-Ab and CTLA-4-Ab	Low dose gemcitabine with FAKi and mPD-1-Ab and CTLA-4-Ab	Increased survival, 2/15 mice are long-term survivors	
	Interleukin 6 (IL-6) targeting	Isolated cancer cells from $KP^{R172H}C$ mice and Pan02 cells were subcutaneously transplanted, KPC-luc cells orthotopically transplanted into C57BL/6 mice	Either isotype control or anti-IL-6 or mPD-1-Ab	Anti-IL-6 and mPD-1-Ab in combination	Reduced tumor growth	(169)
		KPC-Brca2 autochthonous mouse model	Isotype control	Anti-IL-6 and mPD-1-Ab in combination	Extended overall survival	
	Hyaluronan depletion	Orthotopic transplanted $KP^{R172H}C$-luc cells or KPC-Brca autochtonous mice	Either *Salmonella*-based sh-IDO (shIDO-ST) delivery or PEGPH20	*Salmonella*-based sh-IDO (shIDO-ST) delivery and PEGPH20	Reduced tumor burden, increased overall survival	(177)
Myeloid compartment	Cluster of differentiation 40 (CD40) agonist	Subcutaneously transplanted $KP^{R172H}C$ cells	Either gemcitabine/nab-paclitaxel or CD40 agonist-Ab	Gemcitabine/nab-paclitaxel and CD40 agonist-Ab	Higher tumor regression, enhanced survival, reduced overall tumor growth rate, maintained T cell memory	(203)
	CXCR2 inhibitors	$KP^{R172H}C$ autochthonous mouse model	mPD-1-Ab treatment with vehicle	mPD-1-Ab treatment with CXCR2 SM (AZ13381758)	Extended survival, 2/14 mice long-term survivors	(212)
	CSF1R inhibitors	Orthotopic transplantation of KC-$INK4A/Arf^{lox/lox}$	Gemcitabine with either vehicle or CTLA-4-Ab or CSF1Ri (PLX3397)	Gemcitabine with CTLA-4-Ab and CSF1Ri	More than 90% reduced tumor progression	(216)
			Either vehicle or CTLA-4-Ab and mPD-1-Ab combination, or CSF1Ri	CTLA-4-Ab, mPD-1-Ab, and CSF1Ri combination	Completely blocked tumor progression, 15% tumor regression	
			Gemcitabine with either vehicle or CTLA-4-Ab and mPD-1-Ab combination, or CSF1R-Ab	Gemcitabine with CTLA-4-Ab, mPD-1-Ab, and CSF1R-Ab combination	Completely blocked tumor progression, 85% tumor regression	
Metabolic regulation	Glucocorticoid treatment	Pre-cachectic $KP^{R172H}C$ autochthonous	Isotype and PBS treatment	CXCR4i (AMD3100) with mPD-L1-Ab	Arrested PDA growth	(254)
			Isotype, PBS, and corticosterone treatment	CXCR4i (AMD3100), mPD-L1-Ab, and corticosterone	PDA is no more arrested, tumor growth in control and experimental groups was same	
Radiotherapy	Radiation with ICI	Subcutaneous transplantation of $KP^{R172H}C$ cell line	Either treatment of CTLA-4-Ab or mPD-1-Ab or radiation, or dual combinations	CTLA-4-Ab, mPD-1-Ab, and radiation triple combination	Extended survival	(269)
	Radiation with CD40 agonist-Ab	Subcutaneous and orthotopic transplantation of $KP^{R172H}C$ cell line	Radiation with CTLA-4-Ab and mPD-1-Ab	Radiation, CTLA-4-Ab, mPD-1-Ab, and CD40 agonist-Ab	Increased abscopal effect, extended survival	(270)

Cytolytic activity is determined by the transcription levels of granzyme A (GZMA) and perforin (PRF1), which are known cytotoxicity markers of $CD8^+$ T cells (131). Interestingly, genetic amplification of MYC and/or deletion of CDKN2A/B were associated with reduced cytolytic activity in TCGA PDAC datasets (125). Mutant Kras-mediated immunosuppression *via* GM-CSF or IL17R production might be another reason for impaired cytolytic activity in PDAC (132–134). Other than oncogenic drivers, stromal composition may have an impact: PDAC with so-called "normal" stroma (127) (i.e., a good version of stroma, characterized by high ACTA2, VIM, and DES pancreatic stellate cell-PSC markers) was associated with a higher cytolytic activity (125). Considering various factors determine cytolytic activity other than neoantigenic capacity, it is important to adapt individualized precision immunotherapy covering different determinants of immunogenicity in PDAC (125, 135). For an ease of understanding, the determinants of immunogenicity can be divided into two: intrinsic and extrinsic factors.

Intrinsic Determinants of Immunogenicity

Antigenic tumors can still evade ICI therapy *via* downregulation of tumor cell-intrinsic immunogenicity (97). In various cancers, stimulation of oncogenic pathways such as PI3K (136, 137), MYC (138), TAZ (139), and JAK-STAT (35) through either excessive ligand production or their mutations induces constitutive PD-L1 production (**Figure 2**). Myeloid cell induction of EGFR and MAPK signaling in PDAC cells enhanced PD-L1 production inhibiting $CD8^+$ T cell infiltration (140). The expression of PD-L1 in various tumors was associated with higher immune cell infiltration and the presence of lymphoid aggregates, and tumors with naturally high levels of PD-L1 in these showed comparably high response rates to anti-PD-1 or anti-PD-L1 (38, 83, 141). Regulation of PD-L1 and other checkpoint inhibitors or oncogenic signaling cascades in cancer cells also constitute an important place for the regulation of tumor immunogenicity (142–144).

Although initial IFNγ production is favorable for CTL activity, chronic exposure may lead to immunoediting in tumor cells. As a result of this, tumors develop genetic or epigenetic modifications in IFNγ signaling components such as IFNγ receptors (IFNGR1 and IFNGR2), JAK-STAT pathway components and IRF1 transcription factors (145, 146). A loss of function mutation on Apelin receptor has recently been identified impairing IFNγ induced JAK-STAT signaling cascade in melanoma (147). Although IFNγ is considered to be antitumorigenic, its induction of PD-L1 transcription in cancer cells might positively correlate to anti-PD-1 or PD-L1 therapy response in established tumors (148). Since IFNγ exposure of cancer cells induces PD-L1 production, mutations in IFNγ signaling components JAK1 and JAK2 would lead to clonal evolution of PD-L1-negative tumor cells, which are not responsive to anti-PD-1 treatment (149). Although no such mutations have been identified in PDAC, personalized medicine can favor the prediction of checkpoint inhibition response through identification of these type of mutations.

Several solid tumors including PDAC showed anti-PD-1 resistance signatures (IPRES) such as enhanced mesenchymal transformation, cell adhesion, extracellular matrix modeling, angiogenesis, hypoxia, and wound healing in TCGA datasets (123). Overall, the differential mutational and transcriptional landscape of tumors does not only determine neoantigen quality and quantity but also regulates several signaling pathways responsible for intrinsic and extrinsic properties of immunogenicity in cancer.

Extrinsic Determinants of Immunogenicity: Modulation of TME

Cytotoxic T lymphocyte infiltration into the TME is essential for ICI therapy (150). Even if the anti-tumor CTL infiltration is seen in many tumor types, PDAC represents an outlier in this manner (92). Starting from the premalignant lesions, its microenvironment restricts the cytotoxic T cell infiltration. The cytotoxic T cell function is limited through the actions of immunosuppressive cells in the TME such as cancer-associated fibroblasts (CAFs), myeloid cells, and inhibitory actions of some T cell subsets, albeit they infiltrate in the TME (97, 151). In support of this, strategies eliminating immunosuppressive populations in the TME enhanced CTL infiltration in various cancers (152, 153). To shift the immunosuppressive environment to a non-immune-privileged status, it is important to be aware of the individual components of the TME and to know how to modulate them.

Stromal Remodeling

The characteristic abundant desmoplastic stroma of PDAC can be both beneficial and harmful in terms of carcinogenesis. Studies showed that transplantation of PDAC cancer cells with pancreatic stellate cells increased tumorigenic potential and metastasis (154). However, depletion of stroma in preclinical mouse models also revealed further accumulation of T_{regs} in the TME showing the dual nature of stromal compartment (155). In a study performed on human PDAC tissues, the fibrotic reaction did not impair TIL infiltration, rather fibrosis associated collagen-I amount positively correlated with effector T cell presence (156). However, previous studies showed the inhibitory actions of αSMA^+ CAFs on $CD8^+$ CTLs in PDAC TME (157, 158). These results indicated the presence of (?) tumor heterogeneity not only in terms of cancer cells but also stromal compartments of PDAC (159).

One study revealed that depletion of CAFs could actually be employed to increase the immunotherapy response of PDAC: fibroblast activation protein (FAP^+) CAFs were shown to induce chemokine (C-X-C motif) ligand 12 (CXCL-12) mediating immunosuppression through limiting effector T cell infiltration (158). Targeted inhibitors of FAP^+ CAFs or CXCL-12 chemokine (C-X-C motif) receptor (CXCR-4) inhibition *via* AMD3100 increased $CD3^+$ T cell accumulation and revealed a synergistic effect with anti-PD-L1 therapy in mouse models (158).

Further studies focusing on focal adhesion kinase (FAK) showed FAK inhibition in cancer cells can remodel stroma, inhibiting immunosuppressive TME cells (160). Combination of FAK inhibitor with gemcitabine and anti-PD-1 increased $CD8^+$ CTL infiltration, reducing tumor burden and prolonging overall survival (160). Even though single agents targeting FAK inhibition in PDAC showed no objective response in clinic (161–163), trials combining iFAK (vs.-4718) with gemcitabine and anti-PD-1 are ongoing (NCT02758587).

The importance of interleukin 6 (IL-6) signaling in PDAC has been shown by several groups revealing its importance on both carcinogenesis and persistency (164–166). CAFs are also responsible for the production of pro-inflammatory cytokines other than myeloid cells such as IL-6 (167). Unfortunately, clinical trials targeting IL-6 alone demonstrated no benefit (168). However, preclinical studies targeting IL-6 in combination with PD-L1 showed decreased αSMA^+ stromal cells and increased $CD3^+$ lymphocyte infiltration in KPC and Panc02 subcutaneous and orthotopic transplantation models and a survival benefit in the KPC-Brca2 autochthonous mouse model (169).

Hyaluronan, an extracellular matrix component, is a linear glycosaminoglycan in PDAC, associated with multiple markers of aggressiveness of cancer for instance increased cell proliferation, invasion, and metastasis (170). High hyaluronan expression correlates with worse prognosis in PDAC patients (171). Several drugs have been developed to deplete stromal hyaluronan, such as PEGPH20. In preclinical models, hyaluronan depletion *via* PEGPH20 remodeled stroma, decreased interstitial fluid pressure, and increased drug delivery by enhancing micro-vessel permeability (172–174). As PEGPH20 increased delivery of chemotherapeutic agents in PDAC preclinical models, the same was seen for monoclonal antibodies (trastuzumab) in breast cancer (175). With the use of transplanted and autochthonous PDAC mouse models, *Salmonella*-based IDO-1 depletion (176) was also enhanced with (by means of gibi mi?) PEGPH20 treatment (177). Vitamin D receptor (VDR) was identified as a PSC master regulator for dynamic regulation of stromal composition. Treatment with VDR ligand reduced inflammation and enhanced gemcitabine delivery and efficacy in a mode of action similar to hyaluronan depletion (178). Based on these results, hyaluronan depletion or VDR activation appear as promising combination partners of checkpoint inhibitor monoclonal antibodies in clinical trials.

Modulation of Immunosuppressive Myeloid Cells

Tumor-associated macrophages (TAMs) differentiate from resident macrophages or mobile inflammatory monocytes (179). TAM polarization can be both beneficial and harmful in terms of carcinogenesis. M1 differentiation of TAMs is known to be antitumorigenic due to their tumoricidal nature *via* releasing pro-inflammatory cytokines. By contrast, the M2 subtype is pro-tumorigenic, since it suppresses immunosurveillance by secreting anti-inflammatory cytokines, e.g., TGF_β and IL-10 or by remodeling tumor stroma (180). Consistent with this, expression of M2-related markers such as CD204 and CD163 negatively correlate with patient survival (181, 182). Derived from immature cells of myeloid origin, MDSCs are known for their neoangiogenic and immune-suppressive activities in TME. MDSCs have been shown to inhibit CTL activity by recruiting T_{reg} subset, modulating amino acid reserves in TME, and pushing T cells toward apoptosis *via* ROS production (183). Also, the presence of immunosuppressive cells such as M2 macrophages, T_{regs}, and MDSCs in PDAC negatively correlates with overall survival (155, 184–188). Both pro- and antitumorigenic properties of neutrophils in cancer are reported, and their inhibitory action on CTL activity is known to be mediated by various mechanisms (189). Considering the complexity of immune cells in TME and their crosstalk with T cell activity, it is challenging but important to modulate these mechanisms to boost ICI response in cancer.

Cluster of differentiation 40 (CD40) is a member of the tumor necrosis factor receptor superfamily and is expressed on APCs including monocyte subsets, DCs, macrophages, and B cells (190). CD40 signaling is important for licensing APCs (to maximize their capacity to present antigens) followed by cross-priming of $CD8^+$ CTL in lymph nodes (191–193). CD40 agonists mediated an enhancement of adaptive antitumor immunity in preclinical mouse models in various cancer types (194–196). By contrast, treatment of KPC mice with CD40 agonist (FGK45) and gemcitabine transiently blocked PDAC development through re-education of tumor-infiltrating macrophages and stromal remodeling, but was not able to invoke an adaptive antitumor immune response (197). On the other hand, subcutaneous transplantation of KPC cancer cells into syngeneic mice revealed that the same treatment strategy induced an adaptive immune response with $CD4^+$ and $CD8^+$ T cell infiltration. Consequently, the authors used another "two tumor" model, in which intact KPC tumors (cancer cells with intact TME) were transplanted into endogenous tumor-bearing KPC mice. Here, gemcitabine with FGK45 treatment induced a $CD4^+$ and $CD8^+$ T cell infiltration into subcutaneous tumor but only $CD4^+$ infiltration into endogenous tumor. The barrier for $CD8^+$ repletion in spontaneous tumors was exceeded through systemic macrophage depletion. Upon deeper analysis, $Ly6C^{low}$ $F4/80^+$ macrophages residing in vicinity of PDAC TME were identified as the responsible physical barrier for CTL infiltration (198). In a similar manner, CTL-mediated antitumor immune responses were not seen in clinical trials with CD40 agonists in various cancers even with the addition of gemcitabine to increase tumor immunogenicity (199–202). CD40 agonist treatment finally acted as a checkpoint co-activator through its action on APCs inducing T cell priming upon gemcitabine/nab-paclitaxel dual treatment (203). In conclusion, PDAC retains its antigenic properties to induce both innate and adaptive immune response. This antigenicity might be increased through the use of chemotherapeutics or targeted therapy. Yet, since PDAC is immunologically cold (i.e., very scarce resident CTL infiltration) to respond to increased antigenicity, mechanisms to enhance CTL infiltration must be elucidated. Combination of gemcitabine with nab-paclitaxel remodels TME to permissive conditions for CTL infiltration, but not with gemcitabine alone (203). Furthermore, once T cell priming barrier is exceeded through CD40 agonist treatment, CTL activity might be more expedited with checkpoint inhibitor usage.

C-X-C motif chemokine receptor 2 (interleukin 8 receptor beta, CXCR-2) is a G-protein-coupled receptor for various CXCL ligands including IL-8. CXCR-2 in a cell type-specific manner can act both as a tumor suppressor where it induces senescence in premalignant lesions of PDAC (204, 205) and as tumor promoting *via* enhancing neutrophil and MDSC recruitment to TME (152, 206–208). Through inhibition of CXCR-2 either genetically or pharmacologically with CXCR-2 pepducin (209, 210) or AZ13381758 (211) inhibitors, Steele et al. showed an enhanced response to anti-PD-1 therapy and decreased metastasis in PDAC (212). The enhanced therapy response is reasoned by reduced

infiltration of monocytes and MDSCs, which augments T cell infiltration in TME. Also, they propose that stromal remodeling through T cell recruitment might enhance gemcitabine efficacy in tumors (212, 213).

Colony-stimulating factor 1 receptor (CSF1R) is an important regulator of TAMs' differentiation and sustenance in microenvironment (214, 215). Therefore, inhibition of CSF1R is considered to have potential for cancer therapeutics. Yet, single-agent use targeting CSF1R did not yield clinical benefits in various tumor types (215). In mouse models, treatment of PDAC with CSF1R inhibitors enhanced antitumor immune response; however, this effect was diminished due to the production of checkpoint proteins such as PD-L1 and CTLA-4 (216). Combination of checkpoint inhibitors with CSF1R blockage showed regression of tumors in mouse models (216). CSF1R inhibition was shown to have an effect not only on TAMs but also on CAFs in various subcutaneously transplanted mouse models (217). Recently, CSF1R blockage was shown to enhance the production of granulocyte-specific chemokine expression such as CXCL-1 by CAFs increasing polymorphonuclear MDSC (PMN-MDSC) recruitment as a resistance mechanism (217). PMN-MDSC cells are known for their pro-tumorigenic and anti-immunogenic properties (218). Therefore, combination treatment of CSF1R and CXCR2 inhibitors (see above) targeting, respectively, both TAMs and MDSCs enhanced anti-PD-1 therapy response in transplanted tumor models (217). Considering response enhancement by usage of either CXCR2 or CSF1R inhibitor in combination with immune checkpoint inhibitors, simultaneous use of the three might exploit a broader benefit for therapy response also in PDAC (208, 212).

B Cells

Bruton's tyrosine kinase (BTK) is an enzyme expressed in B cells, macrophages, and mast cells, and targeting BTW in combination with ibrutinib was shown to be effective in chronic lymphocytic leukemia, Mantle cell lymphoma, and Waldenstrom's macroglobulinemia (219–221). Besides targeting BTK, ibrutinib also inhibits interleukin-2-inducible T-cell kinase in T cells, skewing Th differentiation toward Th_1 (222). Because of this effect, dual combination of ibrutinib with anti-PD-L1 inhibitor was shown to have a synergistic effect in a T cell-dependent manner, but not MDSC dependently in studies with mouse transplantation models of lymphoma, breast, and colon cancer (219). In various PDAC preclinical mouse models, ibrutinib demonstrated its antitumorigenic effect *via* depletion of macrophage deposition and fibrosis (220). In another study, on the other hand, ibrutinib enhanced macrophage production of Th_1 differentiation cytokines, while inhibiting Th_2, and augmenting the $CD8^+$ cytotoxic T cell deposition in tumors. The effect on macrophage activity was also dependent on B cells, and B cell-specific BTK signaling, still there was no change in fibrosis (221). Based on these results, checkpoint inhibition in combination with BTK inhibitor ibrutinib might enhance the therapeutic benefit of single use of each in PDAC, accordingly clinical trials are ongoing.

γδT Cells (γδT)

T cells are broadly divided into two subtypes based on the antigen receptor types they express: αβT and γδT (223). While 95% of the $CD3^+$ T cells in blood express αβTCR (includes $CD4^+$ and $CD8^+$ T cells) recognizing MHC class I–II, 5% have γδTCR which does not require MHC engagement for activation: γδT are cytolytic through the release of inflammatory cytokines (224, 225). There are conflicting data about the function of γδT in PDAC, with both pro- and antitumorigenic potential. Isolated γδT were shown to be tumoricidal to PDAC cell lines *in vitro* (226). By contrast, in mouse models, pre-neoplastic lesions with $KRAS^{G12D}$ were shown to recruit IL-17-expressing immune cells including γδT, which accelerated carcinogenesis through IL-17 receptor oncogenic signaling (133). In support of this, genetic and therapeutic depletion of γδT in mouse models prolonged survival. Other than the IL-17-mediated oncogenic effect on PanIN lesions, γδT directed checkpoint receptor inhibitory action (through galectin-9 and PD-L1 expression) on αβT cells, accelerating carcinogenesis. While ablation of $CD4^+$ and $CD8^+$ T cells had no impact on PDAC generation and persistency, this was different upon δTCR knock out: γδT cell deletion increased $CD8^+$ CTL and $CD4^+$ Th_1 tumor infiltration, and skewed $CD4^+$ differentiation toward the Th1 type. More importantly, the immunosuppressive action of γδT cell was not due to an effect on MDSCs or TAMs; instead, it was directly dependent on checkpoint co-inhibitory receptor engagement with antitumor T cells. PD-L1 and Galectin-9 checkpoint inhibition was effective in tumors with γδT cell present, but not in their absence. This implies the importance of personalized medicine, through which the γδT cell presence may be characterized in patients, to predict checkpoint inhibition therapy response (227).

Metabolic Regulation

Enhancing checkpoint inhibition efficiency may also be achieved through regulation of metabolic properties of T cells. For cytotoxic and effector T cell activity, a metabolic switch from a catabolic to anabolic state is important (228–231). While naïve T cells rely mostly on oxidative phosphorylation, activated T cells prefer to switch aerobic glycolysis for faster ATP production (230, 231). In support of this, T cells in anergic state even with TCR engagement and costimulator checkpoint activation can retain their hyporesponsive state in a nutrient poor environment (232). The nutrient poor microenvironment with low glucose and amino acid reservoir is regulated by both cancer cells and the TME (233). Cancer cells, for example, outcompete T cells for glucose uptake having implications for intrinsic immunogenicity regulation (234). Furthermore, glutamine usage by cancer cells also limits its presence in TME, limiting its activator function on T cells (235, 236). ARG-1 (Arginase 1) produced by TAMs and MDSCs degrades arginine (237, 238), while indoleamine 2,3-dioxygenase (IDO-1) produced by cancer cells, TAMs, and MDSCs converts tryptophane to an immunosuppressive metabolite kynurenine reducing T cell activity (239–244). Other than limiting nutrient availability, production of immunosuppressive intermediary metabolic products can also impair T cell activation. Cancer cell production of lactate as a result Warburg effect can impair T cell immunity by both decreasing TME pH and lactate shuttling into T cell (245–247). In addition, adenosine produced by cancer and T_{regs} (248–250), and prostaglandin E2 produced by TAMs

and MDSCs are known to inhibit T cell signaling (251). Even though PDAC with its cancer cell and TME components shows similarities in metabolic properties as discussed above, how these metabolic properties effect T cell immunity specifically in PDAC has not been well studied (252). Overall, other than modulation of cytokine–chemokine–receptor axis, nutrient availability and production of immunosuppressive metabolites might also affect the extent of T cell immunity.

The impact of metabolism on checkpoint inhibition efficacy may not be only relevant on a micro environmental but also on a more systemic level. Cachexia is a systemic disorder with an excessive weight loss through the consumption of muscles and adipose tissues (253). Many diseases are associated with cachexia, including cancer in general and PDAC in particular (253). An increase in serum IL-6 levels was shown to impair hepatic ketogenesis inducing cachexia in C26 colon cancer and autochthonous KPC-PDAC mouse models (254–256). Physiologically, the body responded to cachexia with an upregulation of glucocorticoids like (?) corticosterone, which inhibits T cell infiltration into tumors of C26 cells. In support of this, transcriptomics analysis of pre-cachectic and cachectic C26 transplanted mice revealed an impaired immunological phenotype. However, this signature was not seen in the KPC model of PDAC, again implying its innate immunocompromised nature (254). With the use of the CXCR-4 inhibitor AMD3100, this barrier was overcome, increasing T cell infiltration and PD-L1 checkpoint inhibitor efficiency (158, 254). However, with the addition of corticosterone to the AMD3100-PD-L1 combination, the therapeutic effect was diminished (254). These results have multiple implications for PDAC therapeutics: the checkpoint inhibition resistance might be tackled with glucocorticoid synthesis inhibition, though this might first require a prior consideration for CTL infiltration. Second, serum glucocorticoid levels might be important markers for checkpoint inhibition response in PDAC patients. And finally, serum IL-6 depletion might provide further opportunities to increase the checkpoint inhibition efficacy, not only because of its direct effect in the TME but also due to its physiological role in cancer-related cachexia (254).

OTHER COMBINATION STRATEGIES EXPLOITING ANTIGENICITY/ IMMUNOGENICITY OF TUMORS TO ENHANCE CHECKPOINT INHIBITION THERAPY

Approaches including specific inhibitors (small molecules and antibodies) of various signaling pathways are described thus far and listed in **Table 2**. Apparently, combining the classical, untargeted treatment strategies, chemotherapy and RT, with checkpoint inhibition in clinical trials appears reasonable. Other targeted immunotherapeutic options, e.g., oncolytic viruses, vaccines, and chimeric antigen receptor-T cell (CAR-T) therapies aim to treat cancer in a more specific manner with minimal side effects. Selected clinical trials combining immune checkpoint inhibitors with untargeted and other targeted immunotherapeutic options are listed in **Table 3**. Combination therapies can modulate both antigenic and immunogenic landscape of tumors (**Figure 4**). Likely more important than just developing novel combination partners, exact understanding of the mode of action of combination partners, their tolerability and toxicity, a determination of dosing and appropriate sequencing of the combinations are required (257).

Combination of Immune Checkpoint Inhibitors With Untargeted Therapeutic Options

Chemotherapy

The first-line PDAC therapeutics used in clinic are chemotherapeutic agents such as gemcitabine with/without nab-paclitaxel, and FOLFIRINOX (folinic acid, fluorouracil, irinotecan, and oxaliplatin) (258). These agents are known for their ability to induce cytotoxicity due to impaired cell division. The mutagenic effect of chemotherapy (or RT) may enhance neoantigen production and MHC class I antigen presentation on cancer cells, increasing tumor antigenicity (109). Still, even if sub-clones with reactive neoantigenic properties might evolve, they might not be substantial enough to result in a broad clonal response in response to checkpoint inhibition (109). Furthermore, considering that PDAC already retains its antigenic capacity but its immunosuppressive microenvironment is the main barrier to pass as explained above, chemotherapy might exert its effect rather by altering immunogenicity. Immunogenic cell death (ICD) upon chemotherapy releases danger signals and cytokines for the generation of a more immunogenic TME (109). As also seen in the gemcitabine with nab-paclitaxel example above, remodeling of the immunosuppressive TME can bolster up T cell cytotoxicity due to enhanced immunogenicity. Chemotherapy can increase immunogenicity by also its direct action on immunosuppressive cells of TME. For example, fluorouracil and paclitaxel were shown to induce MDSC apoptosis in various tumor models, while low dose gemcitabine was shown to deplete T_{regs} in panc02 orthotopic mouse model (259). Furthermore, it will be important to select chemotherapeutic agents, their dosing and time and sequence of administration with regard to their ability to induce ICD and remodel the microenvironment.

Radiotherapy

Although the use of RT for the treatment of PDAC has been controversially discussed due to rather disappointing results in clinical trials (260), radiation treatment in combination with ICI might be a promising strategy for pancreatic cancer patients. In a phenomenon known as the abscopal response, RT was shown to induce immune responses that mediate regression of metastatic lesions lying outside the field of radiation (261). RT could activate the immune system, increase trafficking of T cells to the tumor, and elicit antitumor immune responses following ICD (262). Several preclinical and clinical studies in different cancer types showed synergistic effects in cohorts treated with RT and immune checkpoint blockade (263–266). Although not many studies have been published thus far, evidence for synergism can also be seen in PDAC and has been related to increased immunogenicity (95, 267, 268). In the PDAC mouse model used by Twyman-Saint

TABLE 2 | Selection of currently ongoing clinical trials evaluating CTLA4 or/and PD1/PD-L1 checkpoint blockade in combination with targeted therapy approaches for pancreatic cancer as indicated.

Combination strategy/ target	Compounds	Entity	Phase	Trial ID
Oncogenic signaling	Cobimetinib (MEK-inh.) + atezolizumab (PD-L1-Ab)	Metastatic PDAC, progressed on chemotherapy	Ib/II	NCT03193190
TME: stroma	Ulocuplumab (CXCR-4-ant.) + nivolumab (PD-1-Ab)	Advanced/metastatic pancreatic cancer (next to SCLC)	I/II	NCT02472977 (terminated 03/2018 due to lack of effic. in short-term ph.)
	BL-8040 (CXCR4-ant.) + pembrolizumab (PD-1-Ab)	(Pretreated) metastatic pancreatic cancer	II	NCT02826486 and NCT02907099
	BL-8040 (CXCR4-ant.) + atezolizumab (PD-L1-Ab)	Metastatic PDAC, progressed on chemotherapy	Ib/II	NCT03193190
	Olaptesed pegol (pegylated oligoribonucleotide, neutralizing CXCL12) ± pembrolizumab (PD-1-Ab)	Metastatic pancreatic cancer (next to CRC)	I/II	NCT03168139
	Defactinib (FAK-inh.) + pembrolizumab (PD-1-Ab)	Advanced pancreatic cancer (next to NSCLC and mesothelioma)	I/II	NCT02758587
	PEGPH20 (pegylated recombinant human hyaluronidase) + atezolizumab (PD-L1-Ab)	Metastatic PDAC, progressed on chemotherapy	I/II	NCT03193190
	PEGPH20 (see above) + avelumab (PD-L1-Ab)	Chemotherapy resistant advanced pancreatic cancer	I	NCT03481920
	Pembrolizumab (PD-1-Ab) ± paricalcitol (vitamin D analog)	Maintenance of pretreated advanced pancreatic cancer in (partial) remission	II	NCT03331562
TME: myeloid	RO7009789 (CD40 ago. Ab) + atezolizumab (PD-L1-Ab)	Locally advanced/metastatic solid tumors	I	NCT02304393
	Cabiralizumab (CSF1R-Ab) + nivolumab (PD-1-Ab)	Advanced solid tumors	I	NCT02526017
	AMG820 (CSF1R-Ab) + pembrolizumab (PD-1-Ab)	Advanced pancreatic cancer (next to CRC and NSCLC)	I/II	NCT02713529
	Pedixartinib (CSF1R-tyrosine kinase inh.) + durvalumab (PD-L1-Ab)	Pretreated advanced/metastatic pancreatic cancer (next to CRC)	I	NCT02777710
	Acalabrutinib (bruton tyrosine kinase inh.) + pembrolizumab (PD-1-Ab)	Metastatic pancreatic cancer	II	NCT02362048
TME: metabolism	Epacadostat (IDO1-inh.) + pembrolizumab (PD-1-Ab)	Previously treated advanced pancreatic cancer (with chromosomal instability/HRRD)	II-withdrawn	NCT03432676

Ab, antibody; inh., inhibitor; ant., antagonist; ago., agonist; CRC, colorectal cancer; HRRD, homologous recombination repair deficiency; IDO1, indoleamine 2,3-dioxygenase 1; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; TME, tumor microenvironment; PD-1, programmed cell death protein 1.

Victor et al., any combination of immune checkpoint inhibitor with RT substantially increased overall survival, compared with immune checkpoint blockade with either CTLA-4 antibody or PD-1 antibody alone. The highest response rate and longest overall survival was seen in the triple combination therapy (two checkpoint inhibitors + RT) group (269).

Recently, CD40 agonist treatment was demonstrated to be beneficial upon a RT + ICI regimen in murine pancreatic cancer models (270). While RT alone or in combination with ICI resulted in reduction of irradiated tumor growth, only the triple therapy, RT + αCD40 + ICI (RCP4), affected the growth of both irradiated and unirradiated tumors. These observations were also reflected in the long-term survival. Furthermore, CD4 and CD8 T cells, as well as short-lived myeloid cells were shown to be necessary for optimal response to RCP4 and that RCP4 antitumor immunity. This immunity was dependent on host CD40, Batf3, and IFNγ but not on B cells and canonical innate immune activation pathways. The three therapies all showed non-redundant impact on the antitumor immune response. While RT triggered an early pro-inflammatory stimulus, αCD40 caused systemic myeloid compartment reorganization and ICI increases intratumoral T cell infiltration, thus improving the $CD8/T_{reg}$ cell ratio.

In conclusion, RT can enhance the "visibility" of tumor antigens and make the tumor more immunogenic. While the combination of RT and ICI shows promise in preclinical and clinical trials in various cancer entities, challenges still exist for the safe and efficacious application of the combination. Tumor-type and immune therapy-specific optimization of radiation dose and timing and the identification of potential biomarkers is likely to further enhance the effectiveness (271). Also, the addition of αCD40 agonists appears to be a promising avenue to pursue in clinical PDAC trials.

Combination of Immune Checkpoint Inhibitors With Other Immunotherapeutic Approaches

Oncolytic Viruses

Tumor-targeted oncolytic viruses (TOVs) are viruses that selectively infect, replicate in, and lyse tumor cells, while leaving healthy, normal tissues unharmed. TOVs can have intrinsic tumor-selectivity, making them naturally nonpathogenic to humans and sensitive to antiviral signaling (272) or depend on oncogenic signaling pathways, e.g., constitutively activated RAS (273, 274). Viral tumor specificity can also be genetically

TABLE 3 | Selection of currently ongoing clinical trials evaluating CTLA4 or/and PD1/PD-L1 checkpoint blockade in combination with untargeted and targeted options including other immunotherapeutic approaches for pancreatic cancer as indicated.

Combination strategy/target	Compounds	Entity	Phase	Trial ID
Chemotherapy	Gemcitabine + ipilimumab (CTLA-4-Ab)	Advanced pancreatic cancer	Ib	NCT01473940
	Nab-paclitaxel (±gemcitabine) + nivolumab (PD-1-Ab)	Advanced/metastatic pancreatic adenocarcinoma (next to NSCLC and mBC)	I	NCT02309177
	mFOLFOX6 + pembrolizumab (PD-1-Ab) [+celecoxib (COX-2-inh.) for non-responders]	Advanced gastrointestinal-cancer including pancreatic cancer	I	NCT02268825
Radiotherapy	SBRT 6 Gy × 5 days + durvalumab (PD-L1-Ab), vs. tremelimumab (CTLA-4-Ab) vs. both combined	Unresectable, non-metastatic pancreatic cancer	Ib	NCT02868632
	SBRT 5 Gy × 5 days vs. 8 Gy × 1 day + durvalumab (PD-L1-Ab), vs. tremelimumab (CTLA-4-Ab) vs. both combined	Unresectable pancreatic cancer	I/II	NCT02311361
	Radiotherapy (not defined) + nivolumab (PD-1-Ab) and ipilimumab (CTLA-4-Ab)	Pancreatic cancer, progressed on chemotherapy (next to CRC)	II	NCT03104439
	45–50.4 Gy + PD-1-Ab (not defined)	Unresectable pancreatic cancer	II	NCT03374293
Vaccines	GVAX/Cy ± nivolumab (PD-1-Ab)	Neoadjuvant/adjuvant for resectable pancreatic cancer	I/II	NCT02451982
	GVAX/Cy + CRS-207 ± nivolumab (PD-1-Ab)	Previously treated metastatic pancreatic adenocarcinoma	II	NCT02243371
	CRS-207 (±GVAX/Cy) + nivolumab (PD-1-Ab) and ipilimumab (CTLA-4-Ab)	Previously treated pancreatic cancer	II	NCT03190265
Chemotherapy + vaccine	Capecitabine + CV301 + durvalumab (PD-L1-Ab)	Metastatic pancreatic cancer (next to CRC)	I/II	NCT03376659
Chemotherapy + Vit. D analog	Paricalcitol (vitamin D analog) + pembrolizumab (PD-1-Ab) ± gemcitabine/nab-paclitaxel	Resectable pancreatic cancer, neoadjuvant setting	I	NCT02930902
Chemotherapy + FAK	Defactinib (FAK-inh.) + gemcitabine + pembrolizumab (PD-1-Ab)	Advanced solid tumors	I	NCT02546531
Chemotherapy + CD40	Gemcitabine/nab-paclitaxel + APX005M (CD40-ago.-Ab) ± nivolumab (PD-1-Ab)	Untreated metastatic pancreatic adenocarcinoma	II	NCT03214250
Chemotherapy + CSF1R	Cabiralizumab (CSF1R-Ab) + nivolumab (PD-1-Ab) ± different chemotherapeutic regimens	Pretreated, progressed metastatic pancreatic adenocarcinoma	II	NCT03336216
Radiotherapy + vaccine	SBRT 6.6 Gy × 5 days + GVAX/Cy + nivolumab (PD-1-Ab)	Borderline resectable pancreatic cancer, no previous therapy	II	NCT03161379
Radiotherapy + vaccine	SBRT 6.6 Gy × 5 days + GVAX/Cy + pembrolizumab (PD-1-Ab)	Locally advanced pancreatic cancer	II	NCT02648282
CSF1R + vaccine	IMC-CS4 (CSF1R-Ab) + GVAX/Cy + pembrolizumab (PD-1-Ab)	Borderline resectable pancreatic adenocarcinoma	I	NCT03153410
IDO1 + vaccine	Epacadostat (IDO1-inh.) + CRS-207 (±GVAX/Cy) + pembrolizumab (PD-1-Ab)	Metastatic pancreatic cancer progressed on prior chemotherapy	II	NCT03006302
ACT	Autologous TIL, ipilimumab (CTLA-4-Ab), nivolumab (PD-1-Ab), proleukin, Cy., fludara	Cancer patients across all diagnoses	I/II	NCT03296137

Ab, antibody; inh., inhibitor; ago., agonist; CRC, colorectal cancer; CRS-207, Listeria-based mesothelin vaccine; CV301, CEA/MUC1 prime-boost vaccine based on modified vaccinia Ankara-Bavarian Nordic (MVA-BN), a recombinant fowlpox viral vector (foe the boost) and TRICOM, which is comprised of three costimulatory molecules B7-1, ICAM-1, and LFA-3; Cy, cyclophosphamide; GVAX, irradiated pancreatic cancer cells, genetically modified to express GM-CSF; IDO1, indoleamine 2,3-dioxygenase 1; mBC, metastatic breast cancer; NSCLC, non-small cell lung cancer; SBRT, stereotactic body radiation therapy; ACT, adoptive cell therapy; TIL, tumor-infiltrating lymphocyte; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; PD-1, programmed cell death protein 1.

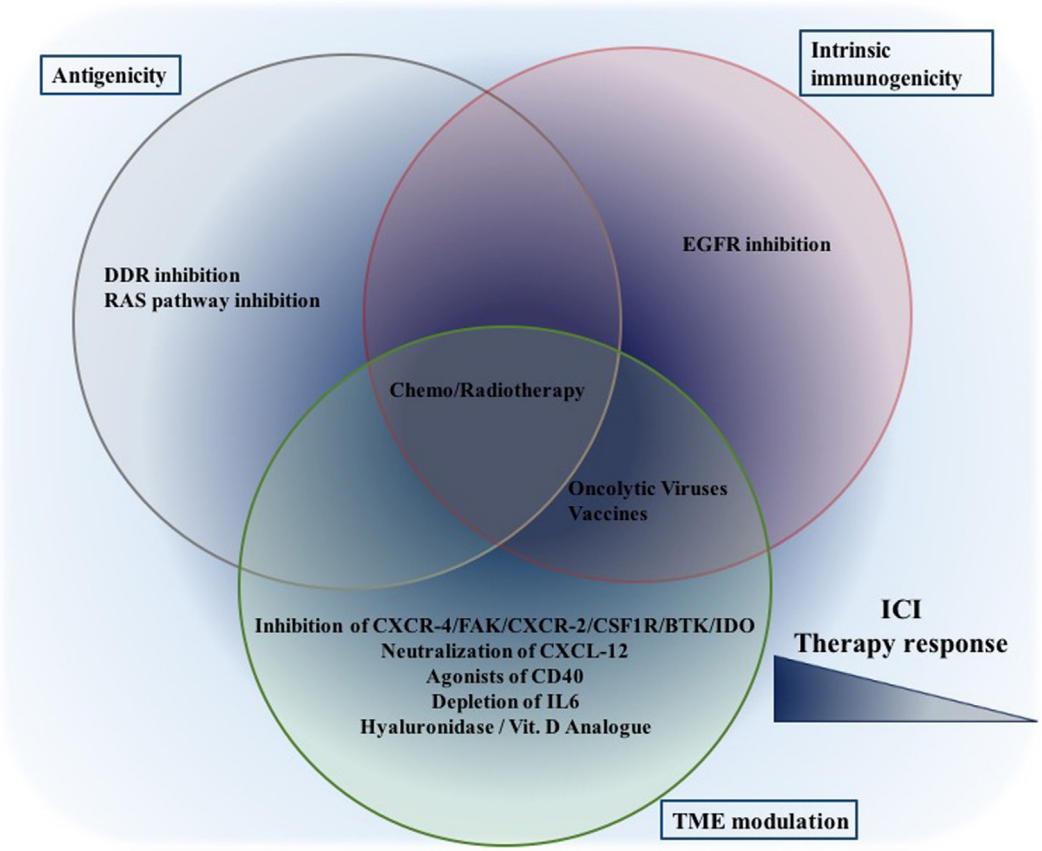

FIGURE 4 | Combination therapeutic options to increase ICI efficiency: while given therapeutic options are placed in the corresponding cluster, only published data thus far are taken into consideration. This still does not eliminate their potential to affect other aspects. While treatments focusing on a single aspect (either one of antigenicity/intrinsic immunogenicity/TME modulation) might be effective, the best synergism will probably be achieved through combinations focusing on all aspects. Abbreviations: ICI, immune checkpoint inhibition, DDR, DNA damage response.

engineered by deleting genes required for replication in normal tissues (273) or by placing viral replication under the control of a tumor-specific promoter (274–276), TOVs can also be designed to express tumor-specific cell surface receptors (277, 278). TOVs can thus be engineered to increase safety, efficacy, and tissue tropism.

The advantages of TOVs are their specificity, modest toxicity, low probability for resistance, and most importantly, their induction of an inflammatory cascade and engagement of the adaptive immune system (273). In contrast to any other drug, the therapeutic dose of TOVs increases over time, as the virus replicates and spreads to neighboring cells (273). Although TOVs directly lyse infected malignant cells, causing acute tumor debulking, it is the ability of the virus to spread from cell to cell and potentiate an inflammatory response through ICD that make oncolytic viruses such promising new therapies (279–281). However, oncolytic virus therapy faces challenges in solid tumors and especially PDAC. These challenges, i.e., overcoming the TME, avoiding neutralization by the host immune system, and acquired resistance in tumor cells culminate in the main problem, i.e., the systemic delivery of TOVs for the targeting of metastatic cancer cells (282). Thus, it is not surprising that thus far, no studies investigating ICI and oncolytic viral therapy in pancreatic cancer have been published. However, Mahalingam et al. (283) conducted a phase II study of pelareorep, a proprietary replication-competent isolate of reovirus type 3 dearing in combination with gemcitabine in advanced PDAC and observed the upregulation of PD-L1 in following treatment. They suggested to investigate the combination of oncolytic virus therapy with anti-PD-L1 inhibitors in PDAC.

Congruent with this finding, recent research in other cancer entities revealed that antiviral immunological events induced by the administration of oncolytic viruses can turn tumors "hot" (284, 285) and establish a TME that is conducive for enhancing the efficacy of checkpoint inhibitors (286–288). Using intravenous infusion of oncolytic human orthoreovirus, Samson et al. (288) found that TOV treatment increases cytotoxic T cell tumor infiltration, upregulates IFN-regulated gene expression, and the PD-1/PD-L1 axis in tumors, *via* an IFN-mediated mechanism. And finally, addition of PD-1 blockade to reovirus treatment enhanced systemic therapy in a preclinical glioma model. In their simultaneously published triple-negative breast cancer

(TNBC) study, Bourgeois-Daigneault et al. (286) reported that TOV therapy sensitizes otherwise refractory TNBC to immune checkpoint blockade, preventing relapse in most of the treated animals.

In conclusion, once the problem of systemic delivery is solved, oncolytic viruses are not only valuable therapies in terms of tumor debulking but are also useful in a "prime and boost" approach in combination with ICIs.

Vaccines

Another promising approach to enhance the immunogenicity of pancreatic cancer cells and boost the antitumor T cell response is the use of cancer vaccines. Vaccines have been designed to generate a humoral/cellular immune response with the aim of stimulating the host immune system to recognize and eliminate tumor cells with specific effector and memory T cells. There are two major categories of tumor vaccines: whole cell vaccines and antigen-specific vaccines (289). A brief review on the different vaccines currently investigated for pancreatic cancer can be found in the publication by Skelton et al. (290). Although early studies using single-agent tumor vaccines against PDAC showed improved immune profiles, they were largely unable to produce a positive clinical response (291). This can be explained by the upregulation of immunosuppressive signaling, as well as other immune modulating mechanisms, which negate the positive effects of the vaccine (267, 292).

The induction of T cell infiltration and PD-L1 expression in the TME by vaccine treatment was hypothesized to prime PDACs for anti-PD-1/PD-L1 therapies. Indeed, the whole cell vaccine GVAX, consisting of two allogeneic irradiated PDAC cell lines engineered to secrete GM-CSF, converted a non-immunogenic or "cold" neoplasm into an immunogenic or "hot" neoplasm by inducing infiltration of T cells and development of tertiary lymphoid structures (267, 285). In a subsequent phase Ib study, Le et al. (293) were able to show that the combination of GVAX with ipilimumab induced objective responses in patients with metastatic PDAC that were not observed with either single therapy alone.

Preclinical data suggested beneficial effects when two vaccination treatments were co-implemented, e.g., GVAX and CRS-207, a live-attenuated *Listeria monocytogenes* vaccine expressing the TAA mesothelin, in a sequential combination—a so-called prime/boost approach. The first vaccine was given to initiate or "prime" the immune system, and this immune response was then "boosted" following re-administration of antigen resulting in the induction of a synergistic enhancement of T cell induction and antitumor effect (289). Based on the preclinical data, a phase II trial (NCT01417000) was conducted resulting in the conclusion that heterologous prime/boost with Cy/GVAX and CRS-207 extended the survival of patients with pancreatic cancer, with minimal toxicity (294). Unfortunately, a subsequent phase IIb trial of CRS-207 and GVAX (NCT02004262) did not show a significant difference in overall survival between the groups treated with either CRS-207/GVAX or CRS207 alone and the group treated with chemotherapy, i.e., physicians' choice of therapies including: gemcitabine, capecitabine, fluorouracil, leucovorin, irinotecan, and erlotinib.

Although the last mentioned study was quite a set-back, the strategy of combining different immunotherapy options with each other still holds a merit, especially for "prime and boost" approach.

Chimeric Antigen Receptor (CAR)—T Cell Therapy

Chimeric antigen receptors are fusion proteins that can be comprised of three major domains. These are the antigen-specific ectodomain, commonly derived from a single-chain variable antibody-fragment (scFv); a transmembrane domain fused to a spacer that links to the ectodomain; and an endodomain consisting of different cytoplasmic proteins responsible for T cell activation (295). Unlike endogenous TCRs, CARs recognize their target antigen in an MHC (or HLA)-independent manner, due to their engineered antibody fragment. Upon antigen recognition, CAR-T cells are activated, leading to cytokine secretion, T cell proliferation, and antigen-specific cytotoxicity (296). The production of CAR-T cells for adoptive T cell transfer requires the isolation, stimulation, expansion, transduction, i.e., viral vector-mediated insertion of specific CAR genes, and ultimately reinfusion of autologous or allogeneic T cells (297–299).

Although impressive clinical activities of CAR-T cells in hematological malignancies were reported, CAR T-cell trials in solid tumors have yet to yield the same level of success (300, 301). The most prominent obstacles standing in the way of successful CAR-T cell therapy are (1) lack of ideal TSAs, (2) inefficient trafficking of CAR-T cells to tumor sites, (3) the immune-suppressive TME, and (4) the risk of developing on-target/off-tumor toxicities, i.e., the attack of normal cells expressing the targeted tumor antigen (296).

While the investigation of CAR-T cells in pancreatic cancer is still in early stages, it is fair to say that the first above mentioned obstacle does not apply. PDAC exhibits a number of TSAs and, conceptually, is a promising candidate tumor for investigating CAR T-cell therapy. Thus far, there have been preclinical studies on various pancreatic cancer cell surface antigens, namely, MSLN, CEA, MUC1, PSCA, CD24, HER2, and natural killer receptors (302).

The main obstacles in pancreatic cancer are most likely the strong immunosuppressive TME, already discussed in this review, and improper homing and inefficient infiltration of CAR T-cells to the tumor bed. Especially challenging is the high number of infiltrating T_{regs} and MDSCs, which can deactivate CAR-T cells through cytokines inhibitory cytokines such as TGF_{β} and IL-10, and the upregulation of inhibitory receptors, e.g., PD-1 on adoptively transferred CAR-T cells after homing to the tumor (95, 302–304). T cell hypofunction was reversed when the cells were isolated from the tumor, or after treatment with a blocking PD-1 antibody (304–306), and there are promising preclinical studies on CAR-T cells engineered to secrete PD-1 checkpoint inhibitors (307, 308) or PD-1 dominant negative receptor (304).

These results provide rationale for combination therapies, with CAR-T cells and checkpoint blockade, as a new strategy to overcome the tumor escape and to further strengthen CAR-T cells, especially in patients with PDAC shown to express high levels of PD-L1.

Adoptive Cell Therapy (ACT) With Endogenous TILs

Adoptive cell therapy using endogenous TILs taken from surgically resected tumors, expanded *in vitro*, and re-infused back into the patient, is a promising approach for otherwise untreatable cancer types (309). In metastatic melanoma patients, for example, TIL-ACT was associated with a 20% complete response lasting beyond 3 years (310). Gastrointestinal tumor patients with $CD3^+$ T cell infiltration showed a higher rate of progression-free survival (311), and pancreatic adenocarcinomas containing both $CD4^+$ and $CD8^+$ T cells correlated with an improved prognosis and significantly greater 5-year survival (181, 312, 313). This evidence of a host T cell immune response in patients with pancreatic adenocarcinoma drove both Hall et al. (309) and Poschke et al. (314) to expand and analyze the T cell repertoire in resected primary PDAC specimen. Contrary to the common description of PDAC as an immunologically "cold" tumor, they found that most resectable PDA tumors actually contained significant numbers of T-cells and, along with that, tertiary lymphoid structures in which clonal T-cell expansion takes place and were able to expand them *in vitro* using high levels of IL-2 (309, 314). The majority of these TILs were $CD4^+$ T cells and were highly activated and resembled those extracted from melanoma samples. Media supplemented with anti-4-1BB significantly increased the TIL yield per fragment and shifted the T cell population to predominantly $CD8^+$ cells compared with control cultures. The population of 4-1BB positive $CD8^+$ lymphocytes represented the population of tumor-resident TILs specific for expressed tumor antigens on the surface of pancreatic adenocarcinoma cells (309, 314).

Thus far, there has been no studies investigating the combination of TIL ACT and ICI in pancreatic cancer. However, pretreating PDAC patients with immune checkpoint inhibitors and thus enriching the population of tumor-specific lymphocytes prior to surgical resection might be a worthwhile strategy. This way the yield of tumor reactive cells could be increased, the expansion time and the time between surgery and infusion shortened, and thus the risk of recurrent growth during the expansion period decreased.

This is exactly the approach taken by Mullinax et al. when analyzing the combination of TIL ACT and Ipilimumab in a clinical pilot study (NCT01701674) for metastatic melanoma, and in clinical trials for metastatic ovarian cancer (NCT03287674). The pretreatment with Ipilimumab followed by ACT in metastatic melanoma patients was reported as feasible, well tolerated, and associated with a low rate of attrition due to progression during cell expansion (315). The investigators are currently recruiting patients for a similar trial in metastatic melanoma, now including 4-1BB (NCT02652455), and another study with a similar design is currently recruiting patients with locally advanced or metastatic cancers of various types (NCT03296137).

Considering the positive results in metastatic melanoma, as well as the similarity in the population of extractable T cells from melanoma and PDAC, the investigation of ICI + TIL ACT in PDAC is recommendable.

CONCLUDING REMARKS

The revolution of immunotherapy is changing our perspective in cancer therapeutics. For some solid tumors, immunotherapy has already entered into clinical practice. While PDAC is unresponsive and refractory to many of the conventional therapies, immunotherapy holds a promise for future improvement. However, single-agent ICI has largely failed. Based on the findings thus far, the decisive drawback for ICI efficiency in PDAC is the initial T cell priming. Only less than 1% of human PDAC samples are projected to show aberrant genomic instability, enabling T cell priming despite the immunosuppressive microenvironment. However, this does not mean the other 99% are not antigenic, rather its antigenic strength likely cannot beat reduced immunogenicity. Each patient, each tumor, and each cancer cell are distinct. T cells might provide the best repertoire for the recognition of each single difference, yet to overcome immunogenic obstacles, combination strategies are required. Development of the best combinations comes along with better characterization of the patient samples. Characterization of these samples might help us to better classify the individual distinctions that patients, tumors, and cancer cells have, and to find the best combination partners with checkpoint inhibition. Even though complete regression of the primary tumor might not be achieved, reduction and control of metastasis can still provide a considerable prognostic value in PDAC patients. While we know that metastatic lesions evade the expression of high quality neoantigens of their cognate primary tumor and antigen presenting machinery, they might still retain their unique antigenic and immunogenic master regulators to be targeted. Most importantly, T cell memory provides the best tool to minimize disease recurrence, therefore strategies exploiting T cell memory may provide long-term disease control. Before achieving T cell memory, to make PDAC responsive to first time checkpoint inhibition, we have to elucidate and exploit the mechanisms discussed above: (1) increasing initial T cell priming, (2) exceeding immunosuppressive TME, and (3) inhibiting compensatory mechanisms of T cell anergy and exhaustion.

AUTHOR CONTRIBUTIONS

DK wrote the manuscript, prepared the figures and tables. KC wrote and edited the manuscript. DR provided the tables and edited the manuscript. HA edited and supervised the manuscript.

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PD1-CD28 Fusion Protein Enables CD4+ T Cell Help for Adoptive T Cell Therapy in Models of Pancreatic Cancer and Non-hodgkin Lymphoma

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Background: Interaction of the programmed death receptor 1 (PD-1) and its ligand, PD-L1, suppresses T cell activity and permits tumors to evade T cell-mediated immune surveillance. We have recently demonstrated that antigen-specific CD8+ T cells transduced with a PD1-CD28 fusion protein are protected from PD-1-mediated inhibition. We have now investigated the potential of PD1-CD28 fusion protein-transduced CD4+ T cells alone or in combination with CD8+ T cells for immunotherapy of pancreatic cancer and non-Hodgkin lymphoma.

Methods: OVA-specific CD4+ and CD8+ were retrovirally transduced with the PD1-CD28 fusion protein. Cytokine release, proliferation, cytotoxic activity, and phenotype of transduced T cells were assessed in the context of Panc02-OVA (murine pancreatic cancer model) and E.G7-PD-L1 (murine T cell lymphoma model) cells.

Results: Stimulation of PD1-CD28 fusion protein-transduced CD4+ T cells with anti-CD3 and recombinant PD-L1 induced specific T cell activation, as measured by IFN-γ release and T cell proliferation. Coculture with Panc02-OVA or E.G7-PD-L1 tumor cells also led to specific activation of CD4+ T cells. Cytokine release and T cell proliferation was most effective when tumor cells simultaneously encountered genetically engineered CD4+ and CD8+ T cells. Synergy between both cell populations was also observed for specific tumor cell lysis. T cell cytotoxicity was mediated via granzyme B release and mediated enhanced tumor control *in vivo*. Transduced CD4+ and CD8+ T cells in co-culture with tumor cells developed a predominant central memory phenotype over time. Different ratios of CD4+ and CD8+ transduced T cells led to a significant increase of IFN-γ and IL-2 secretion positively correlating with CD4+ T cell numbers used. Mechanistically, IL-2 and MHC-I were central to the synergistic activity of CD4+ and CD8+ T cells, since neutralization of IL-2 prevented the crosstalk between these cell populations.

Conclusion: PD1-CD28 fusion protein-transduced CD4+ T cells significantly improved anti-tumoral effect of fusion protein-transduced CD8+ T cells. Thus, our results indicate that PD1-CD28 fusion protein-transduced CD4+ T cells have the potential to overcome the PD-1-PD-L1 immunosuppressive axis in pancreatic cancer and non-Hodgkin lymphoma.

Keywords: adoptive T cell transfer, cancer immunotherapy, costimulation, PD-1-CD28 fusion protein, CD4+ T cells

INTRODUCTION

Cytotoxic T cells specifically recognize tumor antigens presented on major histocompatibility complex-1 (MHC-I). After binding to the tumor antigen in the context of MHC, T cells are activated, which results in the secretion of cytotoxic factors and target cell lysis (1, 2). This concept is utilized therapeutically for adoptive T cell therapy (ACT). Patient-derived, tumor-specific T cells are expanded *ex vivo* or, to further enhance tumor-specificity, are genetically modified. T cell engineering usually follows two main approaches; either by introducing a T cell receptor specific for a given tumor-associated antigen or by equipping T cells with chimeric antigen receptors (CAR), which are synthetic receptors enabling tumor recognition. Following expansion, T cells are infused back to the patient in therapeutic intention (3). Pioneering work for ACT utilized tumor-infiltrating lymphocytes (TIL) for melanoma treatment yielding consistent durable response rates in subsets of patients. The challenges to generate these cells from tumor tissue of individual patients or even across entities has so far refrained this strategy from large scale clinical testing (4). Based on compelling preclinical and clinical data in hematological malignancies, ACT holds great promise for cancer immunotherapy. In 2017, the Food and Drug Administration (FDA) approved the first cellular therapy for refractory B-cell acute lymphoblastic leukemia (B-ALL) and diffuse large B cell lymphoma. Anti-CD19-CAR T cells are now part of the standard of care in the US, based on unparalleled remission rates and prolonged overall survival for patients with an otherwise very poor prognosis (5). In addition, ACT is under investigation for the treatment of other hematologic as well as more frequent non-hematological malignancies. Typically, ACT is performed with a mixture of CD4+ and CD8+ T cells, which is dictated by the patient's own peripheral blood T cell ratio and the differential expansion status in cell culture. Some protocols also adjust for defined ratios, based on own evidence that this might be more beneficial (6–8). When being transduced for tumor specificity both cell types are being modified and in the case of CAR T cells, both cell populations are thought to be therapeutically relevant (9).

However, CD8+ T cells are generally considered more potent and more central for ACT efficacy. CD4+ T cells have a distinct functional and secretory phenotype from CD8+ T cells which is neither redundant nor overlapping. Importantly, CD4+ T cell-derived cytokines play an important role in anti- but also in pro-tumoral immunity (10, 11). While it is established that CD4+ T cells can be cytotoxic on their own, a major function lays in regulating trafficking, activation, proliferation, differentiation, and persistence of tumor-infiltrating cytotoxic CD8+ T cells (12–15). Several studies have confirmed the helper function of tumor-specific CD4+ T cells and showed that the anti-tumor activity of combined treatment with CD4+ and CD8+ T cells is more pronounced than that seen when using individual cell types. The exact mechanism of this synergy remains to be elucidated (16–18).

Despite the clinical success of ACT in defined indications, ACT is inherently limited by antigen-loss variants of tumor cells, side effects resulting from on- and off-target expression of the chosen antigen and low T cell infiltration into the tumor tissue. ACT failure is often associated with an increased expression of the programmed death-1 receptor (PD-1), a marker protein for T cell anergy, on previously activated T cells (19, 20). PD-1 signaling mediates T cell suppression that prevents autoimmunity under physiological conditions and is therefore a key immune checkpoint on CD4+ and CD8+ T cells (21, 22). PD-L1, one of the two known ligands for PD-1, is broadly expressed on epithelial as well as hematological cells and shields these cells from T cell overactivation (23). Along these lines, tumors usurp this mechanism to evade anti-tumor immune responses (24). It is thereby not surprising, that undulating PD-L1 expression is found in most if not all human cancers at different levels and its expression is associated with dismal prognosis in the pre-immunotherapy era (25). Paradoxically, recognition of tumor cells by T cells transferred for ACT will result in T cell activation, upregulation of PD-1 on the said T cell, but also of PD-L1 on the tumor cell. This will ultimately end in abrogation of T cell activity and thereby ACT failure (26). Clinical evidence that this state of anergy might be reverted when antagonizing the PD-1-PD-L1 axis has been shown in several phase III clinical trials testing anti-PD-1 or anti-PD-L1 antibodies in melanoma or non-small cell lung cancer (27–31). Based on these studies, it seems likely that a similar approach might also be of value for ACT. As both checkpoint blockade and ACT have severe side effects on their own, it might be advisable to develop more targeted strategies to overcome T cell anergy than systemically blocking important immune checkpoints.

To overcome PD-1 suppression selectively and to improve ACT, we have developed a therapeutic concept that converts tumor-associated immunosuppression via the PD-1-PD-L1 axis into stimulation of tumor-specific T cells (32). We created a fusion receptor consisting of the extracellular domain of the PD-1 receptor fused to the intracellular, T cell-activating domain of CD28. In the tumor tissue, PD-1-CD28 fusion protein-expressing CD8+ T cells recognize tumor-derived PD-L1 and get locally activated. This results in tumor cell lysis and therapeutic

benefit. It, however, remained unclear if the benefit is specific to CD8+ T cells, and particularly if adding this fusion protein to CD4+ T cells would further accelerate therapeutic activity. We hypothesized that our PD-1-CD28 fusion protein is not only functional in antigen-specific CD4+ T cells but also that simultaneous introduction in CD8+ T cells would further enhance T cell function. Here, we demonstrate that primary murine CD4+ T cells, expressing PD1-CD28 fusion protein, overcome PD-L1-induced T cell anergy in murine models of pancreatic cancer and non-Hodgkin lymphoma. Coculture experiments demonstrate a synergism of gene-modified CD4+ and CD8+ T cells for anti-tumor activity, which was dependent on IL-2 secretion from CD4+ T cells. Our results indicate the potential of PD1-CD28 fusion protein-transduced CD4+ T cells to further improve ACT.

MATERIALS AND METHODS

Cell Lines

Panc02-OVA, a murine pancreatic cancer cell line and E.G7-OVA, a murine T cell lymphoma cell line, were previously described (32, 33). Panc02-OVA-PD-L1 and E.G7-OVA-PD-L1 were generated by transduction of Panc02-OVA or E.G7-OVA cells with pMXs-puro or pMXs (a generous gift from Toshio Kitamura, M.D., PhD, the Institute of Medical Science, University of Tokyo, Japan) encoding the full-length murine PD-L1 (Swiss-Prot accession number Q9EP73). Panc02-OVA-PD-L1 cells were selected based on puromycin resistance. E.G7-OVA-PD-L1 cells were obtained by fluorescence activated cell sorting. Panc02-OVA and Panc02-OVA-PD-L1 were cultured in DMEM3+ (DMEM with 10 % fetal bovine serum [FBS, Life Technologies, USA), 100 U/ml penicillin and streptomycin (PS), and 2 mM L-glutamine (all from PAA, Germany)]. E.G7-OVA-PD-L1 were cultured in RPMI 1,640 supplemented with 10% FBS, 100 U/ml PS and 2 mM L-glutamine, 1 mM sodium pyruvate (PAA, Germany), and 1 mM HEPES (Sigma Aldrich, Germany).The retroviral ecotrophic packaging cell line Platinum-E was purchased from Cell biolabs (USA). DMEM3+ medium for Platinum-E cells additionally contained 10 µg/ml puromycin and 1 µg/ml blasticidin (both from Sigma, Germany). Primary murine T cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml PS and 2 mM L-glutamine, 1 mM sodium pyruvate (PAA, Germany), 1 mM HEPES (Sigma Aldrich, Germany), and 50 µM β-mercaptoethanol.

Mice

Mice transgenic for a T cell receptor specific for ovalbumine (OT-1 or OT-2) were obtained from the Jackson laboratory (Bar Harbor, ME) (stock number 003831 for OT-1 and 004194 for OT-2) and were bred in our animal facility under SPF conditions. Both mouse strains served as T cell donors for primary murine T cell transduction.

Animal Experiments

For *in vivo* studies wild type C57/Bl6 mice were purchased from Charles River. Tumors were induced by subcutaneous injection of 4×10^5 E-G7-OVA-PD-L1 tumor cells. Mice were randomized with regard to tumor size and treated via serial transfer of PTM-transduced or untransduced T cells: First, CD8+ T cells were injected i.v. 48 h later, CD4+ T cells were injected i.v. Tumor growth was assessed every other day in a blinded fashion and tumor volume was estimated according to the following formula: $4/3 \times \pi \times L_1^2 \times L_2$ (with L_1 defined as maximal diameter and L_2 as the diameter perpendicular to L_1). All experiments were approved by the local regulatory agency (Regierung von Oberbayern).

T Cell Transduction

The PD1-CD28 fusion protein was described previously (32). The retroviral vector pMP71 (kindly provided by Christopher Baum, M.D., Institute of Experimental Hematology, Medizinische Hochschule Hannover, Germany) was utilized for all transduction experiments. Detailed protocols for murine T cell transduction have been published (34–37). In brief, pMP71 PD1-CD28 vector was transfected into Platinum-E cells and retrovirus-containing supernatants were collected for transduction of murine T cells. Primary murine T cells were first stimulated with anti-CD3e and anti-CD28 antibody (eBioscience, clones 145-2C11 and 37.51, respectively) and recombinant IL-2 (Novartis, Switzerland). Priot to transduction, anti-CD3- and anti-CD28 beads (Life technologies, USA) were added. Recombinant IL-15 (Peprotech, Germany) was used for T cell expansion. The CD4+ T cell fraction was purified on the day of spleen extraction by magnetic activated cell sorting using a CD4+ T cell isolation kit (Miltenyi Biotec, Germany).

Flow Cytometry

For multi-color flow cytometry, a BD FACS Canto II (BD bioscience, Germany) together with the following antibody panels was used. For purity testing and analysis of transduction efficiencies, anti-PD-1 (APC, clone RMP-30, BioLegend, USA), anti-CD8 (Pacific Blue™, clone 53-6.7, BioLegend, USA) and anti-CD4 (Pacific Blue™ e, clone GK1.5, BioLegend, USA) were used. For analysis of MHC I-, MHCII-, and PD-L1-expression, tumor cells were stained with anti-MHCI (PE, clone M1/42.3.9.8, Elabscience, USA), anti-MHCII (APC, clone M5/114.15.2, eBioscience, USA) and anti-CD274 (PE/Cy7, clone 10F.9G2, BioLegend, USA). Rat IgG2a– (PE, clone #54447, R&D Systems, USA), Rat IgG2b kappa—(APC, clone eB149/10H5, eBioscience, USA) and Rat IgG2b kappa—antibodies (PE/Cy7, clone RTK4530, BioLegend, USA) were applied as isotype control. For proliferation analysis in antibody-stimulation assays, T cells were stained with anti-PD-1 (APC, clone RMP-30, BioLegend, USA), anti-CD28 (APC, clone 37.51, BioLegend, USA), anti-CD4 (Pacific Blue™, clone GK1.5, BioLegend, USA), and Zombie aqua fixable viability dye (BioLegend, USA) prior to fixation and permeabilization with FoxP3/Transcription Factor Staining Buffer Set (eBioscience, USA). For staining of intracellular proteins, anti-Ki67 (PE, clone 16A8, BioLegend, USA) and anti-EOMES (PE/Cy7, clone DAN11mag, eBioscience, USA) were added. Cells were washed and resuspended in PBS (Lonza, Switzerland) containing count bright absolute counting beads (Life technologies, USA). For proliferation analysis in cocultures of T cells and tumor cells, T cells were stained

with anti-PD-1 (APC, clone RMP-30, BioLegend, USA), anti-CD4 (Pacific Blue™, clone GK1.5, BioLegend, USA), anti-CD8 (APC/Cy7, clone 53-6.7, BioLegend, USA), and Zombie aqua fixable viability dye (BioLegend, USA). Equal amounts of counting beads (Life technologies, USA) were added to each sample. The antibody panel for T cell phenotyping consisted of anti-PD-1 (FITC, clone 29F.1a12, BioLegend, USA), anti-CD8 (APC/Cy7, clone 53-6.7, BioLegend, USA), anti-CD4 (PE/Cy7, clone RM4-5, BioLegend, USA), anti-CD62L (Pacific Blue™, clone MEL-14, BioLegend, USA), anti-CCR7 (PerCP/Cy5.5, clone 4B12, BioLegend, USA), and Zombie aqua fixable viability dye (BioLegend, USA).

MHC I-, MHC II-, and PD-L1-Profiling of Tumor Cells

For the analysis of MHC I-, MHC II-, and PD-L1-expression on Panc02-OVA and E.G7-OVA-PD-L1, 5×10^4 tumor cells were stimulated for 48 h with recombinant murine IFN-γ (Peprotech, USA) at increasing concentrations of 2, 20, or 100 ng/ml respectively and analyzed by flow cytometry as described above.

Antibody-Stimulation Assays

For antibody-stimulation assays, T cells were stimulated with anti-CD3 antibody (100 ng/ml, clone 145-2C11, eBioscience), anti-CD3 antibody and recombinant PD-L1-Fc chimera protein (5 μg/ml, R&D Systems) or anti-CD3 antibody and anti-CD28 antibody (2 μg/ml, clone 37.51, eBioscience) for 48 h. Mitotic activity and CD28 surface expression was analyzed by flow cytometry. Cells were stained as indicated and cell numbers were normalized with counting beads (Life Technologies, Germany). Cytokine release was quantified by ELISA (IL-2 and IFN-γ, both BD).

Cocultures of T Cells and Tumor Cells

For T cell-tumor cell cocultures, CD8+ and CD4+ T cells (in a 3:1, 1:1, or 1:3 cell ratio) were prestimulated with anti-CD3 antibody (100 ng/ml, clone 145-2C11, eBioscience) and recombinant PD-L1-Fc chimera protein (5 μg/ml, R&D Systems) for 24 h, as described above. T cells were then cocultured for 16 h with either E.G7-OVA-PD-L1, Panc02-OVA, or Panc-OVA-PD-L1 tumor cells in a 10:1 effector to target cell ratio. Cytokine release was quantified by ELISA (IL-2 and IFN-γ). For cytotoxicity assays, tumor cell-derived LDH release was quantified after 16 h using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, USA). Granzyme B secretion was determined using Mouse Granzyme B DuoSet® ELISA (R&D systems, USA). For T cell phenotyping and proliferation assays, T cells were cocultured with Panc-OVA-PD-L1 for 36 h, as described above. T cell phenotype and proliferation was analyzed by flow cytometry as described above.

MHC I, MHC II, and IL-2 Neutralization Assays

For MHC I, MHC II, and IL-2 neutralization experiments, CD8+ and CD4+ T cells (in a 1:1 ratio) were prestimulated with anti-CD3 antibody and recombinant PD-L1-Fc chimera for 24 h. Subsequently, T cells and Panc02-OVA cells were cocultured at a 10:1 effector to target cell ratio. Anti-mouse MHC class I antibody (10 μg/ml, clone M1/42.3.9.8, *InVivo*MAb), anti-mouse MHC class II antibody (10 μg/ml, clone M5/114.15.2, eBioscience) and LEAF purified anti-mouse IL-2 antibody (10 μg/ml, clone JES6-1A12, BioLegend) were added during prestimulation and co-culture. Supernatants were analyzed for IFN-γ by ELISA.

Statistical Analysis

For statistical analysis, GraphPad Prism software version 7.04 was used. Reported values are continuous. Differences between experimental conditions were analyzed using the unpaired two-sided Student's *t*-test. *P*-values < 0.05 were considered as significant. Data shown are mean values ± SEM of at least three biological replicates representative for three independent experiments as indicated.

RESULTS

Functional Analysis of PD1-CD28 Fusion Protein (PTM) in CD4+ T Cells

To characterize the functionality of PTM in CD4+ T cells, we transduced PTM into primary murine CD4+ T cells. PTM-transduced and untransduced T cells were then stimulated with anti-CD3 antibody, anti-CD3 antibody and recombinant PD-L1 or anti-CD3 antibody and anti-CD28 antibody for 48 h. CD4+ PTM-transduced T cells showed significantly higher IFN-γ release as compared to untransduced T cells (**Figure 1A**). T cell activation was paralleled by an increase in T cell viability and T cell proliferation (**Figures 1B,C**). Untransduced CD4+ T cells were more strongly stimulated by anti-CD3 than PTM-transduced CD4+ T cells, while combination with anti-CD28 antibodies brought PTM-transduced T cells to a similar level of stimulation as untransduced T cells in this control condition. Similarly, expression of the mitogenic marker Ki67 was higher in PTM-transduced T cells than in untransduced T cells (**Figure 1D**). Expression of Eomesodermin (EOMES), a T cell differentiation marker, was highest for anti-CD3 and PD-L1-stimulated, transduced T cells compared to untransduced cells (**Figure 1E**). Together, these results demonstrate that PTM is functional in CD4+ T cells and enhances their functionality.

Functional Analysis of PTM-Transduced T Cells Cocultured With Tumor Cells

To assess the therapeutic potential of PTM-transduced CD4+ T cells *in vitro*, we prestimulated antigen-specific CD4+ or CD8+ PTM-transduced or untransduced T cells at a ratio of 1:1 with anti-CD3 antibody and recombinant PD-L1 for 24 h. Prestimulation was performed to mimic primary antigen contact and to induce partial anergy of the cells, as expected in the tumor environment. CD4+ or CD8+, untransduced, or transduced T cells were then cocultured alone or in different combinations with either Panc02-OVA cells or E.G7-PD-L1 cells. PTM-transduced CD4+ and CD8+ T cells produced more IFN-γ in contact with either cell line compared to untransduced T cells (**Figure 2A**). Highest IFN-γ secretion was measured for both tumor cell lines when PTM-transduced CD4+ and PTM-transduced CD8+ were combined. The same effect was observed

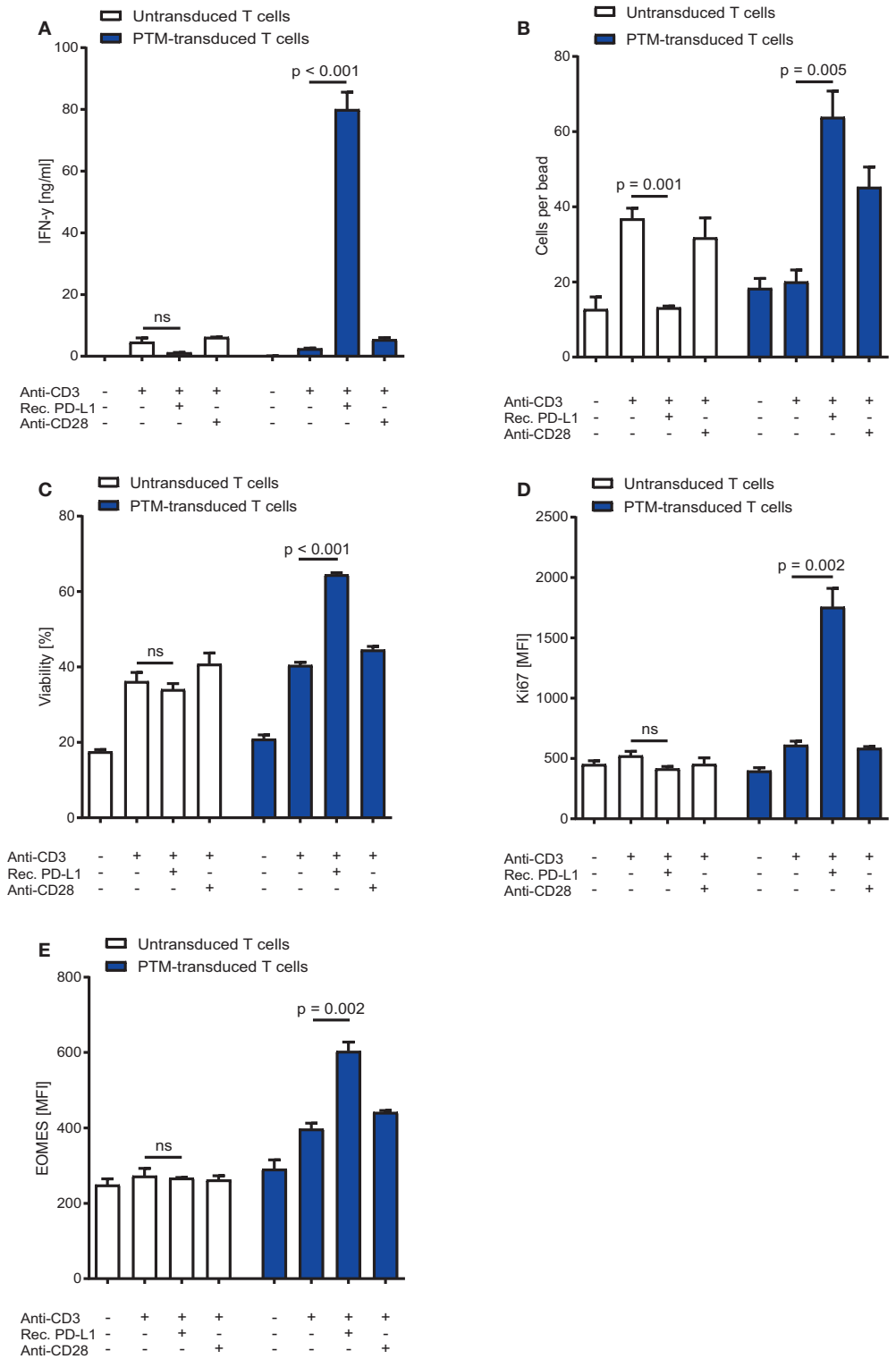

FIGURE 1 | *In vitro* characterization of PD1-CD28 fusion protein (PTM)-transduced CD4+ T cells. PTM-transduced or untransduced primary murine CD4+ T cells were either stimulated with anti-CD3 antibody, anti-CD3 antibody, and recombinant PD-L1 or anti-CD3 antibody and anti-CD28 antibody. **(A)** Interferon-γ (IFN-γ) secretion was measured by enzyme linked immunosorbent assay (ELISA). **(B)** T cell number was analyzed by flow cytometry and normalized to standardized counting beads. **(C)** Viability of T cells was assessed by flow cytometry. **(D)** After 48 h of stimulation T cells were intracellularly stained for Ki67, a mitosis marker or **(E)** for the differentiation marker eomesodermin (EOMES). Experiments **(A–E)** are representative of three independent experiments each performed in triplicates. Bars represent SEM and *P* values from Student's *t*-test are shown. All tests are two-sided.

for IL-2 release (**Figure 2B**). T cell activation was followed by a similar effect on T cell-mediated cytotoxicity. CD4+ and CD8+ PTM-transduced T cells, prestimulated individually, induced significant lysis of Panc02-OVA and E.G7-PD-L1 cells as compared to untransduced T cells (**Figure 2C**). Similar to cytokine production, cytotoxic activity was highest, when CD4+ and CD8+ PTM-transduced T cells were cocultured with tumor cells as compared to control conditions. Mechanistically, T cell cytotoxicity correlated with granzyme B release indicating that T cell degranulation is the mode of action, which is boosted by PTM transduction (**Figure 2D**). T cell cytotoxicity was accompanied by an increase in the number of CD8+ T cells in coculture with CD4+ T cells and Panc02-OVA (**Figure 2E**). PTM-transduced CD4+ T cells in coculture with Panc02-OVA-PD-L1 cells developed a predominant central memory phenotype, defined by CCR7+ and CD62L+ expression, over time (**Supplementary Figure 1A**). The effect on CD4+ T cells was strongest in the presence of untransduced or PTM-transduced CD8+ T cells. However, PTM-expression on CD8+ T cells alone, did not have an influence on the CD4+ T cell phenotype. CD8+ T cells, in contrast, differentiated into central memory T cells within the same experimental setting (**Supplementary Figure 1B**). In these cocultures, the amount of effector memory T cells was reduced in both, CD4+ and CD8+ T cells transduced with PTM (**Supplementary Figures 1C,D**). Our results suggest that CD4+ PTM-transduced T cells have therapeutic activity *in vitro* and point toward a synergistic collaboration of CD4+ and CD8+ T cells. Of note, this effect was highest when PTM was expressed by both T cell subsets. *In vivo,* combined treatment of OT1-PTM with OT2-PTM T cells mediated enhanced tumor control over PTM-transduced OT1 T cells, OT1 plus OT2 T cells and OT1 plus PTM-OT2 T cells in the EG7-PD-L1 model (**Figure 2F**). These results indicate the potential value of the strategy *in vivo*.

CD4+ to CD8+ T Cell Ratio Positively Influence the Activity of PTM-Transduced T Cells via IL-2 in Coculture With Tumor Cells

To test the CD4+ to CD8+ T cell ratio with the highest synergistic potential, we prestimulated antigen-specific, untransduced, or PTM-transduced CD8+ T cells and increasing numbers of antigen-specific, untransduced, or PTM-transduced CD4+ T cells with anti-CD3 antibody plus recombinant PD-L1. CD4+- or CD8+-, untransduced or transduced T cells were then cocultured alone or in different combinations with either Panc02-OVA or E.G7-PD-L1. In both tumor models, IFN-γ secretion, as indicator for T cell activation, was highest when PTM+ CD4+ and PTM+ CD8+ T cells were combined (**Figures 3A,B**). IFN-γ level positively correlated with the number of CD4+ T cells present in the coculture, accompanied by comparable IL-2 levels (**Figures 3C,D**). IL-2 levels were highest when PTM+ CD4+ and PTM+ CD8+ were cocultured with target cells. IL-2 production was tightly correlated with the number of CD4+ cells, pointing toward a potential role of IL-2 in their collaborative activity. To test this hypothesis, T cells were prestimulated and incubated with Panc02-OVA cells in the presence of anti-IL-2 neutralizing antibody. T cell activation, measured by IFN-γ release, was almost abrogated through neutralization of IL-2 (**Figure 3E**). Similarly, synergy in T cell cytotoxicity was also blocked by anti-IL-2 neutralizing antibody in cocultures of Panc02-OVA cells with PTM+ CD4+ and PTM+ CD8+ T cells (**Supplementary Figure 2**). Taken together, our results demonstrate that the synergistic effect of transduced CD4+ and CD8+ T cells is dose-dependent and is mediated by IL-2.

Synergistic Activity Is Dependent on PD-L1 and MHC I but Not on MHC II Expression

To further delineate the synergistic action of OT1-PTM and OT2-PTM T cells, we addressed the expression of potential components of the system on the tumor cell side. We therefore analyzed MHC I for OT1 T cell recognition, PD-L1 for PTM-T cell activation and MHC II for OT2 T cell activation. In both models—Panc02-OVA and EG7-PD-L1—we found strong expression of MHC I but not of MHC II (**Figures 4A,B**). Not surprisingly, PD-L1 was constitutively overexpressed on EG7-PD-L1 and could be induced on Panc02-OVA upon IFN-γ stimulation (**Figures 4A,B**). Functionally, the observed synergy on EG7-PD-L1 of OT1-PTM and OT2-PTM T cells (**Figure 4C**) was entirely abrogated on OVA negative EL4 T cells (**Figure 4D**). Importantly this was not due to lack of MHC I or PD-L1 expression (not shown). Identical results were found when EG7-PD-L1 were pretreated with MHC I-blocking antibodies. As in the absence of OVA, T cell activity was entirely abrogated (**Figure 4E**). In contrast, MHC II-blockade did not impact on T cell recognition by combined OT1-PTM and OT2-PTM T cells (**Figure 4F**). These results indicate that both PD-L1 and MHC I but not MHC II are essential for the activity of our proposed strategy.

DISCUSSION

ACT, especially for solid tumors, is often limited by the immunosuppressive tumor milieu. Tumor cells evade an efficient tumor immune response especially via the PD-1-PD-L1 axis. Here, we report that CD4+ T cells, expressing a PD1-CD28 fusion receptor, have the potential to overcome PD-L1-mediated T cell suppression. We hypothesized that PTM-transduced CD4+ T cells might further boost the efficacy of CD8+ T cells *in vitro*, pointing toward potential avenues for translation of the approach.

Inhibitory receptors, such as PD-1 and CTLA-4, are important checkpoint molecules that prevent autoimmunity under physiological conditions. However, when expressed by tumor-infiltrating T cells these molecules strongly prevent an effective anti-tumor response. Following a similar strategy, a costimulatory CTLA-4–CD28 fusion receptor was shown to induce large amounts of IL-2 and high proliferation of CD4+ T cells when introduced in the latter, strengthening the idea of such fusion proteins to support CD4+ T cell activity (38). We previously described a PD1-CD28 fusion protein

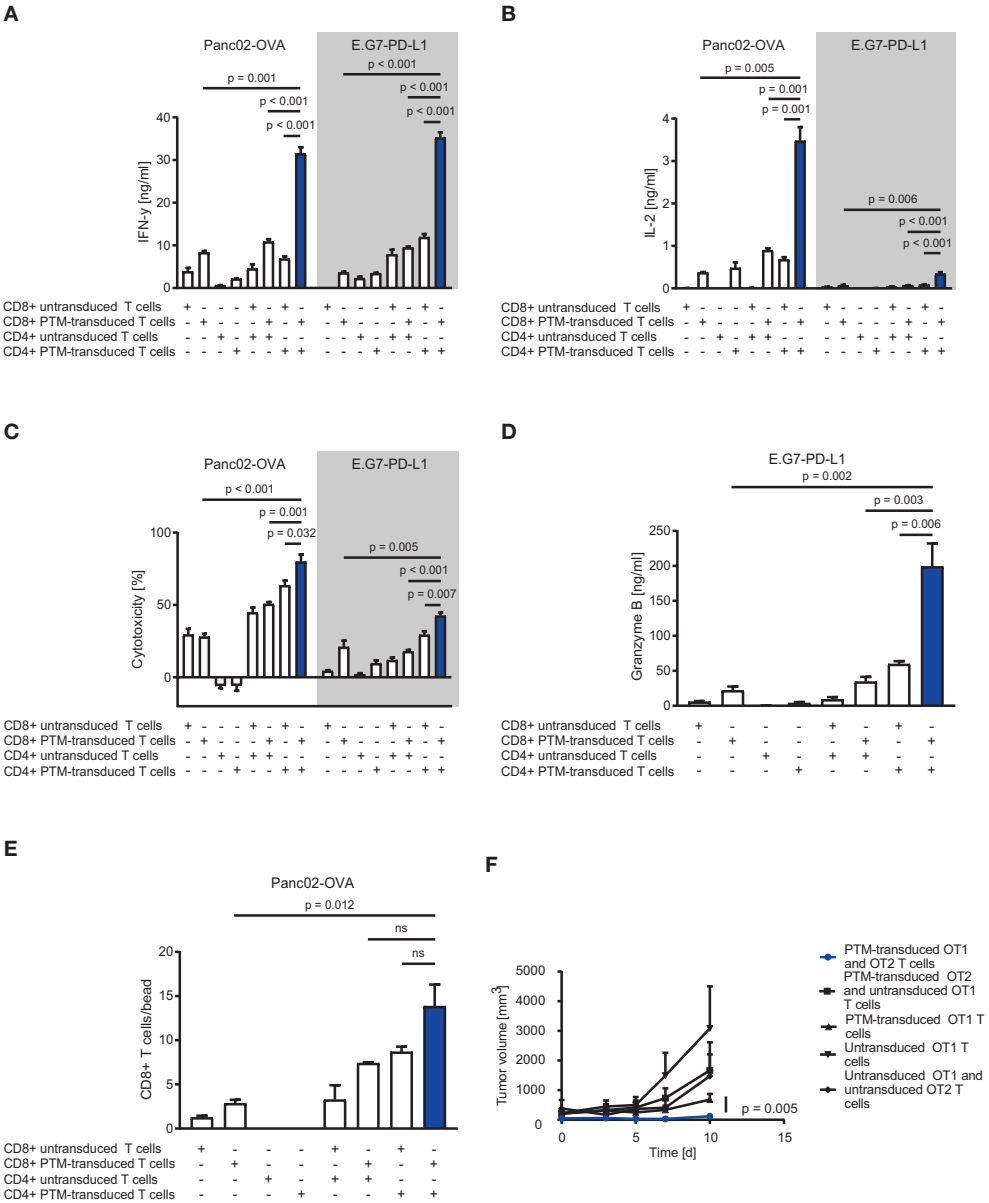

FIGURE 2 | *In vitro* and *in vivo* assessment of anti-tumor efficacy of PD1-CD28 fusion receptor (PTM receptor)-transduced CD4+ and CD8+ T cells. **(A)** PTM-transduced, untransduced primary murine OT-1, PTM-transduced, untransduced primary murine OT-2 T cells, or OT-1 together with OT-2 T cells were prestimulated for 24 h with anti-CD3 antibody plus recombinant PD-L1. T cells were then cocultured with Panc02-OVA or E.G7-PD-L1 cells. Interferon-γ (IFN-γ) secretion was measured by enzyme linked immunosorbent assay (ELISA). **(B)** Interleukin-2 (IL-2) release was measured by ELISA. **(C)** PTM-transduced, untransduced primary murine OT-1, PTM-transduced, untransduced primary murine OT-2 T cells, or OT-1 together with OT-2 T cells were prestimulated for 24 h with anti-CD3 antibody and recombinant PD-L1. In the meantime, Panc02-OVA or E.G7-PD-L1 cells were seeded and grown prior to the addition of T cells. LDH release measurement from lysed tumor cells was performed after 16 h of coculture. **(D)** Granzyme B secretion by T cells cocultured with E.G7-PD-L1 cells for 16 h measured by ELISA. **(E)** PTM-transduced, untransduced primary murine OT-1, PTM-transduced, untransduced primary murine OT-2 T cells or OT-1 together with OT-2 T cells were prestimulated for 24 h with anti-CD3 antibody plus recombinant PD-L1 and then cocultured with Panc02-OVA cells. T cell numbers were analyzed by flow cytometry and normalized to standardized counting beads. **(F)** 30 mice were subcutaneously injected with E.G7-OVA-PD-L1 tumor cells in two independent experiments. As soon as all tumors were established, the mice were randomized, assigned to five different treatment groups and treated with either PTM-transduced ($n = 6$) or untransduced primary murine OT1 T cells ($n = 7$) or with PTM-transduced ($n = 4$) or untransduced ($n = 4$) primary OT2 T cells in combination with OT1 T cells or PTM-transduced OT-1 T cells ($n = 9$). Tumor growth was assessed every other day in a blinded fashion and tumor volume was calculated as indicated. Pooled data from two independent experiments is shown here. Curves are censored by the time the first mice had to be taken out of the experiment either due to tumor size or ulceration (day 10). Experiments **(A–E)** are representative of three independent experiments each performed in triplicates. Experiment **(F)** represents pooled data of two independent experiments. Bars represent SEM and *P* values from Student's *t*-test are shown. All tests are two-sided.

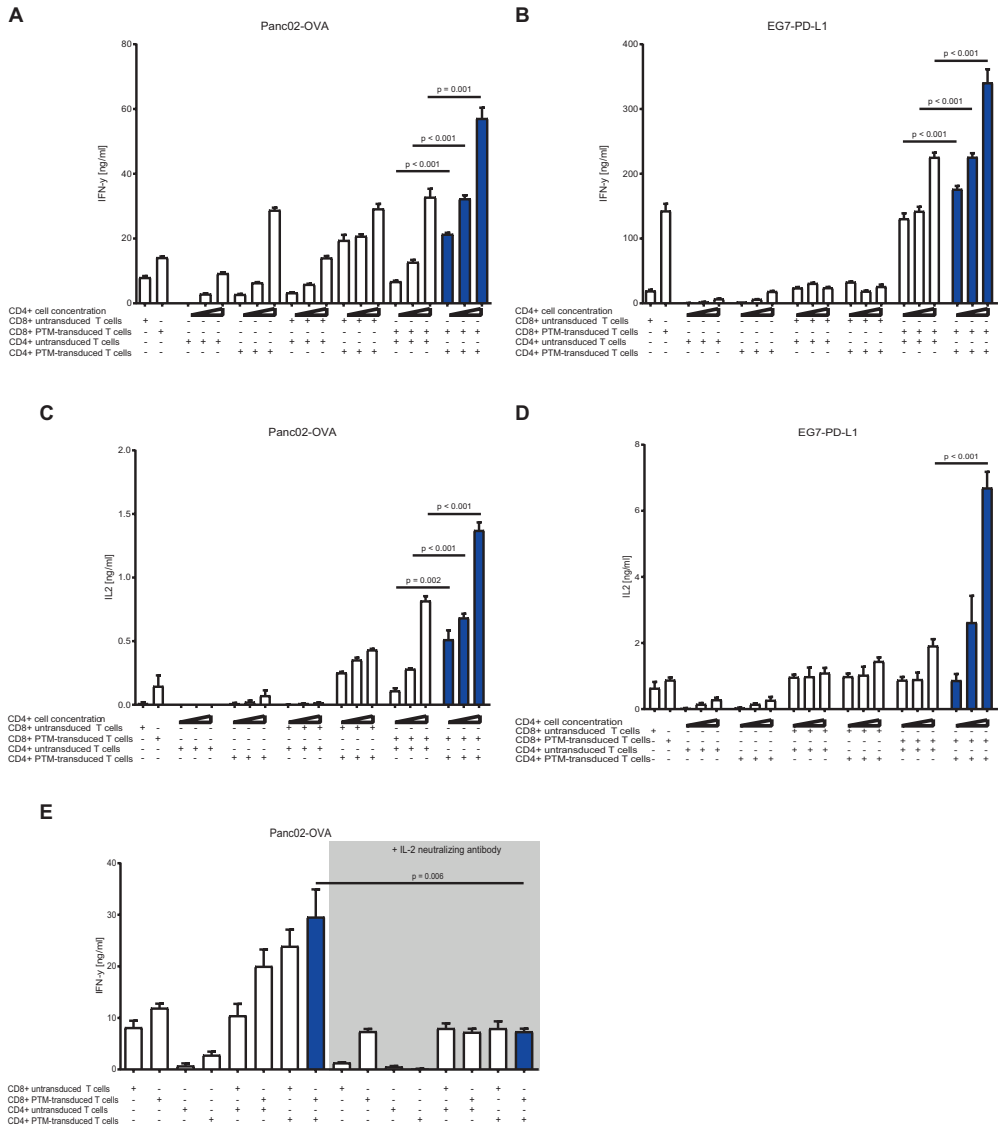

FIGURE 3 | *In vitro* characterization of PD1-CD28 fusion protein (PTM)-transduced CD4+ and CD8+ T cells in T cell-tumor cell cocultures at different CD4+ to CD8+ T cell ratios in the presence or absence of a neutralizing anti-IL-2-antibody. **(A,B)** PTM-transduced or untransduced primary murine OT-1, PTM-transduced or untransduced primary murine OT-2 T cells or combinations of these were prestimulated for 24 h with anti-CD3 antibodies plus recombinant PD-L1. Three different ratios of CD4+ to CD8+ T cells were applied (i.e., 3:1, 1:1, or 1:3 CD4+ to CD8+ T cell ratio). After prestimulation, the T cells were cocultured with Panc02-OVA or E.G7-PD-L1 cells for a further 48 h. The resulting Interferon-γ (IFN-γ) release was measured by enzyme linked immunosorbent assay (ELISA). **(C,D)** The concentration of interleukin-2 (IL-2) in the supernatants was measured by ELISA. **(E)** PTM-transduced or untransduced primary murine OT-1, PTM-transduced or untransduced primary murine OT-2 T cells or combinations of these were prestimulated for 24 h with anti-CD3 antibodies plus recombinant PD-L1. T cells were then cocultured with Panc02-OVA. In the blocking condition, a neutralizing anti-IL-2 antibody was present during the period of prestimulation and coculture. The resulting IFN-γ release was measured by ELISA. Experiments **(A–E)** are representative of three independent experiments each performed at least in triplicates. Bars represent SEM and *P* values from Student's *t*-test are shown. All tests are two-sided.

that rendered antigen-specific CD8+ T cells resistant to PD-1-PD-L1-mediated anergy. Thus, we wondered if this would also apply to CD4+ T cells in a similar fashion (32). In the present manuscript, we could indeed transfer the activity of PTM to CD4+ T cells boosting T cell proliferation and cytokine production in the presence of cancer cells, which further underlines underpinning previous data using a CTLA-4-CD28 fusion receptor.

CD4+ T cells exert potent anti-tumoral effects on their own right (10, 15). This can be mediated either through direct recognition of MHC II+ tumor cells or indirectly through secretion of IFN-γ and activation of bystander myeloid cells (15). In addition, CD4+ T cells can support and contribute to CD8+ T cell function (39–42). If transferred adoptively, CD4+ T cells can even rescue anergic tumor infiltrating CD8+ T cells by T cell help (43). As our fusion protein essentially

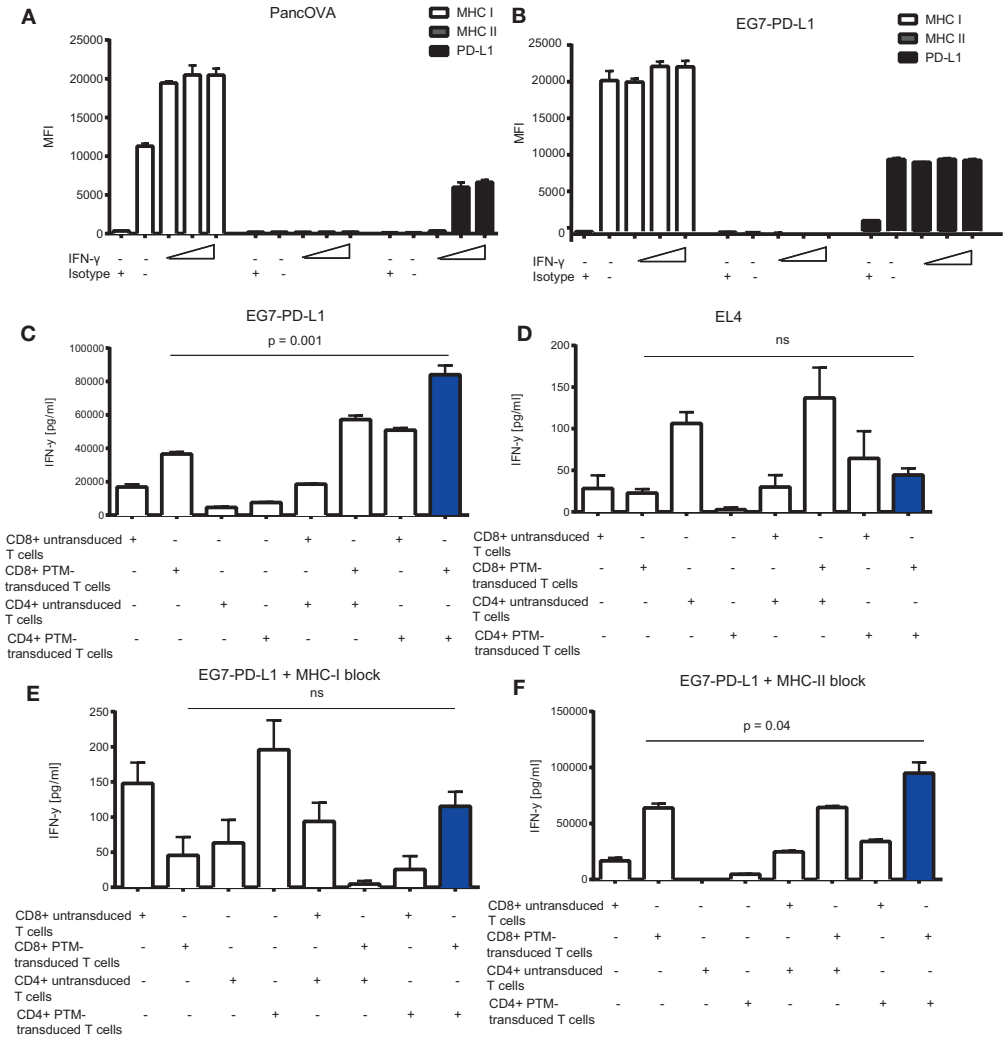

FIGURE 4 | *In vitro* characterization of the MHC I, MHC II, and PD-L1 expression on tumor cells and its effect on interferon-γ (IFN-γ) release by PD1-CD28 fusion protein (PTM)- transduced CD4+ and CD8+ T cells in T cell-tumor cell cocultures. **(A)** Panc02-OVA or **(B)** E.G7-OVA-PD-L1 cells were stimulated with increasing concentrations of recombinant murine IFN-γ (2, 20, 100 ng/ml) during a 48 h period. MHC I, MHC II, and PD-L1 expression was assessed by flow cytometry. **(C–F)** PTM-transduced or untransduced primary murine OT-1, PTM-transduced or untransduced primary murine OT2 T cells or OT1 plus OT-2 T cells were prestimulated for 24 h with anti-CD3 antibody plus recombinant PD-L1. T cells were then cocultured with EL4 **(D)** or with E.G7-OVA-PDL-1 in the absence **(C)** or presence of neutralizing anti-MHC. I antibody **(E)** or of neutralizing anti-MHCII-antibody **(F)**. The resulting interferon-γ (IFN-γ) release was measured by enzyme-linked immunosorbent assay (ELISA). Experiments **(A–F)** were performed in triplicates. Experiments **(C–F)** are representative of two independent experiments. Bars represent SEM and *P* values from Student's *t*-test are shown. All tests are two-sided.

seems to further boost the function of the cell subsets either alone or in combination, we indeed observed that also the collaboration between CD4+ and CD8+ T cells was enhanced through introduction of PTM in both cell types. Interestingly, this effect was dependent on an optimal CD4+ to CD8+ T cell ratio, which is also in line with clinical observations observed with CAR T cells (7, 8). This is further confirmed in multiple studies dealing with mixtures of CD4+ and CD8+ T cells for ACT (16–18). Notwithstanding the role of PD-1-mediated anergy, we argue and show that this brake is released by our PD1-CD28 fusion protein. Similar observations were reported with CTLA-4-CD28-expressing CD4+ and CD8+ T cells (38). Mechanistically, IL-2 derived from CD4+ T cells seems to mediate the synergistic effect of PD1-CD28 fusion receptor-transduced CD4+ and CD8+ T cells. As IL-2 improves CD8+ T cell activation, proliferation, and persistence one could assume that the additional transfer of CD4+ T cells would allow a lower dose of CD8+ T cells per patient. This would come with the additional advantage, that systemic IL-2 administration which often accompanies ACT protocols and causes significant side effects, could be prevented (44, 45). CD4+ T cells are also important for long-term protective anti-tumoral immunity

(46, 47). In our hands, transduced CD4+ and CD8+ T cells predominantly developed a central memory phenotype. At least for CD8+ T cells longer persistence of CD8+ clones isolated from central memory T cells as compared to clones from CD8+ effector cells was observed *in vivo* after T cell transfer. This further indicates the importance of specific T cell subset functions for effective adoptive immunotherapy (48). An open question remains how CD4+-T cells would sense their antigen *in vitro*. We could demonstrate that OVA expression by the tumor cells, MHC I presentation and recognition of MHC I presented peptide by cocultured CD8+ T cells was mandatory for CD4+ T cell action. CD4+ T cells in general and OT-2 T cells in particular can be stimulated MHC II independently in the presence of large amounts of soluble antigen (49). OVA is known to be secreted by cells stably transfected with it and additional antigen release by CD8+-OT-1-T cells might lead to the level of antigen required for CD4+ T cells *in vitro*. The exact role of this known mechanism *in vivo* is currently unclear but has been repeatedly shown in several models (15). In any case, the *in vivo* activity observed strongly suggests translational potential for this strategy. An open question is how much data from the OT-1-OT-2 system will be transferrable to endogeneous antigens and to TCRs with different affinities. This antigen system is one of the most widely tested systems in T cell research. A significant amount of our knowledge has been generated in these models. Several studies suggest that data gathered from such preclinical studies will actually translate to clinical studies, corroborating the value of the OT-1-OT-2 system for translational T cell research (50, 51).

Antibodies, such as nivolumab, targeting the PD-1-PD-L1 axis can revive exhausted CD8+ T cells and have demonstrated impressive clinical activity (52, 53). However, more than 50% of PD-L1-positive tumors do not respond to anti-PD-L1/PD-1 antibody treatment (54). In addition, treatment protocols using those antibodies often require multiple injections and cause significant toxicities to the patient (55). Based on our previous data we assume that a single dose of PD1-CD28 fusion receptor-transduced CD4+ and CD8+ T cells would induce tumor regression *in vivo*, significantly lowering potential side effects due to systemic T cell activation (32). Even PD-L1 negative tumors could be targeted by our combinatorial approach. Transduced CD4+ T cells can also be activated by interaction with PD-L2, another ligand of PD-1, expressed on antigen-presenting cells present in the tumor microenvironment.

In summary, our results indicate that PD1-CD28 fusion protein transduced CD4+ T cells have the potential to overcome the PD-1-PD-L1 immunosuppressive axis in pancreatic cancer and non-Hodgkin-lymphoma. Collectively, inhibiting PD-1 signaling in both CD4+ and CD8+ T cells might be the most effective way to enhance antitumor immunity. This data will need to be further investigated in other models while moving the approach toward translation.

AUTHOR CONTRIBUTIONS

FR designed experiments, supervised experiments, discussed data, and wrote the manuscript. FK designed experiments, conducted experiments, analyzed data, and wrote the manuscript. MC, SG, CH, and BC conducted experiments. PD and SE wrote the manuscript; SK designed and conceptualized the research, supervised the experiments, discussed data, and wrote 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.2018.01955/full#supplementary-material

Supplementary Figure 1 | Differentiation of PD1-CD28 fusion protein (PTM)-transduced CD4+ and CD8+ T cells in T cell-tumor cell cocultures. **(A–D)** PTM-transduced, untransduced primary murine OT-1, PTM-transduced, untransduced primary murine OT-2 T cells or OT-1 together with OT-2 T cells were prestimulated for 24 h with anti-CD3 antibody and recombinant PD-L1. T cells were then cocultured with Panc02-OVA-PD-L1. CCR7 and CD62L expression on T cells was analyzed prior to and after 36 h of coculture by flow cytometry. Experiments **(A–D)** are representative of three independent experiments each performed at least in duplicates. Bars represent SEM.

Supplementary Figure 2 | Cytotoxic activity of PD1-CD28 fusion protein (PTM)-transduced CD4+ and CD8+ T cells in T cell-tumor cell coculture in the presence of an Interleukin-2 (IL-2) neutralizing antibody. PTM-transduced, untransduced primary murine OT-1, PTM-transduced, untransduced primary murine OT-2 T cells or OT-1 together with OT-2 T cells were prestimulated for 24 h with anti-CD3 antibody and recombinant PD-L1. T cells were then cocultured with Panc02-OVA in the presence or absence of neutralizing anti-IL-2 antibody and LDH release from lysed tumor cells was measured. The experiment was performed in quadruplicates. Bars represent SEM and *P* values from Student's *t*-test are shown. All tests are two-sided.

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Myeloid-Derived Suppressor Cells Hinder the Anti-Cancer Activity of Immune Checkpoint Inhibitors

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Immune checkpoint inhibitors (ICI) used for cancer immunotherapy were shown to boost the existing anti-tumor immune response by preventing the inhibition of T cells by tumor cells. Antibodies targeting two negative immune checkpoint pathways, namely cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed cell death-ligand 1 (PD-L1), have been approved first for patients with melanoma, squamous non-small cell lung cancer (NSCLC), and renal cell carcinoma. Clinical trials are ongoing to verify the efficiency of these antibodies for other cancer types and to evaluate strategies to block other checkpoint molecules. However, a number of patients do not respond to this treatment possibly due to profound immunosuppression, which is mediated partly by myeloid-derived suppressor cells (MDSC). This heterogeneous population of immature myeloid cells can strongly inhibit anti-tumor activities of T and NK cells and stimulate regulatory T cells (Treg), leading to tumor progression. Moreover, MDSC can contribute to patient resistance to immune checkpoint inhibition. Accumulating evidence demonstrates that the frequency and immunosuppressive function of MDSC in cancer patients can be used as a predictive marker for therapy response. This review focuses on the role of MDSC in immune checkpoint inhibition and provides an analysis of combination strategies for MDSC targeting together with ICI to improve their therapeutic efficiency in cancer patients.

Keywords: myeloid-derived suppressor cells, immunosuppression, cancer immunotherapy, immune checkpoint inhibition, combination therapy

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Abbreviations: ATRA, all-trans retinoic acid; ARG-1, arginase-1; bFGF, basic fibroblast growth factor; CCR, C-C chemokine receptor; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; CCRK, cell cycle-related kinase; CSF-1R, colony stimulating factor 1 receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DAMP, damage-associated molecular pattern; DC, dendritic cells; GM-CSF, granulocyte-macrophage colony stimulating factor; ICI, immune checkpoint inhibitors; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; IFN, interferon; IL, interleukin; JAK, Janus kinase; MMP, matrix metalloproteinases; MyD88, myeloid differentiation primary response 88, MDSC, myeloid-derived suppressor cells; NK, natural killer; NO, nitric oxide; NSCLC, non-small cell lung cancer; NF-κB, nuclear factor "kappa-light-chain-enhancer" of activated B-cells; NY-ESO-1, New York esophageal squamous cell carcinoma-1; PBMC, peripheral blood mononuclear cells; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PDE5, phosphodiesterase-5; PD-1, programmed cell death protein 1, PD-L1, programmed cell death-ligand 1; ROS, reactive oxygen species; Treg, regulatory T cells; STAT, signal transducer and activator of transcription; TCR, T cell receptor; TLR, toll-like receptor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

INTRODUCTION

Cancer immunotherapy has become a promising approach to treat patients over the past decade (1). Furthermore, new types of cancer immunotherapy that are currently under investigation will impact the treatment of cancer patients in the future. Among immunotherapeutic approaches, immune checkpoint inhibition is very promising. However, other types of immunotherapies such as monoclonal antibodies against tumor-associated antigens, cancer vaccines, cell therapy, and unspecific boosting of the immune system with interleukins (IL), interferons (IFN), or toll-like receptor (TLR) ligands are also used and/or under investigation (2).

Immune checkpoint pathways are important to restrict excessive immune responses (3). However, under cancer conditions, tumor cells can exploit these mechanisms to impair or prevent the tumor-targeted immune response. Signals transmitted to T cells either *via* programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) promote T cell anergy and thereby switch off the immune response. Therefore, blockers of these immune checkpoint molecules have been shown to restore an immune response against cancer and increase patient survival (4, 5).

Ipilimumab (monoclonal antibody against CTLA-4) is used for the therapy of cutaneous melanoma. Nivolumab and pembrolizumab (monoclonal antibodies against PD-1) are approved for the therapy of cutaneous melanoma, non-small cell lung cancer (NSCLC), kidney cancer, bladder cancer, head and neck cancers, and Hodgkin lymphoma. Atezolizumab [monoclonal antibody against programmed cell death-ligand 1 (PD-L1)] is approved for the treatment of NSCLC and bladder cancer and avelumab (monoclonal antibody against PD-L1) is approved for gastric cancer and Merkel cell carcinoma therapy. Despite the fact that these immune checkpoint inhibitors (ICI) have proved to be effective, therapeutic resistance occurs in the majority of patients, leading to tumor progression (5, 6). This occurs due to the immunosuppressive tumor microenvironment represented by several immunosuppressive factors and cells, including myeloid-derived suppressor cells (MDSC) (7–10). Importantly, the efficacy of cancer immunotherapy has been reported to be negatively correlated with an increased MDSC frequency and function (11–15).

Myeloid-derived suppressor cells play a leading role in immunosuppression in various cancer types. Accumulating evidences in recent years have even highlighted them as a major driver of an immunosuppressive tumor microenvironment (7–10, 16). Therefore, MDSC could be a promising target in cancer immunotherapy especially in combination with ICI. In this review, we discuss the phenotypic and functional properties of MDSC as well as strategies for their therapeutic targeting. In particular, we address the role of MDSC in immune checkpoint inhibition and provide an analysis of the combination strategies for MDSC targeting together with ICI to improve their therapeutic efficiency in cancer patients.

PHENOTYPIC AND FUNCTIONAL PROPERTIES OF MDSC

Myeloid-derived suppressor cells represent a heterogeneous population of myeloid cells that fail to differentiate into granulocytes, macrophages, or dendritic cells (DC) but expand during cancer and chronic infection (17–20). They can strongly suppress the activity of T cells, natural killer (NK) cells, and some myeloid cells such as DC (8). MDSC have been identified to expand and play an important role in various cancer types, for example, in patients with melanoma (15, 21–24), multiple myeloma (25), hepatocellular carcinoma (26), NSCLC (27), renal cell carcinoma (28), breast cancer (29), prostate cancer (30), and colorectal cancer (31).

MDSC Phenotype

In mice, MDSC were characterized by the expression of CD11b and Gr1. However, the use of these markers is no longer sufficient, since MDSC can be divided into two subpopulations in mice: $CD11b^{+}Ly6G^{-}Ly6C^{high}$ monocytic MDSC (M-MDSC) and $CD11b^{+}Ly6G^{high}Ly6C^{low}$ polymorphonuclear MDSC (PMN-MDSC) (32). Human M-MDSC are defined as $Lin^{-}CD11b^{+}CD14^{+}CD15^{-}HLA\text{-}DR^{-/low}$ and PMN-MDSC as $Lin^{-}CD11b^{+}CD14^{-}CD15^{+}HLA\text{-}DR^{-}$ or $Lin^{-}CD11b^{+}CD14^{-}CD66b^{+}$ (32, 33). One-third subtype of human MDSC, containing more immature $HLA\text{-}DR^{-}CD33^{+}CD15^{-}CD14^{-}$ MDSC, has been recently proposed and was termed early stage MDSC (eMDSC) (32).

MDSC Expansion and Activation

Myeloid-derived suppressor cells are absent in the circulation under homeostatic conditions, but they can be accumulated under pathological conditions like chronic inflammation and cancer (34–39). The expansion and activation of MDSC are controlled by a complex network of soluble factors like IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-10, M-CSF, G-CSF, and vascular endothelial growth factor (VEGF) as well as TLR ligands (8, 17, 20, 32, 40). The process of MDSC generation is supposed to be divided into two phases that include MDSC accumulation and activation (8, 18–20, 40). MDSC enrichment is mediated by the blockade of the terminal differentiation of immature myeloid cells into granulocytes, macrophages, and DC due to an alteration of the growth factor composition, where G-CSF, GM-CSF, and VEGF play a major role. MDSC activation is mediated by the long-term secretion of cytokines like IL-6, IL-10, IL-1β, and IFN-γ, as well as TLR ligands, such as damage-associated molecular pattern molecules produced under chronic inflammation (8, 18–20, 40).

The production of immunosuppressive factors is driven *via* the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and myeloid differentiation primary response 88/nuclear factor "kappa-light-chain-enhancer" of activated B-cells signal transduction cascades in MDSC (17, 40).

MDSC Function

Activated MDSC produce elevated levels of nitric oxide (NO) *via* inducible nitric oxide synthase (iNOS) and upregulate the expression of arginase-1 (ARG-1), both leading to cell cycle arrest in T cells *via* depletion of the amino acid L-arginine from the tumor microenvironment (41, 42) and to T cell anergy induced by the downregulation of T cell receptor (TCR) ζ-chain expression (16, 43). Moreover, NO and reactive oxygen species produced by MDSC can induce T cell apoptosis or TCR nitrosylation (44, 45). In addition, activated MDSC express high levels of PD-L1

(46, 47) that interacts with PD-1 on T cells and causes their exhaustion (48). MDSC also express elevated levels of indoleamine 2,3-dioxygenase (IDO), an enzyme degrading L-tryptophan into N-formylkynurenine (49). The starvation from the amino acid L-tryptophan can lead to T cell arrest and anergy (50). Furthermore, it has been shown to drive the differentiation of $CD4^+$ T cells into immunosuppressive regulatory T cells (Tregs) (51). MDSC can also induce Treg expansion and reduction of the anti-tumor activity of effector T cells *via* the expression of CD40 (52) and the secretion of transforming growth factor-β and IL-10 (53–55). Furthermore, MDSC impair the Fc receptor-mediated functions of NK cells by the production of NO (56).

In addition to their immunosuppressive properties, MDSC can have other tumor promoting effects. In particular, they stimulate tumor angiogenesis by secreting VEGF and basic fibroblast growth factor (57, 58). By secreting matrix metalloproteinases (MMP), especially MMP9, they mediate a lower integrity of the extracellular matrix and the basal membrane, which enables tumor cells to enter the blood stream and form metastasis (59, 60). MDSC were also shown to play an important role in the formation of the pre-metastatic niche, a microenvironment in a secondary organ, facilitating metastasis (61).

MDSC AS A PREDICTIVE MARKER IN IMMUNE CHECKPOINT INHIBITION FOR CANCER THERAPY

Myeloid-derived suppressor cells have been reported to be an important prognostic marker for ICI treatment. Interestingly, MDSC levels could be used to predict therapy response or resistance to ipilimumab treatment in metastatic melanoma patients (62). Clinical responders to ipilimumab therapy showed a significantly lower percentage of $Lin^-CD14^+HLA\text{-}DR^-$ M-MDSC in the peripheral blood as compared to non-responders. This finding suggests the use of circulating M-MDSC frequency as a marker of response, since low frequencies identified patients who could benefit from ipilimumab treatment (62). These data are in agreement with the results from another study, showing that a higher M-MDSC frequency prevented ipilimumab-induced activation and expansion of tumor-specific T cells resulting in the lower clinical response (23). It was shown by three more studies that a lower frequency of circulating MDSC at baseline can be used as a predictive marker for ipilimumab treatment of malignant melanoma patients (14, 15, 63). Moreover, in prostate cancer patients treated with a cancer vaccine in combination with ipilimumab, a lower frequency of circulating MDSC was found to correlate with an increased overall survival of patients (64).

STRATEGIES FOR MDSC THERAPEUTIC TARGETING TO OVERCOME RESISTANCE TO ICI

Due to important role of MDSC in tumor-induced immunosuppression, these cells could be a promising target for a combination therapy with ICI. There are three different approaches to target MDSC, namely the inhibition of (i) MDSC accumulation; (ii) MDSC trafficking; and (iii) MDSC-mediated immunosuppression.

Reduction of MDSC Frequency

To reduce MDSC frequency, the process of myelopoiesis has to be normalized and MDSC accumulation has to be blocked. Some chemotherapeutics were shown to affect MDSC in tumor-bearing hosts. Using the *RET* transgenic mouse model of malignant melanoma, it was demonstrated that ultra-low non-cytotoxic doses of paclitaxel induced a reduction of MDSC numbers and immunosuppressive activity, resulting in an increased survival of melanoma-bearing mice (65). Furthermore, the treatment of pancreatic cancer patients with gemcitabine led to a reduced number of PMN-MDSC (66). In colorectal cancer patients, the treatment with FOLFOX (folinic acid, 5-fluorouracil, and oxaliplatin) resulted in a reduced immunosuppression and a better clinical outcome that could be attributed to a decrease in MDSC frequency and restored anti-tumor immunity (67).

It has been described that the blockade of retinoic acid signal transduction by all-trans retinoic acid (ATRA) led to the differentiation of MDSC into macrophages and DC in murine and human cell samples (68). ATRA has been applied in two clinical trials, including patients with metastatic renal cell carcinoma and late stage small cell lung cancer, leading to a reduction of MDSC frequencies and an improvement of the patient survival (69, 70).

Blockade of MDSC Recruitment

To exhibit their immunosuppressive phenotype, MDSC have to be recruited to the tumor site. This process is mediated mainly by chemokines secreted in the tumor microenvironment and chemokine receptors expressed on MDSC (71, 72). The role of C-C motif chemokine ligand (CCL)2 and its receptors C-C chemokine receptor (CCR)2 and 4 in the recruitment of M-MDSC has been well-documented (71, 73). Moreover, it was recently found that CCR5 is expressed on MDSC in *RET* transgenic melanoma-bearing mice and melanoma patients, playing an important role in their recruitment to the tumor microenvironment *via* the CCR5 ligands (CCL3, CCL4, and CCL5) (74, 75). Interestingly, $CCR5^+$ MDSC were reported to display higher immunosuppressive potential than their $CCR5^-$ counterpart both in mice and patients (74). Moreover, the blockade of the interaction of CCR5 with its ligands by a mCCR5-Ig fusion protein significantly improved the survival of melanoma-bearing animals (74). In addition, in a prostate cancer mouse model, the recruitment of $CD11b^+Gr1^+$ MDSC could be blocked by a CXC chemokine receptor 2 antagonist, thereby potentiating the therapeutic effect of the chemotherapeutic drug docetaxel (76).

Inhibition of MDSC-Mediated Immunosuppression

Phosphodiesterase-5 inhibitors (sildenafil, tadalafil, and vardenafil) are currently in clinical use for non-tumor conditions (77). However, sildenafil was already shown in several transplantable tumor mouse models to downregulate ARG-1 and iNOS expression in MDSC reducing thereby their immunosuppressive capacity and leading to an enhanced intratumoral T cell infiltration

and activation, a reduction of tumor growth, and an improvement of the anti-tumor efficacy of adoptive T cell therapy (78). In the *RET* transgenic melanoma mouse model, sildenafil could also prolong mouse survival that was associated with reduced levels and activity of MDSC in the tumor microenvironment and, therefore, with a restored $CD8^+$ T cell infiltration and function (79). Furthermore, in an inflammation-dependent murine colon cancer model, sildenafil prevented tumorigenesis by inhibiting tumor infiltration with MDSC (80).

In clinical trials, tadalafil was applied in patients with head and neck squamous cell carcinoma and metastatic melanoma (81–83). It improved clinical outcome and augmented the anti-tumor immune response of patients due to the reduction of peripheral and tumor-infiltrating MDSC, highlighting thereby its potential application in combined immunotherapy (81–83).

Another promising approach is targeting of STAT3, since it is a main regulator of MDSC immunosuppressive activity (8, 18–20, 40, 84). Systemic administration of the STAT3 antisense oligonucleotide inhibitor AZD9150 was already tested in a phase I clinical trial in patients with lung cancer and lymphoma (85). It has been recently developed a strategy aiming to target STAT3 decoy oligonucleotides specifically to myeloid cells by coupling them to the TLR9 ligand CpG, which led to a reduced ARG-1 expression and to the restoration of T cell functions in patients with acute myeloid leukemia (86).

COMBINATION OF ICI AND MDSC NEUTRALIZATION

In recent years, the combination of MDSC targeting with ICI treatment has been applied in preclinical tumor models and cancer patients. **Figure 1** illustrates the effect of combination of ICI and MDSC-targeted therapy to enable an anti-tumor immune response. Interestingly, it was shown that anti-PD-1

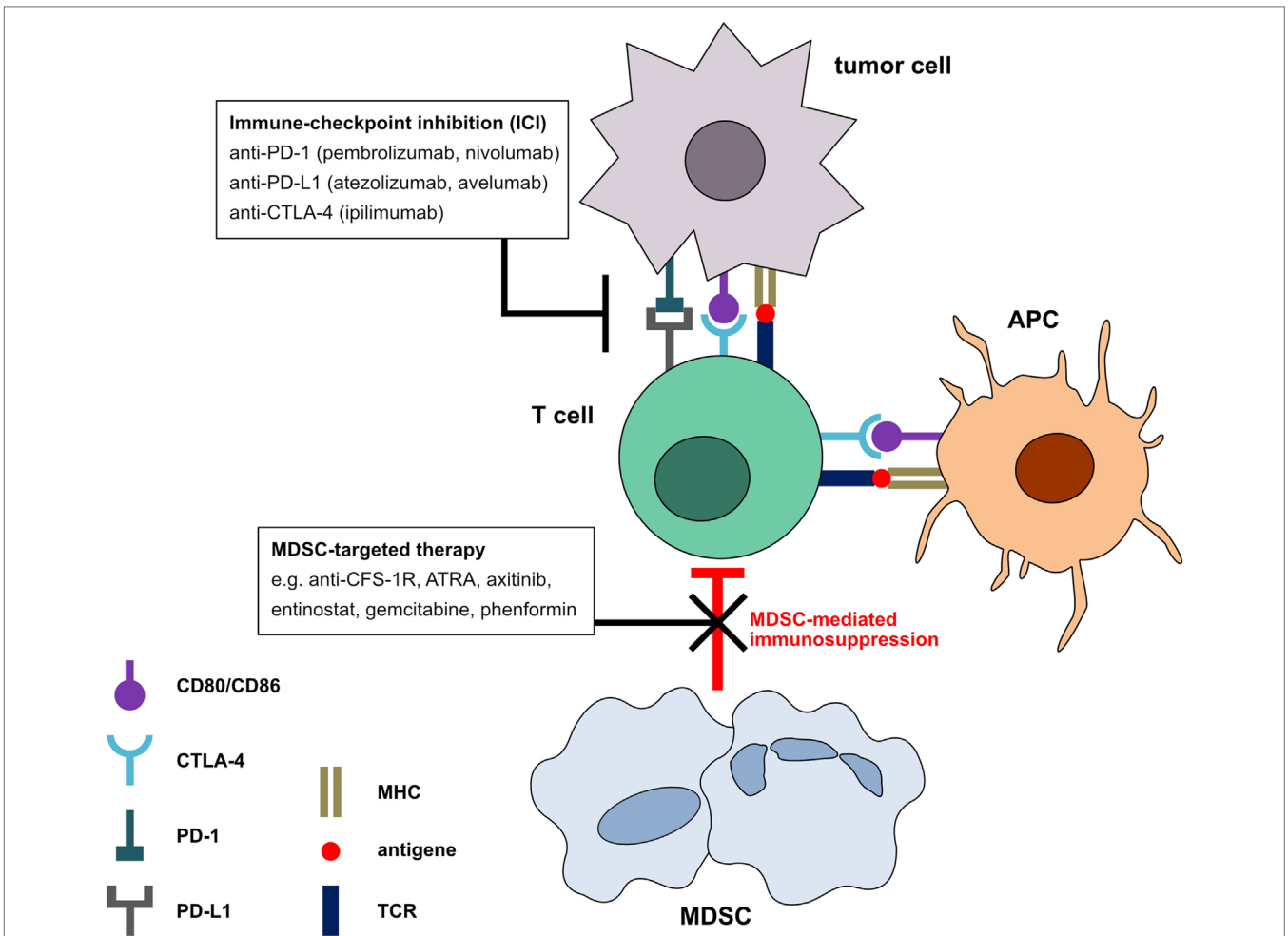

FIGURE 1 | Mechanism of immune checkpoint inhibitors (ICI) in combination with myeloid-derived suppressor cell (MDSC) neutralization. In the tumor microenvironment, tumor and antigen-presenting cells express the ligands for programmed cell death protein 1 and cytotoxic T-lymphocyte-associated protein 4 receptors on T cells. Signals transmitted *via* these receptors induce a T cell arrest and the termination of the anti-tumor immune response. Blockade of these negative checkpoint molecules can restore the anti-tumor activity of T cells. However, MDSC can induce T cell inhibition by mechanisms different from negative checkpoint molecules. The combination of MDSC inhibition with ICI could further increase T cell-mediated anti-tumor immune responses and the clinical outcome of cancer patients.

antibodies themselves seem to have a direct effect on peripheral blood mononuclear cells (PBMC) from cancer patients. It was reported that anti-PD-1 antibodies stimulated *in vitro* PBMC proliferation induced by anti-CD3 antibodies and inhibited the induction of MDSC in the same experimental settings (87).

ICI Plus Reduction of MDSC Frequency

In two different tumor mouse models, the reduction of MDSC by a histone-deacetylase inhibitor, entinostat, in combination with antibodies against CTLA-4 and PD-1 led to 80% tumor eradication although the application of these ICI alone failed to induce anti-tumor effects (88). In Lewis lung and renal cell carcinoma mouse models, MDSC blocking by entinostat in combination with PD-1 blockade resulted in a significantly increased survival in comparison to anti-PD-1 therapy alone (89). Furthermore, MDSC inhibition by phenformin, an antidiabetic drug from the biguanide class, was able to enhance the effect of PD-1 blockade reflected by an increased $CD8^+$ T cell infiltration in the BRAF V600E/PTEN-null melanoma mouse model (90).

In a murine oral cancer model, anti-Ly6G antibodies were applied to deplete PMN-MDSC that resulted in the restoration of antigen-specific T cell responses but failed to improve mouse survival (91). However, the combination of anti-Ly6G and anti-CTLA-4 antibodies induced a complete tumor rejection (91).

ICI Combined With an Alteration of MDSC Function

In a B16 melanoma mouse model expressing IDO, it has been shown that the blockade of colony stimulating factor 1 receptor (CSF-1R) by the kinase inhibitor PLX647 could inhibit tumor-infiltrating MDSC and enhance anti-tumor T cell responses (92). Moreover, this therapy sensitized the tumor for anti-PD-1 and anti-CTLA-4 antibodies, since the combination therapy led to an increased tumor regression and prolonged mouse survival as compared to the therapy with ICI alone (92). The same effect could be shown in CT26 colon and 4T1 breast cancer mouse models, where the combination of anti-CTLA-4 treatment with CSF-1/CSF-1R blockade enhanced the beneficial effect by reprogramming MDSC (93). Moreover, the expression of CSF-1 on tumor cells in melanoma and NSCLC patients correlated with the enrichment of MDSC that could be inhibited *in vitro* by the blockade of CSF-1/CSF-1R signaling (93). This observation was supported by another study, demonstrating that the blockade of M-CSF/CSF-1R interaction by BLZ945 could result in an improved efficacy of PD-1 blockade by inhibiting MDSC in mice with neuroblastoma (94).

The blockade of the VEGF receptor by axitinib in combination with anti-CTLA-4 antibodies increased survival of mice with subcutaneous melanoma and intracranial melanoma metastasis (95). This effect was due to an increased antigen-presenting capacity of DC and to a reduced suppressive capacity of M-MDSC, inducing the stimulation of $CD8^+$ and $CD4^+$ T cells (95).

Importantly, ICI treatment of head and neck cancer was reported to be noneffective due to the recruitment of MDSC (96). However, the treatment of mice bearing head and neck tumors with IPI-145, an inhibitor of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)δ and PI3Kγ isoforms, in combination with anti-PD-L1 antibodies resulted in the inhibition of MDSC activity associated with $CD8^+$ T cell-dependent delay of tumor growth and with an improved survival (97).

It has been demonstrated that cell cycle-related kinase (CCRK) from human hepatocytes stimulated an expansion of $CD11b^+CD33^+HLA\text{-}DR^-$ MDSC *via* an NFκB/IL-6-dependent mechanism (98). Similarly, in CCRK transgenic mice, PMN-MDSC frequency and activity were shown to be increased. Thus, upon inhibition of CCRK, PMN-MDSC numbers were decreased, an increased infiltration of $IFN\text{-}\gamma^+TNF\text{-}\alpha^+CD8^+$ T cells was observed, and tumor progression was impaired (98). The beneficial effect was even stronger upon the combination with anti-PD-L1 antibodies (98).

Ongoing Clinical Trials

Some strategies modulating MDSC frequency and immunosuppressive function are already used in various clinical trials in combination with ICI (**Table 1**). Thus, a combined therapy with the anti-PD-L1 antibody atezolizumab and the histone-deacetylase inhibitor entinostat is currently under investigation in a phase I/II clinical trial in renal cell carcinoma patients. Furthermore, ATRA was applied in combination with ipilimumab in a phase II clinical trial in melanoma patients, inducing an improvement of clinical outcome associated with increased tumor antigen-specific T cell

TABLE 1 | Clinical trials combining myeloid-derived suppressor cell (MDSC) targeting with immune checkpoint inhibitors (ICI) in cancer patients.

No	Title	Disease or conditions	Interventions	Trial number
1	Atezolizumab in combination with entinostat and bevacizumab in patients with advanced renal cell carcinoma	Advanced renal cell carcinoma	Atezolizumab, entinostat, bevacizumab	NCT03024437
2	Ipilimumab and all-trans retinoic acid (ATRA) combination treatment of stage IV melanoma	Melanoma	ATRA, ipilimumab	NCT02403778
3	Depletion of MDSC to enhance anti-programmed cell death protein 1 therapy	Non-small cell lung cancer	Nivolumab, gemcitabine	NCT03302247
4	SX-682 treatment in subjects with metastatic melanoma concurrently treated with pembrolizumab	Melanoma	SX-682, pembrolizumab	NCT03161431
5	RTA 408 capsules in patients with melanoma—REVEAL	Melanoma	Omaveloxolone, ipilimumab, nivolumab	NCT02259231
6	Antibody DS-8273a administered in combination with nivolumab in subjects with advanced colorectal cancer	Colorectal neoplasm	DS-8273a, nivolumab	NCT02991196

responses and decreased MDSC frequencies as compared to ipilimumab alone (99). Two other clinical trials in melanoma patients are utilizing the combination of ICI treatment with MDSC targeting by SX-682, a small-molecule dual-inhibitor of C-X-C motif chemokine ligand 1 and 2, or by the antioxidative and anti-inflammatory drug omaveloxolone (RTA 408). Since it was shown that gemcitabine induced a reduction in MDSC numbers in pancreatic cancer patients (66), potentially increasing thereby the efficacy of nivolumab treatment, the combination of these drugs is applied in a phase II clinical trial in NSCLC patients. Furthermore, the tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptor 2 blocking antibodies DS-8273a, targeting MDSC in cancer patients (100), were applied in a phase I clinical trial in colorectal cancer patients in combination with nivolumab.

CONCLUSION

Immune checkpoint inhibitors for cancer therapy are approved for the treatment of cutaneous melanoma, NSCLC, kidney cancer, bladder cancer, head and neck cancers, Merkel cell carcinoma, gastric cancer, and Hodgkin lymphoma and could significantly improve the clinical outcome of cancer patients. However, the resistance to ICI after initial response or total lack of response is still a problem. Resistance can be mediated by MDSC, which makes these cells a promising target for combination therapy.

In various preclinical tumor models, it has been reported that MDSC targeting potentiated the effect of ICI and led to a significantly increased survival and even to full tumor regression, which was not observed upon the treatment with ICI alone. However, only six early phase clinical trials are running to date to improve ICI outcome in cancer patients by reducing MDSC-mediated immunosuppression.

Therefore, more combinatorial trials are needed to use the strategies of MDSC neutralization to further improve the outcome of cancer immunotherapy by ICI.

AUTHOR CONTRIBUTIONS

RW: writing, review, and revision of the manuscript, preparation and revision of the figure and table. XH: preparation of the figure. VN: preparation of the table. VF, CG, PA, and JU: review and revision of the manuscript. VU: writing, review, and revision of the manuscript and revision of the table and figure.

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Immune Checkpoint-Mediated Interactions Between Cancer and Immune Cells in Prostate Adenocarcinoma and Melanoma

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Prostate adenocarcinoma (PCa) and melanoma are paradigmatic examples of tumors that are either poorly or highly sensitive to therapies based on monoclonal antibodies directed against regulatory pathways in T lymphocytes [i.e., immune checkpoint blockade (ICB)]. Yet, approximately 40% of melanoma patients are resistant or acquire resistance to ICB. What characterize the microenvironment of PCa and ICB-resistant melanoma are a scanty cytotoxic T cell infiltrate and a strong immune suppression, respectively. Here, we compare the tumor microenvironment in these two subgroups of cancer patients, focusing on some among the most represented immune checkpoint molecules: cytotoxic T lymphocyte-associated antigen-4, programmed death-1, lymphocyte activation gene-3, and T cell immunoglobulin and mucin-domain containing-3. We also report on several examples of crosstalk between cancer and immune cells that are mediated by inhibitory immune checkpoints and identify promising strategies aimed at overcoming ICB resistance both in PCa and melanoma.

Keywords: prostate cancer, melanoma, immunity, immune checkpoint, immunotherapy, cytotoxic T lymphocytes

INTRODUCTION

Activated T lymphocytes require mechanisms that timely and properly shut them down to prevent excessive damage at the inflammation site. Inhibitory immune checkpoint molecules, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed death-1 (PD-1), lymphocyte activation gene-3 (LAG-3), and T cell immunoglobulin and mucin-domain containing-3 (TIM-3), are progressively upregulated on activated T cells, and, by interacting with their ligands, switch inhibitory pathways on in T cells (1). Interactions between immune checkpoint molecules on T cells and their ligands on target cells may also signal in the latters, thus generating a crosstalk between T lymphocytes and other cells (2–4). These mechanisms are crucial for self-tolerance, but also represent the Achilles' heel of cancer immunity, as ligands for inhibitory immune checkpoint molecules are expressed on neoplastic and other cells within the tumor microenvironment. In addition, a growing tumor may condition secondary lymphoid organs, thus limiting expansion of tumor-specific T cells (5).

Building on these evidences, monoclonal antibodies (mAbs) directed against regulatory pathways in T lymphocytes [i.e., immune checkpoint blockade (ICB) (6)] have been developed. Phase III clinical trials with anti-PD-1/programmed death-ligand 1 (PD-L1) or anti-CTLA-4 mAbs documented excellent efficacy, and ICB has been approved for the treatment of various solid and

hematological malignancies (7). Because several inhibitory checkpoints act simultaneously, the combination of two or more mAbs can improve ICB therapeutic outcomes (8).

Although melanomas are generally sensitive to ICB, also because of their heavy cytotoxic T lymphocyte (CTL) infiltrate, approximately 40% of melanoma patients are resistant to ICB even when two mAbs are combined (9). ICB resistance was recently reviewed [e.g., Ref. (10, 11)]. Other tumors like prostate adenocarcinoma (PCa) are intrinsically resistant to ICB (12), and either anti-PD-1/PD-L1 or anti-CTLA-4 monotherapy did not impact PCa patients' overall survival (13, 14). ICB resistance in PCa is attributed to tumor cell intrinsic mechanisms and a scanty immune infiltrate (15) dominated by macrophages. In addition, soon after ICB, immune cells upregulate other inhibitory molecules such as V-domain Ig suppressor of T cell activation [VISTA; (16)], a phenomenon not limited to PCa (17). Interestingly, orally available small molecules targeting both PD-L1 and VISTA are investigated in patients affected by advanced tumors (ClinicalTrials.gov Identifier: NCT02812875).

Therefore, melanoma and PCa epitomize two classes of ICB-resistant tumors, in which tumor cell-intrinsic mechanisms of ICB resistance associate with heavy but immunosuppressed or modest immune infiltrates, respectively. Thus, while in the former the combination of two or more ICB mAbs should succeed, in the latter strategies to improve tumor infiltration by CTLs will be needed to improve ICB sensitivity. We will analyze differences and similarities in ICB-resistant melanoma and PCa, focusing on immune checkpoint-mediated interactions between tumor and immune cells. We will also highlight strategies that might improve sensitivity to ICB.

T CELL EXHAUSTION

Prolonged antigen exposure progressively impairs T cell proliferation and effector functions (18) through epigenetic mechanisms (19). In the early dysfunctional state, which is plastic and reprogrammable, $CD8^+$ T cells express PD-1 and LAG-3 and low TIM-3 levels. Later on, T cells enter fixed exhaustion characterized by TIM-3 upregulation, and the additional expression of high CD38 and CD101 and low CD5 levels. The latter cells are no longer reprogrammable by ICB (20). Partially exhausted $CD8^+$ T cells, expressing high levels of PD-1 and CTLA-4 predicted response to anti-PD-1 in metastatic melanoma patients (21). Because also activated T cells express PD-1, this molecule cannot be used as marker of exhaustion, which should instead be functionally defined (22). Additional exhaustion markers (e.g., 2B4 and TIGIT) cannot be discussed here because of space constraint.

Also $CD4^+$ T cells undergo exhaustion (23), loosing helper function and releasing IL-10 (24). CTLA-4 on $CD4^+$ Tregs is an additional mechanism of immune suppression in cancer (25).

CYTOTOXIC T LYMPHOCYTE-ASSOCIATED ANTIGEN-4

Cytotoxic T lymphocyte-associated antigen-4 has been the first immune checkpoint investigated in clinic. Because of higher affinity for CD80 and CD86 than CD28, CTLA-4 impairs T cell co-stimulation (26). Whereas CTLA-4 is expressed on activated effector T cells (27), it is constitutively expressed on Tregs and contributes to their immunosuppressive activity. Thus, anti-CTLA-4 mAbs mainly act in secondary lymphoid organs, also causing Treg depletion through antibody-dependent cellular toxicity (28).

IFN-γ signaling activates expression of CTLA-4 in melanoma cells, and after ipilimumab (anti-CTLA-4) treatment, human melanomas upregulated IFN-γ responsive genes, including CTLA-4, which associated with durable response (29). Thus, anti-CTLA-4 mAbs can directly affect melanoma cells (30). CTLA-4 on tumor cells might also act as local mechanism of immune escape. Of relevance, mutations in the IFN responsive genes associate with resistance to ICB (31). Restifo and collaborators found that also mutations in genes indirectly correlated to the IFN response (e.g., *APLNR*), profoundly affected *in vivo* sensitivity to both adoptive T cell therapy (ACT) and anti-CTLA-4 blockade (32). It is anticipated that investigations on such comprehensive lists of genes will identify new drugs overcoming ICB resistance.

An alternative strategy to increase sensitivity to ICB is to combine them with other therapeutic strategies, such as chemotherapy, hormonal therapy, vaccines, etc. (**Tables 1** and **2**). As an example, both in mice and humans, the combination of local chemotherapy and systemic ICB increased tumor infiltration by effector T cells, and clinical response rates (NCT01323517) (33). Others have shown that targeting myeloid-derived suppressor cells (MDSCs), which are relevant immunosuppressive populations in PCa (34, 35), with tyrosine kinase inhibitors increased sensitivity to ICB in castration-resistant PCa (36). Both in orthotopic melanoma and autochthonous PCa, even the combination of anti-CTLA-4 and anti-PD-1 exerted modest antitumor effects (37), and required the addition of fresh T cells (i.e., ACT) and minute amounts of TNF-α targeted to tumor-associated vessels to favor endothelial cell activation, tumor infiltration by fully effector T cells, and tumor debulking (38, 39). Interestingly, only this triple-combined treatment guaranteed a prolonged overall survival of the mice affected by autochthonous PCa, thus suggesting the treatment generated a potent tumor-specific memory response (37). Additional strategies can be implemented to favor access of both T cells and mAbs to the tumor (40).

Overall, these data support the concept that several therapeutic strategies need to be combined to overcome ICB resistance.

PD-1/PD-L1

Programmed death-1 is upregulated on T cells upon antigen recognition, and by interacting with either PD-L1 expressed on tumor, stromal and immune cells or PD-L2 expressed on myeloid cells, impairs T cell activation (41). An exhaustion-specific enhancer regulates PD-1 expression in T cells (42), and editing exhaustion-specific enhancers might improve the therapeutic efficacy of ACT. Similarly, blocking *de novo* DNA methylation in chronically stimulated $CD8^+$ T cells allowed retention of their effector functions (43).

Programmed death-1 blockade with nivolumab, lambrolizumab, or pembrolizumab has led to relevant clinical benefits

TABLE 1 | Clinical trials of immune checkpoint blockade (ICB) combined with other strategies in prostate adenocarcinoma (PCa).

Target	ICB drug	Partner drug	NCT number	Status
CTLA-4	Ipilimumab	Evofosfamide	NCT03098160	Recruiting
CTLA-4	Ipilimumab	Sipuleucel-T	NCT01804465	Recruiting
CTLA-4	Ipilimumab	Cryoimmunotherapy ciclophosphamide	NCT02423928	Recruiting
CTLA-4	Ipilimumab	PROSTVAC V/F	NCT02506114	Recruiting
PD-1	PDR001	NIS793[a]	NCT02947165	Recruiting
PD-1	M7824[b]	ALT-803[c]; NCB024360[d]	NCT03493945	Recruiting
PD-1	Nivolumab	PROSTVAC V/F	NCT02933255	Recruiting
PD-L1	MDI4736	Olaparib cedinarib	NCT02484404	Recruiting
CTLA-4 and PD-L1	Tremelimulab and durvalumab		NCT02788773	Recruiting
CTLA-4 and PD-L1	Tremelimulab and durvalumab	polyICLC[e]	NCT02643303	Recruiting
CTLA-4 and PD-1	Ipilimumab and nivolumab		NCT03061539	Recruiting
CTLA-4 and PD-1	Ipilimumab and REGN2810	Stereotactic body radiation	NCT03477864	Not yet recruiting
LAG-3 and PD-1	LAG525 and PDR001		NCT03365791	Recruiting

Selected clinical trials combining ICB and/or other therapies in PCa.

[a]*Anti-TGF-β monoclonal antibody.*

[b]*Bifunctional fusion protein consisting of an anti-programmed death-ligand 1 (PD-L1) antibody and the extracellular domain of TGF-β receptor type 2.*

[c]*IL-15/IL-15Rα superagonist complex.*

[d]*IDO1 inhibitor.*

[e]*Toll-like receptor agonist.*

TABLE 2 | Clinical trials of immune checkpoint blockade (ICB) combined with other strategies in melanoma.

Target	ICB drug	Partner drug	NCT number	Status
CTLA-4	Ipilimumab	Dabrafenib	NCT01940809	Recruiting
CTLA-4	Ipilimumab	6MHP[a] peptide vaccine	NCT02385669	Recruiting
PD-1		INT230-6[b]	NCT03058289	Recruiting
PD-1	Pembrolizumab	iMIQUIMOD[c]	NCT03276832	Recruiting
PD-1	Pembrolizumab	Dabrafenib; trametinib	NCT02130466	Recruiting
PD-1	Pembrolizumab	Navarixin[d]	NCT03473925	Recruiting
PD-1	Nivolumab	PD-L1/IDO peptide vaccine	NCT03047928	Recruiting
PD-1	Pembrolizumab	IMP321[e]	NCT02676869	Recruiting
PD-L1	Atzolizumab	RO719857[f]	NCT03289962	Recruiting
CTLA-4 and PD-1	Ipilimumab and nivolumab		NCT03354962	Not yet recruiting
CTLA-4 and PD-1	Ipilimumab and pembrolizumab	Aspirin®	NCT03396952	Recruiting
PD-1 and TIM-3	PDR001 and MBG453		NCT02608268	Recruiting
CTLA-4 and PDL-1	Durvalumab and tremelimumab	IMCgp100[g]	NCT02535078	Recruiting

Selected clinical trials combining ICB and/or other therapies in melanoma.

[a]*High-dose IFN-α2b.*

[b]*Supermolecular complex of cisplatin, vinblastine, and an amphiphilic penetration enhancer.*

[c]*Synthetic agent with immune response modifying activity.*

[d]*CXCR2 antagonist.*

[e]*LAG-3Ig fusion protein.*

[f]*Messenger RNA based individually personalized cancer vaccine.*

[g]*Soluble gp100-specific T cell receptor with anti-CD3 single chain antibody fragment.*

in cancer patients, mainly by rejuvenating cytotoxicity and cytokine secretion capability of T cells (44). However, as mentioned above, T cells undergoing fixed exhaustion are no longer reprogrammable by ICB. An interesting study compared the epigenetic regulation of tumor- or virus-specific T cells in melanoma-bearing mice. Only melanoma-infiltrating, tumor-specific T lymphocytes (TILs) upregulated PD-1, LAG-3, and TIM-3 and showed reduced TNF-α, IFN-γ, and IL-2 secretion ability when compared with virus-specific cells. Exhausted T cells displayed more accessible chromatin in proximity to PD-1 and LAG-3 gene promoters. Treatment with anti-PD-1 mAbs had a positive impact on effector functions of exhausted T cells and on tumor growth, but induced only limited changes in gene expression and chromatin accessibility (45). Similar findings have been reported in a transplantable model of PCa, in which tumor-specific $CD8^+$ T cells showed *de novo* methylation in *Tcf7*, *Ccr7*, *Myc*, and *IFN-γ* genes, and impaired proliferation and effector functions that could not be restored by ICB. Only combination of decitabine, inhibiting the DNA methyltransferase DNMT3A, and anti-PD-1 mAbs re-established proliferation capability of exhausted T cells, thus resulting in delayed tumor growth (43).

Clinical trials evaluating the efficacy of pembrolizumab in combination with epigenetic drugs are ongoing in advanced melanoma patients (NCT03278665, NCT02816021, and NCT02437136). Also in PCa, PD-1 blockade is clinically investigated in combination with ipilimumab (NCT02601014), anti-PD-L1 (NCT03170960, NCT03061539), and other therapies

including hormone, vaccine, and cryosurgery (NCT02787005, NCT02499835, and NCT02489357).

Programmed death-1 can be found expressed also on tumor cells, and PD-1 triggering on melanoma cells increases three-dimensional growth capability with concomitant activation of the mTOR pathway (3). Interestingly, treatment with BRAF and MEK inhibitors associated with increased frequency of PD-1$^+$ tumor cells in melanoma patients, and PD-1 expression sensitized melanoma to PD-1 blockade in immunodeficient mice (46). The same authors also noticed a correlative expression of PD-1 and the stem cell marker Oct-4, thus linking PD-1 to cancer stem cells (46).

Also anti-PD-L1 mAbs may directly affect tumor cells by impacting tumor metabolism, reducing extracellular acidification, phosphorylation of mTOR, and glycolysis (4). *mTORC1* expression has been associated with PD-L1 expression in melanoma cells, and PD-L1^{low} cells showed decreased levels of *mTORC1*, and an altered autophagy pathway. Furthermore, treatment of immunodeficient mice with anti-PD-L1 mAbs delayed melanoma growth, reduced metastases, and prolonged animal survival (2). PD-L1 has also been found overexpressed in melanoma tumor-initiating cells, and the lack of PD-L1 significantly reduced the frequency of these cells in melanoma-bearing mice (47). Thus, interfering with the PD-1/PD-L1 axis may impact both tumor and immune cells.

LYMPHOCYTE ACTIVATION GENE-3

Lymphocyte activation gene-3 is closely related to CD4, is expressed on dysfunctional T cells (48), and TILs in melanoma patients express LAG-3 (49). Because LAG-3 binding to MHC class II molecules activates myeloid cells (50), and MHC class II can be expressed by melanoma cells (51), engagement of LAG-3 with MHC class II might provide a survival signal to tumor cells. LAG-3 also binds LSECtin and Galectin-3 (Gal-3) (49, 52) and associates with the CD3/TCR complex, thus impairs TCR signaling (18, 52). Conversely, LAG-3 binding on Tregs increases their immunosuppressive activity (53).

Lymphocyte activation gene-3 may synergize with other immune checkpoints, and the combination of anti-LAG-3 and anti-PD-1 resulted in more potent inhibition of murine tumor growth than single treatments (54). Anti-LAG-3 mAbs or LAG-3 fusion proteins are being tested in melanoma patients resistant to anti-PD-1/PD-L1 ICB as single agent (NCT01968109), or in combination to anti-PD-1 (NCT02676869).

Drake and collaborators originally reported that in PCa, tumor-specific CD4$^+$ and CD8$^+$ T cells rapidly upregulate LAG-3 upon *in vivo* antigen encounter. Treatment with anti-LAG-3 mAbs enhanced the number and effector function of tumor-specific CD8$^+$ T cells in TRAMP mice, and delayed tumor growth (55). Also Tregs in human PCa lesions upregulate both CTLA-4 and LAG-3 (56). The latter finding has been challenged by recent data showing low expression of LAG-3 in Tregs infiltrating PCa lesions (57). Further investigation is needed to better define the role of LAG-3 in T cell exhaustion and/or Treg function in PCa. One clinical trial is ongoing that investigates efficacy of anti-LAG-3 mAbs in combination with anti-PD-1 in castration-resistant PCa (NCT03365791).

T CELL IMMUNOGLOBULIN AND MUCIN-DOMAIN CONTAINING-3

Programmed death-1 expression in TILs is often associated with TIM-3, and its transient or persistent expression relates to short or chronic antigen stimulations, respectively (58). Indeed, PD-1$^+$TIM-3$^+$ T cells are functionally more exhausted than PD-1$^+$TIM-3$^{-/low}$ T cells (59), and TIM-3 can be considered a marker of terminally differentiated T cells.

T cell immunoglobulin and mucin-domain containing-3 is expressed on dysfunctional, tumor-specific CD8$^+$ T cells in melanoma (60) and PCa patients (61), and in ipilimumab-treated melanoma patients, increased expression and frequency of TIM-3 and PD-1 on both peripheral NK and T cells associated with poor prognosis (62). Correlative data on TIM-3 in PCa patients are conflicting. Whereas one report showed that high TIM-3 expression on PCa cells predicted short recurrence-free and progression-free survival in chemotherapy and radiotherapy naïve PCa patients (63), others found that negative TIM-3 expression was an independent prognostic factor of poor prognosis in advanced metastatic PCa (64). Outcome differences might be brought back to the different subpopulations of PCa patients analyzed in the two studies. The latter also showed that silencing TIM-3 in PCa cell lines reduced tumor cell proliferation and invasion *in vitro* (63), thus, suggesting that TIM-3 has a functional role in PCa cells. Interestingly, the combined targeting of TIM-3 and PD-1 pathways is more effective in controlling tumor growth than targeting either pathway alone (59).

Mechanistically, the interaction between TIM-3 on T cells and one of its ligands [i.e., Galectin-9 (Gal-9)] triggers cell death in effector T cells (65). Ceacam-1, an additional TIM-3 ligand, is co-expressed on exhausted T cells, can bind TIM-3 both in *cis* and *trans*, and both interactions drive the inhibitory function of TIM-3 (66). TIM-3 also enhances FoxP3$^+$ Tregs inhibitory functions (59), and is expressed and upregulated upon activation on human NK cells. In contrast to effector T cells, Gal-9-mediated TIM-3 triggering in NK cells induces IFN-γ production (67). Interestingly, it has been shown that MHC class I downregulation or deficiency in mouse tumors induces upregulation of PD-1 and TIM-3 on NK cells and their exhaustion. PD-1$^+$TIM-3$^+$ NK cells were also found in human melanoma samples, and correlated with low HLA expression (68). Because *in vitro*, TIM-3 blockade reversed NK cell exhaustion (69), it will be interesting to investigate the *in vivo* effects of mAbs against both PD-1 and TIM-3 on NK cells.

While TIM-3 is higher and more precociously upregulated on tumor-associated dendritic cells (DCs) than on CD8$^+$ T cells, its role in innate immunity is controversial (70). By interacting with phosphatidylserine, TIM-3 favors DC uptake of apoptotic cells and cross-presentation (71). Conversely, interaction of the alarmin high mobility group protein B1 with Tim-3 on DCs limits their release of pro-inflammatory cytokines, thus blunting type-1 immunity (72). TIM-3 is also expressed on tumor-associated macrophages (72), and TIM-3 negatively modulates the production of pro-inflammatory cytokines in human CD14$^+$ monocytes (73). Finally, TIM-3 can suppress the antitumor immunity by promoting induction of MDSCs (74).

Clinical trials are investigating safety and tolerability of anti-TIM-3 mAbs given either alone (NCT03489343) or in combination with anti-PD-1 (NCT02817633 and NCT02608268) or anti-PD-L1 (NCT03099109) in cancer patients.

GALECTINS

Apart from being ligands for LAG-3 and TIM-3, galectins also exert relevant pro-tumor functions (75). Increased expression of Gal-3 in melanoma lesions correlates with tumor progression (76), and Gal-3 activates NFAT1 (77), which also regulates IL-8 and MMP3 expression in melanoma cells, thus promoting a malignant phenotype (78). Gal-3 released by melanoma cells can also capture IFN-γ, thus reducing its antitumor activity (79). At odds, others reported that tumor cell expression of Gal-3 or myeloid cell expression of Gal-9 in melanoma lesions associated with a longer survival (80). The latter findings are counterintuitive and deserve further investigation.

Inhibiting Gal-3 together with anticancer vaccination restores the effector function of melanoma TILs (81). Therefore, Gal-3 not only contributes to melanoma tumor growth and metastasis but also dampens the antitumor immune response. Gal-3 inhibition is currently investigated in combination with ICB and vaccine in melanoma (NCT02575404, NCT02117362, and NCT01723813).

Galectin-3 is also expressed in PCa lesions, exerts direct pro-tumor and pro-metastatic functions, and correlates with biochemical recurrence (82). Indeed, administration of a Gal-3 inhibitor suppressed PCa lung metastasis (83).

Galectin-3 is a marker of cancer stem cells (84) and maintains stemness of carcinoma progenitor cells (85). In the TRAMP model, we found that PCa stem-like cells endowed with immunosuppressive activities express Gal-3 (86). We have also evidence that Gal-3 favors growth and metastasis of tumors generated by PCa stem-like cells (Caputo et al., manuscript in preparation). It will be interesting to investigate if Gal-3 also contributes to their immunosuppressive activity.

CONCLUDING REMARKS

Inhibitory immune checkpoint triggering in TILs cripples cancer immune surveillance. As consequence of local inflammation, immune checkpoints are also upregulated on cancer cells, supporting tumor growth and aggressiveness. Thus, the effect of ICB goes beyond rescuing of exhausted/dysfunctional TILs and may directly impact tumor cells.

To overcome TIL exhaustion, several promising combined approaches are envisioned among many others: coupling two or more mAbs against immune checkpoints; increase tumor immunogenicity by exploiting conventional chemotherapy and targeted anticancer agents (87); transiently modify the tumor vasculature to favor T cell infiltration (88–90); combine additional immunotherapeutic approaches such as vaccines and ACT (37); abolish additional mechanisms of local immune suppression (91). Several high throughput analyses (e.g., methylomics and metabolomics) and microbiota sequencing will likely define novel areas of therapeutic intervention in the field of ICB. Finally, it will be essential to focus on adverse events that increase along with therapeutic efficacy (92).

AUTHOR CONTRIBUTIONS

AE, SC, and MB wrote and reviewed the manuscript.

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REVIEW
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Biomarkers for Clinical Benefit of Immune Checkpoint Inhibitor Treatment—A Review From the Melanoma Perspective and Beyond

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Background: Immune checkpoint inhibition (ICI) with anti-CTLA-4 and/or anti-PD-1 antibodies is standard treatment for metastatic melanoma. Anti-PD-1 (pembrolizumab, nivolumab) and anti-PD-L1 antibodies (atezolizumab, durvalumab, and avelumab) have been approved for treatment of several other advanced malignancies, including non-small-cell lung cancer (NSCLC); renal cell, and urothelial carcinoma; head and neck cancer; gastric, hepatocellular, and Merkel-cell carcinoma; and classical Hodgkin lymphoma. In some of these malignancies approval was based on the detection of biomarkers such as PD-L1 expression or high microsatellite instability.

Methods: We review the current status of prognostic and predictive biomarkers used in ICI for melanoma and other malignancies. We include clinical, tissue, blood, and stool biomarkers, as well as imaging biomarkers.

Results: Several biomarkers have been studied in ICI for metastatic melanoma. In clinical practice, pre-treatment tumor burden measured by means of imaging and serum lactate dehydrogenase level is already being used to estimate the likelihood of effective ICI treatment. In peripheral blood, the number of different immune cell types, such as lymphocytes, neutrophils, and eosinophils, as well as different soluble factors, have been correlated with clinical outcome. For intra-tumoral biomarkers, expression of the PD-1 ligand PD-L1 has been found to be of some predictive value for anti-PD-1-directed therapy for NSCLC and melanoma. A high mutational load, particularly when accompanied by neoantigens, seems to facilitate immune response and correlates with patient survival for all entities treated by use of ICI. Tumor microenvironment also seems to be of major importance. Interestingly, even the gut microbiome has been found to correlate with response to ICI, most likely through immuno-stimulatory effects of distinct bacteria. New imaging biomarkers, e.g., for PET, and magnetic resonance imaging are also being investigated, and results suggest they will make early prediction of patient response possible.

Conclusion: Several promising results are available regarding possible biomarkers for response to ICI, which need to be validated in large clinical trials. A better understanding of how ICI works will enable the development of biomarkers that can predict the response of individual patients.

Keywords: biomarker, checkpoint inhibition, PD-1 antibody, PD-L1 antibody, CTLA-4 antibody, cancer, melanoma

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INTRODUCTION

In the last decade, treatment of metastatic melanoma and other malignancies has improved significantly. In addition to targeted treatment options, immunotherapy with immune checkpoint inhibitors (ICI) has contributed greatly to this development.

The anti-CTLA-4 antibody (CTLA4ab) ipilimumab was first approved by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic melanoma in 2011 (1), followed by the anti-PD-1 antibodies (PD1ab) pembrolizumab and nivolumab in 2014 (2–4). Combined ICI with CTLA4ab and PD1ab for melanoma was introduced with enormous success, but was also accompanied by significant immune-related adverse events (irAEs) (5, 6). PD1ab treatment is currently approved for treatment of several other advanced malignancies including non-small-cell lung cancer (NSCLC), urothelial cancer, renal cell carcinoma (RCC), squamous cell carcinoma of head and neck (SCCHN), gastric carcinoma, hepatocellular carcinoma, and classical Hodgkin lymphoma (7–12). The anti-PD-L1 antibodies (PD-L1ab) atezolizumab (urothelial carcinoma and NSCLC), durvalumab (urothelial carcinoma), and avelumab [Merkel-cell carcinoma (MCC) and urothelial carcinoma] have also recently been approved by the FDA (13–18). In 2017 the FDA also announced a biomarker-based approval for pembrolizumab for patients with unresectable or metastatic solid tumors, and for colorectal carcinoma (CRC) with high microsatellite instability or mismatch repair deficiency (dMMR) (19).

Despite this enormous success, ICI does not achieve long-lasting responses for all patients. Response varies between different entities, and between different patients. For melanoma, PD1ab monotherapy can achieve a response of 26–32% (2, 4) and the combination of PD1ab and CTLA4ab achieves a response as high as 60% (5). Some subsets of patients achieve durable responses with PD1ab monotherapy and do not require combined ICI, and could therefore be protected from the higher risk of irAEs.

There remains the medical need to find reliable biomarkers that could help to identify both, the patients who would benefit from ICI and the primary resistant patients. Biomarkers are also needed to help decide the type of first-line treatment, e.g., whether BRAF-mutant melanoma should be treated by use of targeted or immunotherapy, therapy sequencing, and/or by a combination of treatments. Here, we review biomarkers in the field of ICI therapy for metastatic melanoma and other malignancies. We have not performed a review of pre-analytic, analytic, and clinical validation techniques for biomarkers because these have been reviewed elsewhere (20, 21).

CLINICAL BIOMARKERS

Tumor Burden

Tumor burden and metastatic site, e.g., liver or brain metastases, significantly affect patient prognosis, particularly in terms of overall survival (OS), as described in the TNM classification (22). Because the prognostic effect of tumor burden and metastatic site is well known, they are used to stratify clinical trials and are the object of sub-group analyses. Several authors have found an association between metastatic site and incidence of response, progression-free survival (PFS), and OS for PD1ab treatment of melanoma (23–26) (**Table 1**). Response to PD1ab therapy is better for lung and skin metastases than for metastases in other organs, particularly those in the liver. Response for melanoma brain metastases is lower compared with response for extracranial sites, particularly for PD-1ab monotherapy (27). This could be because T cell infiltrate in cerebral metastases is less dense compared with other anatomic sites (28). For combined ICI with ipilimumab and nivolumab, response for brain metastases that were asymptomatic was similar to response for extracranial sites (27, 29). Peripheral blood biomarkers which correlate with tumor burden, such as serum lactate dehydrogenase (LDH), circulating tumor cells (CTCs), and circulating tumor DNA (ctDNA), are of significance for biomarker investigations, as described below.

Clinical Condition (Performance Status)

A good clinical condition expressed by the ECOG (Eastern Cooperative Oncology Group) performance status (ECOG PS 0) is associated with prolonged OS for patients receiving PD1ab treatment, as well as for patients receiving other melanoma treatments such as BRAF inhibitors (30, 70). For other entities such as NSCLC (71), association between performance status and OS is also well known. Because of its prognostic character, performance status is frequently used as a biomarker in enrichment designs of clinical trials, i.e., only biomarker-positive patients are included, in this case only patients with good performance status. Because patients with poorer performance are not included and are therefore unavailable for further analysis, enrichment design prevents information from being gathered on the prognostic versus predictive value of performance status (20). Significance of other patient characteristics for ICI response, such as sex and age, has only been found in single studies with PD1ab for melanoma (24).

Immune-Related Adverse Events

Immune-Related Adverse Events are side effects caused by the activated immune system and are, therefore, a possible sign of successful immune checkpoint blockage. Several retrospective analyses have reported an association between CTLA4ab-induced irAE and a more favorable clinical outcome (**Table 1**). From a cohort of 86 patients, occurrence of irAE $\geq$ grade 2 CTC-AE was associated with improved response, PFS, and OS (31). In contrast, other studies focusing on irAE of any grade could not find this association for large ipilimumab-treated cohorts (37, 38). The development of autoimmune hypophysitis was found to be associated with prolonged OS (33). It has been observed in retrospective analyses of several groups receiving PD1ab therapy that incidence of irAEs is associated with a more favorable outcome. In a large cohort of 576 PD1ab-treated patients, response but not PFS was associated with irAE manifestation of any grade (32). For certain adverse events, vitiligo was found to be associated with response (34) and, similar to exanthema, with longer OS (35). Arthralgia of any grade was associated with response and significantly longer PFS (36). Here, median onset of arthralgia was 100 days after start of treatment and was caused by either arthritis or reactivated osteoarthritis in pre-damaged joints. It is worth noting that all these analyses were performed retrospectively and that there is a risk of guarantee-time bias, i.e., patients with early progression are less likely to develop irAEs because of

TABLE 1 | Clinical, blood, and stool biomarkers for clinical outcome under checkpoint blockage for metastatic melanoma patients.

Biomarker	Number of patients	Treatment	Results	Reference
Clinical Biomarkers				
Metastatic sites	*n* = 177–593	Pembrolizumab, nivolumab	Liver metastases associated with lower response rate and shorter PFS	(26) (24) (25)
	n = 177–257	Pembrolizumab	Soft-tissue and/or lung metastases associated with longer OS	(23)
ECOG performance status	*n* = 50	Nivolumab	ECOG PS ≥ 1 associated with shorter OS	(30)
Immune-related adverse events (irAEs)	*n* = 86	Ipilimumab, nivolumab	irAEs associated with response	(31) (32)
	n = 154	Ipilimumab	Hypophysitis associated with longer OS	(33)
	n = 65–118	Pembrolizumab, nivolumab	Vitiligo associated with response and longer OS	(34) (35)
	n = 196	Pembrolizumab, nivolumab	Arthralgia associated with response and longer OS	(36)
	n = 298–833	Ipilimumab	No association of irAEs with response or survival	(37) (38)
Blood Biomarkers				
LDH	*n* = 50–257	Ipilimumab, nivolumab, pembrolizumab, ipilimumab + nivolumab, or pembrolizumab	Elevated LDH associated with shorter OS	(39) (40) (41) (31) (30) (42) (23) (43) (44)
CRP	*n* = 95–196	Ipilimumab	CRP within normal limits associated with longer OS	(43) (45)
	n = 50	Nivolumab	CRP not significant for OS in multivariable analysis	(30)
Neutrophils	*n* = 50–720	Ipilimumab	Elevated neutrophils associated with shorter OS	(40) (30) (46)
Lymphocytes	*n* = 50–257	Ipilimumab, pembrolizumab, nivolumab	Absolute lymphocyte counts (LC) ≥1,000/ml, high relative LC or increasing LC with treatment associated with longer OS	(45) (23) (30) (41, 47)
NLR	*n* = 58–720	Ipilimumab, nivolumab	Baseline NLR ≥ 3–4 associated with shorter OS	(46) (48) (49)
	n = 90	Nivolumab	Baseline NLR ≥ 2.2 associated with non-response	(50)
Eosinophils	*n* = 59	Ipilimumab	Increase in eosinophil count (week 3) associated with response	(51)
	n = 177–209	Ipilimumab, pembrolizumab	High eosinophils associated with longer OS	(41) (23)
Monocytes, mo-MDSCs	*n* = 20–209	Ipilimumab, nivolumab, pembrolizumab	Elevated mo-MDSCs/monocytes associated with non-response and shorter PFS/OS	(51) (41) (52) (49) (53)
T cell subsets	*n* = 95–209	Ipilimumab	High Treg count associated with longer OS	(45) (41)
	n = 67–82	Ipilimumab	Relative numbers of $CD4^+$ and $CD8^+$ T cells correlated with response and longer OS	(47) (54)
	n = 37–190	Ipilimumab	Higher PD-L1 expression on peripheral T cells correlated with non-response, shorter PFS and OS	(55)
	n = 67	Nivolumab, pembrolizumab	NK cell subsets associated with response to PD1ab	(54)

(*Continued*)

TABLE 1 | Continued

Biomarker	Number of patients	Treatment	Results	Reference
Human leukocyte antigen class I genotype (HLA-I)	*n* = 1535 (mainly melanoma, NSCLC)	Ipilimumab ± nivolumab, nivolumab, pembrolizumab	Maximum heterozygosity at HLA-I loci correlated with longer OS, melanoma only: HLA-B44 supertype associated with longer OS, HLA-B62, or somatic loss of heterozygosity at HLA-I associated with shorter OS	(56)
T cell receptor (TCR) repertoire	*n* = 12	Ipilimumab	TCR repertoire richness prior to therapy correlated with clinical benefit	(57)
sCTLA4	*n* = 14	Ipilimumab	sCTLA4 higher in responders, associated with longer OS	(58)
sPD-L1	*n* = 251	Ipilimumab (±bevazizumab or sargramostim), pembrolizumab	High pretreatment levels associated with disease progression	(59)
sULBP-1, sULBP-2	*n* = 194 (ICI) *n* = 65 (other treatments)	Ipilimumab ± nivolumab or pembrolizumab, nivolumab	sULBP-1 and 2 associated with disease control and longer OS in ICI, but not in other treatments	(44)
sCD25	*n* = 27	Ipilimumab	High baseline sCD25 associated with shorter OS	(60)
CXCL11	*n* = 48–247	Ipilimumab or gp100 peptide vaccine	Pre-treatment elevated serum CXCL11 level associated with shorter OS	(61)
Cytokine levels	*n* = 35	Nivolumab	Serum IFN-γ, IL-6, and IL-10 levels higher for responders	(62)
Protein signature (multimarker assay)	*n* = 119–170	Ipilimumab ± nivolumab, nivolumab, nivolumab ± vaccine, pembrolizumab	Baseline protein signature of 209 proteins discovered by use of MALDI–TOF and computational algorithms correlated with OS	(63)
CTC count	*n* = 7 (ICI) *n* = 42 (other treatments)	Ipilimumab, chemotherapy, targeted therapy	CTC count correlated with OS	
CTC (droplet digital PCR)	*n* = 49	Ipilimumab, nivolumab, pembrolizumab	Decrease in CTC within the first 7 weeks of ICI was linked to longer PFS and OS	(64)
Plasma ct-DNA: BRAF V600E/K, NRAS Q61K/R	*n* = 19 (ICI) *n* = 29 (targeted therapy)	Ipilimumab, nivolumab, pembrolizumab, targeted therapy	Pre-treatment ctDNA <10 copies/ml associated with response and longer PFS, decrease in ctDNA levels in responders of targeted therapy but not immunotherapy	(65)
Stool Biomarkers				
Gut microbiome	*n* = 26	Ipilimumab	Faecalibacterium and other Firmucutes associated with improved response, higher representation of Bacteroidetes related to poor response	(66)
	n = 89	PD1ab, not specified	Enrichment of Ruminococcaceae and Clostridiales found in responders, Bacteriodales in non-responders; Bacteroidales associated with shorter PFS	(67)
	n = 39	Ipilimumab + nivolumab, pembrolizumab	Enrichment of *Bacteroides caccae* in responders	(68)
	n = 39	PD1ab, not specified	Relative abundance of *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* in responders	(69)

CRP, C-reactive protein; CTLA4ab, anti-CTLA-4 antibody; LDH, lactate dehydrogenase; MALDI–TOF, matrix-assisted laser desorption/ionization–time-of-flight; mo-MDSCs, monocytic myeloid-derived suppressor cells; NLR, neutrophil-to-lymphocyte ratio; PD1ab, anti-PD-1 antibody; PFS, progression-free survival; OS, overall survival; TNF-α, tumor necrosis factor-alpha; CTC, circulating tumor cells; ctDNA, circulating tumor DNA; sCTLA4, soluble CTLA4; sPD-L1, soluble PD-L1; sCD25, soluble CD25.

a shorter treatment period. This bias can be controlled to some degree by use of landmark analysis, which was used in the above-mentioned reports.

BLOOD BIOMARKERS

Blood-based biomarkers have several preferential characteristics and are, therefore, the focus of biomarker research. First, they are easily accessible, which enables analysis at several time-points. Second, they might be independent from intra and inter-tumor heterogeneity. Third, they might reflect multiple sites of interest, e.g., tumor cells, tumor microenvironment, and the patient's immune system.

Serum Biomarkers Correlating With Tumor Load

Lactate dehydrogenase (LDH) is a house-keeping enzyme which is released by rapidly growing tumors. Serum LDH therefore correlates with tumor burden. For melanoma, the prognostic significance of this biomarker is expressed by its inclusion in the American Joint Committee on Cancer classification (72). Serum LDH levels correlate with patients' OS in various treatment regimens (73), including ICI (**Table 1**) (23, 30, 31, 39–45). Nearly all studies have found no correlation between baseline LDH and response. Only a dynamic change in LDH from baseline to week 12 was found to be associated with response (31, 45). Hence, despite the prognostic value of LDH, patients with elevated serum

LDH can respond to ICI; LDH elevation does not, therefore, lead to an exclusion of patients from ICI treatment. Patients with very high levels of more than twice the upper limit of normal, however, did not benefit from either CTLA-4ab or PD1ab monotherapy (23, 31, 39). Importantly, even though targeted treatments by use of BRAF/MEK inhibitors are known to lead to fast tumor responses in highly advanced patients with BRAF-V600-mutated melanoma, patients with normal LDH still achieve the better clinical outcome from treatment (74). Hence, the best treatment sequencing of targeted and immunotherapy for patients with BRAF-mutated melanoma and normal LDH is not clear.

Another serum biomarker which correlates with tumor burden is the acute-phase protein C-reactive protein (CRP). It is a prognostic marker for melanoma, and elevated concentrations are linked to worse PFS and OS (75). CRP also significantly affects prognosis for other malignancies such as renal, gastrointestinal, lung, pancreas, hepatocellular, and bladder cancer (76). For ICI, only retrospective analyses are available. A normal CRP level at the start of treatment was associated with longer OS for ipilimumab-treated melanoma patients (43). Decreasing CRP levels from baseline to week 12 of CTLA4ab therapy were, as for LDH, associated with longer PFS and OS (45). In multivariate analysis of a small study of PD1ab-treated melanoma patients, baseline CRP levels were no independent biomarkers for OS (30). For PD1ab treatment of NSCLC, in contrast, elevated baseline CRP levels were shown to be associated with shorter PFS (77).

Differential Blood Count Biomarkers

Immune checkpoint inhibition works *via* activation of T lymphocytes. Hence, the number of lymphocytes and other immune cells circulating might affect its efficacy. Several retrospective analyses have focused on this question. The role of neutrophils, which can display heterogeneous phenotypes and diverse functionality, is also important (78). Increased levels of neutrophils have been found in the peripheral blood of cancer patients; they might possibly be induced by cytokines such as granulocyte-colony stimulating factor (G-CSF), although no definite cause for neutrophilia in malignancies has been clearly shown (78). Pretreatment elevation of neutrophil count has been found to correlate with worse OS in ICI treatment of melanoma (**Table 1**). Increasing lymphocyte counts, in contrast, correlated with prolonged OS in ICI-treated patients (**Table 1**). The neutrophil-to-lymphocyte ratio (NLR) has been more frequently reported to be of prognostic, and potentially predictive, value by several authors using various cutoffs (NLR > 2–5). For ipilimumab-treated melanoma patients, high baseline NLR was associated with shorter PFS and OS (46, 48). For PD1ab treatment, high baseline NLR was linked to non-response (50) and to worse OS for melanoma (49, 79) and for several other types of cancer being investigated in phase I studies with PD1ab/PD-L1ab treatment (79). For example, NLR was associated with lower incidence of response, poor PFS, and OS for NSCLC (71, 80) and for RCC (81). It is worth noting that an association was also found between NLR and prognosis for melanoma patients treated by use of BRAF inhibitors (82). Overall, NLR certainly has prognostic value but is probably not treatment specific and no predictive ability has been observed so far. It has, however, also been shown that eosinophils correlate with clinical outcome in ICI treatment of melanoma. A high pre-ICI absolute or relative eosinophil count was associated with prolonged OS (23, 41). Dynamically, for melanoma patients treated by use of ipilimumab, eosinophil counts that increased with treatment correlated with response to ipilimumab (51).

Myeloid-derived suppressor cells (MDSCs) are important in melanoma and other malignancies. MDSCs have immunosuppressive potential, particularly by inhibiting activated T cells, and can be divided into two subgroups: granulocytic and monocytic myeloid-derived suppressor cell (mo-MDSC) (78). The number of mo-MDSC in the peripheral blood in particular has been correlated with prognosis for melanoma patients (51). In CTLA4ab treatment, the number of mo-MDSC has been found to negatively affect incidence of response and survival (41, 47, 51). In addition, mo-MDSC was negatively correlated with OS for CTLA4ab-pretreated melanoma patients receiving PD1ab (52) (**Table 1**). The development of cytometry by time-of-flight (CyTOF) has enabled in-depth analysis of peripheral blood immune cells. CyTOF can measure up to 50 proteins per cell. Use of CyTOF for ICI patients has shown that high incidence of classical monocytes ($CD14^+CD16^-HLA\text{-}DR^{hi}$) are associated with response and improved PFS in PD1ab therapy for melanoma (53).

It is worth mentioning that all these potential markers have been found by retrospective exploratory analyses. They potentially have prognostic features but their predictive potential remains unclear. Furthermore, the above-mentioned publications used several different cutoffs. Prospective studies are needed to investigate a possible predictive value of these biomarkers.

Biomarkers on Peripheral T Cells

T cells are the effector cells of ICI treatment. Therefore, in addition to the pure cell number of several subsets of T cells in the peripheral blood, a more detailed analysis might be beneficial. Retrospective examination of peripheral blood T cell subsets in ipilimumab-treated melanoma patients revealed that higher pre-treatment $CD4^+/CD25^+/FoxP3^+$ Tregs was associated with favorable survival (41, 45) (**Table 1**). Tregs express high levels of CTLA-4 and might, therefore, be one of the main targets of ipilimumab. It was shown that more melanoma-reactive $CD8^+$ cytotoxic T cells in the peripheral blood were detected in patients after treatment than before treatment (83). Preexisting immune responses were only infrequently boosted.

Most studies have focused on PD-L1 expression on tumor cells and macrophages in the tumor microenvironment, whereas PD-L1-expression on peripheral T cells has been studied to a lesser extent. High PD-L1 expression on peripheral T cells ($CD4^+$ and $CD8^+$) has been shown to be associated with worse PFS and OS for CTLA4ab treatment of melanoma (55). For an NSCLC cohort treated mainly by chemotherapy, high PD-1/PD-L1/PD-L2 expression on peripheral blood T cells was associated with shorter OS (84). PD-L1 expression on peripheral T cells might, therefore, be a mechanism for tumor immune escape. Expression of co-stimulatory molecules on peripheral T cells was also studied. Detectable levels of $CD137^+CD8^+$ cytotoxic T cells in the peripheral blood were found in patients with relapse-free status after adjuvant combined ICI, but this was not investigated in the therapeutic setting (55). CyTOF analysis revealed that

high pre-treatment incidence of memory T cells was a potential marker for response to CTLA4ab, whereas higher incidence of distinct NK cell subsets was found to be associated with response to PD1ab treatment for melanoma (54).

Tumor antigen presentation by human leukocyte antigen class I (HLA-I) molecules is a prerequisite for cancer cell attack by cytotoxic T cells. Maximum heterozygosity at HLA-I loci (A, B, C) opposed to homozygosity for at least one HLA locus was shown to be associated with longer OS after ICI for mainly NSCLC and melanoma (56). Furthermore, HLA-B44 supertype was linked to prolonged OS, whereas the HLA-B62 supertype or somatic loss of heterozygosity at HLA-I were associated with worse OS for melanoma (56). Although assessed in test and validation cohorts, these biomarkers have also not been prospectively tested for their potential predictive versus prognostic value. Nevertheless, this investigation indicates that diversity in antigen presentation might improve tumor defense. Tumor cell antigens presented on MHC molecules are recognized by T cells *via* the T cell receptor (TCR). The TCR is, therefore, of great interest in ICI. TCR diversity and clonality can be investigated by use of sequencing methods. Most investigations focus on TCR sequencing in tumor tissue specimens (see "Tissue Biomarkers"). TCR sequencing data in the peripheral blood are limited. In ipilimumab-treated melanoma patients, patients with a positive clinical outcome had a higher degree of TCR repertoire richness prior to therapy (57). In patients with urothelial carcinoma, TCR sequencing in peripheral blood was done before and after atezolizumab administration. Here, a pretreatment TCR clonality below the median was associated with improved PFS and OS (85). Furthermore, a long-lasting clinical benefit was found in patients with a more substantial expansion of tumor-associated TCR clones after three weeks (85). T cells carrying the γδ-TCR-subtype—as opposed to the more common αβ-subtype—play a distinct role in anti-tumor immunology. A study found that higher incidence of $V\delta2^+$ cells (versus $V\delta1^+$ cells) was linked to longer OS in melanoma patients, and suggested that $V\delta2^+$ cells potentially have tumor-killing capability (86). However, this has not yet been investigated for ICI.

In summary, T cells as the effector cells of ICI are the focus of biomarker research for melanoma and other malignancies. Some approaches are promising, but no biomarker has yet been evaluated in a prospective clinical setting. Their predictive ability therefore remains to be determined.

Soluble Serum Biomarkers

Soluble serum biomarkers that might correlate with clinical benefit of ICI treatment include immune regulatory molecules such as cytokines or soluble checkpoint receptors and binding partners. Biomarker potential in ICI treatment of melanoma (**Table 1**) and other malignancies has been found for several soluble serum factors.

Soluble CTLA-4 (sCTLA4), which is mainly secreted by regulatory T cells (Tregs), has inhibitory effects on T cell immune responses (87). An association has been found between higher sCTLA4 levels and both response and prolonged OS for a small cohort of ipilimumab-treated melanoma patients; this was not found for patients who did not receive ipilimumab (58). In view of its inhibitory function on T cells, neutralization by CTLA4ab therapy might be responsible for this finding. Higher levels of soluble PD-L1 (sPD-L1) can be found in tumor patients compared with healthy individuals (88, 89). Its physiological role has not yet been identified (89). It holds some prognostic value because high pre-treatment concentrations are associated with shorter OS for NSCLC (88), and for hepatocellular carcinoma (90), and it is linked to disease progression in ICI for melanoma (59). sPD-L1 is, however, likely to be only prognostic and not predictive for melanoma, because assessments pre- and during early ICI did not reveal significant associations with response or OS (59). In addition to sPD-L1, a soluble form of PD-1 (sPD-1) also exists (89) which is currently being investigated in a clinical trial (NCT03197636).

Soluble ligands of the transmembrane receptor NKG2D (sULBP-1, sULBP-2), which affect induction or reactivation of T cell responses, were associated with OS for ICI-treated but not for BRAF inhibitor-treated melanoma patients (44). They are interesting biomarkers with treatment-specific potential. Further data are, however, needed to confirm the significance of these markers. In addition, soluble CD25 (sCD25), the alpha unit of the IL-2 receptor, was found to be a biomarker in ipilimumab therapy. The interleukin (IL)-2/IL-2 receptor pathway is essential for the antitumor activity of CTLA4ab (91). High pre-treatment serum levels of sCD25 were shown to be associated with shorter OS for CTLA4ab-treated patients (60). A possible explanation for this finding could be direct binding of sCD25 to IL-2, which would amplify Tregs and inhibit tumor immune response.

It has been shown that other serum factors including vascular epithelial growth factor and chemokines such as C-X-C chemokine motif ligand (CXCL)8 are of prognostic significance for PFS and OS of melanoma of different stages, regardless of treatment (92). For CTLA4ab therapy, elevated pre-treatment levels of CXCL11 were associated with poor OS (61). The gene expression of CXCL11 is induced by interferon-(IFN)-γ, and CXCL11 binds to its chemokine receptor CXCR3, which is mainly expressed on activated T cells. CXCR3 is highly important for the migration of cytotoxic T cells, and its tissue expression correlates with poorer prognosis for several malignancies (93). Elevated CXCL11 in blood has been linked to poorer outcome for ipilimumab-treated melanoma (61). For PD1ab treatment, serum IFN-γ, IL-6 and IL-10 levels were significantly higher for responders than for non-responders (62).

In contrast to single-biomarker searches, serum-based multimarker assays are of current and future interest. One group developed a test based on 119 patients with pre-PD1ab therapy for metastatic melanoma using matrix-assisted laser desorption/ionization-time of flight (MALDI–TOF) mass spectrometry, which was validated in four independent cohorts (63). Computational algorithms were used for data analysis, resulting in a protein signature of 209 proteins that appears to differentiate patients with three-year OS of over 50% from patients with three-year OS of less than 20%. Further analysis revealed that acute phase proteins, complement, and wound healing pathways were associated with poor outcome (63). Because this test has also not been prospectively evaluated yet, distinction of prognostic versus predictive ability is warranted.

Liquid Biopsy

Liquid biopsy, including CTCs, ctDNA, and circulating tumor RNA (ctRNA) has only been studied in small patient cohorts treated by use of ICI (mainly CTLA4ab) for melanoma. An association between treatment response and a decrease in CTCs and ctDNA has been found for targeted therapy, but not for ICI (94). Methodological improvements offer new opportunities for CTC detection, as has been very recently described for microfluidic enrichment of melanoma CTCs combined with RNA-based droplet digital PCR quantitation (64). That study found that a decrease in CTCs within the first seven weeks of ICI was linked to prolonged PFS and OS in CTLA4ab or PD1ab-treated melanoma patients (64). Another approach to CTCs is characterization of subsets of CTCs that express specific markers. The heterogeneity of melanoma CTCs and the significance of CTC subsets (e.g., receptor activator of NF-κβ (RANK) expressing CTCs) as biomarkers has been found to affect targeted treatment; this was not, however, observed for ICI (65). For NSCLC, high expression of carcinoembryonic antigen and telomerase reverse transcriptase was linked to non-response to nivolumab (95). For urothelial carcinoma, CTCs with high PD-L1 expression were associated with worse OS (96), and for chemotherapeutically treated SCCHN, high levels of CTCs with PD-L1 expression were linked to poor PFS and OS (97). CTCs are being studied as part of a recruiting clinical trial on prospective biomarkers for melanoma, and this will hopefully shed light on a potential predictive function of CTCs for ICI.

Not only CTCs but also ctDNA has been investigated in PD1ab therapy. A proof-of-concept study found that detectable levels of ctDNA in week 8 of PD1ab therapy were linked to worse PFS and OS for NSCLC, uveal melanoma, and microsatellite-instable colorectal cancer (98). Furthermore, an association was found between high hypermutated ctDNA levels and response, PFS, and OS for diverse malignancies treated by use of ICI (99).

Other Blood Biomarkers

Several other blood biomarkers have been investigated in ICI patients; for example, blood-based testing of gene-expression profiles of cathepsin D, phopholipase A2 group VII, thioredoxin reductase 1, and interleukin 1 receptor-associated kinase 3 were found to be associated with OS for CTLA4ab-treated patients (100). Ongoing studies on blood biomarkers for ICI treatment of melanoma include assessment of different T cell subsets, cytokines, and CTCs (**Table 1**). The challenge is to select the most promising biomarkers, ideally identified by several different investigators, and to study them in prospective clinical trials.

STOOL BIOMARKERS

The effect of gut microbiota on anti-tumor response has recently been observed in both murine and human studies for several cancers, including melanoma, NSCLC, and RCC. Unsurprisingly, microorganisms are prevalent in primary CRC, but distant metastases are also colonized with Fusobacterium and its associated microbiome, including Bacteroides, Selenomonas, and Prevotella species (101). Gut flora composition can stimulate or inhibit immune response. Immunostimulatory effects of Bacteroidales, particularly *Bacteroides fragilis*, have been observed for CTLA4ab therapy in mice (102). Similarly, Bifidobacterium improved anti-tumor responses for PD-1/PD-L1 blockade in a murine melanoma model (103). In contrast, ICI treatment itself can affect the population of gut microbiota (102). Baseline gut microbiota have been investigated in small CTLA4ab-treated melanoma cohorts (**Table 1**). Enrichment with Faecalibacterium and other Firmucutes was associated with improved response and with development of colitis, whereas a higher representation of Bacteroidetes was related to poorer response to CTLA4ab therapy (66). For melanoma patients receiving PD1ab therapy, enrichment of Ruminococcaceae and Clostridiales was found in responders whereas Bacteriodales were enriched in non-responders (67). Shortened PFS was observed for patients with high abundance of Bacteroidales, which is in agreement with another publication on melanoma patients treated with CTLA4ab (66). Another analysis found enrichment of *Bacteroides caccae* in all ICI responders, and specifically *Faecalibacterium prausnitzii*, *Bacteroides thetaiotaomicron*, and *Holdemania filiformis* if treated with ipilimumab plus nivolumab combination therapy. *Dorea formicogenerans* was enriched in pembrolizumab responders (68). Other authors found relative abundance of *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* in PD1ab responders with melanoma (69). An imbalance in gut microbiota correlating with impaired immune cell activity was observed for non-responders. Treatment by use of antibiotics before or shortly after ICI was associated with poorer response and worse OS for patients with RCC and NSCLC; in this analysis, a higher percentage of non-responders (69%) had a particularly low level of *Akkermansia muciniphila* compared with responders (34%) (104). It is worth nothing that in a mouse model, fecal transplants of responders into germ-free mice restored the anti-tumor effect of PD-1/PD-L1 blockade (67, 69, 104).

In summary, gut microbiota affect anti-tumor immune response. However, there is only partial overlap between the potentially relevant microorganisms (**Table 1**). It is unclear if this is because of methodological reasons, or if it depends on the individual tumor entity, or the geographical region and associated dietary habits of the investigated patients. Further prospective studies are needed to evaluate the prognostic or predictive effect of gut microbiota on ICI outcome (currently ongoing: NCT02960282, NCT03370861). A study on fecal microbiota transplantation for metastatic melanoma patients who failed ICI is also being conducted (NCT03353402).

TISSUE BIOMARKERS

PD-L1 Expression

In the initial phase I study of nivolumab for patients with solid tumors, an association between PD-L1 expression and probability of response was observed for NSCLC, melanoma, and RCC (105, 106) (**Table 2**). Because clinical significance was greatest for NSCLC, this led to further PD1ab studies using enrichment designs with different antibodies and expression cutoffs (107–109). Because patients with PD-L1 negative (or PD-L1 expression below cutoff) value cannot be followed in clinical trials of enriched design, it is not possible to distinguish between

TABLE 2 | Tissue and imaging biomarkers for clinical outcome under checkpoint blockage for metastatic melanoma patients.

Biomarker	Number of patients	Treatment	Results	Reference
Tissue Biomarkers				
PD-L1 expression	*n* = 41–43 (melanoma, NSCLC, renal cell carcinoma, and others)	Nivolumab, atezolizumab	PD-L1 expression on tumor or TILs associated with response	(105) (106) (113)
	n = 945 (stratified for PD-L1 expression)	Ipilimumab, nivolumab, ipilimumab plus nivolumab	Patients with PD-L1 negative tumors had longer PFS and OS under combined ICI compared to nivolumab monotherapy	(5) (114)
Tumor-infiltrating lymphocytes (TILs)	*n* = 82	Ipilimumab	High baseline FoxP3 and IDO expression and increase in TILs from week 0 to week 3 associated with disease control	(115)
	n = 20	Nivolumab, pembrolizumab	Partially exhausted (PD-1^{high}CTLA-4^{high}) tumor-infiltrating CD8^{+} T cells correlated with response and longer PFS	(116)
	n = 16–46	Pembrolizumab	Cytotoxic T cells at tumor margins associated with response, higher clonal expansion of TCR in responders	(117)
	n = 32–33	Nivolumab, pembrolizumab, atezolizumab	TCR clonality not associated with outcome	(118, 119)
Mutational load, neoantigen load	*n* = 38–110	Ipilimumab, tremelimumab, nivolumab, pembrolizumab	High mutational and neoantigen load associated with clinical benefit (response, DCR > 6 months, PFS, OS)	(85, 120) (121) (122) (123) (124)
	n = 68	Nivolumab	Mutational and neoantigen load decreased with treatment in responders	(125)
Single mutations	*n* = 229	IL-2, CTLA4ab, PD1ab, PD-L1ab, not specified	NRAS mutation correlated with disease control and longer PFS	(126)
	n = 32–33	Nivolumab, pembrolizumab, atezolizumab	NF-1 mutation associated with mutational load and response, NRAS-mutations not associated with clinical outcome	(118, 119)
	n = 38	Nivolumab, pembrolizumab	Tumors from responders were enriched for BRCA2 mutations	(122)
Histological subtype	*n* = 60 (desmoplastic melanoma)	Nivolumab or pembrolizumab ± ipilimumab, PD-L1ab, not specified	Desmoplastic melanoma showed higher response rates as reported in the literature (probably because of high mutational burden)	(127)
MHC-I/II expression	*n* = 23–30	Nivolumab, pembrolizumab, atezolizumab	MHC-II positivity on tumor cells associated with response, PFS, and OS	(118, 119)
Gene expression	*n* = 21–45	Ipilimumab	High IFN-γ expression and of IFN-γ-inducible genes (e.g., CXCL9, CXCL10, and CXCL11) correlated with longer PFS, OS	(128) (129)
	n = 43 (melanoma only)	Atezolizumab	Expression of baseline T helper type 1, CTLA4, and IFN-inducible genes (e.g., IDO1, CXCL9) as well as the absence of CX3CL1 associated with response	(113)
Imaging Biomarkers				
Tumor burden measured by CT (RECIST1.1)	*n* = 593	Pembrolizumab	Lower baseline tumor burden (RECIST 1.1) associated with longer OS	(26)
FDG-PET/CT	*n* = 22	Ipilimumab	FDG-PET/CT (EORTC criteria) at week 5 predicts disease progression while response could not be identified	(130)
	n = 20	Ipilimumab (*n* = 16), nivolumab (*n* = 1), PD-L1ab BMS-936559 (*n* = 3)	FDG-PET/CT at week 3–4 predicted best response at ≥4 months [using RECIST 1.1, immune-related response criteria, EORTC criteria, and PET response criteria in solid tumors (PERCIST)]	(131)
	n = 41	Ipilimumab	Cutoff of four newly emerged FDG-avid lesions on PET/CT after 12 weeks indicates treatment failure, SUV changes did not correlate with clinical outcome	(132)
FDG-PET/MRI (PERCIST)	*n* = 10	PD1ab, not specified	Metabolic response at week 2 might indicate response at 3 months	(133)

CT, computerized tomography; CTLA4ab, anti-CTLA-4 antibody; FDG, ^{18}F-fluoro-deoxy-glucose; MRI, magnetic resonance imaging; PET, positron emission tomography; PD1ab, anti-PD-1 antibody; PFS, progression-free survival; OS, overall survival; SUV, standard uptake value; DCR, disease control rate; TCR, T cell receptor.

prognostic and predictive value (20). It is worth mentioning that in a retrospective analysis of metastatic melanoma patients, PD-L1 expression was linked to improved OS irrespective of treatment type; this raises the possibility of a prognostic rather than a predictive value for ICI (28). Throughout several clinical trials for NSCLC and urothelial cancer, no association was observed between PD-L1 expression and ICI therapy outcome (8, 110, 111). There was one exception: for urothelial carcinoma, a composite biomarker of either ≥25% positive tumor cells or ≥25% positive immune cells indicated tumor response to durvalumab, and is a pre-requisite for treatment according to FDA-approval (15). PD-L1 positivity was also associated with higher probability of response in a subgroup analysis of PD-L1ab therapy for MCC (14). In addition, PD-L1 expression on tumor and immune cells was linked to higher incidence of response for SCCHN (112). There is growing evidence that response is associated more with PD-L1 expression on tumor-infiltrating immune cells than it is with tumor cell PD-L1 expression (113).

PD-L1 expression on tumor cells was used to stratify the design of the Checkmate-067 trial investigating the combination of ipilimumab plus nivolumab compared with nivolumab and ipilimumab monotherapies (5). Although not designed for this purpose, this study revealed that patients with tumors expressing PD-L1 had similar PFS and OS compared with PD1ab monotherapy and the combination of CTLA4ab plus PD1ab (5, 114). Response to combined ICI was still higher, however, compared with response to PD1ab monotherapy. The study was not designed to compare the two nivolumab-containing treatment arms, but it shows possible limitations of PD-L1 as a biomarker for treatment decisions for melanoma.

General problems associated with PD-L1 as a biomarker are: use of different immunohistochemical (IHC) assays, different cut-offs, intra-tumor heterogeneity, and dynamic changes of PD-L1 expression. In summary, there are conflicting data in diverse tumor entities. It is worth noting that treatment responses can be found in PD-L1-negative tumors.

Tumor-Infiltrating Lymphocytes (TIL)

The presence of TILs has prognostic potential for different tumor entities regardless of tumor stage (134, 135). For patients with metastatic melanoma, TILs were associated with a better outcome for primary melanoma and metastatic disease, irrespective of treatment type (28, 136). A prospective biomarker study of ipilimumab-treated patients with melanoma found an association between early increase in TILs and disease control (115) (**Table 2**). A more detailed assessment of the T cell infiltrate at baseline revealed an association between high baseline FoxP3$^+$ Tregs and indoleamine-2,3-dioxygenase (IDO) expression and favorable outcome (115). For PD1ab-treated melanoma patients, no association was found between baseline TILs and response to PD1ab (106). Abundance of partially exhausted (PD-1^{high}CTLA-4^{high}) tumor-infiltrating CD8$^+$ T cells correlated with response and PFS in PD1ab therapy (116). Cytotoxic T cells at tumor margins were also linked to response to PD1abs (117). Preliminary investigations on TCR clonality of TILs in melanoma metastases revealed higher clonal expansion of TCR for PD1ab-responders compared with non-responders (117). This finding was not, however, confirmed by other authors (119).

Interferon-γ is one of the cytokines secreted by activated T cells and is known to upregulate PD-L1 expression. This might be one reason why PD-L1 expression could be co-localized with TIL infiltrates in melanoma metastases (117, 137). In a retrospective analysis, pretreatment tumor samples from NSCLC and melanoma patients treated with PD1ab were evaluated for IFN-γ expression (129). A significantly longer PFS and OS were observed for patients with high IFN-γ expression. High pre-treatment expression of IFN-inducible genes (e.g., IDO1, CXCL9, CXCL10, and CXCL11 among others) was associated with response and prolonged OS for PD-L1ab treatment of melanoma, but this was less pronounced for NSCLC or RCC (113, 128). Primary mutations in IFN-γ signaling pathways (e.g., JAK1 and JAK2 mutations) have been described for several tumor entities. For cutaneous melanomas, JAK1/2 mutations were detected before treatment in 21% of tissue specimens (138). Interestingly, patients with resistance to ICI were found to harbor JAK mutations with consecutive loss of IFN-γ pathways (139, 140).

The following challenges apply for all potential tissue biomarkers described above: possible dependency on biopsy site, the specific time of the biopsy, and intratumor-heterogeneity. It should be noted that, when considering multiple potential biomarkers, large multivariable analyses are required to exclude a significant overlap of markers (138). Moreover, future models might include transcriptome-derived stromal and immune cell scores exceeding a pure TIL assessment (141).

Mutational Analysis

The first notion that mutational changes might affect tumor response came with the observation that melanoma patients with a high mutation rate benefitted more from ipilimumab treatment than patients with a low one, resulting in longer OS (120). In agreement with this, tumors with a naturally high mutation rate because of exogenous cancerogens, such as UV light, smoking, and alcohol (melanoma, lung cancer, SCCHN, and bladder cancer), belong to the entities that respond best to ICI treatment (123, 142–145). A small biomarker-stratified trial was performed for non-colorectal CRC and mismatch-repair deficient cancers. Stratification according to mismatch repair deficiency and mismatch-repair proficiency revealed a 40% response for patients with mismatch-repair deficiency (MSI high, dMMR), whereas mismatch-repair proficient patients did not respond at all (146). Whereas mismatch-repair deficiency can be found in gastrointestinal and genitourinary tumors (147, 148), it is of minor significance for melanoma. Colli et al. suggested a cutoff of 192 nonsynonymous mutations for a potential clinical benefit of ICI (149). Here, a high mutational load seems to result in prolonged OS in particular, whereas no correlation with response to PD1ab was observed (122). This is in agreement with the observation that melanoma patients treated with PD1ab survive longer even when not responding to the treatment (150). This was found to change, however, for ipilimumab treatment prior to PD1ab therapy; in contrast to ipilimumab-naïve patients, no association between tumor mutational burden and response/OS was observed (125). Most likely, the difference is not because of the number of mutations, but because of the increasing chance of tumor neoepitopes which might be easier recognized by the immune system. Clonal

neoantigens in particular might significantly affect ICI outcome (120, 123).

A considerable difficulty of using mutational load as a biomarker is its practical implementation in clinical routine. In addition to the high costs incurred by whole exome sequencing, most neoantigens are probably patient-specific and not recurrent (121). Specific types of mutations might be more frequent, e.g., the frameshift insertion and deletion count was found to be associated with ICI response for melanoma (124). Furthermore, several groups have proposed fitness models to describe neoantigen qualities that could be possibly employed as biomarkers in the future (151, 152). It is not clear, however, that neoantigen burden will add significant value to mutational burden testing, as shown in the examples of urothelial carcinoma and melanoma (85, 153).

Tumor antigen presentation is essential for the immune defense of cancer, and mutations affecting pathways important for antigen presentation, e.g., beta-2-microglobuline (B2M) loss, might result in ICI resistance (139). B2M mutations are more frequent for melanoma, bladder, gastric, and lung cancer in particular, with 27–50% found for these cancers in The Cancer Genome Atlas dataset compared with 1.8% across all tumor types (138). The MHC-II-expression itself (HLA-DR$^+$) was found to be associated with PD1ab/PD-L1ab response in melanoma (118).

It would be easy to use biomarkers for mutations that are routinely assessed in clinical care. BRAF-V600 mutations, which are found in 40–50% of melanomas, are not associated with ICI outcome (154–157). A subgroup survival analysis of combined ipilimumab plus nivolumab versus PD1ab monotherapy showed a trend toward longer OS for combined ICI treatment of BRAF-mutant patients, but this needs to be addressed further (114). In a retrospective analysis from the pre-ICI era, NRAS mutations, which are seen in up to 20% of melanomas, were found to be associated with worse OS (158). After introduction of ICI, a retrospective study investigating patients treated by immunotherapy, including IL-2, CTLA4ab, PD1ab, and PD-L1ab revealed greater disease control and longer PFS for NRAS-mutant melanoma (126). This result was not, however, found for a smaller cohort of patients with PD1ab/PD-L1ab therapy (119). The NF-1 mutation, which is associated with UV damage and high mutational load, was linked to higher incidence of response and prolonged survival for PD1ab-treated patients (119). A more favorable response to PD1ab therapy was observed for desmoplastic melanomas which are characterized by a high mutational load and frequent NF-1 mutations (127); it should be mentioned that this observation was also made from retrospective assessment. For NSCLC, single-gene mutation analysis showed that the presence of an EGFR-mutation seems to be a negative predictor for PD1ab response (159). NGS data, however, revealed a lower mutational burden for EGFR-mutant NSCLC, which could be one reason for this finding (160).

Objectives for the future include exploration and validation of a panel of genetic biomarkers detected by next-generation sequencing. Definition of cutoffs is a current challenge because absolute values depend on the depth of sequencing. Furthermore, gene translocations/fusions and other variants will not be detected by use of targeted sequencing techniques. Development of multi-marker assays is more complex and specific computational algorithms must be used for validation (20).

IMAGING BIOMARKERS

Anatomic Imaging

The use of imaging enables non-invasive assessment of tumor dimensions and can also provide biologic tumor data. The current standard assessment procedure for metastatic melanoma and other advanced malignancies is computerized tomography (CT) with iodine contrast dye, evaluated according to response evaluation criteria in solid tumors (RECIST) 1.1 (161). It has been shown that the size of baseline tumor lesions is associated with OS (26, 162). This is probably of prognostic value because it correlates with tumor load. However, RECIST 1.1 might be insufficient to evaluate response to ICI therapy, in particular cases of initial tumor progression or occurrence of new lesions during ICI. To overcome this problem, immune-related response criteria (irRC and irRECIST) have been introduced as alternative response criteria (163). Pure anatomic imaging is, however, unlikely to be sufficient to predict tumor response to ICI. New imaging biomarkers for metabolic and immune imaging are discussed below.

Metabolic Imaging

In addition to anatomic imaging, metabolic imaging by use of ^{18}F-fluoro-deoxy-glucose-positron emission tomography (FDG-PET) can add clinically meaningful data when imaging malignancies. Two different response criteria for FDG-PET imaging are currently used in clinical routine: European Organisation for Research and Treatment of Cancer (EORTC) criteria and positron emission tomography response criteria in solid tumors (PERCIST) (164, 165). It has been shown that FDG-PET combined with CT is of clinical value for assessment of ICI responses in melanoma (130–132) (**Table 2**). The assumption for metabolic imaging is that metabolic changes in tumors occur prior to anatomic changes, which might enable prediction of response to ICI earlier during the course of treatment. This can be prevented by the failure to discriminate between inflammation and tumor metabolism (166). For ipilimumab, one clinical trial showed that FDG-PET/CT 5 weeks after treatment initiation could predict disease progression to CTLA4ab; patients responding to treatment could not be identified at this time (130). In another trial it was possible to predict best response by use of PERCIST and EORTC criteria (131). To evaluate ICI response, a new PET-CT classification, the PET response evaluation criteria for immunotherapy criteria, was developed to reflect the fact that single new lesions do not define disease progression. The absolute number of new lesions was, however, more important than changes in standardized uptake values (SUV) (132). During PD1ab therapy for melanoma, use of FDG-PET/magnetic resonance imaging (MRI) as early as 2 weeks after the start of treatment might identify patients with complete response at the 3-month time-point (133). For FDG-PET/CT for NSCLC, maximum SUV at 4 weeks after commencement of PD1ab therapy was associated with PFS and OS (167). However, these are case series and small prospective studies. Larger prospective trials are needed to investigate these findings further.

^{18}F-fluorothymidine-PET (FLT-PET) uses a thymidine analog that accumulates in proliferating tissues, including malignant and immune cells (168). Positive findings were published for e.g., differentiation of cerebral radionecrosis from glioma progression (169), and correlation of mean SUV with OS for resectable pancreatic carcinoma (170). In contrast, no association was found between early changes in FLT uptake after the first cycle of chemotherapy for CRC and the response evaluated from subsequent CT scans (171). Its value as a biomarker for ICI in melanoma has only been reported in one case of FLT-PET/MRI; this precludes general conclusions (172). Further clinical evaluation of FLT-PET in ICI is, therefore, warranted. O-(2-[18F]fluoroethyl)-L-tyrosine (FET-)PET can be used to specifically image primary brain tumors and metastases to the brain which can be differentiated from healthy, inflamed, and radionecrotic tissue (173, 174). FET-uptake correlates with ki-67 expression and could be a potential biomarker for early response assessment (175). For melanoma, a case report showed that pseudo-progression of melanoma brain metastases could be detected by use of FET-PET (176) but more data are needed to assess the value of FET-PET in ICI.

Modern MRI techniques, including dynamic contrast-enhanced MRI, are also available for high resolution imaging of tumor perfusion or cell-membrane permeability (177). This technique could be particularly useful for assessment of specific metastatic sites, e.g., hepatic metastasis (178). Another potential application could be MRI-based immune-cell tracking and assessment of drug delivery (179).

With the exception of FDG-PET/CT, all the methods described above have been studied in only a few patients, and rarely in the setting of ICI. Results of ongoing studies will reveal a potential prognostic or predictive value of PET-biomarkers.

Immuno-Imaging

Several immuno-PET tracers, namely monoclonal antibodies, scaffold proteins, or peptides have been evaluated in preclinical tumor models. Potential targets are, for example, CD8$^+$ cytotoxic T cells, PD-1, and PD-L1. Immuno-PET tracers have been studied in preclinical models. A ^{89}Zr-labeled PEGylated single-domain anti-CD8 antibody was used for longitudinal evaluation of CTLA4ab treatment in the B16-melanoma mouse model. A homogeneous distribution of the anti-CD8 PET signal was observed for responding animals, whereas a heterogeneous signal was associated with lower response and faster tumor growth (180). Another group studied a ^{89}Zr-desferrioxamine-labeled anti-CD8 cys-diabody in PD1ab treatment of Balb/c mice with CRC. They found a higher SUV in responding animals compared with non-responding ones. They also found that uptake for responders tended to be intra-tumoral, whereas uptake for non-responders was in the margins of the tumor (181); this is in agreement with the role of intra-tumoral CD8$^+$ cytotoxic T cells in PD1ab response.

Another T cell imaging approach is *via* visualization of PD-1. The feasibility of this method has been proven in murine studies. Natarajan et al. developed the anti-PD-1 tracers ^{89}Zr-keytruda and ^{64}Cu-keytruda; these were evaluated in a humanized NOD-scid mouse model, and uptake in tumors and lymphoid tissue was observed for human melanoma tumors (182). Other groups developed radiotracers which target PD-L1 expressed on tumor cells and on immune cells of the tumor microenvironment (183–185). The feasibility of PD-L1 imaging was shown by use of ^{64}Cu-atezolizumab in mice with tumors constitutively expressing PD-L1, and in two breast cancer mouse models (183). Investigation of irradiated versus non-irradiated tumors in a HPV + SCHNN and a B16F10 melanoma mouse models by use of an ^{89}Zr-labeled anti-PD-L1 monoclonal antibody revealed PD-L1 upregulation in irradiated tumors specifically (184). In a patient-derived xenograft model of NSCLC, the ^{89}Zr-C4-PD-L1 antibody revealed PD-L1 changes after chemotherapy (185).

These immuno-PET tracers have been investigated in animal models, which can certainly improve understanding of response or non-response mechanisms to ICI treatment. Studies in humans are under way. A possible predictive ability of immuno-PET in the setting of ICI, however, needs to be explored in the future (NCT03313323, NCT02760225).

CONCLUSION

Several factors might affect response to ICI treatment, including mutational load, tumor microenvironment, and stool microbiome. Upfront exclusion of metastatic melanoma patients from ICI therapy on the basis of biomarkers is not currently possible. It also remains unclear which patients will need combined ICI and which patients will benefit from use of PD1ab only. Although there are several potential biomarkers, their predictive versus prognostic abilities have not yet been validated by prospective clinical trials. In particular, the best sequence of treatment to follow, e.g., targeted versus immunotherapy for melanoma, cannot be answered on the basis of the biomarker data currently available.

Peripheral blood immune-cell analysis, e.g., by use of CyTOF, enables investigation of multiple markers, and will hopefully reveal predictive biomarkers in future. A multi-marker assay is more likely than a single biomarker. Future challenges include the development and validation of multi-marker assays, which will require detailed pre-analytics, computation algorithms, and, most importantly, well-designed clinical trials with large numbers of patients.

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KB-B and JH contributed to the conception and design of the article, acquisition and interpretation of data, drafting of the article, critical revision, and final approval. Both are accountable for the content of this manuscript.

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Expression of the Circadian Clock Gene *BMAL1* Positively Correlates With Antitumor Immunity and Patient Survival in Metastatic Melanoma

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Introduction: Melanoma is the most lethal type of skin cancer, with increasing incidence and mortality rates worldwide. Multiple studies have demonstrated a link between cancer development/progression and circadian disruption; however, the complex role of tumor-autonomous molecular clocks remains poorly understood. With that in mind, we investigated the pathophysiological relevance of clock genes expression in metastatic melanoma.

Methods: We analyzed gene expression, somatic mutation, and clinical data from 340 metastatic melanomas from The Cancer Genome Atlas, as well as gene expression data from 234 normal skin samples from genotype-tissue expression. Findings were confirmed in independent datasets.

Results: In melanomas, the expression of most clock genes was remarkably reduced and displayed a disrupted pattern of co-expression compared to the normal skins, indicating a dysfunctional circadian clock. Importantly, we demonstrate that the expression of the clock gene aryl hydrocarbon receptor nuclear translocator-like protein 1 (*BMAL1*) positively correlates with patient overall survival and with the expression of T-cell activity and exhaustion markers in the tumor bulk. Accordingly, high *BMAL1* expression in pretreatment samples was significantly associated with clinical benefit from immune checkpoint inhibitors. The robust intratumoral T-cell infiltration/activation observed in patients with high *BMAL1* expression was associated with a decreased expression of key DNA-repair enzymes, and with an increased mutational/neoantigen load.

Conclusion: Overall, our data corroborate previous reports regarding the impact of *BMAL1* expression on the cellular DNA-repair capacity and indicate that alterations in the tumor-autonomous molecular clock could influence the cellular composition of the surrounding microenvironment. Moreover, we revealed the potential of *BMAL1* as a clinically relevant prognostic factor and biomarker for T-cell-based immunotherapies.

Keywords: skin cancer, melanoma, circadian rhythms, clock genes, ARNTL/BMAL1 immunotherapy

SIGNIFICANCE

Here, we provide a first glimpse regarding the impact of a disrupted tumor-autonomous molecular clock on the cellular composition of the tumor microenvironment through the modulation of DNA-repair capacity. Within this line, our data revealed the potential of *BMAL1* as a clinically relevant biomarker for immunotherapy response and overall survival of patients with metastatic melanoma.

INTRODUCTION

Melanoma is the most lethal type of skin cancer, with increasing incidence and mortality rates worldwide (1, 2). It represents only 4% of skin cancer but accounts for approximately 80% of skin cancer-related death (3). Although complete surgical resection is often curative for melanomas detected at initial stages, patients with metastatic disease have an overall survival of approximately 5 months (4). Therapeutic options for patients with metastatic melanoma have dramatically changed in the past years, with the introduction of more effective agents such as proto-oncogene, serine/theronine kinase (BRAF), mitogen activated protein kinase kinase (MAPK), and immunotherapeutic antibodies directed to cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell-death protein 1 (PD-1) and its ligand (PD-L1) (5–8). Melanoma etiology is multifactorial and includes risk factors such as ultraviolet radiation exposure, genetic susceptibility, high nevus density, reduced skin pigmentation, and immunosuppression (9, 10).

Proper temporal control of physiological functions is crucial for maintaining the homeostasis of multi-cellular organisms (11–13). In mammals, the molecular machinery of timekeeping and circadian rhythm generation is based on interconnected positive and negative transcriptional–translational feedback loops. The central hypothalamic clock (suprachiasmatic nuclei, SCN) and clocks located in peripheral tissues share the same molecular architecture, engaging core genes such as aryl hydrocarbon receptor nuclear translocator-like protein 1 (*BMAL1* also known as *ARNTL*), cryptochrome 1 and 2 (*CRY1/2*), circadian locomotor output cycles kaput (*CLOCK*), period 1, 2, and 3 (*PER1/2/3*), receptor subfamily 1, group D, member 1/2 (*NRD1/2* also known as *REV-ERB*α/β), and RAR-related orphan receptor A and B (*RORA/B* also known as NR1F1/2). In healthy conditions, CLOCK–BMAL1 heterodimers translocate to the nucleus and induce the gene expression of their own inhibitors, PER and CRY proteins. This core oscillatory pathway is augmented and stabilized by a secondary loop involving *NRD1/2* and *RORA/B*, nuclear receptors that modulate *BMAL1* expression. Importantly, CLOCK–BMAL1 heterodimers also regulate the expression of several clock-controlled genes, which are tissue- and cell type-specific (11–13).

Many epidemiologic studies have demonstrated that the disturbance of biological rhythms through shift work, increased light exposure at night, and irregular feeding regimens (14–16) is associated with increased risk of developing several types of cancers (17–19). In fact, alterations in the cellular circadian machinery have been shown to affect cancer-related processes such as cell proliferation (20, 21), DNA damage response (22, 23), and metabolism (24–27) in a tumor-specific manner. Accordingly, the aberrant expression of clock core genes such as *CRY1*, *PER1*, and *PER2* has been shown to impact tumor progression in colorectal, prostate, and breast cancers, respectively (28–30).

In melanoma, mRNA levels and nuclear immunopositivity for CLOCK, CRY1, and PER1 are reduced compared to adjacent non-tumorous skin and present a significant association with clinicopathological features such as Breslow thickness (31). Moreover, the expression of RORA is lower in melanomas than in nevi, and positively correlates with overall survival and disease-free survival (32). Interestingly, enhancing the circadian clock function of melanoma cells impairs cell cycle progression and inhibits tumor growth *in vivo* (21). In this sense, we have previously demonstrated that the expression of clock core genes in murine melanoma cells can be activated by different stimulus, such as white light exposure (33), UVA radiation (34), estradiol (35), and thermal energy (36). Recently, we have demonstrated that a non-metastatic model of melanoma leads to a systemic chronodisruption in tumor-adjacent skin, lungs, liver, and SCN, as in these tissues the rhythmic expression of *Bmal1* was lost in tumor-bearing mice (37). These data reinforce that the modulation of tumor-autonomous clock might represent a novel and promising therapeutic strategy.

To further characterize the pathophysiological relevance of the molecular clock in skin cancer, we investigated the clinical value of clock core genes expression in metastatic melanoma, using public high-throughput molecular data. Overall, we revealed the robust prognostic power of *BMAL1* expression and provided evidence into its underlying biological processes.

MATERIALS AND METHODS

Datasets of Melanoma and Normal Skin Samples

Gene expression, somatic mutation, and clinical data from 340 metastatic melanomas from The Cancer Genome Atlas (TCGA) and gene expression data from 234 Genotype-Tissue Expression (GTEx) normal skin (not sun exposed) samples were downloaded from the UCSC XENA Browser (http://xena.ucsc.edu) in January of 2017. TCGA and GTEx gene expression data were originally generated by TCGA (38) and GTEx consortia (39), respectively, using the Illumina HiSeq 2000 RNA sequencing platform, quantified using RSEM, upper quartile normalized and $\log_2(x+1)$ transformed. TCGA somatic mutation data were generated using the Illumina GAIIx DNA sequencing platform and somatic variants (SNPs and small indels) were identified using MuTect2. Neoantigen load information for TCGA metastatic melanoma samples was obtained from Rooney et al. (40). Briefly, for each metastatic melanoma patient, all novel amino acid 9–10mers resulting from missense mutations in expressed genes (median > 10 TPM) were identified. Mutant peptides with a HLA-binding affinity <500 nM, predicted by NetMHCpan (v2.4), were considered antigenic (41). Clinical information and gene

expression data of pretreatment biopsies from 49 patients who received anti-PD1 immunotherapy (nivolumab) were obtained from Riaz et al. (42). Expression data were generated using the Illumina HiSeq 2000 RNA sequencing platform, counted using Rsamtools v3.2, upper quartile normalized and $\log_2(x + 1)$ transformed. Treatment response for patients was defined by RECIST v1.1.

Co-Expression Network Analysis

Undirected weighted co-expression networks were constructed based on the pairwise Spearman's correlation coefficients between the expression of clock core genes *BMAL1*, *CRY1*, *CRY2*, *NRD1*, *PER1*, *PER2*, *PER3*, and *RORA*. Using the CoGA R package (43), we compared the structural properties of co-expression networks from normal skin and metastatic melanomas by testing the equality in their spectral distributions (44, 45). The spectrum of a graph, defined as the set of eigenvalues of its adjacency matrix, describes several structural features and represents a comprehensive characterization of networks (44, 46). *P*-values were calculated based on 1,000 phenotype permutations and networks were visualized using the gplots R package.

Gene Set Enrichment Analysis (GSEA)

Genes in the TCGA expression dataset were ranked according to the Spearman's correlation coefficient between their expression and the expression of *BMAL1*. GSEA was performed using GSEA v3.0 and Reactome pathways (47, 48). Enrichment scores (ES) were calculated based on a weighted Kolmogorov–Smirnov-like statistic and normalized (NES) to account for the size of each gene set. *P*-values corresponding to each NES were calculated based on 1,000 phenotype permutations and corrected for multiple comparisons using the false discovery rate (FDR) procedure. Adjusted *P*-values < 0.05 were considered statistically significant.

Single Sample Gene Set Enrichment Analysis (ssGSEA)

Single sample gene set enrichment analysis, an extension of GSEA, was used to estimate the degree of enrichment of gene sets in individual samples within the TCGA gene expression dataset (49). For each sample, gene expression values were rank-normalized, and ESs were calculated based on the difference between weighted Empirical Cumulative Distribution Functions of genes inside and outside the gene sets. We performed ssGSEA using the GSVA R package (50) and DNA repair-related KEGG pathways (51), namely: base excision repair (hsa03410), nucleotide excision repair (hsa03420), mismatch repair (hsa03430), homologous recombination (hsa03440), and non-homologous end joining (hsa0345).

Statistical Analysis

We used the two-sided Wilcoxon–Mann–Whitney test to perform two-group comparisons, the Spearman's correlation test to assess ordinal associations, and the Chi-square test to analyze the relationship between two categorical variables. The impact of clock core genes expression on patient overall survival was evaluated using univariate Cox regressions. The prognostic power of *BMAL1* expression was further investigated using Kaplan–Meier curves, combined to the log-rank test, and multivariate Cox regressions. Hazard Ratios, including 95% confidence intervals, were calculated. Statistical analyses were performed with GraphPad Prism 6 and R (www.r-project.org). *P*-values < 0.05 were considered statistically significant. Where indicated, *P*-values were adjusted for multiple comparisons using the FDR procedure.

RESULTS

Clinical Relevance of Clock Core Genes Expression in Metastatic Melanomas

We first analyzed the expression of clock core genes in normal skin and in metastatic melanomas. Compared to normal skin, metastatic melanomas demonstrated a remarkably decreased expression of *BMAL1*, *CRY1*, *CRY2*, *NRD1*, *PER1*, *PER2*, *PER3*, and *RORA* and an increased expression of *CLOCK* (**Figure 1A**). In normal skin, we have found a classic pattern of clock gene expression: *PER*s and CRYs are concomitantly expressed (in phase) and are in antiphase with *BMAL1* and *CLOCK* expression, as expected; on the other hand, in metastatic melanomas such correlations are severely attenuated (**Figure 1B**), which further corroborates a dysfunctional circadian clock within the tumor. In metastatic melanomas, male presented increased percentage of tumor showing high expression of *NRD1*, *PER2,* and *PER3* ($P = 0.015$, $P = 0.028$, and $P < 0.001$, respectively; **Table 1**; Table S1 in Supplementary Material). Patients with high *PER3* expression were also significantly older and more frequently diagnosed with stage I–II tumors ($P = 0.002$ and $P = 0.037$, respectively; **Table 1**; Table S1 in Supplementary Material).

Next, using univariate Cox regressions we evaluated the clinical relevance of clock core genes in metastatic melanoma. Among all nine genes analyzed, only *BMAL1* showed a significant prognostic value: high *BMAL1* expression was associated with longer overall survival (HR = 0.678, $P = 0.002$; **Figures 1C,D**). Importantly, multivariate Cox regression adjusting for age, gender, tumor pathologic stage, ulceration status, mitotic count, and Breslow thickness revealed *BMAL1* expression as an independent prognostic factor (**Table 2**). Additionally, the prognostic value of *BMAL1* expression in metastatic melanomas was confirmed in two other independent datasets (GSE6590 and GSE54467; Figures S1A,B and Table S2 in Supplementary Material).

BMAL1 Expression and the Overall Biological Profile of Metastatic Melanoma

To investigate the biological mechanisms that likely underlie the impact of *BMAL1* expression on patient survival, we performed GSEA using genes ranked according to their Spearman's correlation with *BMAL1* expression. Significantly enriched pathways presented positive NES and were mainly involved in the activation of the immune system (**Figure 2A**). In fact, in metastatic

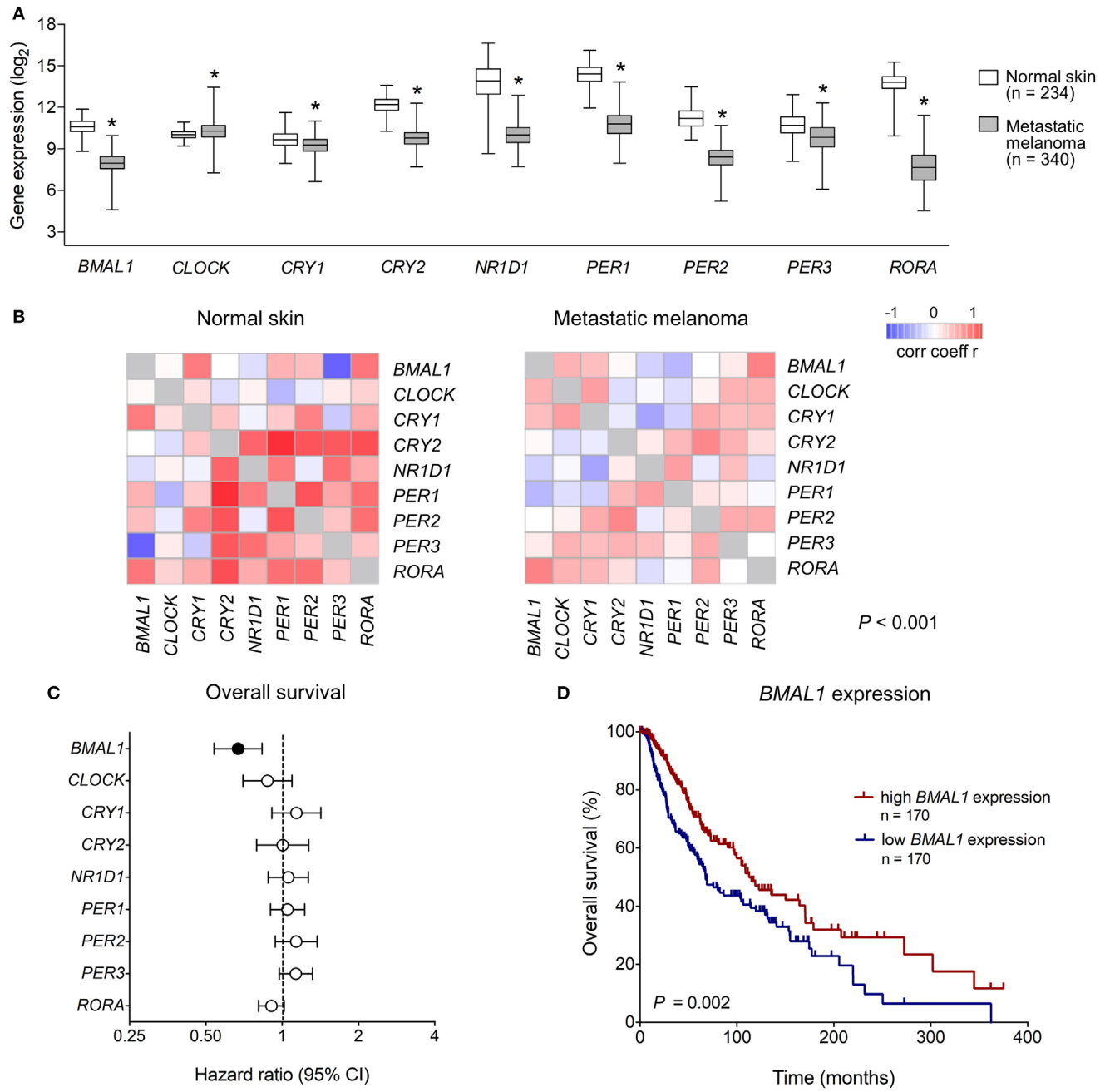


FIGURE 1 | Clinical relevance of clock genes expression in metastatic melanomas. **(A)** RNAseq analysis of clock genes expression in genotype-tissue expression (GTEx) normal skins (n = 234) and The Cancer Genome Atlas (TCGA) metastatic melanoma (n = 340). Expression values were estimated using RSEM and $\log_2(x + 1)$ transformed. The boxes extend from the 25th to the 75th percentile, the central bold line shows the median, and whiskers are drawn from minimum to maximum values. Comparisons were performed using the two-sided Wilcoxon–Mann–Whitney test. *Significantly different from normal skin ($P < 0.05$). **(B)** Co-expression matrix showing pairwise Spearman's correlation coefficients of clock core genes in GTEx normal skins (n = 234) and TCGA metastatic melanomas (n = 340). Networks were compared using the CoGA software. **(C)** Univariate Cox analysis of overall survival according to the expression of clock core genes in TCGA metastatic melanomas. Hazard Ratios including 95% confidence intervals are shown. Genes with a significant prognostic value ($P < 0.05$) are marked in black. **(D)** Kaplan–Meier survival curve according to the expression of aryl hydrocarbon receptor nuclear translocator-like protein 1 (*BMAL1*) in TCGA metastatic melanomas. The median expression of *BMAL1* was used as the cutoff to dichotomize the population. Comparisons were performed using the log-rank test.

melanomas, *BMAL1* expression exhibited a strong positive correlation with the expression of dendritic cell markers, T-cell markers *CD4* and *CD8A*, and T-cell activation/differentiation markers (**Figure 2B**). This robust intratumoral activation of leukocytes was accompanied by the expression of T-cells exhaustion markers (**Figure 2B**), such as *CTLA4*, *PD1*, and *PDL1*,

TABLE 1 | Clinicopathological features according to the expression of clock genes in The Cancer Genome Atlas metastatic melanomas.

	P-values*								
Variables	***BMAL1***	***CRY1***	***CRY2***	***CLOCK***	***NR1D1***	***PER1***	***PER2***	***PER3***	***RORA***
Age	0.59	0.592	0.998	0.151	0.057	0.19	0.754	**0.002**	0.286
Gender	0.659	0.271	0.269	0.269	**0.015**	0.06	**0.028**	**<0.001**	0.269
Pathologic stage	0.817	0.643	0.644	0.083	0.247	0.418	0.132	**0.037**	0.417
Ulceration status	1	0.404	0.094	0.889	1	0.78	0.267	0.889	1
Mitotic count	0.769	0.175	0.07	0.801	0.465	0.256	0.276	0.613	0.963
Breslow thickness	0.731	0.545	0.65	0.847	0.179	0.816	0.823	0.961	0.838

P-values in bold are statistically significant.

**Two-sided Wilcoxon–Mann–Whitney (continuous variables) or Chi-square exact test (categorical variable) comparing tumors with high vs. low expression.*

corroborating the fact that T-cell were chronically exposed to antigens (52, 53). Accordingly, patients with high *BMAL1* expression in pretreatment biopsies demonstrated improved response to anti-PD1 immunotherapy in comparison to patients expressing low *BMAL1* levels (**Figure 2C**).

The correlation between *BMAL1* expression and antitumor immune response was also confirmed in two additional independent datasets (GSE6590 and GSE54467; Figures S1C,D in Supplementary Material). Importantly, the expression of *BMAL1* was a prognostic factor independent of the percentage of leukocyte, monocyte, and neutrophil infiltration in TCGA melanomas (**Table 3**).

TABLE 2 | Multivariate Cox regression analysis of survival in The Cancer Genome Atlas metastatic melanomas.

	Overall survival	
Variables	**HR (95%CI)**	***P*-value**
Age	1.024 (1.007–1.041)	**0.006**
Gender		
Male vs. female	1.158 (0.655–2.051)	0.612
Pathologic stage		
III-IV vs. I–II	2.405 (1.427–4.053)	**<0.001**
Ulceration status		
Present vs. absent	0.994 (0.556–1.769)	0.985
Mitotic count	1.015 (0.986–1.045)	0.301
Breslow thickness	1.080 (1.004–1.161)	**0.038**
BMAL1 expression	0.525 (0.369–0.746)	**<0.001**

P-values in bold are statistically significant.

HR, hazard ratio; CI, confidence interval.

BMAL1 Expression and the Mutational Load in Metastatic Melanomas

Tumor somatic mutations can generate major histocompatibility complex Class I-associated neoantigens expression that plays a central role in inducing T-cell meditated antitumor cytolytic activity (54, 55). Interestingly, in metastatic melanomas, *BMAL1* expression positively correlated with the number of total somatic mutations and predicted neoantigens (**Figure 3A**). With that in mind, we investigated whether the expression of *BMAL1* was associated with the activation of different DNA-repair pathways. Using ssGSEA, we demonstrated that base excision repair is likely impaired in tumors expressing high *BMAL1* (**Figure 3B**). No significant differences were observed regarding the nucleotide excision repair, mismatch repair, homologous recombination, and non-homologous end joining DNA-repairing mechanisms. Importantly, the expression of base excision repair-related genes, such as *NTHL1*, *XRCC1*, and *SMUG1*, and the expression of general DNA repair-related genes, such as *POLD1*, *POLD2*, and *LIG1,* were downregulated in tumors expressing high *BMAL1* in all three datasets analyzed (**Figure 3C**; Figure S2 in Supplementary Material). High *BMAL1* expression was also associated with impaired DNA-repair capacity in human melanoma cell lines from the Cancer Cell Line Encyclopedia (Figure S3 in Supplementary Material).

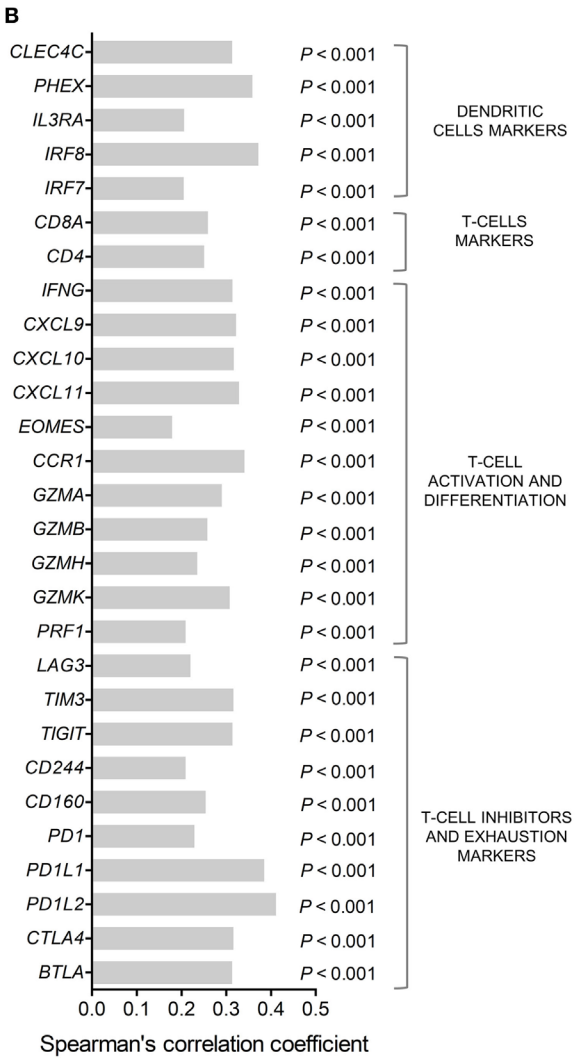
DISCUSSION

Cancer onset, development, and progression have been linked to circadian disruption (17–19); however, the complex role of the tumor-autonomous molecular clock within these processes is yet poorly understood. Here, confirming previous reports in humans and in mice (32, 33, 35, 37), we showed that the expression of core components of the molecular clock machinery is severely repressed in melanomas. Moreover, we demonstrated that, for such tumors, high mRNA levels of *BMAL1* are associated with decreased gene expression of base excision repair enzymes and increased mutation load and predicted neoantigen presentation. The high incidence of antigenic peptides observed in metastatic melanomas with high *BMAL1* expression was accompanied by increased expression of cytotoxic T-cell activity markers in the tumor bulk and better prognosis. Even though our data do not provide a detailed mechanistic perspective, the present findings strongly support a role for *BMAL1* as a clinically relevant biomarker of DNA damage repair deficiency and intratumoral T-cell response. Thus, confirming such findings using common molecular techniques would be of great relevance for prognosis prediction and proposition of personalized therapeutic strategies.

Accumulating evidence implicates cell autonomous-circadian clocks in cancer development, as the disruption of peripheral systems of timekeeping seems to be a common event in malignant tissues (17, 18). As demonstrated here for metastatic melanomas, the expression of most clock core genes is downregulated in several types of cancers when compared to normal tissue (28, 32,

A

Reactome pathway	NES	Adjusted *P*-value
CYTOKINE SIGNALING IN IMMUNE SYSTEM	2.355	0.027
SIGNALING BY ILS	2.314	0.021
NUCLEOTIDE BINDING DOMAIN LEUCINE RICH REPEAT CONTAINING RECEPTOR NLR SIGNALING PATHWAYS	2.275	0.023
INNATE IMMUNE SYSTEM	2.256	0.024
NOD1 2 SIGNALING PATHWAY	2.212	0.035
IL1 SIGNALING	2.211	0.030
ADAPTIVE IMMUNE SYSTEM	2.210	0.025
TOLL RECEPTOR CASCADES	2.201	0.026
ACTIVATED TLR4 SIGNALLING	2.200	0.023
INTERFERON SIGNALING	2.194	0.023
MYD88 MAL CASCADE INITIATED ON PLASMA MEMBRANE	2.170	0.027
TCR SIGNALING	2.156	0.030
G ALPHA1213 SIGNALLING EVENTS	2.135	0.037
DOWNSTREAM TCR SIGNALING	2.135	0.034
REGULATION OF SIGNALING BY CBL	2.116	0.042
G ALPHA I SIGNALLING EVENTS	2.112	0.041
GASTRIN CREB SIGNALLING PATHWAY VIA PKC AND MAPK	2.105	0.041
TRAF6 MEDIATED INDUCTION OF NFKB AND MAP KINASES UPON TLR7 8 OR 9 ACTIVATION	2.093	0.045
INTERFERON GAMMA SIGNALING	2.080	0.049
IL 3 5 AND GM CSF SIGNALING	2.078	0.048
RIG I MDA5 MEDIATED INDUCTION OF IFN ALPHA BETA PATHWAYS	2.077	0.046
TRIF MEDIATED TLR3 SIGNALING	2.075	0.045



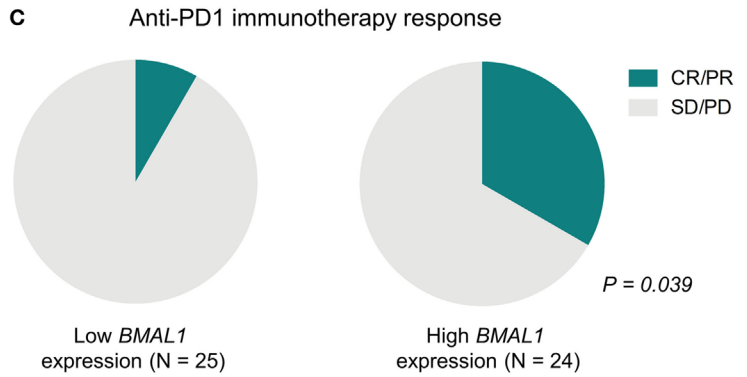


FIGURE 2 | Aryl hydrocarbon receptor nuclear translocator-like protein 1 (*BMAL1*) expression positively correlates with antitumor immunity in metastatic melanomas. **(A)** Gene Set Enrichment Analysis (GSEA) using genes ranked according to the Spearman's correlation coefficient between their expression and the expression of *BMAL1* in The Cancer Genome Atlas (TCGA) metastatic melanomas ($n = 340$). Normalized enrichment scores (NES) and *P*-values corrected by false discovery rate (FDR) were calculated using GSEA v3.0 and Reactome pathways. Only significantly enriched pathways (adjusted $P < 0.05$) are shown. **(B)** Spearman's correlation coefficient between the expression of *BMAL1* and immune cells markers in TCGA metastatic melanomas. *P*-values were corrected by FDR. **(C)** Association between *BMAL1* expression (pretreatment biopsies) and clinical benefit of melanoma patients from anti-PD1 immunotherapy (nivolumab). RNAseq data and treatment response information were obtained from Ref. (42). Comparisons were performed using the Chi-square test. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

TABLE 3 | Multivariate Cox regression analysis of overall survival in metastatic melanomas adjusted for the percentage of immune cell infiltration in The Cancer Genome Atlas metastatic melanomas.

	Overall survival	
Variables	**HR (95% CI)**	***P*-value**
% Lymphocyte infiltration	0.975 (0.932–1.019)	0.261
% Monocyte infiltration	1.001 (0.902–1.111)	0.974
% Neutrophil infiltration	0.956 (0.673–1.357)	0.901
BMAL1 expression	0.685 (0.550–0.854)	**<0.001**

P-values in bold are statistically significant.
HR, hazard ratio; CI, confidence interval.

56–61). Moreover, the overexpression of *PER1* and *PER2* has been shown to impair tumor proliferation and induce apoptosis in lung, prostate, and pancreatic cancer (29, 62, 63), reinforcing the idea that the molecular clock machinery may be considered as a new therapeutic target.

The protein encoded by *BMAL1* belongs to the family of the bHLH-PAS structural domain transcription factors and it is estimated to control the expression of more than 150 target genes, including the clock genes *CRY1*, *CRY2*, *NR1D1*, *PER1*, *PER2*, and *PER3* (64). *BMAL1* has also been revealed as a candidate gene for susceptibility to hypertension, diabetes, and obesity, and mutations in *BMAL1* have been linked to infertility and metabolic dysfunctions (65–70). Here, we demonstrated that, in metastatic melanomas, the expression of *BMAL1* is a robust positive prognostic factor of overall survival and has a negative association with the expression of key DNA-repair enzymes, such as *POLD1*, *POLD2*, and *LIG1*. Accordingly, in colorectal cancer, downregulation of *BMAL1* gene expression accelerates cell proliferation *in vitro*, promotes tumor growth in mice, and decreases DNA damage induced by cisplatin (71). Moreover, high *BMAL1* expression is associated with increased sensitivity of colorectal cancer cells to oxaliplatin *in vitro* and *in vivo*, and predicts favorable outcome for patients treated with oxaliplatin-based chemotherapy (72). *BMAL1* expression also positively correlates with patient survival in pancreatic ductal adenocarcinomas (61), causes growth inhibition in lymphoma/leukemia cells (58), negatively impacts DNA-repair capacity of mice fibroblast (73), but promotes proliferation in malignant pleura mesothelioma (74), suggesting that its role in tumorigenesis is complex and tissue-specific.

Although it has been shown that alterations in the tumor molecular clock impact some parameters of tumor progression (28–30, 62, 63), the influence of endogenous oscillatory systems on the cellular composition of the tumor microenvironment is largely unknown. In this sense, our data indicate that the prolonged survival of metastatic melanoma patients with high *BMAL1* bulk expression is associated with a robust intratumoral T-cell infiltration/activation, which can be partially explained by the increased neoantigen load that likely reflects the impaired DNA-repair capacity. Previous reports have also linked DNA-repair deficiency to increased mutational load and antitumor immune response in melanomas, lung, colorectal, and endometrial cancers (75–78). It is now clear that DNA repair and genomic instability have a pivotal role in the modulation of antitumor immune responses (79); thus, understanding their interplay with tumor-autonomous clocks may provide clinically relevant insights.

Immunotherapies that boost the ability of T lymphocytes to combat tumor cells have demonstrated therapeutic efficiency in a variety of solid tumors. Monoclonal antibodies against T-cell checkpoint proteins, such as CTLA-4, PD-1, and PD-L1, have now been approved for melanoma treatment and are associated with robust durable responses, but only in a subset of tumors (80–82). Thus, there is a need to identify biomarkers that will allow the selection of treatment-responsive patients, avoid unnecessary toxicity, and help personalize therapy regimens (83). Metastatic melanomas presenting high *BMAL1* expression have impaired DNA-repair capacity combined with increased mutation/neoantigen load, T-cell intratumoral infiltration, and T-cell expression of exhaustion markers, all of which have been shown to predict good clinical response to the treatment with immune checkpoint inhibitors (78, 84–87). In fact, we showed that high *BMAL1* expression in pretreatment melanoma samples is associated with clinical benefit from anti-PD1 immunotherapy. Considering that whole-genome and -transcriptome sequencing is expensive and time-consuming, profiling a smaller fraction of genes could serve as a useful tool to help translate those findings into routine clinical practices (88). Therefore, the present data indicate that *BMAL1* expression in melanoma patients must be considered as a relevant marker for immunotherapy efficacy. Nevertheless, larger clinical studies are necessary to validate the potential of *BMAL1* alone, or along with other biomarkers, in discriminating responders from non-responders in immunotherapy regiments.

CONCLUSION

The molecular characterization of melanomas using high-throughput approaches has the potential to generate insights into their biological heterogeneity, having important implications for prognosis and therapy. In this sense, our data highlight the relevance of further studies focusing on the biological and clinical relevance of the tumor-autonomous molecular clock machinery. Overall, we demonstrated that, in metastatic melanoma, a high bulk *BMAL1* expression seems to be associated with a "too tumorigenic" program and could be a marker for immunotherapy response.

ETHICS STATEMENT

All data presented in this manuscript are public and freely available. We did not perform any human or animal related experiments. All analyses and conclusions were drawn from the following public datasets: The Cancer Genome Atlas (TCGA), Genotype-Tissue Expression (GTEx), Gene Expression Omnibus, and datasets from Ref. (40, 42). In all mentioned

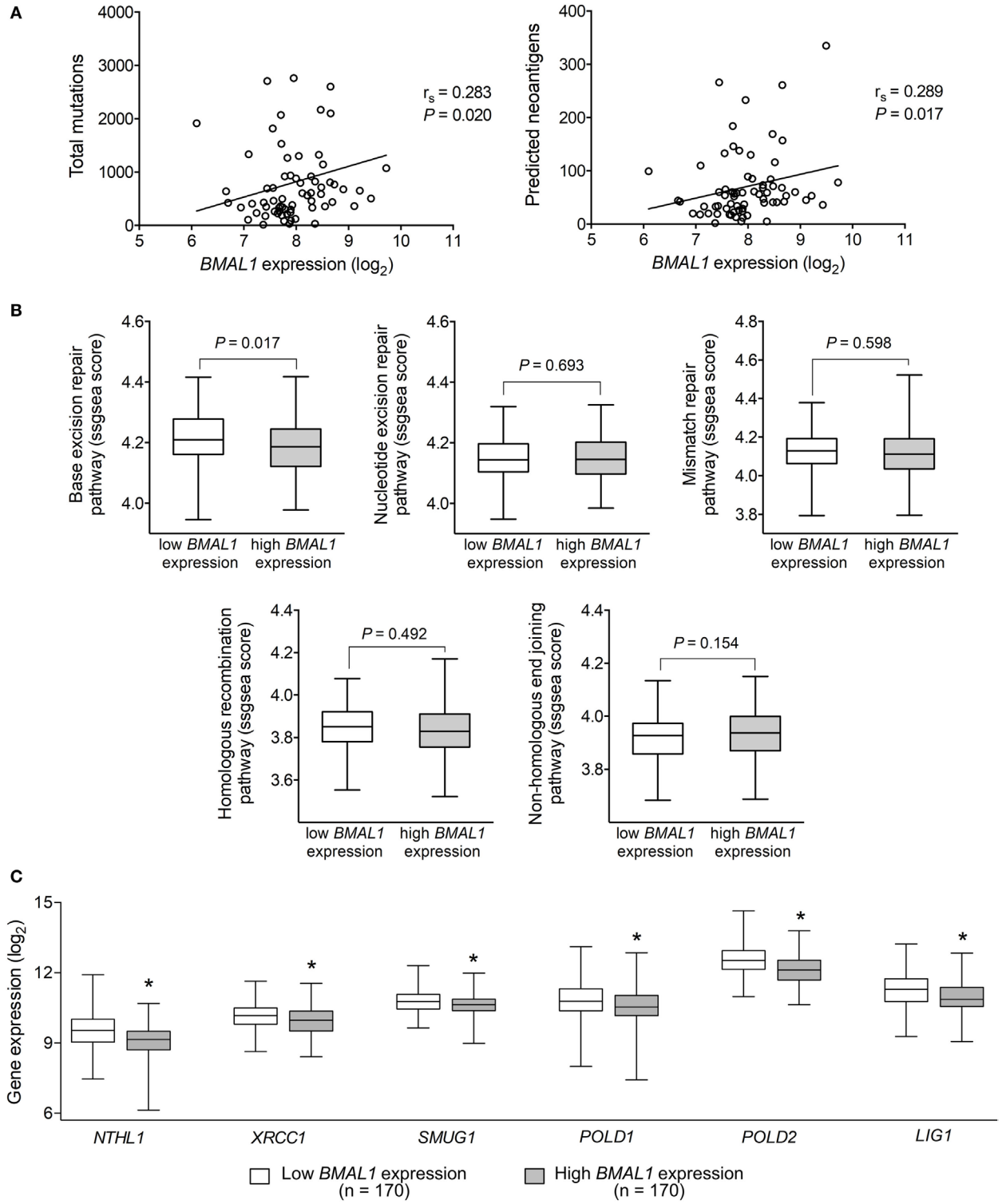


FIGURE 3 | Aryl hydrocarbon receptor nuclear translocator-like protein 1 (*BMAL1*) expression positively correlates with the mutational and neoantigen load in metastatic melanomas. **(A)** Spearman's correlation between the expression of *BMAL1* and the number of total mutations and predicted neoantigens in The Cancer Genome Atlas (TCGA) metastatic melanomas (n = 68). NetMHCpan-predicted neoantigens were obtained from Ref. (40). Gene expression of **(B)** DNA-repair programs and **(C)** selected base excision repair enzymes according to the expression of *BMAL1* in TCGA metastatic melanomas (n = 340). Pathway scores were calculated using single sample Gene Set Enrichment Analysis available in the GSVA R package. The median expression of *BMAL1* was used as the cutoff to dichotomize the population. The boxes extend from the 25th to the 75th percentile, the central bold line shows the median, and whiskers are drawn from minimum to maximum values. Comparisons were performed using the two-sided Wilcoxon–Mann–Whitney test. *Significantly different from the low *BMAL1* group.

papers, the authors stated that all procedures were carried out according ethical rules.

AUTHOR CONTRIBUTIONS

LA and GK designed the study, analyzed the data, and drafted the manuscript. All authors provided insightful discussion during data acquisition and aided in the writing process of the manuscript. All authors critically revised the manuscript. All authors have approved the definitive version of the manuscript and agreed to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the study are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fonc.2018.00185/full#supplementary-material.

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Indoleamine 2,3-Dioxygenase Expression Pattern in the Tumor Microenvironment Predicts Clinical Outcome in Early Stage Cervical Cancer

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The indoleamine 2,3-dioxygenase (IDO) enzyme can act as an immunoregulator by inhibiting T cell function *via* the degradation of the essential amino acid tryptophan (trp) into kynurenine (kyn) and its derivates. The kyn/trp ratio in serum is a prognostic factor for cervical cancer patients; however, information about the relationship between serum levels and IDO expression in the tumor is lacking. IDO expression was studied in 71 primary and 14 paired metastatic cervical cancer samples by various immunohistochemical (IHC) techniques, including 7-color fluorescent multiparameter IHC, and the link between the concentration of IDO metabolites in serum, clinicopathological characteristics, and the presence of (proliferating) T cells (CD8, Ki67, and FoxP3) was examined. In addition, we compared the relationships between *IDO1* and *IFNG* gene expression and clinical parameters using RNAseq data from 144 cervical tumor samples published by The Cancer Genome Atlas (TCGA). Here, we demonstrate that patchy tumor IDO expression is associated with an increased systemic kyn/trp ratio in cervical cancer ($P = 0.009$), whereas marginal tumor expression at the interface with the stroma is linked to improved disease-free (DFS) ($P = 0.017$) and disease-specific survival ($P = 0.043$). The latter may be related to T cell infiltration and localized IFNγ release inducing IDO expression. Indeed, TCGA analysis of 144 cervical tumor samples revealed a strong and positive correlation between *IDO1* and *IFNG* mRNA expression levels ($P < 0.001$) and a significant association with improved DFS for high *IDO1* and *IFNG* transcript levels ($P = 0.031$). Unexpectedly, IDO+ tumors had higher $CD8^+Ki67^+$ T cell rates ($P = 0.004$). Our data thus indicate that the serum kyn/trp ratio and IDO expression in primary tumor samples are not clear-cut biomarkers for prognosis and stratification of patients with early stage cervical cancer for clinical trials implementing IDO inhibitors. Rather, a marginal IDO expression pattern in the tumor dominantly predicts favorable outcome, which might be related to IFNγ release in the cervical tumor microenvironment.

Keywords: cervical cancer, indoleamine 2,3-dioxygenase, kynurenine, tryptophan, serum, T cells, mRNA, The Cancer Genome Atlas

INTRODUCTION

In cervical cancer, a persistent infection with high-risk human papillomavirus strains (mainly types 16 and -18) is responsible for initiating carcinogenesis (1). Expression of the viral E6 and E7 oncogenes is instrumental in this process, and thereby, cervical cancer is a relatively immunogenic disease, employing various escape mechanisms to avoid the host's immune attack (2).

One of these putative tumor escape mechanisms is the expression of indoleamine 2,3-dioxygenase (IDO), which might be induced by IFNγ secretion by cytotoxic $CD8^+$ T cells in the tumor microenvironment (3, 4). IDO is an intracellular enzyme that is able to catabolize tryptophan along the kynurenine pathway. Tryptophan is an essential amino acid, necessary for protein synthesis and other metabolic cell functions. Contradictory results have been reported about the actual effect of tryptophan depletion (5). Mostly, *in vitro* and *in vivo* mice studies have shown that particularly activated, not resting, T- and natural killer (NK) cells seem to be sensitive to tryptophan-depletion and the presence of kynurenine and its derivates in the microenvironment (6–11). Upon tryptophan depletion, arrest of the cell cycle takes place in the G-phase, which in turn renders T cells more sensitive to apoptosis (6, 12, 13). In addition, it has been shown that IDO-expressing tumors promote differentiation and activation of regulatory T cells (Tregs) (9, 14, 15), which in turn can induce IDO expression in myeloid cells *via* cytotoxic T-lymphocyte-associated protein-4 (CTLA-4)–CD80/86 interactions (16) and recruit myeloid-derived suppressor cells (MDSCs) to the tumor site (17, 18). Whereas the majority of reports point to a detrimental effect of IDO expression and activity on patient outcome in various tumor types (19), others have shown IDO to be associated with favorable outcome (20–26).

In cervical cancer, IDO expression has been observed in primary and metastatic tumor cells and in immune cells, like macrophages, dendritic cells, and NK cells (27–30). In addition, IDO activity, measured by the kynurenine/tryptophan (kyn/trp) ratio, in cervical cancer patients' pretreatment sera has been reported by us and by others to be linked to disease stage and poor prognosis (31, 32). Currently, clinical trials in various tumor types are performed to explore the implementation of IDO inhibitors for cancer therapy (19), but to our knowledge, this does not yet include cervical cancer patients.

Here, for the first time, we searched for a link between IDO expression patterns in the tumor microenvironment and the presence of systemic IDO metabolites in early stage squamous cervical cancer. To this end, we have examined the association between IDO expression patterns in formalin-fixed, paraffin-embedded (FFPE) tumor tissue and the concentrations of IDO metabolites in serum. In addition, we studied the association of IDO expression patterns with clinicopathological features and the presence of proliferating cytotoxic $CD8^+$ T cells and Tregs. Also, we compared the relationships between *IDO1* and *IFNG* gene expression and linked this to survival outcome using RNAseq data from cervical tumor samples published by The Cancer Genome Atlas (TCGA).

Our findings may contribute to the development of predictive biomarkers for clinical trials using IDO inhibitors and to the development of new and more effective immunotherapy strategies for cervical cancer.

MATERIALS AND METHODS

Patient Cohort

Previously, we reported on the measurement of serum levels of IDO metabolites (tryptophan, kynurenine, and 3-hydroxykynurenine) in 251 cervical cancer patients (32). From this cohort, we selected all squamous cell carcinoma patients, diagnosed between 2003 and 2008, with surgery as primary treatment and with sufficient FFPE material available for our study. FFPE tissue blocks with 71 primary tumors (PTs) and 14 paired metastatic lymph nodes were obtained from the archives of the Department of Pathology at the Academic Medical Center (AMC) Amsterdam, The Netherlands. The main clinicopathological features of these patients are summarized in **Table 1**. None of the patients underwent chemotherapy or radiotherapy before surgery. The specimens were anonymously processed and selection of blocks was guided by initial diagnosis and review by the pathologist. Ethical approval was waived according to the regulations in The Netherlands (33).

Immunohistochemistry

Immunohistochemical staining of 71 PTs and 14 metastatic lymph nodes was performed as previously described (34) using Tris/EDTA buffer pH 9.0 for antigen retrieval, mouse-IgG1 anti-IDO antibody (1F8.2, Millipore), and ready to use Poly-HRP-GAM/R/R IgG (ImmunoLogic, The Netherlands). Complexes were visualized using 3,3′-diaminobenzidine tetrahydrochloride (Sigma, USA). Slides were counterstained with hematoxylin.

Multiplex Immunohistochemistry

On a representative subset of patients, quadruple immunofluorescence staining was performed as previously described (35) using Tris/EDTA buffer pH 9.0 for antigen retrieval. Primary antibodies mouse-IgG1 anti-FoxP3 (236A/E7; Abcam, UK), rabbit anti-Ki67 (SP6; ThermoFisher, USA), mouse-IgG2b anti-CD8 (4B11; Novocastra, UK), and secondary antibodies goat anti-mouse IgG1 Alexa Fluor 488, goat anti-Rabbit IgG Alexa Fluor 546, and goat anti-mouse IgG2b Alexa Fluor 647 (all from Thermo Scientific, USA) were used for T cell phenotyping ($n = 35$). Primary antibodies mouse-IgG1 anti-IDO (1F8.2, Millipore), mouse-IgG2a CD14 (clone 7, Abcam), rabbit anti-HLA-DR (ab137832, Abcam), and secondary antibodies goat anti-mouse IgG1 Alexa Fluor 488, goat anti-mouse IgG2a Alexa Fluor 546, and goat anti-Rabbit IgG Alexa Fluor 647 (all from Thermo Scientific, USA) were used for IDO-positive myeloid cell identification ($n = 6$). 4',6-diamidino-2′-phenylindole, dihydrochloride (DAPI; Thermo Scientific, USA) was used as a counterstain, slides were enclosed with mounting medium and coverslips.

Multiplexed immunofluorescence staining was performed on eight patients in order to identify the type of tumor-associated vessels expressing IDO, using the OPAL 7-color fluorescence immunohistochemistry (IHC) Kit (Perkin Elmer, USA). A blocking step for endogenous peroxidase was introduced with

TABLE 1 | Patient distribution according to indoleamine 2,3-dioxygenase (IDO) expression in relation to clinicopathological characteristics.

			Tumor cells IDO expression			Tumor cells IDO expression pattern				Tumor-infiltrating immune cells			Stromal immune cells			Tumor-associated vessels		
Clinicopathological characteristics		**Total *n* (%)**	**IDO− *n* (%)**	**IDO+ *n* (%)**	***P***	**Patchy *n* (%)**	**Patchy + margin *n* (%)**	**Margin *n* (%)**	***P***	**IDO− *n* (%)**	**IDO+ *n* (%)**	***P***	**IDO− *n* (%)**	**IDO+ *n* (%)**	***P***	**IDO− *n* (%)**	**IDO+ *n* (%)**	***P***
Number of patients		71 (100)	15 (21)	56 (79)	–	33 (63)	14 (26)	6 (11)	–	33 (54)	28 (46)	–	7 (10)	61 (90)	–	60 (87)	9 (13)	–
Age in years*		44.9	39.0	46.5	***0.010***	45.6	49.4	44.8	*0.499*	43.3	48.1	*0.116*	44.7	45.5	*0.911*	44.8	48.4	*0.276*
FIGO stage[#]	IBI	55 (77)	10 (14)	45 (63)	*0.260*	25 (47)	11 (21)	6 (11)	*0.535*	23 (38)	24 (39)	*0.222*	4 (6)	50 (74)	*0.147*	48 (70)	6 (9)	*0.396*
	≥IBII	16 (23)	5 (7)	11 (16)		8 (15)	3 (6)	0 (0)		10 (16)	4 (7)		3 (4)	11 (16)		12 (17)	3 (4)	
Tumor size[#,a]	≤4 cm	59 (84)	10 (14)	49 (70)	***0.034***	28 (54)	13 (25)	6 (12)	*1.000*	27 (45)	23 (38)	*1.000*	6 (9)	51 (76)	*1.000*	51 (75)	7 (10)	*0.611*
	>4 cm	11 (16)	5 (7)	6 (9)		4 (8)	1 (2)	0 (0)		6 (10)	4 (7)		1 (2)	9 (13)		8 (12)	2 (3)	
Parametrium invasion[#]	No	57 (80)	13 (18)	44 (62)	*0.719*	26 (49)	10 (19)	5 (9)	*0.886*	25 (41)	22 (36)	*0.795*	5 (7)	50 (74)	*0.611*	53 (77)	3 (4)	***0.001***
	Yes	14 (20)	2 (3)	12 (17)		7 (13)	4 (8)	1 (2)		8 (13)	6 (10)		2 (3)	11 (16)		7 (10)	6 (9)	
Vaginal involvement[#]	No	65 (91)	13 (18)	52 (73)	*0.600*	31 (58)	12 (23)	6 (11)	*0.739*	28 (46)	27 (44)	*0.205*	5 (7)	57 (84)	*0.112*	56 (81)	7 (10)	*0.172*
	Yes	6 (9)	2 (3)	4 (6)		2 (4)	2 (4)	0 (0)		5 (8)	1 (2)		2 (3)	4 (6)		4 (6)	2 (3)	
Lymph node metastases	No	49 (69)	10 (14)	39 (55)	*0.825*	22 (42)	9 (17)	5 (9)	*0.824*	18 (30)	24 (39)	***0.012***	4 (6)	45 (66)	*0.390*	43 (62)	6 (9)	*0.712*
	Yes	22 (31)	5 (7)	17 (24)		11 (21)	5 (9)	1 (2)		15 (25)	4 (6)		3 (4)	16 (24)		17 (25)	3 (4)	

FIGO: International Federation of Gynecology and Obstetrics.

[a]Of one case we do not have information on tumor size.

[#]P-value measured with Asymptotic Pearson's and Fisher's exact test was used when counts were <5.

**P-value was calculated with Mann–Whitney U test. IDO+, IDO-positive; IDO–, IDO-negative. NB: in three cases, we found it difficult to score IDO+ tumors for their expression pattern (patchy/margin) due to small tumor fields. In some cases (n = 10), we found it difficult to distinguish between IDO-positive immune cells and IDO-positive tumor cells and excluded those cases for scoring expression in infiltrating cells. In some cases (n = 3), we found the staining pattern not convincing due to small stromal fields in the stained tissue section and excluded those cases for scoring stromal IDO+ cells. In some cases (n = 2), we found it difficult to score IDO expression in vessels and excluded those cases.*

P value in bold italic is <0.05.

P value in italic is >0.05

0.3% H_2O_2/methanol for 20 min and an extra fixation step was included for 20 min with 10% neutral buffered formalin (Leica Biosystems, Germany), followed by 2 min in Milli-Q water and 2 min in 0.05% Tween20 in 1× Tris-buffered saline (TBST). The following primary antibodies were used: mouse-IgG1 anti-CD34 (QBEND-10; Cell Sciences), mouse-IgG2a anti-α-smooth muscle (α-sma) actin (1A4; DAKO), mouse-IgG1 anti-CD31 (JC70A; DAKO), mouse-IgG1 anti-IDO (1F8.2, Millipore), mouse-IgG1 anti-podoplanin (D2-40; BIO-RAD), and rabbit anti-galectin-1 (500-P210, PeproTech). Steps were repeated for each primary antibody; microwave treatments were carried out by placing the slides in a plastic tray, after which they were heated in 0.05% ProClin300/Tris-EDTA buffer at pH 9.0 in an 800 W standard microwave at 100% power until boiling point, followed by 15 min at 30% power. Slides were cooled down in ice water, washed in Milli-Q water and in 1× TBST, and were blocked with Normal Antibody Diluent (Immunologic, The Netherlands) for 10 min at room temperature (RT). After that, slides were incubated with primary antibody diluted in Normal Antibody Diluent for 30 min at RT and 30 rounds per minute (rpm) on a shaker. Next, slides were washed 3 × 2 min in 1× TBST at RT and 30 rpm and were subsequently incubated with SuperPicture Polymer Detection Kit—HRP—broad spectrum (Life Technologies, USA) for 20 min at RT and 30 rpm. Afterward, slides were washed 3 × 2 min in 1× TBST and were incubated with Opal fluorochromes (Opal520, Opal570, Opal650, Opal690 Opal540, and Opal620) diluted 1:150 in amplification buffer (all provided by the OPAL 7-color fluorescence IHC Kit) for 10 min at RT and 30 rpm. Slides were then washed 3 × 2 min in TBST. Finally, microwave treatment with AR6 buffer was performed and slides were washed for 2 min in Milli-Q water and for 2 min in TSBT. DAPI working solution (provided by the OPAL 7-color fluorescence IHC Kit) was applied for 5 min at RT and the slides were washed again in Milli-Q water and in 1× TSBT, and then mounted under coverslips with ProLong Diamond antifade mounting medium (Life Technologies, USA).

Imaging and Scoring

The standard IHC stained sections were scored for IDO expression by tumor cells, immune cells, and tumor-associated vessels using an Olympus BX50 bright-field microscope (Olympus, USA) by two investigators (A. Marijne Heeren and Ekaterina S. Jordanova). Primary- and metastatic tumors were designated IDO-negative (<1% of tumor cells expressed IDO) or IDO-positive (≥1% of the tumor cells expressed IDO). Also, tumor cells were divided in different groups: 0, 1–10, 10–50, and >50% positive for IDO, also used by others (27, 30). Furthermore, a distinction was made between patchy (patchy IDO expression throughout the whole tumor field) or marginal (focal staining, on the border between tumor and stroma) expression by tumors. Also, the presence of IDO-expressing tumor-infiltrating immune cells was scored in primary- and metastatic tumor samples either as absent (−) or present (+). Furthermore, IDO expression by stromal immune cells was scored as either present in low numbers (−) or high numbers (+) in stroma of PTs. In the metastatic lymph nodes, scores for IDO-positive immune cells were obtained, high (+) or low (−) numbers, for peritumoral area or in resident lymph node tissue distant from the tumor metastases. Finally, IDO expression by tumor-associated vessels was scored as 0/a few IDO-positive vessels (−) or all vessels positive for IDO (+).

Quadruple immunofluorescence stainings were imaged and analyzed using a digital imaging fluorescence microscope (Axiovert-200M; Zeiss, Germany) and SlideBook 6 Reader [Intelligent Imaging Innovations (3I), USA]. DAPI staining was used to morphologically distinguish tumor fields from stromal and healthy tissue. From each PT, three to five representative areas, including both tumor and stroma, were randomly selected and imaged with a 20× dry objective with 0.3 numerical aperture (NA). CD8-positive, FoxP3-positive, and Ki67-positive cells from digital images were manually enumerated and results were expressed as number of positive cells per square millimeters.

Seven-color multiplex stainings were visualized with Leica TCS SP8 microscope (Leica, Germany), tilescan (3 × 3, 40× oil objective with 1.3 NA) images were generated and viewed using LAS AF Lite software (Leica, Germany). IDO-positive tumor-associated vessels were analyzed for colocalization with the markers CD34, podoplanin, α-sma, galectin-1, and CD31.

TCGA RNAseq Patient Cohort

Level 3 RSEM normalized, log-transformed RNAseq data, profiled using the Illumina HiSeq RNAseq v2, were retrieved from the TCGA data portal (36). Results of the TCGA RNAseq analysis have been described in detail by TCGA Research Network (37). For our analysis, data on 144 cervical SCC patients were used, including downloaded survival data (38), *IDO1* mRNA, and *IFNG* mRNA expression in PT samples.

Statistical Analysis

Statistical analyses were performed using IBM SPSS (IBM, USA) and GraphPad Prism 5 (GraphPad Software, USA) software. Associations between IDO expression patterns in the tumor microenvironment and serum concentrations were performed using the same cutoff "low" (quartiles 1–3) and "high" (quartile 4) as previously been ascribed for survival analysis (32). Fisher's exact test was used to study the association between IDO expression in the tumor microenvironment and serum concentrations of IDO metabolites for (sub)groups with three or more patients. The Mann–Whitney *U* test, Asymptotic Pearson's- or Fisher's exact tests were used for the comparison of IDO expression patterns and clinicopathological characteristics. The log-rank test was performed for survival analyses. Before association, analyses between local IDO protein expression and T cells were carried out, normal distribution was tested using the D'Agostino and Pearson omnibus normality test. Then, based on the observed distribution, Mann–Whitney *U* test or unpaired *t* test were used or the Kruskal–Wallis or one-way ANOVA, with *post hoc* Dunn's Multiple Comparison or Bonferroni's Multiple comparison tests, respectively. Furthermore, correlation analysis between *IDO1* and *IFNG* mRNA levels, retrieved form the TCGA database, was performed by Pearson's correlation. Hierarchical cluster analysis was carried out using Euclidean distance and Ward.D2 clustering methods with the function heatmap.plus in RStudio Version 1.1.423 (RStudio, USA). Survival analysis for *IDO1* mRNA and *IFNG* mRNA were performed using the two acquired clusters (low and high) or using the median as cutoff (low and high).

Comparisons and associations with *P*-values below 0.05 were considered statistically significantly different.

RESULTS

IDO Expression in PTs

Indoleamine 2,3-dioxygenase protein expression was analyzed by immunohistochemistry. In the PT samples, we observed IDO positivity in tumor cells in a patchy and/or marginal expression pattern. Also, IDO expression was seen in tumor-infiltrating immune cells, stromal immune cells, and tumor-associated vessels. Among patients, various heterogeneous IDO expression patterns were observed (**Figures 1A–F** for representative images and Table S1 in Supplementary Material for IDO IHC scores per patient).

Next, we aimed to further delineate the specific cell subpopulations and vessel types expressing IDO. We hypothesized that most IDO-positive immune cells were monocytic MDSCs or tumor-associated macrophages and tried to identify these cells using multicolor fluorescent immunohistochemistry for IDO, CD14, and HLA-DR in PT section from six cervical cancer patients. IDO-positive tumor- and stroma-infiltrating cells represented a heterogeneous population of immune cells consisting of HLA-DR$^-$CD14$^+$IDO$^+$ MDSC-like cells, HLA-DR$^+$CD14$^+$IDO$^+$ dendritic/macrophage-like cells, HLA-DR$^-$CD14$^-$IDO$^+$-, and HLA-DR$^+$CD14$^-$IDO$^+$ cells (**Figure 2A**). IDO-positive tumor-associated vessels were studied by 7-color multiplex immunohistochemistry using the markers CD31/CD34 (endothelial cell markers), podoplanin (lymphatic endothelial cell marker), α-sma (perivascular cell marker), galectin-1 (activated endothelial cell marker), and IDO. The IDO-positive tumor-associated vessels were predominantly identified as mature blood vessels since most vessels stained positive for α-sma, a marker of pericytes that cover mature vessels (**Figure 2B**). In two patients, IDO expression was also observed in lymphatic (podoplanin-positive) vessels.

IDO Expression in Metastatic Lymph Nodes

In the 14 metastatic lymph node specimens available, we observed IDO positivity in tumor cells, tumor-infiltrating immune cells, immune cells surrounding metastatic tumor cells, and in resident lymph node tissue (**Figures 1G,H**). No IDO-positive vessels were observed. See Table S2 in Supplementary Material for an overview of the IDO IHC scores.

We found no evidence for elevated expression of IDO in the metastatic tumors as compared to the corresponding primary lesions. In one out of 14 metastatic lymph nodes, tumor cells were not detectable in the available tissue sections. In 8 out of 14 metastatic samples, IDO-positive tumor cells were detected. Interestingly, 7 out of 8 metastatic tumors showed a patchy IDO expression pattern. Paired analysis showed that six IDO patchy/patchy + margin expressing PTs retained patchy expression in the metastatic tumor cells, one marginal IDO-expressing PT had a patchy IDO-expressing metastatic tumor, one patchy IDO-expressing PT had a marginal IDO-expressing metastatic tumor, two IDO-negative PTs remained negative for IDO in the metastatic tumor cells and for three IDO-positive (patchy) PTs, corresponding metastatic tumors were negative for IDO (data not shown).

Association Between IDO Expression at the Tumor Site and kyn/trp Ratio in Serum

To determine, in our patient cohort ($n = 71$), whether IDO-positivity in the PT microenvironment correlates with serum levels of IDO metabolites tryptophan, kynurenine, and 3-hydroxykynurenine, we used previously measured serum levels from a cohort of 251 cervical cancer patients where a high kyn/trp ratio was shown to be detrimental for survival (32). The interquartile concentrations of tryptophan, kynurenine, and 3-hydroxykynurenine and the kyn/trp ratio for the current patient cohort are summarized in **Table 2** and were used for analysis.

We analyzed whether IDO expression in the local tumor microenvironment influenced the levels of IDO metabolites in serum. Notably, we found a significant association between IDO positivity in the PT and a high kyn/trp ratio in serum ($P = 0.008$, Fisher's exact test) (**Figure 3A**), independent of IDO expression by immune cells (infiltrate and stroma) (**Figure 3B**). Furthermore, patients with both IDO-positive tumors and vessels had significantly more often a high kyn/trp ratio in serum compared to patients with both IDO-negative tumors and vessels ($P = 0.001$, pairwise Fisher's exact test) and patients with IDO-positive tumors and IDO-negative vessels ($P = 0.025$, pairwise Fisher's exact test) (**Figure 3C**). Interestingly, we found that the dominance of tumor cell expression on systemic serum levels was independent of the percentage IDO-positive tumor cells (**Figure 3D**), but that the serum kyn/trp ratio was apparently determined by the different IDO expression patterns of the PT. All patients with IDO-negative tumors and marginal IDO-expressing tumors had a low kyn/trp ratio, whereas patients with patchy/patchy + marginal IDO expression had more often a high kyn/trp ratio in serum, which was significantly elevated when compared to patients with IDO-negative tumors ($P = 0.009$ and $P = 0.017$ respectively, pairwise Fisher's exact test) (**Figure 3E**).

No associations were found for IDO positivity in the PT microenvironment and the individual IDO metabolites tryptophan, kynurenine, and 3-hydroxykynurenine in serum. Of note, the number of metastatic lymph nodes analyzed was too small for association analysis of IDO expression with serum kyn/trp levels.

IDO Expression in Relation to Clinicopathological Characteristics and Survival

In **Table 1**, the associations between IDO expression patterns and clinicopathological characteristics of the patient cohort are shown.

Interestingly, patients with IDO-positive tumors were older (46.5 vs. 39 years old) ($P = 0.010$, Mann–Whitney *U* test) and manifested more often with smaller tumors (≤4 cm) ($P = 0.034$, Asymptotic Pearson's χ^2-test). However, no difference was observed in survival outcome between patients with IDO-negative and IDO-positive tumors *per se* (**Figures 4A,B**). Remarkably, all patients with marginal, including combined patchy + margin,

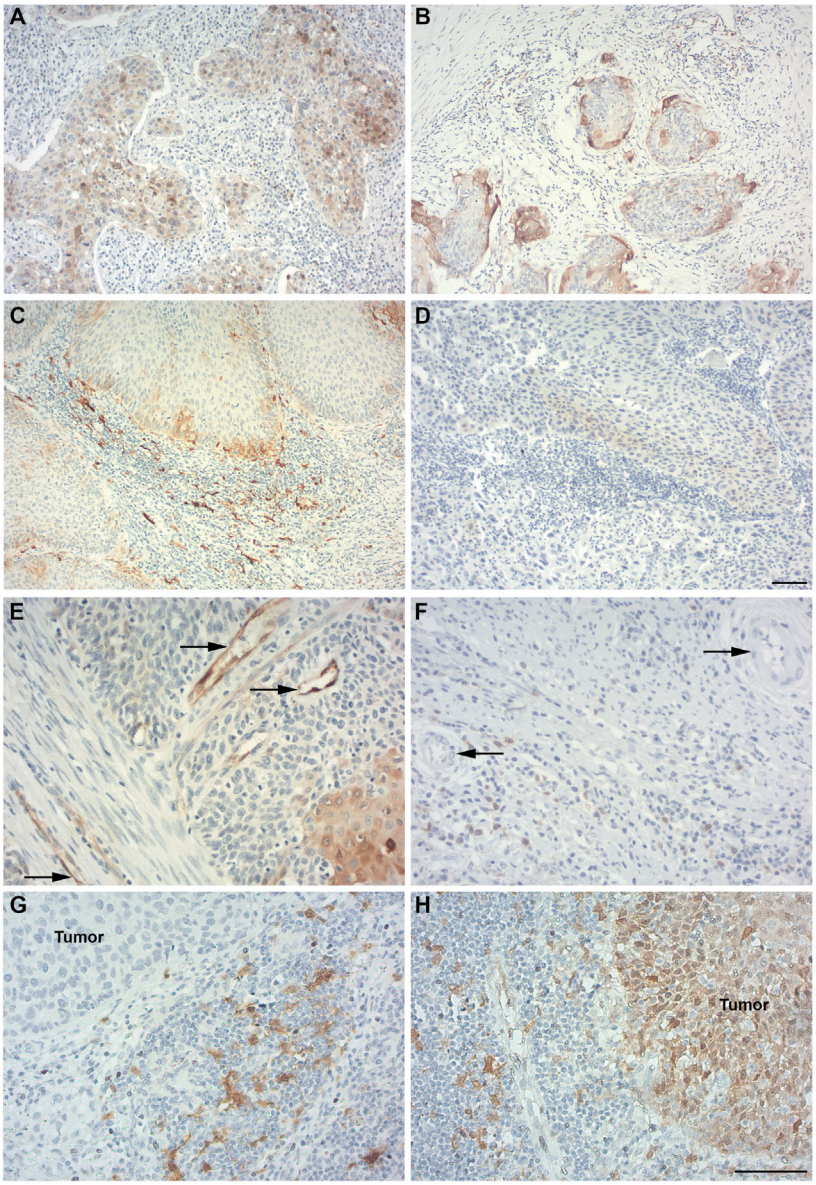

FIGURE 1 | Indoleamine 2,3-dioxygenase (IDO) expression in primary cervical cancer and metastatic lymph nodes. Different patterns for IDO expression (in brown) were detected in primary- and metastatic cervical squamous cell carcinoma. **(A)** Patchy pattern with cytoplasmic IDO expression by primary tumor (PT) cells. **(B)** Marginal IDO expression by PT cells. **(C)** High numbers of IDO-expressing stromal immune cells in a marginal IDO-expressing tumor. **(D)** IDO-negative tumors, with low IDO expression in PT cells and stromal immune cells. **(E)** IDO-positive tumor-associated vessels (indicated by black arrows). **(F)** IDO-negative tumor-associated vessels (indicated by black arrows). **(G)** Metastatic lymph node sample showing metastatic tumor cells negative for IDO and IDO-positive immune cells surrounding the tumor fields. **(H)** Metastatic lymph node sample showing nuclear and cytoplasmic IDO expression by metastatic tumor cells and IDO-positive immune cells. Magnification for **(A–D)** is 100× [scale bar in **(D)** is 100 μm] and for **(E–H)** [scale bar in **(H)** is 100 μm] is 200×.

IDO expression were disease free and still alive after a median follow-up of 60 months. These patients had improved disease-free survival (DFS) ($P = 0.017$, log-rank test) and disease-specific survival (DSS) ($P = 0.043$, log-rank test) as compared to patients with patchy IDO expression only (**Figures 4C,D**).

In addition, patients with IDO-positive tumor-infiltrating immune cells had less often lymph node metastases ($P = 0.012$, Fisher's exact test). Interestingly, patients with IDO-negative tumor-associated vessels had less often parametrium invasion ($P = 0.001$, Fisher's exact test). No further significant correlations were found. IDO expression in tumor-infiltrating immune cells and tumor-associated vessels did not affect survival (data not shown).

IDO Expression in Relation to the Distribution and Localization of T Cells

Next, in order to study the effect of IDO expression on tumor-infiltrating T cell numbers, we quantified cytotoxic

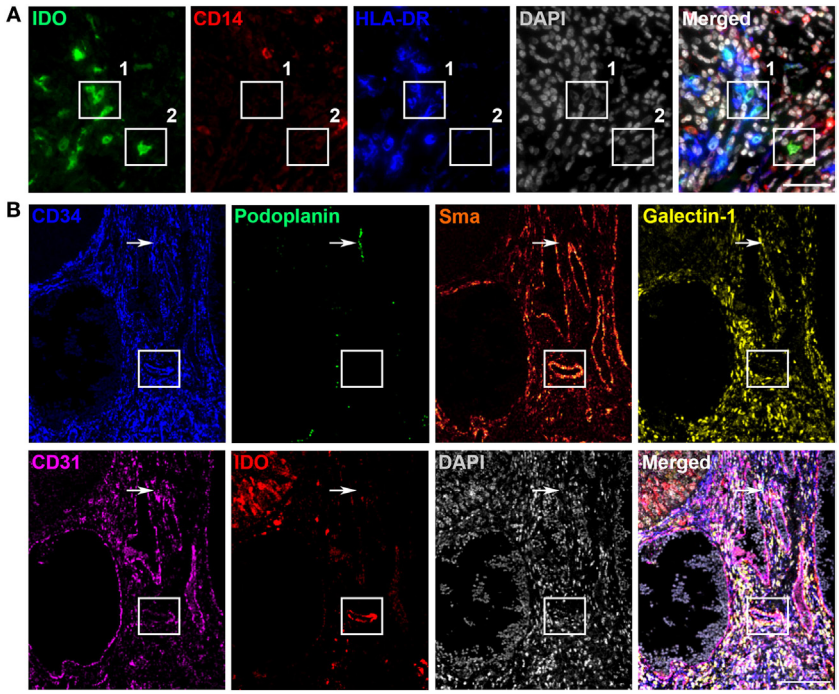

FIGURE 2 | Characterization of indoleamine 2,3-dioxygenase (IDO)-positive immune cells and tumor-associated vessels in primary cervical cancer. Different immune cells expressing IDO were detected in primary cervical cancer. **(A)** Representative immunofluorescence images showing monochromatic IDO (in green), CD14 (in red), HLA-DR (in blue), DAPI (in gray), and the merged panel with IDO, CD14, HLA-DR, and DAPI in the stromal compartment. Box 1 shows IDO$^+$CD14$^-$HLA-DR$^+$ cells and box 2 shows an IDO$^+$CD14$^-$HLA-DR$^-$ cell. Scale bar is 100 µm. **(B)** Representative immunofluorescence tilescan of a IDO-positive tumor showing monochromatic CD34 (in blue), podoplanin (in green), α-sma (in glow), galectin-1 (in yellow), CD31 (in pink), IDO (in red), DAPI (in gray), and the merged image. Box indicates an IDO-positive vessel expressing CD34, α-sma, galectin-1, and CD31. Arrow indicates an IDO-negative podoplanin-positive lymphatic vessel. Scale bar is 95 µm.

TABLE 2 | Concentration of indoleamine 2,3-dioxygenase metabolites in serum ($n = 71$).

	Q1	Q2	Q3	Q4
Tryptophan (µmol/L)	10.86–42.36	42.37–49.73	49.74–55.37	55.38–79.88
Kynurenine (µmol/L)	0.22–1.33	1.34–1.50	1.51–1.64	1.65–2.54
3-Hydroxykynurenine (nmol/L)	3.20–24.89	24.90–32.09	32.10–37.79	37.80–84.90
Kyn/Trp ratio	16.78–25.40	25.41–28.62	28.63–34.91	34.92–52.37

Q, quartile.

CD8$^+$ T cells, FoxP3$^+$(CD8$^-$) Tregs, proliferating CD8$^+$Ki67$^+$ T cells, proliferating FoxP3$^+$Ki67$^+$(CD8$^-$) T cells (proliferative Tregs), and FoxP3$^+$CD8$^+$ T cells per square millimeters in a representative subset of patients ($n = 35$) (**Figure 5A**). Nuclear DAPI stain was used to distinguish tumor tissue from stromal tissue. Unexpectedly, we observed higher counts of intratumoral CD8$^+$Ki67$^+$ T cells in IDO-positive tumors as compared to IDO-negative tumors ($P = 0.004$, Mann–Whitney U test) (**Figure 5B**). No significant differences were found between IDO-negative- and IDO-positive tumors for any of the other T cell subtypes (**Figure 5C** and data not shown). Also, the IDO expression patterns, marginal, patchy + marginal, or patchy did not affect infiltrating T cell numbers (**Figures 5D,E** and data not shown).

Furthermore, we observed higher rates of intratumoral cytotoxic CD8$^+$ T cells ($P = 0.041$, Mann–Whitney U test), a higher intratumoral CD8$^+$ T cell/FoxP3$^+$ Treg ratio ($P = 0.012$, unpaired t test), higher rates of CD8$^+$Ki67$^+$ T cells both in the stromal ($P = 0.004$, Mann–Whitney U test) and intratumoral ($P < 0.001$, Mann–Whitney U test) compartment, in tumors with IDO-positive tumor-infiltrating immune cells (Figures S1A–C in Supplementary Material). Significantly higher rates of intratumoral CD8$^+$Ki67$^+$ T cells were observed in total IDO-positive PTs (PT+stroma+infiltrate+) vs. partly IDO-positive PTs (PT+stroma+infiltrate−) (Figure S1D in Supplementary Material, both $P < 0.01$). No further significant associations were found.

IDO1 vs. *IFNG* mRNA Expression

To test whether RNAseq data of PT samples could be used for the validation of IDO protein expression and to study a possible link between IDO and IFNγ (39), we retrieved *IDO1* and *IFNG* gene expression data from 144 cervical SCC patients from TCGA Research Network database (37). Hierarchical clustering revealed two groups: patients with both low *IDO1* and *IFNG* mRNA expression ("Low" group) and patients with both high *IDO1* and *IFNG* mRNA expression ("High" group) (**Figure 6A**). For DSS analysis, no significant associations were found between the two

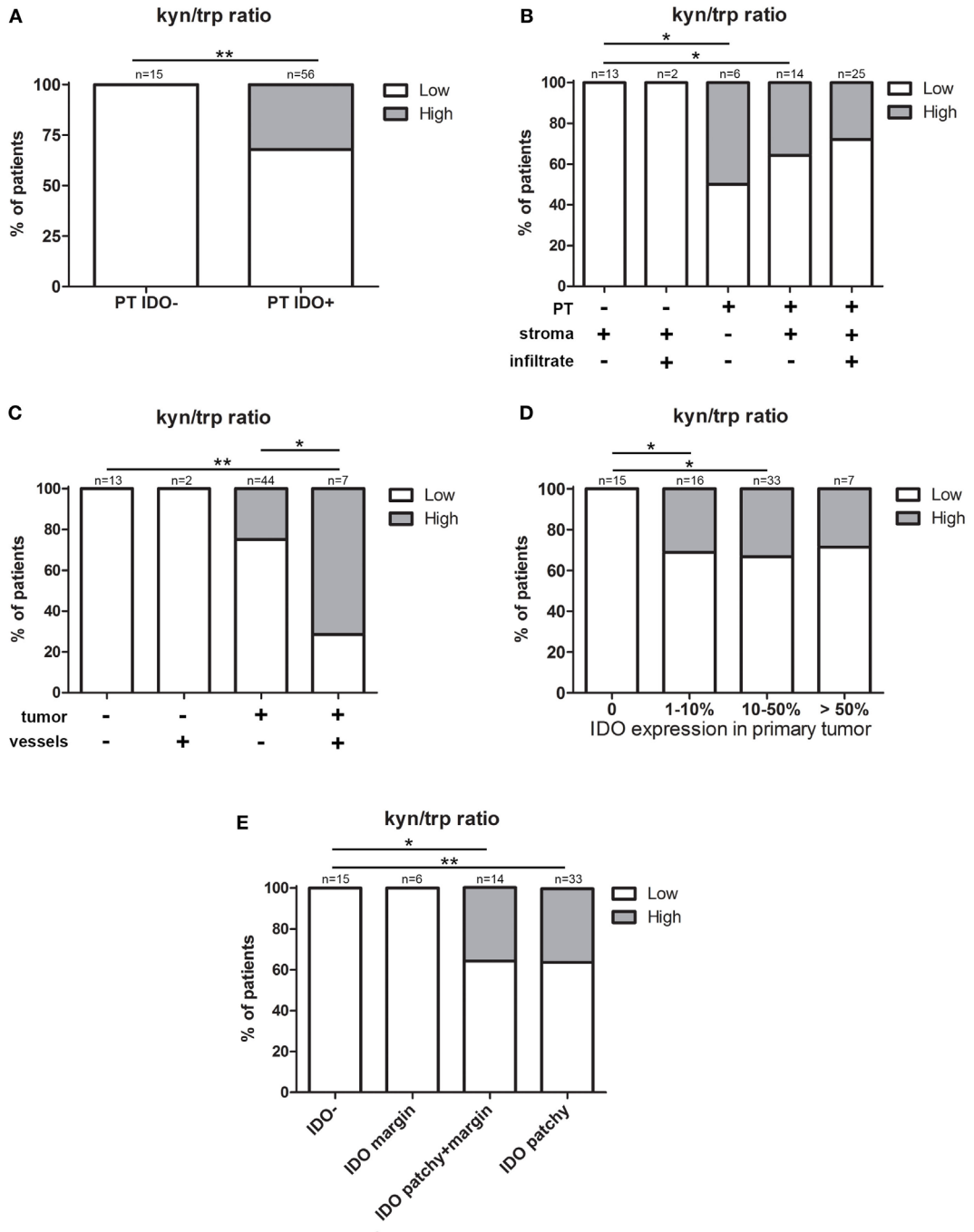

FIGURE 3 | The influence of indoleamine 2,3-dioxygenase (IDO) expression in the local tumor microenvironment on the kyn/trp ratio in serum. Graphs show low (16.78–34.91, white) and high (34.92–52.37, gray) serum kynurenine/tryptophan (kyn/trp) ratio for **(A)** patients with IDO expression (IDO–/IDO+) in primary tumor (PT). Further stratification for expression patterns in **(B)** stroma and infiltrating immune cells (stroma and infiltrate) and **(C)** tumor-associated vessels (vessels). **(D)** Patients with IDO-negative tumors (0%) and patients with IDO-positive tumors divided into groups of 1–10, 10–50, and more than 50% of IDO positivity in tumor cells, and for **(E)** patient groups with different IDO expression patterns including IDO-negative (IDO–), IDO margin, IDO patchy + margin, and patchy IDO expression by PTs. *P* values were calculated excluding subgroups with $n = 2$ or smaller, using (pairwise) Fisher's exact test. $^{*}P = 0.01–0.05$ and $^{**}P = 0.01–0.001$.

patient groups (**Figure 6C**). However, DFS analysis showed an improved outcome for patients with "High" *IDO1* and *IFNG* as compared to patients with "Low" *IDO1* and *IFNG* mRNA expression ($P = 0.031$, log-rank test) (**Figure 6B**). Interestingly, *IDO1* and *IFNG* mRNA expression were strongly and significantly correlated ($P < 0.001$, Pearson's correlation) (**Figure 6D**).

Also, when TCGA tumors were divided into two groups based on above- or below-median *IDO1* mRNA (9.92) and *IFNG*

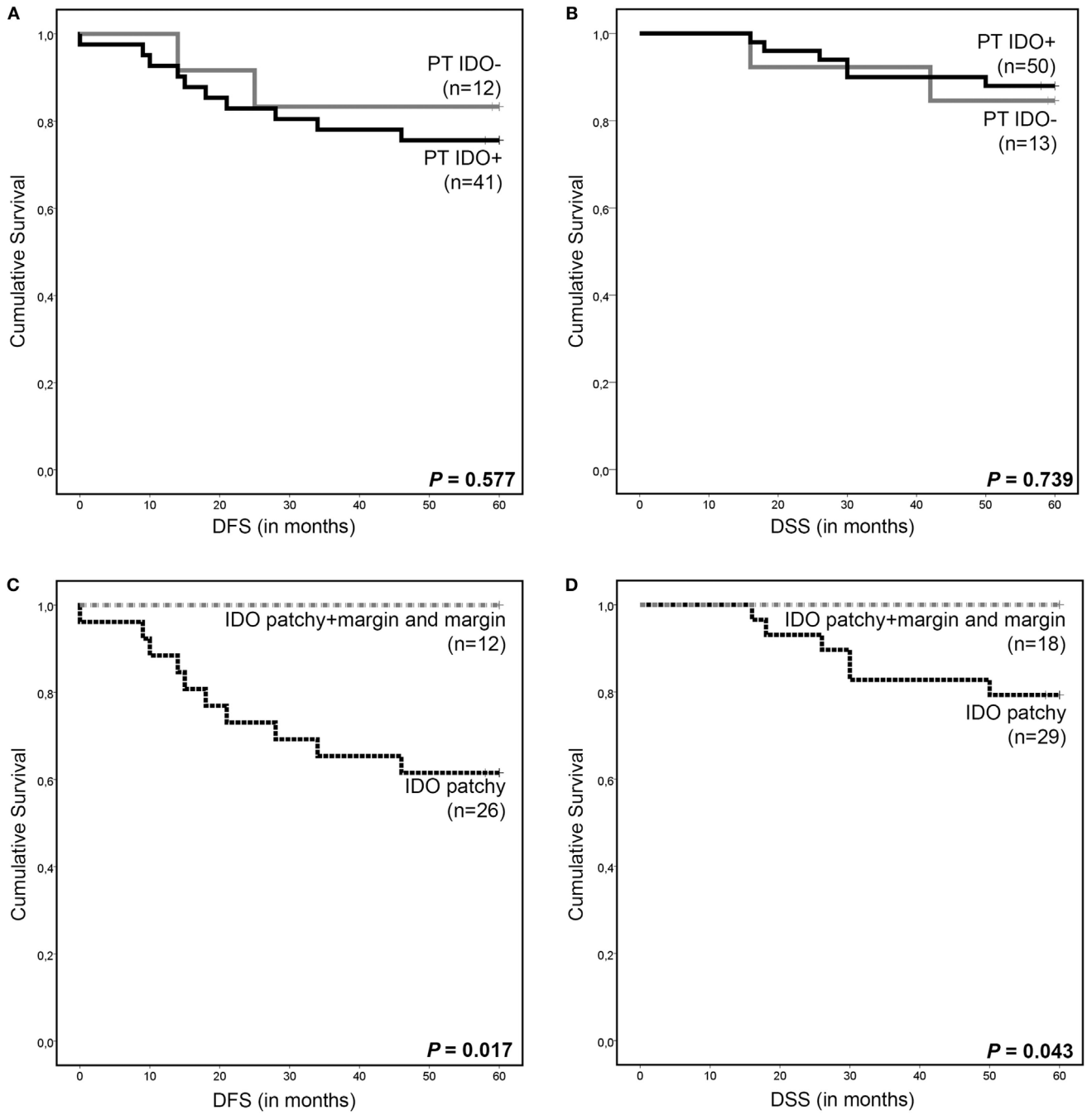

FIGURE 4 | Survival analysis according to indoleamine 2,3-dioxygenase (IDO) expression in cervical cancer. Kaplan–Meier 5-year survival curve shows **(A)** disease-free survival (DFS) and **(B)** disease-specific survival (DSS) for patients with IDO-positive primary tumors (PT IDO+, black line) and IDO-negative (PTs IDO–, gray line). Kaplan–Meier 5-year survival curves show **(C)** DFS and **(D)** DSS for patients with patchy IDO expression (black dotted line), for patients with marginal, including patchy + marginal, IDO expression (gray dotted line) by PT cells. *P* values were calculated between the different groups using the log-rank test. NB: for some patients, DFS and DSS data are missing due to loss of follow-up.

mRNA (3.71) expression levels, *IDO1* mRNA expression was not linked to survival outcome (**Figures 7A,B**), whereas for patients with above median *IFNG* mRNA expression, an improved DFS ($P = 0.008$, log-rank test) and DSS ($P = 0.039$, log-rank test) was observed (**Figures 7C,D**).

DISCUSSION

Expression of the metabolic enzyme IDO is one of the many immune escape mechanisms employed by tumor cells (40). Many clinical trials have investigated, or are currently

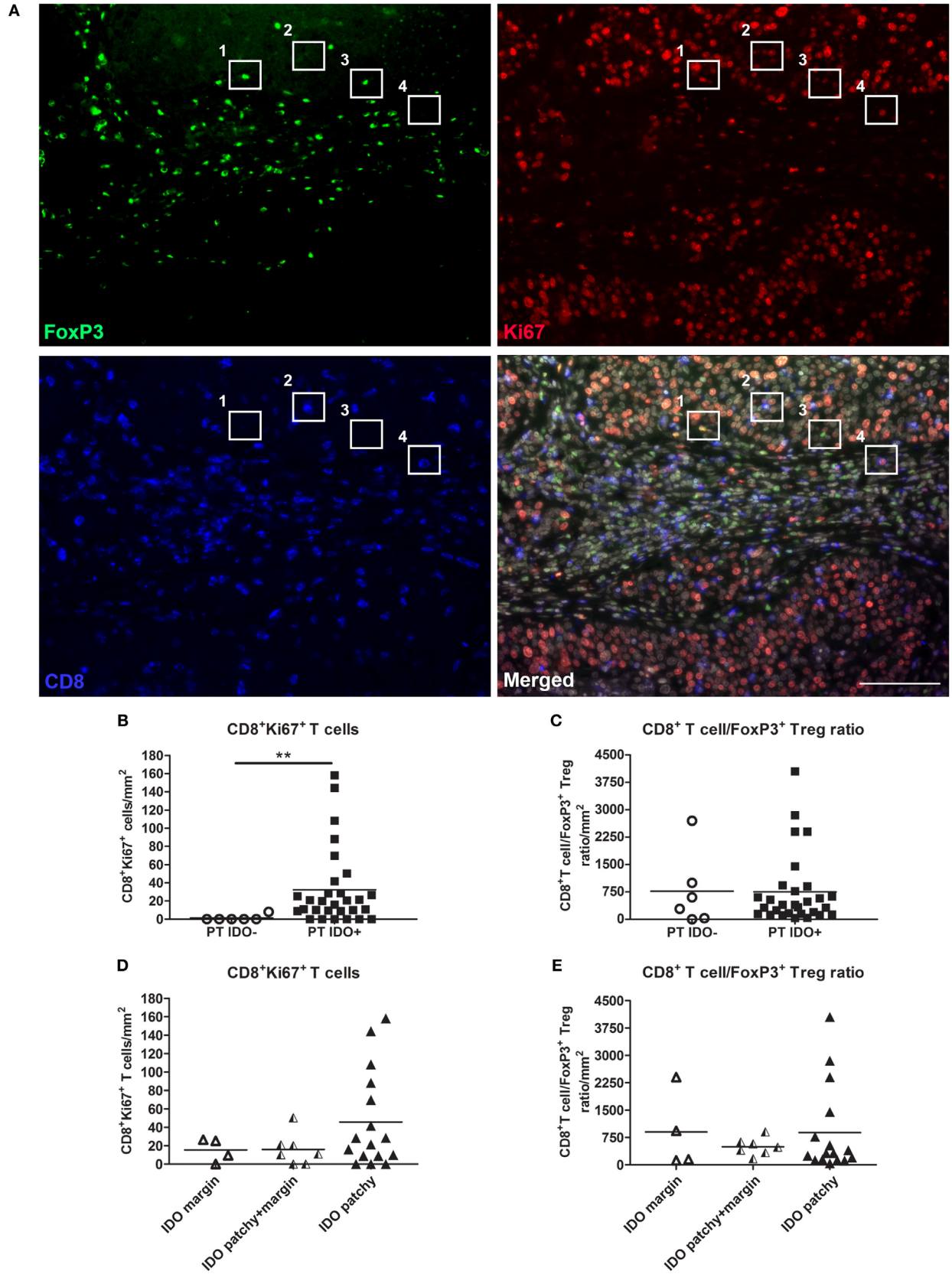

FIGURE 5 | Continued

FIGURE 5 | T cell numbers in relation to indoleamine 2,3-dioxygenase (IDO) expression by primary cervical tumor cells. **(A)** Representative immunofluorescence images showing monochromatic FoxP3 (in green), Ki67 (in red), CD8 (in blue), and the merged panel with FoxP3, Ki67, CD8 and DAPI. In box 1 a proliferating $Ki67^+FoxP3^+$ T cell is depicted, in box 2 a cytotoxic $CD8^+$ T cells is depicted, in box 3 a $FoxP3^+$ Treg is depicted, and in box 4 a proliferating $CD8^+Ki67^+$ T cell is depicted. Scale bar is 100 μm. Scatter plots show intratumoral numbers per square millimeters for **(B)** $CD8^+Ki67^+$ T cells and **(C)** $CD8^+$ T cell/$FoxP3^+$ Treg ratio in IDO-negative ($n = 6$, white dots) and IDO-positive ($n = 29$, black squares) primary tumors (PTs). Scatter plots show intratumoral numbers per square millimeters for **(D)** $CD8^+Ki67^+$ T cells and **(E)** $CD8^+$ T cell/$FoxP3^+$ Treg ratio in PTs with marginal IDO ($n = 4$, white triangles), patchy + marginal IDO ($n = 7$, black/white triangles), and patchy IDO expression ($n = 16$, black triangles). *P* values were calculated using Mann–Whitney *U* test. $^{**}P = 0.004$.

investigating, the effect of IDO inhibitors [i.e., Epacadostat and Indoximod (1-Methyl-D-Tryptophan)], and IDO peptide vaccination in cancer patients (19, 41–44). Currently, patients entering these clinical trials are not stratified for IDO positivity in tumor biopsies and/or systemic kyn/trp levels. Such information could be helpful in order to achieve higher immunotherapy response rates and avoid unnecessary over-treatment. Moreover, it was suggested that IDO activity in serum can be influenced by other factors such as chronic infection, neuropsychiatric diseases, and diet (45–48). Regrettably, extensive studies on the systemic effect of local IDO protein expression are lacking, except for a study in patients with diffuse large B cell lymphoma, which did not find an association between serum kynurenine level and IDO expression in the tumor (49) and a study in prostate cancer wherein a positive correlation between *IDO1* mRNA in PT samples and the kyn/trp ratio in serum was observed (50).

In this study, for the first time, the association between IDO expression in the tumor microenvironment and systemic concentrations of IDO metabolites in cervical cancer patients was comprehensively investigated, using a validated IDO-specific antibody (51). In the current IHC study, we included a subset of patients from the previously reported serum cohort where association between IDO activity and poor survival was observed (32). Interestingly, we did find increased systemic kyn/trp ratio levels in cervical cancer patients with IDO expression by PT cells rather than IDO expression by immune cells. Moreover, the dominance of tumoral IDO expression on kyn/trp serum levels was independent of the percentage of IDO-positive tumor cells, but rather related to patchy IDO expression, with or without marginal IDO expression (at the tumor/stroma interphase), in the PT. Remarkably, this did not directly impact patient survival. This can be explained by the fact that the current cohort consists of patients with early stage of disease (FFPE material is not available for patients with advanced disease), while in the previous serum study, a patient group comprising various disease stages was analyzed. Interestingly, survival analysis showed that patients with marginal IDO expression in the tumor, including combined patchy + marginal expression, manifested with a significantly improved outcome (DFS: $P = 0.017$; DSS: $P = 0.043$). These data are in concordance with another IDO study in cervical cancer by Inaba and colleagues (30). The marginal IDO effect was proposed to be indicative of an effective IFNγ antitumor T cell response inducing, among others, immunomodulatory factors like PD-L1 and IDO expression in tumor cells (39, 52). In line with this hypothesis, we previously reported on an association between marginal PD-L1 expression and improved prognosis in cervical cancer patients (53). In contrast, and in keeping with our PD-L1 data, patchy IDO expression may result from activation of oncogenic signaling pathways leading to intrinsically elevated expression (4, 54). Interestingly, 7 out of 8 metastatic tumors exhibited patchy IDO expression suggesting that this oncogenic signaling is more pronounced in tumors with an aggressive phenotype and poor patient outcome. Possibly, as indicated by the high kyn/trp serum ratio, IDO expression relating to a patchy expression pattern and putative oncogenic signaling occurs at higher levels than the T cell/IFNγ-induced marginal IDO levels. Although, we did not find higher numbers of (proliferating) T cells in tumors with marginal IDO expression, we did confirm a significant correlation between *IDO1* and *IFNG* mRNA expression by analyzing the available TCGA cervical cancer RNA expression data (37). To draw firm conclusions on the role of IFNγ-producing T cells on IDO expression in the complex tumor microenvironment, more in-depth analysis of the location of these cells and corresponding levels of IFNγ relative to IDO-expressing tumor cells should be performed.

Indoleamine 2,3-dioxygenase has been designated as one of the major immune escape mechanisms employed by tumors. In the cervical tumor microenvironment, IDO positivity was observed in tumor cells, immune cells, and in tumor-associated vessels making it a potential therapeutic target. Although the first clinical results on IDO inhibitors show that they are safe and well-tolerated by patients with different tumor types, no major responses have been observed yet (19, 41, 42, 44). IDO inhibitors are not tested yet in cervical cancer. In contrast to other studies that have shown a correlation between IDO expression and lower cytotoxic T cell infiltration rates and higher frequencies of Tregs, as well as an association of IDO levels with poor prognosis in different tumor types, including colorectal cancer (55), endometrial cancer (56, 57), ovarian cancer (58), and breast cancer (59), our findings did not point to a clear-cut association between IDO protein expression and poor patient outcome.

The finding in the current IHC study rather point to IDO expression in tumor cells and in immune cells as a favorable prognostic factor based on association with disease stage (tumor size and lymph node metastases), survival, and infiltration by actively proliferating cytotoxic T cells. In keeping with this notion, we observed a significant correlation between *IDO1* and *IFNG* mRNA expression, with a survival benefit for patients with high levels of *IFNG*, whether or not combined with high levels of *IDO1* expression. A prognostically favorable association for IDO expression has also previously been observed in breast cancer (22, 24), ovarian cancer (60), renal cell cancer (21), vulvar cancer (61), and lung cancer (51). Notably, in literature, there are contradictory results about the actual effect of tryptophan depletion

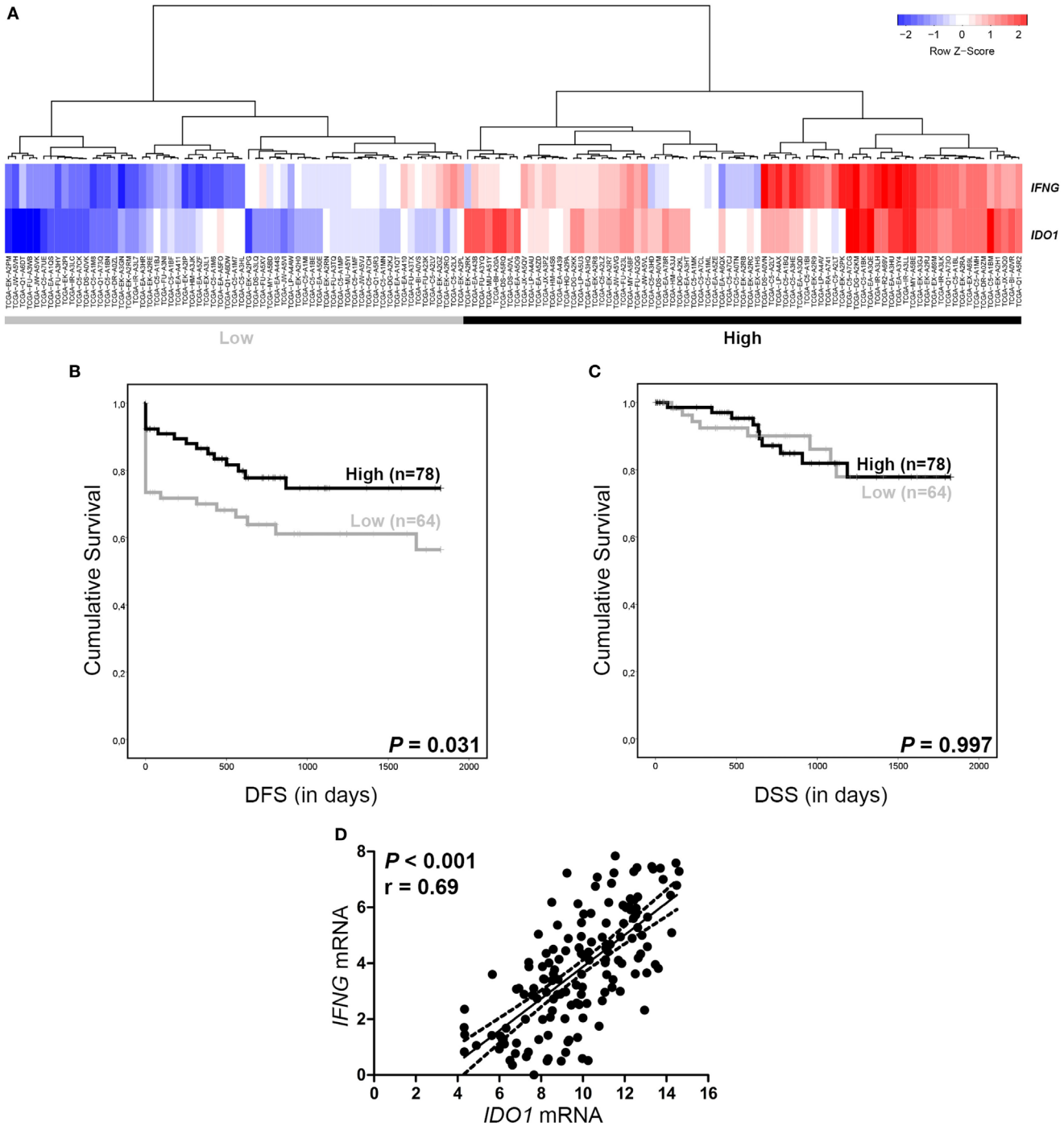

FIGURE 6 | *IDO1* and *IFNG* RNAseq data from The Cancer Genome Atlas. **(A)** Hierarchical clustering of *IDO1* and *IFNG* mRNA (rows) measured on primary tumor samples from 144 cervical SCC patients (columns) reveals a "High" (for both *IDO1* and *IFNG*) and a "Low" (for both *IDO1* and *IFNG*) patient group. Kaplan–Meier 5-year survival curve shows **(B)** disease-free survival (DFS) and **(C)** disease-specific survival (DSS) for patients with both high *IDO1* and *IFNG* (black line) and patients with both low *IDO1* and *IFNG* (gray line), based on hierarchical cluster analysis. **(D)** Graph shows correlation between *IDO1* and *IFNG*. NB: survival data was missing for two patients. *P* values for survival analysis were calculated using the log-rank test. *P* value for correlation analysis was calculated using Pearson's correlation.

on proliferating cells (7, 13, 62), and proof is yet lacking for an immunoregulatory role *in vivo* (5). A recent study using 27 cervical cancer punch biopsies showed a correlation between *IDO1* mRNA levels and a high kyn/trp ratio in primary cervical cancer tissue (63), suggesting the presence of functionally active IDO. However, tryptophan depletion *via* IDO might not be efficient

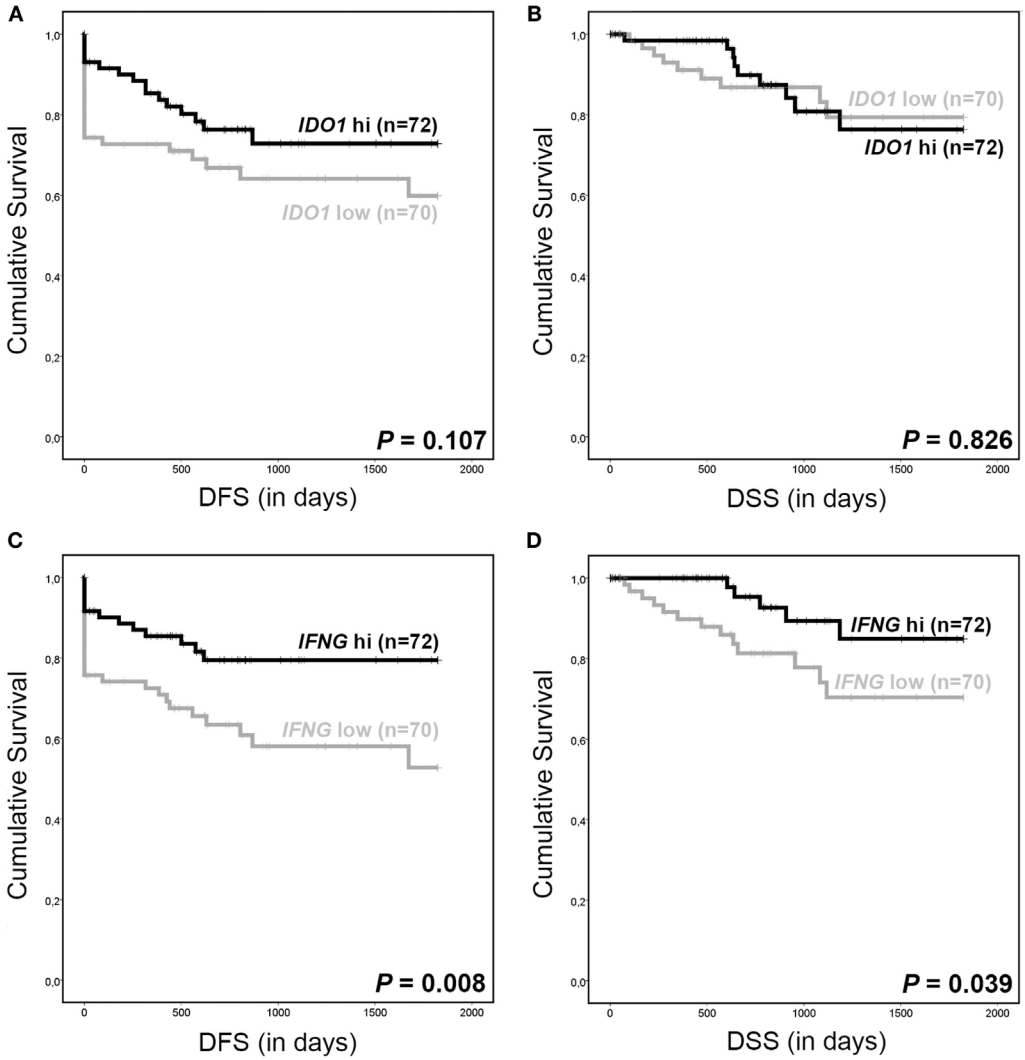

FIGURE 7 | *IDO1* and *IFNG* RNAseq data from The Cancer Genome Atlas. Kaplan–Meier 5-year survival curve shows **(A)** disease-free survival (DFS) and **(B)** disease-specific survival (DSS) for patients with low (gray line, median as cutoff, <9.92) and high (black line, median as cutoff, ≥9.92) *IDO1* mRNA expression. Kaplan–Meier 5-year survival curve shows **(C)** DFS and **(D)** DSS for patients with low (gray line, median as cutoff, <3.71) and high (black line, median as cutoff, ≥3.71) *IFNG* mRNA expression. NB: survival data were missing for two patients. *P* values for survival analysis were calculated using the log-rank test.

enough since tryptophan is able to diffuse rapidly from surrounding tissues into the tumor area (5), or can directly negatively affect the tumor cells themselves (64). This might explain why studies investigating IDO inhibitors in combination with other (immunomodulatory) drugs, like chemotherapy, α-PD-1, and α-CTLA-4, are more promising (43, 65, 66).

This is the first study to apply a multiplex fluorescent immunohistochemistry panel with six different vascular markers for vessel characterization in paraffin-embedded tissue sections. Endothelial IDO expression has previously been reported as an immunoregulatory mechanism in the context of the fetal–maternal interface and of organ transplantation (67–69). In tumors, IDO expression has also been observed in vessels in lymphoma (25, 26), melanoma (70), prostate cancer (50), and renal cell cancer (21). Here, IDO expression was predominantly observed in mature ($CD31^+/CD34^+/\alpha\text{-}sma^+$) tumor-associated blood vessels and in two patients in lymphatic (podoplanin-positive) vessels. IDO-positive vessels were associated with parametrium invasion and higher kyn/trp levels in serum. This is in contrast with another study, which showed IDO to be mainly located in neoangiogenic (CEACAM1-positive) micro-vessels and to correlate with lower rates of tumor cell proliferation (21). However, the number of cases with IDO-positive vessel is small in our study: further analysis on larger cohorts should prove the possible negative effect of IDO-positive vessels on tumor progression.

In conclusion, the effect of IDO in early stage cervical cancer appear to be highly complex. There are several tumor cell expression patterns, many different IDO-positive myeloid cell subtypes as well as varying IDO expression in the vasculature in the tumor microenvironment. Despite this complexity, we have found a dominant effect of patchy IDO expression

by PT cells on kyn/trp ratio in serum. Remarkably, marginal IDO expression in tumor fields, independent of the presence of simultaneous patchy IDO expression, was associated with 100% 5-year DSS and DFS. In these patients, the ongoing IFNγ T cell response most likely outweighs any putatively detrimental effect of tryptophan depletion and resulting IDO metabolites. In conclusion, the kyn/trp ratio in serum and *IDO1* mRNA and protein expression *per se* in PTs cannot be used as a clear-cut biomarker for prognosis or to identify early stage cervical cancer patients eligible for clinical trials targeting IDO. Rather, the IDO protein expression patterns in the PT seem vital in this regard.

ETHICS STATEMENT

The specimens were anonymously processed and selection of blocks was guided by initial diagnosis and review by the pathologist. Ethical approval was waived according to the regulations in The Netherlands (http://www.federa.org, 2011).

AUTHOR CONTRIBUTIONS

AH performed the experiments, analyzed the data, and wrote the manuscript. ID performed the experiments and analyzed the data. DB and MK performed the experiments. JK and RM performed technical assistance for microscopy. CM, GK, DF and MB contributed to patient inclusion and the final review of the manuscript. VT contributed to data interpretation and to the final review of the manuscript. EJ and TG conceived and designed the experiments, data interpretation, and final review of the manuscript. 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.2018.01598/full#supplementary-material.

FIGURE S1 | T cell numbers in relation to IDO expression patterns in primary cervical cancer. Scatter plots show stromal and intratumoral numbers per square millimeters for **(A)** $CD8^+$ T cells, **(B)** $CD8^+$ T cell/$Foxp3^+$ Treg ratio, and **(C)** $CD8^+Ki67^+$ T cells in tumors without IDO-positive tumor-infiltrating immune cells (IDO– infiltrate, white dots, $n = 14$) and with IDO-positive tumor-infiltrating immune cells (IDO+ infiltrate, black squares, $n = 17$). Scatter plot shows **(D)** intratumoral numbers of $CD8^+Ki67^+$ T cells in different groups of primary tumor expression patterns. *P* values were calculated in **(A–C)** using Mann–Whitney *U* test and unpaired *t* test in case of normal distribution of data. *P* values were calculated excluding subgroups with $n = 2$ or smaller (*) in **(D)** using Kruskal–Wallis test. $^*P = 0.01$–0.05, $^{**}P = 0.01$–0.001, and $^{***}P < 0.001$.

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Circulating T Cell Subpopulations Correlate With Immune Responses at the Tumor Site and Clinical Response to PD1 Inhibition in Non-Small Cell Lung Cancer

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Agents targeting the PD1–PDL1 axis have transformed cancer therapy. Factors that influence clinical response to PD1–PDL1 inhibitors include tumor mutational burden, immune infiltration of the tumor, and local PDL1 expression. To identify peripheral correlates of the anti-tumor immune response in the absence of checkpoint blockade, we performed a retrospective study of circulating T cell subpopulations and matched tumor gene expression in melanoma and non-small cell lung cancer (NSCLC) patients. Notably, both melanoma and NSCLC patients whose tumors exhibited increased inflammatory gene transcripts presented high $CD4^+$ and $CD8^+$ central memory T cell (CM) to effector T cell (Eff) ratios in blood. Consequently, we evaluated CM/Eff T cell ratios in a second cohort of NSCLC. The data showed that high CM/Eff T cell ratios correlated with increased tumor PDL1 expression. Furthermore, of the 22 patients within this NSCLC cohort who received nivolumab, those with high CM/Eff T cell ratios, had longer progression-free survival (PFS) (median survival: 91 vs. 215 days). These findings show that by providing a window into the state of the immune system, peripheral T cell subpopulations inform about the state of the anti-tumor immune response and identify potential blood biomarkers of clinical response to checkpoint inhibitors in melanoma and NSCLC.

Keywords: T cells subpopulations, melanoma, lung cancer, checkpoint blockade, PD1 and PDL1

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INTRODUCTION

Since their initial approval for the treatment of melanoma in 2014, anti-PD1 agents have transformed cancer therapy, more than doubling median overall survival rates for melanoma (1) and non-small cell lung cancer (NSCLC) (2). It is clear that not every patient or cancer type benefits from an anti-PD1 agent. As the PD1/PDL1 regulatory pathway inhibits the effector activity of T cells, the efficacy of an anti-PD1 agent depends not only on the presence of a counter-ligand to inhibit but also more importantly, on the availability of tumor-specific T cells whose activity can be unleashed by the therapeutic agent (3).

The quest to identify cancer patients who will benefit from therapy includes several companion and complementary diagnostic assays performed on tumor biopsies. These assays aim to identify PDL1 expression in the tumor and tumor microenvironment (4), and tumor mutation burden

(TMB) as a surrogate measure of neoantigen availability (5). In recent findings, the presence of an active immune infiltrate, evaluated through the expression of transcripts associated with $CD8^+$ T cell function, correlates highly with a positive clinical outcome toward anti-PD1 agents (6).

The determination of a patient's probability of response to anti-PD1/PDL1 agents is critical to inform a course of treatment and requires the identification of readily assessable biomarkers. While tissue biopsies provide a window into the immune response unfolding within the tumor microenvironment, tumor heterogeneity and the presence of multiple tumor sites can lead to mischaracterization of the magnitude of the anti-tumor immune response (7). In addition, the extent of this response depends on the state of the host's immune system. Factors such as genetic background, age, gender, and therapies such as chemotherapy and radiotherapy affect the immune system (8). This heterogeneity creates a need to improve the evaluation of the status of the immune system in cancer patients and its associated clinical outcomes. The dynamic nature of tumor evolution in response to therapy means that long-lasting clinical responses require an immune system fit to adapt to this changing environment (9).

An effective immune response toward a tumor requires neoantigen availability (5) and presentation to T cells, and subsequently the entry of antigen-exposed, activated T cells to the tumor. The tumor, in turn, can downregulate the immune response by expressing PDL1, which activates a regulatory mechanism in the T cell through its interaction with PD1 (3).

To determine if blood T cell subpopulations reflect the immune response against the tumor, we performed a cross-sectional, retrospective analysis of peripheral T cells and matched tumor gene expression in melanoma and NSCLC samples collected before checkpoint inhibitors became part of the standard of care. We observed a correlation between the degree of expression of inflammatory transcripts in the tumor and the percentages of circulating central memory (CM) and effector (Eff) $CD4^+$ and $CD8^+$ T cells, expressed as independent $CD4^+$ and $CD8^+$ CM/Eff T cell ratios. High CM/Eff T cell ratios correlate with inflamed tumors. Given that tumor inflammation correlates with good clinical response to checkpoint inhibitors, we tested whether high CM/Eff T cell ratios correlate with clinical outcome in a cohort of nivolumab-treated NSCLC patients. In this cohort, those patients with high CM/Eff T cell ratios experienced more prolonged progression-free survival (PFS). Given that melanoma and NSCLC patients with inflamed tumors, as well as NSCLC patients with longer PFS have high CM/Eff T cell ratios, we propose that measurement of these ratios in an easily accessible peripheral blood sample is a convenient biomarker of the state of the T cell arm of the immune system. These findings represent progress in the characterization of peripheral immunity, immune state, and its relationship to the inflammatory status of the tumor.

MATERIALS AND METHODS

Tissues and PBMC

Banked PBMC and matched flash frozen tumor samples from melanoma and NSCLC patients were obtained in collaboration with M2GEN and Moffitt Cancer Center (Tampa, FL, USA) and consented through their Total Cancer Care protocol. Control PBMC were obtained from the Bristol-Myers Squibb employee volunteer blood donation program (**Table 1**).

For the second NSCLC cohort, we obtained blood samples from 57 patients with NSCLC from a commercial vendor (MT group, CA, USA). A subset of these samples ($n = 22$) are from patients before receiving nivolumab as part of their clinical care. A second blood sample and clinical evaluation was obtained between 8 and 12 weeks after the start of the treatment. Control blood samples were obtained from the BMS employee volunteer blood program and processed simultaneously.

Flow Cytometry

PBMC were stained for viability with Near Infrared dye (Molecular Probes), blocked and incubated in an antibody mix containing anti-CD127-AF488 (Clone A0195D5), anti-PD1-PE (Clone EH12), anti-CD8-APC-R700 (RPA-T8), anti-CD28-BV650 (CD28.2), anti-CCR7-BV421 (GO43H7), anti-CD25-PECy7 (M-A251), anti-PD-1-PE (EH12), anti-CD45RA-BUV395 (HI100) anti-CD4-BUV495 (SK3), and anti-CD3 BUV737 (SK7).

Whole blood samples were collected and shipped overnight. Whole blood was then stained for viability with Near Infrared dye (Molecular Probes) followed by wash and surface staining with an antibody mix containing: anti-CD45-BV480 (Clone HI30), anti-CD4-AF700 (SK3), anti-CD8-BUV395 (RPA-T8), anti-CD3-BUV496 (UCHT1), anti-CCR7-BV711 (GO43H7), anti-PD-1-APC (MIH4), and anti-CD45RO-BV421 (UCHL1). All samples were read on a BD Fortessa instrument and analyzed with FlowJo. Spanning Tree Progression of Density Normalized Events (SPADE) analysis (10) were implemented on Cytobank (www.cytobank.org). Independent clustering of either $CD4^+$ or $CD8^+$ T cells used CD45RA, CCR7, and CD28. Both the circles and color scale denote the number of cells in the cluster.

Gene Expression and Inflammatory Signature

Total RNA was isolated from frozen tumor using AllPrep DNA/RNA/miRNA kit (Qiagen, Valencia, CA, USA) following manufacturer's recommended protocols. After assessing RNA quality, sequencing libraries were made using the TruSeq Stranded mRNA HT kit (Illumina, San Diego, CA, USA). Libraries were run on an illumina HiSeq 2500 at EA Genomics Services. Paired end FASTQ files were stored in AWS S3, and all analysis took place on AWS EC2 c3.8× large instances created by StarCluster (11).

TABLE 1 | Patient characteristics and demographics.

Cohort	Patients (*n*)	Mean age (SD), years	Male, *n*	Stage II, *n* (%)	Stage III, *n* (%)	Stage IV, *n* (%)
Control	27	54.9 (8.5)	12			
Melanoma	43	63.7 (15.0)	31	1 (2.3)	17 (39.5)	25 (58.1)
Nonsquamous NSCLC	40	65.7 (11.8)	15	16 (4.00)	12 (30.0)	12 (30.0)

Gene and isoform expression were calculated using RSEM (12) v1.1.13 and the UCSC hg19 genome annotation. An additional step of calculating gene and isoform quantile normalized read counts was performed using a custom Perl script. Inflammation gene expression scores were calculated based on the gene signature described in Spranger et al. (6) by calculating the mean of the log2, centered normalized data. Genes included in the signature include *CD8A*, *CCL2*, *CCL3*, *CCL4*, *CXCL9*, *CXCL10*, *ICOS*, *GZMK*, *IRF1*, *HLA-DMA*, *HLA-DMB*, *HLA-DOA*, and *HLA-DOB*. The scores where then split based on quantiles of the normal distribution as inflamed, intermediate, and non-inflamed.

Statistics and Visualizations

Comparisons of T cell subpopulations were performed using Student's *t*-test. For non-normal distributions, data were log-transformed before *t*-test. All reported *p*-values were corrected for multiple comparisons (**Figures 1A** and **3A**).

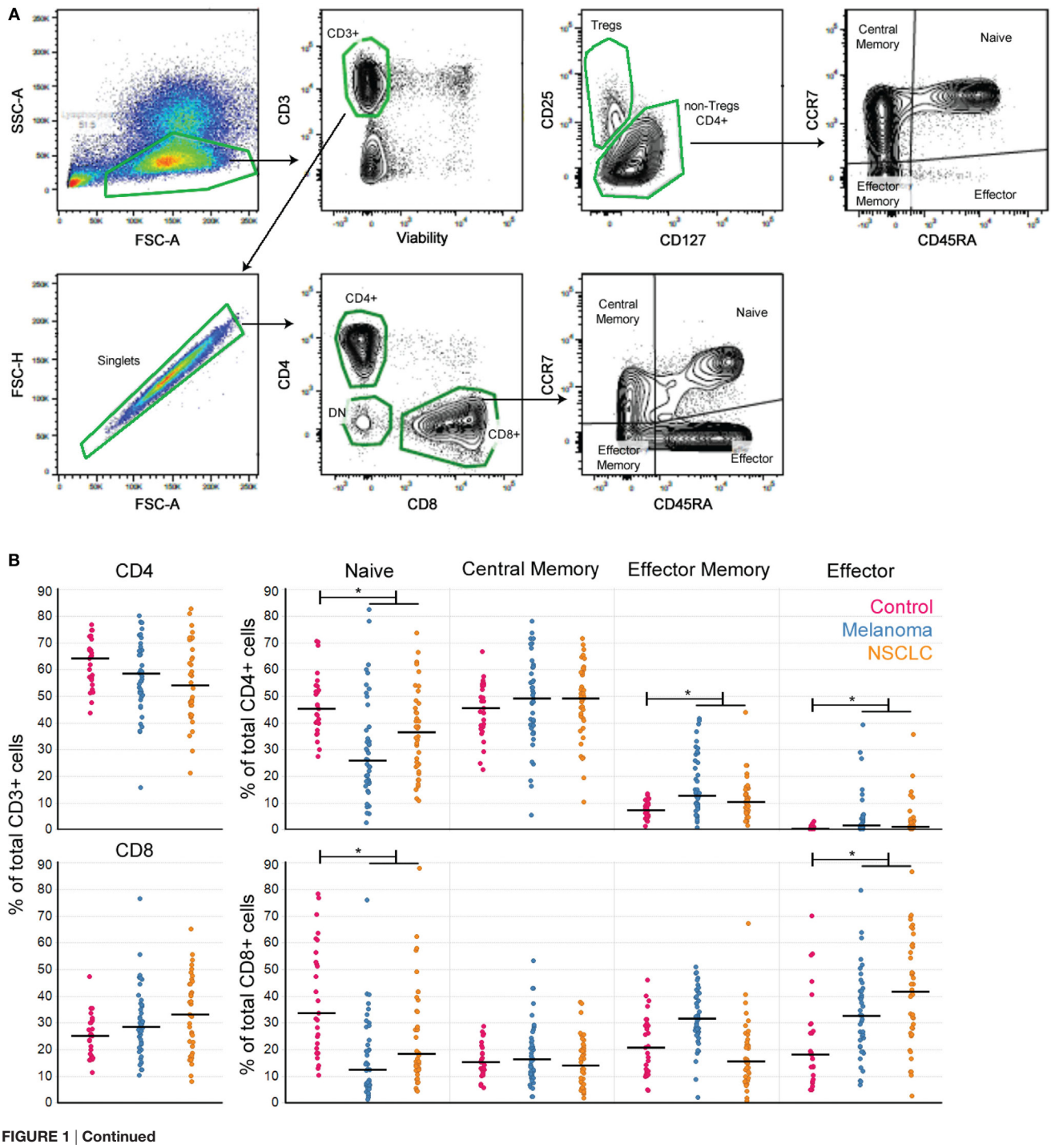

FIGURE 1 | **Continued**

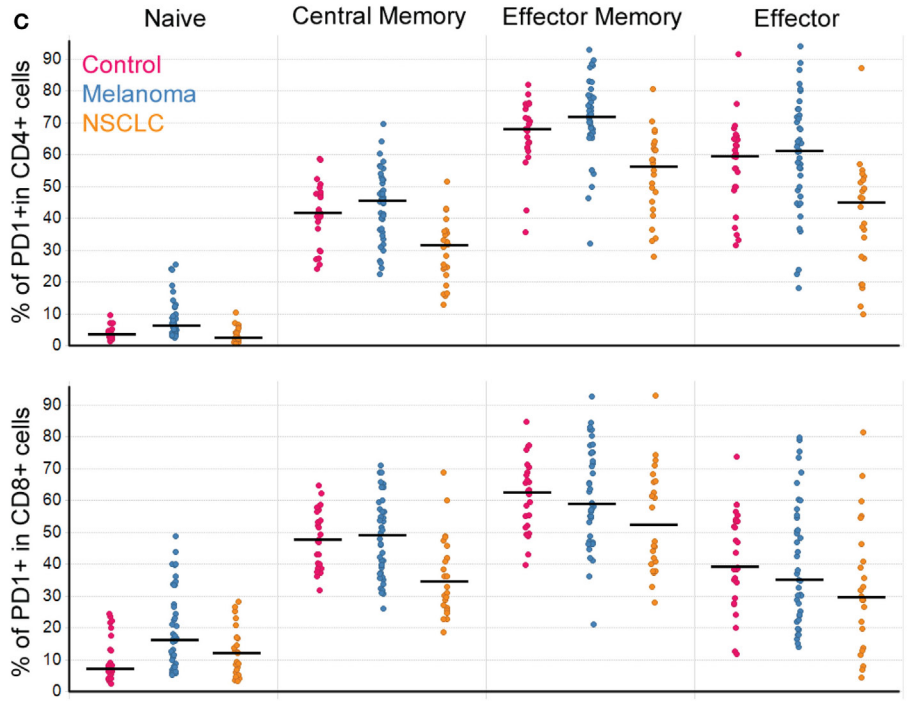

FIGURE 1 | Peripheral T cell subpopulations show evidence of an ongoing immune response in cancer patients. **(A)** Gating strategy to define T cell subpopulations in PBMC. **(B)** $CD4^+$ and $CD8^+$ T cell subpopulations in PBMC from melanoma and NSCLC patients. **(C)** Percentage of PD1+ cells per T cell subpopulation (controls $n = 27$, melanoma $n = 43$, NSCLC $n = 40$; Bonferroni-corrected p-values: *<0.001). Line marks the median.

Fischer's exact test was used for the analysis of 2 × 2 contingency tables for CM/Eff T cell ratios by inflammation state and PDL1 tumor proportion score (TPS) (separately).

For PFS analysis of patients undergoing treatment with nivolumab, all patients had at least 90 days of follow-up after first dose. PFS was calculated from the first day of nivolumab infusion until physician-confirmed disease progression (clinical or CT confirmed) by a scientist blind to the patient's biomarkers characteristics. Right-censored data were used to obtain Kaplan–Meier survival estimates and Wilcoxon p-values.

All statistical analysis were performed in JMP 13 (SAS, NC).

RESULTS

Circulating T Cells in Melanoma and Nonsquamous NSCLC Patients Show Evidence of Ongoing Immune Responses

Patients with cancer have circulating T cells specific for tumor antigens (13). Consequently, we hypothesized that the circulating T cell pool would reflect the immune responses to melanoma and NSCLC. To evaluate this premise in the absence of checkpoint inhibitors, we performed a cross-sectional, retrospective study of T cell subpopulations in archived PBMC from 43 melanoma and 40 NSCLC patients (all of them nonsquamous NSCLC). All of the patients had available matched tumor tissue, and none of them had prior treatment with checkpoint agents (**Table 1**; **Figure 1**).

Analysis of T cell subpopulations revealed that as a group, PBMC from cancer patients presented a decrease in the percentages of both $CD4^+$ and $CD8^+$ naïve T cells, accompanied by an increase in the percentages of EM and Eff $CD4^+$ and Eff $CD8^+$ T cells compared to control samples (**Figure 1B**). These findings are consistent with the presence of an ongoing immune response in these patients similar to that observed in patients with autoimmunity (14).

Association of Circulating T Cell Profiles With the Local Immune Response in Melanoma and Nonsquamous NSCLC

To assess the T cell differentiation patterns present in these patients, we implemented SPADE on the flow cytometry data (see Materials and Methods). Clustering of either $CD4^+$ or $CD8^+$ T cells using the differentiation markers CD45RA, CCR7, and CD28 showed that in cancer patients, the circulating antigen-experienced T cells present either CM-early Effector Memory or Eff phenotypes (**Figure 2A**), also reflected by the inverse relationships between CM and Eff subpopulations.

Next, we evaluated how the circulating T cell subpopulations reflect the local immune state observed in the tumors. We used matched frozen tumor tissues to evaluate gene expression profiles of immune-associated genes. We defined the tumors as inflamed, intermediate, and non-inflamed based on quantiles of inflammation gene signature scores. Through further analysis of

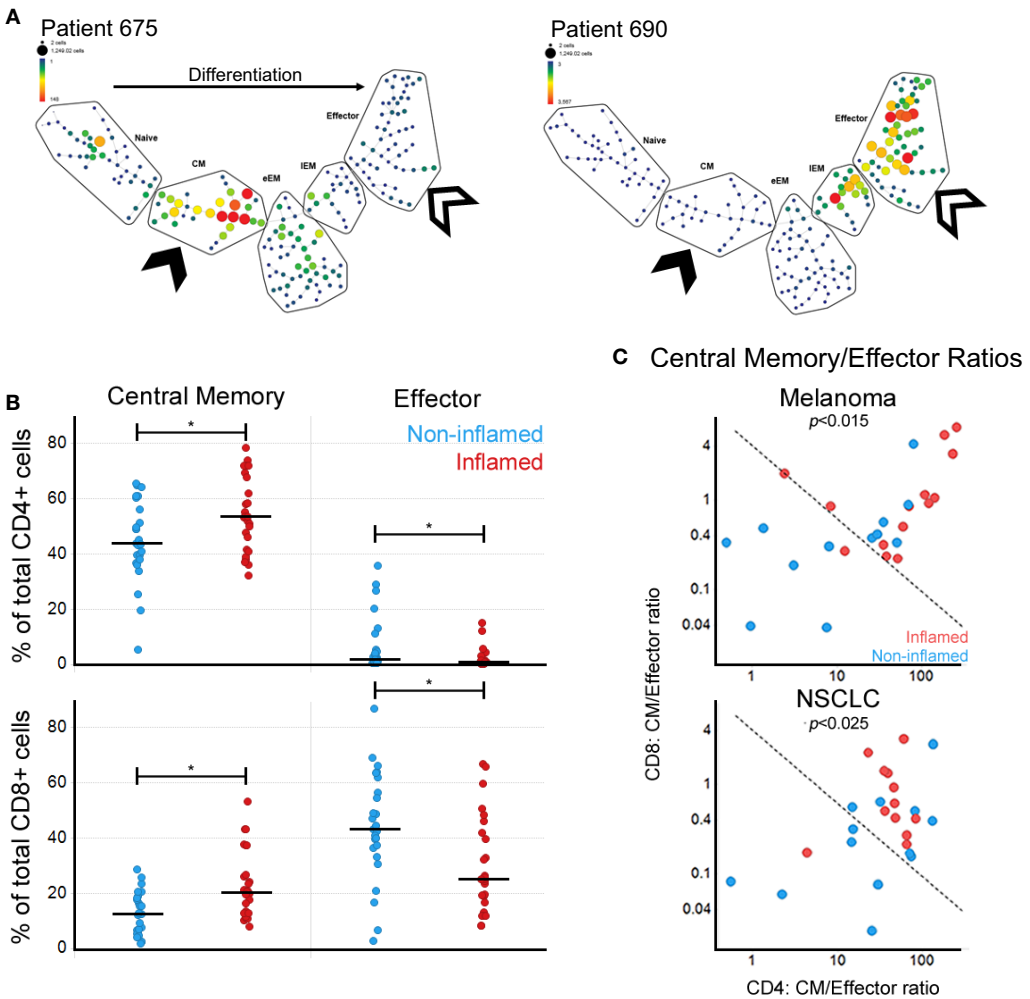

FIGURE 2 | Local immune responses in melanoma and NSCLC correlate with circulating central memory (CM)/effector (Eff) T cell ratios. **(A)** SPADE-generated maturation profiles of CD8⁺ T cells for two melanoma samples showing the divergent patterns of T cell subpopulations. Both have a reduction in naïve CD8⁺ T cells but show an expansion in either CM (solid arrow) or the Eff (open arrow) compartments. eEM, early Effector Memory; EM, Effector Memory. **(B)** Correlation between circulating CM and Eff CD4⁺ and CD8⁺ T cells and tumor inflammation state (*$p < 0.05$). **(C)** Correlation between CM/Eff T cell ratios by inflammation state in melanoma and NSCLC. Fisher's exact test p-value for a 0.05 significance level. Dividing line generated based on the 90th percentile of controls.

the inflamed vs. non-inflamed tumors we observed a correlation between tumor inflammation and the percentages of circulating central memory and effector T cells, which while similar in magnitude, showed a different direction. Surprisingly, the peripheral blood populations which showed a positive correlation with inflamed tumors were not the effector T cell subpopulations, but CM, for both CD4⁺ and CD8⁺ T cells (**Figure 2B**).

Given the inverse relationship between CM and Eff T cells, we calculated CM/Eff ratios for both CD4⁺ and CD8⁺ T cells (**Figure 2C**). Patients with inflamed tumors by gene expression had a tendency toward high CM/Eff ratios (upper right corner). Interestingly, CM/Eff ratios in patients with high inflammation scores are similar to those of the healthy control samples used in this study. Consequently, we used the 90th percentile of control samples to distinguish between low and high CM/Eff ratios (dotted line), observing that the inflamed melanoma and NSCLC tumors have high CM/Eff ratios compared to those with non-inflamed tumors.

Circulating CM/Eff T Cell Ratios in NSCLC Are Associated With Longer PFS in Response to Checkpoint Inhibitors

To evaluate CM/Eff T cell ratios as a tool to evaluate the status of the T cell arm of the immune system, we collected blood from a second cohort of NSCLC patients ($n = 57$). We were able to observe that the reduction of the naïve compartment and the expansion of Eff T cell subpopulations, both in CD4⁺ and CD8⁺ T cells is a reproducible finding (**Figure 3A**).

We then hypothesized that the interferon gamma produced during anti-tumor immune responses would lead to the upregulation of PDL1, as this is an interferon gamma-induced gene

(15). In the 23 patients where PDL1 expression was measured, we observed a bimodal distribution in the percentage of PDL1 tumor proportion score (%TPS) (**Figure 3B**). This pattern made us divide the patients at the antimode (25% TPS) as PDL1$^{neg/low}$ and PDL1^{high}. High CM/Eff T cell ratios, which we had previously found associated with higher inflammatory signature (**Figure 2C**), correlate with high PDL1 expression in the tumor (**Figure 3C**, Fisher's exact $p < 0.025$).

A subset of these NSCLC patients went on to receive nivolumab as part of their clinical care ($n = 22$). Those patients with high CM/Eff T cell ratios at baseline had an extended PFS compared to those patients with low CM/Eff T cell ratios (**Figure 3D**, Wilcoxon test $p < 0.05$, median survival time "*low*" ratio: 91 days, "*high*" ratio 215 days). A second blood sample, obtained around 3 months after the initiation of nivolumab treatment did not show major changes in CM/Eff T cell ratios in patients categorized as

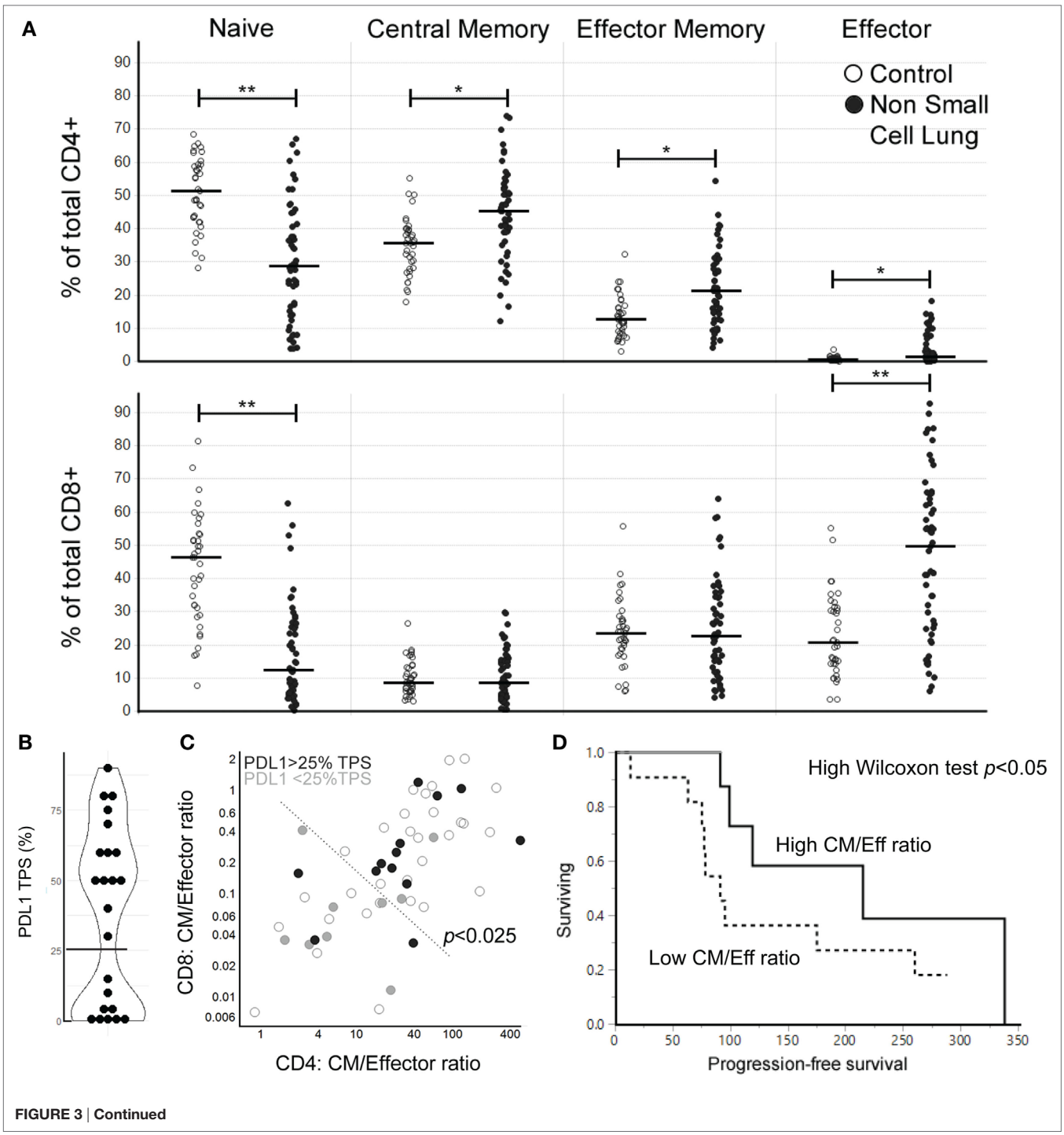

FIGURE 3 | **Continued**

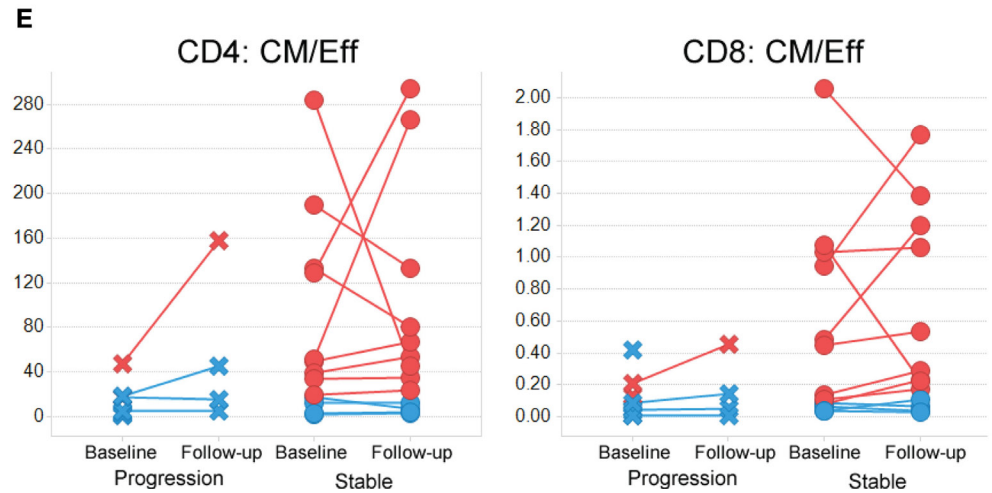

FIGURE 3 | High central memory (CM)/effector (Eff) T cell ratios at baseline are associated with longer progression-free survival (PFS) in response to nivolumab treatment for NSCLC. **(A)** Peripheral T cell profiles in a second cohort of NSCLC and control samples (Bonferroni-corrected *p*-values: *<0.001, **<0.0001, line marks the median for subpopulation). **(B)** Distribution of PDL1 tumor proportion score (TPS) ($n = 23$, the horizontal line marks the cutoff at the antimode: 25% TPS). **(C)** CM/Eff T cell ratios in the NSCLC cohort coded by PDL1 TPS (open circles: PDL1 expression not evaluated). CM/Eff T cell ratios high vs. low division line is drawn using the 90th percentile of the control samples. Fischer's *p*-value <0.025. **(D)** PFS after nivolumab treatment ($n = 22$) (*p*-value < 0.05, median survival by CM/Eff ratio: low, 91 days; high 215 days). **(E)** Change in CM/Eff T cell ratios three months after nivolumab-treatment initiation. Patients are classified by physician-reported response to treatment at three months.

"*low*," in contrast to those patients classified as "*high*" (**Figure 3E**). It is important to mention that because of disease progression, only 7 of the 11 "*low*" patients were still in nivolumab treatment, in contrast to 10 of the 11 "high" patients.

DISCUSSION

Here, we report that high circulating CM/Eff T cell ratios associate with tumor inflammation in melanoma and NSCLC, as well as with increased PDL1 expression at the tumor and longer PFS in response to nivolumab treatment in NSCLC. To the best of our knowledge, this is the first time that circulating T cell subpopulations are proposed as predictive biomarkers of response to checkpoint inhibitors in NSCLC.

The association between higher frequency of CM T cells (CD4 and CD8) and an increased tumor inflammatory profile is congruent with reports that CM T cells are the primary repository of the immunogenic experiences of a lifetime (16, 17). The inverse relationship between the frequency of Eff T cells in circulation and the inflammation signature in the tumor was nevertheless surprising and could reflect the presence of terminally differentiated T cells that are unable to reach the tumor. Rather than reflecting the immune response against the tumor, we hypothesize that CM/Eff ratios are a way to evaluate the status of the immune system. In this model, immune state evaluated by CM/Eff ratios would be associated with the capacity of a subject to mount an immune response against the tumor that checkpoint inhibitors can potentiate. This model is consistent with the high sensitivity of this analysis to detect cancer patients who have inflamed tumors (>90%, **Figure 2C**). Nevertheless, its low specificity highlights the multifactorial nature of the anti-tumor response, as other factors, such as TMB, also play a role in the anti-tumor response (18).

These findings provide a window into how the status of the immune system affects the anti-tumor response. Extended clinical responses to checkpoint inhibitors depend on the presence of tumor-specific T cells, and the ability of the immune system to co-evolve with the tumor. Thus, the predominant T cell response shifts as the dominant antigen disappears or mutates (9, 19). Under this model, increased immunological pressure toward the tumor (increased inflammation signature) may drive the upregulation of PDL1 as an immunosuppressive tumor-survival mechanism (20), as observed in the patients with high CM/Eff T cell ratios.

These results align with previous reports that the percentages of CD4 and $CD8^+$ T cell memory correlate with clinical response in melanoma patients treated with ipilimumab (21, 22). Moreover, a recent analysis of four melanoma patients (two with stable disease, one progressive disease, and one partial response) show an increase of central memory $CD4^+$ T cells in the two patients with longer survival times (23). These data are in line with a recent report of peripheral immune cells and its correlation with response to checkpoint inhibitors in melanoma which also found an association between increased $CD8^+$ CM T cells and clinical response (24). However, the highly overlapping ranges of the populations limit their use to identify patients with higher probabilities of responding to checkpoint inhibitors. Our data show how $CD4^+$ and $CD8^+$ CM and effector T cells are a bellwether of responses to checkpoint inhibitors, presumably because all of them contribute to the anti-tumor responses (25, 26). The integration of all these correlates of T cell status into a simple and novel parameter (CM/Eff T cell rations), allows a better separation between

responders and non-responders and identification of those NSCLC patients most likely to experience clinical benefit from checkpoint inhibitor therapy.

There is a clear need to elucidate the mechanisms underlying primary resistance and short-lived clinical responses to checkpoint inhibitors. Our data suggest that the state of the T cell arm of the immune system, measured by the relative frequency of CM/Eff T cell ratios can be a contributing mechanism. Even more, improving the number of patients who can benefit from immune therapy requires a comprehensive analysis of the relative contributions of T cell subpopulations to anti-tumor responses. This challenge includes understanding whether a reduced naïve T cell repertoire contributes to functional T cell depletion, and the capacity of CM T cells to replenish the T cell repertoire (26). At a functional level, high levels of the pro-apoptotic molecule Bim in $PD1^+CD11a^+CD8^+$ T cells of melanoma patients associate with shorter survival after anti-PD1 treatment, presumably because Bim may induce apoptosis of anti-tumor-specific T cells (27). Early pharmacodynamics effects of anti-PD1 associated with clinical benefit are the extent of expression of the proliferation marker ki67 in $PD1^+$ T cells (28, 29) or of particular memory subtypes (30). An integrated analysis of these T cell subpopulations and their relationship to each other would provide a better understanding of the mechanisms behind primary resistance to anti-PD1 therapy. Along this line, a comprehensive analysis of the TCR repertoire together with gene expression in patients during checkpoint therapy would shed light on this particular question. Although there are still unanswered questions, this method to evaluate the immune system provides an easily accessible circulating biomarker to add to a comprehensive evaluation that already includes TMB and PDL1. Altogether, these assays may enable a better prediction of which patients will respond to checkpoint inhibitors, as well as those who may obtain more benefit from other agents.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of The Moffitt Cancer Center's Institutional Review Board and the Bristol-Myers Squibb Institutional Biosafety Committee. The protocol was approved by the Moffitt Cancer Center's Institutional Review Board and the Bristol-Myers Squibb Institutional Biosafety Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

NM-O, LM, SK, PF, BK, CJ, JC, PK, PK, SB, SS, and SN planned and designed experiments; SK, PF, BK, CJ, VP, and MY performed experiments; NM-O, LM, SK, PF, BK, SS, MC, DG, RG, SB, and SN analyzed data; DG, PK, SL, and HD performed gene expression analysis; SS and SN provided intellectual input and helped preparing this manuscript; all authors approved the 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.2018.01613/full#supplementary-material.

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CASE REPORT
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Squamous Cell Carcinomas of the Head and Neck Cancer Response to Programmed Cell Death Protein-1 Targeting and Differential Expression of Immunological Markers: A Case Report

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Targeting the programmed cell death protein-1 (PD-1)/PD-1 ligand (PD-L1) pathway has been shown to enhance T cell-mediated antitumor immunity. Clinical responses are limited to subgroups of patients. The search for biomarkers of response is a strategy to predict response and outcome of PD-1/PD-L1 checkpoint intervention. The NY-ESO-1 cancer testis antigen has been considered as a biomarker in head and neck squamous cell carcinoma (HNSCC) patients and can induce both specific NY-ESO-1 antibody and T cells responses. Here, we correlated clinical responsiveness to anti-PD-1 (nivolumab) treatment with immunity to NY-ESO-1 in a patient with recurrent HNSCC. The patient was treated with second-line treatment of nivolumab and had a stable disease for over 7 months. His NY-ESO-1 antibody was found to be lower after the third (****$p < 0.0001$) and the fifth (****$p < 0.0001$) cycles of treatment compared to base line, and this was in line with the stability of the disease. The NY-ESO-1-specific T cells response of the patient was found to be increased after the third and the fifth (**$p = 0.002$) cycles of treatment but had a significant decline after progression (**$p = 0.0028$). The PD-1 expression by the patient's T cells was reduced 15-folds after nivolumab treatment and was uniquely restricted to the $CD8^+$ T cells population. Several cytokines/chemokines involved in immune activation were upregulated after nivolumab treatment; two biomarkers were reduced at progression [interleukin (IL)-10: ****$p < 0.0001$ and CX3CL1: ****$p < 0.0001$]. On the other hand, some cytokines/chemokines contributing to immune inhibition were downregulated after nivolumab treatment; two biomarkers were increased at progression (IL-6: ****$p < 0.0001$ and IL-8: ****$p < 0.0001$). This data support the notion that the presence of anti-NY-ESO-1 integrated immunity and some cytokines/chemokines profile may potentially identify a response to PD-1 blockade in HNSCC patients.

Keywords: head and neck squamous cell carcinoma, programmed cell death protein-1, nivolumab, NY-ESO-1 antibody, NY-ESO-1-specific T cells, cytokine profile

INTRODUCTION

Head and neck Squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, accounting for approximately 6% of all cases and is responsible for an estimated 1–2% of all cancer deaths (1, 2). More than 90% of tumors in the head and neck are squamous cell carcinomas (3). The majority of HNSCC patients present with advanced-stage disease characterized by significant rates of local failure and distant metastases subsequent to radiotherapy (4, 5). Advances in surgery, chemotherapy, and radiotherapy have not altered the survival rates of patients with HNSCC over the past two decades (6). Emerging evidence supports an important role of the immune system in the development and evolution of HNSCC in which the status of the immune system is likely to be of prognostic value. It has been demonstrated that patients with HNSCC have either a downregulation of their antitumor immune responses and tumor progression or relapse that correlates with immune dysfunction (7). Immunotherapy has emerged as a promising treatment approach for cancer with extraordinary survival in selected patients. Immunotherapy using immune-modulating antibodies, which is based on reconstitution of the efficacy of pre-existing immune responses in patients, is used to help counteracting various tumor evasion strategies (8). Nivolumab is an immune-modulating antibody against the programmed cell death protein-1 (PD-1). PD-1 is an immune checkpoint receptor found on the surface of T cells that downregulates their activation (8, 9). Nivolumab has been recently approved by the FDA as an option for the second-line treatment of recurrent and/or metastatic HNSCC (10). The results from a very recent prospective randomized trial using this antibody heralded a new era of anti-cancer therapy in HNSCC (11, 12). Because such immune-modulating antibodies are known to *unleash the brake of the immune system* (13, 14), the presence of a pre-existing immune response is essential for the success of such therapy. Therefore, the identification of target antigens for such immune responses has become precedence. The NY-ESO-1 cancer testis antigen has been shown to be expressed in HNSCC patients and to exhibit the capacity to induce both natural antibody and T cell responses (15). Because of its high tumor-specificity and immunogenicity, the NY-ESO-1 antigen may represent an attractive target for specific immunotherapy of HNSCC. Indeed, it has been demonstrated that melanoma patients treated with ipilimumab had an increased rate of NY-ESO-1-specific immunity that was associated with improved clinical benefit of the treatment, especially in patients developing both NY-ESO-1-specific antibody and specific $CD8^+$ T cells (16).

We therefore speculate that such pre-existing immunity to the NY-ESO-1 antigen should be enhanced after anti-PD-1 treatment leading to improved clinical benefit of the patient. We showed here that anti-PD-1 (nivolumab) treatment of an HNSCC patient modulated his immune response to the NY-ESO-1 antigen. We have also showed differential expression of important cytokines/chemokines markers that correlated with the patient clinical outcome.

CASE REPORT

A 71-year-old Qatari male patient was diagnosed with oral cavity HNSCC with stage cT4 N0 M0 in 1997 and underwent radiotherapy in London, UK. He developed post-radiation necrosis and neck fistula, which was treated with a skin flap. After initial chemo-radiation in 2016, a recurring HNSCC involving the supraglottic region and tongue base was identified. On the 12th of January 2017, a second-line treatment with nivolumab was started (3 mg/kg every 2 weeks for five cycles) after declining chemotherapy. However, due to non-compliance the patient refused further treatment. Two CT scans of the patient neck were taken before treatment and 10 days after the fifth cycle of the treatment. PET CT scan was carried out 239 days after the fifth cycle (7 months, 25 days) of treatment. The antibody response to the NY-ESO-1 antigen was measured in the plasma using enzyme-linked immunosorbent assay (ELISA) against a known immunogenic NY-ESO-1 peptide. The cellular response to the NY-ESO-1 antigen was investigated in patient's peripheral blood mononuclear cells (PBMCs) using an enzyme-linked immunospot (ELISPOT) assay for interferon-gamma (IFN-γ) production by T cells against the NY-ESO-1 overlapping peptides. Flow cytometry was used to determine the expression of PD-1 in the patient $CD3^+$ T cells before and after nivolumab treatment. A panel of 27 plasma biomarkers (cytokines and chemokines) was analyzed by multiplex analysis.

Clinical Response to Nivolumab

After the fifth cycle of nivolumab treatment, the patient's bleeding from the tumor site at the neck stopped and CT scan follow-up showed stable disease, no progression, or distant metastasis (**Figure 1A**). It showed a mild increase in size, measuring about 5.1 cm × 4.6 cm, 10 days after the fifth cycle (**Figures 1A–C**) compared to 4.5 cm × 4.3 cm before nivolumab treatment (**Figures 1A–A**) which suggests of pseudo-progression. On the other hand, 163 days (5 months, 10 days) after the fifth cycle of nivolumab treatment, the patient was seen by an oncologist and found to be in a fair general condition. Because the patient declined to have any follow-up CT scans and blood tests, a mobile medical team visited him several times and evaluated him as feeling well with an on and off cough. The patient also complained of limited pain on the left sub-mandibular angle but physical examination showed no palpable mass in that area. The patient was again seen by the medical team 194 days (6 months, 10 days), 209 days (6 months, 25 days), and 226 days (7 months, 12 days) after the fifth cycle of nivolumab treatment. On all visits, the patient had some cough with blood, a small soft tissue mass was observed on the left side of the neck. However, no hard mass was observed. The patient was admitted to the National Center for Cancer Care and Research (NCCCR) 234 days (7 months, 20 days) after the fifth cycle of nivolumab treatment with left mandibular pain and swelling. He had productive cough of copious whitish sputum with no fever. PET CT scan was carried out at day 239 after the fifth cycle (7 months, 25 days) and the patient was found to have progressed (**Figures 1B–F**).

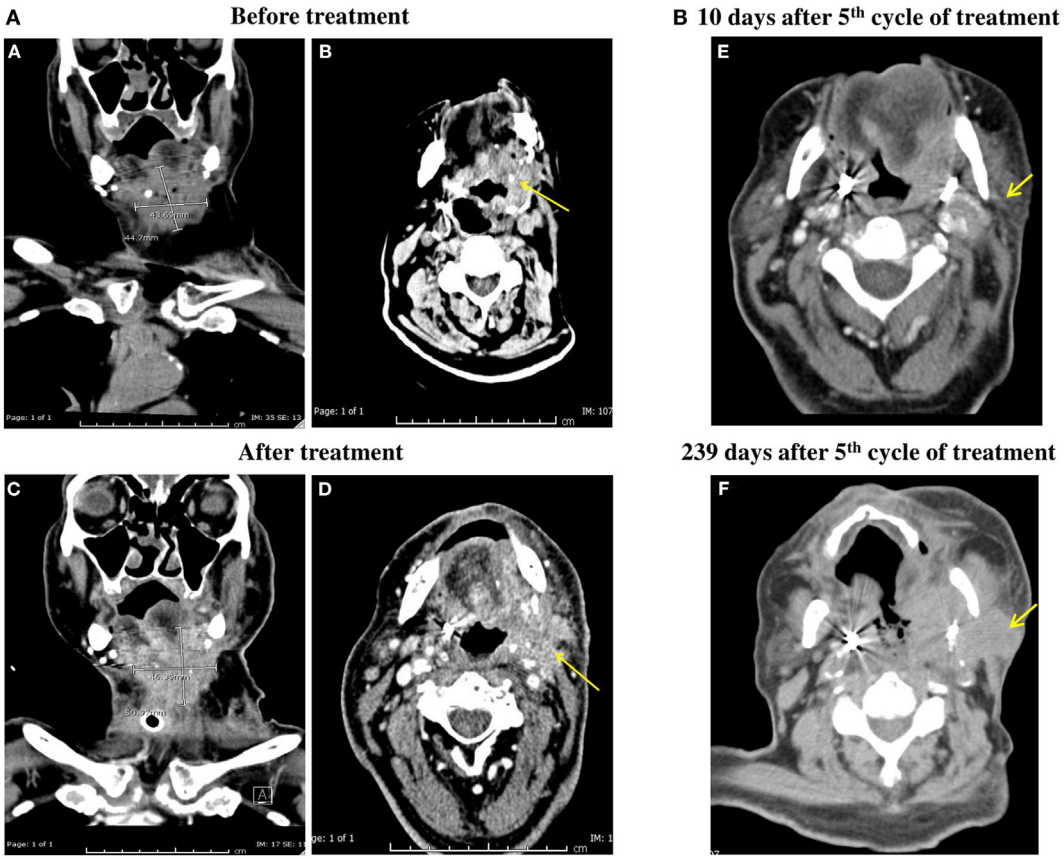

FIGURE 1 | **(A)** CT scan of the patient neck with IV contrast. Irregular infiltrative mass in the left side of the neck adjacent to the base of the tongue, invading the oropharynx and extending caudally to supraglottic and glottic larynx was shown both before and after the fifth cycle of anti-programmed cell death protein-1 (PD-1) treatment [**(B,D)** respectively]. It shows mild increase in size measuring about 5.1 cm × 4.6 cm 10 days after the fifth cycle **(C)** compared to 4.5 cm × 4.3 cm before anti-PD-1 treatment **(A)**. **(B)** PET CT carried out at day 239 after fifth cycle (7 months, 25 days) of anti-PD-1 treatment showing progression of the disease **(F)** compared tp PET CT obtained at 10 days after the fifth cycle **(E)**.

Determination of Antitumor Immune Response

The humoral immune response to the NY-ESO-1 antigen was measured. ELISA results showed that out of the four different plasma dilutions tested (1:100, 1:400, 1:1,600, and 1:6,400), 1:100 and 1:400 were found to be the optimum dilutions to differentiate the anti-NY-ESO-1 antibody level before and after nivolumab treatment (**Figure 2A**). ELISA results showed that the NY-ESO-1 antibody levels at 1:400 plasma dilution were significantly higher before nivolumab treatment compared to samples taken 11 days after the third cycle (third cycle-11 days, $^{****}p < 0.0001$), 8 days after the fifth cycle (fifth cycle-8 days, $^{****}p < 0.0001$), and at progression stage (fifth cycle-226 days, $^{****}p < 0.0001$) (**Figure 2B**). We used pooled plasma from five healthy donors as a negative control. Interestingly, the patient plasma recognized the NY-ESO-1 (11–30 amino acids) peptide which represents one of the most known immunogenic epitope of the NY-ESO-1 antibody. No response was obtained with the non-immunogenic long peptide (amino acids 85–111) (data not shown). The ELISA result was confirmed using Western Blot analysis which showed the recognition of an 18 kDa band by the NY-ESO-1 antibody in the patient plasma (data not shown).

The cellular immune response to the NY-ESO-1 antigen was measured in the PBMCs of the patient before and after nivolumab treatment for IFN-γ secretion using ELISpot assay. Specific IFN-γ secretion was demonstrated against a pool of 43 peptides representing the whole length of the NY-ESO-1 protein (PepMix) (**Figure 2C**). IFN-γ secretion was slightly increased in T cells tested 11 days after the third cycle (third cycle-11 days) and was significantly higher 8 days after the fifth cycle (fifth cycle-8 days, $^{**}p = 0.002$) of nivolumab treatment compared to control before treatment. Interestingly, there was a significant decrease in IFN-γ secretion by the patient T cells collected at progression (fifth cycle-226 days, $^{**}p = 0.0028$) (**Figure 2C**). No IFN-γ secretion was obtained in the presence of the negative control, PSA PepMix (data not shown).

The PD-1 expression by T cells was investigated in the PBMCs of the patient before and after the fifth (fifth cycle-8 days) cycle of nivolumab treatment using flow cytometry analysis. Nivolumab treatment demonstrated a 15-fold decrease in the expression of

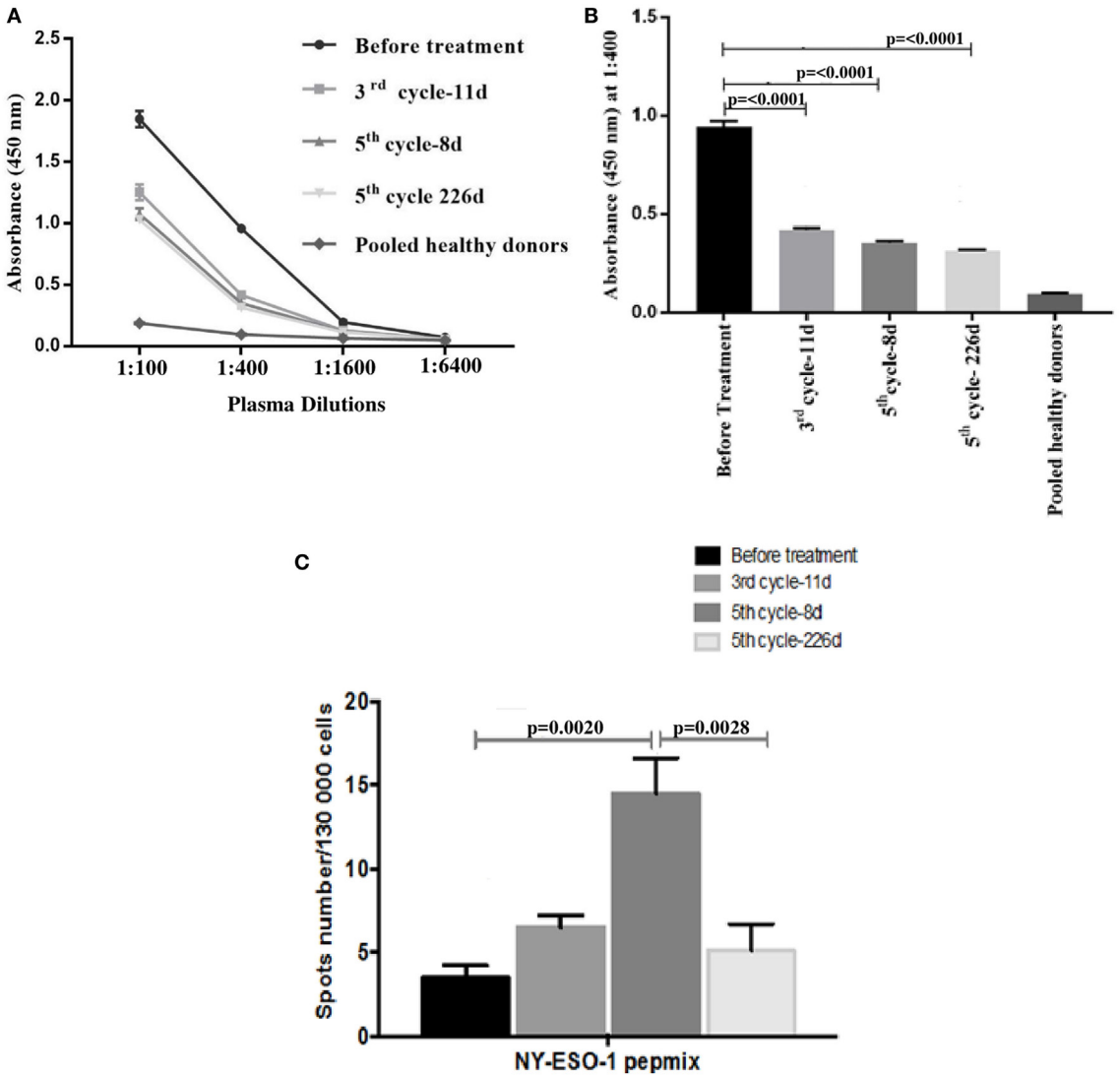

FIGURE 2 | Antibody response to the NY-ESO-1 antigen as measured in the plasma by enzyme-linked immunosorbent assay (ELISA). **(A)** The results are expressed as the mean OD value and error bars indicate the SD for the triplicate values in each dilution. Out of the four different plasma dilutions tested (1:100, 1:400, 1:1,600, and 1:6,400), 1:100 and 1:400 were found to be the optimum dilutions to differentiate the anti-NY-ESO-1 antibody level before and after nivolumab treatment. **(B)** Bar graph represents the mean OD values were measured at 1:400 dilution. Each ELISA experiment was repeated six times and the shown data corresponds to one representative experiment. **(C)** Enzyme-linked immunospot (ELISPOT) assay for interferon-gamma production to investigate T cell response to the NY-ESO-1 antigen in patient's peripheral blood mononuclear cells against NY-ESO-1 overlapping peptides (PepMix). The assay was repeated three times and the shown data corresponds to one representative experiment. Statistical analysis for ELISA and ELISPOT were performed using non-parametric unpaired ANOVA followed by multiple comparison Dunnet's test and p values <0.05 were considered statistically significant.

PD-1 by the $CD3^+$ T cells when compared to the value obtained before treatment (**Figure 3B**). Although both subsets of $CD4^+$ and $CD8^+$ T cells expressed the PD-1 molecule, its expression was dominant in the $CD4^+$ T cells population before treatment (**Figure 3D**). The expression of PD-1 in the $CD4^+$ and $CD8^+$ T cells population was below detection limits after nivolumab treatment (data not shown).

The cytokine/lymphokine profile was investigated in the plasma of the patient before and after the third cycle (third cycle-11 days) and the fifth cycle (fifth cycle-8 days) of nivolumab treatment as well as at progression (fifth cycle-226 days) using multiplex analysis. We have classified the cytokine/lymphokine profile, based on its upregulation or downregulation status after nivolumab treatment, into two groups (**Tables 1** and **2**). Group 1 comprises 10 biomarkers that were significantly upregulated after the third cycle-11 days (**Table 1**). These are IFN-γ, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein-1β (MIP-1β), chemokine C-X3-C motif ligand 1 (CX3CL-1), CXCL-11, and soluble CD137 (sCD137). It is important to mention that four of the biomarkers (IL-10, GM-CSF, CX3CL-1, and sCD137)

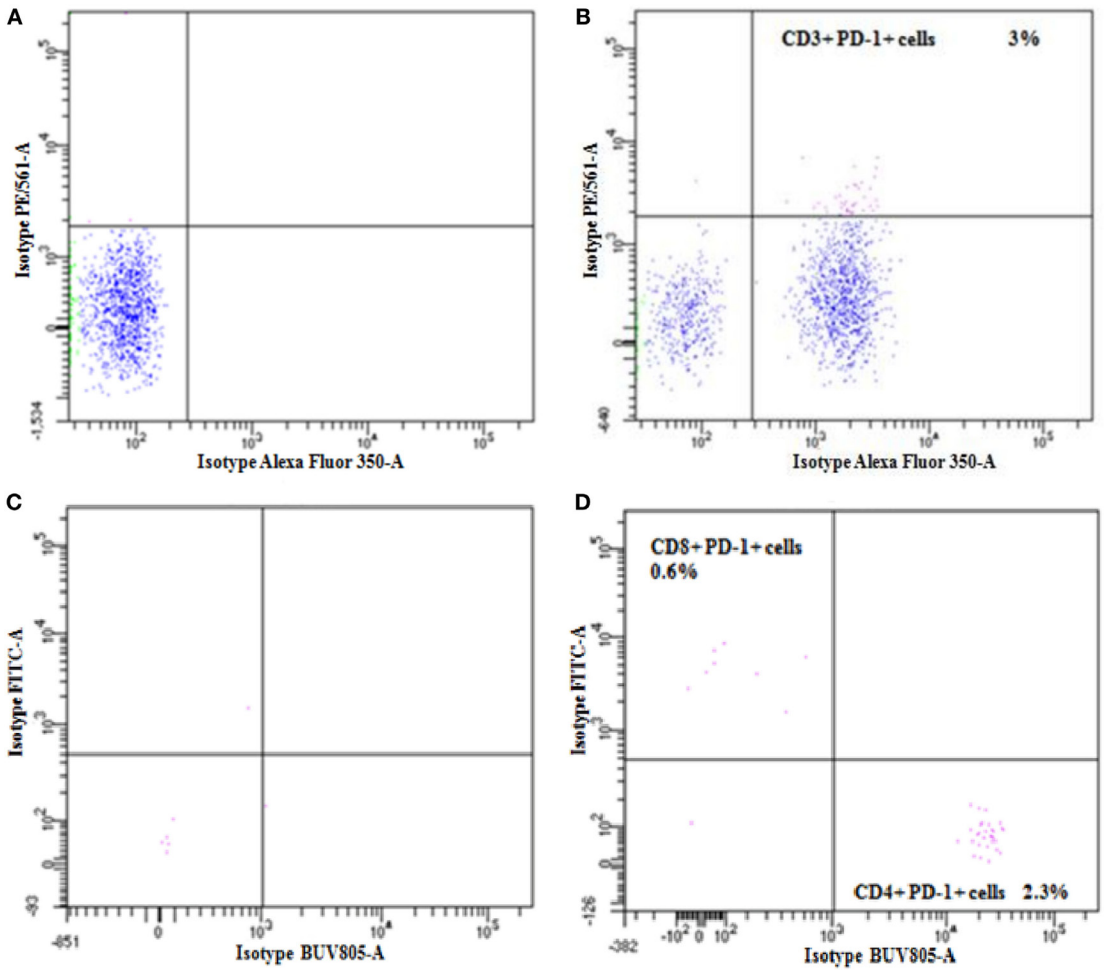

FIGURE 3 | Flow cytometry was used to determine the expression of programmed cell death protein-1 (PD-1) in the patient CD3+, CD4+, and CD8+ T cells before nivolumab treatment. Panels **(A,B)** are dot plots for isotype control and for PD-1 staining in CD3+, respectively. Isotype control and PD-1 staining in CD4+ and CD8+ cells are represented in panels **(C,D)**.

TABLE 1 | Plasma concentrations of upregulated cytokines/chemokines after nivolumab treatment.

Cytokines/ chemokines	[a]Before treatment Conc. (ng/ml)	[a]After third cycle (third cycle-11 days) Conc. (ng/ml)	*p*-Value	[a]After fifth cycle (fifth cycle-8 days) Conc. (ng/ml)	*p*-Value
IFN-γ	0.014 ± 1.15	0.0356 ± 1.05	*****0.0001*	0.018 ± 1.12	*****0.0001*
TNF-α	0.156 ± 0.57	0.575 ± 0.61	*****0.0001*	0.152 ± 1.52	*NS*
IL-6	0.038 ± 0.75	0.171 ± 0.63	*****0.0001*	0.047 ± 1.5	*****0.0001*
IL-8	0.313 ± 1.20	0.709 ± 1.22	*****0.0001*	0.316 ± 1.10	***0.004*
IL-10	0.0316 ± 1.01	0.076 ± 1.1	*****0.0001*	0.058 ± 1.04	*****0.0001*
GM-CSF	0.018 ± 0.57	0.022 ± 1.0	***0.003*	0.028 ± 1.5	*****0.0001*
MIP-1β	0.108 ± 1.2	0.125 ± 1.6	*****0.0001*	0.110 ± 1.2	***0.007*
CX3CL-1	0.068 ± 1.32	0.103 ± 1.02	*****0.0001*	0.096 ± 1.51	*****0.0001*
CXCL-11	1.885 ± 0.7	2.002 ± 0.8	*****0.0001*	1.889 ± 0.7	*NS*
sCD137	50.6 ± 057	65.5 ± 1.32	*****0.0001*	79.1 ± 1.39	*****0.0001*

[a]*Values represent concentration (mean ± SD).*

Statistical analysis was carried out using non-parametric unpaired ANOVA followed by Dunnet's multiple comparison test.

p Value: <0.05 significant.

***Indicates significant.*

*****Indicates highly significant.*

NS, not significant; Conc, concentration; ng/ml, nanogram per milliliter; IFN-γ, interferon-gamma; TNF, tumor necrosis factor-alpha; IL-6, interleukin-6; IL-8, interleukin-8; GM-CSF, granulocyte-macrophage colony-stimulating factor; MIP-1β, macrophage inflammatory protein-1β; CX3CL-1, chemokine C-X3-C motif ligand 1; CXCL-1, C-X-C motif chemokine 11; sCD137, soluble CD137.

TABLE 2 | Plasma concentrations of downregulated cytokines/chemokines after nivolumab treatment.

Cytokines/ chemokines	[a]Before treatment Conc. (ng/ml)	[a]After third cycle (third cycle-11 days) Conc. (ng/ml)	*p*-Value	[a]After fifth cycle (fifth cycle-8 days) Conc. (ng/ml)	*p*-Value
Granzyme A	193.5 ± 0.12	21.6 ± 0.15	*****0.0001*	23.8 ± 0.28	*****0.0001*
Granzyme B	2,111 ± 1.05	1,186.8 ± 1.05	*****0.0001*	1183.2 ± 1.02	*****0.0001*
Perforin	9,367 ± 0.25	5,236 ± 1.04	*****0.0001*	6,483 ± 1.03	*****0.0001*
sFAS	135.6 ± 1.6	63.6 ± 1.05	*****0.0001*	115.6 ± 1.23	*****0.0001*
IL-17A	17.3 ± 0.57	9.6 ± 1.04	*****0.0001*	6.5 ± 1.02	*****0.0001*

[a]*Values represent concentration (mean ± SD).*

Statistical analysis was carried out using non-parametric unpaired ANOVA followed by Dunnet's multiple comparison test.

p Value: <0.05 significant.

*****Indicates highly significant.*

Conc, concentration; ng/ml, nanogram per milliliter; IL-17A, interleukin-17A; sFAS, soluble first apoptosis signal.

also continued to rise after the fifth cycle (fifth cycle-8 days) of nivolumab treatment (**Table 1**). Group 2 comprises five biomarkers that were significantly downregulated after the third cycle-11 days and also continued to decline after the fifth cycle (fifth cycle-8 days) of nivolumab treatment (**Table 2**). These are granzyme A, granzyme B, perforin, soluble first apoptosis signal (sFAS), and IL-17A. Two biomarkers (IL-10 and CX3CL-1 also known as Fractalkine), important for immune activation, were significantly reduced at progression (fifth cycle-226 days, **Figures 4A,B**). Moreover, two biomarkers (IL-6 and IL-8), important for immune inhibition, were significantly upregulated at progression (fifth cycle-226 days, **Figures 4C,D**). The remaining 12 biomarkers analyzed [IL-2, IL-4, IL-5, IL-7, IL-12 (p70), IL-13, IL-21, IL-23, MIP-1α, MIP-3α, MIP-1β, and sFASL] showed no significant change (data not shown).

METHODS

Sample Collection and PBMCs Isolation

Peripheral blood samples were obtained in lithium heparin tubes 12 days before nivolumab treatment, 11 days after the third cycle, then 8 and 226 days after the fifth cycles of nivolumab treatment. Plasma was obtained after centrifugation of the blood at 1,200 rpm, frozen at −85°C and used subsequently for ELISA and multiplexing assays. PBMCs were isolated by density gradient centrifugation using Ficoll® Paque Plus (GE Healthcare) and SepMate™ tubes (STEM Cells technologies) according to the manufacturer's instructions. The obtained PBMCs were frozen at −150°C and used in ELISpot assay.

Enzyme-Linked Immunosorbent Assay

96-well microtiter plates (Thermo Scientific) were coated with bicarbonate/carbonate buffer containing 10 µg/ml of the NY-ESO-1 peptide spanning its region of 11–30 amino acids (JPT Peptide Technologies GmbH, Germany) representing the most immunogenic epitope of the NY-ESO-1 antibody (17). We used a non-immunogenic long peptide (amino acids 85–111) from NY-ESO-1 as a negative control. After overnight incubation at 4°C, plates were washed with PBS containing 0.05% Tween-20 (PBS-T) (Sigma Aldrich) and blocked for 2 h at room temperature (RT) with PBS containing 5% skimmed milk powder (Sigma Aldrich). The Plasma was incubated for 2 h at RT at different dilutions, 1:100, 1:400, 1:1,600, and 1:6,400, in PBS containing 5% skimmed milk powder. Plates were washed with PBS-T and incubated for 1 h at RT with HRP-conjugated goat-anti-human antibody (Abcam) diluted 1:4,000 in PBS containing 5% skimmed milk powder, followed by measurement of the HRPO activity using 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution (Sigma Aldrich) at 450 nm. The NY-ESO-1 antibody was considered positive if the mean OD value of the 1:100 dilution of the plasma is higher than the mean OD value of the healthy donor plus three times the SD (18).

Western Blot Analysis

The anti-NY-ESO-1 antibody response were tested in the plasma by a standard western blot analysis using 1 µg of recombinant NY-ESO-1 protein and plasma at 1:100, 1:500, 1:1,000, 1:2,000, and 1:4,000 dilutions. HRPO-conjugated goat-anti-human antibody (Abcam) diluted at 1:4,000 was used as secondary reagent. The protein band of NY-ESO-1 was detected using chemiluminescence (19). Western blot bands from three independent experiments were quantified using the software Image J (http://rsb.info.nih.gov/ij/) (20).

ELISpot Assay

Enzyme-linked immunospot assay was performed to enumerate the patient NY-ESO-1-specific T cells secreting IFN-γ after an *in vitro* re-challenge with the NY-ESO-1 antigen. A pool of 43 Peptides (15mers with 11 aa overlap) representing the whole length of the NY-ESO-1 protein (PepMix™ Human NY-ESO-1, JPT Peptide Technologies) were used. The human IFN-γ ELISpotPLUS kit (Mabtech) was used according to the manufacturer's protocols. PBMCs were plated in duplicates at 130,000 cells/well/200 µl in complete RPMI media (Life Technologies) supplemented with 10% heat inactivated fetal bovine serum (Life Technologies), 1% Penicillin and Streptomycin cocktail antibiotics (Life Technologies) and 1% GlutaMAX (Life Technologies). The NY-ESO-1 PepMix was added at 1 µg/ml. Anti-CD3 mAb (Mabtech) was used as a positive control. We used the PepMix pool for the prostate-specific antigen (PM-PSA, from JPT technologies) as negative control. After 48 h of incubation, plates were developed and analyzed using an automated ELISPOT reader (AID, Strasberg, Germany). The frequency of NY-ESO-1-specific T cells was expressed as specific spot forming unit (SFC) per input cell numbers.

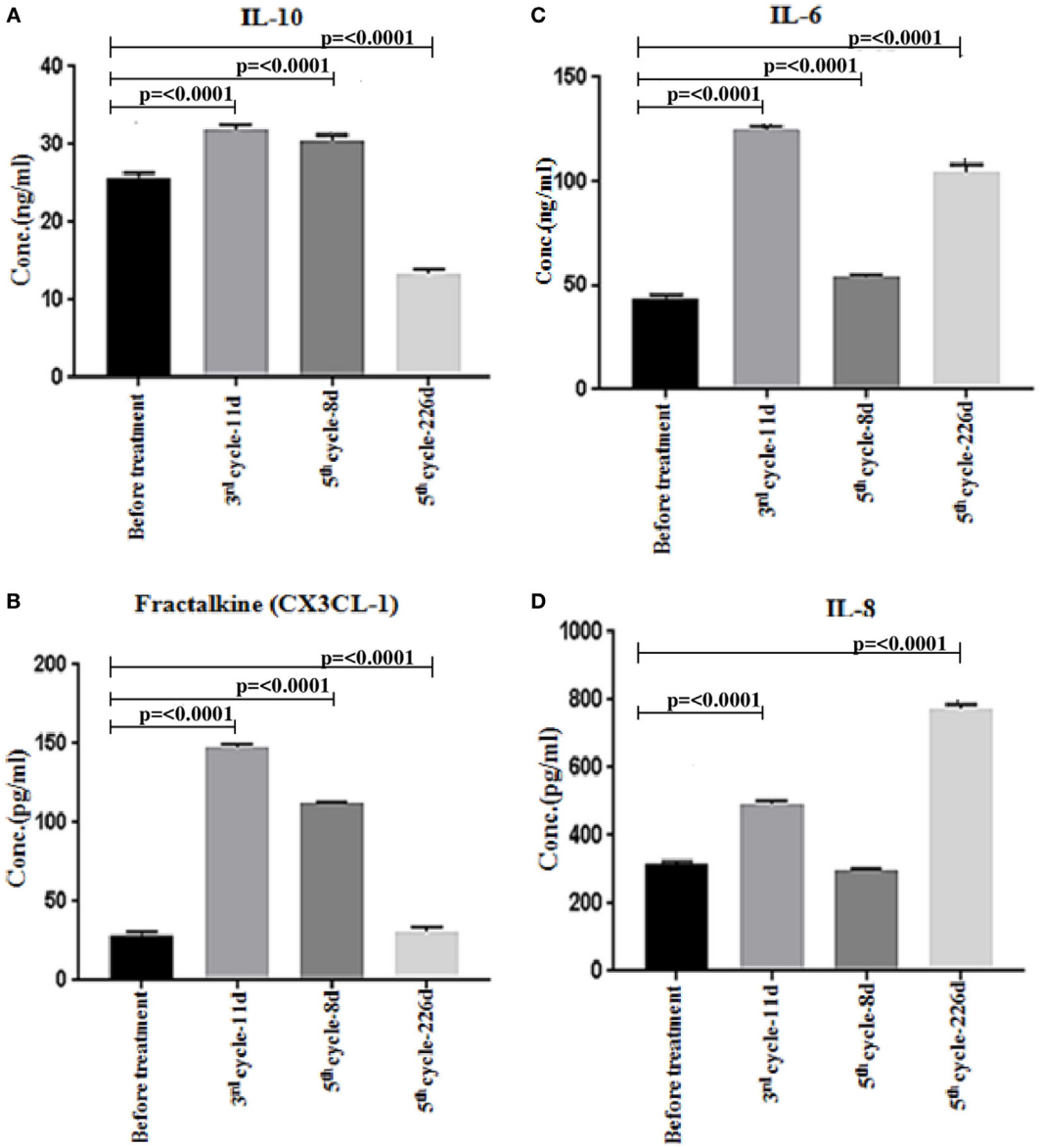

FIGURE 4 | Multiplex analysis of cytokines/chemokines in patient plasma before and after nivolumab treatment, and after progression. **(A,B)** Significant downregulation of the immune activation biomarkers (IL-10 and CX3CL-1 also known as Fractalkine) at progression (fifth cycle-226 days). **(C,D)** Significant upregulation of the immune inhibition biomarkers (IL-6 and IL-8) at progression (fifth cycle-226 days). The assay was repeated three times and the shown data corresponds to one representative experiment. Statistical analysis was performed using non-parametric unpaired ANOVA followed by multiple comparison Dunnet's test and *p* values <0.05 were considered statistically significant.

Flow Cytometry Analysis

Flow cytometry analysis was carried out to determine PD-1 expression by the patient T cells before and after nivolumab treatment. Cryopreserved PBMCs were thawed, washed, and suspended in cold PBS (Gibco). The cells were stained with CD3-BUV395 (BD Biosciences), CD4-BUV805 (BD Bioscience), CD8-FITC (eBioscience), and PD-1-PE (eBioscience) antibodies. Isotype controls corresponding all the tested antibodies were used. After 30 min incubation at RT, the stained cells were washed then acquired on BD FACSCalibur flow cytometer and the data was analyzed using FACSDiva 8.0.1 software (BD Biosciences).

Multiplex Analysis

The quantification of the patient cytokines and chemokines (granzyme A, granzyme B, perforin, MIP-1 α, MIP-1 β, MIP-3α, ITAC, sFASL, sFAS, sCD137, GM-CSF, IFN-γ, TNF-α, IL-1 β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, IL-21, and IL-23) was performed using the human Cytokine/Chemokine Magnetic Bead multiplex assay ($CD8^+$ T cell magnetic

Bead panel and Human high sensitivity T cell magnetic bead panel kits) (MILLIPLEX MAP, EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Fluorescent signals generated were detected using the multiplex array reader Bio-Plex 200 System (Bio-Rad Laboratories, Inc.). Briefly, raw data were initially measured as relative fluorescence intensity then converted to cytokine concentrations based on the standard curve generated from the reference concentrations supplied with the kit. All samples and standards were performed in duplicates and data were analyzed as mean of duplicates.

Statistical Analysis

For ELISpot analysis, Multiplex analysis and ELISA a non-parametric unpaired ANOVA followed by multiple comparisons Dunnet's test were used. Statistical analysis was performed using Graph Pad Prism 7.0 and p values <0.05 were considered statistically significant.

DISCUSSION

It has been recently demonstrated that patients with HNSCC have downregulation of their antitumor immune responses, and tumor progression or relapse correlates with immune dysfunction (7). PD-1 is an immune checkpoint receptor that is expressed mainly on T cells and limits T cell effector functions within the tumor site (8, 9). Furthermore, tumor cells can upregulate the expression of the PD-1 ligand (PD-L1) and further block the antitumor immune response (21). In this study, it has been shown that PD-L1 was expressed in 50–60% of HNSCC patients (21). Interestingly, blocking the PD-1/PD-L1 interaction with anti-PD-1 antibodies was reported to improve survival in recurrent HNSCC patients (9, 22). Indeed, treatment of recurrent HNSCC patients with nivolumab, in a recent phase III CheckMate 141 clinical trial, resulted in longer overall survival compared to treatment with a standard single agent therapy (11, 12). The trial was stopped early due to this survival advantage (11, 12). Based on this indication, nivolumab was recently approved to treat recurrent HNSCC patients (10). In the present report, nivolumab was used to treat a 71-year-old male patient with a long and recurrent history of HNSCC. It has been reported that pseudo-progression with an initial increase in total tumor burden; stable disease state with a slow steady decline in total tumor volume; or a presence of new lesions, were all associated with patients responding to anti-CTLA-4 treatment and were linked to favorable survival (23). The patient reported in our study had an initial increase in total tumor burden and displayed a stable disease with slow steady decline in total tumor volume over 7 months demonstrating an initial pseudo-progression phenomenon. The patient progressed after 7 months of nivolumab treatment. Although immunotherapy has advocated to continue after the disease first progression provided that the general condition of the patient had improved, our patient refused to continue nivolumab treatment after the fifth cycle.

It has been demonstrated that changes in NY-ESO-1 antibody levels correlate with the evolution of NY-ESO-1 positive disease and tend to disappear with tumor resection or therapy-induced regression (24). Furthermore, the humoral immune response against the NY-ESO-1 antigen is frequently observed in patients with NY-ESO-1 expressing tumors and no NY-ESO-1 antibody has been detected in healthy controls and/or patients with NY-ESO-1 negative tumors (24). Plasma samples collected from the patient before nivolumab treatment expressed significant levels of NY-ESO-1 antibody compared to a pool of five plasma samples collected from healthy controls. This confirms the presence of NY-ESO-1 antigen in the patient's tumor, as the induction and maintenance of NY-ESO-1 antibody was shown to be dependent on the presence of NY-ESO-1 expressing tumors. Moreover, we have shown in this report significant reduction in NY-ESO-1 antibody levels after the third (third cycle-11 days) and fifth (fifth cycle-8 days) cycles of nivolumab treatment compared to levels obtained before treatment. We have also demonstrated that the patient at progression stage (fifth cycle-226 days) did not have elevated NY-ESO-1 antibody levels raising the possibility of immune selection of NY-ESO-1 antigen negative variants (25).

We have shown that the patient's T cells response to the NY-ESO-1 antigen was slightly increased after the third cycle (third cycle-11 days) and was significantly higher at the fifth (fifth cycle-8 days) cycle of nivolumab treatment. This is in line with another study demonstrating that treatment with an anti-PD-1 antibody increased the NY-ESO-1-specific T cells expansion in melanoma patients (26). Moreover, it has been demonstrated that T cells response to NY-ESO-1 antigen correlates with the patients' clinical benefit in melanoma treated with anti-CTLA-4 antibody (16). Our data also showed that the T cells response detected after anti-PD-1 treatment was significantly declined after the stage of progression (fifth cycle-226 days). It has been shown recently that the expression of PD-1 on $CD4^+$ T cells has a prognostic value in NSCLC patients, as high expression of this molecule was associated with a shorter progression-free survival/overall survival (27). In line with this, we showed here that the majority of all $PD\text{-}1^+/CD3^+$ T cells analyzed before nivolumab treatment were of the $CD4^+$ T cells population (**Figure 3**). Treatment with an anti-PD-1 antibody was shown to increase NY-ESO-1-specific $CD8^+$ T cells expansion in melanoma patients and in contrast to EBV, influenza, or Melan-A/MART-1-specific $CD8^+$ T cells, NY-ESO-1-specific $CD8^+$ T cells uniquely express the PD-1 molecule (26). In line with this, nivolumab treatment may expand NY-ESO-1-specific $CD8^+$ T population expressing the PD-1 molecule in our patient (**Figures 3A–D**).

It has been previously demonstrated that in addition to the direct tumor lytic activity of $CD8^+$ T cells, $CD4^+$ T cells provide a protective function by cytokines secretion and inflammatory reactions. Our data showed that both Th_1 and Th_2 T cells are involved in the immune response of the patient and this was correlated with the cytokines/lymphokines profile produced upon response to nivolumab treatment (**Figure 4**). We have shown that several cytokines/chemokines, important for immune activation were upregulated after nivolumab treatment in which one important cytokine (IL-10) and chemokine (CX3CL1/Fractalkine) were significantly reduced at tumor progression. IL-10 has been known as an anti-inflammatory cytokine produced primarily by antigen-presenting cells, which exerts negative regulatory effects on pro-inflammatory cytokines by downregulating their synthesis (28). However, it

has been shown recently that IL-10 plays also a major role as an immune-activating cytokine in cancer immunotherapy (29). In this respect, IL-10 induces production of IFN-γ that strongly induces the expression of MHC and costimulatory molecules, therefore both IL-10 and IFN-γ stimulate TCR signaling enabling T cells activation and proliferation (29). Interestingly, it has been recently demonstrated in advanced melanoma that IL-10 levels in nivolumab responders were significantly higher than those in the non-responders (30) supporting our current findings. CX3CL1 is a chemokine induced by inflammatory cytokines such as TNFα, IL-1β, and IFN-γ and its role is to recruit immune cells at tumor sites and to boost antitumor immune responses (31). Indeed, high expression of the CX3CL1 molecule by tumor cells was found to correlate with a good prognosis and with increased tumor-infiltrating $CD8^+$ T cells, natural killer cells, and dendritic cells in breast carcinoma (31). It has been demonstrated recently that high expression of CX3CL1 in colorectal cancer was significantly associated with more favorable patients' prognosis whereas low expression identifies a subset of patients with significantly higher risk of developing distant metastasis and rapid tumor progression (32). Furthermore, a recent study has shown that CX3CR1, a receptor for CX3CL1, is exclusively expressed in a subset of $CD8^+$ effector memory T cells infiltrating tumor tissues in patients responding to anti-PD-1 therapy. This study has also demonstrated that PD-1 is mainly expressed in such T cells population (33). This is in line with our data showing the significant induction of CX3CL1 after nivolumab treatment and its downregulation at tumor progression (**Figure 4B**).

We have also shown that some cytokines/chemokines contributing to immune response inhibition were downregulated after nivolumab treatment in which one important cytokine (IL-6) and chemokine (IL-8) were significantly increased at progression. IL-6 is a pleiotropic cytokine that plays an important role in cell proliferation, survival, differentiation, migration, and invasion. It regulates tumor progression and tumor metastasis by modulating tumor angiogenesis and tumor lympho-angiogenesis (34). It has been demonstrated recently that IL-6 levels were markedly upregulated in HNSCC patients and high IL-6 expression independently predicts tumor recurrence, metastasis, and poor survival (34). We have shown here that IL-6 levels were significantly upregulated at both pseudo-progression and progression stages (**Figure 4C**). IL-8 is a chemokine secreted by malignant cells and tumor stroma cells across many different tumor types (35). It has been shown very recently that melanoma and NSCLC patients treated with anti-PD-1 antibody illustrated an early decrease in the levels of serum IL-8 and this was associated with longer overall survival (35). Moreover, high serum IL-8 levels in cancer patients presenting pseudo-progression also reflected true response to anti-PD-1 antibody treatment (35). We have shown in this report that IL-8 levels were significantly upregulated at both pseudo-progression and progression (**Figure 4D**). Finally, the significant low levels of cytolytic factors such as granzymes A and B, perforin, and sFAS in serum may be explained by the fact that activated T cells must migrate to come into contact with the tumor and after recognition of antigens, they release such cytolytic enzymes which recruit other cells of the immune system to destroy the tumor.

CONCLUDING REMARKS

We have analyzed the expression of immunological markers before and after anti-PD-1 treatment in a patient with a long recurrent history of HNSCC and spontaneous immunity to the NY-ESO-1 antigen. The patient showed a transient regression and stability of the tumor after anti-PD-1 treatment for a period of seven and half months. The analysis of immunological markers in this patient showed a differential expression before and after anti-PD-1 treatment and at progression. Although we recognize one drawback of including only one patient in this current study, we suggest that this immunological monitoring would help in providing a critical understanding of the predictive value of NY-ESO-1 antibody, NY-ESO-1-specific T cells response, and the cytokines/chemokines cascade as biomarkers of the response to anti-PD-1 treatment. Further studies are needed to be performed in a larger number of cases from HNSCC patients treated with anti-PD-1 therapy to confirm our findings.

ETHICS STATEMENT

The report was approved by the ethical board of the Hamad Medical Corporation, Doha, Qatar. The patient gave informed consent to carry out the laboratory analysis and to publish this report.

AUTHOR CONTRIBUTIONS

SD designed and supervised the study; SD, MM, AR, VI, RK, and SU performed research; MH, NA, AN, and AG collected clinical data; and SD wrote the paper. MM, AR, and VI contributed equally in writing sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Controlling the Immune Suppressor: Transcription Factors and MicroRNAs Regulating CD73/NT5E

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The NT5E (CD73) molecule represents an ecto-5′-nucleotidase expressed on the cell surface of various cell types. Hydrolyzing extracellular adenosine monophosphate into adenosine and inorganic phosphate, NT5E performs numerous homeostatic functions in healthy organs and tissues. Importantly, NT5E can act as inhibitory immune checkpoint molecule, since free adenosine generated by NT5E inhibits cellular immune responses, thereby promoting immune escape of tumor cells. MicroRNAs (miRNAs) are small non-coding RNA molecules regulating gene expression on posttranscriptional level through binding to mRNAs, resulting in translational repression or degradation of the targeted mRNA molecule. In tumor cells, miRNA expression patterns are often altered which in turn might affect NT5E surface expression and eventually influence the efficacy of antitumor immune responses. This review describes the diverse roles of NT5E, summarizes current knowledge about transcription factors controlling NT5E expression, and highlights the significance of miRNAs involved in the posttranscriptional regulation of NT5E expression.

Keywords: checkpoint molecule, CD73, NT5E, microRNA, transcription factor, T cell, tumor, A2A receptor

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FUNCTIONS OF NT5E IN HEALTHY TISSUE AND TUMORS

The membrane bound NT5E (CD73) is an ecto-5′-nucleotidase (NT5E) hydrolyzing extracellular adenosine monophosphate (AMP) into adenosine and inorganic phosphate (1) (**Figure 1A**). The enzyme consists of a homodimer inserted into the cellular membrane by glycophosphatidylinositol anchors. Besides hydrolyzing AMP to adenosine, NT5E has nucleosidase activity as shown for nicotinamide adenine dinucleotide and nicotinamide mononucleotide (2, 3). NT5E works in concert with ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1), which is also referred to as CD39, representing another ectonucleotidase acting upstream of NT5E catalyzing the hydrolysis of adenosine triphosphate (ATP) into AMP through two reversible reaction steps, whereas the final NT5E-mediated reaction from AMP to adenosine is largely irreversible (4) (**Figure 1A**). The two C-terminal domains of the NT5E molecule mediate noncovalent homodimer association and harbor the substrate binding sites (2). The molecular structure of NT5E can exhibit open or closed conformation and transition between these two stages occurs during substrate cleavage involving conformational changes enabled by the flexible α-helix connecting the C-terminal domains with the Zn^{2+} binding N-terminal domains (3), the latter being N-glycosylated at four distinct asparagine residues either by mannose saccharide chains or by a mixture of complex glycans and high mannose (2). Besides expression of the full-length molecule NT5EL (NT5E-201, 574 aa), a spliced version lacking exon 7 designated NT5ES (NT5E-203, 525 aa) can be detected in various human tissues and was found intracellularly overexpressed in human hepatocellular carcinoma cell lines (5).

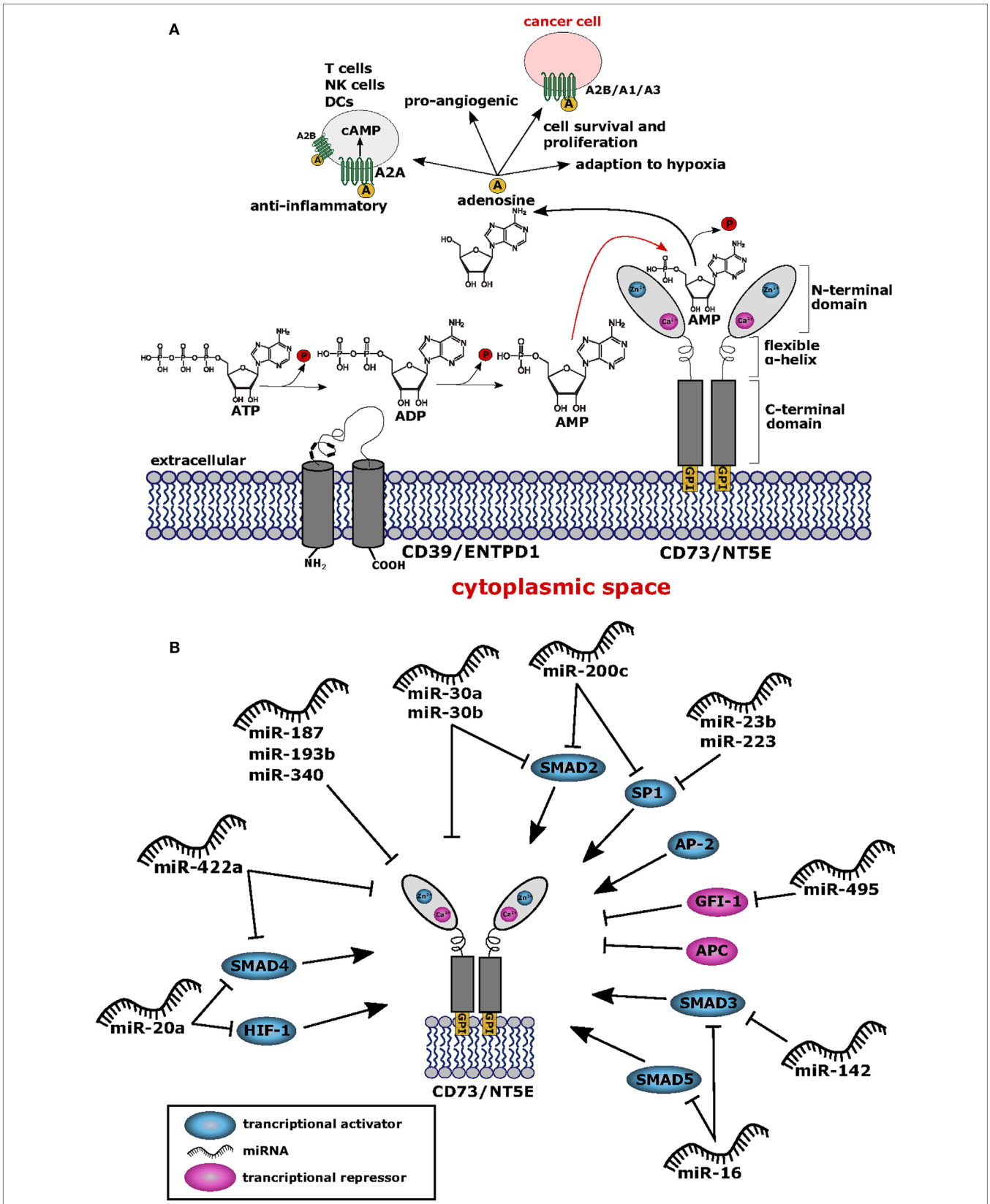

FIGURE 1 | Continued

FIGURE 1 | **(A)** Structure and function of CD73/NT5E. The membrane bound ecto-5′-nucleotidase NT5E hydrolyzes extracellular adenosine monophosphate (AMP) into adenosine and inorganic phosphate (P). Upstream of NT5E, adenosine triphosphate (ATP) is hydrolyzed *via* two reaction steps into AMP by the enzyme ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1) (CD39). Adenosine thus produced exerts anti-inflammatory effects by binding to the adenosine A2A receptor (ADORA2A) expressed by T cells, natural killer (NK) cells, and dendritic cells (DCs) resulting in cAMP mediated blocking of their effector functions. To some extent, the A2B receptor (ADORA2B) is also expressed on DCs and macrophages which are suppressed by adenosine. Thus, cancer cells can evade the immune system by upregulating NT5E protein levels. Furthermore, adenosine binds to the A2B receptor expressed by cancer cells leading to tumor cell survival and proliferation. Cancer cells also express the adenosine A1 receptor (ADORA1) and A3 receptor (ADORA3) and binding of adenosine to these receptors leads to tumor cell migration and proliferation *via* signaling through Gαi proteins. Adenosine is also involved in the adaption to hypoxia and shows pro-angiogenic potential. All adenosine receptors are depicted as stylized green transmembrane proteins. Adenosine is also symbolized as yellow circles marked with "A". **(B)** Network of transcription factors and microRNAs (miRNAs) regulating NT5E expression. This network summarizes the current knowledge on regulation of NT5E on transcriptional (TFs) and posttranscriptional level by TFs and miRNAs, respectively. Transcriptional activators are depicted in blue and transcriptional repressors are highlighted in magenta. miRNAs targeting NT5E directly are shown, as well as miRNAs with indirect impact on NT5E expression through targeting of transcriptional regulators.

As depletion of amino acids 404–453 encoded by exon 7 prevents homo dimerization, NT5ES shows impaired substrate binding resulting in abrogated 5′-nucleotidase activity and lack of surface expression. Importantly, overexpression of NT5ES was shown to cause proteasome-mediated degradation of intracellular NT5EL, without affecting expression levels of native NT5EL dimers. Thus, altered splicing patterns commonly observed in many tumors (6–8) might contribute to aberrant NT5E expression levels in cancer cells.

Considering healthy tissue, expression of NT5E is detectable in epithelial cells of the respiratory tract, smooth muscle cells, and cardiac myocytes and other tissues, as can be deduced from the bioGPS mRNA expression data base (9) using the data set of Primary Cell Atlas (10). Under physiological conditions, NT5E has been described as a regulator of epithelial ion transport, thereby preserving mucosal hydration (11). Moreover, NT5E can act as gate keeper on endothelial cells as free adenosine facilitates "resealing" of gaps between vascular endothelial cells left behind by transmigrating neutrophils (12). Furthermore, adenosine generated through NT5E was described to restrict inflammatory immune responses through a negative feedback loop on adenosine receptor expressing neutrophils (13). NT5E has been found expressed on regulatory T cells (T_{reg}) and at even higher levels on anergic $CD4^+$ T cells, thereby preserving self tolerance in healthy individuals and protecting the fetus from maternal immune attack during pregnancy (14, 15).

Interestingly, qualitative differences in NT5E function have been described depending on the NT5E expressing cell type (16, 17). Comparing epithelial cells and lymphocytes, both expressing NT5E, lymphocyte NT5E was found susceptible to phosphatidylinositol phospholipase to greater extent compared to NT5E expressed by epithelial cells (16). In the same study, antibody binding to NT5E triggered shedding from the surface of lymphocytes, but not in the case of epithelial cells. A similar observation was reported by others who proposed NT5E shedding from the surface of B16F10 cells as explanation for absent cell surface staining on murine B16F10 melanoma cells despite detection of intracellular NT5E expression (18). The study by Airas et al. (16) demonstrated also signal transduction activity of NT5E expressed on lymphocytes, whereas NT5E expressing epithelial cells lacked this function. Signal transduction by NT5E appears unexpected since this molecule lacks intracellular signaling domains (**Figure 1A**); however, it has been suggested that NT5E might associate with src protein kinases, thereby facilitating cellular signal transduction as proposed by Wang and colleagues (19, 20). Alternatively, NT5E might also mediate signal transduction directly (19).

In pathophysiological situations, NT5E activity was found relevant for the generation of cardio-protective adenosine in the ischemic myocardium (21) or for adaption to hypoxia (see below). Importantly, NT5E is involved in tumor development. Thus, NT5E has been described to sustain tumor angiogenesis in murine tumor models of breast cancer and prostate cancer (20, 22) as well as in xenograft models of humans breast cancer (23). Likewise, NT5E expression promoted invasion and metastasis of murine and human melanoma cells (24) and of human breast cancer cells (25).

Notably, NT5E plays a significant role as immune-inhibitory checkpoint molecule (26). Thus, infiltration of tumors by NT5E expressing regulatory immune cells such as T_{reg} (27), MDSCs (28), or dendritic cells (DCs) (29) results in accumulation of immunosuppressive adenosine that can activate cAMP signaling in T cells expressing A2A adenosine receptors (ADORA2A). Moreover, adenosine receptors were found to be expressed on DCs, macrophages, MDSCs, and natural killer (NK) cells, implying that adenosine can repress the function of these immune cells (30, 31). Recently, an interesting phenomenon was described showing that T_{reg} undergoing apoptosis within the metabolically abnormal tumor microenvironment release substantial amounts of ATP, that is degraded by the nucleotidases of the faded T_{reg} resulting in accumulating adenosine levels (32). Adenosine can then trigger immune suppressive downstream effects among T cells like inhibition of chemotaxis, proliferation, activation, and effector function (33, 34). In light of its immune suppressive function and due to its expression by various tumor entities, such as melanoma (35–37), triple-negative breast cancer (34, 38), colorectal cancer (CRC) (39), and non-small cell lung cancer (40), NT5E has been considered as target checkpoint molecule for novel tumor immunotherapy approaches (41, 42). Indeed, injection of blocking NT5E-specific ab into tumor bearing mice resulted in reduced outgrowth of NT5E expressing tumors as shown for various tumor entities (43–45). Noteworthy, tumor cells can express adenosine A1 receptor (ADORA1) and ADORA3 receptors coupled to Gαi proteins, fostering tumor cell proliferation and migration (46). Moreover, therapeutic targeting of NT5E using specific inhibitors or blocking antibodies, respectively, has been proposed (22, 34, 38, 47–50) and is presently tested in a phase I clinical trial (NCT02503774).

In addition to its enzymatic function, NT5E can act as a receptor molecule shown to mediate cell–cell adhesion between lymphocytes and endothelial cells (51). Moreover, it was demonstrated that NT5E was can interact with extracellular matrix components (ECM) (34, 52, 53). This interaction occurred independently from enzymatic activity of NT5E, as blocking of ectonucleotidase function by concanavalin A did not affect interaction with ECM components like fibronectin, tenascin C, or collagen 1. In fact, NT5E turned out to mediate cell adhesion and migration *via* interaction with tenascin C (53).

Thus, NT5E appears to support tumor growth at multiple levels, i.e., by suppression of antitumoral immune responses *via* supply of adenosine and through facilitated dissemination of malignant cells from the primary tumor.

TRANSCRIPTIONAL REGULATION OF NT5E EXPRESSION

The promoter region of NT5E contains binding sites for the transcription factors SP1, AP-2, and SMAD proteins as well as cAMP-responsive elements (54, 55) (**Figure 1B**; **Table 1**). Chromatin immunoprecipitation showed that transcription factors SMAD2, SMAD3, SMAD4, SMAD5, and SP1 bind to the rat NT5E promoter, with SMAD5 and SP1 being most efficient (55). As rat and human NT5E transcripts share 89% identity (56), it appears possible that human NT5E expression might be regulated by SMAD transcription factors as well.

Interestingly, hypoxia-inducible factor-1 (HIF-1) can directly bind to the NT5E promoter thereby activating NT5E expression (57), which is in line with the functional role described for NT5E in hypoxia adaptation (79). Thus, hypoxia resulting from uncontrolled tumor cell proliferation (80) might induce HIF mediated upregulation of NT5E expression on tumor cells. Another biochemical cascade often altered in tumors is the β-catenin-dependent Wnt signaling pathway (81). The promoter core sequence of NT5E is flanked upstream by a regulatory region containing consensus motifs for T cell factor 1 (TCF-1), representing a component of Wnt/β-catenin signaling pathway. In fact, expression of β-catenin could drastically enhance expression of NT5E. This upregulation was found to be dependent on the presence of TCF-1. Interestingly, the authors could also show that the antagonist of β-catenin, adenomatous polyposis coli protein, inhibits NT5E expression (59). The activation of NT5E

TABLE 1 | List of transcription factors and miRNAs regulating NT5E.

Target	Regulator	Effect on NT5E	Host cell	Reference
NT5E	SP1	Activation	Human WI-L2	Hansen et al. (54)
			Rat hepatocytes	Fausther et al. (55)
NT5E	TFAP2A	Activation	Human WI-L2	Hansen et al. (54)
NT5E	SMAD2	Activation	Rat hepatocytes	Fausther et al. (55)
NT5E	SMAD3	Activation	Rat hepatocytes	Fausther et al. (55)
NT5E	SMAD4	Activation	Rat hepatocytes	Fausther et al. (55)
NT5E	SMAD5	Activation	Rat hepatocytes	Fausther et al. (55)
NT5E	HIF1A	Activation	Human T84 epithelial cells	Synnestvedt et al. (57)
			Human HepaRG cells	Tak et al. (58)
NT5E	TCF-1/β-catenin	Activation	Human HeLa and Jurkat cells, monkey Cos-7 cells	Spychala and Kitajewski (59)
NT5E	APC	Inhibition	Human SW480 colon cancer cells	Spychala and Kitajewski (59)
NT5E	NFκB/TNFα	Activation	Human HT29 colon cancer cells	Pagnotta et al. (60)
NT5E	PPARγ	Inhibition	Human HT29 colon cancer cells	Pagnotta et al. (60)
NT5E	GFI-1	Inhibition	Murine Th17 cells	Chalmin et al. (61)
NT5E	STAT3	Activation	Murine Th17 cells	Chalmin et al. (61)
NT5E	FOXP3	Activation	Murine T_{reg} cells	Zheng et al. (62)
NT5E	miR-422a	Inhibition	Human SCC61, SQ20B and HaCaT cells	Bonnin et al. (63)
NT5E	miR-30 family	Inhibition	Human colorectal cancer	Xie et al. (64)
			Human gallbladder cancer	Wang et al. (65)
NT5E	miR-340	Inhibition	Human gallbladder cancer	Wang et al. (65)
NT5E	miR-187	Inhibition	Human colon cancer SW480, RKO and SW620	Zhang et al. (66)
NT5E	miR-193b	Inhibition	Human pancreatic cancer	Ikeda et al. (67)
SP1	miR-23b	Inhibition	Human MM and WM tumor cells	Fulciniti et al. (68)
SP1	miR-223	Inhibition	Human gastric cancer MGC-803, SGC-7901 and BGC-823	Hu et al. (69)
SP1	miR-200c	Inhibition	Human gastric cancer MGC-803 and AGS	Tang et al. (70)
SMAD2	miR-200c	Inhibition	Human ATC-derived cells	Braun et al. (71)
SMAD2	miR-30 family	Inhibition	Human ATC-derived cells	Braun et al. (71)
SMAD3	miR-16	Inhibition	Human Osteosarcoma	Jones et al. (72)
SMAD3	miR-142	Inhibition	Human HT29 colon cancer cells	Chanda et al. (73)
			MDA-MB-231 breast cancer cells	Ma et al. (74)
SMAD4	miR-20a	Inhibition	Human HT29 and HCT116 colon cancer cells	Cheng et al. (75)
HIF1A	miR-20a	Inhibition	HeLa cells, primary human macrophages	Poitz et al. (76)
GFI1	miR-495	Activation	Human DAOY and D283 (medulloblastoma) cells	Wang et al. (77)
SMAD4	miR-422a	Inhibition	Human LHCN-M2 muscle cells	Paul et al. (78)

MM, multiple myeloma; WM, Waldenstrom's macroglobulinemia; ATC, anaplastic thyroid carcinoma; miRNAs, microRNAs; HIF-1, hypoxia-inducible factor-1; TCF-1, T cell factor 1; APC, adenomatous polyposis coli; T_{reg}, regulatory T cells.

expression by β-catenin was also confirmed by Pagnotta et al. who furthermore identified NFκB/TNFα as positive transcriptional regulators of NT5E (60). Seeking biomarkers for CRC, the authors applied a translational pathology approach and identified NT5E among others as a prognostic marker. In line with these findings, NT5E levels were found significantly upregulated in tumor specimens compared to normal colonic mucosa samples. Spranger et al. found that active β-catenin signaling was negatively associated with T cell infiltration in human melanoma samples. This was confirmed in authochtonous tumor models with inducible β-catenin expression, where absence of T cells was observed selectively in β-catenin expressing tumors (82). It is tempting to speculate that this immune suppressive effect on T cell infiltration might result from enhanced NT5E expression induced through β-catenin signaling. Of note, besides activating mechanisms on NT5E expression *via* NFκB/TNFα signaling pathways, also negative effects on NT5E expression through of PPARγ have been described (60).

In murine Th17 cells differentiated with IL-6 and TGF-β *in vitro*, IL-6 was found to activate Stat3, while TGF-β suppressed the transcription factor Gfi-1. As shown by the authors Stat3 sustained, whereas Gfi-1 repressed expression of ENTPD1 and NT5E through specific promoter binding, thus demonstrating transcriptional regulation of these exonucleotidases in Th17 cells through IL-6 and TGF-β (61).

A genome-wide analysis to identify forkhead box transcription factor (Foxp3) target genes in mouse led to the identification of Nt5e as one target gene of Foxp3 in mature T_{reg} cells (62). Foxp3 is a specific transcription factor expressed in murine and human T_{reg} cells and in recently activated human T cells (83). Thus, regulation of Nt5e by Foxp3 appears cell type specific and does not necessarily apply the same way to cancer cells. Noteworthy, high expression levels of FOXP3 in ovarian cancer has been identified as a prognostic marker for poor survival of patients (84).

MICRORNAs (miRNAs) REGULATING NT5E EXPRESSION

MicroRNAs are small non-coding RNA molecules that bind to the 3′ untranslated region (3′-UTR) of target mRNAs, thereby blocking translation or inducing degradation of the targeted mRNA molecule, respectively, depending on the degree of complementary among the interacting nucleotide sequences (85). In cancer cells, aberrant miRNA expression patterns resulting in impaired regulation of target mRNA expression is commonly observed. Tumor cell-derived miRNAs have therefore gained relevance as biomarkers and as prognostic factors as described (86–88). Of note, the 3′-UTR of NT5E comprises 1,774 nucleotides (89) (NM_001204813.1), exceeding the average size of a human 3′-UTR (90) approximately threefold. Thus, regulation of NT5E expression by miRNAs appears to be particularly restrictive. To date, only a few miRNAs have been described that directly regulate NT5E expression (**Table 1**). Bonnin and co-workers reported the regulation of NT5E by miR-422a in head and neck squamous cell carcinoma (HNSCC) patients. The authors found a significant negative correlation between expression levels of miR-422a levels and NT5E mRNA. Blocking of endogenous miR-422a by specific antagomiRs resulted in increased NT5E protein levels with enhanced enzymatic activity. Reduced levels of miR-422a correlated with shorter relapse free survival times in HNSCC, potentially due to overexpression of NT5E (63).

Recently, miR-30a was found to target NT5E in CRC (64). In this study, transfection with miR-30a reduced NT5E expression on the mRNA and on protein level and direct interaction of miR-30a with the NT5E 3′-UTR was demonstrated *via* luciferase reporter assays. Similarly, direct regulation of NT5E was described through miR-30a-5p in non-small cell lung cancer (91). Enhanced expression of NT5E was accompanied by reduced miR-30a-5p expression, whereas miR-30a-5p overexpression resulted in downregulated NT5E expression on mRNA and protein levels. At the same time, proliferation, cell migration, and invasion of these cells were significantly reduced. These effects were mimicked by silencing NT5E expression using shRNA directed against NT5E.

Interestingly, the miR-30 family shares the same seed sequence (92). Thus, other miRNAs from this family might also regulate NT5E. Indeed, direct regulation of NT5E by miR-30b was shown by Wang et al. in gall bladder carcinoma (GBC). Including miR-340 in their study, the authors found that overexpression of miR-30b or miR-340 reduced GBC cell proliferation, migration, and invasion. For both miRNAs, direct interaction with the NT5E 3′-UTR could be verified and NT5E overexpression partially reverted these miRNA-mediated effects in GBC cells (65). In CRC, miR-187 levels were found strongly downregulated compared to adjacent normal tissue leading to the establishment of miR-187 expression levels as prognostic marker for CRC patients. In fact, transfection of miR-187 reduced cell proliferation and migration *in vitro* and decelerated tumor growth of CRC lines *in vivo*. In the same study, direct targeting of NT5E by miR-187 was demonstrated (66). Studies focused on miRNAs involved in the MAPK pathway of human pancreatic cancer cell lines revealed miR-193b as a direct binder of the NT5E 3′-UTR. However, in this study, binding specificity using a mutated reporter plasmid was not controlled and effects of miR-193 overexpression on NT5E expression on mRNA and protein level were not analyzed (67).

Considering the extraordinary size of the NT5E 3′-UTR region, the restricted number of validated miRNAs identified so far that directly target NT5E mRNA most likely represent just the tip of the iceberg. Further studies are needed to broaden the spectrum of known miRNAs that directly regulate NT5E surface expression, thereby potentially affecting the tumor cells' vulnerability toward immune attack.

On the other hand, miRNAs can also function *via* indirect circuits, for example, by targeting transcription factors of NT5E, opening an alternative route for miRNA-mediated regulation of NT5E expression. In fact, miR-23b was found to directly suppress expression of transcription factor SP1 in multiple myeloma cells (68), and in gastric cancer, an inhibiting effect of miR-223 on epithelial to mesenchymal transition *via* direct posttranscriptional silencing of SP1 was reported (69). Other authors described miR-200b and miR-200c as direct inhibitors of SP1 transcription within this tumor entity (70). Whether the miRNA-mediated inhibition of SP1 expression resulted also in downstream reduction of NT5E expression levels was not investigated in these studies.

In a study by Braun et al., the authors focused on the identification of miRNAs affecting the invasive potential of anaplastic thyroid carcinoma and found miR-200c and miR-30a–e to target SMAD2 (71), representing another transcriptional activator of NT5E. Similarly, miR-16 might indirectly downregulate NT5E expression, as this miRNA was shown to inhibit expression of the transcription factors SMAD3 and, to lesser extent, SMAD5 in human osteosarcoma lines (72). Furthermore, SMAD3 is also targeted by miR-142-5p as shown in human rotavirus infected cells as well as in human breast and lung cancer cell lines (73, 74).

Further examples of miRNA-targeted transcription factors of NT5E are SMAD4 and HIF1A downregulated by miR-20a-5p (75), and Poitz et al. showed the direct downregulation of HIF1A by miR-20a (76). As mentioned above, miR-422a was shown to directly regulate NT5E, however, SMAD4 has also been described as a direct target of miR-422a (78), suggesting that miR-422a has the capacity to decrease NT5E levels both directly and indirectly. One example for a miRNA that could indirectly lead to an upregulation of NT5E levels is miR-495, which was shown to target one of NT5E's transcriptional repressor GFI1 in medulloblastoma cells (77).

CONCLUSION

NT5E (CD73) has emerged as a novel target for tumor immunotherapy approaches, since functional inhibition of NT5E reversed its immunosuppressive effects resulting in tumor immune attack and eradication of cancer cells by cytotoxic $CD8^+$ T cells and NK cells. Knowledge about NT5E regulation on the transcriptional and posttranscriptional level might provide a deeper understanding how cancer cells acquire aberrant NT5E expression to facilitate immune escape. We suggest a complex regulatory network of activatory and inhibitory transcription factors acting in conjunction with miRNAs to control NT5E expression. Interestingly, certain regulators such as miR-422a exert their effect on NT5E expression both directly as well as indirectly, i.e., through binding to the 3′-UTRs of NT5E mRNA and SMAD4 mRNA, the latter representing a transcriptional activator of NT5E. Even though the 3′-UTR region of NT5E is of extraordinary size, only few miRNAs have been described so far that regulate NT5E expression. Identification of further miRNAs targeting NT5E will help to unravel the complex regulation of NT5E expression in cancer cells.

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Checkpoint Blockade Rescues the Repressive Effect of Histone Deacetylases Inhibitors on γδ T Cell Function

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Histone deacetylases (HDAC) are one of the key epigenetic modifiers that control chromatin accessibility and gene expression. Their role in tumorigenesis is well established and HDAC inhibitors have emerged as an effective treatment modality. HDAC inhibitors have been investigated for their specific antitumor activities and also clinically evaluated in treatment of various malignancies. In the present study, we have investigated the effect of HDAC inhibitors on the effector functions of human γδ T cells. HDAC inhibitors inhibit the antigen-specific proliferative response of γδ T cells and cell cycle progression. In antigen-activated γδ T cells, the expression of transcription factors (Eomes and Tbet) and effector molecules (perforin and granzyme B) were decreased upon treatment with HDAC inhibitors. Treatment with HDAC inhibitors attenuated the antitumor cytotoxic potential of γδ T cells, which correlated with the enhanced expression of immune checkpoints programmed death-1 (PD-1) and programmed death ligand-1 in γδ T cells. Interestingly, PD-1 blockade improves the antitumor effector functions of HDAC inhibitor-treated γδ T cells, which is reflected in the increased expression of Granzyme B and Lamp-1. This study provides a rationale for designing HDAC inhibitor and immune check point blockade as a combinatorial treatment modality for cancer.

Keywords: gamma delta (γδ) T cells, phosphoantigen, histone deacetylases inhibitors, effector functions, programmed death-1, programmed death ligand-1

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INTRODUCTION

Gamma delta T cells, the enigmatic brethren of alpha beta (αβ) T cells were discovered coincidently during cloning the αβ T-cell receptor (TCR) locus (1). This small subset of T cells, γδ T cells constitute about 5–10% of the circulating T cell population, which express the variant form of TCR heterodimer (2). γδ T cells manifest the features of both innate and adaptive immunity (3). TheVγ9Vδ2 T cell subset of γδ T cells predominates in peripheral blood, and these cells play an important role in the defense against microbial pathogens, stressed cells, and tumor cells of various origin (4, 5). γδ T cells differ from αβ T cells by their TCR gene usage, tissue tropism, and MHC-independent antigen recognition (6, 7). γδ T-cells display broad functional plasticity, like regulatory potential, antigen-presenting capacity, B-cell helper activity, and have the potential for diverse cytokine production (8). γδ T cells recognize non-peptide phosphoantigens such as isopentenyl pyrophosphate (IPP) or 4-hydroxy-3-methyl-but-2-eneyl pyrophosphate (HMBPP), which are produced through the mevalonate pathway

in mammalian cells or non-mevalonate/Rohmer pathway in non-mammalian cells, respectively (9). γδ T cells are also activated indirectly by aminobisphosphonates such as Zoledronate. Aminobisphosphonates inhibit the key enzyme of mevalonate pathway, farnesyl pyrophosphate synthase and lead to accumulation of IPP. Tumor cells treated with aminobisphophonates show increase in the intracellular level of IPP and, therefore, are easily targeted by γδ Tcells (10, 11).

Activated γδ T cells are known to produce large amounts of the pro-inflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) as well as the chemokines MIP-1 (macrophage inflammatory protein) and RANTES (regulated on activation, normal T cell expressed, and secreted) (12). In addition, cytolytic mediators such as granzyme B and perforin are produced to induce specific lysis of cells with elevated phosphoantigen levels (13). Transcription factors like Eomes and Tbet are known to be expressed upon activation by γδ T cells and are essential for antitumor effector functions (14).

Nucleosome is the basic structure of eukaryotic chromatin, composed of histones and DNA. Each nucleosome comprises 146 bp of DNA wrapped around an octamer of core histones (two H2A–H2B dimers and a H3–H4 tetramer) (15). Histone proteins are rich in basic amino acids lysine and arginine. It is through interaction with these histone proteins that massive DNA is packed inside the nucleus. The tails of histone proteins undergo different complex and coordinated posttranslational modifications like histone acetylation, methylation, phosphorylation, and ubiquitination. According to histone code hypothesis, these modifications are read by specific factors, which ultimately lead to downstream events (16). Histone modifications are reversible in nature and influence many fundamental biological processes. Histone acetylation are directed by histone modifying enzymes, histone acetyl transferases (HAT), and histone deacetylases (HDAC), which participate in potential cross-talk between different modifications (15). Normal physiological functions require a balance between HAT and HDAC. Abrupt alterations that skew this balance can give rise to different pathophysiological conditions like cancer (17, 18).

Histone deacetylases inhibitors, including Trichostatin-A (TSA) and sodium valproate (VPA), can alter the acetylation of histones in chromatin and enhance gene transcription. In the recent decades HDAC inhibitors have received attention as antineoplastic treatment. Extensive evidence suggests that HDAC inhibitors play a role in antitumor immunity (19). HDAC inhibitors lead to growth arrest, induction of apoptosis, and differentiation in tumors. Pan HDAC inhibitors like VPA, TSA, and suberoylanilidehydroxamic acid (SAHA) target Class I (HDAC 1, 2, 3, and 8), Class II (HDAC 4, 5, 7, 9, 6, and 10) HDACs. Hence, their anticancer activities are pleotropic in nature, mediated by altering the expression of various genes that are regulated by class I and II HDACs. Additionally, they also target several non-histone proteins such as transcription factors (p53, E2f1) and cytoplasmic proteins (tubulin, hsp, β-catenin). Hyperacetylation of these histone and non-histone proteins brought about by HDAC inhibiton culminate in induction of cell-death pathways in cancer cells. Several studies have established effective tumor reduction *in vitro* as well as *in vivo* upon HDAC inhibitor treatment (20).

Moreover, HDAC inhibitors inhibit angiogenesis and increase the tumor cell antigenicity (21, 22). HDAC inhibitors mediate elevated expression of antigens on tumor cells so that they can be easily targeted by immune cells (23, 24). Due to these promising antitumor functions, HDAC inhibitors are now assessed in clinical trials and some of them have been approved for treatment (25, 26). Recent reports have demonstrated that HDAC inhibitors enhance response to immune checkpoint blockade in triple negative breast cancer, lung adenocarcinoma, melanoma, and multiple myeloma (27–30).

Although the impact of HDAC inhibitors on tumor cells is well studied, their effect on immune cells has recently surfaced. HDAC inhibitors have been shown to have a dual effect on immune cells, either enhancing their activation in cases of CD4 T cell and Tregs whereas dampening the effector functions of NK cells and CD8 T cells. HDAC inhibitors are also known to inhibit the cytotoxic potential of NK cells. HDAC inhibitors are also reported to downregulate the co-stimulatory molecules and cytokine signals in antigen-presenting cells (31). Previous studies have shown that HDAC inhibitor treated tumor cells are easily targeted by γδ T cells (32), but the impact of HDAC inhibitors on the functional responses of human γδ T cells are not well understood.

For successful immunotherapy, T cell responses are essential. Besides the TCR signal, co-stimulatory signal also determines the functional response of T cells. Co-stimulatory signal may be of positive or negative. Negative co stimulatory signals may be from different receptors like programmed death-1 (PD-1) and PD ligand-1 (PD-L1) interaction. PD-1 and PD-L1 are the members of immunoglobin family like that of CD28. Interaction of PD-1 and PD-L1 leads to functional impairment in T cells (33). It is well-known fact that tumors use this mechanism to escape the immune attack. Blocking antibodies for these immune check points can enhance antitumor responses, and these immune-modulating antibodies have achieved clinical success with FDA approved treatments for several malignancies (34). It has been shown that γδ T cells express PD-1 and PD-L1 and blocking of this signaling lead to increase in the antitumor potential of γδ T cells (35).

The present study focuses on investigating the direct impact of HDAC inhibitors on human γδ T cells. We have studied the effect of three different HDAC inhibitors, TSA, SAHA, and VPA on γδ T cells. We observed that HDAC inhibitors suppress the antigen-specific proliferative responses of γδ T cells and their antitumor effector functions by increasing the expression of immune checkpoints (PD-1 and PD-L1). The study further demonstrates that blocking of immune checkpoints on γδ T cells is capable of augmenting their antitumor cytotoxic potential. The present study will open new avenues in the field of cancer immunotherapy using HDAC inhibitors.

MATERIALS AND METHODS

γδ T Cell Separation

Heparinized peripheral blood was collected from healthy individuals. Peripheral blood mononuclear cells (PBMCs) were isolated by differential density gradient centrifugation using Ficoll Hypaque (Sigma-Aldrich, St. Louis, MO, USA). The study was

approved by the Institutional Ethics Committee (TMC-IECIII Project no. 166) and written informed consent was obtained from the donors prior to collection of blood samples. The experimental conditions and procedures for handling blood samples were performed as per the biosafety guidelines of the Institute Biosafety Committee. In short, blood samples were handled in biosafety cabinets and personnel handling blood samples were vaccinated against Hepatitis B. γδ T cells were purified from PBMCs using immunomagnetic MicroBeads (Miltenyi Biotech, Bergish Gladbach, Germany) by positive selection, as per manufacturer's instructions. The purity of separated γδ T cells was >95% as confirmed by flow cytometry (FACS Aria, BD Biosciences, USA). Isolated γδ T cells were cultured in RPMI 1640 supplemented with 10% heat inactivated AB serum, 2 mM glutamine, and antibiotics.

Cell Viability Assay

The viability of γδ T cells upon treatment with HDAC inhibitors was evaluated with MTT assay and Annexin V and 7-AAD staining. Briefly, 0.1×10^6 γδ T cells, seeded in 96-well flat bottom plates (Nunc), were treated with the following HDAC inhibitors for the given concentration range: VPA (4–0.25 mM; Sigma-Aldrich), TSA (200–25 nM; Sigma-Aldrich), and SAHA (4–0.25 μM; Sigma-Aldrich) along with HDMAPP (1 nM; Echelon) and rIL-2 (50 IU/ml; Peprotech) for 72 h. γδ T cells treated only with HDMAPP (1 nM) and rIL-2 (50 IU/ml) were used as control. DMSO was used as vehicle control. Following 72 h of treatment, MTT (5 mg/ml) was added and incubated for 4 h at 37°C. After incubation, the spent medium was discarded, the formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm by microplate reader (TECAN, Switzerland). Untreated γδ T cells were used as reference for calculating the viability. Concentrations of HDAC inhibitors, which had no impact on viability of γδ T cells were further validated by Annexin V and 7-AAD staining. The concentration of HDAC inhibitors showing viability more or equal to 90% in γδ T cells were used for all the further experimental procedures.

Quantitative Real-Time PCR (qPCR)

The purified γδ T cells, activated with HDMAPP (1 nM) and rIL-2 (50 IU/ml) were treated in the presence or absence of HDAC inhibitors at the given concentrations VPA (2, 1, 0.5 mM), TSA (100, 50, 25 nM), and SAHA (1, 0.5, 0.25 μM) for 72 h. DMSO was used as vehicle control. Total cellular RNA was isolated by using Trizol reagent (Invitrogen Life Technologies, NY, USA) in accordance with the company's instructions and cDNA was synthesized by High-Capacity cDNA Reverse transcription kit (Applied Biosystems) according to manufacturer's instructions. The gene expression of T-bet, Eomes, perforin, granzyme B, IFN-γ, and TNF-α was evaluated by Quantstudio 15k Flex system (Applied Biosystems) using Power SYBR Green reagents (Applied Biosystems) as per manufacture's procedure. All samples were analyzed with the following sequence specific primers: Perforin forward and reverse primer 5′-GACACACAAAGGTTCCTGCG-3′and5′-GACTTTGGCCCTGGTTACAT-3′, respectively, Granzyme B forward and reverse primer 5′-CAACCAATCCTGCTTCTGCT-3′ and 5′-GTCGTCTCGTATCAGGAAGC-3′, respectively, Eomes forward and reverse primer 5′-ATTCCACCGCCACCAAAC TG-3′ and 5′-GCACCACCTCTACGAACAC-3′, respectively, Tbet forward and reverse primer 5′-GTGACCCAGATGATTGTG CT-3′ and 5′-ATGCGTGTTGGAAGCGTTGC-3′, respectively, IFN-γ forward and reverse primer5′-GCATCGTTTTGGGTT CTCTTG-3′ and 5′-AGTTCCATTATCCGCTACATCTG-3′, respectively, TNF-α forward and reverse primer 5′-ACTTTG GAGTGATCGGCC-3′ and 5′-GCTTGAGGGTTTGCTACA AC-3′, respectively, and 18S rRNA forward and reverse primer 5′-AACGGCTACCACATCCAA-3′ and 5′-TTCCAATTACACG GCCTC-3′, respectively. The gene expression was determined by threshold cycle (C_T) method by applying $2^{-\Delta\Delta Ct}$. All the values were normalized to the expression of 18S rRNA as endogenous control.

Western Blot Analysis

1×10^6 freshly isolated γδ T cells were cultured with HDMAPP (1 nM), rIL-2 (50 IU/ml), and with or without HDAC inhibitors at the given concentrations VPA (2, 1, 0.5 mM), TSA (100, 50, 25 nM), and SAHA (1, 0.5, 0.25 μM) at 37°C. After 72 h of treatment, cells were harvested and whole cell lysates were prepared with SDS lysis buffer (1 M Tris–HCl pH 6.8, 10%w/v SDS, glycerol, β-mercaptoethanol, 1M DTT, and bromophenol blue). 10% Polyacrylamide gels were used to resolve the protein samples and transferred to Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The primary antibodies to T-bet (1:1,000) (Cell Signalling Technology), p21 (1:1,000) (Abcam), Eomes (1:1,000) (Abcam), p53 (1:500) (Santa Cruz), NF-κB (1:1,000) (Abcam), total H3 (Abcam) (1:1,000), total H4 (Abcam) (1:1,000), acetyl H3 (Abcam) (1:1,000), acetyl H4 (Abcam) (1:1,000), and β-actin (1:4,000) (Sigma-Aldrich) as loading control were added at different dilution. Immunostaining was performed using appropriate secondary antibody at a dilution of 1:10,000 and developed with ECL plus Western blot detection system (Amersham Pharmacia).

Immunostaining and Cell Cycle Analysis

The magnetically sorted γδ T cells were kept overnight in RPMI supplemented with 10% FBS and were stained for various cell surface markers such as Vδ2 TCR, CD14, CD19, and CD56. Briefly, the cells were harvested from culture, washed with ice cold PBS, and fixed with 1% paraformaldehyde at 4°C for 15 min. The cells were washed with FACS buffer and then labeled with fluorophore tagged antibodies Vδ2-PE, CD3-PECy7, CD14-PerCP, CD19-FITC, and CD56 PerCP Cy5.5 (BD Biosciences, USA) for 30 min at 4°C. Further, the cells were washed and acquired on FACS Aria (BD Biosciences, San Jose, CA, USA). γδT cells treated with or without HDAC inhibitors for 72 h as described earlier were stained with live–dead (LD) fixable dead cell stain kit (Thermo Fischer) as per manufacturer's protocol. After staining with LD dye, the cells were fixed with paraformaldehyde and permeabilized with 1% saponin. Cells were washed and stained with γδ TCR-PE, CD25-PerCPCy5.5, CD69-APC (BD Biosciences, USA), Perforin-BV421, Granzyme B-PECF594, PD-1-PECF594, and PD-L1-PerCP Cy5.5 (BioLegend, San Diego, CA, USA), NKG2D-APC, CD16-BV421, KIRD2L2/3-PE (Miltenyi Biotech,

Bergish Gladbach, Germany). For determination of degranulation marker, Lamp-1 (CD107a) and effector molecule Granzyme B release, purified γδ T cells were activated with rIL2 (50 IU/ml) and HDMAPP (1 nM) in the presence and absence of TSA (100 nM), SAHA (1 μM), and VPA (2 mM) for 72 h at 37°C. Additionally, for PD-1 blockade, anti-PD1 antibody (3 μg/ml; BioLegend, San Diego, CA, USA) was added along with HDAC inhibitors. These effectors were then cocultured with zoledronate treated tumor targets (AW13516 Oral cancer cell line, COLO-205 Colon cancer cell line and Raji B lymphoblastic cell line) for 4 h in polypropylene tubes (BD Biosciences, USA) at effector target ratio of 4:1 in presence of monensin (5 mg/ml; Sigma-Aldrich) as described previously (36). Anti CD107a APC Ab (BioLegend, San Diego, CA, USA) was added at the start of coculture assay. After 4 h, cells were washed, fixed, and stained with anti-human TCR γδ PE and Granzyme B-PECF-594 (BD Biosciences, USA). Cells were acquired on FACS Aria (BD Biosciences, USA) and analysis was done by using FlowJo software (Tree Star, Ashland, OR, USA). The expression of various cell surface markers and intracellular proteins were analyzed on the γδ TCR^+ cells gated populations.

For cell cycle analysis, 1×10^6 γδ T cells treated with HDAC inhibitors VPA (0.5, 1, 2 mM), TSA (25, 50, 100 nM), and SAHA (0.25, 0.5, 1 μM) for 72 h or kept untreated, were harvested, and fixed by adding chilled 70% ethanol. Next day, cells were washed with PBS and stained with DNA intercalating dye propidium iodide (PI) along with RNAse A at a concentration of 40 and 10 μg/ml, respectively. Cells were incubated at room temperature for 30 min. The samples were acquired on FACS Calibur (BD Biosciences, USA) and analyzed using ModFit software.

Proliferation Assay

Proliferation of γδ T cells was analyzed using ^{3}H-Thymidine (3HTdR) incorporation assay. A total of 5×10^4 γδ T cells were treated in the presence or absence of HDAC inhibitors VPA (0.5, 1, 2 mM), TSA (25, 50, 100 nM), and SAHA (0.25, 0.5, 1 μM) along with HDMAPP (1 nM) and rIL2 (50 IU/ml) for 72 h in 96-well tissue culture plates. The cultures were pulsed with 1 μCi [^{3}H] thymidine (Board of Radiation and Isotope Technology, Mumbai) 18 h prior to termination of the assay. Following the incubation, cells were transferred upon glass-wool filters using cell harvester (Perkin Elmer, UK). The radioactivity incorporated into the DNA was measured in a liquid beta scintillation counter (Packard, Meriden, CT, USA). Data were expressed as counts per minute (cpm).

Cytokine ELISA

For cytokine ELISA, supernatants were collected from γδ T cells treated in the presence of different concentrations of HDAC inhibitors VPA (0.5, 1, 2 mM), TSA (25, 50, 100 nM), and SAHA (0.25, 0.5, 1 μM) along with HDMAPP (1 nM) and rIL2 (50 IU/ml) for 24 h. The concentration of secreted cytokines IFNγ and TNFα was measured by human IFN-γ and TNF-α ELISA kit, respectively (BD Biosciences, USA) as per manufacture's procedure.

Cytotoxicity Assay

Cytotoxic potential of γδ T cells against panel of tumor cell lines, oral tumor cell line (AW13516), colon tumor cell line (COLO-205), and B lymphoblastic cell line (Raji) was performed using lactate dehydrogenase (LDH) release assay as described previously (37). Tumor cell lines were treated for 18 h with zoledronate (100 μM; Sigma-Aldrich). γδ T cells were treated with HDMAPP (1 nM) and rIL-2 (50 IU/ml) in presence and absence of HDAC inhibitors, VPA (2 mM), TSA (100 nM), and SAHA (1 μM) for 72 h at 37°C were used as effectors. Additionally, for PD-1 blockade, anti-PD1 antibody (3 μg/ml) was added to HDAC inhibitor treated γδ T cells for 72 h at 37°C and were also used as effectors. Briefly, tumor cell lines were cocultured with effectors at 40:1 effector target (E/T) ratio for 4 h at 37°C in 96-well plates (Nunc, Denmark). After 4 h of coculture, an aliquot of 50 μl of media was used in LDH cytotoxic assay using the LDH cytotoxic assay kit (Thermo Fisher Scientific, USA) according to manufactures protocol. γδ T cell cytotoxicity was defined as % specific lysis = Experimental value − Effector cells spontaneous control − Target cells spontaneous control/Target Cell Maximum Control − Target cells spontaneous control.

Chromatin Immunoprecipitation (ChIP) qPCR Assays

Chromatin Immunoprecipitation assays were performed using MAGnify TM Chromatin Immunoprecipitation System (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Specific anti-acetyl histone H3 (Abcam) and anti-acetyl histone H4 (Abcam) were used to determine the promoter acetylation of perforin and granzyme-B. Normal rabbit IgG was used as negative control. DNA was extracted and analyzed by quantitative real time PCR (qPCR) with specific primers for perforin (region I forward:5′-GATGAGGGCTGAGGACAG-3′; region I reverse:5′-TCTTCACCGAGGCTCCTG-3′; region II forward:5′-CTGCTGGCCTGTTCATCAAC-3′; region II reverse: 5′-CTGTCCTCAGCCCTCATC-3′) and granzyme B (region I forward: 5′-GGGTGGGCAGCATTTACAG-3′; region I reverse: 5′-TTCTCAGGAAGGCTGCCC-3′; region II forward: 5′-CACT TCATAGGCTTGGGTTCC-3′; region II reverse: 5′-CCTCTGG TTTTGTGGTGTCTC-3′). 1% of starting chromatin was used as input. Relative data quantification was performed using $2^{-\Delta\Delta Ct}$ method, using formula: % Input = 2 (Ct Input − Ct ChIP) × Input dilution factor × 100 and expressed in the form of % input as described earlier (38).

Statistical Analysis

Data analysis was done by Student's *t*-test using GraphPad Prism software (GraphPad Software Inc., CA, USA). The comparative CT method was applied in the quantitative real time RT-PCR according to $2^{-(\Delta\Delta Ct)}$ method. Results were indicated as means ± SE and considered significant at $p < 0.05$.

RESULTS

Effect of HDAC Inhibitors on Viability of γδ T Cells

We first studied effects of HDAC inhibitors VPA (0.25–4 mM), TSA (25—200 nM), and SAHA (0.25–4 μM) on the viability of γδ T cells. Magnetically sorted γδ T cells from peripheral blood

of healthy individuals were activated with HDMAPP (1 nM) and rIL2 (50 IU/ml) in presence and absence of above mentioned HDAC inhibitor concentrations for 72 h. HDMAPP is a synthetic analog of IPP and potent activator of γδ T cells. Immunomagnetically sorted γδ T cells were positive for γδ TCR (90%), CD56 (53%), and negative for αβ TCR, CD14, and CD 19 (Figure S2A in Supplementary Material). Viability of γδ T cells was assessed by MTT assay. It was observed that higher concentrations of HDAC inhibitors were toxic to γδ T cells. γδ T cells showed least viability at VPA (3–4 mM); TSA (150–200 nM), and SAHA (3–4 μM). At lower concentrations, these HDAC inhibitors were not toxic and γδ T cell were viable (>90%) (Figure S1 in Supplementary Material). For further validation of viability, γδ T cells activated with HDMAPP and rIL2 in the presence or absence of HDAC inhibitorsVPA (0.5–2 mM), TSA (25–100 nM), and SAHA (0.25–1 μM) were stained with Annexin V and 7-AAD. We observed that at these concentrations HDAC inhibitors did not induce any significant apoptosis. Since HDAC inhibitor concentrations, VPA (0.5–2 mM), TSA (25–100 nM), and SAHA (0.25–1 μM) showed least effect on the viability of γδ T cells (Figures S2B,C in Supplementary Material), these were selected in further experiments.

HDAC Inhibitors Inhibit the Antigen-Driven Proliferation and Cell Cycle Progression of γδ T Cells

γδ T cell show robust proliferation when stimulated with phosphoantigen (HDMAPP) in presence of rIL2. In order to investigate the effect of HDAC inhibitors on proliferation of γδ T cells, γδ T cells were stimulated with phosphoantigen HDMAPP and rIL2 in the presence or absence of different concentration of HDAC inhibitors (VPA; 0.5–2 mM, TSA; 25–100 nM, and SAHA; 0.25–1 μM) and proliferation was monitored using ^{3}H thymidine incorporation assay. γδ T cells showed robust proliferative responses to phosphoantigen HDMAPP in presence of rIL-2, compared to unstimulated γδ T cells. However, in the presence of various concentrations of VPA, TSA, and SAHA, the proliferative responses of γδ T cells were significantly reduced in a concentration-dependent manner (Figure S3A in Supplementary Material), with maximum decrease in proliferation of γδ T cells observed at higher concentration of HDAC inhibitors, VPA 2 mM, TSA 100 nM, and SAHA 1 μM, respectively. Further, we also evaluated the role of HDAC inhibitors on cell cycle progression of γδ T cells. Freshly isolated γδ T cells were stimulated with HDMAPP and rIL2 in presence or absence of different concentrations of HDAC inhibitors. Upon stimulation with HDMAPP and rIL2, significant number of γδ T cells were in S phase and G2/M phase. However, upon treatment of HDAC inhibitors, γδ T cells were arrested in G0/G1 phase (Figures S3B,C in Supplementary Material). This inhibition of cell cycle progression in γδ T cells upon HDAC inhibitor treatment was reflected in the increased expression of p53 and its downstream target p21, suggesting that HDAC inhibitors impede the G0/G1-S phase transition in γδ T cells in p53-dependent manner (**Figures 1A–C**) and (**Figures 1D–I**).

HDAC Inhibitors Regulate Cytokine Production and Activation in γδ T Cells

γδ T cells upon activation secrete copious amount of cytokines such as IFN-γ and TNF-α (36). We examined the effect of HDAC inhibitors on expression of these cytokines in γδ T cells. Marked increase in the expression of cytokines IFN-γ and TNF-α was observed upon stimulation of γδ T cells with HDMAPP and rIL2 compared to unstimulated γδ T cells. Expression of IFN-γ (**Figures 2A,B**) and TNF-α (**Figures 2C,D**) was decreased significantly when treated with HDAC inhibitors TSA, SAHA, and VPA. This inhibition was observed both at mRNA and protein levels. It was observed that inhibition of cytokine expression was concentration dependent for HDAC inhibitors. We also evaluated the effect of HDAC inhibitors on the expression of early activation marker CD69 and late activation marker CD25 on γδ T cells. Treatment of γδ T cells with HDAC inhibitors led to decrease in the expression of early activation (**Figures 3A,B**) and late activation marker on γδ T cells (**Figures 3C,D**). The expression of these activation markers on γδ T cells were significantly reduced in a concentration-dependent manner, with maximum decrease at VPA 2 mM, TSA 100 nM, and SAHA 1 μM, respectively. Percentage of γδ T cells positive for these markers was also less in HDAC inhibitor treated γδ T cells as compared to untreated γδ T cells. To investigate the role of HDAC inhibitors on the expression of other activating receptors like NKG2D, CD16, and inhibitory receptors like KIR2DL2/3 (CD158b), γδ T cells were treated with HDAC inhibitors VPA 2 mM, TSA 100 nM, and SAHA 1 μM for 72 h. We found that HDAC inhibitor-treated γδ T cells show decreased expression of NKG2D (Figure S4A in Supplementary Material) as compared to untreated γδ T cells. On the contrary, γδ T cells treated with HDAC inhibitors VPA 2 mM, TSA 100 nM, and SAHA 1 μM show increase in the expression of inhibitory receptor KIR2DL2/3 (CD158b) (Figure S4B in Supplementary Material). However, we did not observe any change in CD16 expression (Figure S4C in Supplementary Material). Collectively, the data advocate the role of HDAC inhibitors in abating the expression of activation markers (CD69, CD25, NKG2D) and cytokine (IFN-γ, TNF-α) production in γδ T cells.

HDAC Inhibitors Suppress the Expression of Transcription Factors Eomes and Tbet in γδ T Cells

Eomes and Tbet are two main T-box transcription factors expressed in T cells. They are the main transcription factors, which regulate the effector functions of CD8 T cells through the expression of effector genes perforin and granzyme B (39, 40). Besides CD8 T cells, γδ T cells also express Eomes and Tbet (41). Upon activation with phosphoantigen (HDMAPP) and rIL2, γδ T cells show increased expression of these two transcription factors. We hypothesized that HDAC inhibitors may have an impact on the expression of these two transcription factors in γδ T cells. Therefore, the role of HDAC inhibitors was analyzed in regulating expression of Eomes and Tbet in γδ T cells activated with phosphoantigen (HDMAPP) and rIL2. γδ T cells treated with HDAC inhibitors showed decrease in the expression of Eomes and Tbet at both mRNA (**Figures 4A,B**) and protein level (**Figure 4C**).

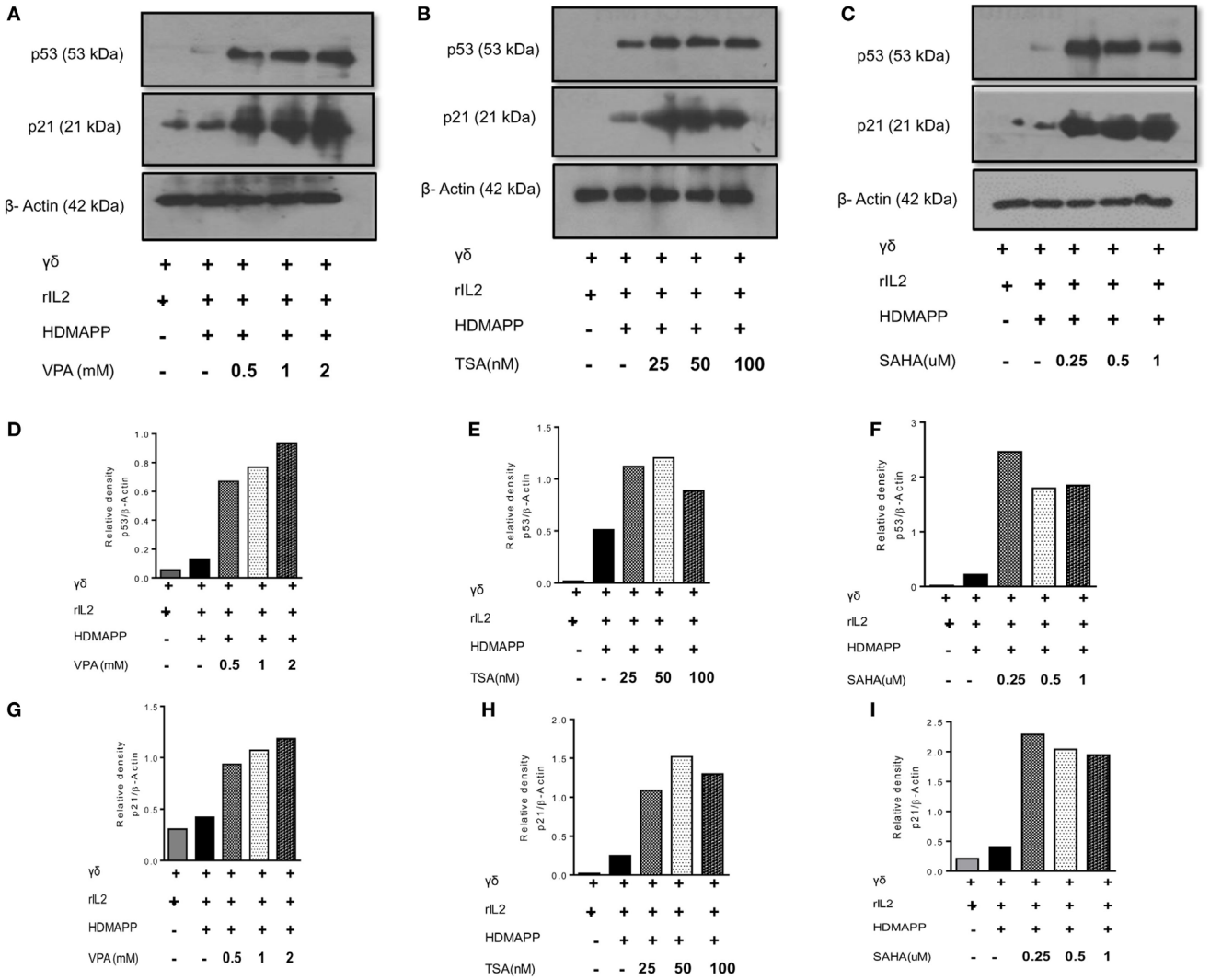


FIGURE 1 | Histone deacetylases (HDAC) inhibitors increase the expression of cell cycle checkpoint proteins p53 and p21. Protein expression of p53 and p21 by γδ T cells upon treatment with **(A)** sodium valproate (VPA), **(B)** Trichostatin-A (TSA), and **(C)** suberoylanilidehydroxamic acid (SAHA) as detected by western blotting. Cell lysates of γδ T cells, stimulated with HDMAPP after treatment with HDAC inhibitors at different concentrations for 72 h were prepared and p53, p21 proteins were detected. β-actin was used as loading control. Densitometry quantification of p53 **(D–F)** and p21 **(G–I)** expression in γδ T cells upon treatment with VPA, TSA, and SAHA, relative to β-actin.

In addition to Eomes and Tbet transcriptional factors, γδ T cells treated with HDAC inhibitors also show decreased expression of NF-κB (Figures S4D–F in Supplementary Material) as compared to untreated γδ T cells. Inhibition of Eomes, Tbet, and NF-κB by HDAC inhibitors clearly demonstrates that HDAC inhibitors regulate the effector functions of γδ T cells.

HDAC Inhibitors Inhibit the Antitumor Cytotoxic Potential of γδ T Cells

To evaluate the role of HDAC inhibitors in modulation of antitumor potential of γδ T cells, we analyzed the expression of effector molecules Perforin and Granzyme B in γδ T cells at mRNA and protein level. Perforin and Granzyme B are the effector molecules, which are responsible for the antitumor functions of CD8 and γδ T cells (42, 43). Freshly isolated γδ T cells activated with phosphoantigen HDMAPP and rIL2 show increased expression of these two effector genes; however, γδ T cells activated in presence of HDAC inhibitors showed decrease in the expression of perforin (**Figures 5A–C**) and granzyme B. (**Figures 5D–F**). Maximum effect on the expression of perforin and granzyme B was observed with VPA 2 mM, TSA 100 nM, and SAHA 1 μM. These concentrations of HDAC inhibitors were used in further cytotoxicity experiments. We next evaluated whether decrease in expression of effector molecules perforin and granzyme B are regulated by histone modifications in γδ T cells. To investigate this, we checked the total histone H3 and H4 acetylation in γδ T cells treated with HDAC inhibitors VPA 2 mM, TSA 100 nM, and SAHA 1 μM. We observed that the total level of H3 acetylation and H4 acetylation increases in γδ T cells after treatment of

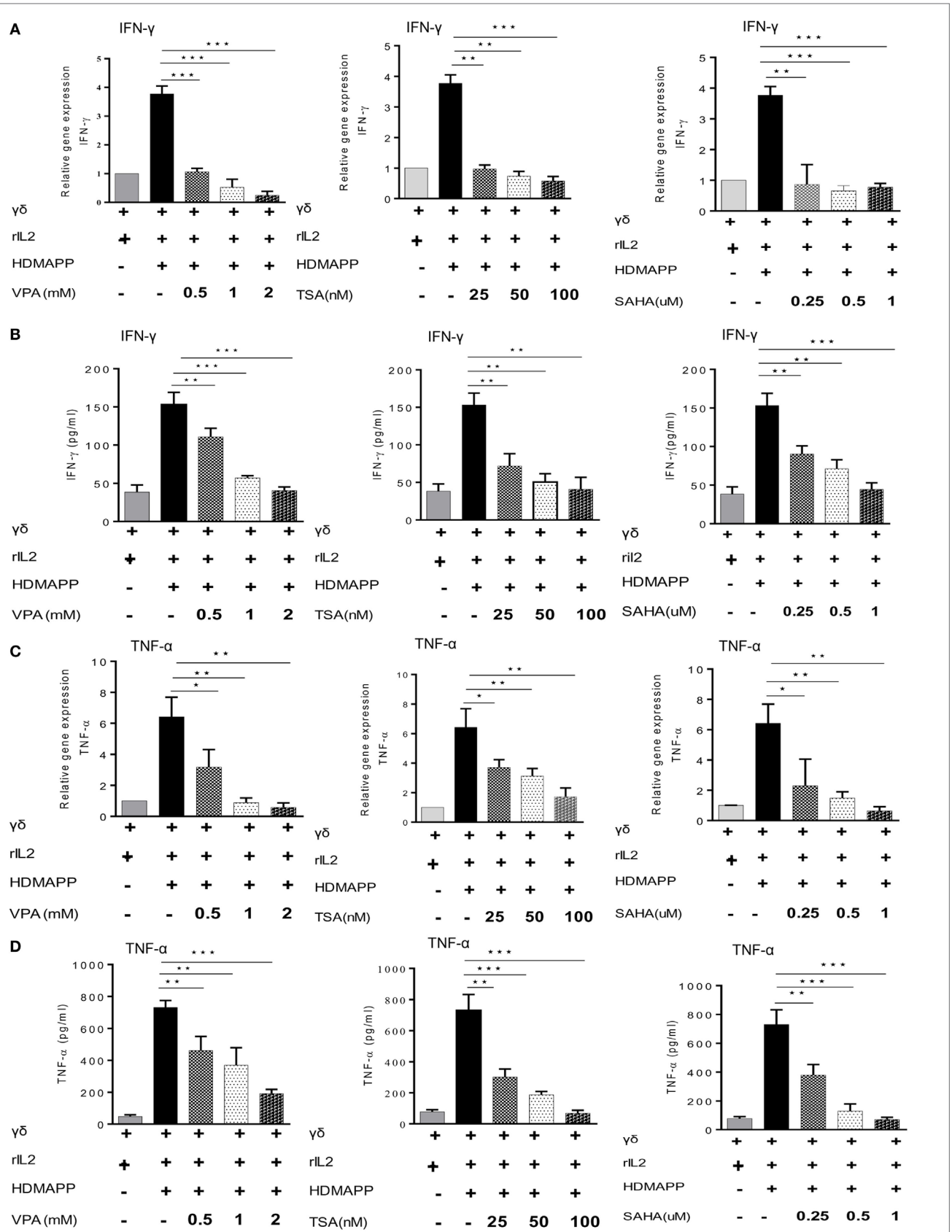


FIGURE 2 | Continued

FIGURE 2 | Histone deacetylases (HDAC) inhibitors regulate cytokine production. Expressions of IFN-γ and TNF-α were detected by quantitative real-time PCR and sandwich ELISA. **(A,B)** IFN-γ expression by γδ T cells stimulated with HDMAPP, treated with or without HDAC inhibitors sodium valproate (VPA), Trichostatin-A (TSA), and suberoylanilidehydroxamic acid (SAHA) at different concentrations at mRNA and protein levels, respectively. **(C,D)** Expression of TNF-α in the supernatants collected from HDMAPP stimulated γδ T cells in the presence or absence of HDAC inhibitors VPA, TSA, and SAHA at different concentrations at mRNA and protein levels, respectively. The expression of different m-RNA transcripts was normalized to 18S r-RNA. All the results indicated are mean ± SEM of three independent experiments, where $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

HDAC inhibitors as compared to untreated γδ T cells (Figure S5A in Supplementary Material). However, HDAC inhibitor-treated γδ T cells show less histone H3 acetylation and H4 acetylation on promoters of perforin and granzyme B compared to untreated γδ T cells determined by ChIP qPCR assay. Histone acetylation is positively correlated with transcriptional activity. Thus, our data show that epigenetic changes on promoters of effector molecules perforin and granzyme B control the expression of these molecules in HDAC inhibitor treated γδ T cells (Figures S5B,C in Supplementary Material). The cytotoxic potential of HDAC inhibitor treated γδ T cells was evaluated against panel of zoledronate-treated tumor cells lines (AW13516, COLO-205, and Raji). At different E/T ratios starting from 5:1 to 40:1, HDMAPP-activated γδ T cells in the presence of IL-2 efficiently lysed zoledronate-treated tumor cells lines (AW13516, COLO-205, and Raji). Maximum cytotoxicity of γδ T cells was observed at E/T ratio of 40:1 (**Figures 6A–C**). This ratio of E: T was used in further experiments, to assess the effect of HDAC inhibitors TSA, VPA, and SAHA on cytolytic ability of γδ T cells. γδ T cells stimulated with HDMAPP and rIL2 in presence of HDAC inhibitors VPA (2 mM), TSA (100 nM), and SAHA (1 μM) for 72 h were used as effector against zoledronate-treated tumor cell lines (AW13516, COLO205 and Raji) as targets at E/T ratio of 40:1. Zoledronate, an aminobisphonate drug, inhibits the enzyme farnesyl pyrophosphate synthase in the mevalonate pathway leading to accumulation of IPP, which stimulates γδ T cell activation *via* TCR signaling. γδ T cells treated with different HDAC inhibitors showed significant decrease in their cytotoxic potential against zoledronate treated tumor targets (AW13516, COLO 205, and Raji) (**Figures 6D–F**). It was observed that all the three HDAC inhibitors (VPA, TSA, and SAHA) significantly inhibited the ability of γδ T cells to kill zoledronate treated tumor cell lines.

HDAC Inhibitors Abrogate the Effector Functions of γδ T Cells by Upregulating the Immune Checkpoint Proteins PD-1 and PD-L1

Programmed death-1 receptor and its ligand PD-L1 are commonly expressed on immune cells. PD-1 and PD-L1 belong to the family of immune checkpoint proteins that act as co-inhibitory signaling inducers. Upon activation, T cells show enhanced expression of immune check point PD-1. Interaction between PD-1 and PD-L1 halt the T cell activation, thus maintaining the immune homeostasis. Tumor cells exploit this pathway to evade immune response. The effect of HDAC inhibitors on the expression of PD-1 and PD-L1 on γδ T cells was studied. γδ T cells were treated with different concentrations of HDAC inhibitors and expression of PD-1 and PD-L1 was analyzed by flow cytometry. Upon activation with antigen HDMAPP and rIL-2, expression of PD-1 and PD-L1 increases on γδ T cells. However, the expression of PD-1 and PD-L1 on γδ T cells substantially increased upon treatment with HDAC inhibitors. Maximum increase in the expression of PD-1 (**Figures 7A,B**) and PD-L1 (**Figures 7C,D**) on HDMAPP and rIL-2 activated γδ T cells was observed after treatment with VPA (2 mM), TSA (100 nm), and SAHA (1 μM). To assess the role of PD1/PD-L1 signaling in HDAC inhibitor treated γδ T cells, γδ T cells were activated with HDMAPP and rIL2, treated or untreated with HDAC inhibitors for 72 h. PD-1 blocking antibody was added at the start of culture. After 72 h, these γδ T cells were cultured with zoledronate-treated tumor cell lines AW13516, COLO-205, and Raji for 4 h at E/T ratio of 4:1. Blockade of PD-1 in HDAC inhibitor treated HDMAPP activated γδ T cells rescued the expression of effector molecules Lamp-1 (CD107a) (**Figure 8A**) and granzyme B (**Figure 8B**) as compared to only HDAC inhibitor treated γδ T cells. To further evaluate the role of HDAC inhibitors on the PD1/PD-L1 signaling axis in γδ T cells, we did the similar experiment by coculturing the effectors and above mentioned tumor targets to analyze the cytotoxic potential by LDH release assay at a ratio of 40:1 for 4 h. Blocking of PD-1 in HDMAPP-activated γδ T cells treated with HDAC inhibitorsVPA (2 mM), TSA (100 nM), and SAHA (1 μM) improves the cytolytic potential of γδ T cells as compared to γδ T cells treated with HDAC inhibitor only (**Figure 8C**). Thus, the results shows that blockade of PD-1 and PD-L1 signaling in HDAC inhibitor treated γδ T cells rescue their effector functions.

DISCUSSION

γδ T cell immunotherapy has become the emerging lead in the landscape of cancer immunotherapies due to their distinctive immune features and potent antitumor effector functions. They have been extensively targeted against diverse tumors such as melanoma, renal cell carcinoma, as well as B cell malignancies and have shown promising results in clinical settings (44). While these therapies have encountered modest clinical success, they have to overcome certain challenges such as limited availability of γδ T cells and rapid exhaustion upon repeated *in vitro* activation. Hence, combinational approaches have been envisaged with chemotherapeutics, monoclonal antibodies, small molecule inhibitors, etc. Newer treatment modality may include combining γδ T cell immunotherapy with antitumor drugs and other immune-modulating antibodies.

Epigenetic dysregulation is one of the hallmarks of cancer. Hence, epigenetic modifiers such as HDAC inhibitors are being comprehensively explored for their anticancer potential. Besides anticancer properties, HDAC inhibitors have also shown promising results in controlling the other pathological conditions such as neurological disorders and viral infections and are well tolerated

(45, 46). Currently, VPA along with other short-chain fatty acids HDAC inhibitors are being clinically evaluated as anticancer drugs (47). HDAC inhibitors employ wide range of antitumor mechanisms such as induction of apoptosis, senescence, differentiation, or inhibition of cell cycle (48, 49). Vorinostat (SAHA), is among the first HDAC inhibitor to be approved by United

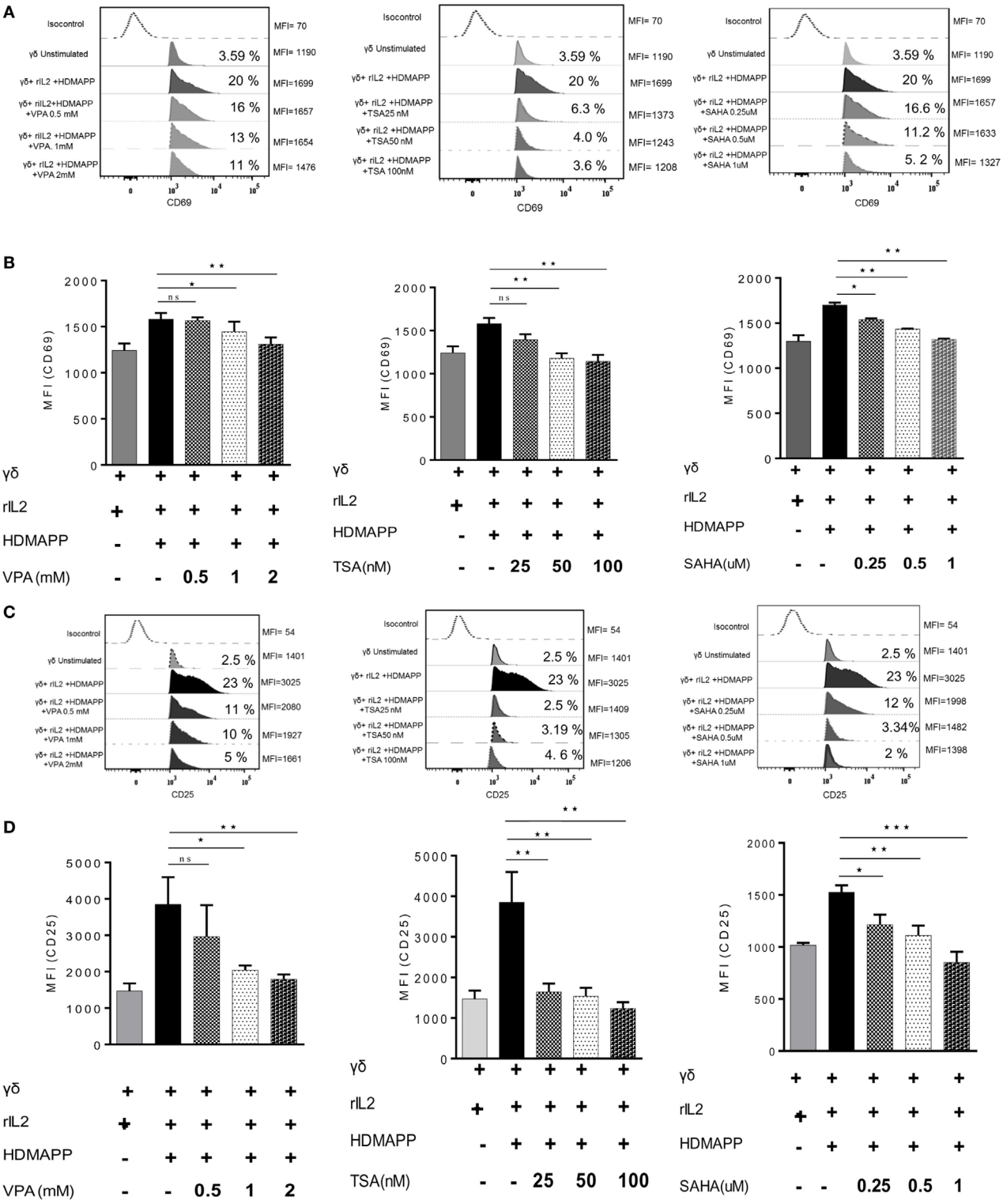


FIGURE 3 | Continued

FIGURE 3 | Histone deacetylases (HDAC) inhibitors affect the activation markers on γδ T cells. **(A)** The expression of early activation marker (CD69) on unstimulated γδ T cells and HDMAPP and rIL-2 stimulated γδ T cells with or without HDAC inhibitor treatment was analyzed by multi-color flow cytometry. Values on right side indicate the median fluorescence intensity (MFI) of CD69, while the values inside the histogram represent the percent CD69-positive γδ T cells. The histograms shown are representative of three independent experiments. **(B)** The cumulative MFI of CD69 expression on γδ T cells is represented as bar graphs. Data shown are representative of three independent experiments where $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$. **(C)** The effect of HDAC inhibitors on expression of late activation marker CD25 was assessed by flow cytometry. Unstimulated γδ T cells and HDMAPP stimulated γδ T cells with or without HDAC inhibitors at different concentrations, after 72 h were stained with the flurophore-tagged antibody and acquired on FACS Aria. Values on right side indicate the MFI of CD25, while the values inside the histogram represent the percent CD25-positive γδ T cells. The histograms depicted are representative of three independent experiments. **(D)** The results shown are cumulative MFI of CD25 expression on γδ T cells. HDAC inhibition decreases expression of CD25. Data shown are representative of three independent experiments where $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

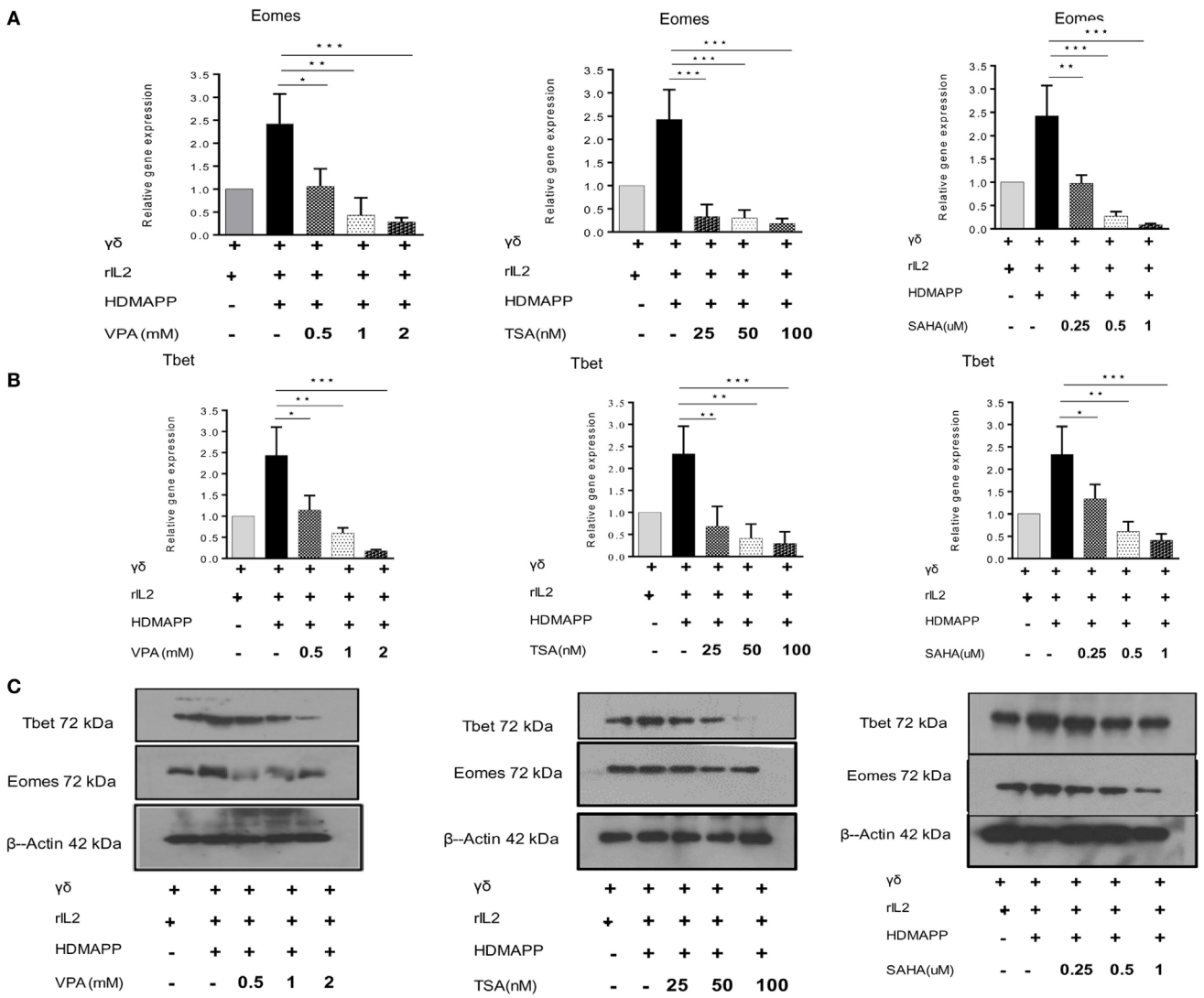
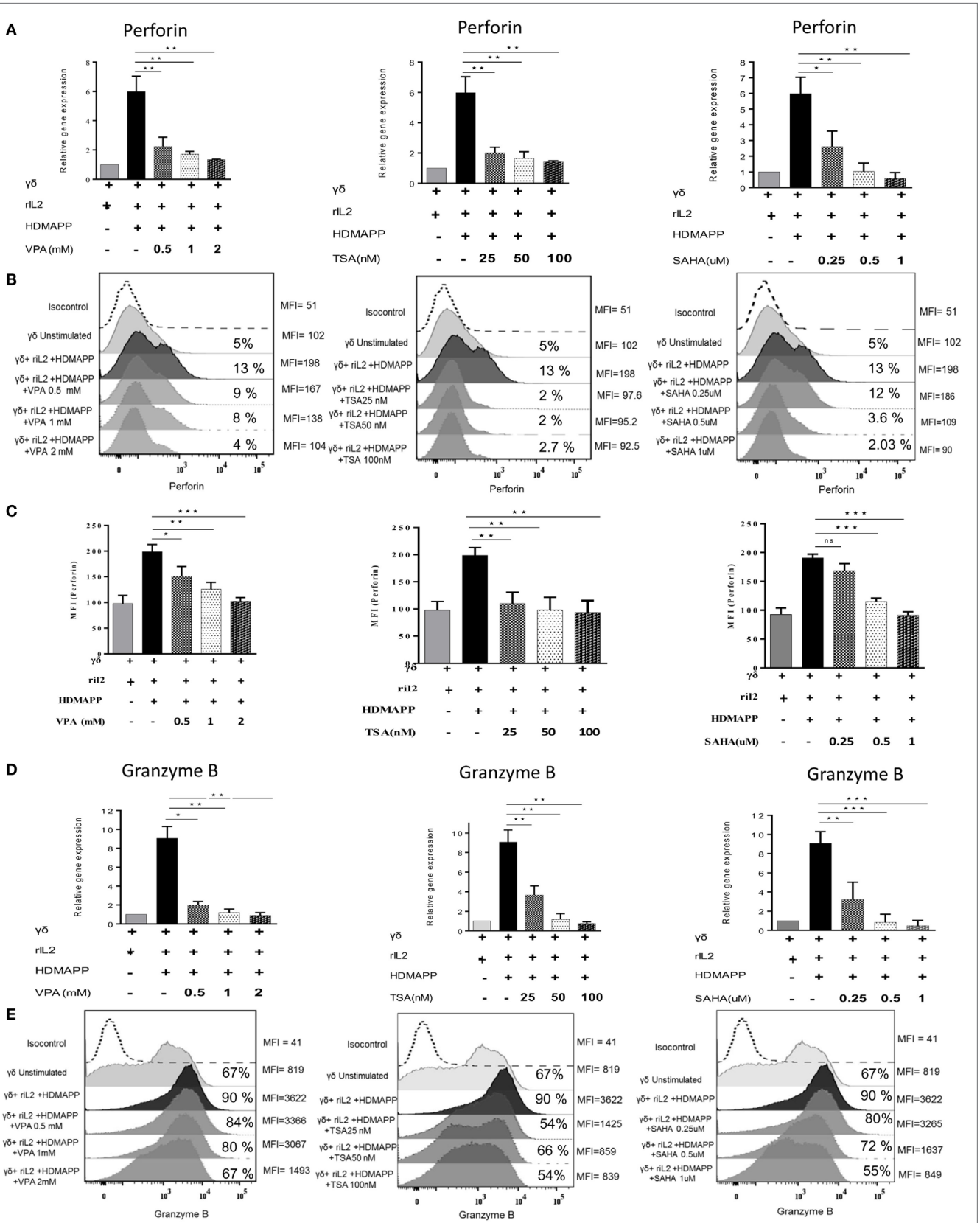


FIGURE 4 | Histone deacetylases (HDAC) inhibition abrogates expression of transcription factors regulating effector functions of γδ T cells. The m-RNA expression of Eomes **(A)** and T bet **(B)** in γδ T cells activated with HDMAPP, in the presence or absence of HDAC inhibitors sodium valproate, Trichostatin-A, and suberoylanilidehydroxamic acid at different concentrations was quantified by quantitative real-time PCR. The results indicated are cumulative mean of relative gene expression normalized to 18S r-RNA where $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, compared with γδ T cells activated with HDMAPP. The data shown are representative of three independent experiments. **(C)** The protein level expression of T bet and Eomes was detected by western blotting. HDAC inhibitor treatment decreases the expression of T-bet and Eomes. β-actin was maintained as loading control. The blots shown are representative of three experiments.

States Food and Drug Administration (FDA) for the treatment of relapsed and refractory cutaneous T-cell lymphoma (50). Although HDAC inhibitors are approved for hematological malignancies, but clear proof-of-concept data for the clinical efficacy of HDAC inhibitors in solid tumors remains to be established (51). Recent studies have demonstrated that HDAC inhibitors exhibit higher



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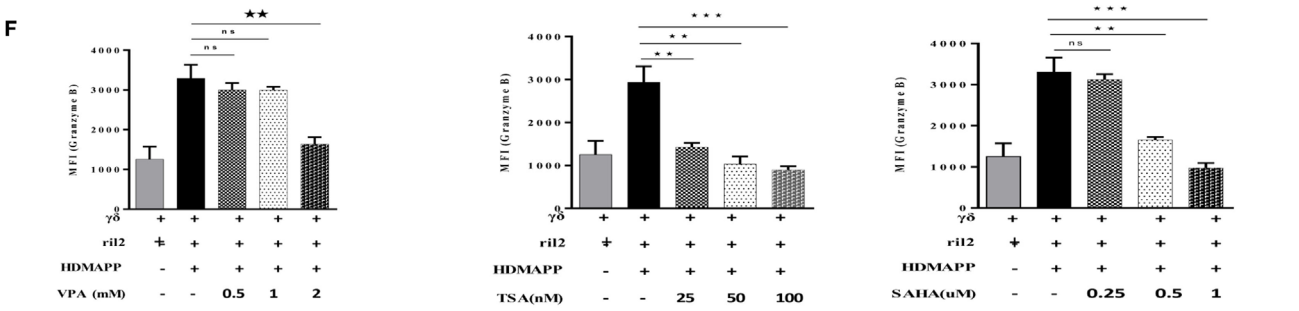

FIGURE 5 | Histone deacetylases (HDAC) inhibitor treatment abrogates the antitumor potential of γδ T cells. Expression of perforin and granzyme was studied at mRNA and protein levels using quantitative real-time PCR and flow cytometry, respectively. The gene expression of **(A)** perforin and **(D)** granzyme B by HDMAPP-stimulated γδ T cells with or without HDAC inhibitor treatment was quantified by quantitative real-time PCR. The results shown are cumulative means of relative gene expression, normalized to 18S r-RNA, and representative of three independent experiments. **(B)** γδ T cells activated by HDMAPP with or without sodium valproate, Trichostatin-A, and suberoylanilidehydroxamic acid at different concentrations were stained after 72 h with corresponding flurophore tagged antibody and expression of perforin was analyzed by flow cytometry. The values on right side of histograms indicate median fluorescence intensity (MFI) of perforin, while the values inside the histogram represent the percent positive γδ T for perforin. **(C)** Bar graphs represent cumulative MFI values where $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$, compared with γδ T cells activated with HDMAPP. **(E)** Expression of granzyme B by unstimulated γδ T cells and HDMAPP activated γδ T cells with or without HDAC inhibitor treatment was assessed by flow cytometric analysis. The values on right side of histograms indicate MFI of granzyme B, while the values inside the histogram represent the percent positive γδ T for granzyme B. The histograms are representative of three independent experiments. **(F)** The graphs represent MFI values of granzyme B where $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

therapeutic efficiency when combined with other antineoplastic agents (52). Hence, there is growing interest in exploring other combined therapeutic strategies with HDAC inhibitors.

Emerging evidence suggest that HDAC play a crucial role in T cell differentiation and effector functions. A number of studies have demonstrated that HDAC inhibitors suppress the immune response of T cells in severe inflammatory conditions and induce tolerance in organ transplantation (53). Specifically, HDAC inhibitors have shown to induce the regulatory T cell (Tregs) generation or stabilization of Tregs in inflammatory microenvironment due to which they have shown promising responses in experimental colitis (54). HDAC inhibitors increase the immunogenicity of tumors by increasing the expression of tumor antigens recognized by the immune cells. The antitumor responses of cytotoxic T lymphocytes like γδ T cells are mediated through recognition of stress molecules (ULBP, HSPs) or danger signals like MICA/B expressed on tumor cells by class of activating receptors known as NKG2D (55–57). Studies have demonstrated that HDAC inhibitors upregulate the NKG2D ligands on tumor cells, thereby sensitizing tumor cells to cytotoxicity mediated by γδ T cells in bladder cancer as well as NK cells in other malignancies such as osteosarcoma, pancreatic cancer, and multiple myeloma (32, 58–60). However, the causal effect of HDAC inhibitors on immune scenario is not well investigated and is contradictory. Several studies have shown that HDAC inhibitors affect each immune subset distinctly either leading to activation as in the case of CD4 T cells and CD8 T cells or by abrogating the effector functions of cells such as NK cell (61–63). Furthermore, for a particular immune cell type, the nature of immune regulation differs based on the type of HDAC inhibitor (64, 65). A recent study demonstrated that NKG2D expression in NK cells is inhibited by VPA (66).

Most of the studies have focused on investigating the impact of HDAC inhibitors on tumor cell lines and immune cells other than γδ T cells. Report by Suzuki et al. demonstrated that the antitumor effect of γδ T cells on bladder cancer was enhanced by treatment with VPA (32). The study focuses only on the impact of HDAC inhibitor, VPA on bladder cancer cell line. VPA leads to increase in the expression of MICA and MICB, which are recognized by NKG2D receptor on γδ T cells. The study does not explain the direct effect of HDAC inhibitors on γδ T cells. Earlier study by Kabelitz et al. reported that HDAC inhibitor VPA induces differential modulation of cell surface markers on γδ T cells compared to αβ T cells (67). Although the study shows the direct effect of VPA on γδ T cells, the functional responses of γδ T cells were not investigated in detail. In the present study, we have used three different HDAC inhibitors to delineate their effect on the functional responses of pure and sorted population of γδ T cells. We used clinically relevant concentrations of VPA, TSA, and SAHA in our study, which have been used in *in vitro* studies by other investigators (68, 69). We showed that three different HDAC inhibitors used suppressed the antitumor effector functions of γδ T cells.

We observed that γδ T cells activated with the phosphoantigen, HDMAPP in the presence of HDAC inhibitors showed decreased proliferative potential. One of the mechanism by which HDAC inhibitors exhibit their anticancer properties is through induction of cell differentiation and cell cycle arrest at G1 phase (48, 49). Besides affecting histone proteins, these inhibitors also have several non-histone protein substrates like p53, p21, Rb, and E2F1 in tumors (70, 71). On the other hand, it was demonstrated that downmodulation of p53 in T cells enhances their antigen-specific proliferative response and also augments antitumor cytotoxic functions (72, 73). Studies from our lab have shown that CD3-activated T cells upon activation show robust proliferative capacity and decreased expression of p53 and its downstream target p21 (74). Thus, the decrease in the antigen-specific proliferative response of γδ T cells in presence of HDAC inhibitors incited us to look for effect of HDAC inhibitors on cell cycle

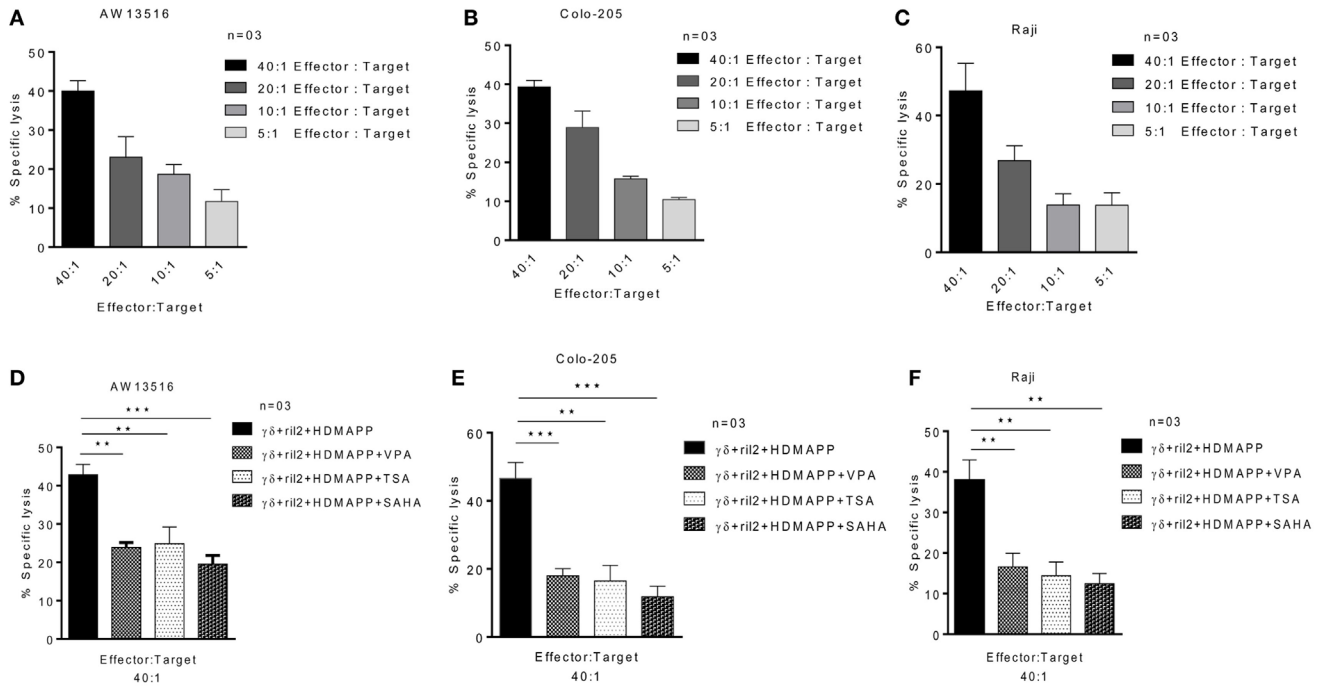

FIGURE 6 | Histone deacetylases inhibitors decrease the cytotoxic effector functions of γδ T cells. Cytotoxic effector function of γδ T cells was assessed against three tumor cell lines **(A)** AW13516, **(B)** COLO205, and **(C)** Raji. Zoledronate-treated tumor targets were cocultured with γδ T cells for 4 h and cytotoxicity was assessed by lactate dehydrogenase (LDH) assay. The results indicated are mean percent specific lysis at different effector:target ratio. Data are representative of three individual experiments. Cytotoxic potential of γδ T cells activated with HDMAPP in the presence of rIL-2 with or without treatment with sodium valproate (2 mM), suberoylanilidehydroxamic acid (1 μM), and Trichostatin-A (100 nM) was assessed against three zoledronate-treated tumor targets AW13516 **(D)**, COLO-205 **(E)**, and Raji **(F)** by LDH cytotoxicity assay. The results indicated are percent specific lysis at the effector to target ratio of 40:1 where $^{**}p < 0.005$ and $^{***}p < 0.0005$ when compared with γδ T cells activated with HDMAPP in the presence of rIL-2 ($n = 3$).

progression and expression of cell cycle regulators p53 and its downstream target p21. Decrease in the proliferation of γδ T cells in presence of HDAC inhibitors was associated with the increase in the expression of p53 and its downstream target p21. γδ T cells show increased expression of activation markers CD69 and CD25 when activated with phosphoantigens (36, 75). We observed that HDAC inhibitors inhibit the expression of CD69 and CD25 activation markers. CD25 is the high-affinity IL-2 receptor subunit and IL-2 signaling is necessary for the proliferation of T cells. It would be logical to conclude that HDAC inhibitors abrogate the IL-2 signaling and thus inhibit the proliferation of γδ T cells. We have used three different HDAC inhibitors VPA, TSA, and SAHA at different concentrations and they showed varied effects on expression of all the γδ T cell markers we studied. The likely explanation for the differences observed in their effects could be their structural diversity and also the biological activities they exert may be cell-type dependent.

Activated γδ T cells express Tbet and eomesodermin (Eomes) transcription factors. The T-box transcription factors T-bet and Eomes are important for acquisition of effector functions in cytotoxic T cells including γδ T cells (41, 76). Eomes and T-bet are highly homologous transcription factors and have cooperative and redundant functions in regulating the expression of different genes involved in the effector functions of CD8 T cells and activated natural killer cells. T-bet and Eomes regulate the expression of perforin, Granzyme-B, and IFN-γ by binding to promoter regions of these effector genes (14, 39). Knowing that HDAC inhibitors decrease the activation and proliferation of γδ T cells, we further hypothesized that HDAC inhibitors may modulate the effector functions of γδ T cells by affecting the expression of transcription factors Eomes and T-bet. We observed that treatment of γδ T cells with HDAC inhibitors lead to decrease in the expression of Eomes and T-bet. To further establish impact of HDAC inhibitors on the antitumor cytotoxic function of γδ T cell, we used panel of tumor cell lines (AW13516, COLO-205, and Raji) treated with zoledronate as target cell line in cytotoxicity assay. Previous work from our laboratory and others has demonstrated that tumor cells treated with zoledronate are aggressively killed by γδ T cells (10, 77). Our data demonstrate that treatment of HDAC inhibitors retard the ability of γδ T cells to kill zoledronate-treated tumor targets. Further, we proved that this inhibition of cytotoxic potential of γδ T cells was due to decrease in the expression of perforin and granzyme-B in these cells.

The activation of T cells initiated through T cell receptor is regulated by balance between co-stimulatory and inhibitory signals (immune checkpoints). Imbalance between these signals lead to different pathological conditions like tumor. Majority of the tumors use these immune checkpoints such as PD-1 or its ligand PD-L1 to escape from the immune surveillance. Immune check point inhibitors have revolutionized the field

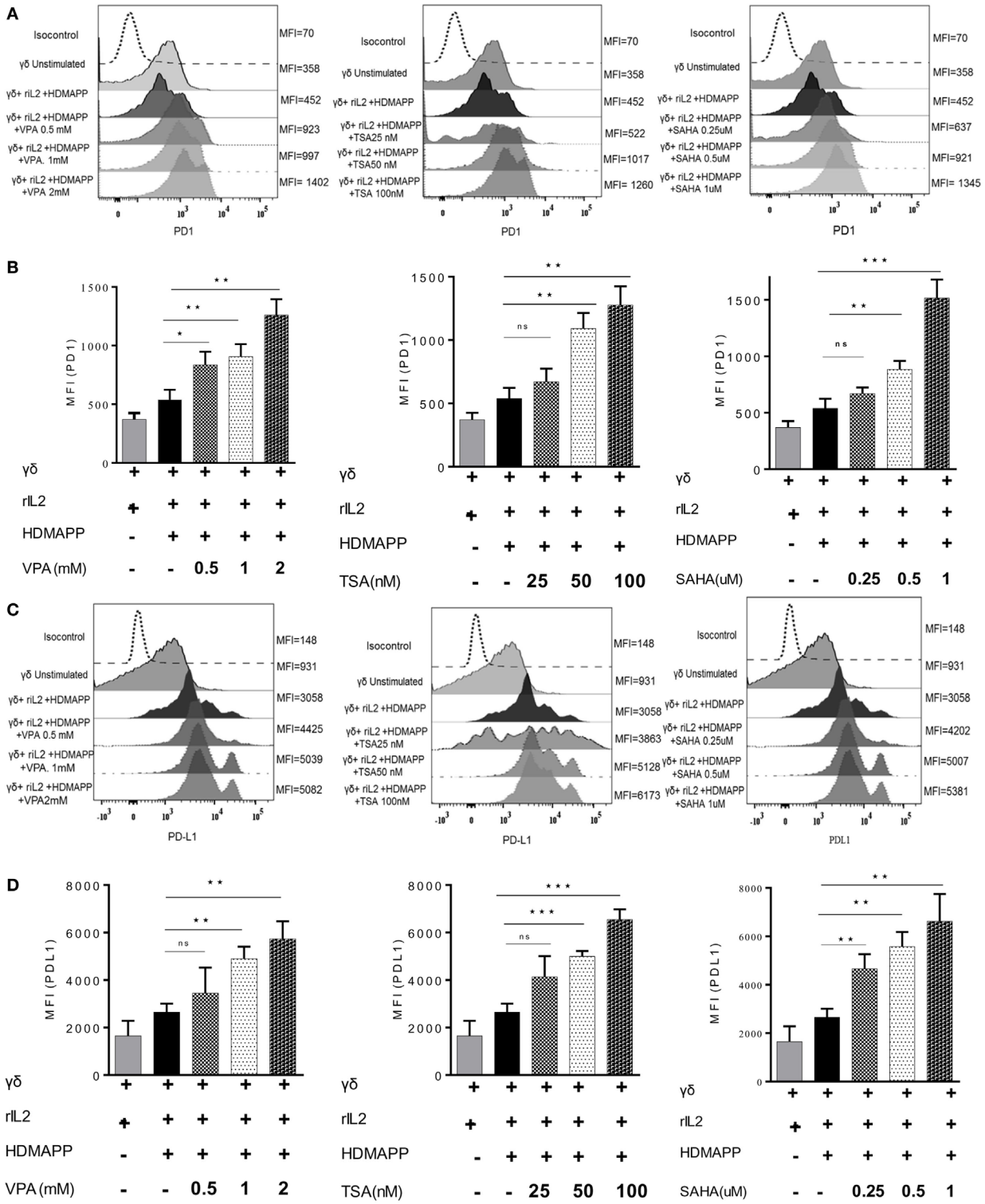

FIGURE 7 | Histone deacetylases (HDAC) inhibitors upregulate the expression of immune checkpoints on γδ T cells. **(A)** The expression of programmed death-1 by HDAC inhibitor-treated γδ T cells at their respective concentration. Histograms are representative of three individual experiments. The values on right side of histograms indicate median fluorescence intensity (MFI) of PD1. **(B)** MFI of PD1 expression as bar graphs where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ and ns, not significant when compared with γδ T cells activated with HDMAPP in the presence of rIL-2. **(C)** The expression of programmed death ligand-1 (PD-L1) by γδ T cells treated with HDAC inhibitors sodium valproate, Trichostatin-A, and suberoylanilidehydroxamic acid at their respective concentration was analyzed by immunostaining. Histograms shown are representative of three individual experiments. The values on right side of histograms indicate MFI of PD-L1. The results indicated in **(D)** are MFI of PD-L1 expression where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ and ns, not significant when compared with γδ T cells activated with HDMAPP.

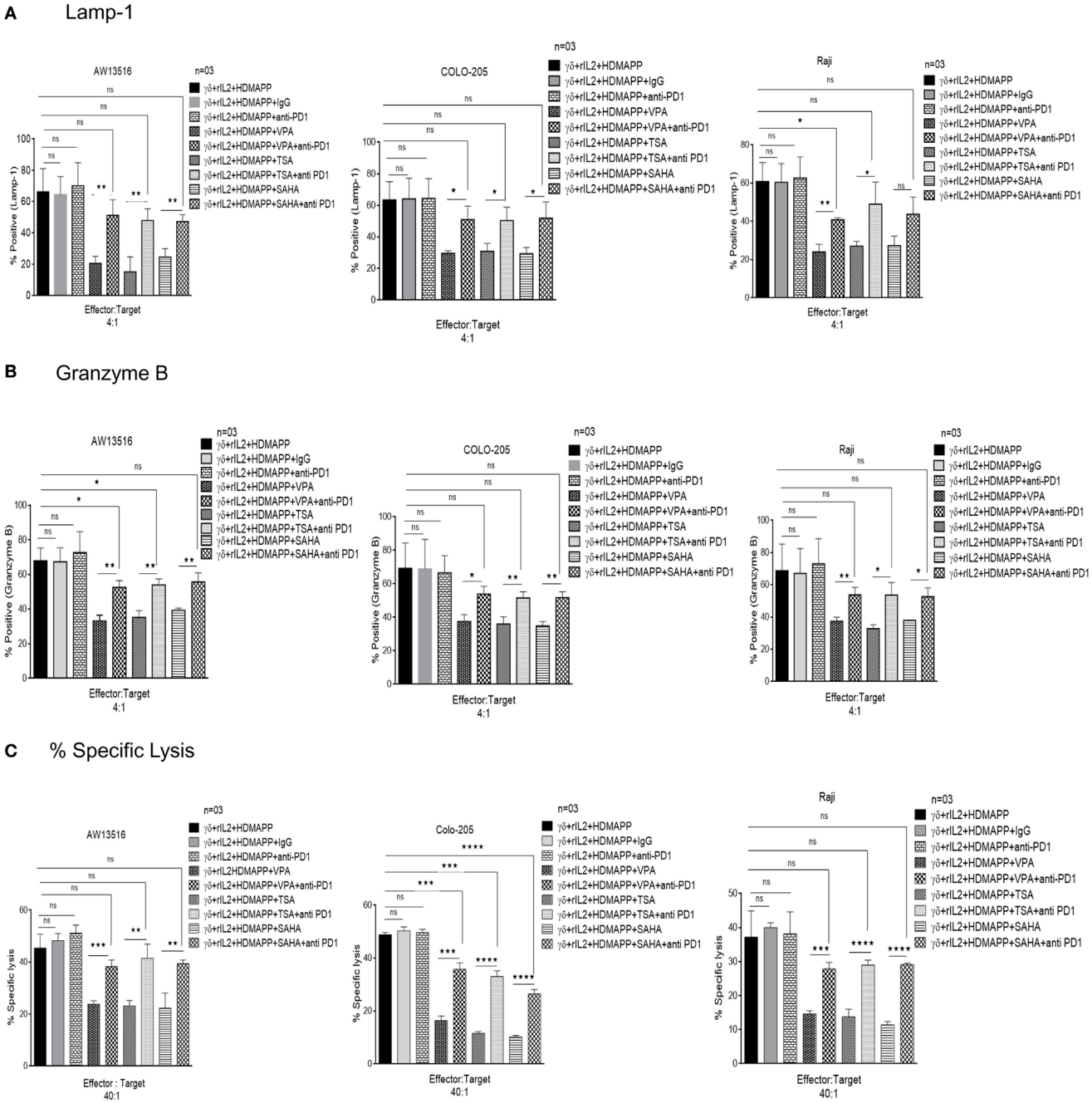

FIGURE 8 | Histone deacetylases (HDAC) inhibitors abrogate effector functions of γδ T cells *via* programmed death-1 (PD-1) upregulation. Expression of of **(A)** degranulation marker CD107a and **(B)** granzyme B by γδ T cells was assessed by flow cytometric analysis. HDMAPP activated γδ T cells upon treatment with sodium valproate (VPA) (2 mM), Trichostatin-A (TSA) (100 nM), and suberoylanilidehydroxamic acid (SAHA) (1 μM) in the presence or absence of PD1 blocking antibody were cocultured with three zoledronate treated tumor targets (AW 13516, Raji, COLO205) cells for 4 h at effector to target ratio of 4:1. The data represent consolidated median fluorescence intensity values of granzyme B and CD 107a expressing cells, indicative of three independent experiments (**$p < 0.005$, *$p < 0.05$, and ns, not significant). **(C)** The cytotoxic ability of γδ T cells treated with HDAC inhibitors TSA, SAHA, and VPA in the presence or absence of PD-1 blocking antibody was assessed against three zoledronate treated tumor targets (AW 13516, Raji, COLO205) by lactate dehydrogenase cytotoxicity assay. HDAC inhibitor treated γδ T cells show increased cytotoxic potential in the presence of PD-1 blocking antibody. The results indicate percent cytotoxicity where **$p < 0.005$, ***$p < 0.0005$, and ns, not significant, when compared with HDMAPP-activated γδ T cells treated with the respective HDAC inhibitor. Data represent three independent experiments.

of tumor immunotherapy (34). Besides surgery, radiation, and chemotherapy, immune check point inhibitors have surfaced as an important immunotherapeutic approach for cancer treatment. Due to their promising antitumor effects in experimental animal models, preclinical studies and successful clinical trials, immune check point inhibitors have been now approved by the U.S Food and Drug Administration (FDA) for treatment of different malignancies. PD-1/PD-L1 blocking strategy has led to tumor regression in patients with melanoma, renal cell carcinoma, non-small cell lung cancer, and bladder cancer (78–82).

Recent reports have shown that tumors associated with PD-1 expressing NK cells show poor survival (83). PD-1/PD-L1 signaling axis along with NKG2D signaling axis determine effector response of NK cells. Blockade of PD1/PD-L1 signaling cascade in NK cells along with other antitumor drugs have shown promising responses in cancer patients (84). This study supports our observation that HDAC inhibitors modulate the effector functions of human γδ T cells against tumors *via* PD1/PD-L1 signaling axis. We observed that γδ T cells show increased expression of immune check points PD1 and PD-L1 upon HDAC inhibitor treatment.

A report by Garcia-Diaz et al. have shown that induction of PD-L1 and PD-L2 on tumor cells is regulated *via* IFN-γ (85). In the present study, we have demonstrated that HDAC inhibitors decrease the expression of IFN-γ and TNF-α in antigen-activated γδ T cells. It has been demonstrated that Tbet transcription factor binds to PD-1 promoter and mediates the suppression of PD-1 expression (86). In the present study, we have shown that upon HDAC inhibitor treatment of γδ T cells, Tbet protein and mRNA is decreased significantly indicating that less Tbet may be available to bind PD-1 promoter to suppress PD-1 expression. This mechanism may explain the IFN-γ independent mechanism of PD-1 expression on γδ T cells.

Activated γδ T cells are known to express PD-1, which was investigated by Iwaski et al., on expanded γδ T cells population. They found that γδ T cells express PD-1 rapidly from day 3 of induction and PD-1$^+$ γδ T cells exhibit attenuated effector functions and decreased cytotoxicity against PD-L1 expressing tumors. However, they observed that zoledronate treatment to tumor cells, which induces IPP release along with PD-L1 blockade, rescued the γδ T cell cytotoxicity (35). While our study also confirms that blocking of PD-1 in γδ T cells increases the antitumor cytotoxic potential, our study reports on the effect of HDAC inhibitors on the freshly isolated γδ T cells activated with antigen for 72 h, whereas Iwaski group used γδ T cells already in activation state for their experimental purposes. Another interesting study by Castella et al. explores the multifunctional role of zoledronate in augmenting γδ T cells responses against multiple myeloma. In this study, zoledronate-treated autologous DCs were found to efficiently activate γδ T cells and enhance their cytotoxic functions against myeloma cells. Additionally, zoledronate was also shown to promote antitumor immunity *via* suppression of regulatory T cell function, downregulation of PD-L1 expression on DCs, and increased proliferation of tumor antigen-specific CD8 T cells. Although, their study has effectively demonstrated role of zoledronate in enhancing antitumor responses γδ T cells, it is specific only to multiple myeloma and uses zoledronate expanded γδ T cells from patient PBMNCs (87). Converse to our observation, they found that DC-activated γδ T cells did not express PD-1, this might be due to the immune modulation by zoledronate, which needs further exploration.

We observed that blockade of PD1/PD-L1 signaling partially restores the antitumor cytotoxic function of γδ T cells in the presence of HDAC inhibitors, which reflected in increased expression of effector molecules granzyme B and Lamp-1. Wei et al. have demonstrated that PD-1 ligation dramatically shifts the dose–response curve, making CD8+ Tcells much less sensitive to TCR generated signals (88). Although, this was shown in CD8+ αβ T cells, it may also apply to γδ T cells. Thus, PD-1 ligation affects TCR signaling and thereby reduces the cytotoxic function of γδ T cells. The role of other activating receptors such as NKG2D interacting with MICA/B and inhibitory receptors KIR2DL2/3 (CD158b) cannot be ignored and it explains the incomplete restoration of cytotoxic effector function γδ T cells upon PD-1 blocking.

Our results implicate that HDAC inhibitors along with the immune checkpoint modulating antibodies could be developed as combination immunotherapy to treat different malignancies. Thus, in future, this strategy may be applied for overcoming the limitations of HDAC inhibitor-based cancer therapies. The underlying mechanistic link of PD-1/PD-L1 may be targeted in developing more efficacious combination γδ T cell-based therapies in the future.

ETHICS STATEMENT

The study was approved by the Institutional Ethics Committee of ACTREC-TMC. All subjects gave written informed consent in accordance with the Declaration of Institutional Ethics Committee, ACTREC-TMC.

AUTHOR CONTRIBUTIONS

SC supervised, contributed conceptionally, and helped to write the manuscript. SB conducted experiments and wrote the manuscript. DV helped in conducting experiments. All authors contributed in final approval of 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.2018.01615/full#supplementary-material.

FIGURE S1 | Histone deacetylases (HDAC) inhibitors and viability of γδ T cells. HDAC inhibitors affect viability of γδ T cells only beyond specific concentrations. γδ T cells stimulated with HDMAPP in the presence of rIL-2 were treated with HDAC inhibitors sodium valproate (4, 3, 2, 1, and 0.5 mM), Trichostatin-A (250,

150, 100, 50, and 25 nM), and suberoylanilidehydroxamic acid (4, 3, 2, 1, 0.5 μM) for 72 h. The viability of γδ T cells was assessed by MTT assay. The results indicated are mean ± SE percent viability of γδ T cells and are representative of three experiments.

FIGURE S2 | The effect of histone deacetylases (HDAC) inhibitors on γδ T cells viability. **(A)** Purity of sorted γδ T cells. γδ T cells were positively sorted from peripheral blood mononuclear cells and were positive for γδ T-cell receptor (TCR) (90.8%), CD56 (53.2%), and negative for αβTCR, CD14, CD19. **(B)** Effect of HDAC inhibitor treatment on viability of γδ T cells. γδ T cells were activated with HDMAPP and rIL-2. HDAC inhibitors sodium valproate (2, 1, and 0.5 mM), Trichostatin-A (100, 50, and 25 nM), and suberoylanilidehydroxamic acid (1, 0.5, and 0.25 μM) were added to the culture and apoptosis was measured after 72 h with Annexin V and 7-AAD staining. Data shown are representative of three independent experiments. **(C)** The graphs show consolidated γδ T cell viability post HDAC inhibitor treatment ($n = 3$). Data represent mean ± SE.

FIGURE S3 | Histone deacetylases (HDAC) inhibitors impede proliferation of γδ T cells in a dose-dependent manner and leads to cell cycle arrest in G0–G1 phase. **(A)** The proliferative response of γδ T cells was assessed by thymidine incorporation assay. Sorted γδ T cells were stimulated with phosphoantigen HDMAPP, with or without treatment with HDAC inhibitors sodium valproate, Trichostatin-A, and suberoylanilidehydroxamic acid at different concentrations for 72 h. The graphs illustrate the cumulative mean cpm and are representative of three independent experiments where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. **(B)** Cell cycle analysis of γδ T cells upon HDAC inhibitor treatment. Freshly isolated γδ T cells were activated with HDMAPP with or without HDAC inhibitors for 72 h, and cell cycle progression was analyzed with propidium iodide (PI) staining using FACS calibur. The histograms are representative of three independent experiments. **(C)** The graphs indicate cumulative mean percentage of PI-positive cells in each phase of cell cycle. The graphs are representative of three independent experiments where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

FIGURE S4 | Histone deacetylases (HDAC) inhibitors differentially affects the expression of activating and inhibitory receptors in γδ T cells. The expression of **(A)** NKG2D, **(B)** KIR2D2L2/3, and **(C)** CD16 by γδ T cells in the presence and absence of HDAC inhibitors after 72 h was analyzed by flow cytometry. The histograms are representative of three individual experiments. The values on right side of histograms indicate median fluorescence intensity. HDAC inhibitors reduce NFκB expression in γδ T cells. Protein expression of NFκB by γδ T cells upon treatment with **(C)** sodium valproate, **(D)** Trichostatin-A, and **(E)** suberoylanilidehydroxamic acid as detected by western blotting. Cell lysates of γδ T cells, stimulated with HDMAPP after treatment with HDAC inhibitors at different concentrations for 72 h were probed with NFκB antibody, β-actin was used as loading control. The blots shown are representative of three experiments.

FIGURE S5 | Histone deacetylases (HDAC) inhibitors decrease the acetylation on promoters of perforin and granzyme B in γδ T cells. **(A)** γδ T cells treated with HDAC inhibitors sodium valproate (VPA), Trichostatin-A (TSA), and suberoylanilidehydroxamic acid (SAHA) show increased acetylation of H3 and H4. Expression of acetyl Histone 3 and acetyl histone 4 in γδ T cells was detected by western blotting and total H3 and total H4 were used as loading control. The blots shown are representative of three experiments. Chromatin immunoprecipitation qPCR was used to analyze the histone H3 acetylation **(B)** and histone H4 acetylation **(C)** on perforin region I (−103 to +27 bp), perforin region II (−309 to −130 bp); granzyme B region I (−130 to +11 bp), and granzyme B region II (−263 to −77 bp) from the transcription start site (TSS) of perforin and granzyme B in γδ T cells treated with or without HDAC inhibitors VPA (2 mM), TSA (100 nM), and SAHA (1 μM) for 72 h. Enrichment of promoter regions of Perforin and Granzyme B was quantified by qPCR and expressed as percentage of input DNA. The images above the graphs are schematic representation of Perforin and granzyme B promoter regions including the TSS. All results indicated are mean + SEM where $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

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Interferon-α Up-Regulates the Expression of PD-L1 Molecules on Immune Cells Through STAT3 and p38 Signaling

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Interferon-α (IFNα) has one of the longest histories of use amongst cytokines in clinical oncology and has been applied for the treatment of many types of cancers. Due to its immune-activating properties, IFNα is also an attractive candidate for combinatory anti-cancer therapies. Despite its extensive use in animal tumor models as well as in several clinical trials, the different mechanisms underlying patient responses and affecting desirable clinical benefits are still under investigation. Here we show that in addition to its immune-activating properties, IFNα induces the expression of a key negative regulator, immunosuppressive PD-L1 molecule, in the majority of the specific immune cell populations, particularly in the dendritic cells (DC). DC can modulate immune responses by a variety of mechanisms, including expression of T-cell regulatory molecules and cytokines. Our results showed that treatment of DC with IFNα-2b led to pronounced up-regulation of surface expression of PD-L1 molecules, increased IL-6 and decreased IL-12 production. Moreover, we present evidence that IFNα-treated DC exhibited a reduced capacity to stimulate interferon-γ production in T cells compared to control DC. This T-cell response after treatment of DC with IFNα was recovered by a pre-treatment with an anti-PD-L1 blocking antibody. Further analyses revealed that IFNα regulated PD-L1 expression through the STAT3 and p38 signaling pathways, since blocking of STAT3 and p38 activation with specific inhibitors prevented PD-L1 up-regulation. Our findings underline the important roles of p38 and STAT3 in the regulation of PD-L1 expression and prove that IFNα induces STAT3/p38-mediated expression of PD-L1 and thereby a reduced stimulatory ability of DC. The augmentation of PD-L1 expression in immune cells through IFNα treatment should be considered by use of IFNα in an anti-cancer therapy.

Keywords: PD-L1 (B7-H1), immunosuppression, IFNα, cancer immunotherapy, dendritic cell, STAT3 signaling

INTRODUCTION

The cytokine interferon-α (IFNα) has been used for a long time for treatment of many types of cancers, such as renal cell carcinoma, malignant melanoma or chronic myeloid leukemia (1, 2). However, the molecular mechanisms affecting patient responses and clinical outcome of IFNα therapy are still under investigation. IFNα is a member of the type I interferon family and is produced by various cell types, including monocytes, macrophages, lymphoblastoid cells, fibroblasts and plasmacytoid dendritic cells (3). Initially utilized as an anti-viral agent and meant for use in tumors where a viral origin was suspected, IFNα initiated a variety of biological activities that warranted further investigation. Indeed, this cytokine manifests direct suppressive effects on tumor cell growth *in vitro* and *in vivo* (4) and enhances tumor recognition by the increase in MHC-1 expression. Additionally, radio- and chemo-sensitizing capacities, as well as anti-angiogenic properties, have been described for IFNα (5, 6). Furthermore, Essers and colleagues have showed that the cytokine activated dormant hematopoietic stem cells *in vivo* (7). We have confirmed this phenomenon in pancreatic cancer, where we found that IFNα exhibited the ability to activate stem cell markers (8). Meanwhile, the immunostimulatory characteristics of IFNα have gained special attention since they can affect the differentiation of DC, survival of T cells, generation of $CD8^+$ memory cells, macrophage activities and activation of natural killer (NK) cells (9). A lot of tumor entities, like pancreatic cancer, are characterized by reduced immunological defense (10, 11). A combination of chemotherapy with immune stimulation could improve therapy efficacy and provide an optimal cancer treatment (12). Armed with its attributes, IFNα could be an attractive candidate for combinatory therapies. Indeed, IFNα-2b (trade name Intron-A), a well-known IFN-based therapeutic (13) that is approved for the treatment of various infectious diseases as well as for many types of cancer including leukemia, lymphoma, multiple myeloma and malignant melanoma, is also actively used in multiple clinical trials (http://www.druglib.com/druginfo/intron-a/trials/).

However, it has become increasingly clear in the last few years that certain cytokines originally described as immunostimulatory and pro-inflammatory, could also up-regulate immunosuppressive molecules. Such molecules are key elements of immune inhibitory pathways, so-called immunological checkpoints, which are crucial for maintaining self-tolerance and modulating the strength of immune responses. The most prominent of them is the PD-L1 (Programmed death-ligand 1, CD274, B7-H1)–PD-1 axis. PD-L1 is a type 1 transmembrane glycoprotein and one of two ligands for the CD28 homolog programmed death-1 receptor (PD-1) (14). The protein expression of PD-L1 can be found on immune cells as well as on non-immune endothelial and epithelial cells and can be up-regulated by different agents, such as cytokines and TLR (toll-like receptor) ligands (15–19). The PD-L1 molecule plays an important role in controlling immune reactions by inhibiting T-cell response and by influencing several other cell types. It is implicated in a number of human and mouse disorders as well as in transplant rejection and pregnancy complications (14, 20, 21). Additionally, it is responsible for the chronification of viral and bacterial infections (22). The expression of this molecule contributes as well to tumor immune evasion and correlates with a poor prognosis for the cancer patient (23–26). This makes PD-L1 and its regulation an important target for on-going investigations that aim to develop new anti-cancer treatment strategies. Interferons have been shown to be able to regulate PD-L1 expression not only on tumor (25) but as well on several non-tumor cell types: IFNγ increases PD-L1 in dermal fibroblasts (15), hepatic stellate cells (27) and DC (28, 29); the up-regulation of PD-L1 in DC by IFNβ contributes to immunomodulatory effects of this cytokine in multiple sclerosis and in lipopolysaccharide-induced immune paralysis (30, 31) and the expression of PD-L1 in hepatocytes and in myeloid cells *in vitro* can be augmented by IFNα (29, 32).

The stimulation of PD-L1 expression by IFNα could make a substantial negative contribution in patient responses and clinical outcomes of IFNα therapy through increased immunosuppression. Therefore, in this work we investigated the mechanisms of regulation of PD-L1 expression in specific immune cell populations by IFNα.

MATERIALS AND METHODS

Antibodies and Reagents

Anti-mouse monoclonal antibodies directly conjugated to fluorophores against the following targets of interest were used: F4/80, PD-L1, and Foxp3 (eBioscience, Germany); CD4, CD3e, CD44, CD8a, CD62L, CD45R, CD11b, CD11c, Gr-1, NK1.1, CD25, Ly6C, and PD-1 (BD Bioscience, Germany). Fc receptor binding inhibitor (anti-mouse CD16/CD32) was purchased from eBioscience (Germany). Anti-human antibodies included anti-PD-L1-PE and purified anti-PD-L1 blocking antibody (both from eBioscience, Germany). For western blot, anti-phospho-ERK42/44, anti-phospho-p38 and anti-phospho STAT3 along with their reference antibodies, anti-ERK42/44, anti-p38, and anti-STAT3 antibody (all from Cell Signaling Technology, USA), were used. Signal transduction inhibitors: UO126 (MEK1/2 inhibitor, Cell Signaling Technology, USA), SB203580 (p38 MAPK inhibitor, Cell Signaling Technology, USA), LY294002 (PI3K inhibitor), CAS457081-03-7 (Jak inhibitor 1, Calbiochem, Germany) and Cucurbitacin/JSI-124 (STAT3 inhibitor, Calbiochem, Germany). Cytokines used included IL-4, GM-CSF, TNF-α, IL-6, IL-1β, PGE2 (Promokine, Immunotools, Strathmann, Germany) and IFNα (R&D; IntronA, Interferon alfa-2b, Schering-Plough; Germany).

Mice

C57BL/6 mice were purchased from Charles River (Germany) and bred and maintained under specific pathogen-free conditions in the animal facility of the University of Heidelberg (IBF, Heidelberg). Animal experiments were carried out after approval by the Karlsruhe regional government council (Regierungspraesidium Karlsruhe, 35-9185.81/G-184/11). The following experimental groups have been used: (1) control group (vehicle injection) and (2) treatment group (injection of IFNα). Healthy mice were intraperitoneally injected three times within 1

week with 50 μl of IFNα (2×10^5 U/ml) or an equivalent volume of vehicle control with subsequent examination of the mouse splenocytes by flow cytometry.

Generation of Human DC

Buffy coats for research purposes were provided by blood donor service BSD Mannheim. Human peripheral blood myeloid DC (mDC) were isolated from freshly-prepared PBMCs from whole blood using MACS isolation kit (Miltenyi, Germany) as described elsewhere (19), according to the manufacturer's protocol. Human monocyte-derived DC (MoDC) were generated as described previously (19). Briefly, PBMCs were prepared from whole blood by Biocoll gradient centrifugation (Biochrom AG, Germany). Isolated plastic-adherent monocytes were cultured in *X-vivo* 15 medium (Bio-Whittaker, Belgium) supplemented with 1.5% human plasma in the presence of the cytokines GM-CSF and IL-4 for 5–6 days.

Cytokine Treatment

The following cytokine concentrations were used for the treatment of DC: standard cytokine cocktail for maturation (10 ng/ml TNF-α, 1,000 U/ml IL-6, 10 ng/ml IL-1β, 1 μg/ml PGE) and 500–6,000 U IFNα for generating the dose-dependent curve. For other experiments, DC were treated with 1,000 U/ml IFNα for 24 h and subjected to FACS analysis, western blot or DC-T-cell co-cultures.

DC-T-Cell Co-cultures

DC and T-cells were co-cultured as described previously (19). Generated or freshly isolated DC were pretreated with 1,000 U/ml IFNα for 24 h and then washed twice with medium. $CD4^+$ cells were isolated from freshly-prepared PBMCs using MACS beads (Miltenyi, Germany). $CD4^+$ cells and DC were cultured together in 96-well round-bottom plates for 5 days, after which the production of cytokines in supernatants was determined in triplicates using Luminex assay.

Immunocytochemistry

Immunocytochemistry was performed as described elsewhere (8). Briefly, cells were immobilized on microscope slides using a cytocentrifuge, fixed with 3.7% formaldehyde, pre-absorbed and incubated with anti-PD-L1 or control antibody. After incubation with secondary biotin-coupled antibodies followed by streptavidin–phosphatase complexes, PD-L1 was detected through the formation of a colored reaction product from the hydrolysis of substrate by alkaline phosphatase. Hematoxylin was used for counterstaining nuclei.

Western Blot Analysis

DC treated with 1,000 U/ml IFNα for the indicated periods of time were harvested, lysed in 2× sample buffer (100 mM Tris, pH 6.8, 4% SDS, 16% glycerol, 0.57 M β-mercaptoethanol, 0.01% bromophenol blue) and heated to 95°C for 5 min. SDS-PAGE was carried out after loading the proteins onto a 10% polyacrylamide gel. After transfer to PVDF membranes, the blots were sequentially blocked for 2 h using 5% milk in TBST solution (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween-20), incubated overnight with primary antibodies in TBST containing 5% BSA, washed three times in TBST and incubated with HRP-conjugated secondary antibodies. Subsequently, the proteins of interest were detected by chemiluminescence produced by HRP-catalyzed oxidation of ECL substrate (Lumigen TMA6, UK).

Flow Cytometry Analysis

Cells were collected, washed and incubated for 15 min at 4°C in FACS buffer containing antibodies directly conjugated to fluorophores. The fluorescence was evaluated using a FACSCanto II flow cytometer (BD Biosciences, Germany) and data were analyzed with Diva and FlowJo Software (BD Biosciences, Germany). All isotype controls had fluorescence values that remained below a threshold of 1×10^3. For analysis of murine leukocytes, a freshly-isolated splenocyte cell suspension was prepared as described previously (33), resuspended in the stain buffer (PBS supplemented with 1% mouse serum and 1 mM EDTA), counted and adjusted to a concentration of 4×10^7 cells/mL. Cells were blocked with anti-mouse CD16/CD32 antibodies at 4°C in the dark for 10 min and then incubated with stain buffer containing various combinations of previously titrated monoclonal antibodies at 4°C in the dark for 15 min. After two washing steps with the stain buffer, the cells were used for flow cytometry analysis. For intracellular staining, Foxp3 buffer set was used according to the manufacturer's instruction. All the gates were set according to the corresponding fluorescence minus one (FMO) controls. For the gating strategy see **Figure S1** and the manuscript from Fritz et al. (33).

Luminex Assay

Analyses of human cytokines IL-6, IL-12(p40), IFNγ, IL-1b,−4,−5,−10,−12(p70), and−17 as well as TNF-α in culture supernatants were performed as described elsewhere (34) using a MILLIPLEX® MAP Kit (Millipore GmbH, Schwalbach/TS, Germany) according to the manufacturer's instructions. The measurements were performed in triplicates using a Luminex® 100/200 System.

Statistical Analysis

The data were analyzed with unpaired Student's *t*-test or one-way ANOVA using GraphPad PRISM 5.0. Values with $p < 0.05$ were considered significant. Quantitative data are expressed as mean ± SEM.

RESULTS

Expression of PD-L1 and PD-1 on Various Populations of Murine Immune Cells

Our first aim was to determine the distribution of PD-L1 and PD-1 expression on extracellular surfaces of different specific immune cell populations. For this purpose, splenocytes from BL6 mice were subjected to a deep FACS analyses (**Figure S1**) to obtain the results summarized in **Table 1**. Expression of PD-L1 was found on all immune cells tested with higher percentages of $PD\text{-}L1^+$ cells (more than 80%) in conventional myeloid DC (cDC, $CD11c^{high}CD11b^+$), macrophage ($CD11b^+Gr\text{-}1^-F4/80^+$), naïve CD8 cell ($CD62L^+CD44^-$), effector memory (em) CD4 cell ($CD62L^-CD44^+$) and regulatory T cell (Treg,

TABLE 1 | Distribution of PD-1 and PD-L1 expression on immune cell subpopulations.

	PD-1		PD-L1	
	Cell frequency, %	**MFI**	**Cell frequency, %**	**MFI**
$CD3^-NK1.1^+CD4^-$	4.02 (±1.35)	992.6 (±407.4)	n.d. (not detected)	n.d.
$CD3^+NK1.1^+CD4^+$	22.55 (±12.98)	2,308 (±298.2)	n.d.	n.d.
$CD11c^{high}CD11b^+$	n.d.	n.d.	91.73 (±4.44)	22,500 (±25.5)
$CD11c^{int}CD45R^+$	n.d.	n.d.	58.3 (±14.79)	5,913 (±233.3)
$CD11b^+Gr\text{-}1^-F4/80^+$	n.d.	n.d.	82.3 (±0.63)	9,223 (±70)
$CD11b^+Gr\text{-}1^+$	n.d.	n.d.	21.58 (±11.69)	7,669 (±2,250)
$CD11b^+Gr\text{-}1^{high}Ly\text{-}6C^{int}$	n.d.	n.d.	15.27 (±6.99)	4,040 (±2,329)
$CD11b^+Gr\text{-}1^{int}Ly\text{-}6C^{high}$	n.d.	n.d.	44 (±10.52)	6,772 (±1,539)
$CD8^+CD3^+$	11.16 (±6.72)	1,103 (±437.7)	81 (±26.73)	683 (±49.5)
$CD62L^-CD44^+CD8^+$	n.d.	n.d.	1.34 (±0.26)	993 (±162.6)
$CD62L^+CD44^+CD8^+$	n.d.	n.d.	64.65 (±0.26)	876 (±86.3)
$CD62L^+CD44^-CD8^+$	n.d.	n.d.	83.35 (±0.26)	554 (±22.6)
$CD62L^-CD44^-CD8^+$	n.d.	n.d.	1.47 (±0.26)	671 (±166.9)
$CD4^+CD3^+$	14. 54 (±4.88)	1,468 (±429.3)	57.87 (±27.65)	1,568 (±1,686)
$CD62L^-CD44^+CD4^+$	n.d.	n.d.	85.8 (±6.79)	1,490 (±16.3)
$CD62L^+CD44^+CD4^+$	n.d.	n.d.	44.05 (±7.99)	1,688 (±359.9)
$CD62L^+CD44^-CD4^+$	n.d.	n.d.	48.75 (±40.94)	801 (±14.1)
$CD62L^-CD44^-CD4^+$	n.d.	n.d.	41.9 (±40.94)	1,494 (±304.1)
$CD25^{dim}FoxP3^+CD4^+$	39.18 (±9.83)	2,226 (±343.5)	54.3 (±40.94)	3,156 (±2,771)
$CD25^+FoxP3^+CD4^+$	24.15 (±6.65)	2,213 (±355.8)	84.45 (±9.12)	3,223 (±2,785)
$CD25^+FoxP3^-CD4^+$	18.68 (±7.59)	1,654 (±266.4)	4.2 (±5.06)	2,632 (±2,722)
$CD25^-FoxP3^-CD4^+$	77.07 (±2.74)	1,400 (±187.3)	70.25 (±0.64)	2,583 (±2,786)

$CD4^+CD25^+FoxP3^+$) populations. The lowest percentages of PD-$L1^+$ cells (lower than 5% positive cells) were found in em cells ($CD62L^-CD44^+$) and naïve $CD8^+$ cells as well as in activated conventional (con) T cells (Tcon, $CD4^+CD25^+FoxP3^-$). Expression of PD-1 was also found on all lymphoid cell populations analyzed (**Table 1**). Thus, PD-L1 and its receptor are commonly present on various murine immune cells.

IFNα Up-Regulates *ex vivo* and *in vivo* Expression of PD-L1 on Mouse Leukocytes

In the next step, isolated splenocytes were treated for 24 h with 1,000 U/ml IFNα and PD-L1 expression on splenocytes was detected with flow cytometry. IFNα treatment led to increased percentages of cells expressing PD-L1 in plasmacytoid DC (pDC), macrophage and $CD11b^+Gr^-1^+$ cell populations, but not in cDC in the myeloid leukocyte population (**Figure 1**). The reason is that cDC are already PD-L1 positive to a high percentage (**Table 1**). However, the level of PD-L1 expression (measured in MFI, mean fluorescent intensity) was positively affected in both DC subpopulations of $CD11b^+$ cell (**Figure 1**).

In the lymphocyte population, IFNα treatment increased a number of PD-$L1^+$ cells and up-regulated the expression of PD-L1 in both $CD4^+$ and $CD8^+$ cells (**Figure 2**). In specific subpopulations of $CD4^+$ cells, the percentage of cells expressing PD-$L1^+$ was increased in eff, em and cm ($CD62L^+CD44^+$) cells and the level of PD-L1 expression was higher after IFNα treatment in all subpopulations (**Figure 2**). Interferon-α was observed to exert a similar effect in Treg (**Figure 2**). For the gated $CD8^+$ cells, an increased number of PD-$L1^+$ cells was found in eff, em and cm populations (**Figure 2**). The level of PD-L1 expression measured in MFI was higher after treatment with IFNα in all subpopulations except eff $CD8^+$ cells (**Figure 2**). Thus, IFNα is capable of up-regulating PD-L1 expression in different murine immune cells.

Since the highest initial percentage of PD-$L1^+$ cells and up-regulation of PD-L1 after IFNα treatment was observed in DC in *ex vivo* splenocytes cultures, the *in vivo* regulation of PD-L1 expression by IFNα was examined and we verified a similar up-regulation in mouse DC (**Figure 3**) as well as in other myeloid cells (data not shown).

IFNα Up-Regulates the Expression of PD-L1, Increases the Production of IL-6 and Decreases the Production of IL-12 by Human DC

To increase the clinical relevance of our study, we extended our research to investigate PD-L1 regulation in human DC facilitated through the use of IFN-based therapeutic IFNα-2b (Intron A). Myeloid DC were isolated from buffy coats of healthy human subjects and cultivated with different concentrations of IFNα as indicated in **Figure 4**. The treatment induced dose-dependent up-regulation of PD-L1 expression in the cells (**Figure 4A**). Similarly, PD-L1 expression was up-regulated in MoDC obtained from buffy coats of human healthy donors

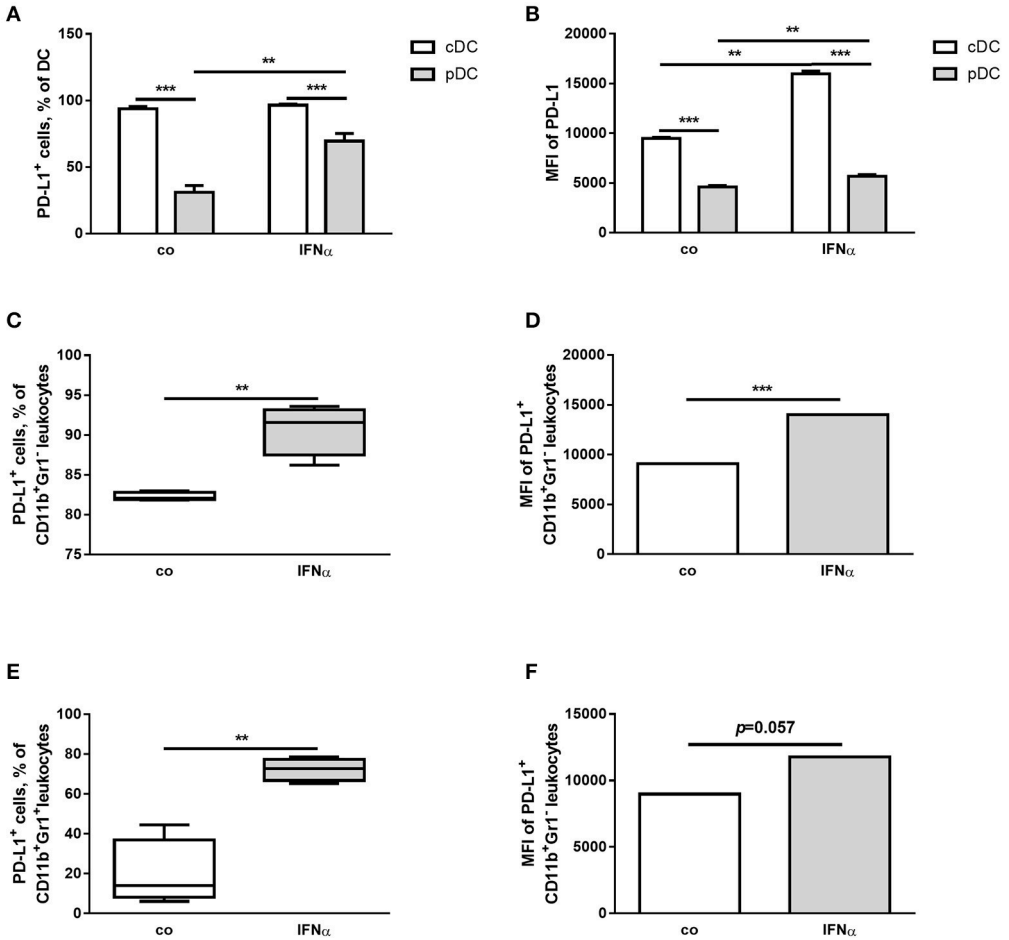

FIGURE 1 | IFNα up-regulates PD-L1 expression on different myeloid immune cell populations *ex vivo*. FACS analysis of PD-L1 expression on the surface of different myeloid immune cells from splenocytes of healthy mice. Splenocytes were isolated, treated for 24 h with 1,000 U/ml IFNα and investigated by flow cytometry. The results are presented as interleaved bars **(A,B)**, as box and whiskers plots **(C,E)** or column bar graphs **(D,F)** and statistically analyzed using two-way ANOVA **(A,B)** or unpaired *T*-tests **(C–F)**, $n = 4$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

(**Figure 4B**). Remarkably, an increased PD-L1 expression was still induced when mDC and MoDC were pulsed with IFNα after incubation with a classical maturation cytokine cocktail (**Figure S2**). Therefore, IFNα can specifically induce PD-L1 overexpression in human DC.

Produced cytokines are important indicators of DC functionality. After the treatment of DC with IFNα, 10 human cytokines were measured in cell culture supernatants using the Luminex platform. We found an increased concentration of IL-6 and a decreased concentration of IL-12(p40) and IL-12(p70) in the supernatants (**Figure 4C**) after IFNα treatment. However, the production of IFNγ, IL-1β,−4,−5,−10, and−17 as well as TNF-α was not affected (data not shown).

Blocking PD-L1 Leads to Recovery of IFNγ Production by CD4$^+$ Lymphocytes Cultivated With IFNα-Treated mDC

The expression of PD-L1 on DC negatively modulates their ability to activate CD4$^+$ lymphocytes and subsequent IFNγ production (19). Based on our findings above indicating that IFNα treatment affects IFNγ production by co-cultured CD4$^+$ lymphocytes but not DC, we aimed to investigate whether the blocking of PD-L1 protein with antibodies [anti (α)-PD-L1] could influence IFNγ production in our co-culture experiments. In order to achieve the above aim, we co-cultivated CD4$^+$ cells with mDC pretreated with IFNα, anti-PD-L1 or both IFNα and anti-PD-L1. As expected, the co-cultivation of CD4$^+$ cells with untreated mDC led to an increase in the amount of IFNγ in the supernatants and addition of anti-PD-L1 antibody further improved the cytokine release (**Figure 5**). Treatment of CD4$^+$ cells with anti-PD-L1 but without mDC did not induce IFNγ production. Pre-incubation of mDC with IFNα resulted in a decrease in the amount of IFNγ in the supernatants of co-cultivated CD4$^+$ cells (**Figure 5**), which could be explained by the up-regulation of PD-L1 expression on mDC. Indeed, blocking of IFNα-induced PD-L1 on DC led to higher IFNγ production from co-cultivated CD4$^+$ cells compared to the co-cultures containing IFNα-DC without an

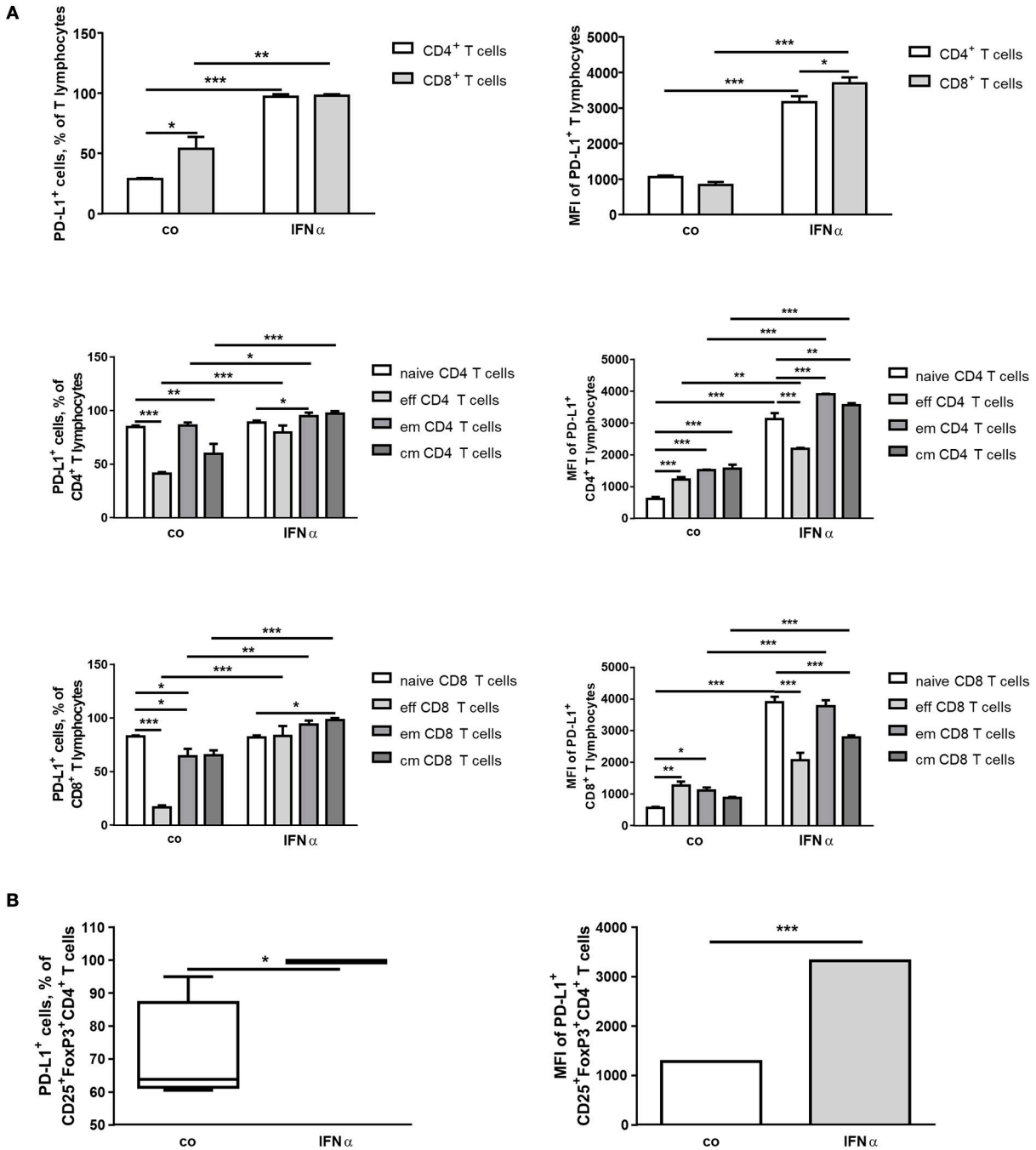

FIGURE 2 | IFNα up-regulates PD-L1 expression on different T-cell populations *ex vivo*. FACS analysis of PD-L1 expression on the surface of different lymphoid immune cells from spleens of healthy mice. Splenocytes were isolated, treated for 24 h with 1,000 U/ml IFNα and investigated by flow cytometry. The results are presented as interleaved bars **(A)** or as box and whiskers plot or column bar graph **(B)** and statistically analyzed using two-way ANOVA or unpaired *T*-tests, $n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

αPD-L1 antibody blockade. The restoration of IFNγ production, resulted in the same amount of the cytokine released into the supernatant as in the samples that were co-cultivated with mDC without any pre-treatment (**Figure 5**). Thus, blocking PD-L1 leads to the recovery of IFNγ production by $CD4^+$ lymphocytes activated with IFNα-treated mDC. The same results were observed when MoDC were used instead of mDC (data not shown).

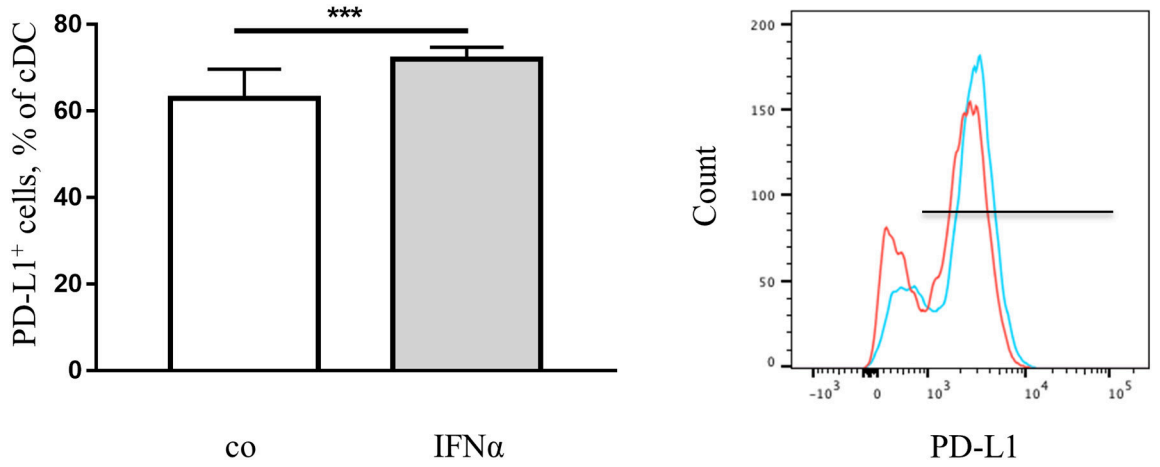

FIGURE 3 | IFNα up-regulates PD-L1 expression on DC *in vivo*. FACS analysis of PD-L1 expression on the surface of DC from spleens of healthy mice with or without treatment with IFNα. Splenocytes were isolated and examined using flow cytometry. The results are presented as percent of positive cells in a column bar graph and statistically analyzed using unpaired *T*-test, $n = 10$, $^{***}p < 0.001$. A representative FACS histogram (red-control, blue-IFNα) is shown.

Inhibitors of STAT3, p38 and Jak Down-Regulate the Expression of PD-L1 Induced by IFNα, Whilst PI3K or ERK Inhibitors Do Not Exert This Effect

Finally, we wanted to identify the molecular mechanisms by which IFNα up-regulates PD-L1 expression. We treated human mDC obtained from buffy coats with inhibitors of different signaling proteins as indicated in **Figure 6**. We found that IFNα-induced PD-L1 expression was down-regulated by inhibitors of p38, Jak and STAT3 but not by inhibitors of PI3K or ERK (**Figure 6A**). However, combinations of two different inhibitors did not show additive inhibitory effects (**Figure 6B**). In line with these observations, IFNα could increase the phosphorylation of p38 and STAT3 as shown by western blot (**Figure 6C**). In addition, we did not observe any effect on PD-L1 expression when NF-kB or STAT1/STAT5 were blocked with specific inhibitors (data not shown). Therefore, we concluded that signaling cascades including Jak/STAT3 and p38 could be involved in the regulation of PD-L1 expression.

DISCUSSION

In this study, we showed for the first time that PD-L1 molecule was expressed and could be up-regulated in the majority of specific immune cell populations by IFNα.

Programmed death-ligand 1, expressed on different immune cells, such as MDSC (35, 36), T cells (37), DC (19, 38), macrophages (39), pDC (40), is able to cause immunosuppression. Taking into account the ubiquitous expression of PD-L1 receptor (PD-1) on non-myeloid specific immune cells (CD8, CD4 and Treg), our results indicate that immunotherapy with IFNα could lead to an undesirable side effect of general immunosuppression and consequently to increased tumor immune evasion or chronification of infection. Our data could also explain the insufficiency of IFNα therapy observed in several models, despite its promising *in vitro* and immunomonitoring results (41–44). We propose that the combination of IFNα with checkpoint inhibitors like PD-L1 blocking antibody, could repress this immunosuppressive path and improve the efficiency of IFNα therapy by uncoiling the immune costimulatory potential of IFNα. While the majority of the DC are already positive for PD-L1, the intensity of this expression could be strongly up-regulated in our study by IFNα *in vivo*, *ex vivo*, and *in vitro*. DC play a crucial role in the control of adaptive tolerance and immunity and modulate immune responses by multiple mechanisms, including the production of cytokines and expression of T-cell regulatory molecules. Their final stimulatory capacity depends on the balance between stimulatory and suppressive pathways, whereby PD-L1 provides one of the most decisive suppressive signals and leads to unfavorable outcomes due to decreased anti-tumor immunity. This becomes particularly evident by documentation of reduced DC functions in several types of cancer and chronic infections (45–53). In cases utilizing vaccination as immunotherapeutic approaches against tumor and viral antigens, it is important that the produced DC display their full activation capacity (54, 55). Therefore, many therapies aim to activate DC to increase their stimulatory potential. IFNα was suggested as one of potential candidates for non-specific immune stimulation or as a replacement of IL-4 in the process of Mo-DC production (56–59). In accordance with our data, the MoDC produced from the monocytes treated with GM-CSF and IFNα (IFNα/GM-CSF MoDC) had a higher expression of PD-L1 molecules compared to those treated with IL-4/GM-CSF (60). In this case, the therapeutic efficacy of DC produced or activated with IFNα

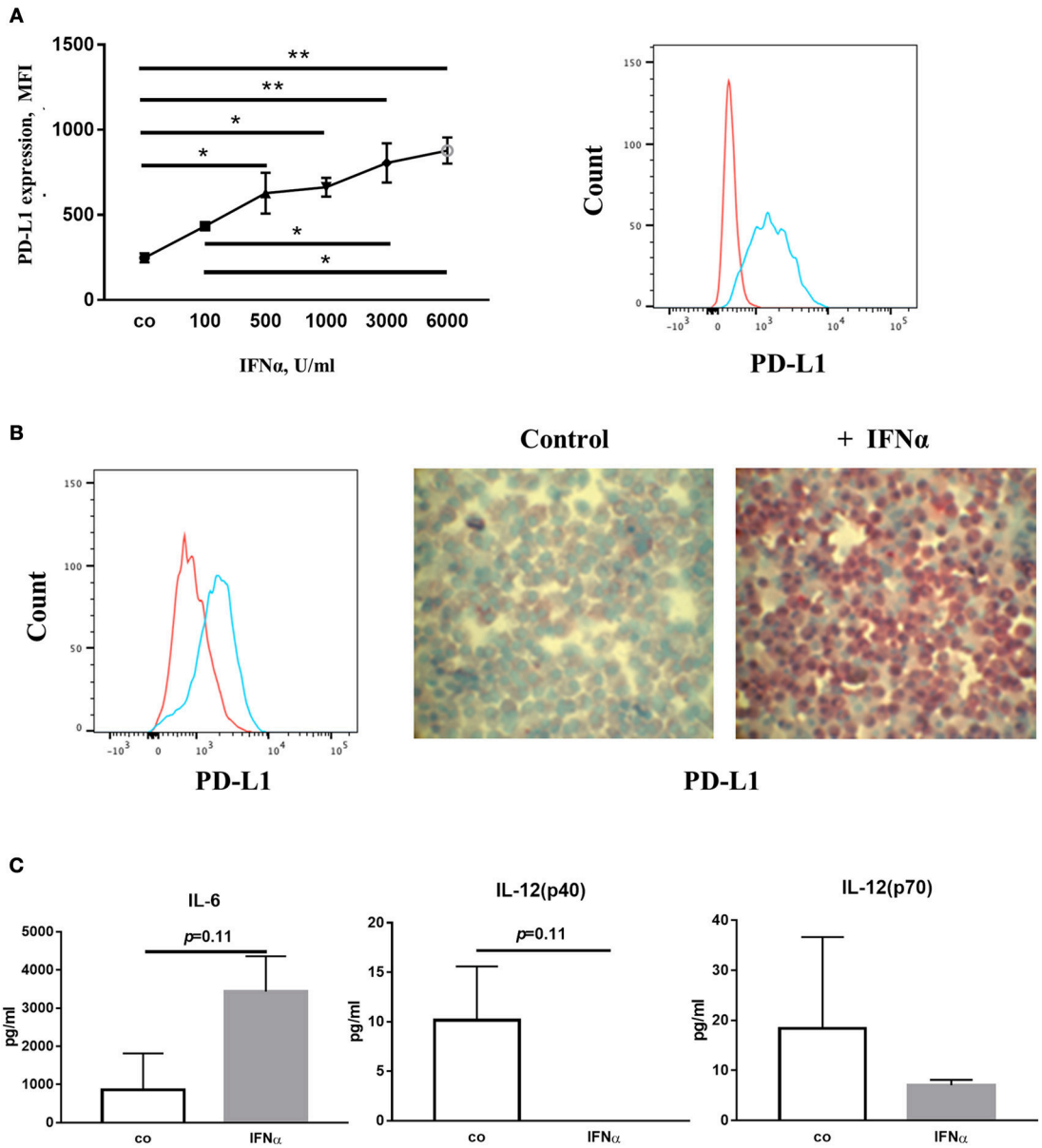

FIGURE 4 | IFNα up-regulates PD-L1 expression on human dendritic cells. **(A)** FACS analysis of PD-L1 expression on the surface of mDC. Myeloid DC were isolated, treated for 24 h with IFNα and analyzed with flow cytometry. The results are presented as column mean, error bars and mean connected and statistically analyzed using ordinary one-way ANOVA with the Tukey's multiple comparisons post-test, $n = 2$, $^{*}p < 0.05$ and $^{**}p < 0.01$. **(B)** FACS analysis and Immunocytochemistry (ICH) of PD-L1 expression on the surface of MoDC. MoDC were treated for 24 h with 1,000 U/ml IFNα and analyzed with flow cytometry. Alternatively, cytospin slides were produced and ICH was performed using an anti-PD-L1 primary antibody followed by a biotinylated secondary antibody. FACS histograms (red-control, blue-IFNα) and ICH pictures are representative for independent experiments. **(C)** Cytokines in the supernatant of the IFNα-treated DC analyzed with Luminex assays. The results are presented as column bar graphs and statistically analyzed using unpaired *T*-test, $n = 2$.

could be improved by the simultaneous reduction of PD-L1 expression.

The IFNα-treated DC demonstrated increased IL-6 and decreased IL-12 production. This is in line with the observation that TLR-antigen presenting cells, which express a high amount of PD-L1 and fail to induce T-cell proliferation, also exhibited increased IL-6 production (61). IFNα can inhibit IL-12 production in mouse splenocytes (62) and IL-12p40 production in human MoDC (57, 63). However, IL-6 has been shown to down-regulate IL-12 production by human MoDC (64). In our model, the cause of decreased IL-12 production, whether directly through IFNα treatment or partially caused as an

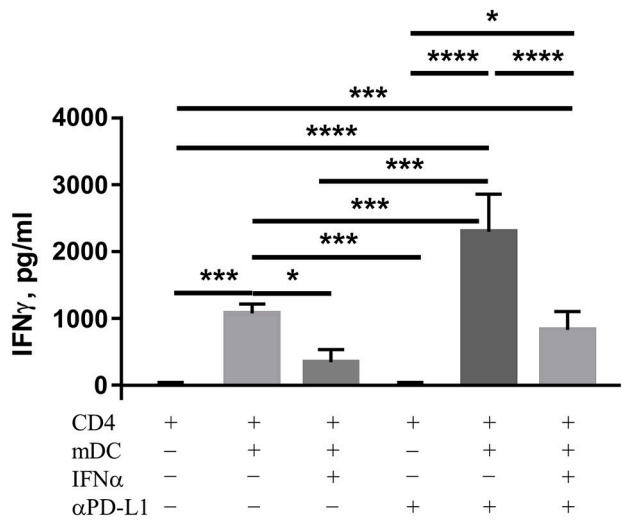

FIGURE 5 | PD-L1 controls IFNγ release from CD4 T cells stimulated by DC. Allogeneic CD4 cells were isolated and cocultured with DC in the presence or the absence of anti-PD-L1 blocking antibody or vehicle control. The level of IFNγ in supernatants was determined by Luminex assay. The results are presented as column bar graphs and statistically analyzed using ordinary one-way ANOVA with the Tukey's multiple comparisons post-test, $n = 3$–6, $^{*}p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

effect of IL-6, remains to be clarified. Moreover, we reported previously that IL-6 is able to induce PD-L1 in DC *per se* (18), thus PD-L1 expression could be boosted additionally by this loop.

In accordance with the findings of other researchers, we have shown previously that specific up-regulation of PD-L1 regulatory molecules on DC surfaces affect the capacity of DC to induce T-cell cytokine production (19, 30). In line with these previous results, we found that IFNα-treated DC strongly down-regulated IFNγ production by T cells. However, IL-12, produced by antigen-presenting cells including DC, can also control the production of IFNγ by T cells (65) and this is as well-evident when IFNγ release by co-cultured CD4 T cells decreased in response to diminished IL-12p70 production by IL-6 treated DC (64). In our study, the decrease in IL-12 production might not have an important role in stimulating IFNγ release in the co-cultures, since blocking PD-L1 on IFNα-treated DC could almost completely restore IFNγ release. Thus, in our experimental settings, the inhibitory ability of IFNα is directly linked to increased PD-L1 expression on DC. Given that IFNγ induces the expression of PD-L1 in DC (28, 29), repression of IFNγ production by T cells by IFNα could negatively regulate de novo expression of PD-L1 on the surface of DC during immune response *in vivo*.

The decisive role held by PD-L1 molecule in controlling immune response and anti-tumor immunity urges the investigation of signaling events controlling its up-regulation. Elucidation of the PD-L1 regulation on DC is an emerging field, as these cells govern the decision between tolerance and immunity. By canonical way, ligand engagement of the IFNα receptor (IFNAR, composed of the IFNAR1 and IFNAR2 subunits) activates Jak1 and tyrosine kinase 2 (TYK2) and can result in the recruitment of STATs as well as MAPKs, PI3K, Akt, NF-kB and PRMT1 (66). There are three predominant STAT complexes that might be formed in response to IFNs: (i) The interferon-stimulated gene factor 3 (ISGF3) complex [STAT1/STAT2 and IFN-regulatory factor 9 (IRF9)], which binds to IFN-stimulated response element (ISRE) sequences and activate antiviral genes, (ii) STAT1 homodimers, which bind to gamma-activated sequences (GASs) and initiate pro-inflammatory genes, and (iii) STAT3 homodimers which indirectly suppress pro-inflammatory gene expression. Our experiments revealed that IFNα regulates PD-L1 expression in a Jak-, STAT3-, and p38-dependent manner. Whether other downstream effectors of IFN1 signaling might be involved in the modulation of PD-L1 expression needs further investigations. Similarly, in mouse IL-27-treated pDC, STAT3-dependent enhancement of PD-L1 was described (40). The TLR-agonist-induced PD-L1 expression was modulated in a MAPK/STAT3-dependent way, whereby STAT3 was rapidly recruited to the PD-L1 promoter and in agreement with our findings, blocking of STAT3 activation prevented PD-L1 expression (61). We showed previously that IL-27-induced specific up-regulation of the PD-L1 regulatory molecule on DC was accompanied by the phosphorylation of another STAT family member, STAT1 protein (19), but we did not observe STAT1 involvement in the IFNα-induced PD-L1 expression.

Furthermore, in several tumor cell types, the role of Jaks/STATs in PD-L1 regulation was highlighted recently. Attenuation of IFNγ-induced PD-L1 expression in melanoma cells was proven to happen via down-regulation of the Jak/STAT/IRF-1 signaling pathway (67, 68), while activation of Jaks led to PD-L1 up-regulation in hematopoietic tumor cell lines and primary tumor cells (69). In contrast to our previous observations, neither PI3K nor ERK activation was essential for IFNα-induced PD-L1 expression (18). Thus, our findings underline the important roles of STATs in the regulation of PD-L1 expression and are in agreement with Barton et al. who states that the stimulatory ability of APCs depends on the degree of STAT3 activation (70). We can also speculate that therapies that target p38 and STAT3 pathways could potentially produce a desirable secondary effect on PD-L1 expression.

Another type I IFN, IFNβ, is also used as an immunomodulatory cytokine in the treatment of multiple sclerosis (71) and in anti-cancer therapies, for example against nasopharyngeal carcinoma (72). Since other type I IFNs bind the same IFNR1/2 receptor complexes and have a similar mode of action (73), one could assume that the effects found in our IFNα study might be valid for them as well and so more attention needs to be paid to these IFNs in future studies. Giving further evidence to support this hypothesis, IFNβ-dependent facilitated increase in PD-L1 expression in DC was documented in multiple sclerosis and in immune paralysis (30, 31).

Our findings underline the important roles of p38 and STAT3 in the regulation of PD-L1 expression and showed that IFNα-2b, which is clinically used for a wide range of indications including cancers, induced STAT3/p38-mediated expression of

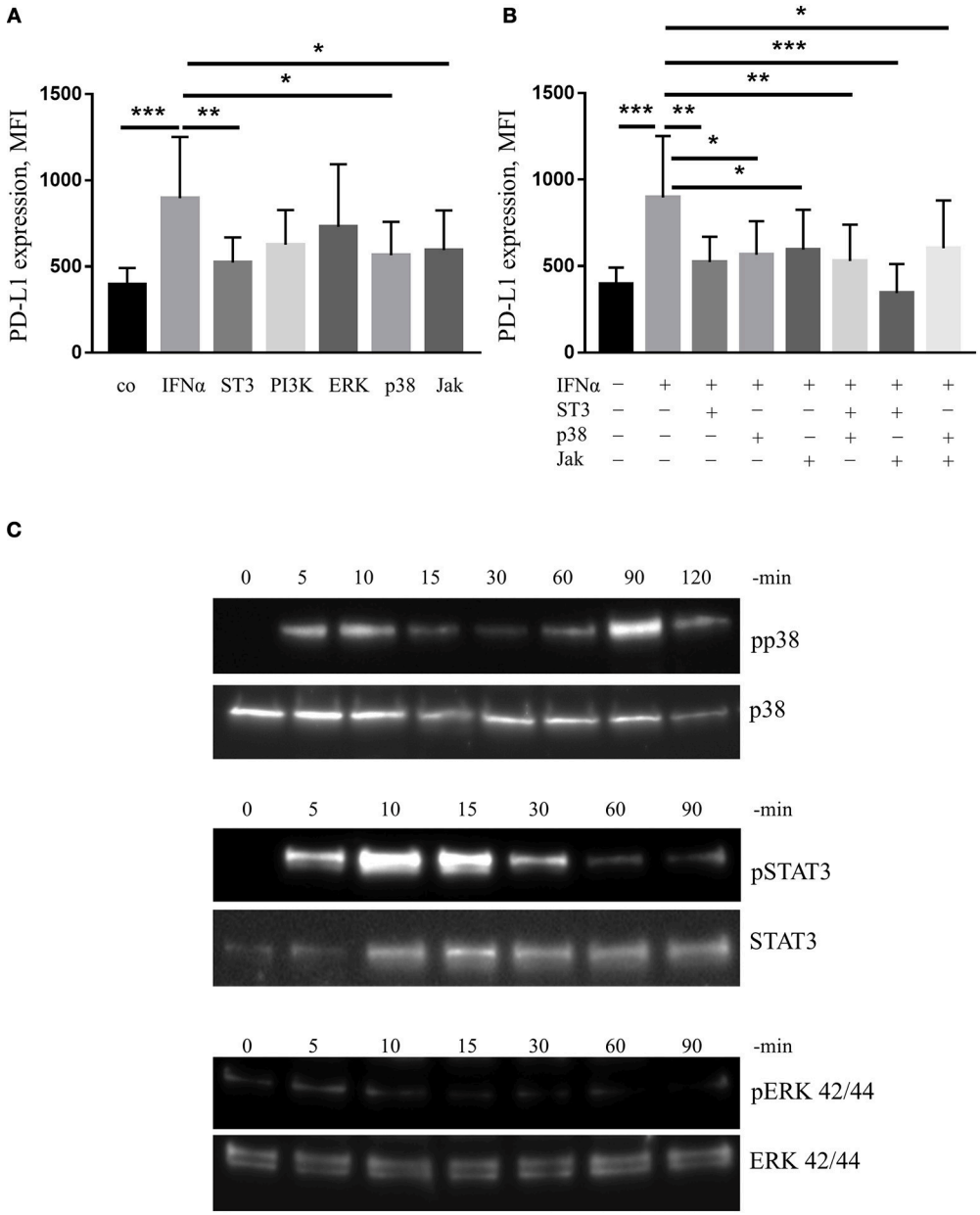

FIGURE 6 | IFNα up-regulates PD-L1 expression in mDC in p38/STAT3-dependent manner. **(A,B)** FACS analysis of PD-L1 expression on mDC activated for 24 h with 1,000 U/ml IFNα with or without 1 h pre-incubation with signal transduction inhibitors of p38, STAT3 (ST3), PI3K, ERK, p38, and Jak. The results are presented as column bar graphs and statistically analyzed using ordinary one-way ANOVA with the Dunnett's multiple comparisons post-test, $n = 8$–10, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. **(C)** Western blot analysis of p38, STAT3, and ERK phosphorylation (pp38, pERK, and pSTAT3 for phosphorylated form) in mDC before and after activation for indicated period of time with IFNα.

PD-L1, favoring a reduction in the stimulatory ability of DC. Particular consideration should be given to the enhanced PD-L1 expression in multiple immune cell types caused by the use of IFNα in anti-cancer therapy in the future.

The obtained results reveal a new avenue for the development of novel and optimization of existing therapeutic strategies with IFNα, in order to precisely modulate PD-L1 expression in DC and other target cells.

AUTHOR CONTRIBUTIONS

SK and AB participated in the research design; All authors participated in carrying out the research and analyzing the data; AB and SK participated in writing the manuscript and critical correction of the manuscript. AB, JW, and SK administered this work. All authors discussed the results and implications and gave constructive feedback on the manuscript at all stages.

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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.2018.02129/full#supplementary-material

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REVIEW
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Modulation of Gut Microbiota: A Novel Paradigm of Enhancing the Efficacy of Programmed Death-1 and Programmed Death Ligand-1 Blockade Therapy

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Blockade of programmed death 1 (PD-1) protein and its ligand programmed death ligand 1 (PD-L1) has been used as cancer immunotherapy in recent years, with the blockade of PD-1 being more widely used than blockade of PD-L1. PD-1 and PD-L1 blockade therapy showed benefits in patients with various types of cancer; however, such beneficial effects were seen only in a subgroup of patients. Improving the efficacy of PD-1 and PD-L1 blockade therapy is clearly needed. In this review, we summarize the recent studies on the effects of gut microbiota on PD-1 and PD-L1 blockade and discuss the new perspectives on improving efficacy of PD-1 and PD-L1 blockade therapy in cancer treatment through modulating gut microbiota. We also discuss the possibility that chronic infections or inflammation may impact on PD-1 and PD-L1 blockade therapy.

Keywords: gut microbiota, programmed death 1, programmed death ligand 1, cancer immunotherapy, efficacy

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INTRODUCTION

The immune system uses various effector cells and molecules to control and eradicate infectious agents and cancer cells. Cytotoxic T cells (CTL) are the major effector cells in anti-tumor immune responses (1, 2). However, the functions of these effector cells are inhibited in the tumor microenvironment, which contributes to cancer cell immune evasion (3). In recent years, the blockade of immune checkpoint proteins and molecules that deliver inhibitory signals to activated T cells, have shown great promise in cancer treatment. However, the beneficial effects of these treatment strategies were seen only in a subgroup of patients (4). In this review, we summarize the emerging evidence of improving immune checkpoint protein blockade therapy efficacy by modulating gut microbiota and discuss the possibility that chronic infections or inflammation may impact on programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) blockade therapy.

PD-1 AND ITS LIGANDS

Programmed death 1, also known as cluster of differentiation 279 (CD279), is a cell surface receptor that was discovered in 1992 (5). PD-L1 and PD-L2, the two molecules that interact with PD-1, were identified in the following years (6, 7). PD-L1 is also known as CD274 or B7 homolog 1 (B7-H1) and PD-L2 known as CD273 or B7-DC.

Programmed death 1 is expressed on T, B cells, and myeloid cells (8). PD-L1 is expressed by a variety of cells in the immune system and non-immune cells. However, the expression level of PD-L1 in normal human tissues is low; despite the presence of PD-L1 mRNA, PD-L1 protein is rarely detected on the cell surfaces in most of normal human tissues except for a subset of human tissue macrophages

(6, 9). PD-L2 is predominately expressed by antigen-presenting cells, such as dendritic cells (DCs) and macrophages (10–13). The expression of both PD-L1 and PD-L2 is regulated by cytokines, for example interferon (IFN)-γ greatly increases the expression of PD-L1 and several cytokines are able to induce the expression of PD-L2 in other immune cells and non-immune cells in addition to the DCs and macrophages (9–11, 14–16).

Programmed death 1 and its ligands are members of the immune checkpoint proteins delivering inhibitory signals to activated T cells. The interaction of PD-1 with PD-L1 or PD-L2 leads to suppression of T cells, a regulatory mechanism to prevent overstimulation of immune responses and autoimmunity (6, 7, 9, 16–21). However, such a mechanism is hijacked in the tumor microenvironment, providing opportunities for tumor cells to evade the attack from the immune system.

PD-1 AND PD-L1 BLOCKADE IN CANCER IMMUNOTHERAPY

In anti-tumor immune responses, the tumor antigens generated by gene mutations, are recognized by the immune system and specific CD8+ CTLs targeting tumor antigens are generated (22). These specific effector CTLs recognize the target tumor cells and induce tumor cell apoptosis.

However, tumor cells employ various strategies to escape the attack from the immune system, one of which is to resist the killing effects from the anti-tumor CTLs by increasing PD-L1 expression in tumor tissues (9, 23, 24). Most normal human tissues do not express detectable PD-L1 on their cell surface, in contrast PD-L1 is abundantly expressed by tumor cells, the immune and non-immune cells in various tumor tissues (6, 9, 25–30). IFN-γ released by the anti-tumor CTLs infiltrating into tumor tissues plays a major role in inducing the expression of PD-L1 (9–11, 14–16). Other cytokines, such as tumor necrosis factor (TNF) -α, interleukin (IL)-4, and IL-10 can also increase PD-L1 expression (31, 32).

The interaction of PD-L1 with PD-1 in the tumor microenvironment enables the tumor cells to resist the endogenous anti-tumor activities from the immune system. PD-L1 expressed in tumor tissues interacting with PD-1 expressed on the activated T cells leads to the dysfunction of the effector T cells, *via* multiple mechanisms, such as promoting T cell apoptosis, anergy, and exhaustion (6, 7, 9, 16–21). More recently, it was found that interaction of PD-L1 with PD-1 expressed on tumor-associated macrophages inhibits the phagocytic potency of macrophages against tumor cells (33). The importance of PD-L1 and PD-1 interaction in tumor cell evasion has led scientists to explore the use of these molecules as therapeutic targets in cancer immunotherapy (**Figure 1**).

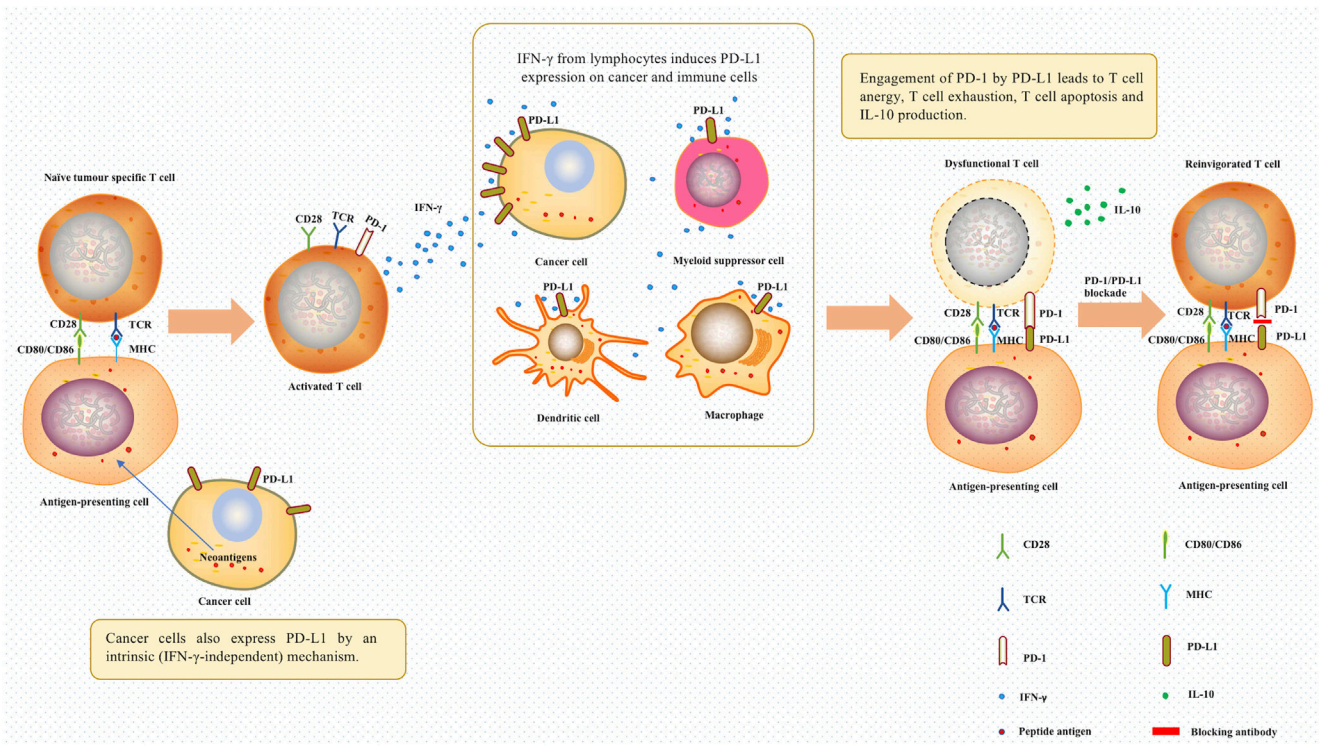


FIGURE 1 | The role of programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) in tumor evasion and cancer immunotherapy. In the tumor microenvironment, T cells were activated after antigen-presenting cells recognized tumor neoantigens. The IFN-γ produced by activated T cells induced the expression of PD-1 ligands on cancer cells and immune cells. Afterward, the engagement of PD-1 by PD-L1 between T cells and antigen-presenting cells will lead to T cell dysfunction. PD-1/PD-L1 blockade using relevant antibodies can inhibit this process, therefore, offering a chance for T cells to continue being effectors. Abbreviations: TCR, T-cell receptor; MHC, major histocompatibility complex; IFN-γ, interferon gamma; IL-10, interleukin 10.

Dong et al. showed that PD-L1 positive human tumor cells induced apoptosis of co-cultured activated effector T cells and this effect was blocked by an anti-human PD-L1 monoclonal antibody (mAb). They also showed that the growth of PD-L1 positive murine tumors in syngeneic mice was suppressed by an anti-murine PD-L1 mAb (9). Other researchers later reported similar findings in examination of different types of cancer cells using mice models (24, 34–36). These important laboratory observations led to numerous clinical trials of using monoclonal antibodies targeting PD-1 or PD-L1 in cancer immunotherapy for a variety of cancers. In addition to affecting the immunological pathways, PD-L1 and PD-1 blockade may also work in part by disrupting autologous PD-1 and PD-L1 signaling within tumors (37, 38).

To date, the U.S. Food and Drug Administration (FDA) has approved the use of five monoclonal antibodies targeting PD-L1 or PD-1 in cancer treatment. The details of the clinical trials of these five monoclonal antibodies are summarized in **Table 1**. Despite the clear benefits of PD-L1 and PD-1 blockade in treating some cancer patients, not all cases responded to treatment (**Table 1**). Given this, strategies to improve the efficacy of cancer immunotherapy are needed. Emerging evidence suggests that modulation of the gut microbiota is a promising approach.

MODULATION OF GUT MICROBIOTA ENHANCES THE ANTI-TUMOR EFFICACY OF PD-1 AND PD-L1 BLOCKADE THERAPY

A very interesting study by Sivan et al. provided strong evidence that the efficacy of PD-L1 blockage therapy can be improved by the modulation of gut microbiota (70). In this study, Sivan et al. examined the subcutaneous growth of B16.SIY melanoma in genetically similar C57BL/6 mice raised in the Jackson Laboratory (JAX) and Taconic Farms (TAC), and found that the tumor growth was more aggressive in TAC mice as compared to that in JAX mice and that TAC mice had a significantly lower intratumoral CD8+ T cell accumulation. They then conducted various experiments, which demonstrated that gut microbiota contributed to this difference.

They first showed that prophylactic transfer of fecal material from JAX mice to TAC mice was sufficient to delay tumor growth. To examine whether microbial community alone was effective as a therapy, they administered fecal material from JAX mice alone or in combination with anti-PD-L1 mAbs to TAC mice. These experiments showed that fecal material alone was sufficient to significantly inhibit tumor growth and that the combination treatment further improved tumor control. To identify the responsible bacterial species, they used 16S ribosomal RNA (16S rRNA) sequencing and identified *Bifidobacterium* species, particularly *Bifidobacterium breve*, *Bifidobacterium longum*, and *Bifidobacterium adolescentis* as the candidate species. The role of these *Bifidobacterium* species in enhancing protective immunity against tumors were further investigated by administering TAC mice bearing established tumors with a cocktail of *Bifidobacterium* species containing *B. breve* and *B. longum* by oral gavage. This experiment resulted in *Bifidobacterium*-treated mice having significantly improved tumor control as compared to mice that did not receive *Bifidobacterium*. Sivan et al. also showed that the possible mechanisms by which *Bifidobacterium* species inhibited tumor growth were through activating DCs, which in turn, improves the effector function of tumor-specific CD8+ T cells. Given that the enhanced anti-melanoma effect from *Bifidobacterium* species had occurred at the innate immunity level, the authors anticipated that *Bifidobacterium* species also provide anti-tumor beneficial effects to other types of tumors. However, the mechanisms by which *Bifidobacterium* species activated DCs improved the effects of anti-tumor CD8+ cells still need to be clarified.

The findings by Sivan et al. using mice models suggest that it is possible to enhance the anti-tumor efficacy of PD-L1 blockade therapy in treating cancer patients by modulating their gut microbiota and their findings are summarized in **Figure 2**. Interestingly, a very recent study by Matson et al. examining the stool samples collected from patients with metastatic melanoma before anti-PD-1 immunotherapy found that *B. longum*, *Collinsella aerofaciens,* and *Enterococcus faecium* were more abundant in the anti-PD-1 immunotherapy responders, supporting the anti-tumor effects of *Bifidobacterium* species (71).

Several additional studies also compared the gut microbiota in patients with metastatic melanoma receiving anti-PD-1 therapy. A recent study by Frankel et al. using metagenomic shotgun sequencing method showed that melanoma patients who responded to immune checkpoint inhibitors were enriched with *Bacteroides caccae* (72). Furthermore, they showed that the bacteria that are enriched within responders are most likely to be antibody dependent. Patients who responded to nivolumab (PD-1 antibody) were enriched with *Fecalibacterium prausnitzii*, *Bacteroides thetaiotamicron*, and *Holdemania filiformis*, whereas patients who responded to pembrolizumab (another PD-1 antibody), their gut microbiota enriched with *Dorea formicogenerans.* However, the mechanisms responsible for these changes are not clear. Studies comparing the gut microbiota changes prior to and following anti-PD-1 therapy of individual patients are required, which will provide information regarding whether anti-PD-1 antibodies directly affect gut bacterial species.

A study by Wargo et al. examined the human gut microbiota and metabolites of metastatic melanoma patients who received anti-PD-1 therapy using 16S rRNA and whole genome shotgun sequencing (73). They found that bacterial diversity and composition in patients that responded to the therapy were significantly different from that in patients who did not respond to the therapy. The responding patients had a higher diversity of bacteria and a higher abundance of *Clostridiales*, and the non-responders had a higher abundance of *Bacteroidales*. In a very recent study with multiple first authors and J. A. Wargo being the responding author, they further compared the gut microbiota of patients with metastatic melanoma receiving anti-PD-1 therapy (74). They found that patients who responded to anti-PD-1 therapy were associated with a significantly higher bacterial diversity and abundance of bacteria from the *Ruminococcaceae* family, which belongs to the *Clostridiales* order, as compared to patients who did not respond to the therapy. Furthermore, they performed fecal microbiota transplantation experiments in germ-free mice,

TABLE 1 | Five monoclonal antibodies targeting programmed death ligand-1 (PD-L1) or programmed death 1 (PD-1) were approved by the U.S. Food and Drug Association to treat cancer.

Commercial name (active ingredient)	Target	Treatment of disease	Targeting patients	Clinical cases	Clinical phase	Overall response rate (95% CI)	Objective response rate (95% CI)	Clinical study (clinical trial ID)	Reference
Bavencio (Avelumab)	PD-L1	Metastatic MCC	Metastatic MCC patients whose disease had progressed on or after chemotherapy administered	88	Phase 2	33% (23.3%, 43.8%)	Not applicable	JAVELIN Merkel 200 Trial (NCT02155647)	(39, 40)
Tecentriq (Atezolizumab)	PD-L1	Advanced or metastatic urothelial carcinoma	Cisplatin-ineligible patients with locally advanced or metastatic urothelial carcinoma	119	Phase 2	23.5% (16.2%, 32.2%)	Not applicable	IMvigor210 (NCT02951767)	(41)
			Previously treated patients with locally advanced or metastatic urothelial carcinoma	310	Phase 2	14.8% (11.1%, 19.3%)	Not applicable	IMvigor210 (NCT02951767)	(41)
		Metastatic NSCLC	Previously treated patients with metastatic non-small cell lung cancer	22	Phase 2	Not applicable	15% (10%, 22%)	POPLAR (NCT01903993)	(42)
Imfinzi (Durvalumab)	PD-L1	Locally advanced or metastatic urothelial carcinoma	Patients with locally advanced or metastatic urothelial carcinoma in total	182	Phase 1 and 2	Not applicable	17.0% (11.9%, 23.3%)	Study 1108 (NCT01693562)	(43–45)
			Patients with locally advanced or metastatic urothelial carcinoma who showed high PD-L1 expression on tumor cells	95	Phase 1 and 2	Not applicable	26.3% (17.8%, 36.4%)	Study 1108 (NCT01693562)	(43–45)
			Patients with locally advanced or metastatic urothelial carcinoma who showed low or non-PD-L1 expression on tumor cells	73	Phase 1 and 2	Not applicable	4.1% (0.9%, 11.5%)	Study 1108 (NCT01693562)	(43–45)
Keytruda (Pembrolizumab)	PD-1	Melanoma	Patients with Ipilimumab-Naïve melanoma (receive KEYTRUDA at a dose of 10 mg/Kg every 3 weeks)	277	Phase 3	33% (27%, 39%)	Not applicable	KEYNOTE-006 (NCT01866319)	(46, 47)
			Patients with Ipilimumab-Naïve melanoma (receive KEYTRUDA at a dose of 10 mg/Kg every 2 weeks)	279	Phase 3	34% (28%, 40%)	Not applicable	KEYNOTE-006 (NCT01866319)	(46, 47)
			Patients with Ipilimumab-refractory melanoma (receive KEYTRUDA at a dose of 2 mg/Kg every 3 weeks)	180	Phase 2	Not applicable	21% (15%, 28%)	KEYNOTE-002 (NCT01704287)	(48)
			Patients with Ipilimumab-refractory melanoma (receive KEYTRUDA at a dose of 10 mg/Kg every 3 weeks)	181	Phase 2	Not applicable	25% (19%, 32%)	KEYNOTE-002 (NCT01704287)	(48)

(*Continued*)

TABLE 1 | Continued

Commercial name (active ingredient)	Target	Treatment of disease	Targeting patients	Clinical cases	Clinical phase	Overall response rate (95% CI)	Objective response rate (95% CI)	Clinical study (clinical trial ID)	Reference
		NSCLC	Metastatic NSCLC patients with first-line treatment with a single agent	154	Phase 3	Not applicable	45% (37%, 53%)	KEYNOTE-024 (NCT02142738)	(49)
			Metastatic NSCLC patients with first-line treatment in combination with pemetrexed and carboplatin	60	Phase 1 and 2	55% (42%, 68%)	Not applicable	KEYNOTE-021 (NCT02039674)	(50)
			Previously treated NSCLC patients (all randomized patients who receive KEYTRUDA at a dose of 2 mg/Kg every 3 weeks)	344	Phase 2 and 3	Not applicable	18% (14%, 23%)	KEYNOTE-010 (NCT01905657)	(51)
			Previously treated NSCLC patients (all randomized patients who receive KEYTRUDA at a dose of 10 mg/Kg every 3 weeks)	346	Phase 2 and 3	Not applicable	19% (15%, 23%)	KEYNOTE-010 (NCT01905657)	(51)
		HNSCC	HNSCC patients whose disease had progressed on or after chemotherapy administered	174	Phase 1	16% (11%, 22%)	Not applicable	KEYNOTE-012 (NCT01848834)	(52)
		Urothelial Carcinoma	Cisplatin-ineligible patients with urothelial carcinoma	370	Phase 2	Not applicable	29% (24%, 34%)	KEYNOTE-052 (NCT02335424)	(53)
			Previously treated urothelial carcinoma patients	270	Phase 3	Not applicable	21% (16%, 27%)	KEYNOTE-045 (NCT02256436)	(54)
		cHL	Patients with cHL	210	Phase 2	69% (62%, 75%)	Not applicable	KEYNOTE-087 (NCT02453594)	(55, 56)
		MSI-H	Patients with MSI-H or mismatch repair deficient (dMMR)	149	Phase 1 Phase 2 Phase 1 Phase 2 Phase 2	Not applicable	39.6% (31.7%, 47.9%)	KEYNOTE-012 (NCT01848834) KEYNOTE-016 (NCT01876511) KEYNOTE-028 (NCT02054806) KEYNOTE-158 (NCT02628067) KEYNOTE-164 (NCT02460198)	(52, 57–59)

(*Continued*)

TABLE 1 | Continued

Commercial name (active ingredient)	Target	Treatment of disease	Targeting patients	Clinical cases	Clinical phase	Overall response rate (95% CI)	Objective response rate (95% CI)	Clinical study (clinical trial ID)	Reference
Opdivo (Nivolumab)	PD-1	Unresectable or metastatic melanoma	Previously treated patients with unresectable or metastatic melanoma in the treatment of OPDIVO	316	Phase 3	Not applicable	40% (34%, 46%)	CheckMate-067 (NCT01844505)	(60, 61)
			Previously treated patients with unresectable or metastatic melanoma in the treatment of OPDIVO plus Ipilimumab (anti-CTLA4 antibody)	314	Phase 3	Not applicable	50% (44%, 55%)	CheckMate-067 (NCT01844505)	(60, 61)
		Metastatic NSCLC	NSCLC patients who had experienced disease progressed during or after one prior platinum doublet-based chemotherapy regimen	272	Phase 3	Not applicable	20% (14%, 28%)	CheckMate-017 (NCT01642004)	(62)
			Patients with metastatic non-squamous NSCLC who had experienced disease progressed during or after one prior platinum doublet-based chemotherapy regimen	292	Phase 3	Not applicable	19% (15%, 24%)	CheckMate-057 (NCT01673867)	(63)
		Renal cell carcinoma	Patients with advanced RCC who had experienced disease progressed during or after one or two prior anti-angiogenic therapy regimes	410	Phase 3	Not applicable	21.5% (17.6%, 25.8%)	CheckMate-025 (NCT01668784)	(64, 65)
		cHL	Adult patients with cHL	258	Phase 2	Not applicable	69% (63%, 75%)	CheckMate-205 (NCT02181738)	(66, 67)
					Phase 1			CA209-039 (NCT01592370)	
		Recurrent or metastatic SCCHN	Patients with metastatic or recurrent SCCHN	240	Phase 3	Not applicable	13.3% (9.3%, 18.3%)	CheckMate-141 (NCT02105636)	(68, 69)

Five monoclonal PD-L1 or PD-1 antibodies granted after May 2017 by FDA for cancer treatments were not included in the table.

MCC, metastatic Merkel cell carcinoma; NSCLC, non-small cell lung cancer; HNSCC, head and neck squamous cell cancer; cHL, classical Hodgkin lymphoma; MSI-H, microsatellite instability-high cancer; dMMR, mismatch repair deficient; SCCHN, recurrent or metastatic squamous cell carcinoma of the head and neck.

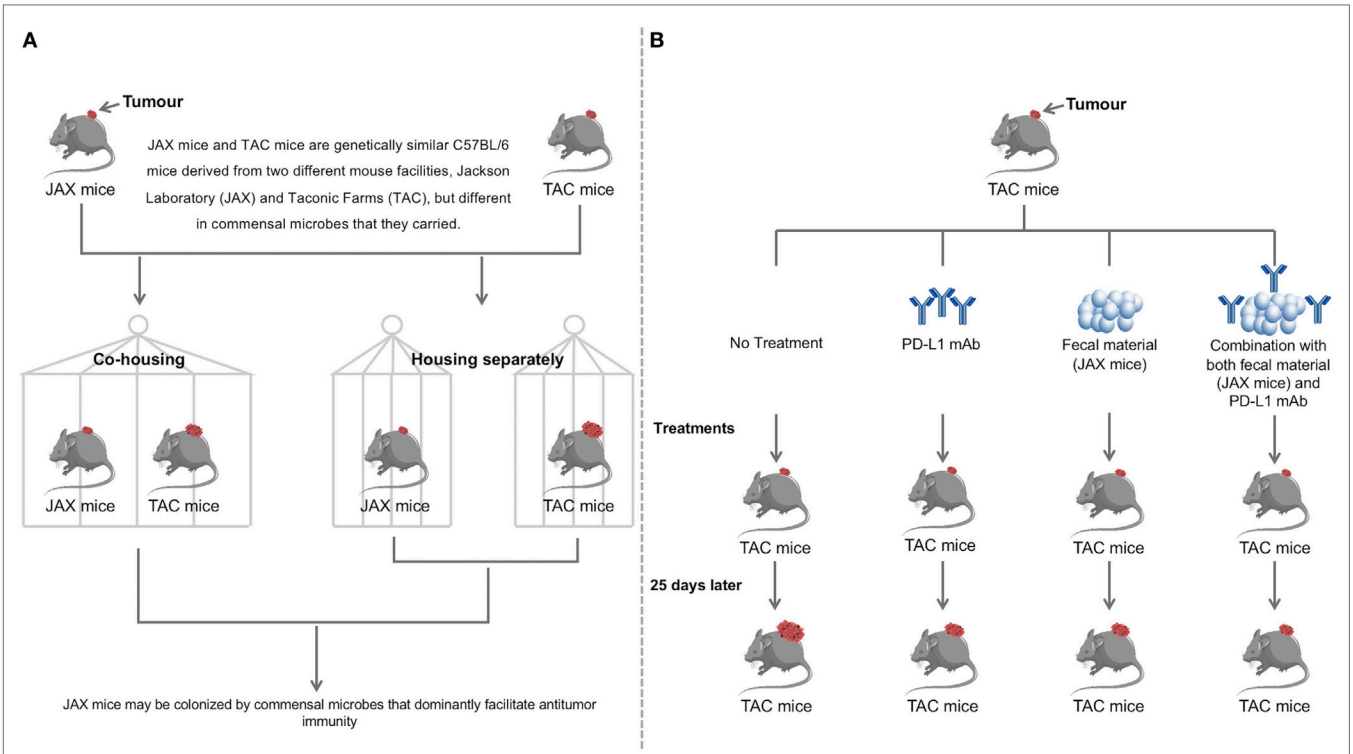


FIGURE 2 | Discovery and validation of the therapeutic significance of commensal microbiota by facilitating anti-programmed death ligand-1 (PD-L1) efficacy. **(A)** Two genetically similar mice, JAX and TAC, differing in commensal microbes carried were cohoused, while another pair was housed separately. Cohousing resulted in the TAC mice obtaining the JAX microbial phenotype, with reduced tumor growth as compared to the TAC mice housed separately. JAX mice had no differences in tumor size when cohoused with TAC mice compared to separate housing. This suggests that JAX mice are colonized by commensal microbes that facilitate anti-tumor immunity. **(B)** TAC mice were treated with PD-L1 mAb, JAX mice fecal material, both the PD-L1 mAb and JAX mice fecal material or not treated. Administration of JAX fecal material alone resulted in slower tumor growth to the same degree as treatment with PD-L1 mAb. Combination treatment with both the PD-L1 mAb and JAX fecal material showed the slowest tumor growth, indicating that commensal microbes play a therapeutic role in anti-tumor immunity. Data were adapted from Sivan et al (38). Further findings in this study have demonstrated that *Bifidobacterium* is the responsible bacterial species that contributes to improving the efficacy of PD-L1 blockade therapy.

in which they showed that germ-free mice transplanted with stool samples from patients who responded to anti-PD-1 and anti-PD-L1 therapy had a significantly reduced tumor growth and improved responses to anti-PD-1 and anti-PD-L1 therapy, coupled with a higher density of CD8+ T cells. However, it is not clear which bacterial species in the *Ruminococcaceae* family has played the role in enhancing the PD-1 blockade therapy.

Another recent study by Routy et al. investigated the effects of gut microbiota in PD-1 blockade therapy (75). In their study, data from 140 patients with advanced non-small-cell-lung cancer, 67 patients with renal cell carcinoma, and 42 patients with urothelial carcinoma were collected, and they found that 69 patients who took antibiotics before or soon after starting the PD-1 blockade therapy had shorter progression-free survival and overall survival. They then explored the composition of the gut microbiota using shotgun sequencing, which showed that *Akkermansia muciniphila* was enriched in patients who responded to anti-PD-1 therapy. This suggests that *A. muciniphila* may enhance patient response to PD-1 blockade therapy. They verified this observation by transplanting the patients stool samples in specific pathogen-free mice or germ-free mice and observed tumor growth in these mice. They also found that *A. muciniphila* alone was able to restore the anti-tumor effects of PD-1 blockade that was inhibited by antibiotics. However, the mechanism by which *A. muciniphila* enhancing PD-1 blockade therapy is not known.

Bacterial species that are positively associated with PD-1 and PD-L1 blockade therapy are summarized in **Table 2**. Some bacterial species have also been demonstrated to affect CTLA-4 blockade immunotherapy, which were not reviewed here (76, 77).

POTENTIAL MECHANISMS OF GUT MICROBES ON IMPROVING THE EFFICACY OF PD-1 AND PD-L1 BLOCKADE THERAPY

Despite the exciting findings in this research field, the underlying molecular mechanisms by which the identified gut bacterial species in the above studies enhance PD-1 and PD-L1 blockade therapy remain largely unknown.

Recently, unmethylated CpG oligodeoxynucleotides, which are abundant in bacterial DNA, were found to enhance CD8+ T cell anti-tumor immunity by downregulating PD-1 expression *via* the IL-12 pathway, suggesting that gut bacterial species that

TABLE 2 | Bacterial species that are positively associated with programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) blockade therapy.

Bacteria	Model	Methods	Main findings	Reference
Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium adolescentis	Mouse	Fecal transplantation Microbial DNA analysis Bacterial administration Cell sorting Gene expression profiling	• Some *Bifidobacterium* species enhanced the efficacy of anti-PD-L1 therapy *in vivo*	(70)
Fecalibacterium prausnitzii, Bacteroides t hetaiotamicron, Holdemania filiformis, Dorea formicogenerans	Human	Metagenomic shotgun sequencing Gut metabolomic profiling	• Melanoma patients who responded to *nivolumab* (PD-1 antibody) were enriched with *F. prausnitzii*, *B. thetaiotamicron*, and *H. filiformis* • Melanoma patients who responded to pembrolizumab (another PD-1 antibody), their gut microbiota enriched with *D. formicogenerans*	(72)
Clostridiales	Human	16S rRNA gene sequencing Whole genome shotgun sequencing Immunohistochemistry Flow cytometry Cytokines assay Gene expression profiling	• Melanoma patients who responded to anti-PD-1 therapy had a higher diversity of bacteria and a higher abundance of *Clostridiales*	(73)
Ruminococcaceae[a]	Mouse Human	16S rRNA gene sequencing Whole genome shotgun sequencing Immunohistochemistry Flow cytometry Cytokines assay Gene expression profiling Fecal microbiota transplantation	• Melanoma patients who responded to anti-PD-1 therapy had a higher diversity of bacteria and a higher abundance of *Ruminococcaceae* • Germ-free mice transplanted with stool samples from patients responded to anti-PD-1 and anti-PD-L1 therapy had a significantly reduced tumor growth and improved responses to anti-PD-1 and anti-PD-L1 therapy coupled with higher density of CD8+ T cells in tumor	(74)
Akkermansia muciniphila	Mouse Human	Metagenomic shotgun sequencing Fecal microbiota transplantation Immunohistochemistry Flow cytometry Cytokines assay	• 27% cancer patients[b] who took antibiotics before or soon after starting the PD-1 blockade therapy had shorter progression-free survival and overall survival • *A. muciniphila* was found enriched in those patients who respond to anti-PD-1 therapy • *A. muciniphila* alone was able to restore the anti-tumor effects of PD-1 blockade that was inhibited by antibiotics.	(75)
B. longum, Collinsella aerofaciens, Enterococcus faecium	Mouse Human	16S rRNA gene sequencing Metagenomic shotgun sequencing Species-specific quantitative PCR Immunohistochemistry Fecal transplantation	• Melanoma patients who responded to anti-PD-1 therapy had a higher abundance of *B. longum, C. aerofaciens,* and *E. faecium* • Germ-free mice transplanted with fecal material from responding patients could lead to improved tumor control, augmented T cell responses, and greater efficacy of anti-PD-L1 therapy	(71)

[a]*Bacteria of Ruminococcaceae family belongs to the Clostridiales order.*

[b]*Patients here include patients with advanced non-small-cell-lung cancer, renal cell carcinoma, and urothelial carcinoma.*

are positively associated with PD-1 and PD-L1 blockade therapy may release components that directly downregulate PD-1 or PD-L1 expression (78, 79).

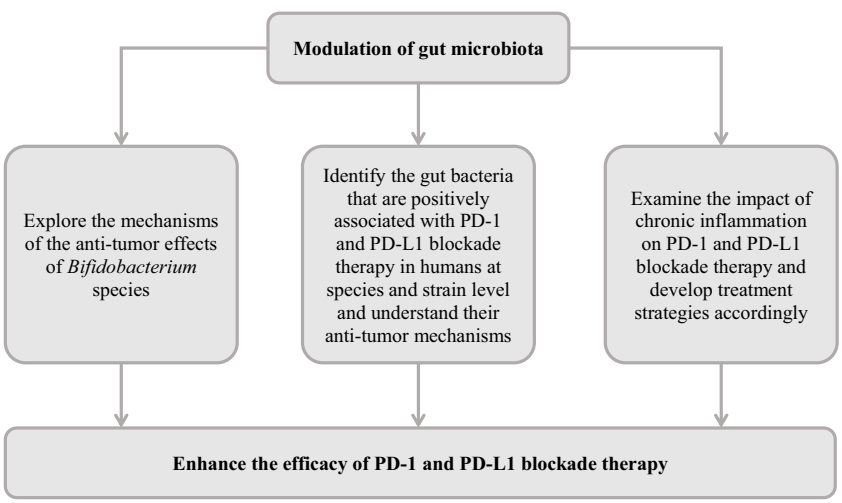
It is also possible that the gut bacterial species indirectly affect PD-1 and PD-L1 expression through locally or systematically regulating immune responses, thereby affecting the efficacy of PD-1 and PD-L1 blockade therapy. Gut microbiota has been shown to impact on both innate and adaptive immune cells. Germ-free animals had a reduced number of intestinal DCs and administration of *Escherichia coli* in these animals was able to recruit sufficient DCs to the intestines (80, 81). In Germ-free pigs, systemic circulating macrophages were also reduced and their functions were compromised (82). Germ-free mice had markedly decreased presence of lamina propria CD4+ T cells and absence of lymphocyte zones in spleens and mesenteric lymph nodes (83, 84). Polysaccharide A from *Bacteroides fragilis* was found to induce the Th1 response (83). Reduction of commensal microbiota in mice by using broad-spectrum antibiotics resulted in defective T and B cell responses against influenza infection (85). The findings that gut microbes can affect the immune functions, both locally and systematically suggest that bacterial species positively associated with PD-1 and PD-L1 blockade may enhance PD-1 and PD-L1 immunotherapy through regulation of the immune response. The previous study by Sivan et al. showed that *Bifidobacterium* species that inhibited tumor growth activated DCs, further supporting this view (70).

THE POSSIBLE IMPACT OF CHRONIC INFECTIONS AND INFLAMMATION ON PD-1 AND PD-L1 BLOCKADE THERAPY

Several microbes cause chronic infections in humans, some of which are known to increase host PD-1 and PD-L1 expression (86–94). However, studies have not examined whether existing chronic infections in patients with cancer affect the efficacy of PD-1 and PD-L1 blockade therapy.

An example of a chronic infection is *Helicobacter pylori* infection. *H. pylori* are a Gram-negative bacterium that colonizes the stomach of more than 50% of the world population. While most of the individuals colonized with *H. pylori* are asymptomatic, some may develop chronic gastritis and peptic ulcers, and *H. pylori* colonization is also a risk factor for gastric cancer (95). Previous studies have shown that patients with *H. pylori* infection have a significantly higher level of pro-inflammatory cytokines, such as TNF-α (96–98). Das et al. showed that *H. pylori* increased the gastric epithelial expression of PD-L1 using a gastric epithelial cell line model (86). Furthermore, they showed that gastric epithelial cells exposed to *H. pylori* inhibited the proliferation of CD4+ T cells isolated from blood and the inhibitory effect can be blocked using antibodies PD-L1. Similarly, Wu et al. observed increased PD-L1 expression in gastric biopsies of individuals infected with *H. pylori*, and co-culture of *H. pylori* infected primary gastric epithelial cells with T cells isolated from blood induced T cell apoptosis (87). These results suggest that *H. pylori* infection may cause the non-specific inhibition of circulating T cells, including tumor-specific T cells. In addition to *H. pylori*, several viruses, such as the hepatitis B virus, hepatitis C virus, human papillomavirus, and Epstein–Barr virus are also able to establish chronic infections in humans and increase host PD-1 or PD-L1 expression (88–94). Future studies are needed to examine whether chronic infections or inflammation impact on the efficacy of PD-1 and PD-L1 blockade. A recent study by Kottke et al. using a mouse model showed that pro-inflammatory cytokine TNF-α promoted tumor recurrence, while TNF-α blockade prevented tumor recurrence (99–102). Some bacterial species that are known to reduce chronic inflammation after administration orally may be examined to see whether they can improve cancer treatment (103–108). If chronic infections or inflammation reduce the efficacy of PD-1 and PD-L1 blockade, it would be through mechanisms other than the induction of the PD-1 and PD-L1 expression in the tumor tissues, as previous studies observed better responses to PD-1 blockade in patients with higher expression of PD-L1 in tumor tissues (51).

TABLE 3 | Suggested future directions.



FUTURE DIRECTIONS

As discussed, despite the clear benefits of PD-1 and PD-L1 blockade in treating some cancer patients, the efficacy and the recurrence of tumor are issues that remain to be tackled. Emerging evidence suggests that modulation of the gut microbiota is a promising approach for improving PD-L1 and PD-1 blockade therapy. However, future studies are needed to further develop this research area.

The *Bifidobacterium* species, particularly *B. longum*, increased anti-PD-L1 efficacy in mice models and was positively associated with anti-PD-1 efficacy in metastatic melanoma patients. Future studies are needed to understand the molecular mechanisms of these *Bifidobacterium* species in enhancing PD-1 and PD-L1 blockade therapy. In addition to the *Bifidobacterium* species, various studies reported positive associations of gut microbes with PD-1 and PD-L1 blockade therapy at genus level. These microbes need to be identified at species and strain level and their potential anti-tumor mechanisms require further investigation.

Several bacterial and viral pathogens are known to cause chronic human infections and the pro-inflammatory cytokines are known to induce host PD-1 and PD-L1 expression. In addition, some of these pathogens are known to directly attach immune cells. Whether chronic infections caused by different pathogens impact on PD-1 and PD-L1 blockade therapy should be investigated, and appropriate strategies to enhance PD-1 and PD-L1 blockade therapy in these patients can then be developed accordingly. A suggested course of action is outlined in **Table 3**.

AUTHOR CONTRIBUTIONS

Wrote the paper: YW, LZ. Figures and tables: YW. Revised the paper: YW, LZ, RM, FL, SL. All authors have approved the final version of the manuscript.

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REVIEW
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Combining Immune Checkpoint Inhibitors With Conventional Cancer Therapy

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Immune checkpoint inhibitors (ICIs) have recently revolutionized cancer treatment, providing unprecedented clinical benefits. However, primary or acquired therapy resistance can affect up to two-thirds of patients receiving ICIs, underscoring the urgency to elucidate the mechanisms of treatment resistance and to design more effective therapeutic strategies. Conventional cancer treatments, including cytotoxic chemotherapy, radiation therapy, and targeted therapy, have immunomodulatory effects in addition to direct cancer cell-killing activities. Their clinical utilities in combination with ICIs have been explored, aiming to achieve synergetic effects with improved and durable clinical response. Here, we will review the immunomodulatory effects of chemotherapy, targeted therapy, and radiation therapy, in the setting of ICI, and their clinical implications in reshaping modern cancer immunotherapy.

Keywords: immunotherapy, chemotherapy, targeted therapy, radiotherapy, combination therapy

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INTRODUCTION

Deeper understanding in the regulatory mechanisms of antitumor immunity, especially the identification of immune checkpoint pathways, has led to the success of modern immunotherapy. The past decade has witnessed a revolution in cancer therapy since the introduction of immune checkpoint inhibitors (ICIs), including anti-CTLA4 antibody and anti-PD-1/PD-L1 antibody. These antibodies have reshaped the landscape of treatments in various types of cancers, including melanoma, renal cell cancer, colorectal cancer (CRC), and non-small-cell lung cancer (NSCLC). However, it is estimated that up to 60–70% of patients do not respond to single-agent ICI therapy (1–7). To address this clinical challenge, different conventional cancer treatment modalities have been tested in combination with ICIs to achieve synergetic effects and to overcome the resistance to immunotherapy. Although some of these approaches have provided clinical benefits, the lack of knowledge in the functional interactions between conventional cancer therapies and immune checkpoint blockades at the molecular level remains a crucial hurdle in developing rational and optimal combination strategies. In this article, we will review the immune-regulatory effects of conventional cancer treatments and their clinical applications in combination with immune checkpoint blockades and future challenges.

COMBINATION OF IMMUNOTHERAPY AND CHEMOTHERAPY

Immunomodulatory Impact of Cytotoxic Chemotherapy

It has long been speculated that the immunoregulatory properties of conventional cytotoxic chemotherapy contribute to the antitumoral effects of these agents, in addition to direct tumor killing

(8). Although the mechanisms are yet to be fully understood, chemotherapy can regulate antitumor T cell response through increasing tumor antigenicity, inducing immunogenic cell death (ICD), disrupting immune suppressive pathways, and enhancing effector T-cell response (9–12).

Chemotherapy executes direct cancer killing *via* multiple mechanisms, including causing DNA damage, inhibiting DNA replication, and preventing mitosis (13). The induced tumor cell death further elicits systemic and intratumoral immune responses, contributing to the antitumor immunity. Chemotherapy enhances the antigenicity of the tumors through the increase of mutation burden and neoantigen load (such as in NSCLC and other various malignancies), which are correlated with higher responses to ICI therapy (14, 15). Some chemotherapy drugs upregulate MHC class 1 expression to increase antigen presentation (16, 17). In addition, chemotherapy drugs promote dendritic cell maturation and enhance the T cell activation by DCs (18). Chemotherapy also promotes ICD by releasing damage-associated molecular patterns, which can generate effector immune response when bound to pattern-recognition receptor. Experiments in animal models have suggested that some chemotherapy drugs induce the expression of PD-L1 on ovarian cancer cells (19).

Cytotoxic chemotherapy is regarded as immunosuppressive due to its dose-limiting myelosuppression. However, recent studies have demonstrated that it also can disrupt suppressive pathways. These immunosuppressive subsets play critical roles in downregulating the antitumor T-cell response and in promoting resistance to ICI treatments. Lymphodepletion resulted after chemotherapy can potentiate antigen-specific T-cell responses, therefore, augment antitumor immunity, particularly during the recovery phase from lymphopenia. Lymphodepletion can eliminate regulatory T cells (Treg) and other immunosuppressive cell subsets, such as myeloid-derived suppressor cells (MDSCs) (20–22) and tumor-associated macrophages (TAMs) (23). For instances, cyclophosphamide eliminates Treg and improves overall survival when combined with immunotherapy in a colon cancer models (24). Doxorubicin eliminates MDSCs and enhances the efficacy of immunotherapy in breast cancer (25). Reductions of these immunosuppressive populations in the tumor microenvironment of glioblastoma, synergize with anti-PD-1 therapy, and enhance the antitumor immunity (26, 27). The elimination of these immunosuppressive cells will increase the availability of survival and proliferative cytokines for T cells and lower the threshold for T-cell activation. Chemotherapeutic reagents also promote the polarization of Th1/Th2 and enhance the proliferation of T-lymphocytes in patients with advanced solid cancers (such as renal cell carcinoma, colon cancer, and ovarian cancer) (28, 29). Over the past decade, multiple studies have shown that different types of chemotherapy drugs can modulate the antitumor immunity in various mechanisms (9).

Given the extensive roles of chemotherapy in regulating the antitumor immune response, it is safe to hypothesize that the addition of chemotherapy to ICI may further enhance the activities of cytotoxic T cells with improved clinical outcomes. Over the past few years, chemoimmunotherapy (CIT) combination has attracted attention from clinicians and researchers and has been investigated in multiple clinical trials.

Clinical Studies With CIT Combinations

Chemotherapy in combination with immunotherapy (CIT) has been studied in multiple solid tumors, largely in NSCLC, providing broadened treatment options with improved outcomes.

The combination of pembrolizumab with pemetrexed and carboplatin has been evaluated in KEYNOTE-021, a multicenter phase 1/2 study, in patients with NSCLC. In the phase 1 study (30), pembrolizumab in combination with either carboplatin and paclitaxel, or carboplatin and paclitaxel plus bevacizumab, or carboplatin and pemetrexed was investigated, with overall response rate (ORR) of 52, 48, and 71%, respectively, irrespective of PD-L1 expression levels. These results led to the phase 2 study, evaluating the clinical outcome of pembrolizumab in combination with carboplatin and pemetrexed (31). A total of 123 chemotherapy-naïve nonsquamous NSCLC patients were randomized to chemotherapy alone, or chemo-pembrolizumab combination. Indefinite pemetrexed maintenance therapy was allowed for patients in chemotherapy alone group, and maintenance therapy with indefinite pemetrexed and up to 24 months of pembrolizumab was allowed for patients in chemo-pembrolizumab combination group. A significantly higher response rate was observed in the CIT combination group (55%) than in the chemotherapy alone group (29%), with progression-free survival (PFS) of 13 vs. 6 months, respectively. The magnitude of adverse effects (grade 3 or above) in both the groups was comparable (39 vs. 26%, respectively). Based on this study, the FDA granted accelerated approval of pembrolizumab in combination with carboplatin and pemetrexed for the treatment of NSCLC adenocarcinoma in the first-line setting. Updated survival data with median follow-up of 18.7 months showed a PFS of 19.0 months in CIT group vs. 8.9 months in chemotherapy group, with OS in CIT group not reached vs. 20.9 months in the chemotherapy arm (32).

In KEYONOTE-021 study, the tumor cell-associated PD-L1 expression level can impact the response rates in patients who received CIT treatment: response rate of 57% in those with <1% PD-L1 expression, 54% in those with ≥1% PD-L1 expression, 26% in those with 1–49% PD-L1 expression, and 80% in those with ≥50% PD-L1 expression (30). Accordingly, higher cutoff of PD-L1 expression was associated with higher response rates. Since pembrolizumab singe-agent is only indicated in NSCLC patients with ≥50% PD-L1 expression in the frontline setting (33, 34), this study established CIT as an alternative first-line therapeutic approach for nonsquamous NSCLC patients with <50% tumor PD-L1 expression, who do not harbor targetable mutations.

The results of the phase 3 trial (KEYNOTE-189) evaluating pembrolizumab in combination with chemotherapy in frontline setting in nonsquamous NSCLC patients without sensitizing EGFR or ALK mutations have been recently reported. A total of 616 patients were randomized in a 2:1 ratio to receive pemetrexed and platinum-based drug plus either pembrolizumab or placebo for 4 cycles, followed by pembrolizumab or placebo (for up to 35 cycles) plus pemetrexed maintenance therapy. With a median follow-up of 10.5 months, the 12-month OS was 69.2% in combination group vs. 49.4% in placebo group, and median PFS was 8.8 vs. 4.9 months, respectively. Interestingly, the survival benefit was seen across all PD-L1 categories (35).

A phase 3 trial evaluating CIT combination in frontline setting is currently ongoing for squamous NSCLC (KEYNOTE-407). The results from a second interim analysis after patients had been followed for a median of 7.8 months were recently presented (36). A total of 559 patients were enrolled and stratified prior to randomization based on tumor PD-L1 expression (<1 vs. ≥1%) as well as the choice of taxane (paclitaxel vs. nab-paclitaxel). Patients were assigned to receive four cycles of carboplatin and paclitaxel/nab-paclitaxel plus either pembrolizumab or placebo, followed by pembrolizumab (for CIT group) or placebo (for placebo group) maintenance therapy. Patients in the placebo group could cross over to receive pembrolizumab monotherapy after disease progression. Significant improvements in OS, PFS, and response rate were observed in CIT group vs. chemo-alone group regardless of PD-L1 expression level: the median OS of 15.9 vs. 11.3 months (HR 0.64, 95% CI [0.49, 0.85]; $p = 0.0008$), PFS of 6.4 vs. 4.8 months (HR 0.56, 95% CI [0.45, 0.70]; $p < 0.0001$), and objective response rate of 58.4 vs. 35.0% at the first interim analysis ($p = 0.0004$), respectively.

Nivolumab has also been tested in multiple clinical trials. In phase 1 CHECKMATE-012 trail, 56 patients with NSCLC received first-line therapy with combination of nivolumab with either gemcitabine and cisplatin, pemetrexed and cisplatin, or paclitaxel and carboplatin, followed by nivolumab maintenance therapy (37). CIT combination demonstrated improved ORR compared with nivolumab monotherapy in the front-line setting, with a manageable, non-overlapping toxicity profile. While nivolumab monotherapy has shown an ORR of 23% in the historical study (38), it demonstrated a higher ORR when combined with gemcitabine and cisplatin (33%), or pemetrexed and cisplatin (47%), or paclitaxel and carboplatin (43%), with acceptable tolerability and toxicity profiles. In addition to that, association between treatment response and PD-L1 expression levels was not observed (ORR 48% in PD-L1 ≥ 1 vs. 43% in PD-L1 < 1%).

A phase 3 study (CHECKMATE-227) is ongoing to evaluate nivolumab in combination with chemotherapy vs. chemotherapy alone in the frontline setting for patients with NSCLC (39). A total of 550 chemo-naive NSCLC patients without known sensitizing EGFR/ALK mutations, with <1% tumor PD-L1 expression were randomized to receive nivolumab 3 mg/kg Q2W + ipilimumab 1 mg/kg Q6W, nivolumab 360 mg Q3W + chemo, or chemotherapy for 2 years. In the recently presented results (40), a total of 177 patients received CIT while 186 received chemotherapy alone. With a minimum follow-up of 11.2 months, an improved PFS was seen in nivolumab-chemo arm vs. chemotherapy alone arm (HR = 0.74, 95% CI [0.58, 0.94]). Part 2 of CheckMate 227 is currently ongoing to evaluate the benefit of nivolumab-chemo combinational irrespective of PD-L1 expression.

The FDA approval of the pembrolizumab-chemotherapy combination in NSCLC has encouraged physicians to investigate various CIT in clinical trials in other types of cancers (**Table 1**). The combination of pembrolizumab with different chemotherapy regimens is being evaluated in PembroPlus study (NCT02331251) for patients with various types of advanced cancers. Pembrolizumab in addition to cisplatin or capecitabine or 5-Fluorouracil is being investigated in KEYNOTE-062 study (NCT02494583) for patients with advanced gastric cancer. Atezolizumab, an anti-PD-L1 antibody, has also been evaluated in combination with chemotherapy in the phase 1 GP28328 study (NCT01633970) in multiple tumor types, demonstrating improved ORR of CIT in patients with NSCLC (41). Phase 3 studies are the currently ongoing to determine the clinical benefit of atezolizumab in combination with chemotherapy (IMpower 130, IMpower132). Durvalumab, another anti-PD-L1 antibody, is also being evaluated in a phase 3 clinical trial (POSEIDON), randomizing untreated NSCLC patients to chemotherapy alone or chemotherapy in combination with durvalumab with or without anti-CTLA4 antibody, tremelimumab (NCT03164616).

Considerations and Challenges

Despite recent clinical success, our limited understanding of the interplay between chemotherapy and immunotherapy hurdles the design of the optimal combination strategy. Different types of chemotherapies execute cytotoxicity against tumors through distinct mechanisms. Similarly, each chemotherapy drug demonstrates unique impact on the systemic and intratumoral immune responses (8). Distinct intrinsic signaling pathways in different types of tumors attribute to the variability in their chemosensitivities. Moreover, the advanced knowledge of immune checkpoint pathways has rapidly expanded the list of ICIs that are acting through different mechanisms (e.g., TIM-3, LAG-3, indoleamine 2,3-dioxygenase, B7-H3). Understanding the impact of chemotherapy in the setting of different types of immunotherapies, as well as the impact of immunotherapy on chemosensitivity (or chemoresistance) of tumors, at both cellular and molecular levels are crucial for the design of rational combination regimens with minimized toxicity.

It is not just the appropriate combination but also the sequence and scheduling of CIT that have to be considered in the clinical scenario. In most of the clinical trials, chemotherapy and immunotherapy are given concurrently, lacking the understanding of the impact of sequencing on the antitumor immunity. Given the fact that antitumor T cell response has different phases that can be targeted by different ICIs (47, 48), and that chemotherapy can modulate immune system while having cytotoxicity against T cells at the same time, it is very likely that the sequence and timing of CIT would significantly impact the treatment outcomes. Both preclinical and clinical studies have shown controversial results regarding the sequence of CIT, and the ideal combination regimens are evolving. Since chemotherapy can cause immunogenic tumor death that promotes T cell priming, some have thought that immunotherapy should be given after chemotherapy to allow maximal T cell proliferation and expansion. In a phase 2 study investigating carboplatin and paclitaxel in combination with concurrent or sequenced ipilimumab in small-cell lung cancer (SCLC), the sequenced treatment is associated with improved PFS compared with chemotherapy alone (49). However, in a mesothelioma mouse model, concurrent treatment with ipilimumab and chemotherapy improved outcomes compared with sequential therapy (50). By contrast, studies in lung cancers and melanoma have shown that chemotherapy administered after immunotherapy can achieve successful clinical responses (51, 52). Our group recently demonstrated that in metastatic melanoma patients who had disease progression after anti-PD1

TABLE 1 | Completed and ongoing clinical trials evaluating chemotherapy in combination with immune checkpoint inhibitors.

Trial	Tumor type	Therapy regimes	Endpoints
Gadgeel et al. (30)	NSCLC (KEYNOTE 021) phase 1	Cohort A: Pembrolizumab (pembro) + carboplatin and paclitaxel (CP) → pembro Cohort B: Pembro + CP + bevacizumab (BEV) → pembro + BEV Cohort C: Pembro + carboplatin + pemetrexed (PEM) → pembro + PEM	Overall response rate (ORR): 52% Progression-free survival (PFS): 10 months ORR: 48% PFS: NR ORR: 48% PFS: 10
Langer et al. (31) Borghaei et al. (32)	NSCLC (KEYNOTE 021) phase 2	Pembro + carboplatin + PEM → pembro + PEM Carboplatin/PEM → PEM	ORR: 55% PFS: 19 months ORR 29% PFS: 8.9 months
Gandhi et al. (35)	NSCLC (nonsquamous) (KEYNOTE-189)	Pembro + platinum + PEM → pembro + PEM Placebo + platinum + PEM → PEM	OS (12 months): 69.2% PFS: 8.8 months OS (12 months): 49.4% PFS: 4.9 months
KEYNOTE-047 (ongoing)	NSCLC (squamous)	Pembro + CP → Pembro Placebo + CP	Primary: OS and PFS Secondary: ORR
Weiss et al. (42)	Advanced, metastatic solid tumors (PEMBRO-PLUS)	Pembrolizumab plus gemcitabine (G), G + docetaxel (D), G + nab-paclitaxel (NP), G + vinorelbine (V), or irinotecan	Standard dose pembrolizumab can be safely combined with G, G + NP, G + V, I, and LD
Rizvi et al. (37)	NSCLC (CHECKMATE 012) phase 1	Nivolumab (Nivo) + gemcitabine (GEM) + cisplatin (CIS) → Nivo Nivo + PEM the + CIS → Nivo Nivo (10 mg/kg) + CP →Nivo Nivo (5 mg/kg) + CP → Nivo	PFS: 5.7 months OS: 11.6 months PFS: 6.8 months OS: 19.2 months PFS: 4.8 months OS: 14.9 months PFS: 7.1 months OS: NR
Paz-Ares et al. (39)	NSCLC (CHECKMATE 227) phase 3 Ongoing	Chemotherapy alone or in combination with Nivo Squamous: CP Nonsquamous: PEM + carboplatin (or cisplatin)	Primary: PFS and OS Secondary: ORR
Liu et al. (41)	NSCLC	Atezolizumab (Atezo) + CP → Atezo Atezo + carboplatin/PEM → Atezo + PEM Atezo + carboplatin + nab-paclitaxel → Atezo	ORR: 36% PFS: 7.1 months OSS: 12.9 months ORR: 68% PFS: 8.4 months OS: 18.9 months ORR: 46% PFS: 5.7 months OS: 17.0 months
NCT02367781 NCT02367794 NCT02657434	IMpower 130 (NSCLC nonsquamous) IMpower 131 (NSCLC Squamous) IMpower 132 (NSCLC nonsquamous) All ongoing	Atezo + carboplatin + nab-paclitaxel → Atezo Atezo + CP → Atezo Atezo + PEM/carboplatin (or cisplatin) → Atezo + PEM	Primary: PFS and OS Secondary: ORR
NCT02537418	NSCLC (PESEIDON) Phase 3 Ongoing	Durvalumab + tremelimumab + chemotherapy (histology-based) Durvalumab + chemotherapy (histology-based) Chemotherapy (histology-based)	Primary: PFS Secondary: OS and ORR
NCT02735239	Metastatic/locally advanced esophageal cancer (neoadjuvant therapy)	Durvalumab in combination with standard of care chemotherapy or chemoradiation	Primary: Adverse events, dose-limiting toxicities Secondary: ORR, PFS, OS
NCT03317496	NSCLC Urothelial Cancer	Avelumab + pemetrexed/carboplatin Avelumab + gemcitabine/cisplatin	Primary: Confirmed OR Secondary: PFS, OS, duration of response, time to tumor response

(*Continued*)

TABLE 1 | Continued

Trial	Tumor type	Therapy regimes	Endpoints
Govindan et al. (43)	Advanced NSCLC	Ipilimumab + CP Placebo + CP	OS: 13.5 months PFS: 5.6 months (with higher toxicities) OS: 12.4 months PFS: 5.6 months
Patel et al. (44)	Metastatic melanoma	Ipilimumab plus temozolomide	6-month PFS was 45% with median OS of 24.5 months. 10 (15.6%) confirmed partial responses and 10 (15.6%) confirmed complete responses. No deaths/unexpected toxicities
Reck et al. (45)	SCLC	Ipilimumab + etoposide and platinum Placebo + etoposide and platinum	OS: 11.0 months PFS: 4.6 months OS: 10.9 months PFS: 4.4 months
Yamazaki et al. (46)	Melanoma	Ipilimumab + dacarbazine	Was not considered tolerable in the Japanese patient population

monotherapy, the addition of chemotherapy to PD-1 blockade induced a significantly improved clinical response, with an ORR of 65% (CR of 25%) (53–55). Using peripheral blood from patients who benefited from the CIT combination, we identified a novel subset of therapy-responsive $CD8^+$ T cells ($CX3CR1^+$) that can survive chemotherapy toxicity with preserved CTL functions (53). This subset of effector T cells is less actively proliferating during the combination therapy, and hence is spared from chemocytotoxicity. In addition, these $CX3CR1^+CD8^+$ T cells demonstrate the ability to efflux chemotherapy drugs. Our preclinical animal model studies also demonstrate that that CIT combination after previous exposure to immunotherapy provides better tumor control with an increase in $CX3CR1^+CD8^+$ T cells population (53). Our results elucidated mechanisms that are responsible for the success of combination, facilitating the rational design of CIT. This subset of T cells may be used as a biomarker in monitoring and predicting clinical response to CIT, especially when tumor PD-L1 levels fail to show direct correlation with the CIT treatment outcomes in multiple clinical trials. Future studies are warranted to define whether the efficacy of CIT is dependent on certain ICI to recruit immune cells into tumor tissues (like $CX3CR1^+CD8$ effector T cells) or to expand local tumor-infiltrating immune cells to reject tumors.

The scheduling and timing of chemotherapy in CIT are also critical for achieving clinical success. The fluctuation of dynamic systemic immunity in metastatic melanoma patients has been reported. Chemotherapy that was delivered in synchronization with unique phase of dynamic immune response tends to correlate with improved response (56). Some chemotherapy drugs work in cell cycle specific manners (e.g., S phase for drugs inhibiting DNA synthesis), suggesting that their direct tumor-killing activities and immunomodulatory effects can be influenced by the schedule of drug administrations. Platinum-based chemotherapy given in different dosing schedules has shown different antitumor immune responses associated with variable clinical outcomes in an ovarian cancer mouse model (57). Our recent preclinical study further demonstrated that the timing of chemotherapy administration after the immunotherapy initiation can affect the frequencies of $CX3CR1^+$ T cell population and the treatment outcomes (53), suggesting the variable chemo-induced immunomodulation in relationship to the timing of the immunotherapy. With the overwhelming possibility of CIT combinations, further preclinical and clinical research is in need to design rational combinations for different types of cancers, while minimizing the therapeutic toxicities.

COMBINATION OF IMMUNOTHERAPY AND MOLECULAR TARGETED THERAPY

The identification of deregulated cellular signaling pathways that are responsible for tumorigenesis has led to the successful development of molecular targeted therapy in recent decades. Medications inhibiting oncogenic pathways, DNA repair response, and angiogenesis pathways have provided effective treatment options for patients with different types of malignancies, although response durability is often lacking. Recent research has demonstrated that these pathways also have immunomodulatory effects on systemic and intratumoral antitumor immune responses, suggesting that the combination of molecular targeted therapy with ICIs can result in synergistic antitumor effects.

BRAF and MEK Inhibitors

Dysregulations in the RAS/RAF/MAPK pathway are commonly seen in oncogenic transformation and tumorigenesis. Mutations in *BRAF*, a proto-oncogene, are associated with various types of cancers, especially melanoma. In patients with BRAF mutant metastatic melanoma, high response rates are observed after treatment with BRAF/MEK inhibitors, although the duration of response is short lasting due to adaptive therapy resistance. Since ICIs provide durable clinical benefit, combinations of ICIs with BRAF/MEK inhibitors may provide fast and long-lasting disease control.

In melanoma cell models, BRAFV600 mutations can lead to decreased antitumor immunity through upregulation of immunosuppressive factors [e.g., IL-10, vascular endothelial growth factor (VEGF)] (58), elevation of PD-L1 expression levels (59), increased tumor infiltration of immunosuppressive cells

(e.g., Treg) (60), and downregulation of melanoma MHC-1 expression (61). In patients with metastatic melanoma, BRAF inhibitors have shown to decrease the immunosuppressive cytokines with resultant increased $CD8^+$ T cell tumor infiltration and antitumor immunity (62). In addition, the treatment response of BRAF inhibitors is $CD8^+$ T-cell dependent (60, 63). Interestingly, in CRC patients, the majority of those who have PD-L1 positive tumor carry BRAF mutations along with microsatellite instability, suggesting that the immunosuppressive tumor microenvironment can be induced by BRAF mutations (64). In a colorectal carcinoma mouse model, MEK inhibitors demonstrated synergistic therapeutic effects with anti-PD-L1 antibody (65). In a melanoma mouse model, dabrafenib treatment alone results in increased TAMs and Treg, while the addition of trametinib further decreases these suppressive cell subsets. When combined with anti-PD-L1 antibody, the triple therapy provides a superior tumor control (66).

The combination of BRAF inhibitors with anti-CTLA-4 antibody has been studied in multiple clinical trials. However, substantial immune-related adverse effects were the main concern in several studies. Liver toxicity and high-grade skin adverse effects were seen in trials with vemurafenib and ipilimumab combination (NCT01400451 and NCT01673854) (67, 68). Severe colitis was seen in patients who received dabrafenib, trametinib, and ipilimumab triple combination (NCT01767454). Anti-PD1/PD-L1 antibodies were also evaluated in combination with BRAF/MEK inhibitors (**Table 2**). In a phase 1 study, the combination of dabrafenib, trametinib, and durvalumab demonstrated tolerable toxicity profiles and encouraging disease response rates (69). Pembrolizumab is also being studied in combination with dabrafenib and trametinib in patients with metastatic melanoma (NCT02130466). Multiple other clinical trials are currently ongoing (NCT01940809; NCT01656642; NCT02027961; NCT02224781) to test similar combinations.

Combining BRAF/MEK inhibitors with ICIs has the potential to overcome resistance to targeted therapy; however, further investigations are needed to understand the underlying molecular interplay and to design the ideal combination regimens. Given the toxicities observed in early trials, the optimum tolerable dose of targeted therapy in combination with ICIs needs to be determined. The rapid development of resistance to BRAF/MEK inhibitors and their dynamic impacts on the tumor microenvironment and systemic antitumor immunity should also be considered to determine the sequencing and scheduling of the combination. One study demonstrated long-term tumor control after short-term targeted therapy with subsequent anti-PD1 antibody in patients with metastatic melanoma, and T cell tumor infiltration was seen in tumor biopsies within 1 week after BRAF/MEK inhibitors administration while less frequent after 2 weeks on therapy (71), suggesting that the timing of combination with PD-1 blockade can impact patient outcomes.

Other oncogenic pathways, such as PI3K–Akt–mTOR pathway and KIT, can also regulate the antitumor immunity in addition to regulating cellular proliferation, providing further options for combination therapy with ICIs. For example, inhibition of PI3kγ can promote T cell infiltration through regulating the balance between stimulatory and suppressive TAMs (72). Inhibition of KIT decreased the INF-γ induced PD-L1 expression (73). Treatments targeting these pathways are also being investigated in combination with ICIs to overcome their limited clinical response. The crosstalk between multiple signaling oncogenic pathways in the setting of immunotherapy should be further investigated to determine the ideal drugs to be combined, with special consideration of the individual's unique intrinsic genetic background.

Poly (ADP-Ribose) Polymerase (PARP) Inhibitors

DNA damage repair machinery plays important roles in cell cycle regulation and tumorigenesis (74). Inhibition of DNA damage repair can potentially increase the tumor mutational burden, especially in tumors with high endogenous DNA damage. PARP plays a critical role in the repair of single-strand DNA break. In tumor cells with BRCA mutations, PARP inhibition can increase the genomic instability and cell death, with resultant increased neoantigen load and antitumor T cell response (75). This synthetic lethality of PARP inhibitors established the foundation for its clinical application in cancer treatment (76). PARP inhibitors also demonstrate immunoregulatory effects in preclinical studies. They can attenuate chronic inflammation and increase T cell infiltration (77).

Olaparib has been recently approved for the treatment of ovarian cancers with BRCA1 and BRCA2 mutations. Olaparib in combination with PD-L1 inhibitor, durvalumab, was recently investigated in a clinical trial for patients with gynecologic

TABLE 2 | Clinical trials of BRAF targeted therapy in combination with immune checkpoint inhibitors.

Trial	Mutation status	Therapy regimens	Outcomes
Puzanov et al. (70)	BRAF V600 mutant melanoma	Dabrafenib ± trametinib + ipilimumab	Triple therapy resulted in severe GI toxicities
Ribas et al. (67)	BRAF V600 mutant melanoma	Vemurafenib + ipilimumab	Combination resulted in severe liver toxicities
Ribas et al. (69)	Both wild-type and BRAF mutant melanoma	Durvalumab + dabrafenib + trametinib Durvalumab + trametinib Trametinib → durvalumab	Tolerable, no unexpected toxicity
Amin et al. (68)	BRAF V600 mutant melanoma	Vemurafenib + ipilimumab	Combination resulted in high-grade GI and skin toxicities
NCT02224781	Metastatic melanoma	Dabrafenib + trametinib followed by ipilimumab + nivolumab at progression vs. ipilimumab + nivolumab followed by dabrafenib + trametinib	Ongoing Primary: OS Secondary: PFS

cancers. Among 26 enrolled women, a disease control rate of 83% was reported, with an acceptable safety profile (78). Several trials evaluating different PARP inhibitors in combination with ICIs are currently ongoing in various solid tumors, including NSCLC and breast cancer (**Table 3**). The combination of niraparib with pembrolizumab was studied in the phase 1/2 TOPACIO/Keynote-162 (NCT02657889) study, and the results from the recurrent ovarian cancer cohort were recently reported (79). Among the 60 evaluable patients, an ORR of 25% was seen in all platinum-resistant ovarian cancer patients with an ORR of 45% in those with BRCA mutations. No new safety concerns were identified.

Given the fact that DNA repair response not only regulates tumorigenesis but also plays role in antitumor immunity, it is imperative to fully understand the interplay between DNA repair inhibitors and ICIs to combine them in a safe and effective manner, since inhibition of DNA repair response can potentially decrease the immune response. Moreover, the intrinsic tumor genetic background and DNA repair response status [e.g., BRCA mutation vs. wild-type (WT)] have positive impact on the PARP inhibitor-induced antitumor immune response during immunotherapy. Furthermore, it is possible that different PARP inhibitors can modulate the antitumor immune response through different mechanisms, which could impact the treatment outcomes when combined with different ICIs.

VEGF Inhibitors

Vascular endothelial growth factor stimulates angiogenesis, tissue remodeling, and fibrosis. Its immunosuppressive effects make VEGF a good target candidate to potentiate the antitumor immune response in combination with ICIs (80, 81). Studies from our group have shown that in patients with stage IV melanoma the baseline Treg concentration positively correlates with baseline VEGF level, which associates with poor clinical outcomes (82). Upregulated VEGF level and chronically Th-2-mediated immune status are observed in patients with metastatic melanoma (83). Animal models have shown that anti-VEGF antibody can increase T cell tumor infiltration with enhanced antitumor response (84). Decreased Treg proliferation and MDSC population are associated with bevacizumab treatment in CRC (85). The hypoxic conditions resulting from anti-VEGF treatment also can upregulate PD-L1 expression.

Tremelimumab in combination with sunitinib has been evaluated in patients with metastatic melanoma. Unfortunately, unexpected dose-limiting renal toxicity was observed (86). High dose of tremelimumab (6 mg/kg) used in this study could contribute to the adverse effects. Bevacizumab was also investigated in combination with ipilimumab in melanoma patients (87). A disease control rate of 67.4% was observed with combination therapy, with increased $CD8^+$ lymphocyte infiltration resulting from more effective lymphocytic trafficking. Multiple clinical trials are currently ongoing investigating the combination of bevacizumab and pembrolizumab in patients with ovarian cancer (NCT02853318) and solid tumor brain metastases (NCT02681549). Other agents targeting VEGF pathway, such as ramucirumab, are also being evaluated in combination regimens (**Table 3**). Lenvatinib, a tyrosine kinase inhibitor, is being studied in combination with pembrolizumab in a phase 1/2 trial (NCT03006926). However, the dynamic immunologic effects of these combinations remain to be elucidated. In a recent report, 10 patients with metastatic renal cell carcinoma were treated with bevacizumab plus atezolizumab after bevacizumab run-in period (88). A partial disease response was observed in four patients, and median time to response was 4.2 months. Following combination therapy, increased intratumoral $CD8^+$ T cells, Th1, and T effector markers are found. Interestingly, increases in intratumoral chemokine, CX3CL1, and peripheral $CX3CR1^+$ (CX3CL1 receptor) $CD8^+$ T cells are observed after combination therapy, similar to the observation in patients received CIT combination (53). Clinical trials are ongoing to evaluate this combination in untreated metastatic renal cell carcinoma (IMmotion151 study)

TABLE 3 | Clinical trials of immune checkpoint inhibitors in combination with poly (ADP-ribose) polymerase inhibitors or vascular endothelial growth factor targeting therapy.

Trial	Tumor type	Treatment regimen	Outcome
Lee et al. (78)	Gynecological cancers	Durvalumab + olaparib Durvalumab + cediranib	83% disease control rate in durvalumab + olaparib group 75% disease control rate in durvalumab plus cediranib group
NCT02734004	Breast, gastric, ovarian and SCLC	Durvalumab + olaparib	Ongoing
NCT02484404	NSCLC, SCLC, breast, ovarian, colorectal, prostate	Durvalumab + olaparib Durvalumab + cediranib Durvalumab + olaparib + cediranib	Ongoing
NCT02657889	Breast and ovarian	Pembrolizumab + niraparib	Ongoing
NCT02944396	NSCLC	Nivolumab + veliparib + platinum-based chemotherapy Veliparib + platinum-based chemotherapy	Ongoing
NCT02849496	Breast	Veliparib Atezolizumab Veliparib + atezolizumab	Ongoing
NCT02443324	Gastric, GEJ adenocarcinoma, NSCLC, transitional cell carcinoma of the urothelium, biliary tract	Pembrolizumab + ramucirumab	Ongoing
NCT02572687	GI or thoracic malignancies	Durvalumab + ramucirumab	Ongoing

(89) and untreated locally advanced or metastatic hepatocellular carcinoma (IMbrave150) (NCT03434379). Further investigation will be critical to design safe and efficacious combinations and to address biomarker selection.

COMBINATION OF IMMUNOTHERAPY WITH RADIOTHERAPY (RT)

Radiotherapy remains to be the backbone modality in the treatment for different types of cancer, either given alone or in combination with chemotherapy. It induces single- and double-strand DNA breaks, triggering multiple signaling pathways including DNA damage responses and activation of cell cycle checkpoints. The RT-induced cell death can further initiate systemic antitumor responses through various immune cell subsets (90). The participation of immune cells is indispensable for the clinical benefit of RT; in turn, RT can also modulate the antitumor immunity. It has been reported that localized tumor radiation can result in distant systemic tumor control in the unirradiated area. This clinical phenomenon, known as the abscopal effect, is a result of RT-induced immune modulations (91). The potential synergistic antitumor activities of RT in combination with ICIs have attracted increased research efforts in this new era of cancer immunotherapy (92).

Increased infiltration of macrophages and monocytes has been observed post-irradiation in multiple humana cancer xenograft models (squamous cell carcinoma, breast, and lung carcinoma), and depletion of the TAMs with antibody to CD11b or inhibitor of SDF-1a receptor CXCR4 (AMD3100) provided further tumor control when combined with irradiation (93, 94). The activation of dendritic cells and their release of type I interferon after irradiation is also critical in $CD8^+$ T cell activity and treatment efficacy in mouse colon carcinoma (MC38), lung carcinoma (LLC), and melanoma models (B16F10) (95, 96). In an animal model of Lewis lung carcinoma (LLC), when the local area was exposed to irradiation, distant tumor control was observed only in p53 WT (vs. in p53 null mice), suggesting that the abscopal effect was mediated through pathways downstream of p53 (97). Multiple studies have shown that cytotoxic $CD8^+$ T cells are required for RT-induced tumor control in mouse breast cancer (4T1), melanoma (B16), lymphoma (EL4), and lung cancer (98, 99).

Although the myelosuppression after irradiation is thought to be immunosuppressive, RT can regulate T cell-mediated immune responses *via* various mechanisms (100). RT regulates key cell surface molecules for cytotoxic immune cell activation (NKG2D), and antigen presentation machinery (MHC class I expression with antigen peptides), therefore augments T cell tumor recognition and activation (101, 102). RT also enhances T cell priming for activation via activation of antigen-presenting cells (dendritic cells) and releasing of immunogenic antigens (95, 103). RT increases the release of chemokines (CXCL16, a chemokine that binds to CXCR6 on Th1 CD4 and effector CD8 T cells) by mouse breast cancer (4T1) cells, therefore enhances the infiltration of cytotoxic T cells (104). In this situation, blockade of CTLA-4 further promotes tumor regression. Irradiation also alters the immunosuppressive tumor microenvironment to a M1 phenotype, favoring accessibility for T cell infiltration (105). In addition, in melanoma and Kras-mutant lung cancer models, irradiation was found to upregulate PD-L1 expression in tumor microenvironment (106, 107). The synergistic relationship between RT and ICI has been further explored in preclinical models. In mice bearing poorly immunogenic breast carcinoma, treatment with CTLA-4 blockade in combination with RT (vs. CTLA-4 blockade alone) resulted in decreased tumor growth and metastasis with improved survival (108). RT in combination with PD-1 blockade also induced improved and durable tumor control in NSCLC mouse models (107). In melanoma and renal cell carcinoma animal models, PD-1 knockout (KO) mice demonstrated higher survival after stereotactic ablative radiotherapy (SABR) compared with their PD-1 WT little mates. The addition of PD-1 blocking antibody to SABR led to the improved antitumor response and survival in PD-1 WT mice. In addition, treatment with SABR and anti-PD1 antibody combination induced significant reduction of non-irradiate tumor. The increased frequency of $CD11a^{high}CD8^+$ tumor-reactive T cells and their enhanced functions were associated with the antitumor response in PD-1 KO mice, suggesting the translation potential of combining RT and PD-1 blockade (109).

Over the recent years, the clinical efficacy of RT in combination with ICIs have been studied in multiple clinical trials were designed to investigate. However, data supporting the routine application of this combination are still limited. The RT and ipilimumab combination demonstrated acceptable toxicity profile in patients with metastatic melanoma, yet failed to provide survival benefit (110). Ipilimumab in combination with radiation was evaluated in a single-arm phase 2 study in melanoma patients with unresectable brain metastases (111). Fifty-eight patients were enrolled in this study; with 1-year OS of 31.8% (95% CI [18.8–44.8%]) that is higher than the historical reported results, without unexpected adverse events.

In a phase 1 KEYNOTE-001 study, 97 patients with advanced NSCLC were enrolled (112). Longer PFS and OS, without higher incidence of grade 3 or above pulmonary toxicity, were seen in patients who underwent RT prior to anti-PD1 therapy compared with those who did not receive RT, supporting the safety and potential synergistic activity of the RT-ICI combination. The phase 3 randomized PACIFIC trial investigated the role of subsequent durvalumab therapy in stage III NSCLC patients after definitive chemoradiation (NCT02125461) (113). A total of 713 patients with locally advanced unresectable NSCLC without disease progression after definitive chemoradiation were randomly assigned in 2:1 to receive durvalumab or placebo irrespective of PD-L1 status. Median progression survival is 16.8 months in durvalumab group vs. 5.6 months in placebo group (16.8 months vs.), with 18-month PFS rate of 44.2 vs. 27.0%, respectively (113). Results from this study led to the FDA approval of durvalumab for the treatment of stage III NSCLC regardless of PD-L1 expression levels. The RT and immunotherapy combination is currently being evaluated in other tumor types, such as metastatic GI malignancies (NCT02830594) and metastatic breast cancer (NCT02730130) (**Table 4**).

Despite the encouraging finding from the PACIFIC trial, future research is urgently needed to define the immunoregulatory mechanisms that cross talk between RT, chemo, and immunotherapy, to design optimized combination strategies. Similar to

TABLE 4 | Clinical trials of immune checkpoint inhibitors in combination with radiotherapy (RT).

Trial	Tumor type	Regimens	Outcomes
Shaverdian et al. (112)	NSCLC (KEYNOTE-001)	Compared patients on pembrolizumab with previous RT to those who did not receive previous RT	Previous treatment with RT results in longer PFS and OS, with an acceptable safety profile
Antonia et al. (113)	NSCLC	Definitive ChemoRT → durvalumab Definitive ChemoRT → placebo	Progression-free survival (PFS): 16.8 months 18 months PFS: 44.2% PFS: 5.6 months 18 months PFS: 27.0%
Levy et al. (114)	Inoperable or metastatic cancers	Concurrent durvalumab + RT	Concurrent palliative RT with the anti-PD-L1 durvalumab was well tolerated
Tang et al. (115)	Metastatic solid tumor	Ipilimumab + stereotactic ablative radiotherapy (SABR)	Combining SABR and ipilimumab was safe with signs of efficacy, peripheral T-cell markers may predict clinical benefit, and systemic immune activation was greater after liver irradiation
Hiniker et al. (116)	Metastatic melanoma	Palliative RT + ipilimumab	Combination therapy was well tolerated without unexpected toxicities. Eleven patients (50.0%) experienced clinical benefit from therapy, including complete and partial responses
NCT03050554	NSCLC	Stereotactic body radiation therapy (SBRT) in combination with Avelumab	Ongoing Tolerability, RFS
NCT02658097	NSCLC	Single fraction nonablative radiation in combination with pembrolizumab	Ongoing RR and best OS
NCT03458455	Brain tumor	Stereotactic radiosurgery plus ipilimumab, nivolumab, or pembrolizumab	Ongoing Treatment response at 18 months
NCT03115801	Metastatic renal cell carcinoma and urothelial carcinoma	Nivolumab/atezolizumab Nivolumab/atezolizumab plus RT	Ongoing Best overall response rate, PFS, toxicities, OS
NCT03176173	NSCLC	Radical-dose image guided radiation therapy daily for up to 10 days (within 2 weeks) while undergoing standard of care immunotherapy Patients who decline to undergo radiation therapy receive standard of care immunotherapy	Ongoing PFS

other combination strategies discussed, the optimum sequencing of the RT and ICI combination has not been elucidated in available studies. In the KEYNOTE-001 study, irradiation prior to pembrolizumab therapy provided improved PFS and OS (112), while in another clinical report palliative RT-induced global disease control in a PD-1 antibody resistant patient (117). In a melanoma mouse model, the addition of adjuvant chemotherapy after ablative RT abrogated the RT-induced $CD8^+$ T cell activation and tumor control, while the addition of immunotherapy can enhance the tumor response (98), suggesting that the treatment modalities and the sequence of their combination need to be carefully investigated to achieve clinical success. In addition, research is needed to identify biomarkers with both predictive and prognostic values in RT-immunotherapy combination.

CONCLUSION

Modern cancer immunotherapies exert their tumor-killing activities through enhancing antitumor immunity while suppressing the tumor-promoting immune process. However, since ICIs do not provide clinical benefits in the majority of cancer patients, it is crucial to design rational and efficacious synergic therapeutic approaches to increase clinical responses to ICI. The combination of chemotherapy, targeted therapy, and RT with ICIs has gained increased attentions from clinicians and researchers over the recent years, given their immunomodulatory effects and potential synergistic antitumor activities. Despite the encouraging clinical results from various clinical studies, further investigations are warranted to elucidate the exact molecular and cellular mechanisms driving these clinical responses. More importantly, the optimal regimens, dose, timing, and schedule of the combination therapy for differently types of tumors are yet to be identified. Molecular interplay between different therapeutic modalities will need to be further investigated given the unlimited possibilities of combining currently available cancer treatments.

AUTHOR CONTRIBUTIONS

YY and HD conceived the outline of the manuscript. YY, HD, AK, HF, and SM wrote and edited the manuscript. SP and RD edited the manuscript.

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Metabolic Checkpoints: Novel Avenues for Immunotherapy of Cancer

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Novel therapies targeting immune checkpoint molecules have redefined the treatment of cancer at advanced stages and brought hope to millions of patients worldwide. Monoclonal antibodies targeting immune-inhibitory receptors often lead to complete and objective responses as well as to durable progression-free survival where all other therapeutic approaches fail. Yet, many tumors show significant resistance to checkpoint blockade through mechanisms that are only starting to come to light. An alluring alternative strategy to reinvigorate anticancer immune responses comes from the emerging field of immuno-metabolism. Over the past few years, numerous studies revealed that many well-known metabolic playmakers also serve as critical checkpoints in immune homeostasis and immunity against tumors. Here, we survey recent insights into the intimate and intertwining links between T cell metabolic programs and environmental cues in the tumor milieu. Transferring these new findings from the bench to the bedside may soon entirely re-shape the field of cancer immunotherapy and significantly improve the lives of patients.

Keywords: metabolic checkpoints, immune checkpoints, cancer immunotherapy, PD-1, checkpoint blockade, mammalian target of rapamycin pathway

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INTRODUCTION

Immunotherapy has become a paradigm-shifting approach showing unmatched efficacy in patients with advanced malignancies. Targeting immune regulatory receptors, such as CTLA-4, PD-1, and PD-1 ligand (PD-L1), leads to spectacular clinical responses (1). Unfortunately, the success of these treatments is often limited to minor cohorts of patients. This calls for the development of alternative strategies for reinvigorating anticancer immunity. Recently, research into immuno-metabolism has emerged as an extraordinarily vibrant and productive area of study that is likely to become a launch-pad for novel therapeutic approaches (2, 3). Over the past few years, the immunological community has witnessed a veritable Cambrian explosion of remarkable studies identifying the critical metabolic programs and checkpoints in the activation, differentiation, and migration of immune cells (2, 4). This research has illuminated the metabolic requirements for successful T cell-mediated effector responses and memory T cell generation in cancer. Furthermore, several groups have revealed the complex effects of multiple micro-environmental factors on T cell functionality in the tumor (5, 6). For instance, the pleiotropic roles of oxygen tension in the regulation of anticancer immunity are now coming to light.

Importantly, pathways controlling T cell responses to external challenges often converge on the same limited set of enzymes, transcription factors, and signaling complexes serving as metabolic checkpoints (7, 8). This highlights the elegant simplicity and dazzling complexity of T cell biology.

Untangling these immuno-metabolic nodes will be essential for the rational design of future therapies for cancer.

IMMUNO-METABOLIC CHECKPOINTS IN T CELL DIFFERENTIATION AND FUNCTION

Metabolic changes occur throughout the lifespan of a T cell and provide the essential energetic currency and building blocks to help the T cell meet the emerging needs (4). Effective T cell responses against tumors strongly depend on the differentiation pathways taken up by individual $CD4^+$ and $CD8^+$ T cells upon their interaction with tumor antigens (9). The lineage commitment of stimulated T cells depends on the integration of the plethora of environmental cues and cell-intrinsic signals during activation, initial proliferation rounds, development of effector functions, and until final differentiation steps (2, 10).

Throughout their developmental path, T cells must strike a balance between increasing energy demands and a growing need for substrates to maintain their functionality and proliferation (2). Naive or quiescent T cells rely mainly on oxidative phosphorylation (OXPHOS), a highly efficient pathway for generating ATP from glucose. Upon activation, however, a switch to aerobic glycolysis takes place. In this rather inefficient process, only two ATP molecules are produced per each molecule of glucose. Switching to aerobic glycolysis might appear very inefficient, due to the low ATP/glucose ratio. Yet, at the same time, aerobic glycolysis yields a higher number of building blocks for anabolism. Importantly, the glycolytic switch is also essential for T cells to acquire diverse effector functions (e.g., production of IL-2, IFN-γ, etc.), since it relieves the blockade of IFN-γ mRNA translation by the glycolytic enzyme GAPDH (11).

The metabolic switch upon T cell activation is governed by a plethora of transcription factors and signaling pathways. Together, TCR engagement, costimulation, and cytokine signaling boost glycolysis by upregulating the expression of nutrient transporters (such as the Glut1 glucose importer) and activating the central metabolic regulator mammalian target of rapamycin (mTOR) complex (12–14). mTOR drives the development of all the effector T cell subsets but hampers peripheral Treg induction. This can be accounted for the requirement of intensified glycolysis during effector T cell expansion, while Treg cells primarily deploy OXPHOS and oxidation of fatty acids. mTOR comprises two distinct complexes, mTORC1 and mTORC2, and orchestrates cellular responses to changes in nutrient levels and energy status (15). Costimulation *via* CD28 activates PI3K recruiting 3-phosphoinositide-dependent protein kinase-1 and Akt, which, in turn, activates mTOR. This pathway leads to the quick upregulation of Glut1 expression and to its increased transport to the plasma membrane. The rapid intensification of glucose import is critical for efficient T cell activation, clonal expansion, and survival.

Of note, a study by Macintyre et al. (16) suggested that Glut1 is only indispensable for the differentiation of Th1, Th2, and Th17 cells, but not $CD8^+$ T cells or Tregs. A plausible explanation might be coming from recent proteomic studies indicating that Glut1 and Glut3 protein levels are comparable in $CD8^+$ T cells (17). Therefore, glucose transporters can act in a somewhat redundant fashion to meet the demands of T cells for glucose.

The multifaceted role of glucose uptake in Treg biology has recently been further elucidated in a study by Rathmell and colleagues who identified toll-like receptor (TLR) signals that drive Treg cell proliferation *via* PI(3)K–Akt–mTORC1 signaling, which intensifies glycolysis and glucose import by Glut1 (18). Conversely, TLR-induced mTORC1 signaling also diminished the ability of Tregs to suppress effector T cell proliferation. In line with previous studies, the transcription factor Foxp3 dampened the effects of PI(3)K–Akt–mTORC1 signaling to hamper glycolysis and anabolism while boosting OXPHOS and catabolic pathways. Likewise, although Glut1 expression promoted Treg expansion, it also reduced their suppressive activity and Foxp3 expression. This indicates that Treg cells might occasionally switch to aerobic glycolysis and expand when they receive inflammatory signals, and subsequently switch back to FAO and OXPHOS to achieve maximal suppressive potency. These findings further highlight glycolysis as a critical metabolic axis in maintaining the immunological balance. Therefore, glycolytic enzymes and nutrient transporters represent attractive targets for future immuno-metabolic therapies of cancer. Specifically, administration of glucose uptake inhibitors or Glut knockdown in adoptively transferred cells could be instrumental for pushing antitumor T cell differentiation toward long-lived memory cell lineage for the generation of enduring antitumor immunity (9, 19).

Migration of activated Treg cells to the site of inflammation is crucial for their immune-inhibitory function (20). Kishore et al. (21) have studied the metabolic needs for migratory Treg. They demonstrated that glycolysis strongly promotes Treg migration and is triggered by a PI3K–mTORC2-mediated pathway driving the activation of the enzyme glucokinase. These findings also suggest a new attractive strategy to avert Treg infiltration into tumors by manipulating their metabolic programs.

The transcription factors c-Myc and hypoxia-inducible factor-1α (HIF-1α) coordinately activate the genes required for the vigorous proliferation of effector T cells during clonal expansion (22, 23). Importantly, both Myc and HIF-1α are under the control of the mTOR complex. C-Myc promotes the expression of enzymes involved in aerobic glycolysis and glutaminolysis and fine-tunes these metabolic pathways to the biosynthesis of lipids, amino acids, and nucleic acids. HIF-1α mediates T cell responses to oxygen levels and also promotes glucose uptake and breakdown (24). Thus, the same transcription master regulators that control such fundamental processes as cell proliferation and cellular response to oxygen tension are also responsible for adjusting T cell metabolism to emerging needs. This underscores the startling universality and efficiency of these most fundamental mechanisms of epigenetic regulation and indicates that a robust reprogramming of antitumor T cell metabolism might be achieved by only targeting a few select molecules.

The dichotomy between glycolysis versus OXPHOS and FAO is not only central to the control of T cell activation and effector function but is also decisive in the fate of differentiating T cells. Most importantly, in precursors of long-lived memory

cells, OXPHOS and FAO are engaged to balance out the effects of aerobic glycolysis (25). A pivotal role for mitochondria (26) in these cells is manifest in the dynamics of their ultrastructure. In memory T cells, mitochondrial cristae fuse into elaborate networks, while mitochondria in effector T cells exhibit extensive fission. The functionality of mature memory cells is also sustained by the higher biomass and spare respiratory capacity of their mitochondria through IL-15-driven upregulation of carnitine palmitoyl-transferase. This enzyme drives FAO and engenders stronger and more protracted OXPHOS and glycolysis upon restimulation.

Importantly, a wealth of recent evidence illuminates the possibilities to improve clinical responses to immune checkpoint inhibitors by combining these therapies with modulation of metabolic pathways. For instance, a series of elegant studies by Chi and colleagues (7, 27, 28) have demonstrated the intimate interplay between immune and metabolic checkpoints in T cell differentiation. Specifically, mTORC1 signaling was established as a key "rheostat" in Treg cell function (28). Treg-intrinsic disruption of mTORC1 led to a drastic slump in Treg suppressive activity and launched a deadly early-onset inflammatory disease. Raptor/mTORC1 signaling in Tregs boosted the metabolism of cholesterol and lipids, while the mevalonate pathway proved essential for orchestrating Treg proliferation and upregulated expression of the checkpoint molecules CTLA4 and ICOS. Another study by the same group highlighted the role of autophagy in the lineage stability and survival of Treg cells (29). Treg cell-specific deficiency in Atg7 or Atg5, two pivotal genes in autophagy, resulted in a diminished Treg compartment, improved antitumor immunity, and development of inflammatory disorders. Autophagy kept in check mTORC1, c-Myc, and glycolytic enzymes, thereby coupling environmental signals to metabolic homeostasis.

METABOLIC COMPETITION IN THE TUMOR MICROENVIRONMENT

Recent studies have directly linked T cell metabolism, T cell exhaustion, and antitumor immunity. In the tumor, the scarcity of nutrients can profoundly affect cell proliferation, survival, and functionality. T cell-infiltrating tumors become enmeshed into teeming metabolic networks established within the hostile microenvironment and are forced to face ruthless competition for nutrients. Cancer cells can express various enzymes that deprive T cells of critical substrates and produce immune-inhibitory metabolites (**Figure 1**). For instance, many tumors contain large amounts of indoleamine 2,3-dioxygenase (IDO), an enzyme that eliminates tryptophan from the microenvironment and hampers T cell proliferation (30). Of note, despite the significant efficacy of IDO inhibitors in mouse models of cancer, these compounds have shown no measurable antitumor efficacy in clinical settings (31). The potential advantages and pitfalls of therapeutically exploiting the metabolic differences between normal and malignant cells have recently been comprehensively surveyed by Martinez-Outschoorn and coauthors (32).

In two pioneering studies, published side by side in *Cell* (5, 6), the groups of Susan Kaech and Erika Pearce reported that tumor cells outcompete T cells for glucose, thereby dampening their effector function and evading immune destruction. Ho et al. (5) demonstrated that unperturbed glucose metabolism in T cells is critical for TCR-induced Ca^{2+} flux. Extracellular glucose promoted accumulation of phosphoenolpyruvate, a glycolytic metabolite that inhibited sequestering of Ca^{2+} from the cytoplasm into the ER, thereby sustaining activation-induced Ca^{2+} flux and T cell effector function. Conversely, increased expression of the glycolysis gatekeeper hexokinase 2 in tumor cells facilitated tumor escape from $CD4^+$ T cell-mediated immune surveillance, further corroborating the importance of metabolic competition between tumor-infiltrating lymphocytes (TILs) and tumor cells.

In the other study, Chang et al. (6) demonstrated that signaling through PD-L1 in tumor cells promotes glycolysis *via* activation of the AKT/mTOR pathway. Therapeutic blockade of PD-L1 decreased glycolysis rate by triggering PD-L1 internalization, restored glucose levels in the microenvironment, and hindered tumor progression. These data provided a breakthrough in our understanding of the deeply intertwined metabolic and immune checkpoint signaling pathways and underscore the potential of future immune-metabolic therapies.

Conversely, rather than targeting the metabolism of tumor cells, Ho et al. (5) put forward an alternative strategy to reinforce T cell function by artificially increasing PEP levels in adoptively transferred tumor-reactive T cells. PEP carboxykinase (PCK1) catalyzes conversion of oxaloacetate into PEP. Overexpression of PCK1 in transferred T cells allowed the authors to restore TCR-driven Ca^{2+} flux and anticancer T cell activity, thus overriding the effects of low glucose levels in the tumor microenvironment. Overall, these reports provided inspiring examples of reprogramming T cell metabolism to enhance the efficacy of adoptive cell therapies for cancer and spawned many further studies exploring T cell metabolic dysfunction in the tumor.

For instance, the latest works by Delgoffe and colleagues shed new light on various aspects of T cell metabolism in cancer. For example, Scharping et al. (33) reported a continuous loss of mitochondrial mass and functionality in tumor-infiltrating T cells, which proved restricted to the tumor milieu and was not a mere consequence of activation. Due to chronic Akt signaling, TILs showed dwindling expression of PPAR-gamma coactivator 1α (PGC1α), a vital factor in mitochondrial biogenesis. Reprogramming TILs through forced expression of PGC1α reinvigorated their metabolic and effector function. Interestingly, in a study by Wherry and colleagues (34), PD-1 was shown to inhibit PGC1α and thereby serve as a dominant-negative regulator of glycolysis in activated T cells.

More recently, it has been reported that T cell activation leads to a rapid glycolytic switch independently of transcription, translation, costimulation through CD28, or Akt signaling and without an increase in glucose uptake or in the activity of glycolytic enzymes (35). Instead, TCR engagement enhances activation of pyruvate dehydrogenase kinase 1 (PDHK1), thereby hampering import of pyruvate into mitochondria and promoting its cleavage into lactate. Intriguingly, inhibiting PDHK1 revealed that the early glycolytic switch is required for immediate cytokine production but not for cytotoxicity. Recently, Menk et al. (36) demonstrated that ligation of 4-1BB (a costimulatory molecule highly expressed

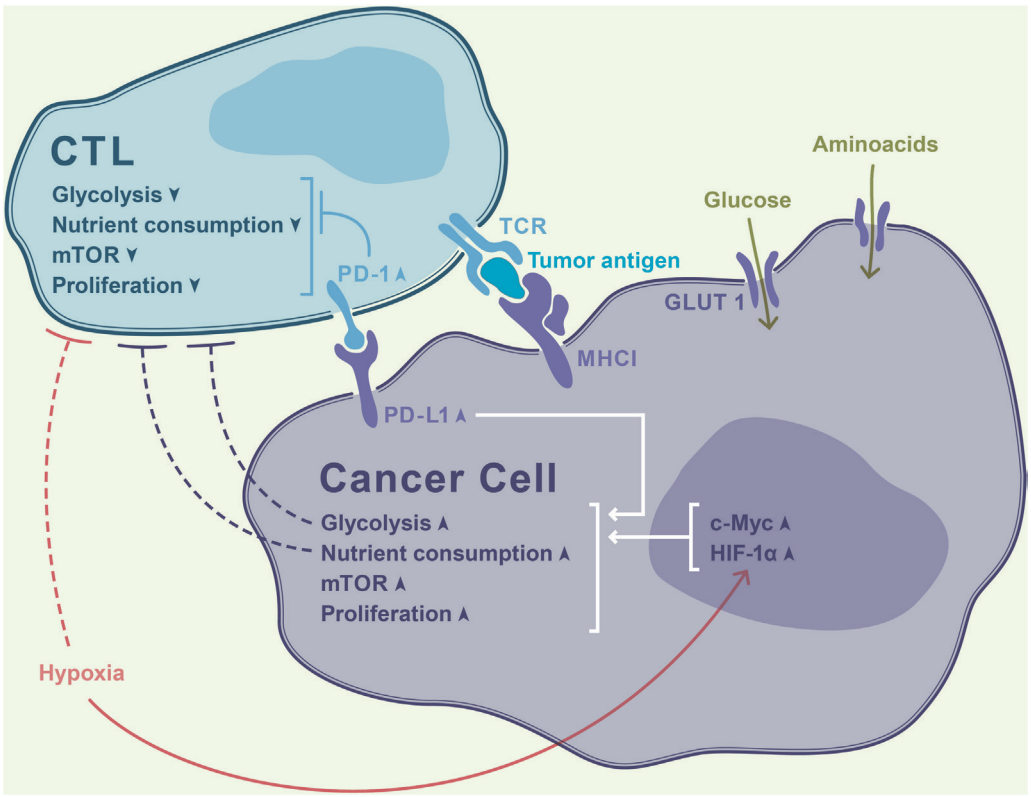

FIGURE 1 | Rapidly proliferating tumor cells avidly consume nutrients from the microenvironment, thereby outcompeting T cells in a contest for metabolic fitness. Increased activity of hypoxia-inducible factor-1α (HIF-1α) driven by tumor hypoxia further intensifies glycolysis and exacerbates the paucity of glucose in the milieu. This results in T cell deprivation of essential fuel for their effector function, resulting in a thwarted antitumor immune response. PD-1 signaling in T cells dampens their glycolytic activity and proliferation, while PD-1 ligand (PD-L1) ligation leads to opposite effects in cancer cells.

on exhausted T cells) provides metabolic support to tumor-infiltrating T cells by enhancing their mitochondrial capacity and engaging PGC1α-mediated pathways. Remarkably, 4-1BB stimulation combined with PD-1 blockade resulted in robust antitumor immunity. This study further highlights the potential of combinatorial strategies targeting immune-metabolic checkpoints for reshaping the immune-inhibitory tumor milieu.

OXYGEN SENSING

The transcription factor HIF-1α is a critical driver of effector T cell responses, which is particularly important for the differentiation of $CD4^+$ Th17 cells (37), $CD8^+$ T cell effector function (22), and interferon IFN-γ production by T regulatory cells. Besides governing the glycolytic switch in Th17 cells, HIF-1α also transactivates the gene encoding the Th17 master transcription factor RORγt (37). It has been demonstrated that HIF-1α-mediated T cell response to hypoxia upregulates the expression of Glut1, as well as glycolytic enzymes, and therefore strongly affects T cell differentiation and function. For instance, Cretenet et al. (38) demonstrated that hypoxia significantly enhances Glut1 upregulation in response to TCR stimulation. Furthermore, $Glut1^{hi}$ T lymphocytes displayed more pronounced Th1 effector phenotype and higher proliferation rate than their $Glut1^{lo}$ counterparts, both under normoxic and hypoxic conditions. Therefore, enhancing glucose uptake in adoptively transferred T cells might allow for efficiently countering hypoxia-driven immune suppression.

The activity of HIF-1a is tightly controlled by the oxygen-sensing prolyl-hydroxylase (PHD) proteins. Under normoxia, PHDs hydroxylate HIF-1α and HIF-2α, thereby targeting them for degradation. In their recent study, Restifo and colleagues (39) found that expression of PHDs in T cells ensures local tolerance for harmless antigens in the lung but markedly facilitates the seeding of circulating tumor cells. In line with their role as HIF-1α inhibitors, PHDs limited pulmonary Th1 responses, promoted Treg cell induction, and dampened $CD8^+$ T cell function. Importantly, it was shown that the effects of the PHD enzymes are primarily mediated by the repression of HIF-driven glycolytic metabolism.

Specifically, T cells stimulated in TGF-β-containing media exhibited a PHD-dependent reduction of glycolytic activity. On the other hand, PHD-deficient $CD4^+$ T cells exhibited accelerated uptake of glucose and switched to an anaerobic metabolic program. PHD proteins also restrained glycolysis in $CD8^+$ T cells. Remarkably, targeting mTOR-driven glycolytic metabolism with rapamycin and 2-deoxyglucose completely blocked spontaneous Th1 development and partially restored iTreg cell differentiation

in PHD-deficient T cells. Hence, oxygen sensing appears to coordinate transcriptional and metabolic programs driving the differentiation of Th1 and iTreg cells. Recent clinical studies have demonstrated the efficacy and overall safety of PHD inhibitors in patients with anemia and other hypoxia-driven pathologies (40). However, the complexity and the near-universal nature of the HIF pathways necessitate thorough evaluation of adverse effects. Collectively, targeting PHD proteins and other links in oxygen sensing is an alluring strategy to tilt the balance between immune activation and immune suppression in the tumor.

CONCLUSION AND FUTURE DIRECTIONS

Metabolism is integral to every biological process. The immune system largely consists of mobile cells that patrol the body and need to adapt to diverse challenging environments. This requires tight and sophisticated coordination of their bioenergetic machinery with their homeostatic pathways and effector functions. Here, we summarized the latest studies shedding light onto the specific roles of particular substrates, enzymes, and metabolic regulators in T cell differentiation and antitumor activity. Despite the startling progress that has been made in the field within just a few years, several critical questions remain unanswered. For instance, the timing and the mutual causality of the major metabolic switches during T cell differentiation remain largely elusive. Specifically, it will be crucial to capture the exact moments when differentiating T cells pass through a given metabolic checkpoint and how this affects the identity and the fate of a T cell. Likewise, further research is necessary to gain a more integrative view of how intratumoral T cells are affected by hypoxia and relentless metabolic competition imposed by cancer cells.

In the same vein, there is a pressing need to assess the therapeutic efficacy of a broader spectrum of genetic modifications targeting diverse metabolic regulators and oxygen sensors in adoptively transferred T cells. Importantly, the ubiquity of many metabolic pathways calls for careful target selection and cautious design of therapeutic regimens based on highly specific small molecule inhibitors. Overall, future immune-metabolic therapies have all the potential to make a critical difference for patients suffering from otherwise untreatable cancers.

AUTHOR CONTRIBUTIONS

IS and AB wrote this manuscript. IS generated the figures.

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