NOVEL STRATEGIES FOR CANCER IMMUNOTHERAPY: TARGETING IMMUNE-MEDIATED SUPPRESSIVE MECHANISMS

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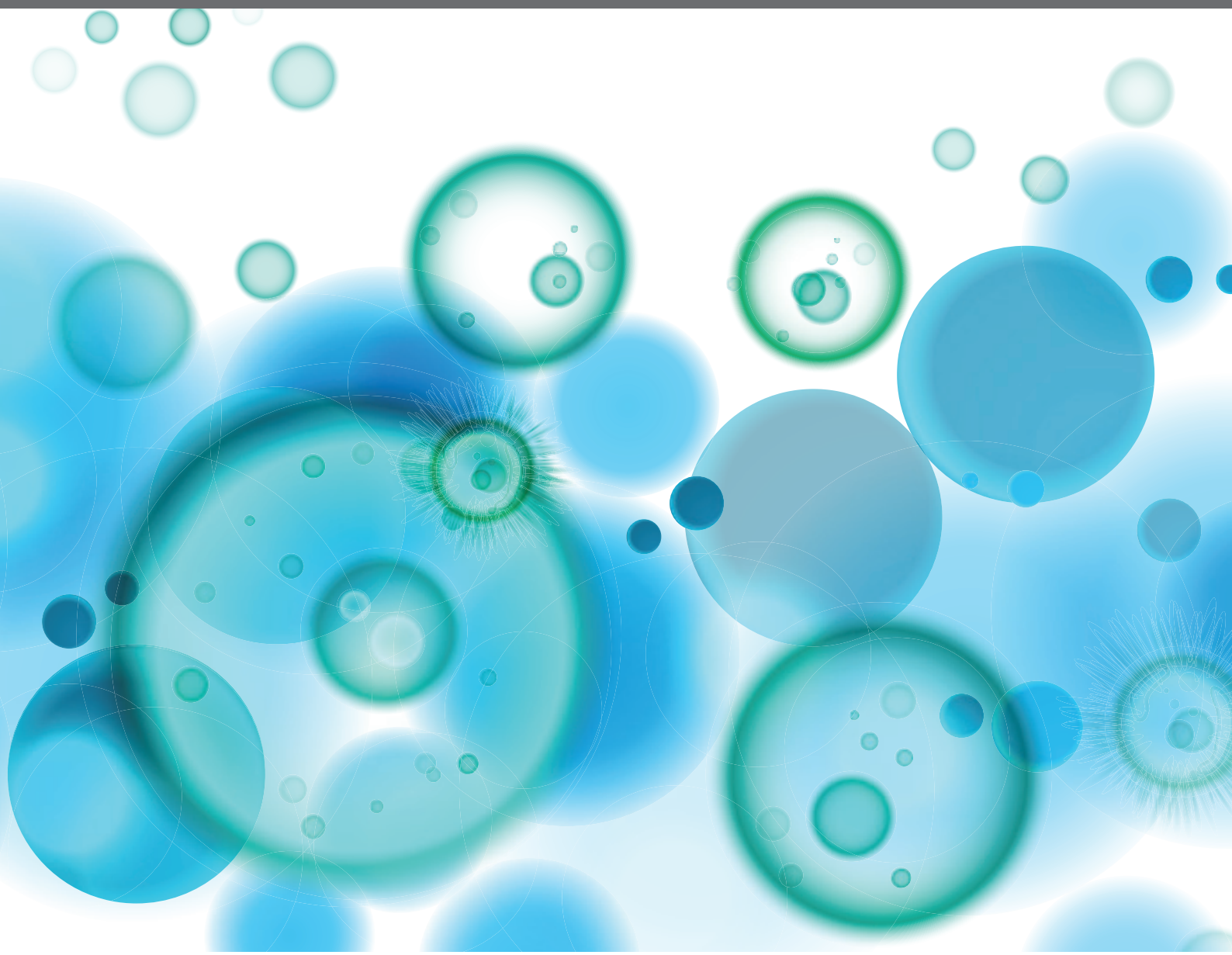

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NOVEL STRATEGIES FOR CANCER IMMUNOTHERAPY: TARGETING IMMUNE-MEDIATED SUPPRESSIVE MECHANISMS

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EDITORIAL
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Editorial: Novel Strategies for Cancer Immunotherapy: Targeting Immune-Mediated Suppressive Mechanisms

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Keywords: tumor environment, immunotherapy, immunosuppression, regulatory cells, tumor escape

Editorial on the Research Topic

Novel Strategies for Cancer Immunotherapy: Targeting Immune-Mediated Suppressive Mechanisms

Over the past decade, novel forms of cancer therapies, collectively referred to as cancer immunotherapies, have emerged and yielded spectacular results, unfortunately in what still remains to date a minority of cancer patients. Immunotherapies mobilize the immune system to promote or restore effective anti-tumor immune responses. Pioneer approaches targeting the CTLA-4/B7 and PD1/PDL1 immune checkpoints (so-called Immune Checkpoint Inhibitors or ICIs) have now broad clinical applications. Although ICIs often offer durable benefits, complete or nearly complete tumor responses occur in only a minority of cases, and resistance is observed in a substantial fraction of patients. Primary or acquired resistance to ICIs are common, with predictive markers of efficacy or resistance remaining difficult to identify. Major efforts are currently made to identify new alternative or complementary targets that activate, unleash or enhance antitumor immune responses.

In this context, developing strategies to target the immunosuppressive tumor microenvironment (TME) is of utmost importance. Current challenges are to decipher at molecular and cellular levels the immunosuppressive mechanisms that most significantly contribute to primary or acquired resistance of cancer cells to anti-tumor immune responses, and provide proof-of-concept that targeting these mechanisms exerts therapeutic anti-tumor activity. A better understanding of the interplay between cancer cells and immune cells is required to develop novel strategies to improve the outcome and increase the proportion of patients responding to cancer immunotherapy. For example regulatory T cells (Tregs), which are important modulators of adaptive immunity and are indispensable to maintain self-tolerance, are well-known to favor local immunosuppression within tumors. Nevertheless, due to the diversity of the immunosuppressive mechanisms used by these cells to exert their functions, the multiplicity of the various Treg subpopulations identified to date, and the existence of many other cell types endowed with immunosuppressive functions, original investigations are required to identify and better define novel therapeutic targets that could help to achieve effective anti-tumor immunity.

The aim of this special issue is to provide an overview of the immunosuppressive mechanisms that seem to prevail within the TME as well as the identification of novel, therapeutically targetable immunosuppressive mechanisms. The various articles illustrate how far the field has advanced, but also remind us of the extent of its complexity.

Thus, in this issue Guo et al. start by reviewing the state-of-the-art on clinical studies and patent applications for PD1/PD-L1 targeted therapies, discussing advantages and disadvantages of new classes of PD1/PD-L1 interaction inhibitors.

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Next, many articles concentrate on immune evasion mechanisms as potential new predictive biomarkers of resistance to immunotherapy or targets for novel combination strategies.

Two review articles focus on immunosuppressive cell subsets found in the TME. Chabab et al. describe the mechanisms of action and the role of regulatory γδ T cells, whereas Ngiow and Young summarize key approaches to target immunosuppressive cells such as Tregs and tumor associated macrophages to induce changes in the TME and reinvigorate anti-tumor immune responses.

Metabolic reprogramming of cancer cells is recognized as a well-established hallmark of cancer (1), and emerges as a key immunosuppressive mechanism of the anti-tumor immune response. This topic is reviewed by Mathew and Torres, focusing on the role of lysophosphatidic acid as an inflammatory lipid that binds to the LPA5 receptor on CD8 T cells, as well as by Jacobs et al., describing how 5'-deoxy-5'-methylthioadenosine, an oncometabolite often present in TME, impairs NK cell activity and function.

Several articles or case reports bring interesting data providing rationale for novel combinations to increase ICI efficacy. Guida et al. describe the potential of combining a somatostatin analog with checkpoint inhibitor immunotherapy in Merkel-cell Carcinoma. Qu et al. report that when combined with IL-36, anti-CTLA-4 mAbs increase the proliferation and IFN-γ production by CD4+ and CD8+ T cells and reduce lung metastasis in murine mammary carcinoma, by comparison to single therapies. Burke et al. report benefits of combining histone deacetylase inhibitors with ICIs in bladder cancer. They show that the inhibition of histone deacetylase renders bladder cancer cells more visible, recognizable and destructible by T cells. Ireland et al. investigate the effects of stromal GAS6 protein in pancreatic ductal adenocarcinoma and showed that it alters cancer cell plasticity, activates NK cells and inhibits pancreatic cancer metastasis. Finally, a case report by Shui et al. describes durable responses and tolerance to a triple combination with a novel PD-1 inhibitor plus two chemotherapy agents (Gemcitabine and nab-paclitaxel) in metastatic pancreatic ductal adenocarcinoma. Novel combinations to increase response rates to ICIs are also discussed in three reviews. In the first, Chuang et al. describe the mechanism of action and impact of a monotherapy with TLR-9 agonists and discuss the rationale and the current status to combine them with ICIs as a novel strategy to treat cancer. In a second review, Skeate et al. discuss the effects of LIGHT (Tumor necrosis factor superfamily member 14) delivered or expressed in tumors on the vascular reorganization and generation of tertiary lymphoid structures, and how this can greatly improve immunotherapeutic strategies against cancer. In a third review, Goruganthu et al. discuss the effects of Notch ligand receptor recruitment on anti-tumor immune responses, as well as major clinical and preclinical findings that highlight the potential therapeutic activity of targeting this pathway.

Finally, mining GEO and TCGA databases, Li et al. identify an immune risk signature that could serve as a predictor of survival and immune activity in colon cancer.

Thanks to this collection of articles, this issue provides insightful perspectives on immunosuppressive mechanisms at play in the TME, their impact on anti-tumor immunity, and the identification of novel targets for the immunotherapy of cancer.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publications. All authors contributed to the article and approved the submitted version.

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REFERENCE

1. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013

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ORIGINAL RESEARCH
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Blockade of Stromal Gas6 Alters Cancer Cell Plasticity, Activates NK Cells, and Inhibits Pancreatic Cancer Metastasis

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Pancreatic ductal adenocarcinoma (PDA) is one of the deadliest cancers due to its aggressive and metastatic nature. PDA is characterized by a rich tumor stroma with abundant macrophages, fibroblasts, and collagen deposition that can represent up to 90% of the tumor mass. Activation of the tyrosine kinase receptor AXL and expression of its ligand growth arrest-specific protein 6 (Gas6) correlate with a poor prognosis and increased metastasis in pancreatic cancer patients. Gas6 is a multifunctional protein that can be secreted by several cell types and regulates multiple processes, including cancer cell plasticity, angiogenesis, and immune cell functions. However, the role of Gas6 in pancreatic cancer metastasis has not been fully investigated. In these studies we find that, in pancreatic tumors, Gas6 is mainly produced by tumor associated macrophages (TAMs) and cancer associated fibroblasts (CAFs) and that pharmacological blockade of Gas6 signaling partially reverses epithelial-to-mesenchymal transition (EMT) of tumor cells and supports NK cell activation, thereby inhibiting pancreatic cancer metastasis. Our data suggest that Gas6 simultaneously acts on both the tumor cells and the NK cells to support pancreatic cancer metastasis. This study supports the rationale for targeting Gas6 in pancreatic cancer and use of NK cells as a potential biomarker for response to anti-Gas6 therapy.

Keywords: Gas6, pancreatic cancer, metastasis, macrophages, fibroblasts, NK cells

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INTRODUCTION

Growth arrest-specific gene 6 (Gas6) is a multifunctional factor that regulates several processes in normal physiology and pathophysiology (1). Gas6 binds to the Tyro3, Axl, and Mer (TAM) family of receptor tyrosine kinases (TAM receptors) with the highest affinity for Axl (2). Gas6 supports erythropoiesis, platelet aggregation, angiogenesis, efferocytosis and inhibits the immune response (3). Gas6 is critical for the maintenance of immune homeostasis and mice deficient in Gas6 or TAM receptors experience severe autoimmune diseases (4). Gas6 and its main receptor Axl are overexpressed in several cancer types including, breast, ovarian, gastric, glioblastoma, lung, and pancreatic cancer and their expression correlates with a poor prognosis (5). Axl is ubiquitously expressed in all tissues (6) but is particularly notable in cancer cells, macrophages, dendritic cells and natural killer cells for its role in driving immunosuppression and tumor progression (7–9). Several cancer studies have focused on the role of Gas6-Axl signaling on the tumor cells and have demonstrated that Axl activation supports tumor cell proliferation, epithelial-mesenchymal

transition (EMT), drug resistance, migration and metastasis (5). Factors secreted within the tumor microenvironment are able to sustain Gas6/Axl signaling. Hypoxia Inducible Factor (HIF) has been shown to bind to the *Axl* promoter region and upregulate its expression on renal cell carcinoma cells (10). Secretion of IL-10 and M-CSF by tumor cells induces tumor associated macrophages to secrete Gas6 (11). However, only a few studies have investigated the role of Gas6-Axl signaling in the immune response to breast cancer, ovarian cancer and melanoma (7, 9).

In solid tumors such as breast or pancreatic cancer, the tumor stroma can represent up to 80% of the tumor mass and actively influences cancer progression, metastasis (12–14) and resistance to therapies (15–17).

Pancreatic ductal adenocarcinoma (PDA) is one of the most lethal cancers worldwide and better therapies are urgently needed (18). Metastasis, therapy resistance, and immunosuppression are key characteristics of pancreatic tumors (19, 20). The Gas6–Axl pathway is activated in 70% of pancreatic cancer patients (21) and is associated with a poor prognosis and increased frequency of distant metastasis (22). Blocking Gas6-Axl signaling inhibits cancer progression (23, 24) and several Axl inhibitors and warfarin (a vitamin K antagonist that blocks Gas6 signaling) are currently being tested in cancer patients, including PDA patients. While the cancer cell autonomous functions of Gas6 are well-documented, the effect of Gas6 signaling in the stroma/immune compartment in pancreatic cancer has not been fully explored. In these studies, we sought to understand the effect of Gas6 blockade in both the tumor and the stroma/immune compartments, *in vivo,* in pancreatic cancer. Gaining a better understanding of how blockade of Gas6 signaling affects pancreatic cancer is important because it will help design and interpret the results of the recently launched clinical trials that are testing anti-Gas6/TAM receptors therapies in pancreatic cancer patients (25).

RESULTS

Pharmacological Blockade of Gas6 Inhibits Spontaneous Pancreatic Cancer Metastasis

To investigate the effect of Gas6 blockade in pancreatic cancer growth and metastasis, we used an orthotopic syngeneic pancreatic cancer model, in which pancreatic cancer cells derived from the gold standard genetic mouse model of pancreatic cancer (LSL-$Kras^{G12D}$; LSL-$Trp53^{R172H}$; Pdx1-Cre mice; KPC model), transduced with a reporter lentivirus expressing zsGreen/luciferase, were orthotopically implanted into the pancreas of syngeneic immuno-competent mice. This model faithfully recapitulates features of the human disease, and tumors are highly infiltrated by macrophages and are rich in fibroblasts (16, 26, 27). Importantly, pancreatic tumors from this mouse model also showed expression and activation of Axl receptor (**Supplementary Figure 1A**). These mice were then treated with isotype control IgG antibody or an anti-Gas6 neutralizing antibody (**Figure 1A**). This anti-Gas6 neutralizing antibody has previously been shown to block Gas6 signaling through the AXL receptor to a similar extent as an anti-AXL antibody (28). Thirty days after implantation, pancreatic tumors, lungs, livers, and mesenteric lymph nodes were surgically removed and analyzed. As expected, control treated mice showed high levels of Axl receptor activation in tumors, whereas the anti-Gas6 treated group showed markedly reduced levels of Axl receptor activation, confirming that anti-Gas6 antibody has reached the tumor and has blocked Axl signaling (**Supplementary Figures 1A,B**). No differences were seen in primary pancreatic tumor growth (**Figure 1B**) between the control and anti-Gas6 treatment groups. However, mice treated with the anti-Gas6 antibody showed reduced metastasis to lungs, livers, and mesenteric lymph nodes, compared to control treated mice, as assessed by biolumiscence *ex-vivo* imaging of these organs (**Supplementary Figures 1C,D**). Since lungs showed the highest level of metastasis in this model, lung tissues were further assessed for metastasis by H&E and cytokeratin 19 (CK19) staining. We observed that both the number of metastatic foci, as well as the size of the metastatic lesions were significantly reduced in control vs. anti-Gas6 treated mice (**Figures 1D,E**, **Supplementary Figures 1E–G**). As a consequence the overall metastatic burden was very significantly reduced in the mice treated with anti-Gas6 blocking antibody compared to control mice (**Figure 1F**). These data suggest that blockade of Gas6 affects the metastatic cascade at different stages, affecting the metastatic spreading and/or initial seeding as well as the metastatic outgrowth of disseminated pancreatic cancer cells.

Tumor Associated Macrophages and Fibroblasts Are the Main Sources of Gas6 in Pancreatic Cancer

Gas6 is a multifunctional protein that is secreted by different cell types. Gas6 has been shown to be produced by macrophages in pre-malignant lesions of a mammary tumor model (29) and in xenograft and orthotopic models of colon and pancreatic cancer (11). Gas6 can also be produced by tumor cells (30) and fibroblasts (31). To determine which cell types produce Gas6 in pancreatic tumors, tumors were harvested at day 23, and tumor cells (CD45–/zsGreen+), non-immune stromal cells (CD45–/zsGreen–), M1-like macrophages (CD45+/F4/80+/CD206–) and M2-like macrophages (CD45+/F4/80+/CD206+) were isolated by flow cytometry (**Figure 2A**, **Supplementary Figure 2A**) and analyzed for the expression of *gas6* (**Figures 2A,B**). We found that both F4/80+/CD206+ (M2-like macrophages) and αSMA+ stromal cells (**Supplementary Figure 2B**) are the main sources of Gas6 in pancreatic tumors (**Figure 2B**). *Ex-vivo*, bone-marrow derived macrophages and pancreatic fibroblasts also produce Gas6 (**Figures 2B,C**). In agreement with these findings, we observed that tumor areas with activated Axl receptor were often surrounded by TAMs and CAFs (**Figure 2D**). Analysis of Axl expression and activation in pancreatic cancer patient samples has been correlated with a poor prognosis (21, 22) and Axl activation in cancer cells has been shown to support EMT, cell proliferation, metastasis and drug resistance (5). While these studies have mainly focused on analyzing the expression and function of Axl on the cancer cells, Axl is also

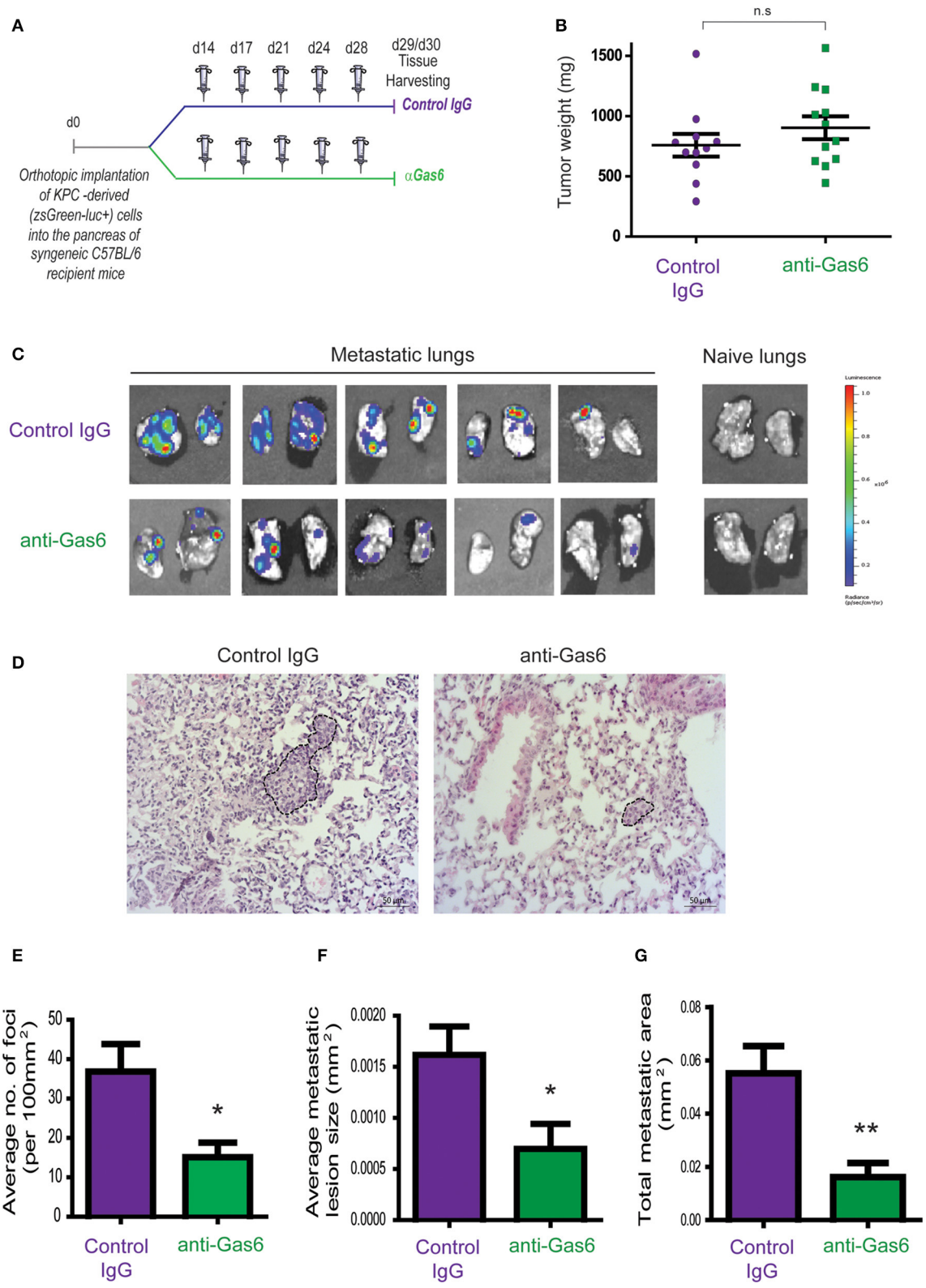

FIGURE 1 | Pharmacological blockade of Gas6 inhibits pancreatic cancer metastasis. **(A)** KPC$^{luc/zsGreen}$ (zsGreen) -derived pancreatic tumor cells (FC1242$^{luc/zsGreen}$) were orthotopically implanted into the pancreas of syngeneic C57BL/6 recipient mice, and mice were treated, starting at day 14 after tumor implantation, twice a

(Continued)

FIGURE 1 | week i.p., with either isotype control IgG antibody or Gas6 blocking antibody (2 mg/kg). Primary pancreatic tumors, livers, lungs, and mesenteric lymph nodes were harvested at day 30. **(B)** Tumor weights ($n = 11$ mice for control IgG treatment group; $n = 12$ mice for anti-Gas6 treatment groups). **(C)** Representative IVIS images of metastatic lungs from control IgG and anti-Gas6 treated mice. **(D)** Representative images of H&E staining of metastatic lungs from control IgG and anti-Gas6 treated mice. Scale bar 50 μm. **(E)** Quantification of number of lung metastatic foci per 100 mm^2 in mice treated with control IgG or anti-Gas6 antibody identified by H&E. $^*p \leq 0.05$, using unpaired student *T*-test, error bars represent SEM ($n = 7$). **(F)** Average size of pulmonary metastatic lesions in mice treated with control IgG or anti-Gas6 antibody identified by H&E. $^*p \leq 0.05$, using unpaired student *T*-test, error bars represent SEM ($n = 7$). **(G)** Quantification of total metastatic burden in mice treated with control IgG or anti-Gas6 antibody identified by H&E. $^{**}p \leq 0.01$, using unpaired student *T*-test, error bars represent SEM ($n = 7$).

expressed in immune cells, endothelial cells and stromal cells and regulates innate immunity (3, 4), angiogenesis (32–34), and fibrosis (31). In agreement with this multi-functional role for Axl, we found that Axl is activated in both the tumor and the stromal/immune compartment in biopsies from pancreatic cancer patients (**Figures 3A,B**).

Gas6 Blockade Alters EMT of Pancreatic Cancer Cells but Does Not Affect Angiogenesis or Collagen Deposition in Pancreatic Tumors

Previous studies have shown that Gas6-Axl signaling promotes tumor cells' EMT (35, 36). To determine whether the reduced metastasis observed when we block Gas6 was caused by an effect on tumor cell EMT, we evaluated the expression of EMT markers and transcription factors on tumor cells from pancreatic tumors treated with isotype control antibody or Gas6 blocking antibody. Tumor cells isolated from pancreatic tumors were analyzed for the expression of the EMT transcription factors *Snail 1, Snail 2, Twist 1, Twist 2, Zeb 1, and Zeb 2* (**Figure 4A**), the epithelial markers *E-cadherin, b-catenin,* and *Epcam* and the mesenchymal markers *Vimentin* and *N-cadherin* (**Figure 4B**). We found that blocking of Gas6 significantly decreased the expression of the EMT transcription factors *Snail 1, Snail 2,* and *Zeb 2*, while *twist 1* and *Zeb 1* levels remained unchanged and *twist 2* was not expressed in pancreatic cancer cells (**Figure 4A**). In agreement with this observation, Gas6 blockade also decreased the expression of the mesenchymal marker *Vimentin,* while *N-cadherin* levels were very low and remained unchanged. *E-cadherin* and *B-catenin* levels were also decreased though upon anti-Gas6 treatment, suggesting that Gas6 signaling partially regulates cancer cell plasticity, a phenomenon previously described in cancer (37, 38). Kirane et al. (23) previously showed that blocking Gas6 signaling with warfarin decreases vimentin expression in a xenograft model of pancreatic cancer.

To further investigate the effect of anti-Gas6 on vimentin expression in pancreatic cancer cells in our *in vivo* tumor model, we analyzed vimentin protein expression in pancreatic tumor tissues from control and anti-Gas6 treated mice (**Figures 4C,D**). We found that blockade of Gas6 partially reduces vimentin protein expression in cancer cells, although this decrease was not statistically significant.

Pancreatic tumors are usually poorly vascularized but since Gas6 signaling can support endothelial cells proliferation and vascularization (33, 39, 40) we next evaluated whether anti-Gas6 therapy could affect angiogenesis in pancreatic tumors. Pancreatic tumor tissues from control and anti-Gas6 treated mice were stained with the endothelial marker CD31. Whole tumor tissues were scanned and quantified for CD31 expression which remained unchanged in both treatment groups (**Supplementary Figures 3A,B**). Fourcot et al. (31) showed, in a liver fibrosis model, that Gas6 is secreted by macrophages and fibroblasts and that Gas6 deficiency decreases TGFb and collagen I production by hepatic fibroblasts. Gas6 also stimulates the proliferation of cardiac fibroblasts (41). Since fibrosis and collagen deposition have been suggested to re-strain the metastatic spreading of pancreatic cancer cells (42–45), we next investigated whether Gas6 blockade could affect fibroblasts and collagen deposition in pancreatic tumors. Pancreatic tumor tissues from control and anti-Gas6 treated mice were stained with picrosirius red to assess collagen deposition (**Supplementary Figures 3C,D**) and for αSMA+ cells (**Supplementary Figures 3E,F**). Whole tumor tissues were scanned and quantified for collagen deposition (Sirius red positive areas) and αSMA+ cells. We observed a slight increase in collagen deposition in tumors from mice treated with anti-Gas6 antibody compared to control but this increase was not statistically significant (**Supplementary Figures 3C,D**). αSMA levels remained the same in both treatment groups (**Supplementary Figures 3E,F**). These findings suggest that the anti-metastatic effect of Gas6 blockade in pancreatic cancer is not due to changes in angiogenesis or fibrosis.

Gas6 Blockade Does Not Affect Myeloid Cells or T Cells Populations at the Primary Tumor Site, in Peripheral Blood or at the Metastatic Site

TAM receptors are also expressed by immune cells and regulate myeloid cell and T-cell functions (3, 46). Thus, next, with the aim to understand the systemic effect of Gas6 blockade in myeloid cells and T cells in pancreatic cancer, we evaluated the number and activation status of myeloid cells and T cells in pancreatic tumors, blood and metastatic tissues using mass and flow cytometry. Mass cytometry analysis of myeloid (CD11b+) cells, neutrophils/MDSCs (CD11b+/Ly6G+), monocytes (CD11b+/Ly6C+), macrophages (CD11b+/F4/80+), MHC-II+, CD206+, and PD-L1+ macrophages (**Figure 5A**) and T cells (CD3+), helper T-cells (CD3+/ CD4+), regulatory T cells (CD3+/CD4+/CD25+), cytotoxic T cells (CD3+/CD8+), activated/exhausted cytotoxic T cells (CD8+/CD69+; CD8+/PD-1+) (**Figure 5B**) from pancreatic tumors from control vs. anti-Gas6 treated mice did not show any significant differences (**Figures 5A,B**,

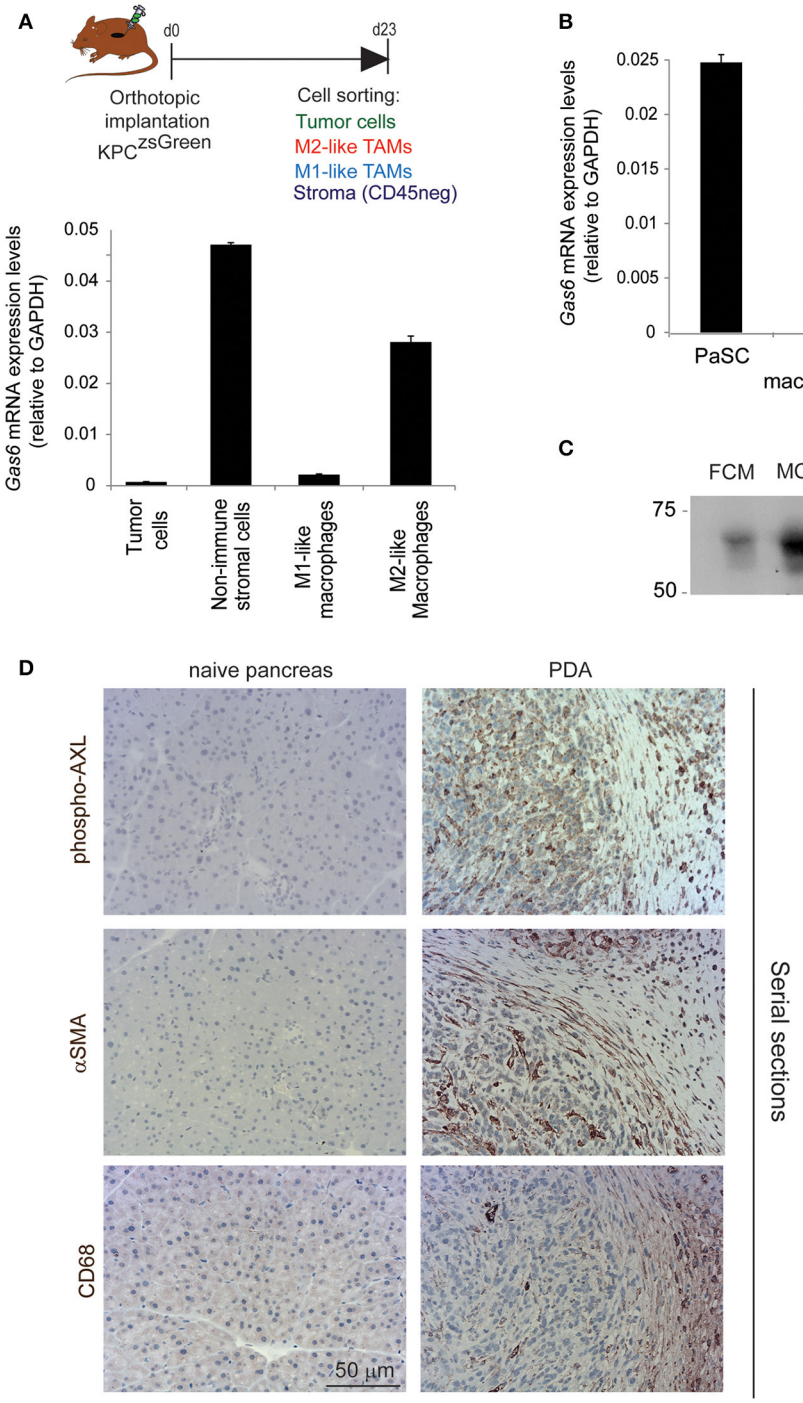

FIGURE 2 | TAMs and CAFs are the main sources of Gas6 in pancreatic tumors. **(A)** KPC$^{luc/zsGreen}$ (zsGreen) -derived tumor cells (FC1242$^{luc/zsGreen}$) were orthotopically implanted into the pancreas of syngeneic recipient (C57/BL6) mice. Tumors were harvested and digested at day 23 after implantation and tumor cells, non-immune stromal cells, M1-like and M2-like macrophages were sorted by flow cytometry. *Gas6* mRNA levels were quantified in CD45–/zsGreen+ tumor cells, CD45–/zsGreen– non-immune stromal cells, CD45+/F4/80+/CD206– M1-like macrophages and CD45+/F4/80+/CD206+ M2-like macrophages sorted by flow cytometry from murine pancreatic tumors. Values shown are the mean and SD ($n = 3$). **(B)** Quantification of *Gas6* mRNA expression levels in *ex vivo* mouse primary isolated macrophages and pancreatic fibroblasts from naïve mice. Values shown are the mean and SD ($n = 3$). **(C)** Immunoblotting analysis of Gas6 secreted protein present in mouse macrophage conditioned media (MCM) and pancreatic fibroblast conditioned media (FCM). **(D)** Images show phospho-Axl, αSMA (fibroblast marker) and CD68 (pan-macrophage marker) staining in naïve mouse pancreas and in serial sections of mouse PDA tissues. Scale bar = 50 μm.

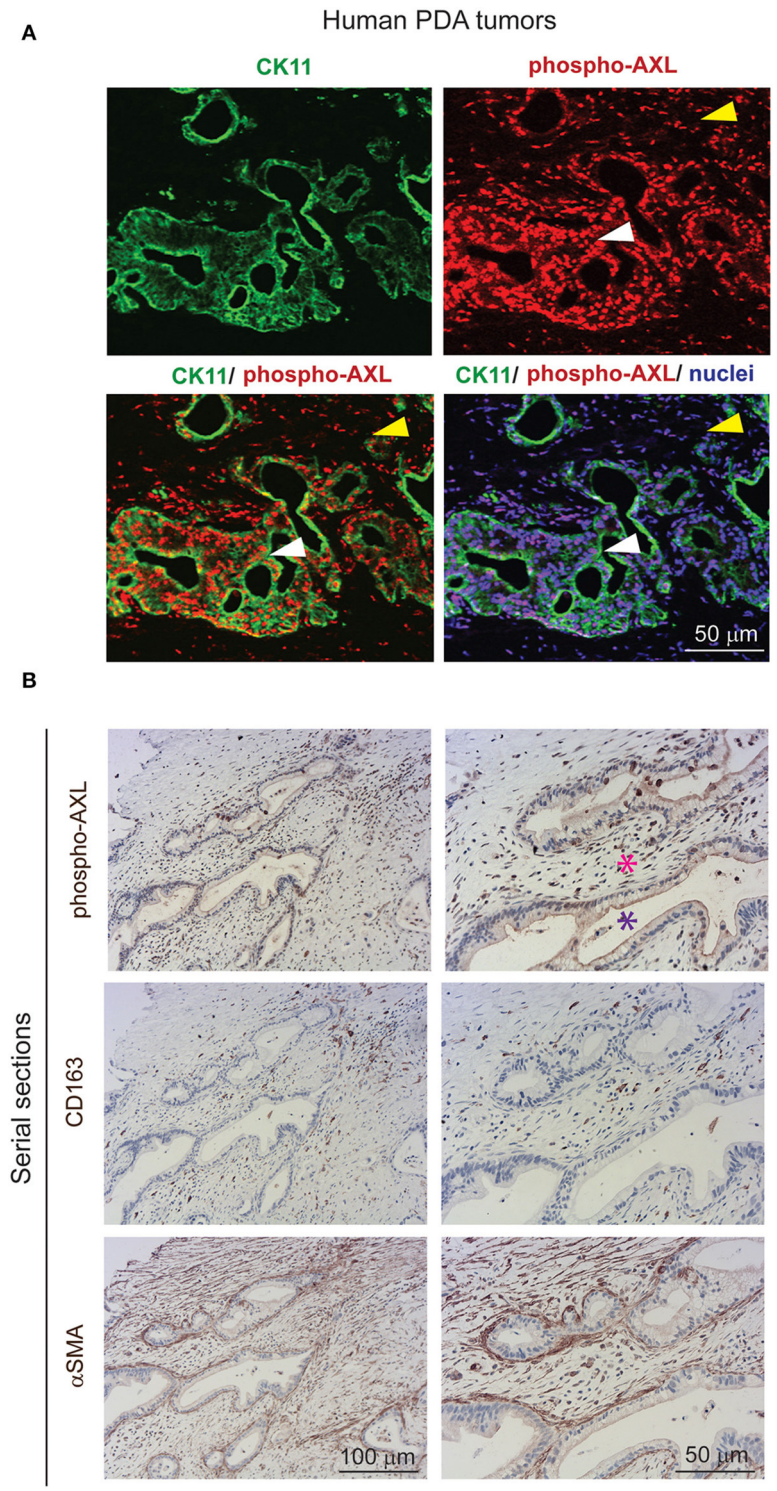

FIGURE 3 | AXL receptor is activated in both the tumor and stromal compartment in biopsies from PDA patients. **(A)** Immunofluorescent staining of human PDA biopsies with CK11 (tumor cell marker, in green), phospho-Axl receptor (in red), and nuclei (in blue). Scale bar, 50 μm. Yellow arrow indicates presence of phosphorylated Axl in the stromal compartment. White arrow indicates presence of phosphorylated Axl in the tumor cells. **(B)** Serial sections of biopsies from human PDA samples immunohistochemically stained for phospho-Axl, CD163 (macrophages) and αSMA (fibroblasts). Cancer cells are indicated by a purple asterisk and tumor stroma is indicated by a pink asterisk. Scale bars, 50 and 100 μm.

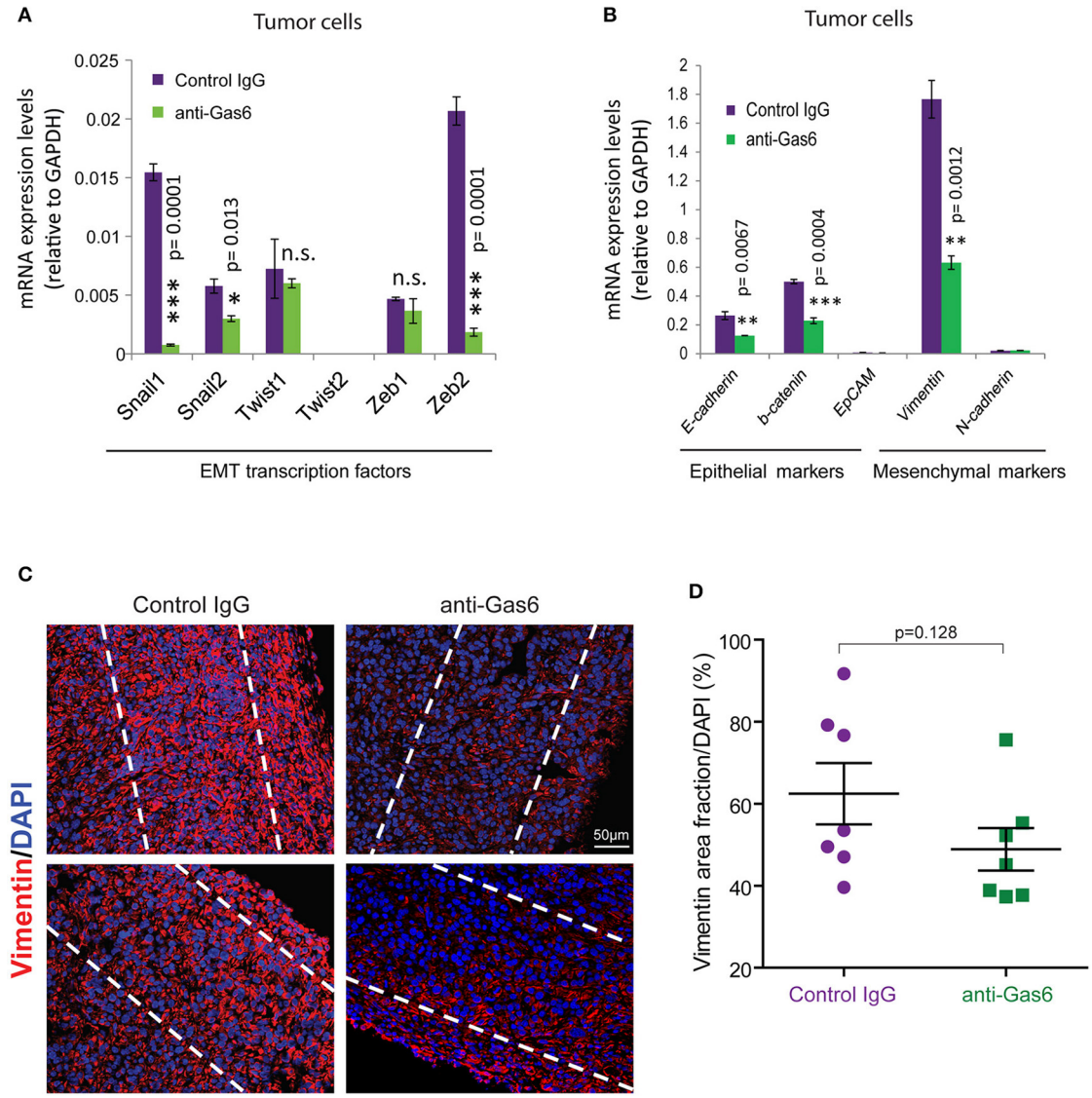

FIGURE 4 | Gas6 blockade in pancreatic tumors partially affects EMT of tumor cells. **(A)** Quantification of the expression levels of the EMT transcription factors: *Snail 1, Snail 2, Twist 1, Twist 2, Zeb 1, and Zeb 2* in tumor cells (zsGreen+) isolated by flow cytometry from mouse PDA tumors. Values shown are the mean and SD ($n = 3$). **(B)** Quantification of the expression levels of the epithelial markers: *E-cadherin, b-catenin, EpCAM,* and the mesenchymal markers *vimentin and N-cadherin* in tumor cells FACS sorted from mouse PDA tumors. Values shown are the mean and SD ($n = 3$). $^{*}p \leq 0.05$, using unpaired student *T*-test; $^{**}p \leq 0.01$, using unpaired student *T*-test; $^{***}p \leq 0.005$, using unpaired student *T*-test. **(C)** Representative immunofluorescent images of vimentin staining at the periphery of mouse pancreatic tumors treated with control IgG or anti-Gas6 antibody. The dashed lines highlight the areas quantified in the tumor tissues. **(D)** Quantification of vimentin protein expression levels in pancreatic cancer cells. Data are displayed as mean and SEM and represent 5 images per mouse, with 7 animals per treatment group. n.s. no statistically significant differences, using unpaired student *T*-test.

Supplementary Figures 4A,B). Similarly, myeloid cell and T cell numbers in blood (**Supplementary Figures 5A,B**) and metastatic lungs from mice treated with control or anti-Gas6 antibody remained the same (**Supplementary Figures 6A,B**).

Gas6 Blockade Restores NK Cell Activation and Infiltration in Metastatic Lesions

TAM signaling is involved in the development of natural killer (NK) cells (47). In an elegant study, Paolino et al. (9) demonstrated that TAM receptor inhibition activates NK cells cytotoxic function and thereby decreases metastasis in mouse models of breast cancer and melanoma. Thus, we next hypothesized that the anti-metastatic effect of Gas6 blockade we observe in our pancreatic cancer model could be due to a re-activation of NK cells. To test this hypothesis we evaluated NK cells in primary pancreatic tumors, tumor draining lymph nodes, and metastatic lesions of mice treated with control IgG or anti-Gas6 antibody. NK cells were almost absent in all primary tumors

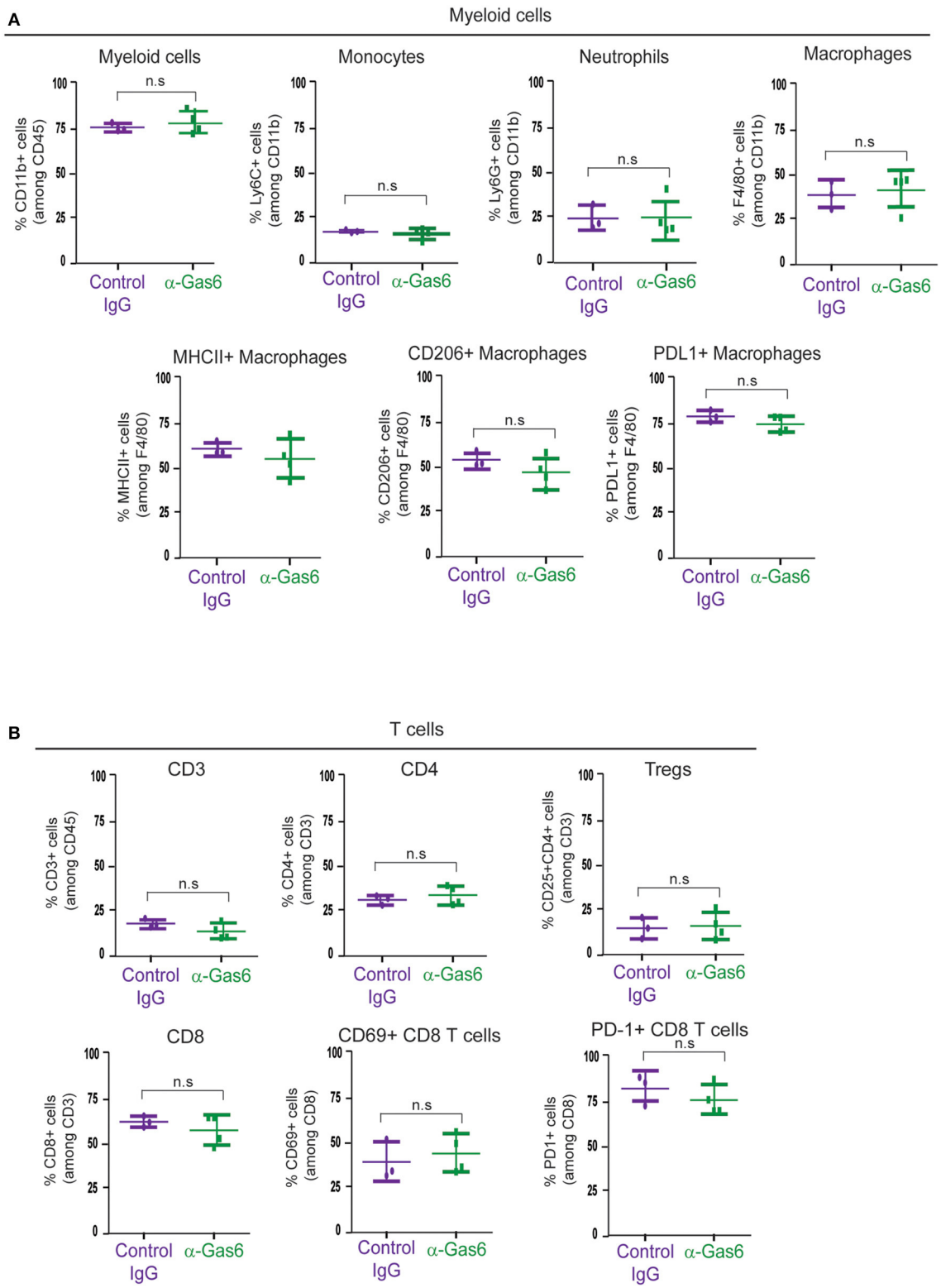

FIGURE 5 | Gas6 blockade does not affect the composition or activation status of myeloid cells and T cells in pancreatic tumors. **(A)** Mass cytometry quantification of CD11b + myeloid cells, Ly6C high/Ly6C low monocytes/MDSCs, Ly6G high/Ly6C low neutrophils/MDSCs, F4/80+ macrophages, MHCII+ macrophages,

(Continued)

FIGURE 5 | CD206+ macrophages, and PD-L1+ macrophages in mouse pancreatic tumors treated with control IgG ($n = 3$) or anti-Gas6 neutralizing antibody ($n = 4$). Values shown are mean and SEM. n.s. no statistically significant differences, using unpaired student *T*-test. **(B)** Mass cytometry quantification of CD3+ T cells, CD4+ T cells, CD4+/CD25+ regulatory T cells (Tregs), CD8+ T cells, CD69+/CD8+ T cells and PD-1+/CD8+ T cells in mouse pancreatic tumors treated with control IgG ($n = 3$) or anti-Gas6 neutralizing antibody ($n = 4$). Values shown are mean and SEM. n.s. no statistically significant differences, using unpaired student *T*-test. Graphs were generated with ViSNE data using Cytobank software.

from both anti-Gas6 and control treated mice (except for one anti-Gas6 treated pancreatic tumor, **Supplementary Figure 7**). However, the number of NKp46+ NK cells in lung metastatic lesions was significantly higher in mice treated with anti-Gas6 antibody compared to control treated mice (**Figures 6A,B**). The number of NK cells, and in particular the number of proliferating NK cells, was also increased in tumor draining lymph nodes from anti-Gas6 treated mice compared to control treated mice (**Figures 6C,D**).

To further investigate the effect of inhibiting Gas6-Axl signaling in pancreatic cancer progression and metastasis, we performed another *in vivo* experiment, using our syngeneic orthotopic KPC model (described in **Figure 1**) using warfarin (instead of a neutralizing anti-Gas6 antibody). Warfarin is a vitamin K antagonist that inhibits the vitamin k dependent γ-carboxylation of Gas6 and prevents it from activating TAM receptors (23, 48). Warfarin is currently being tested in pancreatic cancer patients (NCT03536208). Similar to what we observed with the anti-Gas6 treatment, warfarin reduced pancreatic cancer metastasis to the lungs (**Figures 7C,D**, **Supplementary Figures 8A–C**) and increased the number and activation of NK cells in lungs (**Figures 7E,F**) and mesenteric lymph nodes (**Figures 7G,H**), as shown by the increase in NKp46+ and granzyme B expression. Warfarin treatment also decreased vimentin expression in pancreatic cancer cells, suggesting that warfarin also acts on the cancer cells altering their plasticity (**Supplementary Figures 8D,E**).

DISCUSSION

The data presented in this study describe a dual anti-tumor effect of Gas6 blockade in pancreatic tumors, shedding light on the anti-cancer mechanism of action of inhibitors of the Gas6-Axl pathway and supporting the rationale for using anti-Gas6 therapy in pancreatic cancer patients. In these studies we show that blockade of Gas6 in pancreatic tumors, with either an anti-Gas6 neutralizing antibody or with warfarin, acts simultaneously on both the tumor cells, altering their epithelial-mesenchymal phenotype, as well as on NK cells, promoting their activation and recruitment to the metastatic site (**Figures 6**, **7**). These findings suggest that anti-Gas6 therapy decreases pancreatic cancer metastasis by not only affecting cancer cells' plasticity but also by activating NK cells and supporting their tumoricidal function.

So far many studies have focused on the cancer-cell autonomous role of Gas6 and based on their effect on tumor cell proliferation and plasticity several inhibitors of the Gas6-Axl pathways, including warfarin (clinical trial ID: NCT03536208) are currently being tested in pancreatic cancer patients.

Our studies show that inhibition of Gas6 signaling in pancreatic cancer not only affects the tumor cells but notably affects the NK cells (**Figure 8**). Our findings suggest that the activation status of NK cells should also be assessed in cancer patients and could be used as a biomarker to monitor response to anti-Gas6/Axl therapies.

Gas6/Axl signaling is a negative regulator of the immune system and inhibition of the Gas6-Axl signaling leads to autoimmunity (4). While the function of Gas6-Axl signaling on tumor cell proliferation, EMT, migration and drug resistance has been extensively studied (5), only a few studies have investigated the role of Gas6/Axl signaling in the immune system in the context of cancer (7, 9, 24). Guo et al. (7) found that the Axl inhibitor R428 inhibited tumor growth of subcutaneously implanted murine 4T1 breast cancer cells and intra-peritoneally implanted murine ID8 ovarian cancer cells by activating CD4+ and CD8+ T cells. Inspired by this study, we investigated whether, in our pancreatic cancer model, Gas6 blockade supports the activation of T cells. Unlike Guo et al. (7) we did not observe any statistically significant difference in CD4+ or CD8+ T cells in pancreatic tumors, blood or metastatic tissues, in control vs. anti-Gas6 treated mice. Ludwig et al. (24) found that treating mouse pancreatic tumors with the Axl inhibitor BGB324 decreased the number of tumor associated macrophages (TAMs) in some but not all tumor models. In our study, blocking Gas6 did not significantly affect TAMs, other myeloid cell populations or T cells in primary tumors, blood or metastatic organs. These different results observed in these studies may be explained by the differences in the tumor models used (breast cancer vs. pancreatic cancer; xenograft vs. syngeneic models) and the differences in the therapies used (inhibition of AXL receptor vs. inhibition of Gas6 ligand which binds all TAM receptors). In another study, Paolino et al. (9) showed that TAM receptor inhibition activates NK cells in mouse tumor models of melanoma and breast cancer leading to decreased tumor growth. In agreement with these findings, we found that blocking Gas6 in mice bearing pancreatic tumors, increases NK cell number and activation in tumor draining lymph nodes and lungs, and decreases pancreatic cancer metastasis.

Inhibition of the Gas6-Axl pathway has been shown to reverse EMT, tumor migration and intra-tumoral micro-vessel density in pancreatic cancer (23). In agreement with these findings, we found that inhibition of Gas6 signaling decreases the expression of the EMT transcription factors *Snail 1, Snail 2, Zeb2,* and vimentin expression in pancreatic cancer cells. *E-cadherin* and *b-catenin* levels were also decreased upon anti-Gas6 treatment suggesting that blockade of Gas6 signaling leads to a partial MET or hybrid E/M phenotype. Partial EMT is a phenomenon often observed in cancer, where cancer cells that originate from epithelial cells exhibit both mesenchymal and epithelial

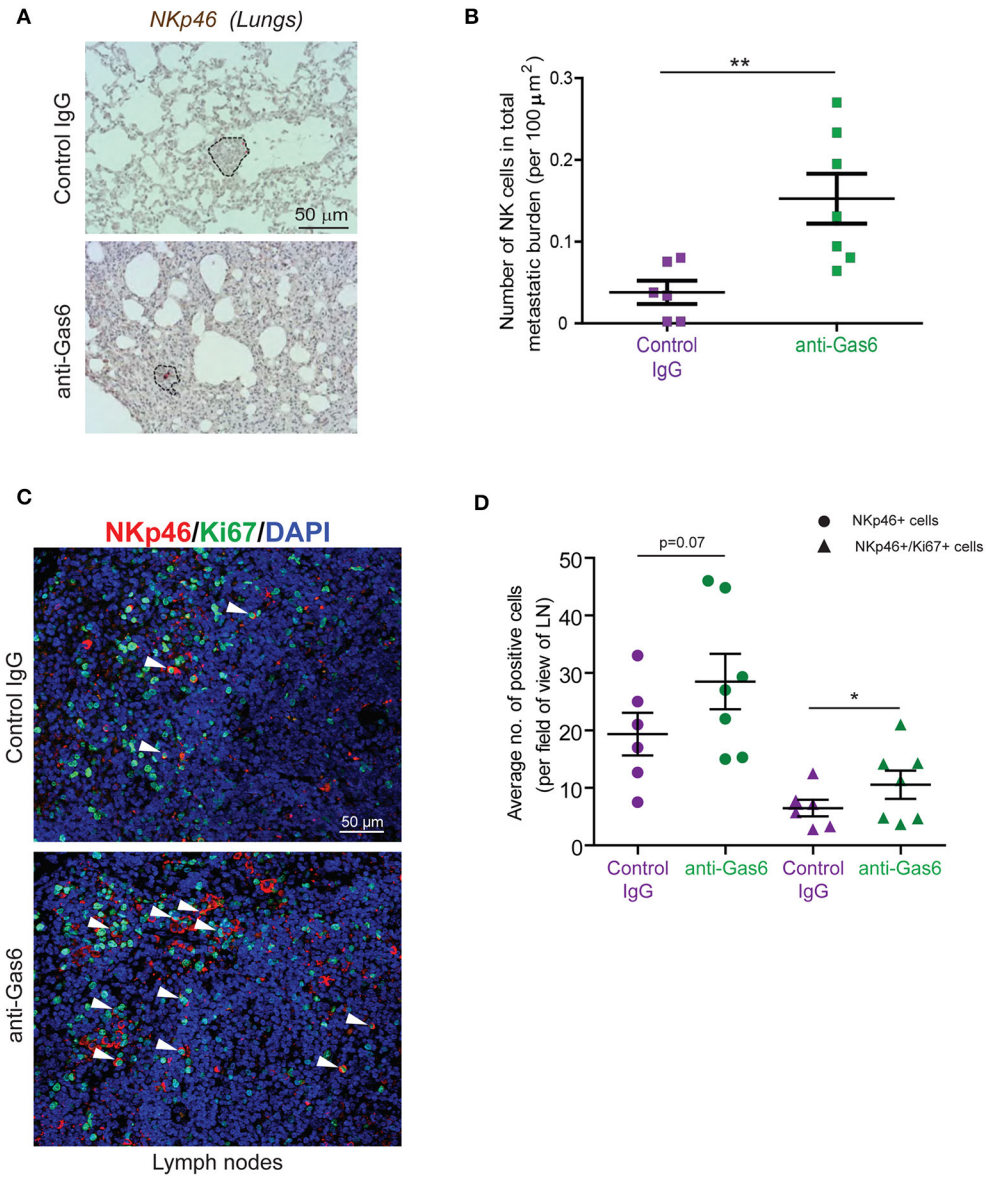

FIGURE 6 | Gas6 blockade increases NK cell numbers in metastatic lungs and in tumor draining lymph nodes. **(A)** Immunohistochemical staining of NK cells in metastatic lungs from pancreatic tumor bearing mice treated with control IgG or anti-Gas6 antibody. Lesions indicated by dashed line and NK cells by red asterisk. Scale bar, 50 μm. **(B)** Quantification of NK cells in metastatic lung tissues from control IgG and anti-Gas6 treated mice. Values shown are the mean and SEM ($n = 6$ mice in IgG treatment group, $n = 7$ mice in anti-Gas6 treatment group). $^{**}p \leq 0.01$, using unpaired student T-test. **(C)** Immunofluorescent staining of NK cells in mesenteric lymph nodes from pancreatic tumor bearing mice treated with control IgG or anti-Gas6 antibody. NK marker NKp46 is shown in red, Ki67 is shown in green and nuclei were stained with DAPI (in blue). Scale bar, 50 μm. **(D)** Quantification of NK cells in tumor draining lymph nodes from control IgG and anti-Gas6 treated mice. Values shown are the mean and SEM ($n = 6$ mice IgG treatment group and $n = 7$ mice anti-Gas6 treatment group, 3–6 fields/ mouse tissue were quantified). $^{*}p \leq 0.05$, using unpaired student T-test.

characteristics. The ability of cancer cells to undergo partial EMT, rather than complete EMT and to maintain the expression of both E-cadherin and vimentin poses a higher metastatic risk (37, 44). Pancreatic tumors are usually hypo-vascularized compared to a normal pancreas and anti-angiogenic therapies have not been successful in pancreatic cancer (49). Similar to the human disease, in our pancreatic mouse tumor model, tumors are poorly vascularized and blocking Gas6 did not show any further decrease in tumor vascularization. Loges et al. (11) previously showed that tumor associated macrophages (TAMs) produce Gas6 in various mouse tumor models. In our study we found that both TAMs and CAFs are the main sources of Gas6 in pancreatic tumors. These findings suggest that the abundance of TAMs and CAFs in pancreatic cancer patients could be

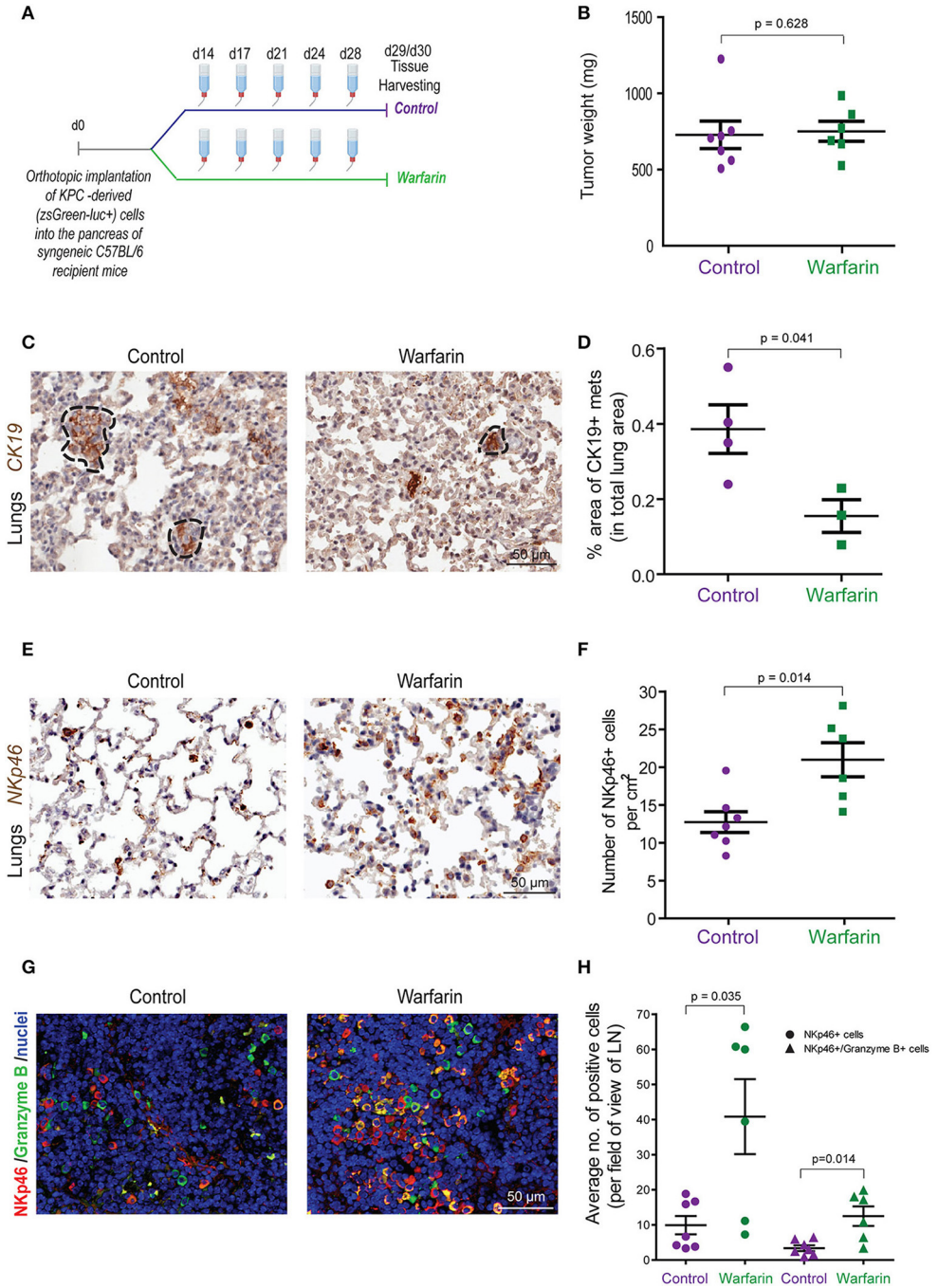

FIGURE 7 | Warfarin decreases pancreatic cancer metastasis and increase NK cell numbers and activation in lymph nodes and at the metastatic site. **(A)** KPC$^{luc/zsGreen}$ (zsGreen) -derived pancreatic tumor cells (FC1242$^{luc/zsGreen}$) were orthotopically implanted into the pancreas of syngeneic C57BL/6 recipient mice. At day 14 the mice were treated with either control drinking water or warfarin sodium in drinking water (0.5 mg/L). Warfarin water was replenished every 3–4 days. Primary tumors, livers, lungs and lymph nodes were harvested at day 29/30. **(B)** Tumor weights from control ($n = 7$) or warfarin ($n = 6$) treated mice. **(C)** Immunohistochemical staining of CK19+ in mice with lung metastases. **(D)** Quantification of the total area of lung metastasis per mouse as a percentage of the total lung area for control ($n = 4$) or warfarin ($n = 3$) treated mice. $^*p \leq 0.05$ using unpaired student T-test. Values shown are mean and SEM. **(E)** Immunohistochemical staining of NKp46+ NK cells in the lungs from pancreatic tumor bearing mice. **(F)** Quantification of the number of NKp46+ NK cells per cm^2 in the lungs of control ($n = 7$) or warfarin ($n = 6$) treated mice. **(G)** Immunoflourescence staining of NK cells in the mesenteric lymph nodes from pancreatic tumor bearing mice. The NKp46 marker is shown in red, granzyme B is shown in green and the nuclei were stained with DAPI (in blue). Scale bar 50 µm. **(H)** Quantification of the number of NKp46+ and granzyme b+ cells in tumor draining lymph nodes for control ($n = 7$) and warfarin ($n = 6$) treated mice. Values shown are the mean and SEM and 3-10 fields/mouse tissue were quantified.

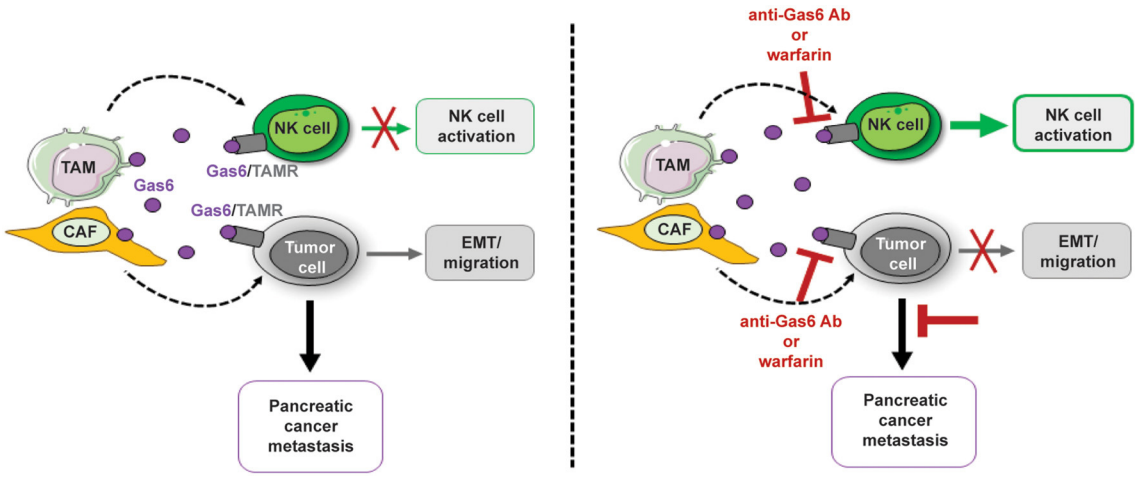

FIGURE 8 | Schematics depicting the multifunctional role of stroma-derived Gas6 in pancreatic cancer. *In vivo* blockade of Gas6 signaling with a neutralizing anti-Gas6 antibody or warfarin, partially reverses tumor cells EMT and activates NK cells, leading to a decrease in pancreatic cancer metastasis.

used to determine which patients would benefit the most from anti-Gas6 therapy.

In conclusion, our studies suggest that in pancreatic cancer, Gas6 is secreted by both TAMs and CAFs and blockade of Gas6 signaling has a dual anti-metastatic effect by acting on both the tumor cells and the NK cells. Thus, inactivation of Gas6 signaling can promote anti-tumor immunity, via NK cell activation, in pancreatic tumors. Since this Gas6-dependent immune regulation of NK cells is also conserved in humans, anti-Gas6-Axl therapies are likely to promote anti-tumor immunity, via NK cell activation, in pancreatic cancer patients. This study provides further mechanistic insights into the mode of action of anti-Gas6 therapies and suggests the use of NK cells as an additional biomarker for response to anti-Gas6 therapies in pancreatic cancer patients.

MATERIALS AND METHODS

Generation of Primary KPC-Derived Pancreatic Cancer Cells

The murine pancreatic cancer cells KPC FC1242 were generated in the Tuveson lab (Cold Spring Harbor Laboratory, New York, USA) isolated from PDA tumor tissues obtained from LSL-Kras^{G12D}; LSL-Trp53^{R172H}; Pdx1-Cre mice of a pure C57BL/6 background as described previously with minor modifications (50).

Generation of Primary Macrophages, Primary Pancreatic Fibroblasts, Macrophage (MCM, and Fibroblasts (FCM) Conditioned Media

Primary murine macrophages were generated by flushing the bone marrow from the femur and tibia of 6–8 week-old C57BL/6 mice followed by incubation for 5 days in DMEM containing 10% FBS and 10 ng/mL murine M-CSF (Peprotech). Primary pancreatic stellate cells were isolated from the pancreas of C57BL/6 mice by density gradient centrifugation, and were cultured on uncoated plastic dishes in IMDM with 10% FBS and 4 mM L-glutamine. Under these culture conditions pancreatic stellate cells activated into myofibroblasts.

To generate macrophage and fibroblast conditioned media, cells were cultured in serum free media for 24–36 h, supernatant was harvested, filtered with 0.45 μm filter, concentrated using StrataClean Resin (Agilent Technologies) and immunoblotted for Gas6 (R&D Systems, AF885).

Immunoblotting

FC1242 cells were plated in DMEM media with 10% FBS for 24 h, harvested and lysed in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.2, 0.1% SDS, 1% Triton X-100, 5 mM EDTA) supplemented with a complete protease inhibitor mixture (SIGMA), a phosphatase inhibitor cocktail (Invitrogen), 1 mM PMSF and 0.2 mM Na_3VO_4. Immunoblotting analyses was performed using phospho-Axl antibody (R&D systems, AF2228).

Syngeneic Orthotopic Pancreatic Cancer Model

1×10^6 primary KPC$^{luc/zsGreen}$ cells (FC1242$^{luc/zsGreen}$) isolated from a pure C57Bl/6 background were implanted into the pancreas of immune-competent syngeneic C57Bl/6 six- to 8 week-old female mice, and tumors were established for 2 weeks before beginning treatment. Mice were administered i.p. with Gas6 neutralizing antibody (R&D systems, AB885) (2 mg/kg), or IgG isotype control antibody, every 3–4 days or warfarin sodium in drinking water (0.5 mg/L) which was replenished every 3–4 days, for 15 days before harvest.

Analysis and Quantification of Immune Cells in Pancreatic Tumors by Mass Cytometry

Pancreatic tumors were resected from the mice and mechanically and enzymatically digested in Hanks Balanced Salt Solution (HBSS) with 1 mg/mL Collagenase P (Roche) Cell suspensions were centrifuged for 5 min at 1,500 rpm, resuspended in HBSS and filtered through a 500 μm polypropylene mesh (Spectrum Laboratories). Cells were resuspended in 1 mL 0.05%Trypsin and incubated at 37°C for 5 min. Cells were filtered through a 70 μm cell strainer and resuspended in Maxpar cell staining buffer (Fluidigm). The samples were centrifuged for 5 min at 450 *x g* and supernatant removed. The cells were subsequently stained with Cell-ID 195-Cisplatin (Fluidigm) viability marker diluted 1:40 in Maxpar PBS (Fluidigm) for 5 min. Cells were centrifuged at 450 *x g* for 5 min and washed twice in Maxpar cell staining buffer. Samples were blocked for 10 min on ice with 1:100 diluted FC Block (BD Pharmingen, Clone 2.4G2) and metal-conjugated antibody cocktail added and incubated for 30 min at 4°C. Antibodies were used at the concentrations recommended by manufacturers. Cells were washed twice in cell staining buffer and stained with 125 μM 191-Intercalator-Ir (Fluidigm) diluted in 1:2,000 Maxpar fix and perm buffer (Fluidigm) overnight at 4°C. The cells were washed twice in Maxpar cell staining buffer and centrifuged at 800 *x g* for 5 min. A post-fix was performed by incubating the cells in 1.6% PFA for 30 min at RT. Cells were washed twice in 18Ω distilled water (Fluidigm), mixed 1:10 with EQTM Four Element Calibration Beads (Fluidigm) and acquired on the Helios CyTOF system (Fluidigm). Samples were acquired at a rate of around 200 cells/s. All generated FCS files were normalized and beads removed (51). All analysis was performed in Cytobank: Manual gating was used to remove dead cells (195Pt+) and debris and to identify single cells (191 Ir+).

viSNE analysis was performed on the data utilizing t-stochastic neighbor embedding (t-SNE) mapping based on high dimensional relationships. CD45+ population selected by manual gating was used as the starting cell population and using proportional sampling viSNE unsupervised clustering was performed. Manual gating was then performed on the viSNE map created to determine cell population percentages. Spanning-tree Progression Analysis of Density-normalized Events (SPADE) analysis was performed in Cytobank using manually gated CD45+ cells, 200 target number of nodes and 10% down sampled events, to equalize the density in different parts of the cloud. In Cytobank SPADE analysis edge number between nodes indicates levels of similarity, with more steps indicating less similarity across channels used to create the tree. Node localization and edge length cannot be used to infer similarity in this analysis. Event number is indicated by both color scale and node size (which is proportional to the number of cells present in each cluster). Gating of cell populations was performed to identify major cell populations and percentages.

FACS Sorting and Analysis of Blood and Lungs by Flow Cytometry

Single cell suspensions from murine primary pancreatic tumors and pulmonary metastasis were prepared by mechanical and enzymatic disruption and tumor cells, tumor associated macrophages and stromal cells were analyzed and sorted using flow cytometry (FACS ARIA II, BD Bioscience). Samples were digested as outlined above, the cells were then filtered through a 70 μm cell strainer and resuspended in PBS + 1% BSA, blocked for 10 min on ice with FC Block (BD Pharmingen, Clone 2.4G2) and stained with Sytox® blue viability marker (Life Technologies) and conjugated antibodies anti-CD45-PE/Cy7 (Biolegend, clone 30-F11) and anti-F4/80-APC (Biolegend, clone BM8).

Blood was collected from mice via tail vein bleed in EDTA-tubes. Red blood cell lysis was performed and resulting leukocytes were resuspended in PBS + 1% BSA and blocked for 10 min on ice with FC block and stained with Sytox® blue viability marker and conjugated antibodies anti-CD45-APC/Cy7 (Biolegend, 103115), anti-CD11b-APC (Biolegend, 101212), anti-Ly6G-PerCP-Cy5.5 (Biolegend, 127616), anti-Ly6C-PE (Biolegend, 128008), anti-CD3-PE-Cy7 (Biolegend, 100320), anti-CD4-PE (Biolegend, 100408), and anti-CD8-PerCP-Cy5.5 (Biolegend, 100734). Cell analysis was performed using FACS Canto II.

Gene Expression

Total RNA was isolated from FACS sorted tumor cells, tumor associated macrophages and non-immune stromal cells from primary pancreatic tumors as described in Qiagen Rneasy protocol. Total RNA from the different cell populations was extracted using a high salt lysis buffer (Guanidine thiocynate 5 M, sodium citrate 2.5 uM, lauryl sarcosine 0.5% in H_2O) to improve RNA quality followed by purification using Qiagen Rneasy protocol. cDNA was prepared from 1 μg RNA/sample, and qPCR was performed using gene specific QuantiTect Primer Assay primers from Qiagen. Relative expression levels were normalized to *gapdh* expression according to the formula $<2\wedge-$(Ct *gene of interest*—Ct *gapdh*) (52).

Quantification of Metastasis

By IVIS Imaging

IVIS spectral imaging of bioluminescence was used for orthotopically implanted tumor cells expressing firefly luciferase using IVIS spectrum system (Caliper Life Sciences). Organs were resected for *ex vivo* imaging coated in 100 μL D-luciferin (Perkin Elmer) for 1 min and imaged for 2 min at automated optimal exposure. Analysis was performed on the Living Image software (PerkinElmer) to calculate the relative bioluminescence signal from photon per second mode normalized to imaging area (total flux) as recommended by the manufacturer.

By H&E Staining

FFPE lungs were serially sectioned through the entire lung using microtome at 4 μm thickness. Sections were stained with H&E and images were taken using a Zeiss Observer Z1 Microscope (Zeiss) to identify metastatic foci. The number of foci were

counted, and the total area of metastatic foci was measured using Zen imaging software. Metastatic burden was calculated by the following equations:

No. of foci per 100 mm^2: *(Average no. foci per section/ average tissue area per section (mm^2) *100*

Average metastatic lesion size (mm^2): *Average total area of metastasis (mm^2)/ average number of foci per section*

Total metastatic burden: *Sum of area of each foci of each section*.

By CK19 Staining

FFPE Lung tissue sections were also stained for cytokeratin 19 (CK19). The slides were scanned with an Aperio slide scanner and the whole lung tissue was quantified for CK19 expression using Image J.

Immunohistochemistry and Immunofluorescence

Deparaffinization and antigen retrieval was performed using an automated DAKO PT-link. Paraffin-embedded pancreatic tumors, lymph nodes, and lung metastasis tissues were immunostained using the DAKO envision+ system-HRP.

Antibodies and Procedure Used for Immunohistochemistry

All primary antibodies were incubated for 2 h at room temperature: αSMA (Abcam, ab5694 used at 1:200 after low pH antigen retrieval), CD31 (Cell signaling technology, CST 77699 used at 1:100 after low pH antigen retrieval), NKp46 (Biorbyt, orb13333 used at 1:200) and AF2225 (used at 1:50 after low pH antigen retrieval), CK19 (ab53119 used at 1:100 after low pH antigen retrieval), and CD68 (Abcam, ab31630 used at 1:400 after low pH antigen retrieval). Subsequently, samples were incubated with secondary HRP-conjugated antibody (from DAKO envision kit) for 30 min at room temperature. All antibodies were prepared in antibody diluent from Dako envision kit. Staining was developed using diamino-benzidine and counterstained with hematoxylin.

Human paraffin-embedded PDA tissue sections were incubated overnight at RT with the following primary antibodies: phospho-Axl (R&D, AF2228, used 1:500 after high pH antigen retrieval), CD163 (Abcam, ab74604 pre-diluted after low pH antigen retrieval), αSMA (Abcam, ab5694 used 1:100 after low pH antigen retrieval).

Antibodies and Procedure Used for Immunofluorescence

After low pH antigen retrieval, lymph node tissue sections derived from mice bearing pancreatic tumors were incubated overnight at RT with the following primary antibodies: NKp46 (R&D systems AF2225, used at 1:25), Ki67 (Abcam ab15580, used at 1:1000), vimentin (Abcam ab92547, used at 1:400), and Granzyme B (ab4059, used at 1:600). Vimentin expression was quantified on cancer cells located at the edge of pancreatic tumors. Samples were washed with PBS and incubated with donkey anti-goat 594 (Abcam ab150132) and donkey anti-rabbit 488 (Abcam ab98473) secondary antibodies, respectively, all used at 1:300 and DAPI at 1:600 for 2 h at RT. Slides were washed with PBS, final quick wash with distilled water and mounted using DAKO fluorescent mounting media.

After low pH antigen retrieval, mouse tissue sections derived from paraffin embedded pancreatic tumors were incubated with vimentin (ab92547, used at 1:400) overnight at 4c. Goat anti-rabbit 594 (ab150080) secondary was used at 1:300 and DAPI at 1:600 for 2 h at RT.

Human PDA frozen tissue sections were fixed with cold acetone, permeabilized in 0.1% Triton, blocked in 8% goat serum and incubated overnight at 4°C with anti-phospho-Axl (R&D, AF2228, diluted 1:200) CK11 (Cell signaling, CST 4545, diluted 1:200), followed by fluorescently labeled secondary antibodies goat anti mouse 488 (Abcam ab98637), goat anti-rabbit 594 (Abcam ab98473) used at 1:300 for 2 h at RT slides were washed with PBS, final quick wash with distilled water and mounted using DAKO fluorescent mounting media.

Picrosirius Red Staining

FFPE PDA tumor sections were deparaffinized in two 5 min xylene washes and through decreasing alcohol washes of 100, 75, and 65% each 5 min. The slides were washed for 5 min in distilled water and incubated in 0.2% phosphomolybdic acid for 5 min. After washing in PBS, were stained with 0.1% Sirius red F3B in saturated picric acid solution for 90 min. After two rinses in acidified water the slides were stained with fast green (0.01%) for 1 min. The sections were rinsed twice in acidified water were rapidly dehydrated using three steps of 100% ethanol and two xylene incubations of 30 s.

Statistical Methods

Statistical significance for *in vitro* assays and animal studies was assessed using unpaired two-tailed Student *t*-test and the GraphPad Prism 5 program. All error bars indicate SD for *in vitro* studies and SEM for animal studies.

Institutional Approvals

All studies involving human tissues were approved by the University of Liverpool and were considered exempt according to national guidelines. Human pancreatic cancer samples were obtained from the Liverpool Tissue Bank from patients that consented to use the surplus material for research purposes. All animal experiments were performed in accordance with current UK legislation under an approved project license (reference number: 403725). Mice were housed under specific pathogen-free conditions at the Biomedical Science Unit at the University of Liverpool.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Liverpool and were considered exempt according to national guidelines. Human pancreatic cancer samples were obtained from the Liverpool Tissue Bank from patients that consented to use the surplus material for research purposes. The patients/participants provided their written informed consent to participate in this study. All animal experiments were performed in accordance with current UK legislation under an approved project license (reference number: 403725). Mice were housed under specific pathogen-free conditions at the Biomedical Science Unit at the University of Liverpool.

AUTHOR CONTRIBUTIONS

LI designed experiments and performed most of the experiments including *in vivo* experiments, mass cytometry/flow cytometry, cell isolations, immunohistochemical stainings, and qPCR experiments. TL designed and performed qPCR experiments, tissue stainings, and *in vivo* experiment with warfarin treatment. AM designed experiments, helped with tissue harvesting and tissue stainings, conceived and supervised the project. MS provided conceptual advice and help with *in vivo* experiments. AM and LI wrote the manuscript. All authors helped with the analysis and interpretation of the data, the preparation of the manuscript, and approved 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.2020.00297/full#supplementary-material

REFERENCES

1. Paolino M, Penninger JM. The role of TAM family receptors in immune cell function: implications for cancer therapy. *Cancers.* (2016) 8:97. doi: 10.3390/cancers8100097
2. Sasaki T, Knyazev PG, Clout NJ, Cheburkin Y, Gohring W, Ullrich A, et al. Structural basis for Gas6-Axl signalling. *EMBO J.* (2006) 25:80–7. doi: 10.1038/sj.emboj.7600912
3. Lemke G, Rothlin CV. Immunobiology of the TAM receptors. *Nat Rev Immunol.* (2008) 8:327–36. doi: 10.1038/nri2303
4. Lu Q, Lemke G. Homeostatic regulation of the immune system by receptor tyrosine kinases of the Tyro 3 family. *Science.* (2001) 293:306–11. doi: 10.1126/science.1061663
5. Wu G, Ma Z, Hu W, Wang D, Gong B, Fan C, et al. Molecular insights of Gas6/TAM in cancer development and therapy. *Cell Death Dis.* (2017) 8:e2700. doi: 10.1038/cddis.2017.113
6. Hafizi S, Dahlback B. Gas6 and protein S. Vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily. *FEBS J.* (2006) 273:5231–44. doi: 10.1111/j.1742-4658.2006.05529.x
7. Guo Z, Li Y, Zhang D, Ma J. Axl inhibition induces the antitumor immune response which can be further potentiated by PD-1 blockade in the mouse cancer models. *Oncotarget.* (2017) 8:89761–74. doi: 10.18632/oncotarget.21125
8. Myers KV, Amend SR, Pienta KJ. Targeting Tyro3, Axl and MerTK (TAM receptors): implications for macrophages in the tumor microenvironment. *Mol Cancer.* (2019) 18:94. doi: 10.1186/s12943-019-1022-2
9. Paolino M, Choidas A, Wallner S, Pranjic B, Uribesalgo I, Loeser S, et al. The E3 ligase Cbl-b and TAM receptors regulate cancer metastasis via natural killer cells. *Nature.* (2014) 507:508–12. doi: 10.1038/nature12998
10. Rankin EB, Fuh KC, Castellini L, Viswanathan K, Finger EC, Diep AN, et al. Direct regulation of GAS6/AXL signaling by HIF promotes renal metastasis through SRC and MET. *Proc Natl Acad Sci USA.* (2014) 111:13373–8. doi: 10.1073/pnas.1404848111
11. Loges S, Schmidt T, Tjwa M, van Geyte K, Lievens D, Lutgens E, et al. Malignant cells fuel tumor growth by educating infiltrating leukocytes to produce the mitogen Gas6. *Blood.* (2010) 115:2264–73. doi: 10.1182/blood-2009-06-228684
12. Ireland L, Santos A, Campbell F, Figueiredo C, Hammond D, Ellies LG, et al. Blockade of insulin-like growth factors increases efficacy of paclitaxel in metastatic breast cancer. *Oncogene.* (2018) 37:2022–36. doi: 10.1038/s41388-017-0115-x
13. Nielsen SR, Quaranta V, Linford A, Emeagi P, Rainer C, Santos A, et al. Macrophage-secreted granulin supports pancreatic cancer metastasis by inducing liver fibrosis. *Nat Cell Biol.* (2016) 18:549–60. doi: 10.1038/ncb3340
14. Qian BZ, Zhang H, Li J, He T, Yeo EJ, Soong DY, et al. FLT1 signaling in metastasis-associated macrophages activates an inflammatory signature that promotes breast cancer metastasis. *J Exp Med.* (2015) 212:1433–48. doi: 10.1084/jem.20141555
15. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov.* (2011) 1:54–67. doi: 10.1158/2159-8274.CD-10-0028
16. Ireland L, Santos A, Ahmed MS, Rainer C, Nielsen SR, Quaranta V, et al. Chemoresistance in pancreatic cancer is driven by stroma-derived insulin-like growth factors. *Cancer Res.* (2016) 76:6851–63. doi: 10.1158/0008-5472.CAN-16-1201
17. Shree T, Olson OC, Elie BT, Kester JC, Garfall AL, Simpson K, et al. Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes Dev.* (2011) 25:2465–79. doi: 10.1101/gad.180331.111
18. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin.* (2019) 69:7–34. doi: 10.3322/caac.21551

19. Ireland LV, Mielgo A. Macrophages and fibroblasts, key players in cancer chemoresistance. *Front Cell Dev Biol.* (2018) 6:131. doi: 10.3389/fcell.2018.00131
20. Zambirinis CP, Miller G. Cancer manipulation of host physiology: lessons from pancreatic cancer. *Trends Mol Med.* (2017) 23:465–81. doi: 10.1016/j.molmed.2017.03.003
21. Song X, Wang H, Logsdon CD, Rashid A, Fleming JB, Abbruzzese JL, et al. Overexpression of receptor tyrosine kinase Axl promotes tumor cell invasion and survival in pancreatic ductal adenocarcinoma. *Cancer.* (2011) 117:734–43. doi: 10.1002/cncr.25483
22. Koorstra JB, Karikari CA, Feldmann G, Bisht S, Rojas PL, Offerhaus GJ, et al. The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target. *Cancer Biol Ther.* (2009) 8:618–26. doi: 10.4161/cbt.8.7.7923
23. Kirane A, Ludwig KF, Sorrelle N, Haaland G, Sandal T, Ranaweera R, et al. Warfarin blocks Gas6-mediated Axl activation required for pancreatic cancer epithelial plasticity and metastasis. *Cancer Res.* (2015) 75:3699–705. doi: 10.1158/0008-5472.CAN-14-2887-T
24. Ludwig KF, Du W, Sorrelle NB, Wnuk-Lipinska K, Topalovski M, Toombs JE, et al. Small-Molecule inhibition of Axl targets tumor immune suppression and enhances chemotherapy in pancreatic cancer. *Cancer Res.* (2018) 78:246–55. doi: 10.1158/0008-5472.CAN-17-1973
25. Gay CM, Balaji K, Byers LA. Giving AXL the axe: targeting AXL in human malignancy. *Br J Cancer.* (2017) 116:415–23. doi: 10.1038/bjc.2016.428
26. Pylayeva-Gupta Y, Das S, Handler JS, Hajdu CH, Coffre M, Koralov SB, et al. IL35-producing B cells promote the development of pancreatic neoplasia. *Cancer Discov.* (2016) 6:247–55. doi: 10.1158/2159-8290.CD-15-0843
27. Zhu Y, Knolhoff BL, Meyer MA, Nywening TM, West BL, Luo J, et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Res.* (2014) 74:5057–69. doi: 10.1158/0008-5472.CAN-13-3723
28. Strange DP, Jiyarom B, Zarandi NP, Xie XP, Baker C, Sadri-Ardekani H, et al. Axl promotes Zika Virus entry and modulates the antiviral state of human sertoli cells. *Mbio.* (2019) 10:2437–50. doi: 10.1128/mBio.01372-19
29. Gomes AM, Carron EC, Mills KL, Dow AM, Gray Z, Fecca CR, et al. Stromal Gas6 promotes the progression of premalignant mammary cells. *Oncogene.* (2019) 38:2437–50. doi: 10.1038/s41388-018-0593-5
30. Baumann C, Ullrich A, Torka R. GAS6-expressing and self-sustaining cancer cells in 3D spheroids activate the PDK-RSK-mTOR pathway for survival and drug resistance. *Mol Oncol.* (2017) 11:1430–47. doi: 10.1002/1878-0261.12109
31. Fourcot A, Couchie D, Chobert MN, Zafrani ES, Mavier P, Laperche Y, et al. Gas6 deficiency prevents liver inflammation, steatohepatitis, and fibrosis in mice. *Am J Physiol Gastrointest Liver Physiol.* (2011) 300:G1043–53. doi: 10.1152/ajpgi.00311.2010
32. Korshunov VA, Mohan AM, Georger MA, Berk BC. Axl, a receptor tyrosine kinase, mediates flow-induced vascular remodeling. *Circ Res.* (2006) 98:1446–52. doi: 10.1161/01.RES.0000223322.16149.9a
33. Melaragno MG, Fridell YW, Berk BC. The Gas6/Axl system: a novel regulator of vascular cell function. *Trends Cardiovasc Med.* (1999) 9:250–3. doi: 10.1016/S1050-1738(00)00027-X
34. O'Donnell K, Harkes IC, Dougherty L, Wicks IP. Expression of receptor tyrosine kinase Axl and its ligand Gas6 in rheumatoid arthritis: evidence for a novel endothelial cell survival pathway. *Am J Pathol.* (1999) 154:1171–80. doi: 10.1016/S0002-9440(10)65369-2
35. Antony J, Tan TZ, Kelly Z, Low J, Choolani M, Recchi C, et al. The GAS6-AXL signaling network is a mesenchymal (Mes) molecular subtype-specific therapeutic target for ovarian cancer. *Sci Signal.* (2016) 9:ra97. doi: 10.1126/scisignal.aaf8175
36. Wilson C, Ye X, Pham T, Lin E, Chan S, McNamara E, et al. AXL inhibition sensitizes mesenchymal cancer cells to antimitotic drugs. *Cancer Res.* (2014) 74:5878–90. doi: 10.1158/0008-5472.CAN-14-1009
37. Diepenbruck M, Christofori G. Epithelial-mesenchymal transition (EMT) and metastasis: yes, no, maybe? *Curr Opin Cell Biol.* (2016) 43:7–13. doi: 10.1016/j.ceb.2016.06.002
38. Saitoh M. Involvement of partial EMT in cancer progression. *J Biochem.* (2018) 164:257–64. doi: 10.1093/jb/mvy047
39. Kim YS, Jung SH, Jung DH, Choi SJ, Lee YR, Kim JS. Gas6 stimulates angiogenesis of human retinal endothelial cells and of zebrafish embryos via ERK1/2 signaling. *PLoS ONE.* (2014) 9:e83901. doi: 10.1371/journal.pone.0083901
40. Zuo PY, Chen XL, Lei YH, Liu CY, Liu YW. Growth arrest-specific gene 6 protein promotes the proliferation and migration of endothelial progenitor cells through the PI3K/AKT signaling pathway. *Int J Mol Med.* (2014) 34:299–306. doi: 10.3892/ijmm.2014.1754
41. Stenhoff J, Dahlback B, Hafizi S. Vitamin K-dependent Gas6 activates ERK kinase and stimulates growth of cardiac fibroblasts. *Biochem Biophys Res Commun.* (2004) 319:871–8. doi: 10.1016/j.bbrc.2004.05.070
42. Leake I. Pancreatic cancer: surprising role for fibrosis. *Nat Rev Gastroenterol Hepatol.* (2014) 11:396. doi: 10.1038/nrgastro.2014.97
43. Ozdemir BC, Pentcheva-Hoang T, Carstens JL, Zheng X, Wu CC, Simpson TR, et al. Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Cancer Cell.* (2015) 28:831–3. doi: 10.1016/j.ccell.2015.11.002
44. Rhim AD, Oberstein PE, Thomas DH, Mirek ET, Palermo CF, Sastra SA, et al. Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. *Cancer Cell.* (2014) 25:735–47. doi: 10.1016/j.ccr.2014.04.021
45. Weniger M, Honselmann KC, Liss AS. The extracellular matrix and pancreatic cancer: a complex relationship. *Cancers.* (2018) 10:316. doi: 10.3390/cancers10090316
46. Cabezon R, Carrera-Silva EA, Florez-Grau G, Errasti AE, Calderon-Gomez E, Lozano JJ, et al. MERTK as negative regulator of human T cell activation. *J Leukoc Biol.* (2015) 97:751–60. doi: 10.1189/jlb.3A0714-334R
47. Walzer T, Vivier E. NK cell development: gas matters. *Nat Immunol.* (2006) 7:702–4. doi: 10.1038/ni0706-702
48. Geng K, Kumar S, Kimani SG, Kholodovych V, Kasikara C, Mizuno K, et al. Requirement of gamma-carboxyglutamic acid modification and phosphatidylserine binding for the activation of Tyro3, Axl, and mertk receptors by growth arrest-specific 6. *Front Immunol.* (2017) 8:1521. doi: 10.3389/fimmu.2017.01521
49. Feig C, Gopinathan A, Neesse A, Chan DS, Cook N, Tuveson DA. The pancreas cancer microenvironment. *Clin Cancer Res.* (2012) 18:4266–76. doi: 10.1158/1078-0432.CCR-11-3114
50. Hingorani SR, Wang LF, Multani AS, Combs C, Deramaudt TB, Hruban RH, et al. Trp53(R172H) and KraS(G12D) cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell.* (2005) 7:469–83. doi: 10.1016/j.ccr.2005.04.023
51. Finck R, Simonds EF, Jager A, Krishnaswamy S, Sachs K, Fantl W, et al. Normalization of mass cytometry data with bead standards. *Cytometry A.* (2013) 83:483–94. doi: 10.1002/cyto.a.22271
52. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C-T method. *Nat Protoc.* (2008) 3:1101–8. doi: 10.1038/nprot.2008.73
53. Ireland L, Luckett T, Schmid MC, Mielgo A. Blockade of stromal Gas6 alters cancer cell plasticity, activates NK cells and inhibits pancreatic cancer metastasis. *bioRxiv [Preprint].* (2019) 732149. doi: 10.1101/732149

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IL36 Cooperates With Anti-CTLA-4 mAbs to Facilitate Antitumor Immune Responses

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Despite the great impact on long-term survival of some cancer patients, the immune checkpoint blockade (ICB) therapy is limited by its low response rates for most cancers. There is a pressing need for novel combination immunotherapies that overcome the resistance to current ICB therapies. Cytokines play a pivotal role in tumor immunotherapy by helping initiating and driving antitumor immune responses. Here, we demonstrated that, besides conventional $CD4^+$ and $CD8^+$ T cells, IL36 surprisingly increased the number of tumor-infiltrating regulatory T (Treg) cells *in vivo* and enhanced proliferation of Tregs *in vitro*. Administration of CTLA-4 monoclonal antibodies (mAbs) strongly enhanced IL36-stimulated antitumor activities through depletion of Tregs. In addition, a cancer gene therapy using the IL36-loaded nanoparticles in combination with CTLA-4 mAbs additively reduced lung metastasis of breast tumor cells. We further showed that the combined therapy of CTLA-4 mAbs and IL36 led to an increase in proliferation and IFN-γ production by $CD4^+$ and $CD8^+$ T cells when compared to single therapy with CTLA-4 mAbs or IL36. Collectively, our findings demonstrated a new combination therapy that could improve the clinical response to ICB immunotherapy for cancer.

Keywords: CTLA-4, IL36, treg, immunotherapy, mAb

INTRODUCTION

Immune checkpoint blockade (ICB) immunotherapy has revolutionized cancer treatment by increasing the overall survival rates of cancer patients. However, clinical response rates are still low for most cancers (1). Higher response rates are achieved when CTLA-4 and PD-1 inhibitors are administered concurrently, demonstrating rational combination therapy will allow more patients to benefit from immunotherapy (2, 3). The antitumor activities of the checkpoint inhibitors are dependent on the number of tumor antigen-specific T cells, which are only abundant in immunogenic tumors. Because inflammatory cytokines play a key role in promoting tumor immunogenicity (4–9), synergistic integration of cytokine- and ICB-based immunotherapy has potential to greatly advance immunotherapy of cancer.

Many recent studies have established a critical role Interleukin 36 (IL36) plays in promoting adaptive and innate immune responses. IL36 consists of IL36α, IL36β, and IL36γ, also known as IL-1F6, IL-1F8, and IL-1F9, respectively, which are members of the IL-1 family of cytokines (10). They share the same receptor complex, which is composed of the IL36 receptor (IL36R) and IL-1RAcP. IL36 can be induced in keratinocytes, bronchial epithelia, brain tissues, and macrophages and is

believed to be an "alarmin" in the damaged tissue (10, 11). IL36 exerts its functions directly on multiple cell types including tissue stromal cells, dendritic cells (DCs), $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and γδ T cells (12–16). Ample evidence supports a crucial role of IL36 cytokines in promoting autoimmunity (17–20). IL36R-deficient mice were protected from imiquimod-induced psoriasiform dermatitis (21). Accumulating evidence supports an important role of IL36γ in driving Th1 immune responses. Pseudomonas, aeroginosa, or TLR3 ligands, induce high levels of IL36γ expression (22, 23) and T-bet is required for the induction of IL36γ in myeloid cells (24). In addition, IL36γ stimulates Th1 differentiation *in vitro* and IL36R is required for protective immune responses to aspergillus and Bacillus Calmette-Guerin infection (14, 25). Recent studies also show that IL36γ promotes antitumor immune responses through enhancing the effector function of type 1 lymphocytes (16, 26–31). All these data have firmly established an important role of IL36 in promoting immune responses. Nonetheless, whether IL36 can participate in immune regulation and enhance the function of immune checkpoint molecules has not been investigated.

Here, we set out to gain a further mechanistic insight of IL36-mediated antitumor immune responses by focusing on its effect on Treg. We first examined whether IL36 promoted Treg proliferation. We also quantified the number of tumoral Treg in IL36-expressing tumors and control tumors. Since one of the antitumor mechanisms of CTLA-4 mAbs is through depletion of tumor infiltrating Treg, we studied the effect of combination therapy of CTLA-4 mAbs and IL36. Our studies further elucidated the cellular mechanisms of IL36-mediated immune responses and also shed light on novel combination immunotherapy of cancer.

MATERIALS AND METHODS

Tumor Cell Culture and Generation of IL36-Expressing Cell Lines

B16 and 4T1.2 cells were cultured in RPMI1640 medium plus 10%FCS. The IL36γ-expression vector was transfected into B16 cells using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions to generate B16 stably expressing IL36. Anempty vector (pcDEF3) was transfected into B16 cells as a control.

Animals

C57BL/6 and BALB/c were purchased from the Jackson Laboratory. All mice were maintained under specific pathogen-free conditions. All mouse experiments were approved by the Institution Animal Care and Use Committee at University of Pittsburgh.

Synthesis of PEG2k-Fmoc Conjugated With IL36 Plasmid

The construction of IL36 expression plasmid has been described before (12–16). Briefly, the IL-36γ expression construct was generated by fusing the nucleotide sequence encoding the human CD8αsignal sequence to the 5' end of IL-36γ (G13-S164) sequence downstream the elongation factor alpha promoter. The detailed procedure of synthesis of POEG-st-Pmor polymer was described previously (29). Briefly, POEG-st-Pmor micelles were prepared by the dialysis method. 10 mg of polymer was dissolved in 5 mL of DMSO. The solution was lyophilized and resolubilized in 1 mL PBS. For plasmid DNA complexation, polymeric micelles were diluted to different concentrations in water and mixed with plasmid DNA solution to obtain the desired N/P ratios. The mixture was filtered and the filtrate was precipitated by ice-cold ether/ethanol twice. The crude product was dissolved in water and filtered through a 450 nm filter, followed by lyophilization to yield the powder of purified POEG-st-Pmor-IL36 (29). Mice were treated intravenously with IL-36γ plasmid/POEG-st-Pmor micelles every 3 days for four times.

Mouse Tumor Experiments

B16 cells were injected intradermally into B6 mice, and the size of tumor was monitored every 2–3 days. B16 and IL36-B16 bearing mice were randomized into two treatment cohorts: (i) control IgG or (ii) CTLA4 monoclonal antibodies (mAbs) (clone 9H10, BioXCell). All antibodies were administered at a dose of 200 μg/mouse through intraperitoneal (i.p.) injection twice per week. Mice were euthanized when the tumor volume reached 2,000 mm^3. The day of euthanasia was used to calculate survival.

To established murine breast tumor lung metastasis model, BALB/c mice were injected *i.v.* with 4T1.2. Treatments with CTLA-4 mAb, POEG-st-Pmor-IL36 nanoparticles or combination were initiated 24 h after tumor cell injection once every 3 days for 4 times *i.v.* On day 15 post tumor cell injection, all mice were sacrificed. Metastatic 4T1.2 tumor nodules were enumerated after the India ink staining procedure, as reported previously. Briefly, India ink solution was injected through the trachea to inflate the lungs, and the lungs were stained for 5 min. The lungs were then removed and placed in Fekete's solution (70% alcohol, 10% formalin, and 5% acetic acid) for destaining. Tumor nodules did not absorb India ink, which resulted in the normal lung tissue staining black and the tumor nodules remaining white. Tumor nodules were counted blindly by two independent investigators (16).

Analysis of Tumor-Infiltrating Lymphocytes

Tumors were dissected and transferred into RPMI medium. Tumors were disrupted mechanically using scissors, digested with a mixture of 0.3 mg/ml DNase I (Sigma-Aldrich) and 0.25 mg/ml Liberase TL (Roche) in serum-free RPMI medium for 25 min, and dispersed through a 40-mm cell strainer (BD Biosciences). Flow cytometric analysis was performed using a FACS flow cytometer (BD Biosciences). Combinations of the following fluorochrome-conjugated antibody were used for cell surface or intracellular staining to define populations of CD8, and subsets of CD4 T cells: CD45, CD8 (clone 53-6.7), CD4 (clone GK1.5), Foxp3, PD-1, Tim-3, CD69, Ki-67, CTLA-4. For *ex vivo* restimulation, freshly isolated single-cell suspension was cultured in complete RPMI 1,640 medium containing PMA (50 ng/ml) and ionomycin (500 ng/ml) for 3 h before it was analyzed for IFN-γ production by intracellular staining with IFN-γ mAbs (XMG1.2). Multi-colored flow cytometry analyses were

performed on LSR II (BD). Data were analyzed with FlowJo software (Tree Star).

Determination of IL36R Expression by RT-Quantitative-PCR

To determine IL36R expression, single-cell suspensions were made from spleens and lymph nodes of C57BL/6 mice. Naive $CD4^+$T ($CD44_{low}$ $CD62L_{high}$), $CD8^+$ T, Treg ($CD4^+CD25^+$) cells were purified by fluorescence-activated cell sorting (FACS). Total RNA was extracted using the TrIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. Total RNA was reverse transcribed using SuperScript II Reverse transcriptase (Invitrogen Life Technologies). The mRNA levels for genes of interest were examined by quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems). Values obtained with the SDS 2.2 (Applied Biosystems) were imported into Microsoft Excel for analyses and gene expression was calculated using the comparative method ($2^{-\delta Ct}$) for relative quantification by normalization to *GAPDH* gene expression.

Primary Lymphocyte Culture and Stimulation

Total $CD4^+$ T cells were washed twice with staining buffer (PBS0.1% BSA), resuspended in staining buffer containing 5 μM CFSE (Molecular Probes), and incubated at 37°C for 15 min. Five volumes of ice-cold culture medium were added to stop labeling, and cells were washed once with culture medium. Cells were then activated with plate-bound anti-mouse CD3 and CD28 antibodies and stimulated with or without IL36 (100 ng/mL), IL-2 (50 U/mL). After 3 days of incubation, the cell division was determined by measuring CFSE fluorescence by flow cytometry.

Statistical Analysis

Data (mean ± SEM) are representative of independent experiments. We used the two-tailed unpaired Student's *t*-test, Mann-Whitney U test or the log-rank test (survival studies). $P < 0.05$ was considered as being significant.

RESULTS

Tumoral Expression of IL36 Increased the Tumor Associated Treg

We have shown that IL36 potently enhanced the effectors function of Th1, $CD8^+$ T, NK, and T cells when over-expressed in the tumor tissues. Whether IL36 can exert direct effect on Treg cells is not known. Interestingly, the percentage of tumoral Treg cells was increased greatly in IL36-B16 when compared to B16 tumors (**Figure 1A**). This was likely due to increases in local proliferation because the percentage of Ki-67^+Treg cells was greater in IL36-B16 when compared to B16 tumors (**Figure 1B**). These data indicated that IL36 also induced a self-limiting mechanism through Treg.

IL36 Promoted the Treg Proliferation *in vitro*

In order to further determine whether IL36 can directly increase Treg proliferation, we examined the expression of IL36R in Treg.

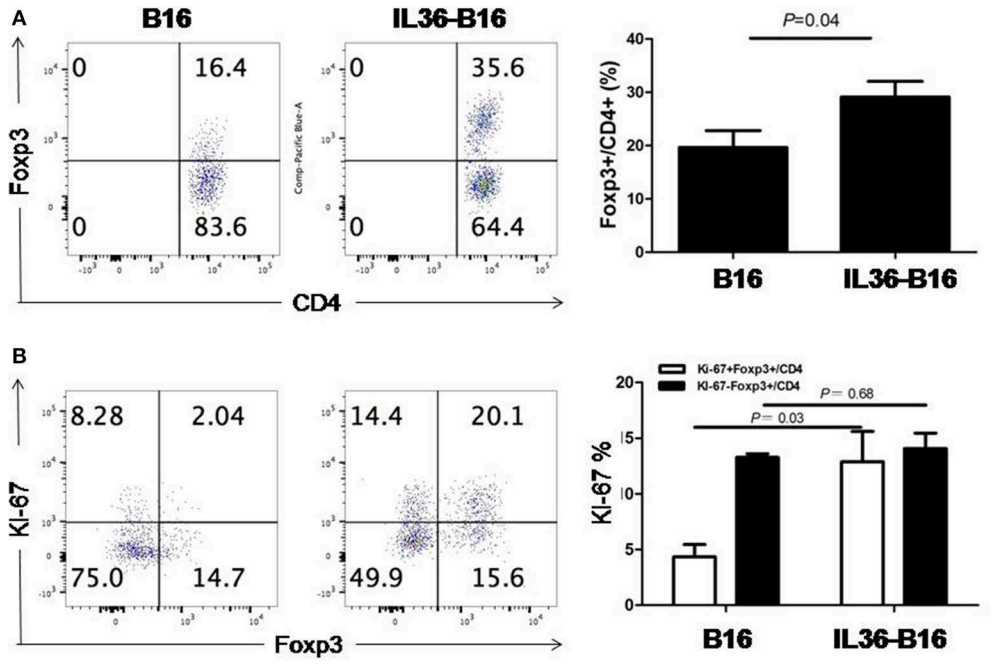

FIGURE 1 | IL-36 increased tumor infiltrated Treg cells and upregulated Ki-67 in Treg cells. A total of 10^4 B16 or IL-36-B16 cells was injected i.d. into C57BL/6j mice ($n = 3$ mice/group). On day 10–12, tumors were resected and processed to generate single cell suspension. Percentages of $Foxp3^+CD4^+$ T cells **(A)** in tumor infiltrating immune cells and percentages of Ki-67^+Foxp3^+ cells **(B)** within the $CD4^+$ TIL population were shown. Results are mean ± SEM of three independent experiments.

Similar to results from previous studies, IL36R could be readily detected in both naïve $CD4^+$ and $CD8^+$T cells (**Figure 2A**). Interestingly, IL36R was also expressed in Treg (**Figure 2A**). This was surprising because it was shown that IL36R inhibits generation of the induced Treg (32). We then tried to determine whether IL36 could enhance proliferation of natural Treg *in vitro* with cultured in the presence of CD3 and CD28 mAbs. We found that IL36 indeed promoted Treg proliferation but did not affect the ratio between Treg and conventional $CD4^+$ T cells after co-culture (**Figure 2B**). These data suggest that IL36 could enhance

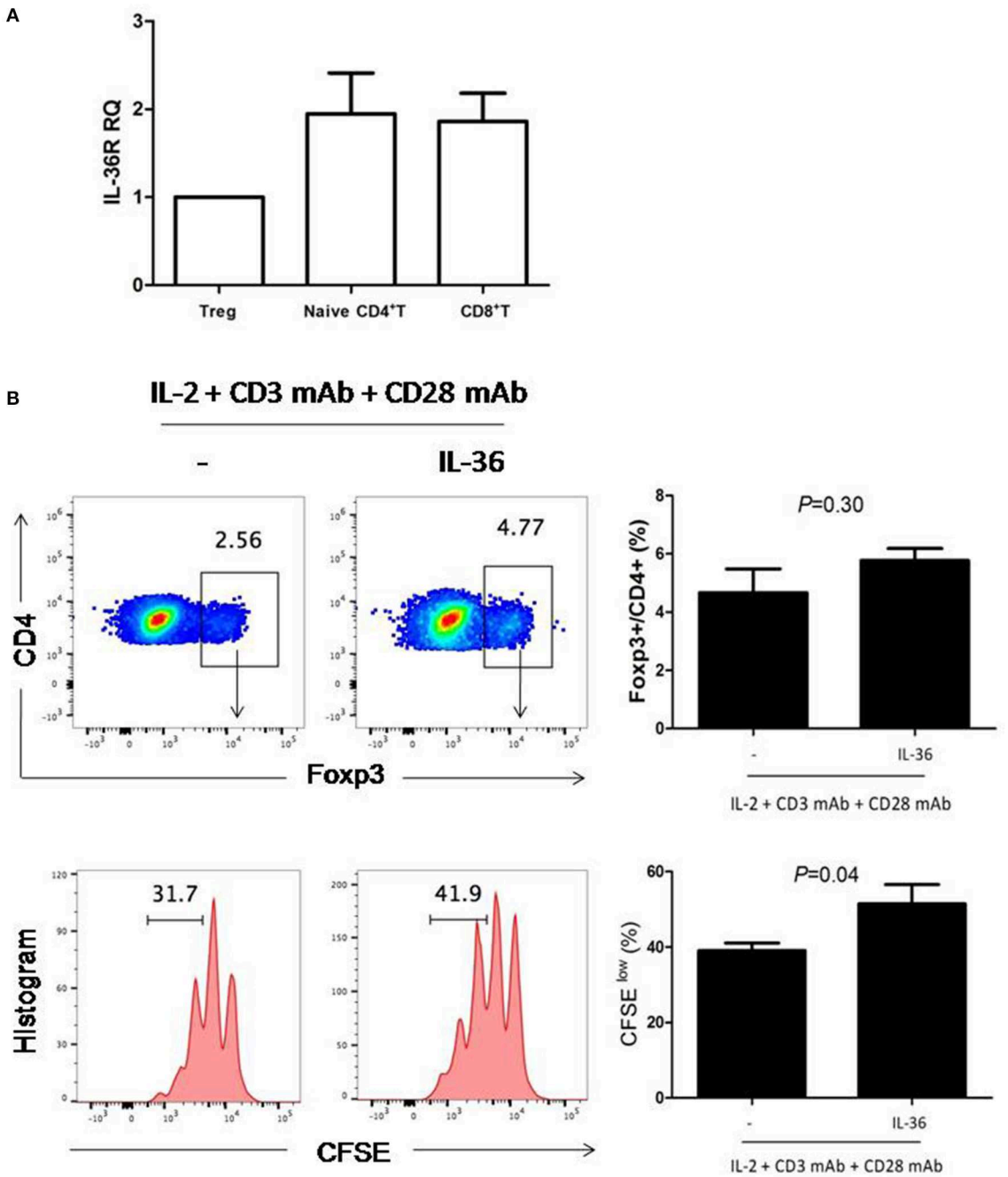

FIGURE 2 | IL-36R was expressed and functional in Treg cells. **(A)**, naïve $CD4^+$T cells (Th0 cells), naïve $CD8^+$ T cell and Treg were purified by FACS. Total mRNA was isolated for analyses by quantitative RT-PCR. Results represented IL-36R mRNA expression levels relative to *GAPDH*. **(B)**, flow cytometric analysis of Treg cells proliferation responses induced by IL-36-stimulated splenic $CD4^+$T cells. Splenic $CD4^+$T cells were stimulated with anti-CD3 and anti-CD28 antibodies with or without IL36 for 72 h. CFSE dilution was used to evaluate T cell proliferation responses 72 h following co-culture. Flow cytometry plot is representative of four independent experiments. Results are mean ± SEM of three independent experiments.

the proliferation of both Treg and conventional T cells. The IL36/Treg axis is likely a natural negative feedback mechanism that limits an overzealous IL36-driven T cell immune response. Such mechanism, although fit for limiting autoimmunity, poses an obstacle for utilization of IL36 in tumor immunotherapy.

IL36 Combined With CTLA-4 mAbs Additively Eradicated Tumors

One of the antitumor mechanisms of CTLA-4 mAbs is depletion of tumoral Treg (33–35). We therefore hypothesize that combination of tumoral expression of IL36 and CTLA-4 mAbs might additively increase antitumor activities. We administered CTLA-4 mAbs 4 days after subcutaneous implantation of B16 cells or IL36-B16 cells, when tumors were established with an average diameter of 2 mm. We used the B16 melanoma cells because they represent an aggressive murine tumor model and are highly resistant to various immunotherapies. Consistent with previous studies, CTLA-4 mAbs failed to control tumor growth, and tumoral expression of IL36 had pronounced antitumor functions. Combination of CTLA-4 mAbs and IL36 expression produced much greater antitumor efficacy with reduced tumor growth rates and much prolonged survival (**Figures 3A–C**).

Our previous data show that drug-loaded PEG2k-Fmoc micelles are stable in the blood and are highly effective in selective delivery gene expressing constructs to the lung tumor tissues (29). Our nanocarrier was designed to target both lungs and distant solid tumors (29). Selective accumulation of the nanocarrier is largely attributed to the leaky tumor vasculature. On the other hand, the effective accumulation in the lung is likely due to the interaction of tertiary amine moiety with negatively charged cell membrane in the lung (36). Amine-containing basic compounds have been reported to be predominantly accumulated in the lung due to the specific binding to acidic phospholipids on the cell membrane, which is abundantly distributed in lung tissue. Using this approach, IL36 is locally specifically expressed in tumor cells and lung tissue cells to avoid potential toxicity of *i.v.* injection of a recombinant IL36 protein. We then determine the effectiveness of this gene therapy strategy for delivery of

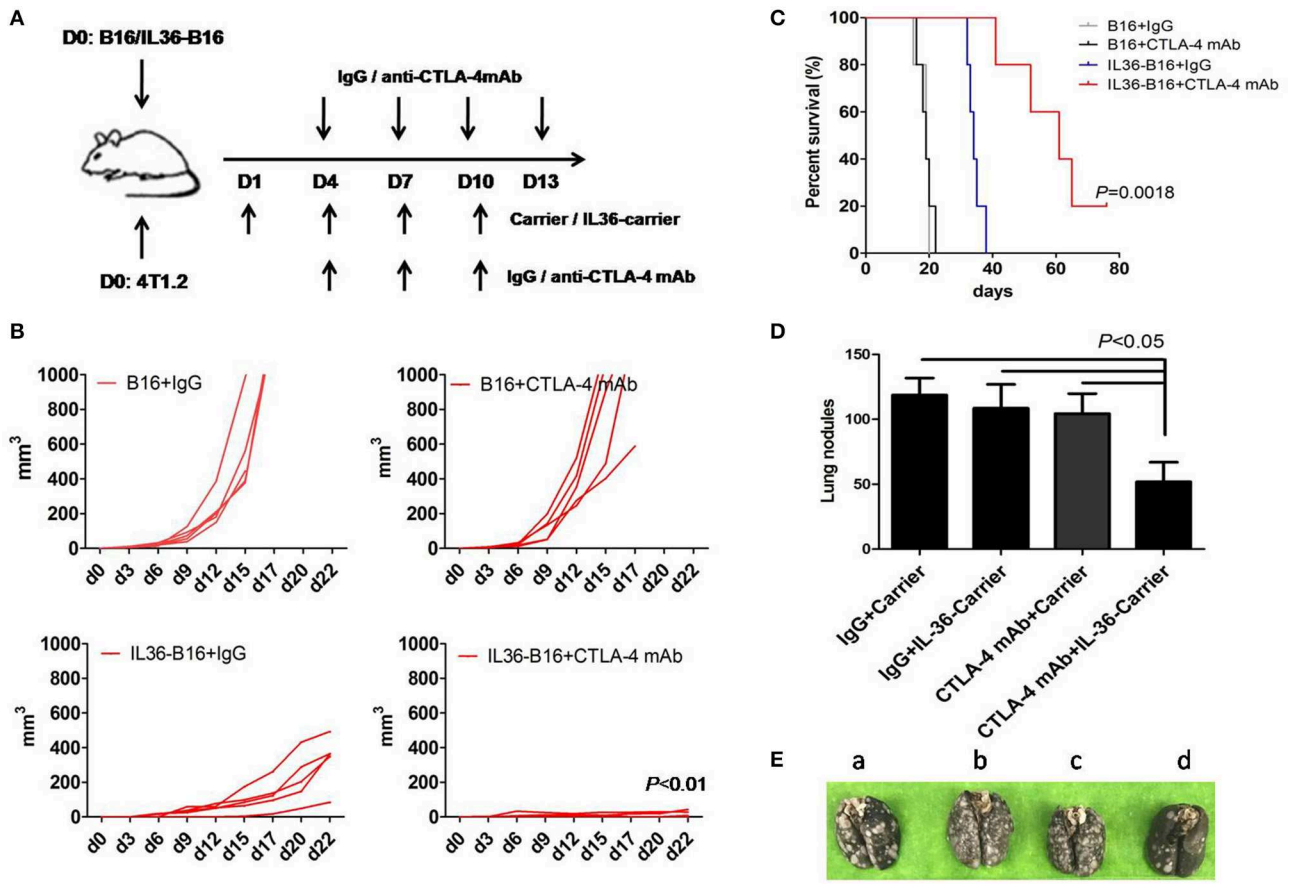

FIGURE 3 | Additively increased antitumor activities with combination of IL36 and CTLA-4 mAbs. **(A)**, Schematic drawing of the experimental setup of IL36 and CTLA-4 mAbs treatment. **(B)**, Individual growth curves of B16 and IL36-B16 tumors with or without CTLA-4 mAbs treatment ($n = 5$ mice/group). **(C)**, Long-term survival of different treatment groups was shown ($n = 5$ mice/group). **(D,E)**, Therapeutic studies were conducted using the 4T1.2 metastatic lung cancer model [$n = 5$ mice/group, **(E)** a, IgG plus Carrier group; b, IgG plus IL36-Carrier group; c, CTLA-4 mAbs plus Carrier group; d, CTLA-4 mAbs plus IL36-Carrier group]. Treatment with CTLA-4 mAbs, PEG2k-Fmoc-IL36 nanoparticles or combination was initiated after tumor cell injection. The presented is representative of samples in one out of three independent experiments. Data (mean ± SEM) are representative of three independent experiments.

IL36 expression plasmids to tumor and synergy between the IL36 gene therapy and CTLA-4 mAbs. The therapeutic studies were conducted in a 4T1.2 lung metastatic model using a nanomicellar carrier that is based on a prodrug conjugate of PEG, Fmoc with the IL36 plasmid (PEG2k-Fmoc-IL36) (29). We evaluated the lungs of these mice for metastatic nodules and found that numerous tumor nodules were visible on the surface of the lungs of control mice, whereas only mice received both PEG2k-Fmoc-IL36 and CTLA-4 mAbs had significantly fewer lung nodules (**Figures 3D,E**). These results suggest that local IL36 expression in combination with CTLA-4 mAbs was sufficient to inhibit the growth of metastatic colonies in the lung.

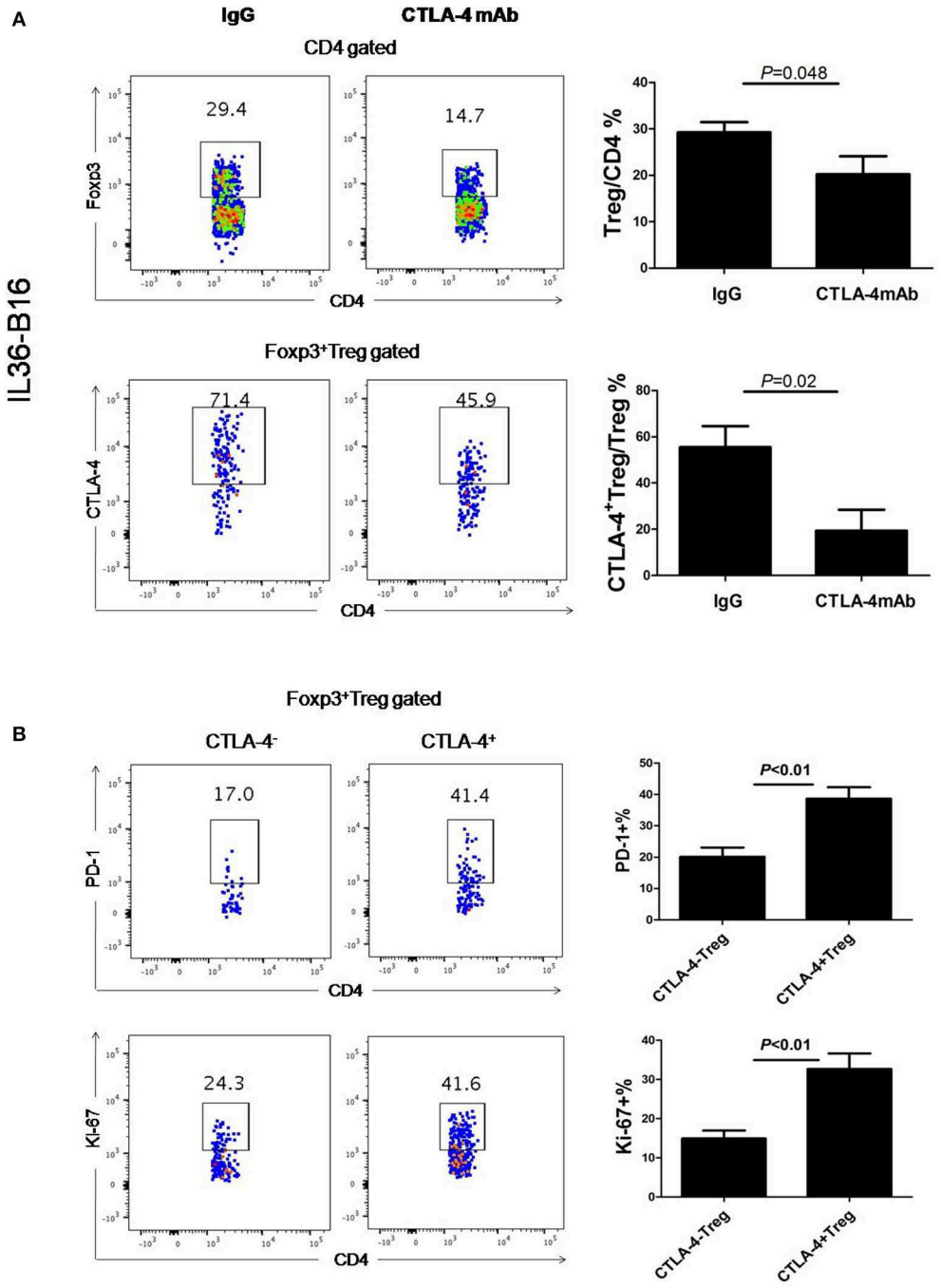

FIGURE 4 | Administration of CTLA-4 mAbs decreased Treg cell population in tumors. **(A)**, Representative flow cytometry plot showing percentages of $Foxp3^{+}CD4^{+}$ TIL and $CTLA\text{-}4^{+}Foxp3^{+}$ TIL in IL-36-B16 tumors after CTLA-4 mAbs treatment ($n = 3$ mice/group). **(B)**, Representative flow cytometry plot showed PD-1 and Ki-67 expression on $CTLA\text{-}4^{+}Foxp3^{+}$ and $CTLA\text{-}4^{-}Foxp3^{+}$ T cell subsets in IL-36-B16 tumors. Data (mean ± SEM) are representative of three independent experiments.

CTLA-4 mAbs Administration Led to Treg Cell Depletion in Tumor Tissues

Consistent with previously data (37), administration of CTLA-4 mAbs after tumor challenge resulted in a reduced frequency of Foxp3$^+$ cells in the B16-IL36 tumor (**Figure 4A**). This is due to the fact that approximately 70% of the tumoral Treg expressed CTLA-4 (**Figure 4A**). Interestingly, CTLA-4$^+$Treg expressed higher levels of inhibitory molecules PD-1 and Ki-67 than their CTLA-4$^-$ counterparts (**Figure 4B**). These data together suggested thatCTLA-4$^+$Treg cells are a more activated Treg subset. Therefore, administration of CTLA-4 mAbs likely resulted in depletion of the activated CTLA-4$^+$Treg subset.

Combination of IL36 and CTLA-4 mAb Resulted in Higher Type 1 Immune Responses in Tumor

In order to further investigate the protective mechanism, we characterized the immune cells from the tumor tissues by flow cytometry. Compared to control tumors, which were infiltrated with low numbers of immune cells, anti-CTLA-4 mAbs treatment alone did not increase the number of CD45$^+$ immune cells (**Figure 5**). Consistent with our prior report, we found that CD45$^+$ immune cells were significantly increased in IL36-B16 tumors when compared to B16 tumors. Combination of IL36 and CTLA-4 mAbs resulted in even greater increases in CD45$^+$ immune cells in tumor (**Figure 5**). Despite increases in the total CD45$^+$ tumor infiltrating immune cells, the percentage of CD4$^+$ and CD8$^+$ T cells were not changed by IL36 or combined administration of IL36 and CTLA-4 mAbs (**Figure 5**). These data suggest that IL36 and CTLA-4 mAbs additively increase the tumor inflammation.

We then examined whether these treatments resulted in alteration of T cell functions and proliferation. Compared to IL36 or CTLA-4 mAbs alone, combination of the two led to further increase of percentages of IFN-γ^+ CD4$^+$ and CD8$^+$ T cells (**Figure 6**). Likewise, combination of IL36 or CTLA-4 mAbs led to an increase of proliferating CD4$^+$ and CD8$^+$ T

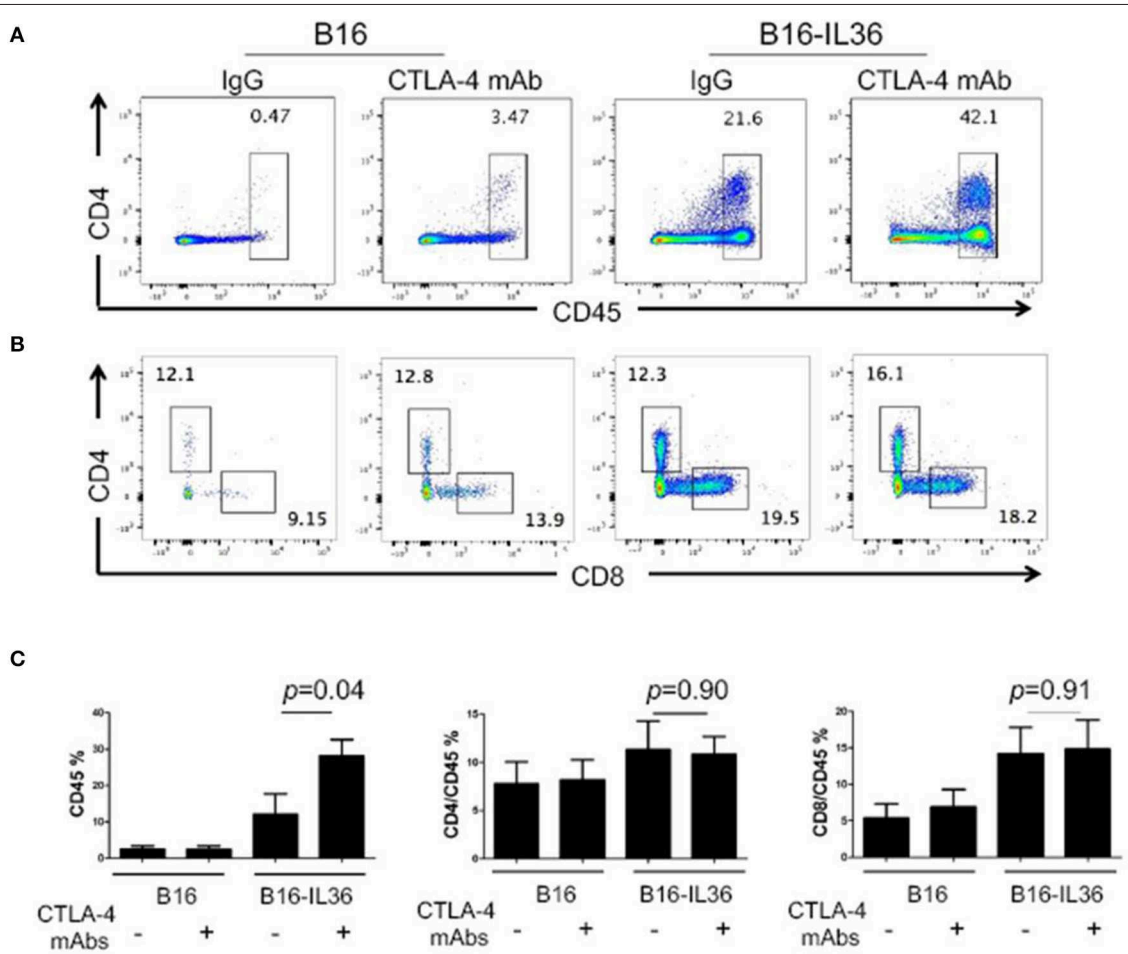

FIGURE 5 | Combination of tumoral expression of IL-36 and anti-CTLA-4 mAbs additively enhanced CD45$^+$immune cell infiltration in the tumor microenvironment. C57BL/6 mice were injected i.d. with 10^4 B16 or B16-IL-36 cells and were subsequently treated with CTLA-4 or control mAbs ($n = 3$ mice/group). Tumors were resected after treatment and subjected to flow cytometry analysis. Representative flow cytometric plots showed CD45$^+$ cells **(A,C)**, CD4$^+$T and CD8$^+$T cells **(B,C)** in tumor. Results are mean ± SEM of 3 independent experiments.

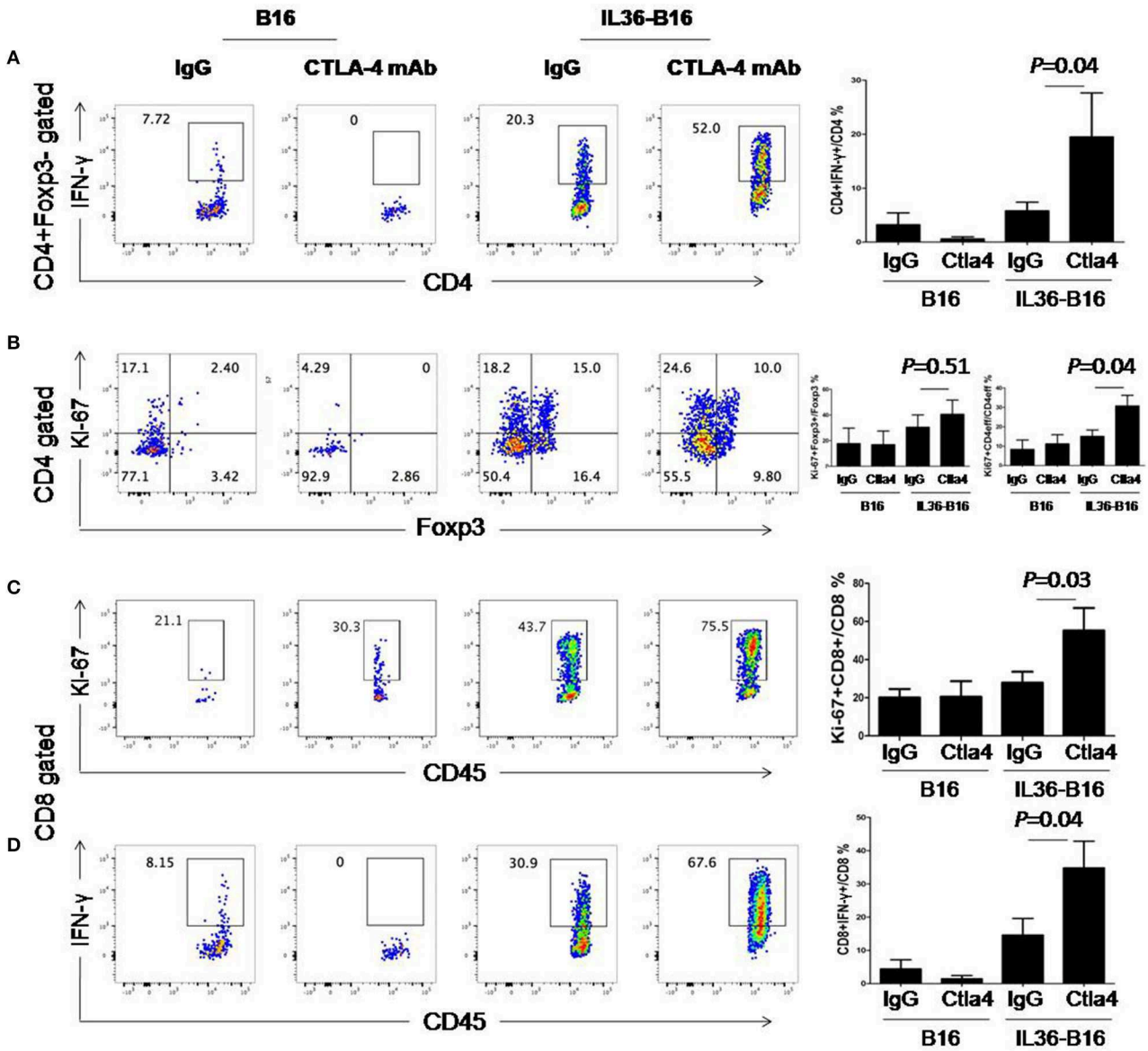

FIGURE 6 | Combination of tumoral expression of IL-36 and anti-CTLA-4 mAbs additively enhanced type 1 immune responses in the tumor microenvironment. C57BL/6 mice were injected i.d. with 10^4 B16 or B16-IL-36 cells and were subsequently treated with CTLA-4 or control mAbs ($n = 3$ mice/group). Tumors were resected after treatment and subjected to flow cytometry analysis. Representative flow cytometric plots showed IFN-γ^+Foxp3$^-$CD4$^+$T cells **(A)**, Ki-67$^+$Foxp3$^-$CD4$^+$T cells **(B)**, Ki-67$^+$CD8$^+$ T cells **(C)**, IFN-γ^+CD8$^+$ T cells **(D)**. Results are mean ± SEM of three independent experiments.

cells (**Figure 6**). These data indicated that IL36 and CTLA-4mAbs additively increased the effector function and expansion of type 1 T cells in the TME.

DISCUSSION

In this study, we found that IL36R was expressed in Treg cells. IL36 promoted Treg proliferation *in vitro* and expansion in tumors *in vivo*. In addition, we showed that CTLA-4 mAbs treatment led to a drastic reduction in Treg in IL36-expressing tumors. Furthermore, we demonstrated that IL36 and CTLA-4 mAbs additively promoted antitumor immune responses and greatly prolonged survival of tumor-bearing mice. Collectively, our data indicate that combination of IL36 and CTLA-4 mAbs is a more effective immunotherapy for tumor than individual treatment with IL36 or CTLA-4 mAbs.

Our study has revealed that IL36 regulates a complex cellular network involving both effector and regulatory T cells. We showed previously that IL36R was expressed on both conventional CD4 and CD8 T cells. We and other have shown that IL36 promotes proliferation and IFN-γ of Th1 and CD8 T cell (14, 16). Besides Th1 cells, IL36 has been shown to promote Th9 differentiation. Interestingly, IL36 was shown to inhibit generation of the induced Treg in culture (32). The role of IL36

on natural Treg is not known. In this study, we showed that Treg also expressed IL36R. In addition, IL36 increased Treg proliferation in culture. Moreover, we observed an increase in the percentage and proliferation of Treg in IL36-expressing tumors when compared to control tumors. These data suggest that IL36 induces a self-limiting mechanism mediated by activation of natural Treg cells to contain immune pathology.

It was initially thought the antitumor mechanism of CTLA-4 mAbs was by removing inhibitory signals in the costimulatory pathway (38, 39). CTLA-4 was established as the first negative checkpoint regulatory molecule expressed on activated conventional $CD4^+$ and $CD8^+$ T cells through a set of experiments using CTLA-4 mAbs (40–43). This concept was further supported by evidence came from analysis of the CTLA-$4^{-/-}$ mice (44–46). CTLA-$4^{-/-}$ mice developed a severe lymphoproliferative disorder and mice die between 18 and 28 days of age. In CTLA-$4^{-/-}$ mice, most of peripheral T cells displayed activated phenotype and secreted effector cytokines and massive lymphocytic infiltration into non-lymphoid tissues are observed. Besides effector T cells, CTLA-4 is also expressed on Treg cells. Ample evidence supports a critical role of CTLA-4 in mediating the function of Treg through downregulating B7/CD28 costimulation (47, 48). Importantly, recent data demonstrated that the antitumor activity of CTLA-4 mAbs seems to be dependent on its Treg-depleting activities (33–35). Therefore, likely both Treg-depletion and reverse of checkpoint inhibition are involved the antitumor function of CTLA-4 mAbs. Our studies support the mechanism of combinatorial effect between IL-36 and CTLA-4 mAbs is at least through Treg. We would expect complete Treg deletion is additive with IL36 treatment and further enhances the antitumor activity of IL36. We have decided to focus on using CTLA4 mAbs in this study due to a clearer pathway for clinical application because there is no other Treg-depletion drug that has been FDA-approved.

Our study suggests that IL-36-based immune therapy of cancer should provide new opportunities for enhancing the immune "checkpoint"-based approach. Our data further demonstrated that depletion of Treg by CTLA-4 mAbs unleashed the power of IL36-mediated tumor immunotherapy. Since CTLA-4 mAbs has been approved for immunotherapy of melanoma and is in clinical trial for combination with PD-1 mAbs (49–51) our data suggest combination of IL36 with CTLA-4 mAbs might be of clinical significance to further increase its efficacy.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this article are not publicly available. Requests to access the datasets should be directed to binfeng@pitt.edu.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institution Animal Care and Use Committee at University of Pittsburgh.

AUTHOR CONTRIBUTIONS

QQ, CC, and BL conceived of the study, participated in its design, coordination, and drafted the manuscript. ZZ, JX, and SL performed 4T1.2 experiments.

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REFERENCES

1. Havel JJ, Chowell D, Chan TA. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nat Rev Cancer.* (2019) 19:133–50. doi: 10.1038/s41568-019-0116-x
2. Camacho LH. CTLA-4 blockade with ipilimumab: biology, safety, efficacy, and future considerations. *Cancer Med.* (2015) 4:661–72. doi: 10.1002/cam4.371
3. Du X, Liu M, Su J, Zhang P, Tang F, Ye P, et al. Uncoupling therapeutic from immunotherapy-related adverse effects for safer and effective anti-CTLA-4 antibodies in CTLA4 humanized mice. *Cell Res.* (2018) 28:433–47. doi: 10.1038/s41422-018-0012-z
4. Cao L, Kulmburg P, Veelken H, Mackensen A, Mezes B, Lindemann A, et al. Cytokine gene transfer in cancer therapy. *Stem Cells.* (1998) 16(Suppl. 1):251–60. doi: 10.1002/stem.5530160831
5. Lord EM, Frelinger JG. Tumor immunotherapy: cytokines and antigen presentation. *Cancer Immunol Immunother.* (1998) 46:75–81. doi: 10.1007/s002620050464
6. Nanni P, Forni G, Lollini PL. Cytokine gene therapy: hopes and pitfalls. *Ann Oncol.* (1999) 10:261–6. doi: 10.1023/A:1008304721889
7. Lu B, Yang M, Wang Q. Interleukin-33 in tumorigenesis, tumor immune evasion, and cancer immunotherapy. *J Mol Med.* (2016) 94:535–43. doi: 10.1007/s00109-016-1397-0
8. Berraondo P, Sanmamed MF, Ochoa MC, Etxeberria I, Aznar MA, Perez-Gracia JL, et al. Cytokines in clinical cancer immunotherapy. *Br J Cancer.* (2019) 120:6–15. doi: 10.1038/s41416-018-0328-y
9. Dwyer CJ, Knochelmann HM, Smith AS, Wyatt MM, Rangel Rivera GO, Arhontoulis DC, et al. Fueling cancer immunotherapy with common gamma chain cytokines. *Front Immunol.* (2019) 10:263. doi: 10.3389/fimmu.2019.00263
10. Gresnigt MS, and van de Veerdonk FL. Biology of IL-36 cytokines and their role in disease. *Semin Immunol.* (2013) 25:458–65. doi: 10.1016/j.smim.2013.11.003
11. Lian LH, Milora KA, Manupipatpong KK, Jensen LE. The double-stranded RNA analogue polyinosinic-polycytidylic acid induces keratinocyte pyroptosis and release of IL-36gamma. *J Invest Dermatol.* (2012) 132:1346–53. doi: 10.1038/jid.2011.482
12. Vigne S, Palmer G, Lamacchia C, Martin P, Talabot-Ayer D, Rodriguez E, et al. IL-36R ligands are potent regulators of dendritic and T cells. *Blood.* (2011) 118:5813–23. doi: 10.1182/blood-2011-05-356873
13. Mutamba S, Allison A, Mahida Y, Barrow P, Foster N. Expression of IL-1Rrp2 by human myelomonocytic cells is unique to DCs and facilitates DC maturation by IL-1F8 and IL-1F9. *Eur J Immunol.* (2012) 42:607–17. doi: 10.1002/eji.201142035
14. Vigne S, Palmer G, Martin P, Lamacchia C, Strebel D, Rodriguez E, et al. IL-36 signaling amplifies Th1 responses by enhancing proliferation and Th1 polarization of naive CD4+ T cells. *Blood.* (2012) 120:3478–87. doi: 10.1182/blood-2012-06-439026
15. Foster AM, Baliwag J, Chen CS, Guzman AM, Stoll SW, Gudjonsson JE, et al. IL-36 promotes myeloid cell infiltration, activation, and inflammatory

activity in skin. *J Immunol.* (2014) 192:6053–61. doi: 10.4049/jimmunol.1301481
16. Wang X, Zhao X, Feng C, Weinstein A, Xia R, Wen W, et al. IL-36gamma transforms the tumor microenvironment and promotes type 1 lymphocyte-mediated antitumor immune responses. *Cancer Cell.* (2015) 28:296–306. doi: 10.1016/j.ccell.2015.07.014
17. Debets R, Timans JC, Homey B, Zurawski S, Sana TR, Lo S, et al. Two novel IL-1 family members, IL-1 delta and IL-1 epsilon, function as an antagonist and agonist of NF-kappa B activation through the orphan IL-1 receptor-related protein 2. *J Immunol.* (2001) 167:1440–6. doi: 10.4049/jimmunol.167.3.1440
18. Blumberg H, Dinh H, Trueblood ES, Pretorius J, Kugler D, Weng N, et al. Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. *J Exp Med.* (2007) 204:2603–14. doi: 10.1084/jem.20070157
19. He Q, Chen HX, Li W, Wu Y, Chen SJ, Yue Q, et al. IL-36 cytokine expression and its relationship with p38 MAPK and NF-kappaB pathways in psoriasis vulgaris skin lesions. *J Huazhong Univ Sci Technolog Med Sci.* (2013) 33:594–9. doi: 10.1007/s11596-013-1164-1
20. Johnston A, Xing X, Wolterink L, Barnes DH, Yin Z, Reingold L, et al. IL-1 and IL-36 are dominant cytokines in generalized pustular psoriasis. *J Allergy Clin Immunol.* (2017) 140:109–20. doi: 10.1016/j.jaci.2016.08.056
21. Tortola L, Rosenwald E, Abel B, Blumberg H, Schafer M, Coyle AJ, et al. Psoriasiform dermatitis is driven by IL-36-mediated DC-keratinocyte crosstalk. *J Clin Invest.* (2012) 122:3965–76. doi: 10.1172/JCI63451
22. Vos JB, van Sterkenburg MA, Rabe KF, Schalkwijk J, Hiemstra PS, Datson NA. Transcriptional response of bronchial epithelial cells to *Pseudomonas aeruginosa*: identification of early mediators of host defense. *Physiol Genomics.* (2005) 21:324–36. doi: 10.1152/physiolgenomics.00289.2004
23. Chustz RT, Nagarkar DR, Poposki JA, Favoreto S Jr, Avila PC, Schleimer RP, et al. Regulation and function of the IL-1 family cytokine IL-1F9 in human bronchial epithelial cells. *Am J Respir Cell Mol Biol.* (2011) 45:145–53. doi: 10.1165/rcmb.2010-0075OC
24. Bachmann M, Scheiermann P, Hardle L, Pfeilschifter J, Muhl H. IL-36gamma/IL-1F9, an innate T-bet target in myeloid cells. *J Biol Chem.* (2012) 287:41684–96. doi: 10.1074/jbc.M112.385443
25. Gresnigt MS, Rosler B, Jacobs CW, Becker KL, Joosten LA, van der Meer JW, et al. The IL-36 receptor pathway regulates *Aspergillus fumigatus*-induced Th1 and Th17 responses. *Eur J Immunol.* (2013) 43:416–26. doi: 10.1002/eji.201242711
26. Pan QZ, Pan K, Zhao JJ, Chen JG, Li JJ, Lv L, et al. Decreased expression of interleukin-36alpha correlates with poor prognosis in hepatocellular carcinoma. *Cancer Immunol Immunother.* (2013) 62:1675–85. doi: 10.1007/s00262-013-1471-1
27. Tsurutani N, Mittal P, St Rose MC, Ngoi SM, Svedova J, Menoret A, et al. Costimulation endows immunotherapeutic CD8 T cells with IL-36 responsiveness during aerobic glycolysis. *J Immunol.* (2016) 196:124–34. doi: 10.4049/jimmunol.1501217
28. Weinstein AM, Chen L, Brzana EA, Patil PR, Taylor JL, Fabian KL, et al. Tbet and IL-36gamma cooperate in therapeutic DC-mediated promotion of ectopic lymphoid organogenesis in the tumor microenvironment. *Oncoimmunology.* (2017) 6:e1322238. doi: 10.1080/2162402X.2017.1322238
29. Chen Y, Sun J, Huang Y, Liu Y, Liang L, Yang D, et al. Targeted codelivery of doxorubicin and IL-36gamma expression plasmid for an optimal chemo-gene combination therapy against cancer lung metastasis. *Nanomedicine.* (2019) 15:129–41. doi: 10.1016/j.nano.2018.09.005
30. Hewitt SL, Bai A, Bailey D, Ichikawa K, Zielinski J, Karp R, et al. Durable anticancer immunity from intratumoral administration of IL-23, IL-36gamma, and OX40L mRNAs. *Sci Transl Med.* (2019) 11:eaat9143. doi: 10.1126/scitranslmed.aat9143
31. Weinstein AM, Giraldo NA, Petitprez F, Julie C, Lacroix L, Peschaud F, et al. Association of IL-36gamma with tertiary lymphoid structures and inflammatory immune infiltrates in human colorectal cancer. *Cancer Immunol Immunother.* (2019) 68:109–20. doi: 10.1007/s00262-018-2259-0
32. Harusato A, Abo H, Ngo VL, Yi SW, Mitsutake K, Osuka S, et al. IL-36gamma signaling controls the induced regulatory T cell-Th9 cell balance via NFkappaB activation and STAT transcription factors. *Mucosal Immunol.* (2017) 10:1455–67. doi: 10.1038/mi.2017.21
33. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, et al. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer Immunol Res.* (2013) 1:32–42. doi: 10.1158/2326-6066.CIR-13-0013
34. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med.* (2013) 210:1695–710. doi: 10.1084/jem.20130579
35. Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang NAS, Andrews MC, et al. Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. *Cell.* (2017) 170:1120–33.e1117. doi: 10.1016/j.cell.2017.07.024
36. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. *Nat Rev Drug Discov.* (2005) 4:581–93. doi: 10.1038/nrd1775
37. Ingram JR, Blomberg OS, Rashidian M, Ali L, Garforth S, Fedorov E, et al. Anti-CTLA-4 therapy requires an Fc domain for efficacy. *Proc Natl Acad Sci USA.* (2018) 115:3912–7. doi: 10.1073/pnas.1801524115
38. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science.* (1996) 271:1734–6. doi: 10.1126/science.271.5256.1734
39. Chambers CA, Kuhns MS, Egen JG, Allison JP. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol.* (2001) 19:565–94. doi: 10.1146/annurev.immunol.19.1.565
40. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity.* (1994) 1:405–13. doi: 10.1016/1074-7613(94)90071-X
41. Kearney ER, Walunas TL, Karr RW, Morton PA, Loh DY, Bluestone JA, et al. Antigen-dependent clonal expansion of a trace population of antigen-specific CD4+ T cells *in vivo* is dependent on CD28 costimulation and inhibited by CTLA-4. *J Immunol.* (1995) 155:1032–6.
42. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med.* (1995) 182:459–65. doi: 10.1084/jem.182.2.459
43. Krummel MF, Sullivan TJ, Allison JP. Superantigen responses and co-stimulation: CD28 and CTLA-4 have opposing effects on T cell expansion *in vitro* and *in vivo*. *Int Immunol.* (1996) 8:519–23. doi: 10.1093/intimm/8.4.519
44. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity.* (1995) 3:541–7. doi: 10.1016/1074-7613(95)90125-6
45. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science.* (1995) 270:985–8. doi: 10.1126/science.270.5238.985
46. Chambers CA, Cado D, Truong T, Allison JP. Thymocyte development is normal in CTLA-4-deficient mice. *Proc Natl Acad Sci USA.* (1997) 94:9296–301. doi: 10.1073/pnas.94.17.9296
47. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science.* (2008) 322:271–5. doi: 10.1126/science.1160062
48. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science.* (2011) 332:600–3. doi: 10.1126/science.1202947
49. Ott PA, Hodi FS, Robert C. CTLA-4 and PD-1/PD-L1 blockade: new immunotherapeutic modalities with durable clinical benefit

in melanoma patients. *Clin Cancer Res.* (2013) 19:5300–9. doi: 10.1158/1078-0432.CCR-13-0143

50. Ribas A, Kefford R, Marshall MA, Punt CJ, Haanen JB, Marmol M, et al. Phase III randomized clinical trial comparing tremelimumab with standard-of-care chemotherapy in patients with advanced melanoma. *J Clin Oncol.* (2013) 31:616–22. doi: 10.1200/JCO.2012.44.6112
51. Wolchok JD, Hodi FS, Weber JS, Allison JP, Urba WJ, Robert C, et al. Development of ipilimumab: a novel immunotherapeutic approach for the treatment of advanced melanoma. *Ann NY Acad Sci.* (2013) 1291:1–13. doi: 10.1111/nyas.12180

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REVIEW
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TNFSF14: LIGHTing the Way for Effective Cancer Immunotherapy

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Tumor necrosis factor superfamily member 14 (LIGHT) has been in pre-clinical development for over a decade and shows promise as a modality of enhancing treatment approaches in the field of cancer immunotherapy. To date, LIGHT has been used to combat cancer in multiple tumor models where it can be combined with other immunotherapy modalities to clear established solid tumors as well as treat metastatic events. When LIGHT molecules are delivered to or expressed within tumors they cause significant changes in the tumor microenvironment that are primarily driven through vascular normalization and generation of tertiary lymphoid structures. These changes can synergize with methods that induce or support anti-tumor immune responses, such as checkpoint inhibitors and/or tumor vaccines, to greatly improve immunotherapeutic strategies against cancer. While investigators have utilized multiple vectors to LIGHT-up tumor tissues, there are still improvements needed and components to be found within a human tumor microenvironment that may impede translational efforts. This review addresses the current state of this field.

Keywords: tumor necrosis factor superfamily member 14 (TNFSF14), LIGHT, CD258, cancer immunotherapy, tumor microenvironment

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INTRODUCTION

Cancer remains as one of the most significant medical challenges for human beings and accounts for 1 out of every 6 deaths (1). In the United States it is estimated that 39% of people will develop cancer, and given the aging population we can assume that the cancer incidence rate will remain a significant burden for humankind (2). As such, the need for new therapies that target cancer remains at the epicenter of medical research. Compared to current standards of care such as chemotherapy, surgery, and radiation; immunotherapies have brought to the table a new set of tools and strategies that have expanded the scope of cancer treatment options. The main goals of cancer immunotherapy can be broken down into three separate approaches: generation of *de novo* anti-cancer immune responses, bolstering/amplification of ongoing immune responses, and the prevention of cancers from shutting down/manipulating anti-tumor responses. While there has been significant progress made in our understanding of how tumors evade immune-based interventions, the generation of specific anti-tumor responses alone remains to be insufficient to clear solid tumors as T cells often fail to traffic to and infiltrate tumor sites. These shortcomings are compounded by the immunosuppressive nature of the tumor microenvironment itself and by associated immune suppressor cells, which makes it difficult for even checkpoint inhibitor-based therapies to be entirely effective. This review addresses how Tumor Necrosis Factor Superfamily

member 14 (TNFSF14/CD258), otherwise known as LIGHT, could potentially be used to counteract these aforementioned shortcomings.

Intratumoral LIGHT expression is highly effective in driving anti-tumor immune responses while also eliciting significant changes to the tumor microenvironment. In this review, we will summarize the known effects that LIGHT has on tumor immunobiology and highlight the findings, expression vectors strategies, and immunotherapy combinations researchers have used over the years to "LIGHT-up" the tumor microenvironment as well as provide considerations that should be taken into account for future LIGHT-based vector designs.

LIGHT

LIGHT (homologous to **l**ymphotoxin, exhibits **i**nducible expression and competes with Herpes Simplex Virus **g**lycoprotein D for **H**erpes Virus Entry Mediator, a receptor expressed by **T** cells), is a protein primarily expressed on activated T cells, activated Natural Killer (NK) cells, and immature dendritic cells (DC) (3, 4). Approximately 29 kD in size, LIGHT can function as both a soluble and cell surface-bound type II membrane protein and must be in its homotrimeric form to interact with its two primary functional receptors: Herpes Virus Entry Mediator (HVEM) and Lymphotoxin-β Receptor (LTβR) (3, 5, 6). LIGHT signaling through these receptors have distinct functions that are cell-type dependent, but interactions with both types of receptors have immune-related implications in tumor biology.

LIGHT-HVEM interaction is responsible for a majority of the immune-stimulating properties of LIGHT (7). Expressed on lymphocytes, NK cells, smooth muscle, and epithelium, HVEM serves as an important T cell costimulatory agent leading to activation, proliferation, and survival (4, 8, 9). HVEM can also trigger NK cells to produce IFNγ through LIGHT-mediated nuclear factor-κB (NFκB) RelA/p50 signaling (7, 8, 10, 11). Furthermore, LIGHT produced by tumor-sensing NK cells is a critical component in the NK-DC crosstalk that occurs in the priming of *de novo* anti-tumor responses (12). To activate T effector cells, HVEM is necessary for LIGHT's costimulatory effect in a CD28-independent T cell to T cell manner (4). Such pro-inflammatory HVEM interactions increase the expression of Th1 cytokines IFNγ and GM-CSF. As such, LIGHT-HVEM mediated T cell co-stimulation and NK-DC crosstalk both play a vital role in generating anti-tumor immunity in a therapeutic context (13).

The other receptor, LTβR, is found on the surface of epithelial, stromal, immature DC, and myeloid cells, but not on lymphocytes (14). During normal biological development LIGHT-LTβR interactions have been identified as a component of lymphoid structure development and maintenance (15). In the context of anti-tumor immune support, LIGHT-LTβR signaling has a wide range of roles that span from influencing cancer cells' susceptibility to immune responses, functioning to repair chaotic tumor vasculature, and to supporting effector cells cell trafficking to and infiltration into tumors. If we consider LIGHT-HVEM the primary driver of anti-tumor immune activity, then LIGHT-LTβR functions to build-out, repair, and maintain the infrastructure needed to support these immune responses.

EFFECTS OF LIGHT ON TUMOR BIOLOGY

The expression of LIGHT within tumors has profound effects on host immune responses against tumors and remodeling of the TME (**Figure 1**). In addition to sensitizing tumor cells to IFNγ-mediated apoptosis, LIGHT induces tumor vasculature normalization, and drives the formation of high endothelial venules which subsequently encourage generation of tertiary lymphoid structures (TLS) (16–18). In addition, LIGHT stimulates effector cell function and antitumor $CD8^+$ T cell entry into tumors, which aids in establishing anti-tumoral memory (19–22). In this section, we will summarize the critical roles that LIGHT can play in remodeling tumor architecture while also driving anti-tumor immunity.

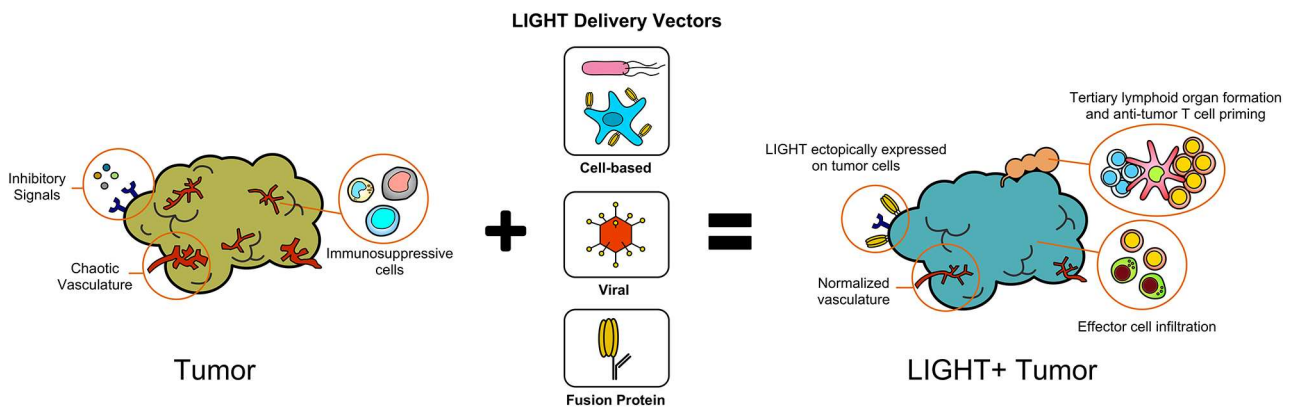

FIGURE 1 | Delivery of LIGHT through different methods modifies the tumor microenvironment.

Tumor Vascular Normalization Occurs With Targeted LIGHT Treatments

Healthy vasculature allows constant blood flow, oxygen perfusion, and circulation of immune cells; features which tumor vasculature lacks (23). As tumor cells divide, hypoxic pockets develop within the tumor mass. Tumor cells within these hypoxic zones respond by overexpressing pro-angiogenic factors such as members of the vascular endothelial growth factor (VEGF) family to modify nearby stromal cells (endothelial cells, pericytes, vascular smooth muscle cells, fibroblasts) (24, 25). Through this mechanism tumors accommodate their increasing metabolic requirements by extending existing healthy blood vessels through angiogenesis, however tumor cells can also undergo trans-differentiation into an endothelial-like phenotype. They use this phenotypic switching mechanism to create a blood circulation network through a process known as vascular mimicry (26). Furthermore, production of VEGF-protein family members downregulates effector lymphocyte attachment molecules such as intracellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs), supporting direct elimination of effector cells by T regulatory (Treg) cells through FAS/FASL interactions due to changes in the ratio of effector to suppressor cells, a problem that is further exacerbated by the tumor recruiting suppressive cells through the release of molecules such as CCL28 and CCL2 (Treg and myeloid derived suppressor cell chemo-attractants) (27–29). The combined effect of this less perfuse, transfigured vascular basement membrane, and enhanced level of suppressive cell recruitment creates a significant barrier that prevents effector cell infiltration and function (24).

When the vasculature within a tumor is normalized toward a non-pathogenic phenotype, it has been shown to alleviate hypoxia, intra-tumoral pressure, and improves almost all treatment options whether they are immunotherapy, radiotherapy, or chemotherapies (30). LIGHT-based therapies developed by Johansson-Percival et al. were found to combat tumor vasculature not by destroying tumor stroma, but by reversing their pathogenic effects through vascular normalization (21–23). Although the exact mechanisms remain unclear, evidence has shown LIGHT, when delivered as a fusion protein linked to a tumor vascular targeting peptide (VTP), can normalize intra-tumoral blood vessels via increased expression of the LTβR dependent pericyte contractile markers ICAM-1, VCAM-1, smooth muscle actin (SMA), calponin, and caldesmon (21–23). Such contractile markers make tighter cellular junctions, thus creating a less "leaky" phenotype. The intra-tumoral macrophages activated by LIGHT were found to secrete TGF-β, which induced a vascular smooth muscle cell (vSMC) phenotype switch and increased adhesion maker expression in a Rho-kinase dependent manner (21). TGF-β is also responsible for the differentiation of pericytes, explaining the increased pericyte contractile markers found in LIGHT treated tumors (8, 31, 32). The researchers hypothesized that the secreted TGF-β was unable to cause pro-tumor effects because macrophage-secreted TGF-β is released so closely to stromal cells that it is unable to diffuse throughout the tumor. Overall, this LIGHT-driven vascular normalization has been shown to improve pericyte and/or vSMC markers in murine pancreatic insulinoma, breast cancer, glioblastoma, melanoma, Lewis Lung carcinoma (LLC), and metastatic B16 melanoma models, in addition to human glioblastoma and astrocytoma models, rendering them more susceptible to cancer treatments (21–23, 33).

Presence of LIGHT Gives Rise to a More Inflamed Tumor Microenvironment

The tumor microenvironment (TME) is the result of biological crosstalk between stromal, cancer, and immune cells within a given tissue (34, 35). Based on the heterogeneity that tumors develop, they take on a sub classification of being either "hot" or "cold," which is ultimately dictated by the ability of the immune system to recognize, infiltrate, and function against their growth. The inability to recognize cold tumors arises from a set of compounding factors in the TME: lack of response to tumor antigens, homing, maturation, and function of antigen presenting cells, or failure of effector responses to infiltrate or function against tumors due to immunosuppressive cell populations [reviewed in Bonaventura et al. (36)]. Immunologically cold tumors are populated with a myriad of immune suppressor cells such as tumor-associated macrophages (TAM), Tregs, and myeloid derived suppressor cells (MDSC). Each of these populations can impair effector cell generation or function through either direct interaction, the production of immunosuppressive cytokines (e.g., TGF-β and IL-10), or a combination of the two (37–39). Additionally, the tumor itself may influence effector cell function through the expression of signals such as PD-L1 in response to exposure of elevated IFNγ levels (39, 40).

As a hallmark of successful LIGHT therapy designs, researchers have repeatedly shown a LIGHT-dependent increase in intratumoral IFNγ, TNFα, MIG, and IP-10, all of which are indicative of effector cell responses and are cytokines that profile tumors as "hot" (10, 19, 20). This direct change of the tumors immunological phenotype is driven by the effects LIGHT exerts on the TME. First, the normalizing of the tumor vasculature through LIGHT-LTβR signaling described in the last section allows for decreased levels of tumor hypoxia and intra-tumoral pressure. This directly limits the tumors ability to recruit and generate immune suppressor cells within the TME while at the same time encouraging effector cell recruitment and ability to function. Second, LIGHT-LTβR signaling is responsible for creating High Endothelial Venules (HEVs), the primary sites for leukocyte extravasation into target tissues (15). Cells that make up LIGHT-driven HEV structures express mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1) as well as peripheral node addressins (PNAd), which bind L-selectin on lymphocytes and facilitate effector cell entry (22). Additionally, the production of CCL21 by the HEV endothelial cells recruits naïve CCR7+ T cells to tumor sites, which are essential in the generation of anti-tumor immunity (15, 41). Given that the presence of tumor infiltrating lymphocytes (TIL) have been posited with better outcomes in cancer models such as melanoma, breast, ovarian, colorectal, and lung (42, 43), LIGHT-LTβR induced construction of HEVs are clinically relevant. Staining for MECA 79 expression (a PNAd marker) to reveal *de*

novo generation of these structures has occurred in pancreatic, breast, and glioblastoma models that have undergone LIGHT-based treatments (24). This increased lymphoid penetration also leads to other structural changes in the tumor microenvironment, such as the development of TLSs (16).

Johansson-Percival et al. demonstrated that one of the indicators of successful anti-tumor immunity in LIGHT therapy was the formation of TLSs within a rat insulin promoter (RIP)1-Tag5 pancreatic insulinoma mouse model (22, 44). TLS (sometimes referred to as tertiary lymphoid organs), are a subset of lymphoid tissues that arise in sites of chronic inflammation and have been associated with autoimmune diseases (45). TLS are similar to secondary lymphoid organs (SLO), such as lymph nodes, as they are made up of compartmentalized T and B cell germinal centers. But unlike SLOs, TLSs are not encapsulated and lack afferent lymph vessels, allowing them to directly interact with external antigens within the immediate environment (8, 45). TLS are formed in association with the overexpression of lymphocyte and DC chemokines CCL21 and CCL19 as well as HEV markers MAdCAM1 and PNAd: all of which are dependent on LTβR signaling (15, 45, 46). Once formed, TLS within or around tumors function as sites for processing tumor antigens, which are released by dying tumor cells or those that are killed by NK cells activated through LIGHT-HVEM interactions (8, 46). Presentation of these tumor antigens by activated DC then results in the generation and expansion of tumor-specific $CD8^+$ effector cells, the population of cells responsible for LIGHT-driven tumor regression. Importantly, mice that received LIGHT-based therapy rejected distal tumors and were resistant to re-challenges after primary tumor clearance, highlighting the existence of memory responses (10, 19, 20, 47, 48). It is worth noting that outside of LIGHT-based therapies the *de novo* generation of TLS in murine tumor models has been limited [reviewed in (49, 50)]. Importantly, however, the presence of TLS has been associated with positive clinical outcomes in a large number of human cancers and has can serve as a biomarker for successful immunotherapeutic approaches (51, 52).

Taken together, LIGHT-mediated correction of tumor vasculature along with generation of sites for lymphocyte entry and effector cell expansion can work together to shift a cold TME to one that is immunologically hot and may be susceptible to proper therapy interventions. In the next section we review the approaches that investigators have taken to deliver LIGHT to tumor sites as well highlight successful combination approaches.

LIGHT DELIVERY SYSTEMS

Over the past two decades, researchers have investigated the use of gene transduction, adoptive transfers, viral vectors, and peptides as delivery systems for LIGHT therapies. Through the development and utilization of these vectors, researchers have been able to piece together how LIGHT mediates its anti-tumor effects and the extent to which it may be combined with other treatment options to overcome challenging tumor models. The details, including the vector, tumor models tested, delivery route, results, and whether the vectors were used in combination with another modality of treatment are summarized in **Table 1**.

Gene Transduction to Create LIGHT-Expressing Tumors

Researchers first assessed LIGHT's *in vivo* abilities to reduce cancer burden via direct transfection of tumor cells and adoptively transferring them into mice. Ag104Ld is an aggressive fibrosarcoma that is unaffected by most immunotherapies, and has been a popular model for testing the effects of LIGHT (57). Papers by Yu et al. and Fan et al. demonstrated that Ag104Ld tumors expressing LIGHT are rejected in an immunocompetent setting and mice become resistant to re-challenge with the parental Ag104Ld cell line at 8-weeks post initial tumor clearance (10, 20, 48). Intratumoral anti-tumor T cell priming and expansion, most likely due to TLS formation, was seen by Yu et al. through the usage of a T cell receptor (TCR)-transgenic cell line, 2C, that can only be activated by interaction with the Ld antigen directly on Ag104Ld tumors. In a primary Ag104Ld or Ag104Ld $LIGHT^+$ tumor challenge followed by a distal Ag104Ld challenge, Yu et al. found up to 100x more intra-tumoral 2C T cells in distal metastasis sites of Ag104Ld $LIGHT^+$ mice than the control (20). This influx of 2C T cells in distal tumor sites demonstrated direct Ag104Ld T cell priming via LIGHT stimulation within primary tumors.

Fan et al. established an additional layer in the priming process that highlights the vital role of LIGHT-HVEM interaction in the Ag104Ld $LIGHT^+$ model. They found that LIGHT activates NK cells through the HVEM receptor, leading to the activation of $CD8^+$ cells in an IFNγ-dependent manner (10). Furthermore, Zhai et al. forced LIGHT expression in MDA-MB-231 human breast carcinoma cells via a retroviral vector and found significant inhibition of tumor growth when compared to controls (58). Qiao et al. transfected CT26 colorectal cancer models to express LIGHT constitutively, resulting in a stunted tumor growth, lower distal liver metastasis burden, and prevention of tumor take in re-challenge events (47). Further investigation showed a marked increase in tumor infiltrating lymphocytes, increased IFNγ levels, and higher concentrations of the DC activation marker CD86 in LIGHT-expressing tumors when compared to control (47). With the literature establishing that the expression of LIGHT by tumors leads to a CD8-dependent clearance of the primary tumor and generates long-lasting memory against LIGHT-negative parental cell lines, additional methods were sought to specifically deliver LIGHT to tumor sites or force express LIGHT in tumors.

Adenovirus Vectors

The use of replication-deficient viruses, such as the adeno-associated virus, have been used to generate potent immunogenic responses with minimal toxicity (59, 60). Given their promiscuity in cell binding, as well as their ability to force cellular expression of target proteins, they represent viable vectors for the forced expression of proteins of interest within targeted sites (8). Following *in vitro* success of adenoviruses carrying LIGHT (Ad-LIGHT) to inhibit tumor growth, researchers have been able to elicit robust anti-tumor responses *in vivo* (61). In 2007, Yu et al.

TABLE 1 | Systems used to deliver LIGHT to tumors with tumor models, delivery routes, combinations, and summary outcomes.

Platform	Construct	Cancer models	Administration route	Combinations	Effects on tumor	References
Bacterial	*Salmonella typhimurium* expressing LIGHT	D2F2 breast carcinoma	IV	-	Reduced tumor volume	(53)
		CT-26	IV	-	Reduced tumor volume	(53)
		Lewis lung carcinoma	IV	-	Reduced tumor volume	(53)
Viral	Adenovirus delivery of LIGHT (Ad-LIGHT)	Ag104Ld	Intratumoral injection (IT)	-	Primary tumor elimination and distal tumor clearance	(20)
		4T1	IT	-	Primary tumor clearance and elimination of metastatic events	(20)
		MC38	IT	-	Primary tumor elimination	(20)
		B16-SIY	IT	-	Primary tumor elimination	(20)
		A20	IT	-	Primary tumor clearance and protection from rechallenge	(54)
		C3.43 HPV16 cervical cancer	IT	Tumor Vaccine (VRP w/HPV16E7)	Tumor size regression, combination showed enhanced efficacy. Therapeutic treatment provided protection from rechallenge	(19)
		TRAMPC2 prostate cancer	IT	Tumor Peptide vaccine	Tumor size regression, LIGHT reduced effect of Tregs. Enhanced anti-tumor effects with combination treatments	(55)
Cells	Mesenchymal stem cells expressing LIGHT	TUBO mammary cancer	IV	-	LTbR and CD8-dependent prophylactic protection against tumor challenge as well as therapeutic efficacy against day-7 tumor growth	(56)
Fusion Protein	LIGHT linked to a vascular targeting peptide (LIGHT-VTP)	Pancreatic insulinoma (RIP1-Tag5)	IV	Tumor vaccine (Tag-CpG-ODN) + Anti-PD-1 & CTLA-4	Significant reduction in tumor burden of mice receiving full combination treatments. LIGHT-therapies enhanced tumor vaccine + dual checkpoint blockade	(22)
		Lewis lung Carcinoma	IV	Anti-PD-1 & CTLA-4	Reduced tumor burden in mice receiving triple therapy compared to controls. No necrosis in tumors indicating improved vasculature	(22)
		NFpp10-Glioblastoma multiforme (GBM)	IV	Anti-VEGF + Anti-PD-1	HEV formation, vasculature normalization, enhanced levels of CD3+ cell infiltration into tumors, upregulations of granzyme B, and reduction in Tregs	(23)
		B16 melanoma	IV	Anti-PD-1	Vascular normalization in both primary and lung metastases. Reduced number of metastases accompanied by TLO and HEV formation at metastatic sites. Sensitization to anti-PD-1 treatments	(33)
Fusion Protein	Three copies of LIGHT linked to scFv targeting EGFR (anti-EGFR-hmLIGHT)	Ag104Ld	IV	Anti-PD-L1	Significant reduction in tumor size within combination group showing the ability to overcome checkpoint-blockade resistance	(57)
		MC38	IV	Anti-PD-L1	Tumor clearance with combination therapy	(57)

showed rejection of established tumors as well as distal metastases with an intra-tumoral adenovirus injection that resulted in the expression of LIGHT (Ad-LIGHT) (20). The tumor models that have been successfully treated through this modality include the aggressive fibrosarcoma Ag104Ld and mammary carcinoma 4T1 cell lines. Within the tumors, researchers found increased

tumor specific $CD8^+$ T cell infiltration and high levels of IFNγ and TNFα when compared to an adenovirus control and no treatment. Our group has specifically shown successful therapy through adenovirus delivery of LIGHT within the HPV-transformed cervical cancer model C3.43 as well as in the TRAMP-C2 prostate cancer model (19, 55). While this vector was able to show gene-transduction of LIGHT and subsequent anti-tumor responses, it relies on direct injection of the vector into primary tumor sites and lacks the ability to be delivered systemically due to target cell binding promiscuity.

Cell-Based Vectors for LIGHT

Adoptive cell transfer methods offer a unique approach to delivering a payload to tumor sites. One such method of LIGHT-delivery that has been investigated took advantage of the tumor targeting properties of *Salmonella.* Specific strains of this bacterium have been shown to colonize and grow within tumors; most likely due to the tumors' hypoxic nature. Low oxygen regions within the TME can nurture the growth of facultative anaerobes and, given the ease in which genetic material of *Salmonella* can be manipulated, this vector has seen success as a drug or payload delivery system in multiple mouse models and has even been used in clinical trials as a method to target IL-2 to metastatic melanoma (53, 62, 63). As a proof of concept study, Loeffler et al. designed an attenuated strain of *Salmonella typhirium* that expresses LIGHT and took advantage of the tumor-targeting characteristics to deliver the vector (53). BALB/c mice bearing 14-day D2F2 breast cancer tumors revealed significant reduction in tumor growth for mice that received Sal+LIGHT, an effect that was also observed in the metastasized D2F2 model through reductions in metastatic scores and lung tumor burden. Additionally, the authors were able to show that multiple treatments with i.v. Sal+LIGHT were effective 9-days post subcutaneous (s.c.) challenge in the CT-26 colon carcinoma model. The group then showed this therapeutic efficacy extended to other tumor models through significant reductions in tumor burden in C57BL/6 mice that had been challenged s.c. with LLC cells 7-days prior to the start of treatment. Mechanistic involvement of the LIGHT receptors HVEM and LTβR was indicated by including anti-LTβR and anti-HVEM antibodies in control groups that led to the loss of the anti-tumor effects of the vector (53).

Other methods that rely on engineered cells to target and deliver LIGHT to tumors have focused on the mesenchymal stem cell (MSC) population. Taking advantage of cancer endothelial cells' ability to attract MSCs (64, 65), Zou et al. developed a technique that utilizes MSCs expressing LIGHT, which resulted in LIGHT-expressing MSC trafficking to tumor sites (56). By inducing LIGHT expression in MSCs through lentiviral delivery of the vector *ex vivo*, Zou et al. utilized MSC-LIGHT in both a prophylactic (injection of MSC-LIGHT 13 days before challenge) and therapeutic manner (injection of MSC-LIGHT 7 days post challenge) in the TUBO mammary cancer model (56). Profound increases in the intra-tumoral $CD4^+$ and $CD8^+$ T cells were found in both treatment schedules as they repressed tumor growth compared to the controls. While tumors were unable to establish growth in the prophylactic setting, therapeutic intervention only controlled tumor growth (64). Interestingly, removing $CD4^+$ T cells ablated MSC-LIGHT's prophylactic efficacy while removing $CD8^+$ T cells removed MSC-LIGHT's therapeutic efficacy, suggesting different roles for each subset within this method of therapy (56). Anti-tumor memory was subsequently demonstrated through the inability of TUBO re-challenged mice to grow tumors. Importantly, this group established the role of LIGHT-LTβR signaling in tumor clearance by showing that an anti-LTβR antibody prevented therapeutic functioning of MSC-LIGHT, directly implicating LIGHT-LTβR interactions.

Antibody and Peptide Fusion Proteins

Rather than using direct injection of virus, tumor homing cells, or bacteria, LIGHT has also been developed in recombinant peptide and fusion protein platforms that aim to combine the immunostimulatory effects of LIGHT with the ability to target tumor tissues. These moieties have used different strategies of fusing LIGHT to short tumor vasculature targeting peptide sequences (VTPs) or single-chain Fragment variable (scFv) antibodies that have historically been used as stand-alone treatments of cancer. In this manner, researchers can not only induce an anti-tumor immune response through LIGHT function, but also benefit from the targeting capabilities of VTP- or scFv-fused LIGHT moieties.

VTPs have been developed in such a manner that they preferentially interact with tumor angiogenic vessels, which are fundamentally different from healthy vasculature. VTP-fusion protein delivery has shown some limited success in clinical trials when the amino acid sequence CNGRCG (known as NGR) was fused to human TNFα. Specifically, when used in refractory solid tumors such as ovarian cancer in combination with doxorubicin, there was a measurable improvement in patient survival (66–72). Researchers sought to use this feature in an effort to deliver LIGHT systemically, thus eliminating the need for invasive delivery strategies such as intra-tumoral injection (32). Through the use of phage libraries, short peptide sequences were discovered that specifically target tumor angiogenic vasculature. Each VTP contains distinct tumor-specific vascular targets, potentially allowing functional delivery of LIGHT in multiple tumor types. As an example of the specificity that VTPs have in binding aberrant vasculature, the amino acid sequence CGKRK has been shown to preferentially bind tumor blood vessels as opposed to healthy vasculature, theoretically via heparan sulfates, phosphatidylserine, VEGF related extracellular matrices, or a combination of the three (69, 73).

Cancer models demonstrating the utility of LIGHT fused to CGKRK (LIGHT-CGKRK) include murine glioblastoma, murine pancreatic insulinoma, human astrocytoma and human grade I meningioma (22, 23, 74). Recently, the LIGHT-CGKRK fusion peptide was utilized to establish vascular normalization and improved perfusion in s.c. LLC and B16 melanoma models. Interestingly, the authors also showed that intravasation of LLC tumor cells into the bloodstream was decreased through early LIGHT- CGKRK interventions while establishment of visual lung metastatic events could be reduced with late LIGHT-CGKRK therapy that begins after surgical removal of primary tumors.

They took this research even further by establishing that vascular normalization could occur within B16 lung metastases, and that LIGHT-CGKRK therapy was able to induce TLS formation at metastatic sites while also reducing metastatic burden (33). Another VTP with amino acid sequence CRGRRST (abbreviated RGR within the literature), binds specifically to platelet-derived growth factor receptor β (PDGFRβ), and is also successful in targeting LIGHT to murine pancreatic insulinoma and murine breast cancer. One additional benefit of the RGR peptide is that it also has the ability to bind to human glioblastoma tumor sections, which is an important finding for future translational efforts (21–23, 75, 76).

Additional approaches to engineering LIGHT-peptide proteins include fusing multiple monomers of LIGHT to tumor targeting antibodies. Tang et al. found success in this method by combining three units of modified LIGHT (hmLIGHT) that are able to bind and signal through both murine and human receptors with a functional chain (Fc) of immunoglobulin G (IgG) recognizing Epidermal Growth Factor Receptor (EGFR) (57). The product (anti-EGFR-hmLIGHT) was used to treat mice bearing Ag104Ld fibrosarcoma and MC38 colon adenocarcinoma (57). Anti-EGFR-hmLIGHT treatment induced complete tumor regression of small (7-days post s.c. injection) Ag104Ld-EGFR$^+$ primary tumors as well as protected against re-challenge, but had little success as a monotherapy when the parental Ag104Ld tumor line was not over-expressing EGFR or tumors were older than 14 days (57). Tang et al. also reaffirmed that treatment was T cell dependent based on the 300 - 500% increase of intratumoral CD8$^+$ T cells as well as increased IFNγ and TNFα levels. LIGHT-LTβR interaction was found to be the principle driver for this therapy due to the complete loss of anti-tumor effects when an anti-LTβR Ig was included.

LIGHT COMBINATION THERAPIES

Although some groups have shown that LIGHT can be used to a reasonable extent as a monotherapy, the most effective LIGHT-based interventions have come out of combinatory LIGHT-vector treatments together with either therapeutic vaccinations or checkpoint inhibitors.

LIGHT + Therapeutic Vaccinations

Tumor vaccines are therapeutic vaccines that are given with the intent to stimulate an immune response directed against identified or neo-antigens occurring within tumors (77). Alone, they have not historically resulted in significant improvements to survival outcomes, however combining them with LIGHT has been shown to enhance effector cell function within tumors (42). To this end, multiple groups have demonstrated the benefits of combining therapeutic vaccines with LIGHT-based therapies (19, 22, 55).

Within the TRAMP-C2 prostate cancer tumor model, our group was able to show that the combination treatment of Ad-LIGHT with a prostate tumor associated antigen tumor vaccine (PSCA trivax) performed much better than Ad-LIGHT treatment alone (55). Mechanistically it was shown that Ad-LIGHT + PSCA trivax combination therapy increased intra-tumoral CD8$^+$ T cells and prevented the maturation and functioning of intra-tumoral Tregs, ultimately creating a more immunologically hot tumor (55). Additional work from our group has illustrated the efficacy of Ad-LIGHT therapy in conjunction with anti-tumoral vaccines against human papillomavirus type (HPV)-transformed cancers (19). Within the HPV16 transformed tumor line, C3.43, the combination treatment of intra-tumoral Ad-LIGHT and HPV16-E7 expressing Venezuelan equine encephalitis virus replicon particles (VRP) as a tumor vaccine yielded significant regression of established tumors compared to Ad-LIGHT alone or HPV-VRP alone. This combination treatment lead to increased intra-tumoral anti-E7 CD8$^+$ T cells as well as the presence of intra-tumoral inflammatory cytokines and activation markers IFNγ, IL-1a, MIG, and MIP-2. Furthermore, mice treated with Ad-LIGHT and the VRP vaccine were able to generate memory as 75% of mice remained tumor-free upon contralateral tumor re-challenge post-surgical resection of primary tumors (19).

LIGHT + Checkpoint Inhibitors

Given that LIGHT-mediated changes to the TME facilitate the shift from a cold to a hot tumor phenotype, IFNγ levels also rise. Exposure to increased IFNγ mediates tumor upregulation of PD-L1 as a way to shut down immune responses (40). Taking advantage of this, researchers have found synergy with the combination treatment of LIGHT and anti-PD-L1 antibodies (78, 79). Tang et al. found that the combination treatment of anti-PD-L1 antibodies with anti-EGFR-hmLIGHT conferred the best treatment outcomes within their cancer models (57). As tumor size increased, LIGHT based therapy lost efficacy due to the tumor's elevated PD-L1 levels. Inhibiting PD-L1 allowed for further functioning of T cells via anti-EGFR-hmLIGHT within the Ag104Ld and MC38 tumor models, inducing complete rejection of established tumors in a therapeutic setting. Notably, monotherapy with either anti-EGFR-hmLIGHT construct or PD-L1 checkpoint inhibitor was ineffective at eliminating tumors (57, 80).

Combining LIGHT-VTP with anti-PD-1 and anti-CTLA-4 checkpoint inhibitors (dual checkpoint therapy) has also shown efficacy in combatting the tumor microenvironment (22). By utilizing LIGHT-VTP (CGKRK) and dual checkpoint therapy, Johansson-Percival et al. were able to confer a 6-week survival advantage along with vascular normalization and production of TLSs containing HEVs using the murine pancreatic insulinoma model (22). Furthermore, by including an anti-Tag-CpG-ODN tumor vaccine within Tag$^+$ tumors, the triple treatment regimen elicited a 13-week survival improvement compared to LIGHT-VTP and dual checkpoint therapy (22). This was the first time LIGHT-VTP was utilized with both checkpoint inhibitors as well as a tumor vaccine. More recently, the effectiveness of LIGHT-VTP combined with an anti-PD-1 antibody was shown to dramatically improve long-term survival of mice bearing metastatic B16 lung tumors through significant reductions in quantifiable metastatic events. In line with the findings from primary tumor studies, the researchers found dramatic increases in HEV and TLS formation in metastatic tumors (33). Given the multiple promising outcomes from this work, further study of checkpoint inhibitor and tumor vaccine combination therapies are necessary for the future of LIGHT-based cancer therapies.

LIGHT-BASED THERAPY CONSIDERATIONS AND FUTURE DIRECTIONS

The autoimmune consequence of LIGHT overexpression is loss of peripheral tolerance, which has several implications for disorders such as inflammatory bowel disease, diabetes, asthma, graft vs. host disease, and even atherosclerosis (14, 81–87). Foundational studies have clearly shown that transgenic mice constitutively expressing LIGHT have a hyper-activated T cell population putting them at increased risk for spontaneous autoimmunity hallmarked by severe infiltration of effector cells within peripheral tissues. Because of this, it is of the utmost importance that measures be taken to ensure proper targeting of LIGHT vectors to desired sites and apply controlled dosages to prevent initiation of self-recognition. This may be especially important in future studies of LIGHT therapies if blood cancers are considered. One specific example of how this may be an issue is in the case of multiple myeloma (MM). Patients experiencing osteolytic lesions as a result of disease progression have shown significantly elevated levels of circulating LIGHT driven by activated $CD8^+$ T cell, $CD14^+$ monocytes, and neutrophils. When overproduced in MM patients, LIGHT synergizes with receptor activator of nuclear factor kappa-B ligand (RANKL) in driving osteoclast formation, resulting in a breakdown of long bones within the immediate areas of bone marrow (88). These recent results suggest that there are going to be certain cancers or individuals with autoimmune-related diseases that do not qualify for LIGHT-based immunotherapies as it may exacerbate disease manifestations.

Methods that will be most effective at minimizing harm from systemic LIGHT treatment will be enhanced targeted delivery to or controlled release of LIGHT treatments within target tissues. Forced expression of LIGHT by tumor tissues through the usage of viral vectors (e.g., Adenovirus) will almost certainly face issues of neutralizing immunity generated against the vector itself after the first treatment, therefore multiple serotypes or vectors will be required for this route of therapy to be effective. Other studies discussed within this review have shown the evolution of delivering LIGHT to the tumor from LIGHT-expressing bacterial cells to fusion protein constructs that have bimodal functions at tumor sites. These targeting strategies have shown great progress as they have the additional benefit of being combined, often successfully, with other immunotherapeutic interventions. It remains, however, that a significant factor needs to be considered in the application of LIGHT-based therapies in humans: decoy receptor 3 (DcR3). DcR3, also known as tumor necrosis factor receptor superfamily member 6b, is a functional attenuator of LIGHT signaling that is found in the genomes of humans but is absent in both mice and rats (89). While DcR3 serum levels are nearly undetectable in healthy individuals, those experiencing inflammatory disease and/or cancer see significant increases within the bloodstream. In the context of cancer, DcR3 has been found to be upregulated in astrocytoma and gliomas (90, 91). Furthermore, a positive correlation exists between expression of DcR3 and the severity of pancreatic carcinoma, colorectal cancer, breast, cervical, and ovarian cancers (89–93). These findings suggest that even if LIGHT therapy does move into the clinic, its effects may be dampened by a $DcR3^+$ TME. As such, future methods that examine forced expression of DcR3 within mouse tumor models may serve to more appropriately represent a human TME and set up LIGHT-based therapies for a successful clinical transition, specifically informing whether a combination with an anti-DcR3 antibody would prevent attenuation of LIGHT functions.

LIGHT has not yet been used as a treatment in clinical trials. As such, translational studies that aim to move these constructs into humans will need to be considerate of the following: usage of human instead of murine LIGHT, validation of successful homo-trimerization of targeted LIGHT expression or recombinant LIGHT constructs, and verification of biological activity both *in vivo* and *in vitro* through the usage of anti-HVEM, anti-LTβR, soluble DcR3, or a combination of the three. The construct created by Tang et al. has made significant strides in these areas as they linked three repeats of a reengineered form of LIGHT that has an affinity for and shows functionality with both mouse and human receptors, a feature lacking in other LIGHT-based designs. Additionally, they were able to show that their fusion protein had direct effects on the activation of relevant immune cell populations *in vitro*. These controls have been lacking in other peptide-based delivery vectors and should not be overlooked. It is the opinion of our group that this design is superior to its predecessors and is more likely to produce a functional LIGHT construct that will function in both mouse and human studies.

Future approaches such as engineered exosomes containing LIGHT decorated with tumor-targeting moieties may provide a method of shielding LIGHT protein from degradation within the blood stream while allowing transport to tumor sites for delivery. This method may effectively deliver LIGHT payloads to tumor sites. However, there may be significant hurdles in maintaining surface expression on target cells as it is not known to what degree exosome endocytosis will occur in different tumor models. Additionally, in combination with next generation chimeric antigen receptor (CAR) T cells, delivery of LIGHT to the TME may finally provide a breakthrough in CAR-T infiltration and activity in solid tumors. Either an effective delivery system combined with CAR-T therapy or generation of an armored CAR-T cell that produces a LIGHT-related construct once engaged with its target should be investigated. Strategies such as this will also see benefits from the generation of neo-antigen responses by the patient's immune system as LIGHT stimulates NK cell activity, DC antigen presentation, and T cell expansion (12). Given the efficacy of CAR-T therapies for blood-based cancers it may be required to include a negative feedback switch, such as a tyrosine-kinase inhibitor (ex. dasatanib), alongside treatment to control responses or the usage of lower-affinity TCRs (94, 95).

CONCLUSIONS

Despite improvements in immunotherapy, eliciting a robust anti-tumor immune response with the ability to infiltrate clear established tumors remains a challenge. LIGHT-based therapies

have shown great effectiveness in reducing tumor burden and generating lasting anti-tumor memory by modifying the TME through normalizing tumor vasculature, driving TLS neo-genesis at tumor sites that contain HEV, and dramatically improving effector TIL infiltration. The insights that LIGHT research has provided in the recent decades warrants continued investigation of its use as a cancer therapeutic, especially since the effects of LIGHT-supported immunotherapy combinations can be seen in both the primary and metastatic settings of multiple tumor types when the vector for delivery functions as intended.

AUTHOR CONTRIBUTIONS

JS conceptualized, outlined, researched, wrote, generated the figure & table, and handled the review/editing process of the manuscript. MO, RP, DF, DD, and WK provided research, input on figure & table, and editing of the manuscript.

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REFERENCES

1. Collaborators GBDCoD. Global, regional, and national age-sex specific mortality for 264 causes of death, 1980-2016: a systematic analysis for the global burden of disease study (2016). *Lancet*. (2017) 390:1151–210. doi: 10.1016/S0140-6736(17)32152-9
2. Howlader NA, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, et al. (Eds). *SEER Cancer Statistics Review, 1975-2016*. Bethesda, MD: National Cancer Institute (2019).
3. Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, et al. LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity*. (1998) 8:21–30. doi: 10.1016/S1074-7613(00)80455-0
4. Wang J, Lo JC, Foster A, Yu P, Chen HM, Wang Y, et al. The regulation of T cell homeostasis and autoimmunity by T cell-derived LIGHT. *J Clin Invest*. (2001) 108:1771–80. doi: 10.1172/JCI200113827
5. Rooney IA, Butrovich KD, Glass AA, Borboroglu S, Benedict CA, Whitbeck JC, et al. The lymphotoxin-beta receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J Biol Chem*. (2000) 275:14307–15. doi: 10.1074/jbc.275.19.14307
6. Granger SW, Butrovich KD, Houshmand P, Edwards WR, Ware CF. Genomic characterization of LIGHT reveals linkage to an immune response locus on chromosome 19p13.3 and distinct isoforms generated by alternate splicing or proteolysis. *J Immunol*. (2001) 167:5122–8. doi: 10.4049/jimmunol.167.9.5122
7. Steinberg MW, Cheung TC, Ware CF. The signaling networks of the herpesvirus entry mediator (TNFRSF14) in immune regulation. *Immunol Rev*. (2011) 244:169–87. doi: 10.1111/j.1600-065X.2011.01064.x
8. Yu P, Fu YX. Targeting tumors with LIGHT to generate metastasis-clearing immunity. *Cytokine Growth Factor Rev*. (2008) 19:285–94. doi: 10.1016/j.cytogfr.2008.04.004
9. Ware CF, Sedy JR. TNF superfamily networks: bidirectional and interference pathways of the herpesvirus entry mediator (TNFSF14). *Curr Opin Immunol*. (2011) 23:627–31. doi: 10.1016/j.coi.2011.08.008
10. Fan Z, Yu P, Wang Y, Wang Y, Fu ML, Liu W, et al. NK-cell activation by LIGHT triggers tumor-specific CD8+ T-cell immunity to reject established tumors. *Blood*. (2006) 107:1342–51. doi: 10.1182/blood-2005-08-3485
11. Ward-Kavanagh LK, Lin WW, Sedy JR, Ware CF. The TNF receptor superfamily in co-stimulating and co-inhibitory responses. *Immunity*. (2016) 44:1005–19. doi: 10.1016/j.immuni.2016.04.019
12. Holmes TD, Wilson EB, Black EV, Benest AV, Vaz C, Tan B, et al. Licensed human natural killer cells aid dendritic cell maturation via TNFSF14/LIGHT. *Proc Natl Acad Sci USA*. (2014) 111:E5688–96. doi: 10.1073/pnas.1411072112
13. Zhang H, Chen X, Li D, Cui L, Li X, Ye X, et al. DcR3 promotes hepatoma cell migration by downregulating E-cadherin expression. *Oncol Rep*. (2017) 38:377–83. doi: 10.3892/or.2017.5685
14. Giles DA, Zahner S, Krause P, Van Der Gracht E, Riffelmacher T, Morris V, et al. The Tumor Necrosis Factor Superfamily Members TNFSF14 (LIGHT), lymphotoxin beta and lymphotoxin beta receptor interact to regulate intestinal inflammation. *Front Immunol*. (2018) 9:2585. doi: 10.3389/fimmu.2018.02585
15. Lu TT, Browning JL. Role of the lymphotoxin/LIGHT system in the development and maintenance of reticular networks and vasculature in lymphoid tissues. *Front Immunol*. (2014) 5:47. doi: 10.3389/fimmu.2014.00047
16. Garcia-Hernandez ML, Uribe-Uribe NO, Espinosa-Gonzalez R, Kast WM, Khader SA, Rangel-Moreno J. A unique cellular and molecular microenvironment is present in tertiary lymphoid organs of patients with spontaneous prostate cancer regression. *Front Immunol*. (2017) 8:563. doi: 10.3389/fimmu.2017.00563
17. Zhang M, Guo R, Zhai Y, Yang D. LIGHT sensitizes IFNgamma-mediated apoptosis of MDA-MB-231 breast cancer cells leading to down-regulation of anti-apoptosis Bcl-2 family members. *Cancer Lett*. (2003) 195:201–10. doi: 10.1016/S0304-3835(03)00148-4
18. Zhang MC, Liu HP, Demchik LL, Zhai YF, Yang DJ. LIGHT sensitizes IFN-gamma-mediated apoptosis of HT-29 human carcinoma cells through both death receptor and mitochondria pathways. *Cell Res*. (2004) 14:117–24. doi: 10.1038/sj.cr.7290210
19. Kanodia S, Da Silva DM, Karamanukyan T, Bogaert L, Fu YX, Kast WM. Expression of LIGHT/TNFSF14 combined with vaccination against human papillomavirus type 16 E7 induces significant tumor regression. *Cancer Res*. (2010) 70:3955–64. doi: 10.1158/0008-5472.CAN-09-3773
20. Yu P, Lee Y, Wang Y, Liu X, Auh S, Gajewski TF, et al. Targeting the primary tumor to generate CTL for the effective eradication of spontaneous metastases. *J Immunol*. (2007) 179:1960–8. doi: 10.4049/jimmunol.179.3.1960
21. Johansson-Percival A, Li ZJ, Lakhiani DD, He B, Wang X, Hamzah J, et al. Intratumoral LIGHT restores pericyte contractile properties and vessel integrity. *Cell Rep*. (2015) 13:2687–98. doi: 10.1016/j.celrep.2015.12.004
22. Johansson-Percival A, He B, Li ZJ, Kjellen A, Russell K, Li J, et al. *De novo* induction of intratumoral lymphoid structures and vessel normalization enhances immunotherapy in resistant tumors. *Nat Immunol*. (2017) 18:1207–17. doi: 10.1038/ni.3836
23. He B, Jabouille A, Steri V, Johansson-Percival A, Michael IP, Kotamraju VR, et al. Vascular targeting of LIGHT normalizes blood vessels in primary brain cancer and induces intratumoural high endothelial venules. *J Pathol*. (2018) 245:209–21. doi: 10.1002/path.5080
24. Johansson-Percival A, He B, Ganss R. Immunomodulation of tumor vessels: it takes two to Tango. *Trends Immunol*. (2018) 39:801–14. doi: 10.1016/j.it.2018.08.001
25. Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, et al. Normalization of the vasculature for treatment of cancer and other diseases. *Physiol Rev*. (2011) 91:1071–121. doi: 10.1152/physrev.00038.2010
26. Maniotis AJ, Folberg R, Hess A, Seftor EA, Gardner LM, Pe'er J, et al. Vascular channel formation by human melanoma cells in vivo

and in vitro: vasculogenic mimicry. *Am J Pathol*. (1999) 155:739–52. doi: 10.1016/S0002-9440(10)65173-5
27. Yang J, Yan J, Liu B. Targeting VEGF/VEGFR to modulate antitumor immunity. *Front Immunol*. (2018) 9:978. doi: 10.3389/fimmu.2018.00978
28. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang LP, et al. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. *Nature*. (2011) 475:226–30. doi: 10.1038/nature10169
29. Chun E, Lavoie S, Michaud M, Gallini CA, Kim J, Soucy G, et al. CCL2 promotes colorectal carcinogenesis by enhancing polymorphonuclear myeloid-derived suppressor cell population and function. *Cell Rep*. (2015) 12:244–57. doi: 10.1016/j.celrep.2015.06.024
30. Li W, Quan YY, Li Y, Lu L, Cui M. Monitoring of tumor vascular normalization: the key points from basic research to clinical application. *Cancer Manag Res*. (2018) 10:4163–72. doi: 10.2147/CMAR.S174712
31. Joshi NS, Akama-Garren EH, Lu Y, Lee DY, Chang GP, Li A, et al. Regulatory T cells in tumor-associated tertiary lymphoid structures suppress anti-tumor t cell responses. *Immunity*. (2015) 43:579–90. doi: 10.1016/j.immuni.2015.08.006
32. Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. *Cell*. (2011) 146:873–87. doi: 10.1016/j.cell.2011.08.039
33. He B, Johansson-Percival A, Backhouse J, Li J, Lee GYF, Hamzah J, et al. Remodeling of metastatic vasculature reduces lung colonization and sensitizes overt metastases to immunotherapy. *Cell Rep*. (2020) 30:714–24 e5. doi: 10.1016/j.celrep.2019.12.013
34. Hui L, Chen Y. Tumor microenvironment: sanctuary of the devil. *Cancer Lett*. (2015) 368:7–13. doi: 10.1016/j.canlet.2015.07.039
35. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. *Nature*. (2013) 501:338–45. doi: 10.1038/nature12625
36. Bonaventura P, Shekarian T, Alcazer V, Valladeau-Guilemond J, Valsesia-Wittmann S, Amigorena S, et al. Cold tumors: a Therapeutic challenge for immunotherapy. *Front Immunol*. (2019) 10:168. doi: 10.3389/fimmu.2019.00168
37. Wu T, Dai Y. Tumor microenvironment and therapeutic response. *Cancer Lett*. (2017) 387:61–8. doi: 10.1016/j.canlet.2016.01.043
38. Allavena P, Germano G, Marchesi F, Mantovani A. Chemokines in cancer related inflammation. *Exp Cell Res*. (2011) 317:664–73. doi: 10.1016/j.yexcr.2010.11.013
39. Umansky V, Blattner C, Gebhardt C, Utikal J. The role of Myeloid-Derived Suppressor Cells (MDSC) in cancer progression. *Vaccines*. (2016) 4:e4040036. doi: 10.3390/vaccines4040036
40. Garcia-Diaz A, Shin DS, Moreno BH, Saco J, Escuin-Ordinas H, Rodriguez GA, et al. Interferon receptor signaling pathways regulating PD-L1 and PD-L2 expression. *Cell Rep*. (2017) 19:1189–201. doi: 10.1016/j.celrep.2017.04.031
41. von Andrian UH, Mackay CR. T-cell function and migration. two sides of the same coin. *N Engl J Med*. (2000) 343:1020–34. doi: 10.1056/NEJM200010053431407
42. Abastado JP. The next challenge in cancer immunotherapy: controlling T-cell traffic to the tumor. *Cancer Res*. (2012) 72:2159–61. doi: 10.1158/0008-5472.CAN-11-3538
43. Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene*. (2010) 29:1093–102. doi: 10.1038/onc.2009.416
44. Dieu-Nosjean MC, Giraldo NA, Kaplon H, Germain C, Fridman WH, Sautes-Fridman C. Tertiary lymphoid structures, drivers of the anti-tumor responses in human cancers. *Immunol Rev*. (2016) 271:260–75. doi: 10.1111/imr.12405
45. Aloisi F, Pujol-Borrell R. Lymphoid neogenesis in chronic inflammatory diseases. *Nat Rev Immunol*. (2006) 6:205–17. doi: 10.1038/nri1786
46. Neyt K, Perros F, GeurtsvanKessel CH, Hammad H, Lambrecht BN. Tertiary lymphoid organs in infection and autoimmunity. *Trends Immunol*. (2012) 33:297–305. doi: 10.1016/j.it.2012.04.006
47. Qiao G, Qin J, Kunda N, Calata JF, Mahmud DL, Gann P, et al. LIGHT elevation enhances immune eradication of colon cancer metastases. *Cancer Res*. (2017) 77:1880–91. doi: 10.1158/0008-5472.CAN-16-1655
48. Yu P, Lee Y, Liu W, Chin RK, Wang J, Wang Y, et al. Priming of naive T cells inside tumors leads to eradication of established tumors. *Nat Immunol*. (2004) 5:141–9. doi: 10.1038/ni1029
49. Colbeck EJ, Ager A, Gallimore A, Jones GW. Tertiary lymphoid structures in cancer: drivers of antitumor immunity, immunosuppression, or bystander sentinels in disease? *Front Immunol*. (2017) 8:1830. doi: 10.3389/fimmu.2017.01830
50. Sautes-Fridman C, Petitprez F, Calderaro J, Fridman WH. Tertiary lymphoid structures in the era of cancer immunotherapy. *Nat Rev Cancer*. (2019) 19:307–25. doi: 10.1038/s41568-019-0144-6
51. Dieu-Nosjean MC, Goc J, Giraldo NA, Sautes-Fridman C, Fridman WH. Tertiary lymphoid structures in cancer and beyond. *Trends Immunol*. (2014) 35:571–80. doi: 10.1016/j.it.2014.09.006
52. Goc J, Fridman WH, Sautes-Fridman C, Dieu-Nosjean MC. Characteristics of tertiary lymphoid structures in primary cancers. *Oncoimmunology*. (2013) 2:e26836. doi: 10.4161/onci.26836
53. Loeffler M, Le'Negrate G, Krajewska M, Reed JC. Attenuated salmonella engineered to produce human cytokine LIGHT inhibit tumor growth. *Proc Natl Acad Sci USA*. (2007) 104:12879–83. doi: 10.1073/pnas.0701959104
54. Hu G, Liu Y, Li H, Zhao D, Yang L, Shen J, et al. Adenovirus-mediated LIGHT gene modification in murine B-cell lymphoma elicits a potent antitumor effect. *Cell Mol Immunol*. (2010) 7:296–305. doi: 10.1038/cmi.2010.15
55. Yan L, Da Silva DM, Verma B, Gray A, Brand HE, Skeate JG, et al. Forced LIGHT expression in prostate tumors overcomes Treg mediated immunosuppression and synergizes with a prostate tumor therapeutic vaccine by recruiting effector T lymphocytes. *Prostate*. (2015) 75:280–91. doi: 10.1002/pros.22914
56. Zou W, Zheng H, He TC, Chang J, Fu YX, Fan W. LIGHT delivery to tumors by mesenchymal stem cells mobilizes an effective antitumor immune response. *Cancer Res*. (2012) 72:2980–9. doi: 10.1158/0008-5472.CAN-11-4216
57. Tang H, Wang Y, Chlewicki LK, Zhang Y, Guo J, Liang W, et al. Facilitating T Cell infiltration in tumor microenvironment overcomes resistance to PD-L1 blockade. *Cancer Cell*. (2016) 29:285–96. doi: 10.1016/j.ccell.2016.02.004
58. Zhai Y, Guo R, Hsu TL, Yu GL, Ni J, Kwon BS, et al. LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *J Clin Invest*. (1998) 102:1142–51. doi: 10.1172/JCI3492
59. Tahtinen S, Gronberg-Vaha-Koskela S, Lumen D, Merisalo-Soikkeli M, Siurala M, Airaksinen AJ, et al. Adenovirus improves the efficacy of adoptive T-cell therapy by recruiting immune cells to and promoting their activity at the tumor. *Cancer Immunol Res*. (2015) 3:915–25. doi: 10.1158/2326-6066.CIR-14-0220-T
60. Cerullo V, Koski A, Vaha-Koskela M, Hemminki A. Chapter eight–Oncolytic adenoviruses for cancer immunotherapy: data from mice, hamsters, and humans. *Adv Cancer Res*. (2012) 115:265–318. doi: 10.1016/B978-0-12-398342-8.00008-2
61. Li J, Shen F, Wu D, Wei LX, Wang YZ, Shi LH, et al. Expression level of Bcl-XL critically affects sensitivity of hepatocellular carcinoma cells to LIGHT-enhanced and interferon-gamma-induced apoptosis. *Oncol Rep*. (2007) 17:1067–75. doi: 10.3892/or.17.5.1067
62. Zheng JH, Min JJ. targeted cancer therapy using engineered salmonella typhimurium. *Chonnam Med J*. (2016) 52:173–84. doi: 10.4068/cmj.2016.52.3.173
63. Zhou S, Gravekamp C, Bermudes D, Liu K. Tumour-targeting bacteria engineered to fight cancer. *Nat Rev Cancer*. (2018) 18:727–43. doi: 10.1038/s41568-018-0070-z
64. Bergfeld SA, DeClerck YA. Bone marrow-derived mesenchymal stem cells and the tumor microenvironment. *Cancer Metastasis Rev*. (2010) 29:249–61. doi: 10.1007/s10555-010-9222-7
65. Weis SM, Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med*. (2011) 17:1359–70. doi: 10.1038/nm.2537
66. Gregorc V, De Braud FG, De Pas TM, Scalamogna R, Citterio G, Milani A, et al. Phase I study of NGR-hTNF, a selective vascular targeting agent, in combination with cisplatin in refractory solid tumors. *Clin Cancer Res*. (2011) 17:1964–72. doi: 10.1158/1078-0432.CCR-10-1376
67. Lorusso D, Scambia G, Amadio G, di Legge A, Pietragalla A, De Vincenzo R, et al. Phase II study of NGR-hTNF in combination with doxorubicin in relapsed ovarian cancer patients. *Br J Cancer*. (2012) 107:37–42. doi: 10.1038/bjc.2012.233

68. Zucali PA, Simonelli M, De Vincenzo F, Lorenzi E, Perrino M, Bertossi M, et al. Phase I and pharmacodynamic study of high-dose NGR-hTNF in patients with refractory solid tumours. *Br J Cancer*. (2013) 108:58–63. doi: 10.1038/bjc.2012.506
69. Johansson A, Hamzah J, Ganss R. License for destruction: tumor-specific cytokine targeting. *Trends Mol Med*. (2014) 20:16–24. doi: 10.1016/j.molmed.2013.10.002
70. Corti A, Pastorino F, Curnis F, Arap W, Ponzoni M, Pasqualini R. Targeted drug delivery and penetration into solid tumors. *Med Res Rev*. (2012) 32:1078–91. doi: 10.1002/med.20238
71. Rangel R, Sun Y, Guzman-Rojas L, Ozawa MG, Sun J, Giordano RJ, et al. Impaired angiogenesis in aminopeptidase N-null mice. *Proc Natl Acad Sci U S A*. (2007) 104:4588–93. doi: 10.1073/pnas.0611653104
72. Curnis F, Sacchi A, Borgna L, Magni F, Gasparri A, Corti A. Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). *Nat Biotechnol*. (2000) 18:1185–90. doi: 10.1038/81183
73. Hoffman JA, Giraudo E, Singh M, Zhang L, Inoue M, Porkka K, et al. Progressive vascular changes in a transgenic mouse model of squamous cell carcinoma. *Cancer Cell*. (2003) 4:383–91. doi: 10.1016/S1535-6108(03)00273-3
74. Agemy L, Friedmann-Morvinski D, Kotamraju VR, Roth L, Sugahara KN, Girard OM, et al. Targeted nanoparticle enhanced proapoptotic peptide as potential therapy for glioblastoma. *Proc Natl Acad Sci USA*. (2011) 108:17450–5. doi: 10.1073/pnas.1114518108
75. Johansson A, Hamzah J, Payne CJ, Ganss R. Tumor-targeted TNF α stabilizes tumor vessels and enhances active immunotherapy. *Proc Natl Acad Sci USA*. (2012) 109:7841–6. doi: 10.1073/pnas.1118296109
76. Joyce JA, Laakkonen P, Bernasconi M, Bergers G, Ruoslahti E, Hanahan D. Stage-specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis. *Cancer Cell*. (2003) 4:393–403. doi: 10.1016/S1535-6108(03)00271-X
77. Patel A, Kaufman HL, Disis ML. Next generation approaches for tumor vaccination. *Chin Clin Oncol*. (2017) 6:19. doi: 10.21037/cco.2017.02.04
78. Blank C, Brown I, Peterson AC, Spiotto M, Iwai Y, Honjo T, et al. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. *Cancer Res*. (2004) 64:1140–5. doi: 10.1158/0008-5472.CAN-03-3259
79. Allen E, Jabouille A, Rivera LB, Lodewijckx I, Missiaen R, Steri V, et al. Combined antiangiogenic and anti-PD-L1 therapy stimulates tumor immunity through HEV formation. *Sci Transl Med*. (2017) 9:aak9679. doi: 10.1126/scitranslmed.aak9679
80. Woo SR, Corrales L, Gajewski TF. Innate immune recognition of cancer. *Annu Rev Immunol*. (2015) 33:445–74. doi: 10.1146/annurev-immunol-032414-112043
81. Zheng QY, Cao ZH, Hu XB, Li GQ, Dong SF, Xu GL, et al. LIGHT/IFN-gamma triggers beta cells apoptosis via NF-kappaB/Bcl2-dependent mitochondrial pathway. *J Cell Mol Med*. (2016) 20:1861–71. doi: 10.1111/jcmm.12876
82. Wang Y, Zhu M, Yu P, Fu YX. Promoting immune responses by LIGHT in the face of abundant regulatory T cell inhibition. *J Immunol*. (2010) 184:1589–95. doi: 10.4049/jimmunol.0901582
83. Kim WJ, Kang YJ, Suk K, Park JE, Kwon BS, Lee WH. Comparative analysis of the expression patterns of various TNFSF/TNFRSF in atherosclerotic plaques. *Immunol Invest*. (2008) 37:359–73. doi: 10.1080/08820130802123139
84. Doherty TA, Soroosh P, Khorram N, Fukuyama S, Rosenthal P, Cho JY, et al. The tumor necrosis factor family member LIGHT is a target for asthmatic airway remodeling. *Nat Med*. (2011) 17:596–603. doi: 10.1038/nm.2356
85. Herro R, Croft M. The control of tissue fibrosis by the inflammatory molecule LIGHT (TNF Superfamily member 14). *Pharmacol Res*. (2016) 104:151–5. doi: 10.1016/j.phrs.2015.12.018
86. Tamada K, Shimozaki K, Chapoval AI, Zhu G, Sica G, Flies D, et al. Modulation of T-cell-mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway. *Nat Med*. (2000) 6:283–9. doi: 10.1038/73136
87. da Silva Antunes R, Mehta AK, Madge L, Tocker J, Croft M. TNFSF14 (LIGHT) exhibits inflammatory activities in lung fibroblasts complementary to IL-13 and TGF-beta. *Front Immunol*. (2018) 9:576. doi: 10.3389/fimmu.2018.00576
88. Brunetti G, Rizzi R, Storlino G, Bortolotti S, Colaianni G, Sanesi L, et al. LIGHT/TNFSF14 as a new biomarker of bone disease in multiple myeloma patients experiencing therapeutic regimens. *Front Immunol*. (2018) 9:2459. doi: 10.3389/fimmu.2018.02459
89. Jiang M, Lin X, He R, Lin X, Liang L, Tang R, et al. Decoy receptor 3 (DcR3) as a biomarker of tumor deterioration in female reproductive cancers: a meta-analysis. *Med Sci Monit*. (2016) 22:1850–7. doi: 10.12659/MSM.896226
90. Huang S, Chen G, Dang Y, Chen LH. Overexpression of DcR3 and its significance on tumor cell differentiation and proliferation in glioma. *Sci World J*. (2014) 2014:605236. doi: 10.1155/2014/605236
91. Lin CK, Ting CC, Tsai WC, Chen YW, Hueng DY. A tissue microarray study of toll-like receptor 4, decoy receptor 3, and external signal regulated kinase 1/2 expressions in astrocytoma. *Indian J Pathol Microbiol*. (2016) 59:294–300. doi: 10.4103/0377-4929.188122
92. Zhou J, Song S, Li D, He S, Zhang B, Wang Z, et al. Decoy receptor 3 (DcR3) overexpression predicts the prognosis and pN2 in pancreatic head carcinoma. *World J Surg Oncol*. (2014) 12:52. doi: 10.1186/1477-7819-12-52
93. Zong L, Chen P, Wang DX. Death decoy receptor overexpression and increased malignancy risk in colorectal cancer. *World J Gastroenterol*. (2014) 20:4440–5. doi: 10.3748/wjg.v20.i15.4440
94. Mestermann K, Giavridis T, Weber J, Rydzek J, Frenz S, Nerreter T, et al. The tyrosine kinase inhibitor dasatinib acts as a pharmacologic on/off switch for CAR T cells. *Sci Transl Med*. (2019) 11:aau5907. doi: 10.1126/scitranslmed.aau5907
95. Ying Z, Huang XF, Xiang X, Liu Y, Kang X, Song Y, et al. A safe and potent anti-CD19 CAR T cell therapy. *Nat Med*. (2019) 25:947–53. doi: 10.1038/s41591-019-0421-7

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Inhibition of Histone Deacetylase (HDAC) Enhances Checkpoint Blockade Efficacy by Rendering Bladder Cancer Cells Visible for T Cell-Mediated Destruction

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Inhibitory checkpoint blockade therapy is an immunomodulatory strategy that results in the restoration of T cell functions, and its efficacy depends on the recognition of tumor cells for destruction. Considering the factors at play, one could propose that anti-tumor responses will not occur if tumor cells are immunologically invisible to T cells. In this study, we tested a strategy based on the modulation of cancer cell's immunovisibility through HDAC inhibition. In a model (heterotopic and orthotopic) of mouse urothelial bladder cancer, we demonstrated that the use of intratumoral or intravesical HDACi in combination with systemic anti-PD-1 was effective at inducing curative responses with durable anti-tumor immunity capable of preventing tumor growth at a distal site. Mechanistically, we determined that protective responses were dependent on CD8 cells, but not NK cells. Of significance, in an *in vitro* human model, we found that fully activated T cells fail at killing bladder cancer cells unless tumor cells were pretreated with HDACi. Complementary to this observation, we found that HDACi cause gene deregulation, that results in the upregulation of genes responsible for mediating immunorecognition, *NKG2D* ligands and *HSP70*. Taken together, these data indicate that HDAC inhibition results in the elimination of the tumor cell's "invisibility cloak" that prevents T cells from recognizing and killing them. Finally, as checkpoint blockade therapy moves into the adjuvant setting, its combined use with locally administrated HDACi represents a new approach to be included in our current therapeutic treatment toolbox.

Keywords: HDAC, bladder cancer, T cells, NKG2D, anti-PD1, immune evasion

INTRODUCTION

Bladder cancer is the sixth most common malignancy in the United States, and it is estimated that 81,190 patients received a new diagnosis of bladder cancer in 2018 (1). Approximately 80% of these patients will be found to have superficial bladder tumors at the time of diagnosis (2). Following transurethral resection, the gold standard adjuvant therapy for patients with intermediate and

high risk non-muscle invasive bladder cancer is intravesical Bacillus-Calmette Guerin (BCG) (3). While BCG has been shown to reduce the risk of recurrence and delay disease progression, ∼50% of patients will fail to respond to therapy (4–6). Further intravesical therapy options are limited in patients with BCG refractory and recurrent non-muscle invasive bladder cancer, and many of these patients will go on to require radical cystectomy or chemoradiotherapy (4–7). Therefore, the investigation into novel approaches in the management of high-grade, noninvasive bladder cancer is imperative.

Checkpoint blockade derives from the immune system's anti-tumor capabilities through the blocking of inhibitory signals that prevent the proper function of T cells (8). Currently, the use of monoclonal antibodies targeting cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), PD-1, and Program Cell Death Ligand 1 (PD-L1) has remained limited to unresectable, locally advanced and metastatic disease. The recent advent of checkpoint blockade immunotherapy in the treatment of urothelial cell carcinoma has provided new avenues for therapy in patients with urothelial cell carcinoma (9). Checkpoint blockade alone has provided a needed therapeutic venue for patients with advanced disease; however, clinical response occurs in only 20–30% of patients (10, 11).

On February 2020, the Food and Drug Administration (FDA) granted accelerated approval checkpoint blockade therapy for the treatment of some patients with for BCG-unresponsive, high-risk non-muscle invasive bladder cancer. However, clinical responses are expected to occur in only 20–30% of patients. It is possible that the anti-tumor effects of checkpoint blockade alone are limited if T cells are unable to recognize tumor cells as targets for destruction. In order to enhance clinical responses, we propose a therapeutic venue that could increase tumor recognition by the host immune system. Physiologically, cells must control the coiling and uncoiling of DNA around histones. This is accomplished in part by two families of enzymes, histone acetyl transferases (HAT), which promote transcription, and histone deacetylases (HDAC) which condense and transcriptionally silence chromatin. Thus, inhibition of HDAC results in an increase in acetylation of histone tails resulting in chromatin remodeling (12). While HDACi were first conceived as cytotoxic chemotherapeutic agents, further examination in several tumor models indicate that HDACi also enhanced tumor immunogenicity (13–22). These studies raised the possibility that HDACi could improve the efficacy of checkpoint inhibition through direct and indirect mechanisms. This hypothesis was tested in several clinical trials with encouraging results (15, 17, 19, 20, 23, 24). In this study, we evaluated the combined use of local HDACi (CI994 or SAHA) and systemic checkpoint blockade therapy (anti-PD-1 mAb) for the treatment of urothelial cell carcinoma of the bladder.

Studies have shown that certain HDAC family members are aberrantly expressed in urothelial bladder cancer (25–30). For example, studies have shown that high-grade tumors and high expression levels of HDAC1 are more likely to progress compared to all other patients ($p < 0.05$) (29). Based on these observations, we first sought to target HDAC1, hence the use CI994 (Tacedinaline), a selective inhibitor of HDAC1 with significant activity in a number of *in vivo* tumor models (31–33). Moreover, studies have shown high HDAC expression levels are found in 40–60% of all investigated urothelial carcinomas (HDAC-1: 40%, HDAC-2: 42%, HDAC-3: 59%) compared to normal urothelium (29). Based on this data, we also tested SAHA, a broad inhibitor of HDACs (class I and II HDACs) (34, 35). In our study, using models of mouse and human bladder cancer, we demonstrated that the combined use of local HDACi and systemic anti-PD-1 blockade was effective at inducing anti-tumor responses with durable anti-tumor immunity that was associated with the upregulation of genes responsible for mediating immunorecognition, *NKG2D* ligands and *HSP70*.

MATERIALS AND METHODS

Bladder Cancer Cell Lines

Human bladder cancer cell line SW780 was purchased from ATCC. Mouse bladder cancer cell line MB49 was purchased from Sigma-Aldrich. MB49-luciferase (MB49-luc), was generated by first transfecting HekT cells with the F-luciferase plasmid using Lipofectamine 2000 (ThermoFisher). Supernatant from successful transfection (positive GFP signal under a fluorescent microscope) combined with polybrene was applied to wells plated with MB49 and centrifuged at 2,000 rpm for 2 h at 32°C. Transduced GFP-positive cells were cell-sorted, expanded and frozen in freezing media [heat-inactivated fetal bovine serum (Seradigm), 10% (DMSO)]. All bladder cancer cells were cultured in DMEM (Corning) supplemented with 10% heat-inactivated fetal bovine serum, 1% Penicillin/Streptomycin, and 2 mM L-glutamine (Corning).

RNA Isolation and Microarray Analysis

To assess changes in gene expression, mRNA was extracted using mirVana mRNA Isolation Kit (Invitrogen) followed by cDNA conversion using SuperScript IV First-Strand Synthesis System at 500 ng/reaction (Invitrogen). Array-based gene expression analysis was performed using the NextSeq 550 System (Illumina).

Anti-CD3-Activated Human T Cells

Healthy donor PBMCs (Key Biologics) were cultured with 100 ng ml-1 anti-CD3 (clone OKT3) for 5 days with IL-2 (300 IU ml-1) and IL-15 (100 ng ml-1). T cell enrichment and activation were corroborated by flow cytometry.

In vitro T Cell Killing Assay

SW780 cells were incubated with or without SAHA at 5 μM. After 12 h, cells were extensively washed to remove traces of HDACi. Treated SW780 cells were incubated with or without OKT3-activated human T cells at a 5:1 (Effector: Target) ratio. Following 24 h, wells were washed, and floating cells removed. Remaining bound cancer cells were stained with DAPI. Each well was photographed under a fluorescence microscope for nuclear staining, DAPI+ cells. The enumeration of the remaining cells per well was conducted by using a computer-based automatic counting algorithm (Image J, NIH).

Mice

Animal experiments were conducted in accordance with Loyola University Chicago Institutional Animal Care and Use Committee guidelines. Six to eight week-old C57BL/6 male and female mice were purchased from The Jackson Laboratory. All mice were housed in a specific-pathogen-free facility at Loyola University Chicago, Cardinal Bernardin Cancer Center.

Intradermal Mouse Tumor Model

Tumor cells were implanted through flank intradermal injection of 2×10^5 MB49 cells. Mice bearing tumors of 0.5 cm in any direction were treated i.p. with 200 μg IgG, 200 μg anti-PD-1 (BioXcell) once per week, intratumoral SAHA (50 of 10 μM SAHA), or combination (SAHA+anti-PD-1). Control mice were intratumorally injected with 50 μL DMSO-PBS. CD8 and NK cell depletions were conducted by i.p injection of 250 μg Clone 2.43 or 250 μg anti-Asialo GM1 antibody (36). Depletion was confirmed by flow cytometry in sentinel mice. Control groups received hamster IgG. To assess for long-term tumor immunity, mice that rejected tumors were rested for an additional 30 days and received a second MB49 tumor challenge in the contralateral flank alongside a control group.

Intravesical Mouse Tumor Model and Intravesical Tumor Treatment

Tumor implantation was conducted as previously described (37). Briefly, Female B6 mice were intravesically catheterized via a 24G catheter while under constant 3% isoflurane gas anesthesia. After bladder emptying, 80 microliters of 0.125% trypsin in DMEM base medium were instilled in the bladder. After 15 min, trypsin was removed and 50 microliters of PBS containing 2×10^5 MB49-Luc cells were intravesically instilled for 50 min. Intravesical and systemic treatments were conducted in anesthetized mice once tumor take has been confirmed by bioluminescence (∼3–5 days after implantation). Briefly, tumor-bearing mice were separated in groups, emptied bladder and simultaneously treated for 45 min with PBS-DMSO (control) or CI-994 in DMSO in 50 microliters. Tumor growth was followed every 5 days by *in vivo* bioluminescent imaging, IVIS Spectrum *in vivo* Imaging System (Perkin Elmer). Control mice received 200 μg IgG or 200 μg anti-PD-1, i.p. once per week. Mice were only treated once.

Histopathologic Assessment of Bladder Integrity Following Treatment

Treated mice underwent surgical removal of the urinary bladder. The bladders were subsequently sectioned in the midsagittal plane and embedded in paraffin. Sections of 5-μm were obtained from tissue blocks and stained with hematoxylin and eosin (H&E). Cell infiltration and the presence of residual tumors were assessed.

Flow Cytometry

Mouse bladders bearing MB49 tumors were surgically excised followed by mechanical and enzymatic (Liberase, Sigma) dissociation. Fluorochrome-conjugated antibodies against Fc-receptor, CD3, NK1.1, CD4, CD8, CD11c, CD19, F4-80 and Gr-1 (eBioscience) were used. Stained cells were analyzed by flow cytometry on LSR Fortessa (BD Biosciences). For all the flow cytometry data analysis, dead cells were excluded from the analysis by using Zombie Aqua viability dye (BioLegend).

Statistical Analysis

Differences in gene expression were determined by ANOVA analysis. Statistical differences in flow cytometric analysis were determined by Student two-tailed *t*-test. Kaplan-Meier curves were generated using GraphPad Prism 8 to detect differences in tumor regression and tumor-free survival.

RESULTS

Local HDACi Treatment in Combination With Systemic Anti-PD-1 Induces Immune-Mediated Tumor Regression and Durable Tumor Immunity

Because of HDACi's role in increasing tumor immunogenicity, we tested if the use of local (intratumoral) HDACi could enhance the systemic effects of anti-PD-1 therapy. Only male mice were used as immune responses against the Y antigen by female mice has been reported in the MB49 model (38). Mice bearing 0.5 cm intradermal tumors received (i.p.) IgG, anti-PD-1, intratumoral SAHA (or DMSO/PBS), or combination anti-PD-1 (systemic)/SAHA (intratumoral) **Figures 1A–C** show that all mice that received control antibody underwent rapid tumor growth. Significant alterations in the tumor growth slope were observed in mice treated with anti-PD-1 antibody or intratumoral SAHA alone ($p < 0.05$); however, no tumor regression was observed. Of the mice that received systemic anti-PD-1 antibody alone, only one developed an effective anti-tumor response. Of the group treated with SAHA alone, 10 of 10 mice developed tumors without regression. In contrast, 10 of 10 mice that received a combination of intratumoral SAHA and systemic anti-PD-1 therapy had complete regression of their tumors and 100% survival ($p < 0.05$).

Anti-Tumor Immunity After Combined HDACi Treatment With Systemic Anti-PD-1 Is Dependent on CD8 T Cells, but Independent of NK Cells

We next sought to determine if the anti-tumor immune responses observed following treatment with the combination of HDACi and anti-PD-1 antibody were mediated by CD8 T cells or NK cells. Tumor-bearing male mice receiving intratumoral SAHA and systemic anti-PD-1 were also treated with CD8 or anti-Asialo GM1 antibody depleting mAbs. We found that none of the ten mice that had undergone CD8-immune depletion derived benefit from combined therapy. All mice that were CD8 depleted developed tumors with a slope that was indistinguishable from the control group ($p > 0.05$). In contrast, all mice that were NK depleted maintained strong anti-tumor immunity (**Figures 2A–C**) ($p < 0.05$). NK depletion was confirmed by flow cytometry (**Supplementary Figure 1**).

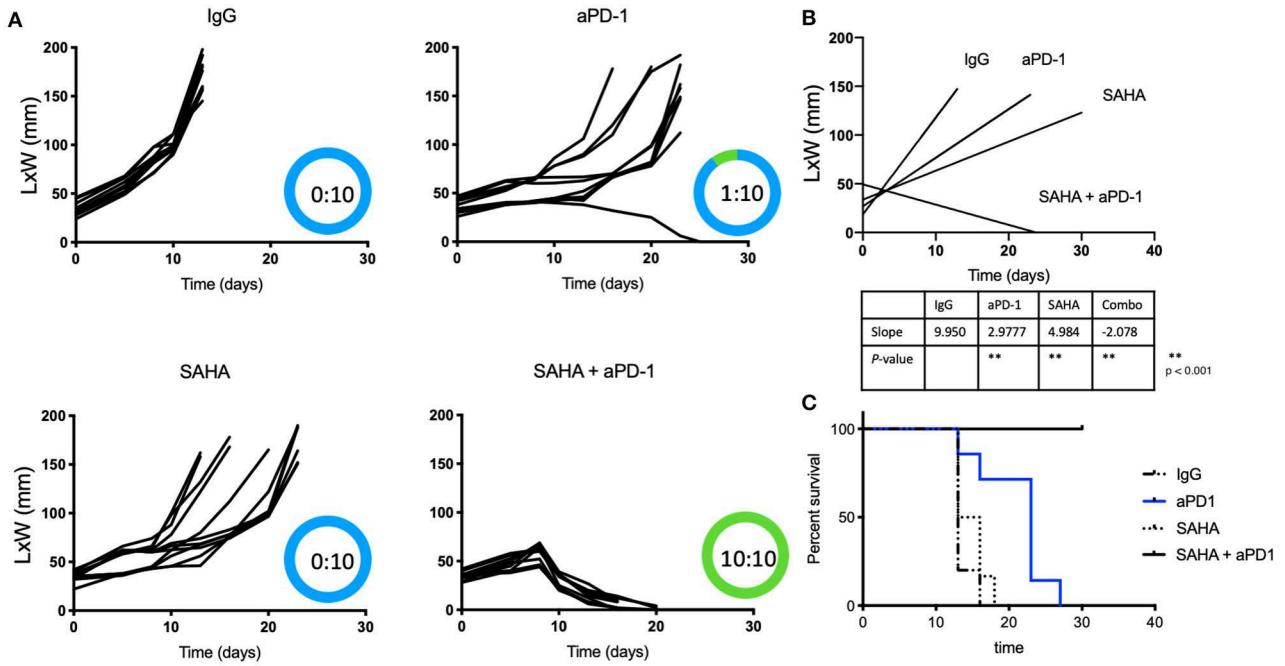

FIGURE 1 | Intratumoral HDACi enhances systemic anti-PD-1 anti-tumor immunity. **(A)** MB49-bearing male B6 mice (10 per group) with tumors of 0.5 cm, were treated as follows: IgG control, systemic anti-PD-1, intratumoral SAHA, combination SAHA and anti-PD-1. Tumor growth was depicted as LxW in mm and plotted against time in days. The number of mice presenting tumor are represented in blue and tumor-free mice in green. Tumor free vs. total mice are indicated inside the circle. **(B)** Tumor growth slopes (L × W mm) are shown comparing the different groups, $^{**}p < 0.001$. **(C)** Kaplan-Meier curves for each treated group are shown. Survival is defined as the tumor reaches 1.5 cm in any direction. Mice were monitored for 30 days. These data are representative of two independent experiments.

Intratumoral HDACi and Systemic Anti-PD-1 Induce Durable Immunity Against a Distal Tumor

To test if the observed anti-tumor immunity was durable and capable of affecting a tumor at a distal site, mice from the combination therapy arm that had undergone complete tumor regression were challenged again, but in the contralateral flank with intradermal MB49 cells after 30 days of resting. These mice received no further therapy after the second inoculation, therefore, changes in tumor growth could be only be attributed to the previously generated immunity. A group of naïve and untreated mice was also challenged to establish MB49 cells *de novo* tumor growth. Not surprisingly, all mice (5 of 5) in the control group rapidly developed tumors. In contrast, seven of 10 in the previously treated mice (HDACi + anti-PD-1 mAb) rejected a second tumor challenge and remained tumor-free 3 months after initial tumor inoculation ($p < 0.05$) (**Figures 3A,B**).

Intravesical HDACi in Combination With Systemic Anti-PD-1 Induces Tumor Regression

To assess the feasibility of intravesical delivery of HDACi for the treatment of bladder cancer, we used an orthotopic bladder cancer model, MB49-luc. In this case, we used female mice given the possibility of catheterizing the urethra. We confirmed intravesical tumor take by *in vivo* bioluminescent visualization (**Figure 4A**). Tumor take was typically observed at day 3–4 after intravesical instillation. As observed in the intradermal model, 10 of 10 female mice treated with the combination of intravesical HDACi (CI-994) and systemic PD-1 blockade developed curative anti-tumor responses in their bladders ($p < 0.05$). Ten of 10 mice that received sham treatment developed tumors. Mice that were treated with CI-994 or anti-PD-1 antibody alone, although generated a tumor size reduction, failed at achieving curative tumor regression (**Figure 4B**). A comparison between the combination treatment and monotherapies indicates no statistically significant differences ($p > 0.005$). MB49-luc maintained luciferase expression for the duration of the *in vivo* experiment (>30 days), as shown by luminescence in tumor-bearing mice that received no treatment. The presence or absence of tumor tissue was corroborated by macro and microscopic analysis of the bladder.

HDAC Inhibitor in Combination With Anti-PD-1 Induce Immune Infiltration and Tumor Destruction *in situ*

Following the completion of intravesical therapy, the bladders of these mice were macroscopically visualized (**Figure 5A**) and surgically removed for histopathologic analysis (H&E) (**Figures 5B,C**). Untreated bladders showed extensive invasive carcinoma. Mouse bladders treated with intravesical CI-994

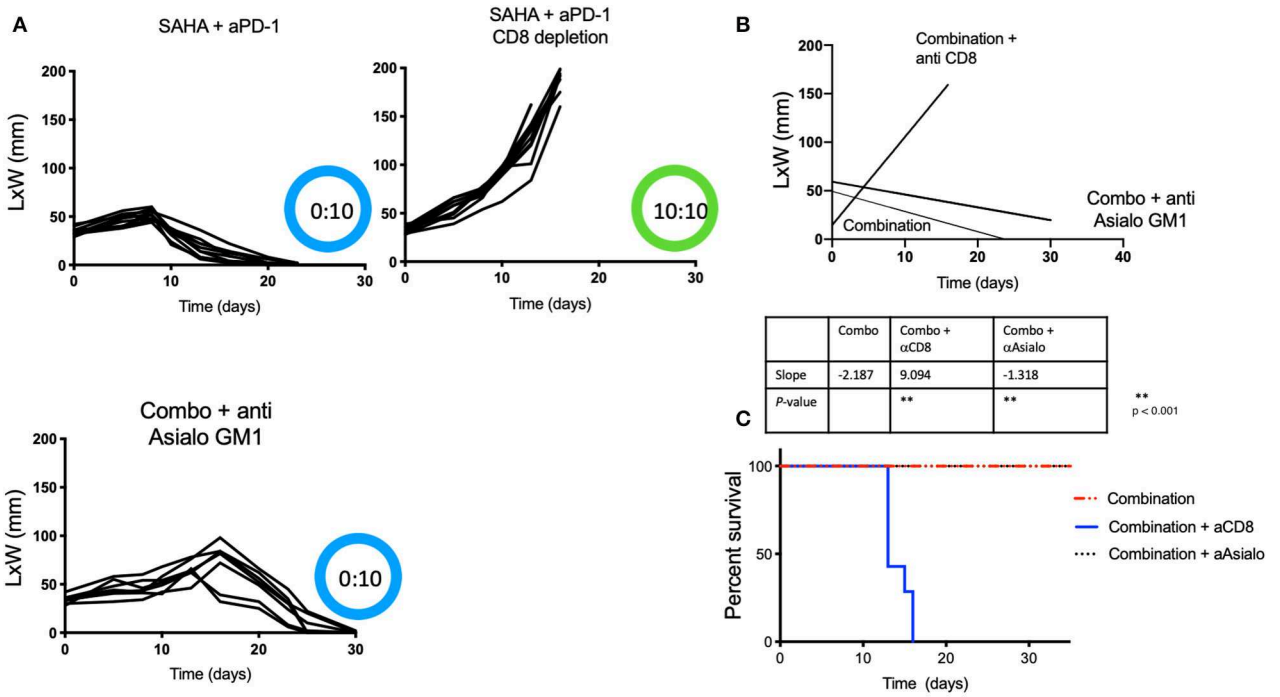

FIGURE 2 | Protective anti-tumor responses by HDACi and PD-1 are CD8-mediated and NK independent. Tumor-bearing mice treated as **Figure 1**, also received depleting anti-Asialo GM1 or anti-CD8. **(A)** Tumor growth was depicted as L × W in mm and plotted against time in days. The number of mice presenting tumor are represented in blue and in tumor-free mice in green. **(B)** Tumor growth slopes (L × W) are shown comparing the different groups, $^{**}p < 0.001$. **(C)** Kaplan-Meier curves for each group are shown. Survival is defined as the tumor reaches 1.5 cm in any direction. Mice were monitored for 30 days. These data are representative of two independent experiments. The number of mice presenting tumor are represented in blue and tumor-free mice in green. Tumor free vs. tumor-bearing mice are indicated inside the circle.

demonstrated a mild or no reduction in invasive carcinoma. Those treated with anti-PD-1 alone showed immune infiltration, but prominent invasive carcinoma remained. Mouse bladders that had received combination therapy demonstrated immune infiltration with minimal or no residual carcinoma. Next, we sought to determine if intravesical HDACi exposure and systemic PD-1 blockade treatment result in alteration in bladder integrity that could preclude their clinical use. We microscopically analyzed (H&E) the bladders of treated mice that received combination therapy. Histopathology analysis revealed no deleterious changes in the integrity of bladders of mice treated with CI-994 and PD-1 blockade compared with mice receiving no treatment (**Figure 5C**, center). To gain further information regarding the cellular composition among the different treatment groups, we analyzed the bladder of tumor-bearing mice 6 days after treatment by flow cytometry. We determined the frequency of T cells, B, macrophages, dendritic cells (DCs) and neutrophils in bladder tumor of mice treated with HDACi, anti-PD-1, combination or no treatment. In **Figure 6** and **Table 1**, we show that treatment with HDACi alone was insufficient to cause significant changes in the immune composition of the bladder. In contrast, mice treated with aPD-1 mAb, the frequency of CD3 and CD8 T cells was higher than in untreated or HDACi-treated mice ($P < 0.005$). These effects of a-PD1 mAb were also observed in the group of mice treated with both aPD-1 and HDACi ($P < 0.05$).

Exposure to HDACi Results in Increased Recognition and T Cell-Mediated Tumor Cell Killing in Human Bladder Cancer Cells

To establish relevance in the human setting, we next examined if exposure of a human bladder cancer cell line SW780 to HDACi (SAHA) would impact their recognition and killing by fully activated human T cells. To do this, T cells and SW780 were both HLA-A2.1 matched. Exposure of SW780 to SAHA alone resulted in a modest cytotoxic effect with a 22% reduction in viable tumor cells compared to control-treated cells ($p < 0.05$). Incubation of SW780 (DMSO treated) with activated T cells alone showed some direct T cell-mediated cytotoxicity with a 30.4% reduction in tumor cells when compared to SW780 cells alone ($p < 0.05$). Notably, tumor cell pretreatment with SAHA augmented activated T cell-mediated cytotoxicity with a 73.9% reduction in viable tumor cells compared to cells treated with DMSO and T cells ($p < 0.05$) (**Figures 7A,B**). Visual analysis of the combined shows fully activated T cells failing to engage tumor cells (SW780 + T cells group), **Figure 7A**. Notably, in the SW780 SAHA treated + T cells group, T cells are clearly seen swarming around tumor cells.

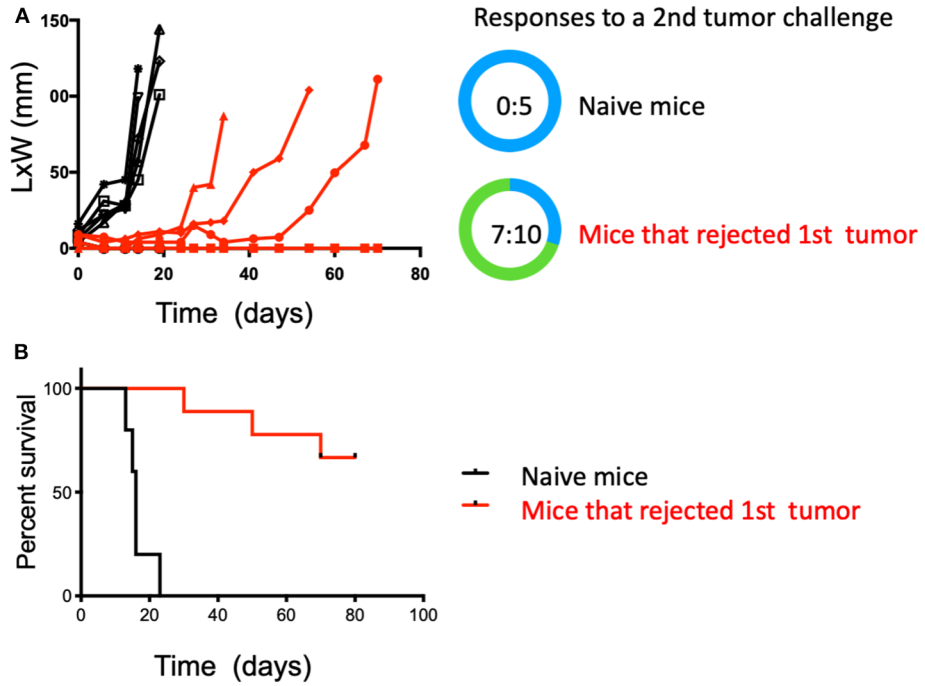

FIGURE 3 | Combined treatment with systemic anti-PD-1 and intratumoral HDACi results in durable anti-tumor immunity that protects against a second tumor challenge. Surviving mice treated with HDACi and anti-PD-1 mAb were rested for 30 days and challenged in the opposite flank with MB49 cells. Non-treated group = 5 mice; treated mice that rejected the first MB49 tumor challenge = 10 mice. **(A)** Five of five non-treated mice developed tumors. In the group of mice that rejected the first tumor (10:10), only three developed secondary tumors. **(B)** Kaplan-Meier curves, surviving mice were monitored for 80 days. Survival is defined as tumor reaching 1.5 cm in any direction. The number of mice presenting tumors are represented in blue and tumor-free mice in green. Tumor free vs. total mice are indicated inside the circle.

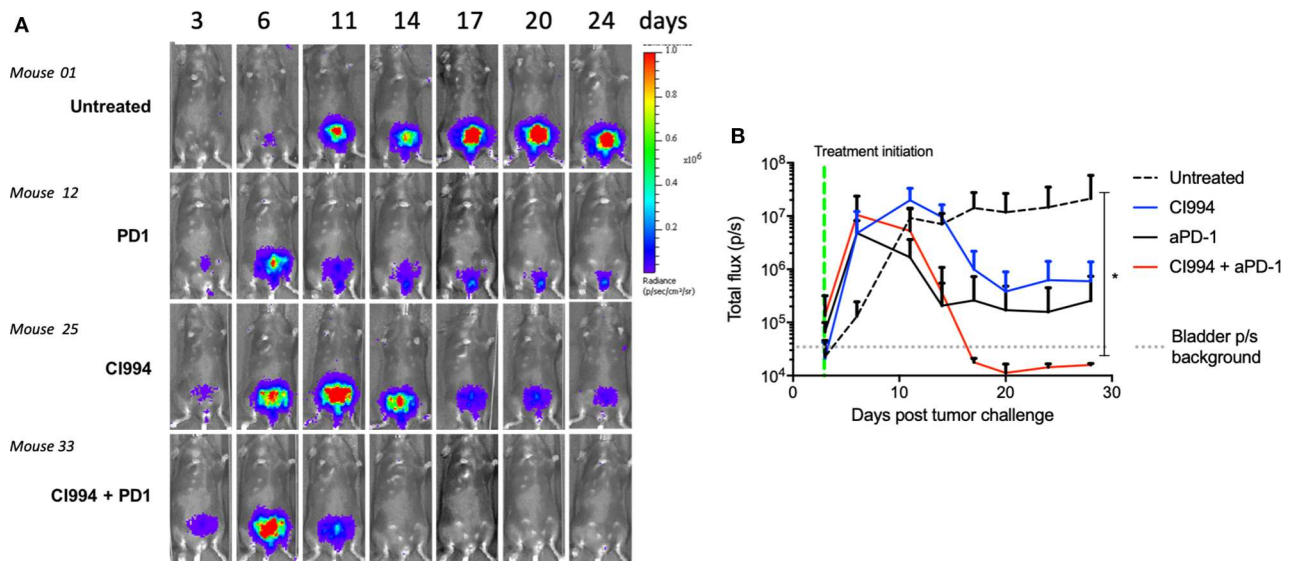

FIGURE 4 | *In vivo* bladder cancer regression following combined intravesical HDACi treatment and systemic T cell immune activation by PD-1 blockade. Once tumor take was confirmed by *in vivo* imaging (day 4), MB49-bearing female B6 mice were treated. Mice were randomized in groups: untreated, intravesical CI-994, systemic anti-PD-1, combination CI-994 and anti-PD-1. Mice were monitored for at least 30 days. These data are representative of two independent experiments. **(A)** *In vivo* imaging examples of intravesical bladder cancer tumor progression in individual mice. **(B)** Graphical representation of intravesical tumor growth (total flux in photons per second) in treated mice. Ten mice per group were used, data are representative of two independent experiments. $^{*}p < 0.05$.

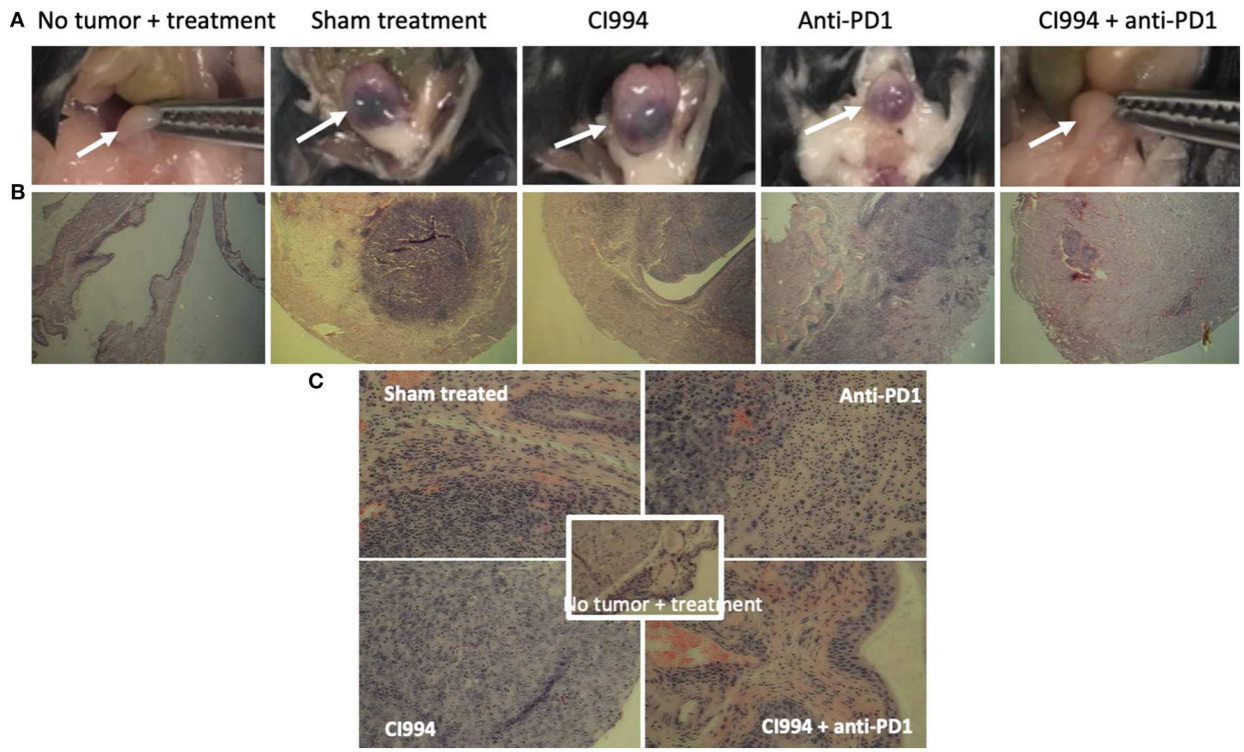

FIGURE 5 | Intravesical HDACi in combination with systemic anti-PD-1 induce immune infiltration and tumor destruction *in situ*. Bladders from treated mice were surgically resected and prepared for H&E staining and histological analysis after tumors were not present by *in vivo* imaging (~d12). Groups: mice were not tumor challenged but received combined treatment; tumor-bearing mice received sham treatment; tumor-bearing mice received intravesical CI-994 or anti-PD-1 or their combination. **(A)** Bladder macroscopic view are shown of representative mice. **(B)** Bladders of mice treated intravesically with CI-994 show the presence of tumor cells. **(C)** Anti-PD-1 alone induces immune infiltration, but tumor cells were also present. Bladder of mice treated intravesically with HDACi (CI-994) and systemic PD-1 antibody combination show immune infiltrate with substantial or complete tumor cell clearance. Combination treatment caused no apparent changes in the bladder integrity.

HDACi Causes Gene Deregulation in Human Bladder Cancer Cells

In order to provide a tentative explanation to the effects observed and perhaps a potential intersection with T cell-mediated anti-tumor pathways, we examined the capacity of HDACi to alter gene expression in human bladder cancer. *In vitro* exposure of the human bladder cancer cell line, SW780 to HDACi (SAHA) results in the alteration of gene expression compared with DMSO-treated cells (**Figure 8**). Baseline gene expression was normalized to 0. Sixty-nine genes were found to have a 2-fold increase or greater, and 19 genes were found to have a 2-fold decrease or greater ($p < 0.05$), (**Supplementary Table 1**). Notably, an in-depth analysis indicates that among the group of genes that were upregulated, the immunologically relevant genes encoding ligands for NKG2D, MICA and ULBP2 and the heat shock protein 70 (HSP70) were among those upregulated.

DISCUSSION

Using a mouse and human model of bladder cancer, we demonstrated that the combined use of local HDACi and systemic anti-PD-1 was effective at inducing curative CD8 T cell immune responses against primary lesions with durable anti-tumor immunity against a secondary and distal tumor. We also demonstrated the relative safety and applicability of this strategy in an orthotopic intravesical bladder tumor model. We show that inhibition of HDAC in a human bladder cancer cell line facilitates their recognition and killing by T cells. Moreover, we show that HDACi causes tumor cell gene deregulation, characterized by upregulated genes responsible for mediating cellular stress, as shown by the increased expression of *NKG2D* ligands and *HSP70*.

HDAC inhibitors were first conceived with the idea of using them as chemotherapeutic/cytotoxic agents. Indeed, some of them, Romidepsin (Istodax; Celgene) and Belinostat (Beleodaq; Spectrum Pharmaceuticals) are FDA approved for the treatment of cutaneous T cell lymphoma (CTCL) and peripheral T cell lymphoma (PTCL) (39), and multiple myeloma (40, 41). HDACi act by affecting the DNA repair machinery, altering gene expression leading to post-translational modifications to proteins HDACi have been shown to stop the proliferation of cancer cells, stimulate apoptosis and induce cell cycle arrest. Moreover, inhibition of HDACs in tumor cells offers additional potential benefits, namely augmentation of cancer immunogenicity (13–22).

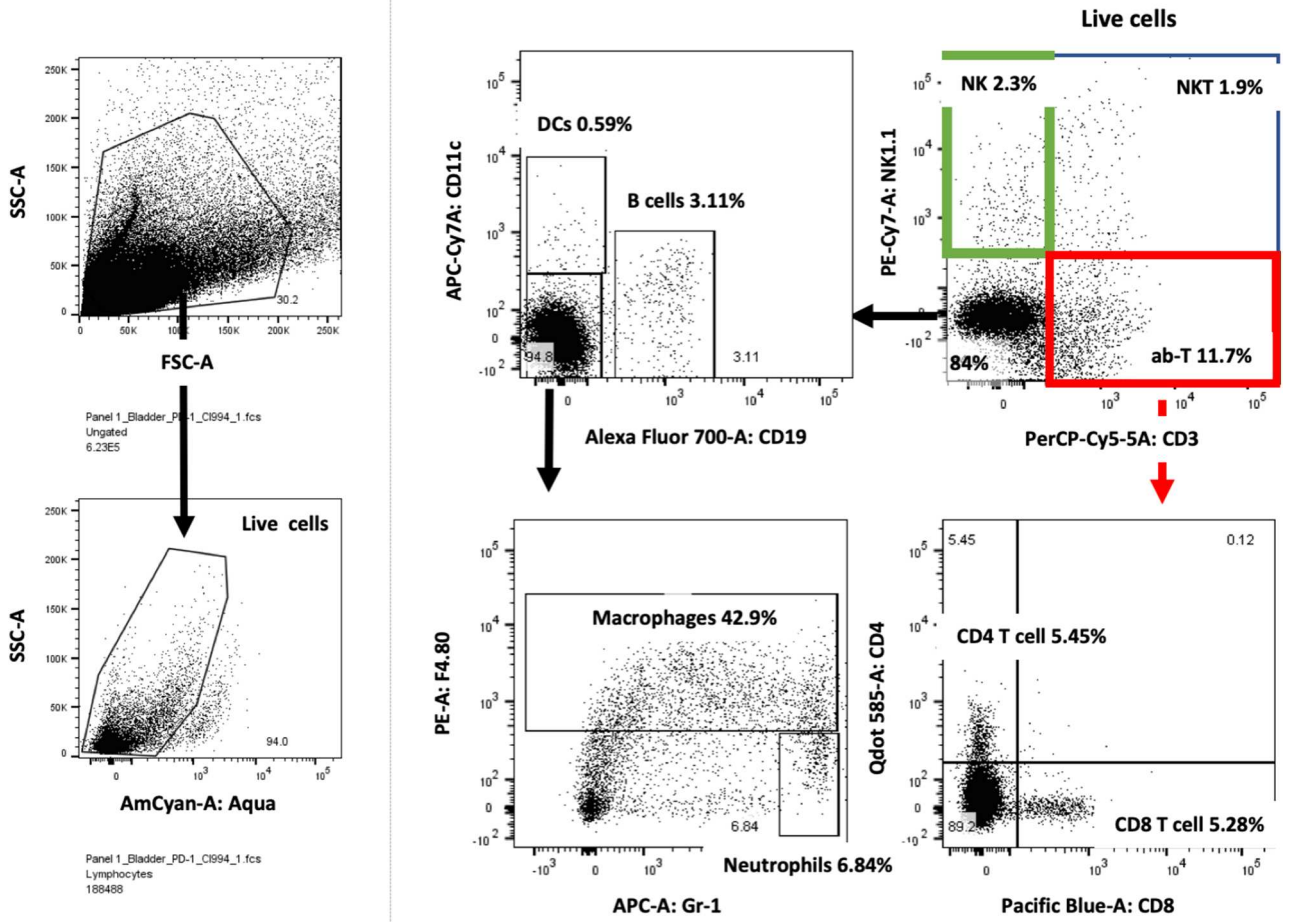

FIGURE 6 | Bladder cancer gating strategy for flow cytometry analysis. Tumor-bearing bladders from treated mice were surgically resected at day after treatment, dissociated and prepared for flow cytometry analysis. Live lymphocytes were analyzed for T cells subdivided into CD4 and CD8 cells, NK, NKT, DCs, macrophages, neutrophils and B cells. Example of one tumor-bearing bladder is shown.

TABLE 1 | Frequency of immune cells in tumor-bearing bladders.

	CD3+CD8+		CD3+CD4+		B cells		DCs		Macrophages		Neutrophils		NK		NKT	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Untreated	1.847	0.8701	4.493	0.8064	4.097	0.8173	0.5567	0.2173	50.6	7.374	5.103	0.7823	2.217	0.08	1.805	0.755
f1994	4.737	2.665	5.69	1.114	5.35	1.074	0.5833	0.0702	44.57	5.404	**8.6***	0.7119	1.983	0.98	2.123	0.989
aPD-1	**6.09***	1.472	**8.757***	1.862	7.15	2.405	0.78	0.0866	43.13	3.758	**11.7***	3.671	2.331	1.44	2.353	1.1124
aPD-1+CI994	**5.287***	1.04	6.437	1.752	6.697	3.116	0.96	0.3804	47.47	4.994	8.753	2.296	2.447	0.89	2.024	1.07

*Bladders from treated mice were surgically resected, dissociated and prepared for flow cytometry analysis as shown in **Figure 6**. The frequency (the % positive cells of a specific phenotype among 10,000 events) of CD3, CD4, CD8, NK, NK T, B cells, DCs, macrophages, and neutrophils are shown. Statistical significance was calculated by student t-test, * $P < 0.05$. Bold indicates statistical significance.*

Studies have shown that certain HDACi can increase the expression of tumor-shared antigens and the expression of MHC class I and class II in melanoma cells (42, 43), or expression of HSP70 and HSP90 on various hematopoietic cancer cells (13, 44). In a panel of NSCLC cell lines *in vitro*, mocetinostat (inhibitor of class I/IV histone deacetylases) upregulated PD-L1 and antigen presentation genes including class I and II human leukocyte antigen (HLA) family members. In a syngeneic tumor model, mocetinostat decreased intratumoral regulatory T cells (Tregs) and potentially myeloid-derived suppressor cell (MDSC) populations and increased intratumoral CD8 T cell populations (20).

Studies have shown that co-treatment with HDACi and checkpoint inhibitors improved treatment outcomes against

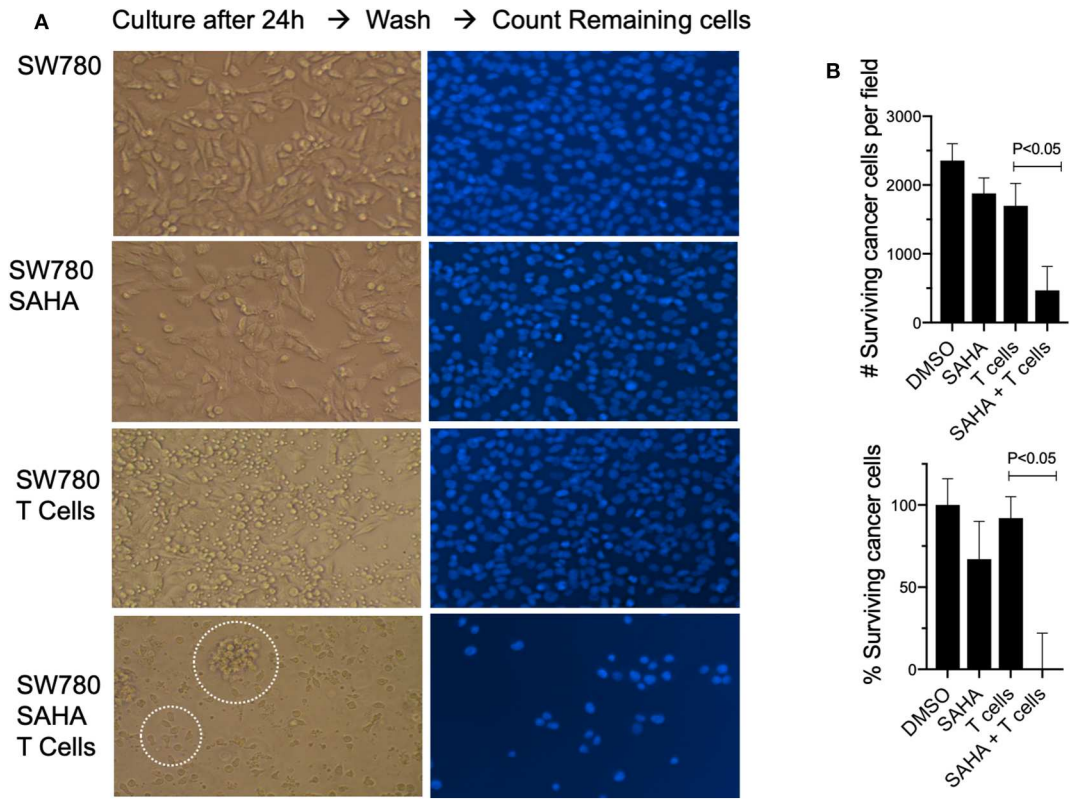

FIGURE 7 | Pretreatment with SAHA enhances T cell-mediated cytotoxicity in human bladder cancer cells. **(A)** SW780 human bladder cancer cells were treated with either DMSO or SAHA and activated T cells were then added to culture (left). T cells were then washed, and remaining cells were stained for DAPI (right). Dotted circles indicate T cells and T cells swarming around a tumor cell. **(B)** Remaining cells following treatment were also counted to determine viability following treatment.

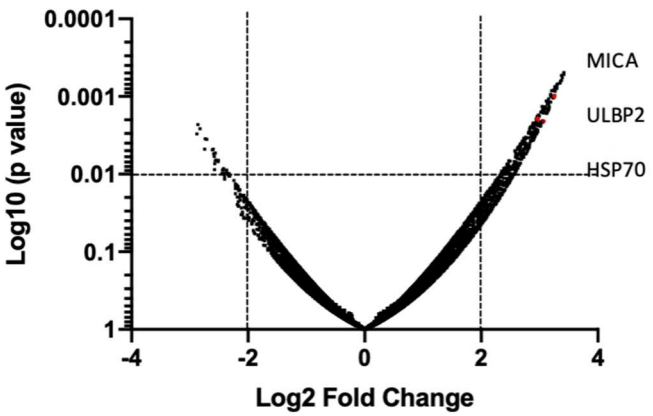

FIGURE 8 | HDACi induces gene expression changes. Global gene array comparing human SW780 cells treated with SAHA vs. DMSO. Volcano plots of genes were created by plotting Log2 fold change in relationship with Log10 (p-value). Significance was considered if $p < 0.01$ was shown. Genes in the upper and left quadrant changed ±2 folds and $p < 0.01$.

colorectal carcinoma, CT-26 and breast cancer 4T1 tumors (16). In these studies, protective responses were associated with the depletion of myeloid-derived suppressor cells (MDSCs). More recently, a study using a mouse model of hepatocellular carcinoma showed that systemic HDACi (Belinostat) combined with the simultaneous blockade of CTLA-4 led to the decrease of regulatory T cells and complete tumor rejection (22). Recently, a study showed that HDAC6 inhibitors improve anti-PD-1 immune checkpoint blockade therapy by decreasing the anti-inflammatory phenotype of macrophages and down-regulation of immunosuppressive proteins in tumor cells (21). A study using prostate (LNCAP) and breast (MDA-MB-231) carcinoma cells

demonstrates that treatment with either the pan-HDAC inhibitor vorinostat or the class I HDAC inhibitor entinostat results in T-cell mediated lysis *in vitro*. Moreover, the authors show that part of the mechanisms responsible for the recognition of HDACi-treated cells is mediated by the ER stress-responsive element (45). Additional studies have shown that the effects of HDACi can extend to changes in the antigen processing machinery. Using melanoma B16 cells, the authors demonstrate that the HDACi, TSA increases the expression of several components of the antigen processing machinery, including TAP-1, TAP-2, LMP-2, and Tapasin (46, 47). Importantly, HDACi have also been shown to increase the expression of costimulatory molecules on the surface of tumor cells, such as CD40, CD80, and 4–1BB. For example, the HDACi, panobinostat, has been shown to enhance immune recognition of melanoma by immune cells. In this study, the effects were correlated to the upregulation of costimulatory molecules CD40 and CD80 (43). Moreover, a study using leukemia cells found that HDAC inhibition results in the upregulation of 4-1BBL/TNFSF9 (48). In our study, we found that, indeed, inhibition of HDACs in bladder tumor cells results in their recognition and killing by CD8 T cells. Giving a possible explanation for the mechanisms, our study suggests that HDACi induces cellular stress in bladder cancer cells, as shown by the increased expression of *NKG2D* ligands and *HSP70*.

Physiologically, NKG2D ligands serve as signals to the immune system of catastrophic cell damage and the need for immune-mediated destruction. This is consistent with prior work from separate studies by L. Lanier and D. Raulet groups where genetically-driven expression of NKG2D ligands in tumors was sufficient to cause tumor rejection (49, 50). Given the canonical function of NKG2D, which is to recognize cells undergoing genotoxic stress response, one can speculate that HDAC inhibitor-treated tumor cells expressing NKG2D ligands would be visible to cytolytic cells for destruction, namely by NK and CD8 T cells. Consistent with this possibility, a study in myeloma cells shows that valproic acid (VPA) upregulates both protein and mRNA expression of NKG2D ligands (MICA/B) and ULBP2 (51). Based on these studies, the increase of NKG2D ligands suggests that HDAC inhibitor-treated cells triggers tumor cell destruction through a suicide by proxy mechanism. Our data support this assumption, as only HDAC inhibitor-treated tumor cells are recognized and killed by CD8 T cells. Interestingly, our data also indicate that HDAC inhibition modulates the expression of HSP70 and ligands for NKG2D in bladder cancer cells. This dual effect is mechanistically consistent, as the expression of both genes is controlled by the heat shock factor 1 (HSF1). This is a transcriptional factor that activates the transcription of genes coding for proteins that protect the cell against harmful stresses (52). Based on studies on the role of HSPs in the cell stress response (13), we interpret the increase in levels of HSP70 as a cellular attempt to survive. However, the function of HSP70 also extends to their participation as a peptide-chaperone to be taken by professional antigen-presenting cells (APC), leading to T cell priming (53). Studies have shown that immunization with a lymphocytic choriomeningitis virus peptide mixed with HSP70 results in protective antiviral immunity and antigen-reactive CD8 T cells (54). Similar results have been shown that vaccination with HSP preparations elicits a CD8 T cell responses *in vivo* (55). Based on these studies and our observations, one can propose that HDACi-related immunity is first mediated by the recognition of NKG2D ligands on the surface of treated tumor cells, and then followed by HSP70-mediated priming of T cells. Moreover, our groups recently demonstrated that NKG2D signaling in CD8 T cells at the time of the effector killing phase results in the acquisition of a transcriptional program that poised T cells with the potential to become long term memory T cells, we termed this process memory certification (56).

We show that the HDACi treated human bladder cancer cell line (SW780) can be recognized by human T cells. In this experiment, it is important to note that while the tumor and T cells were HLA-A2.1 matched, other histocompatibility proteins are expected to be different, leading to some level of allo-responses. However, our data demonstrate that even under these conditions, cancer cells are poorly recognized and destroyed by T cells. Our data clearly show that in contrast with the results observed in HDACi treated cancer cells, killing by T cells was negligible in non-HDACi treated cells. These data indicate that modulation of HDAC in tumor cells is sufficient to render them visible and susceptible to T cells. While our *in vitro* experiments demonstrate that HDACi have similar effects on bladder cancer cell lines, this has yet to be shown in highly heterogeneous tumors, which comprise the vast majority of *in situ* human bladder tumors. As in most preclinical models, a mouse cancer cell line may not completely reflect human bladder cancer patients. However, we believe the benefits of this model are novel as well as practical.

In our therapy experiments, some mice failed to develop curative responses upon combined therapy. However, anti-tumor responses were not absent, the tumor growth curves were significantly different to non-treated mice, tumors appeared later and grew slower. These data indicate the existence of anti-tumor response, however, insufficient to mediate tumor rejection.

However, the mechanisms mediating the tempering of the anti-tumor response could be multiple. It is possible that in this mice, inadequate number of anti-tumor T cells were generated or that immune suppressive mechanisms (i.e., TGF-b or Tregs) dominated the response (57, 58). In any case, it would be uncommon to see any therapy that provides 100% curative responses. We provide non-tangential data demonstrating that CD8 T cells mediate the anti-tumor response. We show that depletion of CD8 T cells results in the complete abrogation of anti-tumor responses upon combined treatment. These data indicate that the cellular mediators of tumor rejection upon combined treatment are CD8 T cells and that responses depend of the combined use of HDACi and anti-PD-1 mAb.

We observed differences in immune infiltration overserved during the flow cytometry and the histologic analysis. This can be explained by the differences in the time points when the samples where studied. The flow cytometry analysis

of tumor-bearing bladders was conducted 3 days after the initiation of treatment. What is seen in the flow cytometry represents the active response to treatment. The histology was conducted 12 days after treatment, once tumors have disappeared in the combination group. The response seen in the histology represents the overall outcome of the treatment (after tumor were no longer detectable by *in vivo* imaging). Under this perspective, the data presented are consistent with the expansion phase, followed by the tumor clearance and contraction phase.

Analysis of the flow cytometry pattern in the bladder shows a population of T cells that are express CD3 but not CD8 or CD4 markers (DN). Studies have shown that these T cells are found in the thymus. In the peripheral, DN T cells have been shown to be involved in immune regulation and tolerance, as well as in host defense and inflammation. While we do not know the function of these population in the observed responses, current literature shows dual effects, inflammatory and suppressor. For example, DN cells have been shown to prevent allograft rejection, graft-vs.-host disease, and autoimmune diabetes (59). In contrast, studies have shown that DN T cells can protect against infections (60, 61). It is plausible that HDACi may also affect the DN cell population, by making less suppressive or more pro-inflammatory. Given these observations and their high frequency in tumor-bearing bladders, we believe that this population merits further studies.

While HDACi are currently available for systemic use in other cancer types, toxicity remains an issue (62, 63). The use of intravesical delivery of this agent may avoid the risks of systemic toxicity while allowing for local delivery directly to target tissue. Furthermore, this reduces the possibility of compounding adverse immune-related events with the administration of both therapeutic agents. It should also be noted that the anti-tumor effects of combination therapy are not only effective in inducing tumor regression but are also long-lasting. This provides a potential advantage in a malignancy with high rates of local recurrence.

The data presented in this study support the hypothesis that HDAC modulation in cancer cells can remove the tumor cells "invisibility cloak" that prevents T cells from recognizing and killing them. Furthermore, these data support a therapeutic route that could increase tumor recognition by the host immune system. Our preclinical investigation of the use of local HDACi in combination with systemic anti-PD-1 provides clinical investigators with an applicable approach that mediates anti-tumor activity with durable clinical response while mitigating the risk of side effects. This novel therapeutic treatment may provide new opportunities for patients with localized bladder cancer and should be further explored in the context of a clinical trial.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Loyola University Chicago IACUC.

AUTHOR CONTRIBUTIONS

JG-P: study concept and design. BB, CE, CP, AB, SH, LP-R, KP, and MD: experiments, analysis, and interpretation of data. BB: drafting of the manuscript. CV-J, EH, GG, and JG-P: critical revision of the manuscript for important intellectual content.

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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.2020.00699/full#supplementary-material

Supplementary Figure 1 | Anti-Asialo GM1 antibody depletes NK cells *in vivo*. **(A)** Dot plot of spleens from sentinel mice that were treated with anti-Asialo GMI antibody. Cells were analyzed for CD3 and NK1.1 marker expression 3 days later by flow cytometry. **(B)** Representative plot of eight mice. $^{****}p < 0.0001$.

Supplementary Table 1 | Gene expression changes by HDAC inhibitor on the human bladder cancer cell line (SW780). Baseline gene expression was normalized to 0. Sixty-nine genes were found to have a 2-fold increase or greater, and 19 genes were found to have a 2-fold decrease or greater ($p < 0.05$).

REFERENCES

1. Cronin KA, Lake AJ, Scott S, Sherman RL, Noone AM, Howlader N, et al. Annual Report to the Nation on the Status of Cancer part I: National cancer statistics. *Cancer*. (2018) 124:2785–800. doi: 10.1002/cncr.31551
2. Sylvester RJ, Van Der Meijden APM, Oosterlinck W, Witjes JA, Bouffioux C, Denis L, et al. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: A combined analysis of 2596 patients from seven EORTC trials. *Eur Urol.* (2006) 49:466–77. doi: 10.1016/j.eururo.2005.12.031
3. Chang SS, Boorjian SA, Chou R, Clark PE, Daneshmand S, Konety BR, et al. Diagnosis and treatment of non-muscle invasive bladder cancer: AUA/SUO guideline. *J Urol.* (2016) 196:1021–9. doi: 10.1016/j.juro.2016.06.049
4. Lamm DL, Thor DE, Winters WD, Stogdill VD, Radwin HM. BCG immunotherapy of bladder cancer: Inhibition of tumor recurrence and associated immune responses. *Cancer*. (1981) 48:82–8. doi: 10.1002/1097-0142(19810701)48:1<82::AID-CNCR2820480117>3.0.CO;2-Z
5. Herr HW, Laudone VP, Badalament RA, Oettgen HF, Sogani PC, Freedman BD, et al. Bacillus Calmette-Guerin therapy alters the progression of superficial bladder cancer. *J Clin Oncol.* (1988) 6:1450–5. doi: 10.1200/JCO.1988.6.9.1450
6. Herr HW, Schwalb DM, Zhang ZF, Sogani PC, Fair WR, Whitmore WF, et al. Intravesical bacillus Calmette-Guerin therapy prevents tumor progression and death from superficial bladder cancer: Ten-year follow-up of a prospective randomized trial. *J Clin Oncol.* (1995) 13:1404–8. doi: 10.1200/JCO.1995.13.6.1404

7. Steinberg G, Bahnson R, Brosman S, Middleton R, Wajsman Z, Wehle M, et al. Efficacy and safety of valrubicin for the treatment of bacillus Calmette-Guerin refractory carcinoma in situ of the bladder. *J Urol.* (2000) 163:761–7. doi: 10.1016/S0022-5347(05)67799-3
8. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science.* (2018) 359:1350–5. doi: 10.1126/science.aar4060
9. Davarpanah NN, Yuno A, Trepel JB, Apolo AB. Immunotherapy: A new treatment paradigm in bladder cancer. *Curr Opin Oncol.* (2017) 29:184. doi: 10.1097/CCO.0000000000000366
10. Bellmunt J, De Wit R, Vaughn DJ, Fradet Y, Lee JL, Fong L, et al. Pembrolizumab as second-line therapy for advanced urothelial carcinoma. *N Engl J Med.* (2017) 376:1015–26. doi: 10.1056/NEJMoa1613683
11. Sharma P, Retz M, Siefker-Radtke A, Baron A, Necchi A, Bedke J, et al. Nivolumab in metastatic urothelial carcinoma after platinum therapy (CheckMate 275): a multicentre, single-arm, phase 2 trial. *Lancet Oncol.* (2017) 18:312–22. doi: 10.1016/S1470-2045(17)30065-7
12. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene*. (2007) 26:5541–52. doi: 10.1038/sj.onc.1210620
13. Jensen H, Andresen L, Hansen KA, Skov S. Cell-surface expression of Hsp70 on hematopoietic cancer cells after inhibition of HDAC activity. *J Leukoc Biol.* (2009) 86:923–32. doi: 10.1189/jlb.0209056
14. Akimova T, Beier UH, Liu Y, Wang L, Hancock WW. Histone/protein deacetylases and T-cell immune responses. *Blood*. (2012) 119:2443–51. doi: 10.1182/blood-2011-10-292003
15. Cycon KA, Mulvaney K, Rimsza LM, Persky D, Murphy SP. Histone deacetylase inhibitors activate CIITA and MHC class II antigen expression in diffuse large B-cell lymphoma. *Immunology*. (2013) 140:259–72. doi: 10.1111/imm.12136
16. Kim K, Skora AD, Li Z, Liu Q, Tam AJ, Blosser RL, et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proc Natl Acad Sci USA*. (2014) 111:11774–9. doi: 10.1073/pnas.1410626111
17. Woods DM, Sodre AL, Villagra A, Sarnaik A, Sotomayor EM, Weber J. HDAC inhibition upregulates PD-1 ligands in melanoma and augments immunotherapy with PD-1 blockade. *Cancer Immunol Res.* (2015) 3:1375–85. doi: 10.1158/2326-6066.CIR-15-0077-T
18. Zheng H, Zhao W, Yan C, Watson CC, Massengill M, Xie M, et al. HDAC inhibitors enhance T-cell chemokine expression and augment response to PD-1 immunotherapy in lung adenocarcinoma. *Clin Cancer Res.* (2016) 22:4119–32. doi: 10.1158/1078-0432.CCR-15-2584
19. Stone ML, Chiappinelli KB, Li H, Murphy LM, Travers ME, Topper MJ, et al. Epigenetic therapy activates type I interferon signaling in murine ovarian cancer to reduce immunosuppression and tumor burden. *Proc Natl Acad Sci USA*. (2017) 114:E10981–90. doi: 10.1073/pnas.1712514114
20. Briere D, Sudhakar N, Woods DM, Hallin J, Engstrom LD, Aranda R, et al. The class I/IV HDAC inhibitor mocetinostat increases tumor antigen presentation, decreases immune suppressive cell types and augments checkpoint inhibitor therapy. *Cancer Immunol Immunother.* (2018) 67:381–92. doi: 10.1007/s00262-017-2091-y
21. Knox T, Sahakian E, Banik D, Hadley M, Palmer E, Noonepalle S, et al. Selective HDAC6 inhibitors improve anti-PD-1 immune checkpoint blockade therapy by decreasing the anti-inflammatory phenotype of macrophages and down-regulation of immunosuppressive proteins in tumor cells. *Sci Rep.* (2019) 9:1–17. doi: 10.1038/s41598-019-42237-3
22. Llopiz D, Ruiz M, Villanueva L, Iglesias T, Silva L, Egea J, et al. Enhanced anti-tumor efficacy of checkpoint inhibitors in combination with the histone deacetylase inhibitor Belinostat in a murine hepatocellular carcinoma model. *Cancer Immunol Immunother.* (2019) 68:379–93. doi: 10.1007/s00262-018-2283-0
23. Persky DO, Li H, Rimsza LM, Barr PM, Popplewell LL, Bane CL, et al. A phase I/II trial of vorinostat (SAHA) in combination with rituximab-CHOP in patients with newly diagnosed advanced stage diffuse large B-cell lymphoma (DLBCL): SWOG S0806. *Blood*. (2015) 93:486–93. doi: 10.1182/blood.v126.23.3931.3931
24. Terranova-Barberio M, Thomas S, Ali N, Pawlowska N, Park J, Krings G, et al. HDAC inhibition potentiates immunotherapy in triple negative breast cancer. *Oncotarget*. (2017) 8:114156. doi: 10.18632/oncotarget.23169
25. Dyrskjøt L, Kruhøffer M, Thykjaer T, Marcussen N, Jensen JL, Møller K, et al. Gene expression in the urinary bladder: a common carcinoma in situ gene expression signature exists disregarding histopathological classification. *Cancer Res.* (2004) 64:4040–8. doi: 10.1158/0008-5472.CAN-03-3620
26. Dyrskjøt L, Zieger K, Real FX, Malats N, Carrato A, Hurst C, et al. Gene expression signatures predict outcome in non - muscle-invasive bladder carcinoma: a multicenter validation study. *Clin Cancer Res.* (2007) 13:3545–51. doi: 10.1158/1078-0432.CCR-06-2940
27. Lindgren D, Frigyesi A, Gudjonsson S, Sjödahl G, Hallden C, Chebil G, et al. Combined gene expression and genomic profiling define two intrinsic molecular subtypes of urothelial carcinoma and gene signatures for molecular grading and outcome. *Cancer Res.* (2010) 70:3463–72. doi: 10.1158/0008-5472.CAN-09-4213
28. Ozawa A, Tanji N, Kikugawa T, Sasaki T, Yanagihara Y, Miura N, et al. Inhibition of bladder tumour growth by histone deacetylase inhibitor. *BJU Int.* (2010) 105:1181–6. doi: 10.1111/j.1464-410X.2009.08795.x
29. Poyet C, Jentsch B, Hermanns T, Schweckendiek D, Seifert H-H, Schmidtpeter M, et al. Expression of histone deacetylases 1, 2 and 3 in urothelial bladder cancer. *BMC Clin Pathol.* (2014) 14:10. doi: 10.1186/1472-6890-14-10
30. Moss TJ, Qi Y, Xi L, Peng B, Kim TB, Ezzedine NE, et al. Comprehensive genomic characterization of upper tract urothelial carcinoma. *Eur Urol.* (2017) 72:641–9. doi: 10.1016/j.eururo.2017.05.048
31. Berger MR, Richter H, Seelig MH, Eibl H, Schmähl D. New cytostatics-more activity and less toxicity. *Cancer Treat Rev.* (1990) 17:143–54. doi: 10.1016/0305-7372(90)90039-I
32. El-Beltagi HM, Martens ACM, Lelieveld P, Haroun EA, Hagenbeek A. Acetyldinaline: a new oral cytostatic drug with impressive differential activity against leukemic cells and normal stem cells preclinical studies in a relevant rat model for human acute myelocytic leukemia. *Cancer Res*. (1993) 53:3008–14.
33. LoRusso PM, Demchik L, Foster B, Knight J, Bissery MC, Polin LM, et al. Preclinical antitumor activity of CI-994. *Invest New Drugs*. (1996) 14:349–56. doi: 10.1007/BF00180810
34. Marks PA, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer: Causes and therapies. *Nat Rev Cancer*. (2001) 1:194–202. doi: 10.1038/35106079
35. Banik D, Moufarrij S, Villagra A. Immunoepigenetics combination therapies: an overview of the role of HDACs in cancer immunotherapy. *Int J Mol Sci.* (2019) 20:2241. doi: 10.3390/ijms20092241
36. Kasai M, Yoneda T, Habu S, Maruyama Y, Okumura K, Tokunaga T. *In vivo* effect of anti-asialo GM1 antibody on natural killer activity. *Nature*. (1981) 291:334–5. doi: 10.1038/291334a0
37. Kasman L, Voelkel-Johnson C. An orthotopic bladder cancer model for gene delivery studies. *J Vis Exp*. (2013) 82:e50181. doi: 10.3791/50181
38. Perez-Diez A, Joncker NT, Choi K, Chan WFN, Anderson CC, Lantz O, et al. CD4 cells can be more efficient at tumor rejection than CD8 cells. *Blood*. (2007) 109:5346–54. doi: 10.1182/blood-2006-10-051318
39. West AC, Johnstone RW. New and emerging HDAC inhibitors for cancer treatment. *J Clin Invest.* (2014) 124:30–9. doi: 10.1172/JCI69738
40. Falkenberg KJ, Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nat Rev Drug Discov*. (2014) 13:673. doi: 10.1038/nrd4360
41. Dicato MA. Multiple myeloma. In: Dicato MA, Van Cutsem E, editors. *Side Effects of Medical Cancer Therapy: Prevention and Treatment. 2nd Edn*. Cham: Springer International Publishing (2018).
42. Vo DD, Prins RM, Begley JL, Donahue TR, Morris LF, Bruhn KW, et al. Enhanced antitumor activity induced by adoptive T-cell transfer and adjunctive use of the histone deacetylase inhibitor LAQ824. *Cancer Res.* (2009) 69:8693–9. doi: 10.1158/0008-5472.CAN-09-1456
43. Woods DM, Woan K, Cheng F, Wang H, Perez-Villarroel P, Lee C, et al. The antimelanoma activity of the histone deacetylase inhibitor panobinostat (LBH589) is mediated by direct tumor cytotoxicity and increased tumor immunogenicity. *Melanoma Res.* (2013) 23:341. doi: 10.1097/CMR.0b013e328364c0ed
44. Kovacs JJ, Murphy PJM, Gaillard S, Zhao X, Wu JT, Nicchitta CV, et al. HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. *Mol Cell*. (2005) 18:601–7. doi: 10.1016/j.molcel.2005.04.021

45. Gameiro SR, Malamas AS, Tsang KY, Ferrone S, Hodge JW. Inhibitors of histone deacetylase 1 reverse the immune evasion phenotype to enhance T-cell mediated lysis of prostate and breast carcinoma cells. *Oncotarget*. (2016) 7:7390. doi: 10.18632/oncotarget.7180
46. de Mora-García ML, Duenas-González A, Hernández-Montes J, De la Cruz-Hernández E, Pérez-Cárdenas E, et al. Up-regulation of HLA class-I antigen expression and antigen-specific CTL response in cervical cancer cells by the demethylating agent hydralazine and the histone deacetylase inhibitor valproic acid. *J Transl Med.* (2006) 4:55. doi: 10.1186/1479-5876-4-55
47. Setiadi AF, Omilusik K, David MD, Seipp RP, Hartikainen J, Gopaul R, et al. Epigenetic enhancement of antigen processing and presentation promotes immune recognition of tumors. *Cancer Res.* (2008) 68:9601–7. doi: 10.1158/0008-5472.CAN-07-5270
48. Vire B, de Walque S, Restouin A, Olive D, Van Lint C, Collette Y. Anti-leukemia activity of MS-275 histone deacetylase inhibitor implicates 4-1BBL/4-1BB immunomodulatory functions. *PLoS ONE*. (2009) 4:e7085. doi: 10.1371/journal.pone.0007085
49. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor *in vivo*. *Proc Natl Acad Sci USA*. (2001) 98:11521–6. doi: 10.1073/pnas.201238598
50. Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature*. (2001) 413:165–71. doi: 10.1038/35093109
51. Wu X, Tao Y, Hou J, Meng X, Shi J. Valproic acid upregulates NKG2D ligand expression through an ERK-dependent mechanism and potentially enhances NK cell-mediated lysis of myeloma. *Neoplasia*. (2012) 14:1178. doi: 10.1593/neo.121236
52. Pockley AG. Heat shock proteins as regulators of the immune response. *Lancet*. (2003) 362:469–76. doi: 10.1016/S0140-6736(03)14075-5
53. Srivastava PK, Menoret A, Basu S, Binder RJ, McQuade KL. Heat shock proteins come of age: Primitive functions acquire new roles in an adaptive world. *Immunity*. (1998) 8:657–65.doi: 10.1016/S1074-7613(00) 80570-1
54. Ciupitu AMT, Petersson M, O'Donnell CL, Williams K, Jindal S, Kiessling R, et al. Immunization with a lymphocytic choriomeningitis virus peptide mixed with heat shock protein 70 results in protective antiviral immunity and specific cytotoxic T lymphocytes. *J Exp Med*. (1998) 187:685–91. doi: 10.1084/jem.187.5.685
55. Udono H, Levey DL, Srivastava PK. Cellular requirements for tumor-specific immunity elicited by heat shock proteins: Tumor rejection antigen gp96 primes CD8+ T cells *in vivo*. *Proc Natl Acad Sci USA*. (1994) 91:3077–81. doi: 10.1073/pnas.91.8.3077
56. Perez C, Prajapati K, Burke B, Plaza-Rojas L, Zeleznik-Le NJ, Guevara-Patino JA. NKG2D signaling certifies effector CD8 T cells for memory formation 11 Medical and Health Sciences 1107 Immunology. *J Immunother Cancer*. (2019) 7:48. doi: 10.1186/s40425-019-0531-2
57. Li MO, Wan YY, Sanjabi S, Robertson A-KL, Flavell RA. Transforming growth factor-β regulation of immune responses. *Annu Rev Immunol.* (2006) 24:99–146. doi: 10.1146/annurev.immunol.24.021605.090737
58. Wan YY, Flavell RA. 'Yin-Yang' functions of transforming growth factor-beta and T regulatory cells in immune regulation. *Immunol Rev*. (2007) 220:199–213. doi: 10.1111/j.1600-065X.2007.00565.x
59. Martina MN, Noel S, Saxena A, Rabb H, Hamad ARA. Double Negative (DN) αβ T Cells: Misperception and overdue recognition. *Immunol Cell Biol.* (2015) 93:305–10. doi: 10.1038/icb.2014.99
60. Viret C, Janeway CA. Self-specific MHC class II-restricted CD4 – CD8 – T cells that escape deletion and lack regulatory activity. *J Immunol.* (2003) 170:201–9. doi: 10.4049/jimmunol.170.1.201
61. Riol-Blanco L, Lazarevic V, Awasthi A, Mitsdoerffer M, Wilson BS, Croxford A, et al. IL-23 Receptor regulates unconventional IL-17–producing T cells that control bacterial infections. *J Immunol.* (2010) 184:1710–20. doi: 10.4049/jimmunol.0902796
62. Marchion D, Münster P. Development of histone deacetylase inhibitors for cancer treatment. *Expert Rev Anticancer Ther*. (2007) 7:583–98. doi: 10.1586/14737140.7.4.583
63. Perry AS, Watson RWG, Lawler M, Hollywood D. The epigenome as a therapeutic target in prostate cancer. *Nat Rev Urol.* (2010) 7:668. doi: 10.1038/nrurol.2010.185

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REVIEW
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Adjuvant Effect of Toll-Like Receptor 9 Activation on Cancer Immunotherapy Using Checkpoint Blockade

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Immunotherapy using checkpoint blockade has revolutionized cancer treatment, improving patient survival and quality of life. Nevertheless, the clinical outcomes of such immunotherapy are highly heterogeneous between patients. Depending on the cancer type, the patient response rates to this immunotherapy are limited to 20–30%. Based on the mechanism underlying the antitumor immune response, new therapeutic strategies have been designed with the aim of increasing the effectiveness and specificity of the antitumor immune response elicited by checkpoint blockade agents. The activation of toll-like receptor 9 (TLR9) by its synthetic agonists induces the antitumor response within the innate immunity arm, generating adjuvant effects and priming the adaptive immune response elicited by checkpoint blockade during the effector phase of tumor-cell killing. This review first describes the underlying mechanisms of action and current status of monotherapy using TLR9 agonists and immune checkpoint inhibitors for cancer immunotherapy. The rationale for combining these two agents is discussed, and evidence indicating the current status of such combination therapy as a novel cancer treatment strategy is presented.

Keywords: adjuvant, cancer immunotherapy, CpG-ODN, innate immune, toll-like receptor, immune checkpoint blockade

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INTRODUCTION

Major advances have been made in the field of cancer immunotherapy in the past two decades (1, 2). Imiquimod, a toll-like receptor (TLR)7 agonist, was FDA-approved in 1997 under the brand Aldara for treating genital warts and later approved for treating superficial basal cell carcinoma in 2004 (3–5). Three anti-cancer vaccines have been approved by the FDA. BCG (TheraCys), was first approved in 1990 for non-muscle invasive bladder carcinoma (6). Subsequently, Sipuleucel-T (Provenge) was approved for metastatic castration-resistant prostate cancer, and talimogene laherparepvec (T-VEC or Imygic), an oncolytic-virus–based vaccine was approved for advanced melanoma (7, 8). The components of BCG and oncolytic viruses activate TLRs in cells to elicit immune responses (9, 10). Further developments include anti-cancer adoptive cell transfer, including dendritic cell and cytotoxic T-cell therapies, in which patients are treated with

ex vivo expanded autologous immune cells (11, 12). Studies of T-cell activation and suppression mechanisms have led to the discovery of key checkpoints for immune suppression, including the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (13–15), programmed cell death protein 1 (PD-1), and the PD-1 ligands programmed death-ligand (PD-L)1 and PD-L2 (16–19). The use of antibody (Yervoy, ipilimumab) for immune checkpoint blockade to increase the anti-cancer effect of T-cells was first approved by the FDA in 2011, and several additional checkpoint blockage drugs were subsequently approved (20–22). These immunotherapies have effectively improved the survival and life quality of cancer patients, resulting in their acceptance as the fourth standard treatment for cancers after surgery, chemotherapy, and radiation therapy. In 2016, the American Society of Clinical Oncology (ASCO) announced "Immunotherapy" as the year's top cancer advance. Further, in 2017, the ASCO named "Immunotherapy 2.0" as advance of the year, emphasizing the recent, rapid progress of research into new agents that enhance the innate abilities of immunity to fight cancers (23). Although cancer immunotherapy is a major achievement in fighting cancer, the efficacy for patient treatment is still limited and unsatisfactory. For example, the response rate of patients with solid tumors to checkpoint inhibitors is only 20–30% (24, 25). Therefore, novel strategies to improve the efficacy of cancer immunotherapy are needed.

Cancer cells are targeted by immune surveillance through a process similar to the host immune response to microbe-infected cells. The human immune system is capable of discriminating and destroying cancer cells that display tumor antigens. These tumor antigens originate from self molecules but exhibit antigenic mutations and/or ectopic expression during tumor development (26, 27). Many cellular and molecular factors are involved in this process of immune suppression of tumor growth. Innate immune cells, including natural killer (NK) cells, monocytes/macrophages, and dendritic cells, mediate direct innate antitumor responses and activate adaptive immune cells such as T and B cells to develop memory and long-term responses to tumor cells. In the innate immune arm, cells release a variety of cytokines to support the immunological activities in the tumor microenvironment. NK cells directly lyse abnormal cells. Monocytes/macrophages and dendritic cells take up debris from dead cancer cells to present peptide fragments of tumor antigens to T-cells through the major histocompatibility complex (MHC) molecules. Such antigen presentation activates the subpopulation of B and T-cells that express tumor antigen recognition receptors to proliferate and differentiate. B cells generate a humoral response by secreting antibodies specific to tumor antigens. T-cells are classified into two major subsets: $CD4^+$ helper T-cells release immunomodulatory cytokines, and $CD8^+$ cytolytic T-cells act as effector cells to directly lyse tumor cells during the adaptive antitumor immune response (28–31).

Thus, the immune system employs coordinated innate immunity and adaptive immunity to fight tumors. This observation provides the rationale for boosting the efficacy (including strength and precision) of an adaptive antitumor immunotherapy such as checkpoint blockade by targeting innate immune cells to activate of the adjuvant response or priming effect (28–31). TLRs are broadly expressed in immune cells for the detection of microbial pathogens to initiate host responses to infection (32–34). Synthetic TLR agonists such as imiquimod have been approved for anti-virus and cancer therapies, and others are being investigated for mono- or combination cancer therapies (10, 35–37). In the following discussion, we will focus on advances in the use of CpG-oligodeoxynucleotide (CpG-ODN), a synthetic TLR9 agonist to increase the efficacy of cancer immunotherapy with checkpoint blockade.

TLR9 FUNCTION, CELLULAR LOCALIZATION, AND SIGNALING

The innate immunity is essential for host defense against microbial infections. Innate immune cells use a diverse variety of pattern recognition receptors (PRRs), including TLRs, to detect various microbial pathogen-associated molecular patterns (PAMPs). Such recognition initiates immediate innate immune responses, leading to the development of adaptive immune responses (33, 38–40). Thirteen TLRs (TLR1–13) have been identified in mammals, and ten (TLR1–10) are expressed in humans. These TLRs recognize a diverse variety of microbial PAMPs via their extracellular domain consisting of multiple leucine-rich repeats (LRRs) (41–45). TLR1, TLR2, TLR6, and TLR10 comprise a subfamily. TLR2 recognizes a broad range of microbial products, including lipoproteins, lipoteichoic acids, lipoarabinomannan, peptidoglycan, glycophosphatidylinositol anchors, zymosan, and prions. TLR2 and TLR1 form a complex that selectively recognizes bacterial lipoproteins and triacyl lipopeptides, whereas a heterodimer composed of TLR2 and TLR6 preferentially recognizes mycoplasma macrophage-activating lipopeptide 2 (46–51). The natural ligand of TLR10 is not yet well characterized; however, a recent study showed that this TLR recognizes double-stranded RNA (dsRNA) (52). TLR4 and TLR5 are closely related. TLR4 recognizes lipopolysaccharides from gram-negative bacteria, and TLR5 recognizes bacterial flagellin (53, 54). The members of the TLR3, TLR7, TLR8, and TLR9 subfamilies recognize nucleic-acid–derived structures. TLR3 detects double-stranded RNA (dsRNA) generated from viral replication in infected cells (55). TLR7 and TLR8 interact with single-stranded RNA viruses such as influenza virus and the vesicular stomatitis virus (56, 57). TLR9 responds to unmethylated CpG-DNA, including microbial DNA from DNA viruses (58, 59). In addition, TLRs recognize a wide variety of endogenous danger-associated molecular patterns (DAMPs) released from dead cells in damaged tissues. These DAMPs are cellular components and stress-induced gene products such as extracellular matrix components, extracellular proteins, intracellular proteins, and nucleic acids (60, 61).

Of the TLRs, TLR9 has the narrowest cell expression profile. In humans, this TLR is constitutively expressed in B cells and plasmacytoid dendritic cells (pDCs) and to some extent is also expressed in activated neutrophils, monocytes/macrophages, cDCs, and T-cells. In addition, TLR9 has been shown to be expressed in some non-immune cells, including keratinocytes and gut, cervical, and respiratory epithelial cells (37, 62, 63).

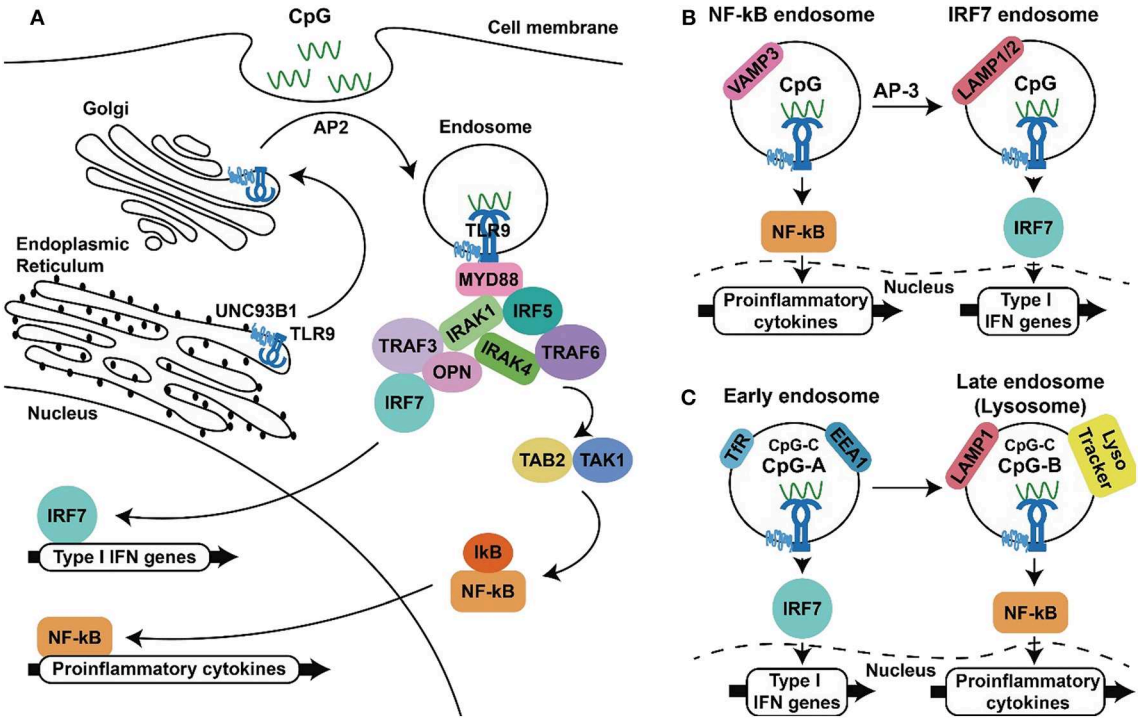

FIGURE 1 | TLR9 signaling to produce inflammatory cytokines and type I IFNs. **(A)** TLR9 synthesized in the endoplasmic reticulum traffics through the ER, Golgi to endosome with the aid of UNC93B and AP2, where TLR9 interact with CpG-ODN, recruits MyD88 and downstream signaling molecules to activate NF-κB and IRF-7, resulting in the production inflammatory cytokines and type I IFNs **(B,C)**. Two proposed models for the spatiotemporal activation of NF-κB and IRF-7 at different type of endosomes. In the first model, TLR9 triggers NF-κB activation from the VAMP3$^+$ endosomal compartments within minutes after activation and initiates IRF-7 activation in the LAMP1/2 endosomal compartment in 30 min to hours **(B)**. In the second model in plasmacytoid dendritic cells, class A CpG-ODNs activate IRF-7 to produce type I IFNs from the EEA1$^+$, TfR$^+$ early endosomes. In contrast, class B CpG-ODNs activate NF-κB for inflammatory cytokine production in LAMP1$^+$ and lysoTracker$^+$ late endosomes/lysosomes **(C)**. The capability of class C CpG-ODNs to activate production of type I IFNs and inflammatory cytokines is in between the capabilities of CpG-ODNs in class A and class C.

Distinct from other TLRs, TLR3, TLR7, TLR8, and TLR9 are located in intracellular vesicles (64–66). In resting cells, TLR9 is localized in the endoplasmic reticulum (ER) and must be trafficked to endosomes for activation by its agonist. The intracellular trafficking of this TLR is regulated by accessory proteins such as UNC-93 homolog B1 (UNC93B1) and specific adaptor proteins (APs). UNC93B1 interacts with TLR9 in the endoplasmic reticulum (ER) and follows the secretory pathway through the Golgi apparatus to the plasma membrane via coat protein complex II (COPII) vesicles. At the cell membrane, UNC93B1 recruits the adaptor protein AP-2 for the endocytosis of TLR9 via clathrin-containing vesicles. In the endosome, TLR9 interacts with its agonist CpG-DNA, which also enters cells via endocytosis [**Figure 1A**, (67–69)].

This engagement culminates in two outcomes: activation of NF-κB to produce inflammatory cytokines and activation of interferon regulatory factors (IRFs) to produce type I interferons (IFNs). Myeloid differentiation primary response 88 (MyD88) is required for TLR9 signal transduction, as MyD88 deficiency abolishes downstream signaling for cytokine productions following TLR9 activation (70). Following recruitment by TLR9, MyD88 in turn interacts with interleukin-1 receptor-associated kinase-1 (IRAK-1) and IRAK-4 through its death domain. IRAK-4 phosphorylates IRAK-1, up-regulating its kinase activity, which leads to the recruitment of tumor necrosis factor associated factor 6 (TRAF6) and the activation of transforming growth factor-β associated kinase 1 (TAK1). This cascade results in activation of the transcription factors NF-κB, which are responsible for the transcription of pro-inflammatory cytokine genes, including IL-6, IL-12 and TNF-α (71–74). Other than this, the transcription factor IRF5 is reported to be indispensable for TLR9-mediated production of pro-inflammatory cytokines. IRF5 interacts with MyD88, and TLR activation induces nuclear translocation of this transcription factor to promote gene expressions (75). In addition to inducing pro-inflammatory cytokine expression, TLR9 activation results in the production of type I IFNs, which are composed of multiple IFN-αs and a single IFN-β. These IFNs play a critical role in TLR9-mediated antitumor responses because they are involved in activation of the adaptive immune response required for tumor-cell killing (76–78). IRF-7 is a transcription factor expressed in pDCs that regulates the expression of type I IFN genes. IRF-7 associates with the complex of MyD88, IRAK1, IRAK4, and TRAF6, where IRF-7 becomes phosphorylated and translocates into the nucleus to induce transcription of type I IFNs (79, 80). In mice, TLR9-mediated production of IFNs is abrogated in cells deficient

in osteopontin (Opn) and TRAF3, whereas the production of IL-12 unaffected, suggesting that Opn and TRAF3 are involved in the signaling pathway that mediates TLR9-induced activation of type I IFN production [**Figure 1A**, (81, 82)].

Two different mechanisms have been proposed for this signal-bifurcated process. One model suggests that TLR9 from the Golgi enters endosomes and then the VAMP3$^+$ (vesicle-associated membrane protein 3) endosomes, leading to inflammatory cytokine expression. Subsequently, with the aid of AP-3, TLR9 is shuttled to LAMP1/2$^+$ (lysomal associated membrane protein 1/2) lysosome-related organelles (LROs) to promote the production of type I IFNs [**Figure 1B**, (83, 84)]. In contrast, another model suggested that TLR9 activation, signalings leading to gene transcription of inflammatory cytokines and type I IFNs come from different type of endosomes. In this model, TLR9 activation in the TfR (transferrin receptor) and EEA1 (early endosomal antigen 1) expressed early endosomes results in IRF-7 activation and production of type IFNs, whereas activation of TLR9 in the LAMP1 and LysoTracker positive late endosome/lysosome lead to the activation of NF-κB and production of inflammatory cytokines [**Figure 1C**, (85–87)]. Although the location of TLR9 required to trigger such signaling is uncertain, the acidic pH of the endolysosomal compartments is thought to be required for ligand recognition of TLR9, as compounds that interfere with endosomal acidification, such as bafilomycin A1 and chloroquine, are inhibitors of TLR9 activation (88).

SYNTHETIC CpG-ODNS FOR TLR9 ACTIVATION

The immunostimulatory activity of microbial DNA was first observed in a DNA fraction of bacillus Calmette–Guerin (89, 90). Further studies revealed that the presence of unmethylated CpG deoxynucleotides in a particular context called the CpG motif is required for such DNA activity (91, 92). *In vivo* studies of gene knockout mice and *in vitro* studies of cell-based TLR9 activation assay later showed that TLR9 is the cellular receptor for CpG-DNA (58, 59, 93). The presence of CG dinucleotides in eukaryotic DNA is lower than in the prokaryotic DNA sequences. Further more the frequency of methylation on CpG sites are higher within eukaryotic DNA than in microbial DNA (94, 95). This difference in CpG-methylation provides a molecular base for TLR9 to distinguish self from non-self DNA in the host defense immune response to microbial infections (91, 96). Synthetic CpG-ODNs mimicking the immunostimulatory nature of microbial CpG-DNA were developed for therapeutic use (77, 96, 97). Natural microbial DNA contains a phosphodiester backbone that is easily degraded by nucleases *in vivo*. Replacement of the oxygen in the nucleic acid phosphate group with sulfur creates CpG-ODNs with a phosphorothioate backbone, making them more resistant to nucleases (98, 99).

CpG-ODNs are classified into three major classes based on their structure. The Class A CpG-ODNs (also known as type D) consist of a central phosphodiester palindromic region with one or more CpG-motifs and contain poly (G) sequences with a phosphorothioate backbone attached to both of the 5' and 3' ends. Class B (type K) CpG-ODNs contain several CpG-motifs and a phosphorothiolate backbone throughout the entire sequence. Class C CpG-ODNs contain one or two CpG-motifs, an entire phosphorothioate backbone, and a palindromic sequence at the 3' end (100–103). More recently, CpG-ODNs with different structural features have been developed to improve their effectiveness and reduce their toxicity. For example, IMO-2125 is generated by linking two CpG-ODN together through their 3' ends (104). MGN1730 contains two loops of CpG-ODN, each containing three CpG-motifs linked by a double-stranded linker (105). Another design employs CpG-ODN conjugated with an antisense oligonucleotide of signal transducer and activator of transcription (STAT3), an oncogenic transcription factor. The first generation of this CpG-STAT3 inhibitor (CSI-1) uses RNA interference for STAT3 silencing. The second generation of this molecule (CSI-2) uses a decoy oligodeoxynucleotide to increase its nuclease resistance (106, 107).

The immunostimulatory activity of a CpG-ODN is dependent on its structure. Class A CpG-ODNs induce maturation of pDCs, have little effect on B cells, and activate the production of large amounts of IFN-α. Class B CpG-ODNs strongly induce B-cell proliferation, activate pDC and monocyte maturation, NK cell activation, and inflammatory cytokine production. These CpG-ODNs also stimulate the production of IFN-α, but to a lesser extent than do the class A CpG-ODNs. The capability of class C CpG-ODNs to induce B-cell proliferation and IFN-α production is between that of class A and B CpG-ODNs (100–103). The distinct abilities of class A and class B CpG-ODNs in induction of type I IFNs is resulted from their higher order structures. Class A CpG-ODNs are able to form multimeric aggregates with a diamteter of about 50 nm. In contrast, Class B CpG-ODNs are monomeric and do not have such a feature (108). Further, a model of spatiotemporal regulation of TLR9 as shown in the **Figure 1C** has been suggested to explain the differential immunostimulatory activities of CpG-ODNs. According to this model, Class A CpG-ODNs activate TLR9 in early endosomes to trigger IRF7 activation, inducing the production of large amounts of IFNs. Class B CpG-ODN is quickly transported to late endosomal/lysosomal compartments for TLR9 activation to activate NF-κB and produce inflammatory cytokines. In contrast, class C CpG-ODNs can be retained in these endosomal compartments, where they activate the production of IFNs and inflammatory cytokines (85–87). In line with these, encapsidation of class B CpG-ODNs into particles allow their retention in eraly endosomes for induction of higer level of type I IFNs (109).

The structure–function relationship of class B CpG-ODNs has been extensively investigated to enable their clinical use. The immunostimulatory activity of class B CpG-ODNs depends on their nucleotide sequence, CpG-dideoxynucleotide–containing hexamer motifs (CpG motif), and the number, spacing, position, and bases surrounding these CpG-motifs (100, 110, 111). Moreover, the activity of these CpG-ODNs differs between species, a phenomenon known as "species-specific activity." This activity of a CpG-ODN is determined by the nucleotide context

of its CpG-motifs. For example, CpG-2007, which contains 22 nucleotides and three copies of the GTCGTT-hexamer motif, is more potent in activating human cells than is CpG-1826, which contains 20 nucleotides and two copies of the GACGTT-hexamer motif. In contrast, CpG-1826 is more potent in activating murine cells than is CpG-2007 (93, 100, 110–112). The nucleotide length of CpG-ODN also plays a significant role in determining its species specificity. In rabbit cells, CpG-C4609, which contains 12 nucleotides and one AACGTT-hexamer motif, generates a stronger immune response than does CpG-2007 or CpG-1826 (113).

CpG-ODNS AS CANCER THERAPEUTICS

The activation of TLR9 by CpG-ODNs induces the immune response in two phases, innate immune and adaptive immune responses (96, 114, 115). Within hours of CpG-ODN stimulation, an antigen-independent innate immune response is elicited for an early immune response and for priming the subsequent adaptive immune responses. During this first innate immune response phase, DCs and B cells are activated. DCs are the most effective antigen-presenting cells (APCs). In addition to presenting extracellular antigens on MHC Class II molecules to $CD4^+$ T-cells, DCs also mediate cross-presentation of extracellular antigens on MHC Class I molecules to $CD8^+$ cytotoxic T-cells. These activities are crucial for establishing effective anti-cancer immunity (116–118). DCs produce inflammatory cytokines and type I IFNs through the activation of NF-κB and IRF. B cells produce cytokines, including IL-6 and IL-12, and chemokines via NF-κB activation. In turn, macrophages and NK cells are activated by IFNs released from pDCs. The macrophages and DCs are major IFN-γ-producing cells and APCs, and NK cells are capable of direct tumor killing during the CpG-ODN-induced antitumor response (119–122). These CpG-ODN–activated early immune responses are followed by a second phase of antigen-specific immune response that occurs several days later. B-cell stimulation by CpG-ODNs increases their sensitivity to antigen stimulation and promotes their differentiation into antibody-secreting plasma cells, increasing their production of antigen-specific antibodies (123, 124). Further, during this stage, CpG-ODN-activated APCs become competent for antigen presentation and the production of Th1-response–promoting cytokines. Increased expression of costimulatory molecules such as cluster of differentiation 80 (CD80), CD86, and molecules of the MHC increases the antigen-presenting activity of these cells to naïve T cells. The produced cytokines (TNF-α, IL-12, and IFNs) promote the T-helper-1 polarization of $CD4^+$ T cells. These result in expension of antigen-specific $CD8^+$ T cells (96, 114, 115, 125–127).

Because these immune responses facilitates eradication of cancer cells from bodies, the antitumor effect of CpG-ODNs has been investigated (76–78). In mouse tumor models, CpG-ODN monotherapy showed modest activity in inducing T-cell-mediated tumor regression. Injection of CpG-ODN into tumor exerted better anti-tumor activity than administration of the CpG-ODN at distant sites such as via intraperitoneal injection or intravenous injection (128, 129). Combining CpG-ODN with other therapeutics such as radiotherapy, chemotherapy, antitumor antibody, or DNA-based vaccination usually achieves greater tumor eradication (130–136). The effects resulting from combination therapy and local administration indicate that CpG-ODN exerts an adjuvant effect in the tumor microenvironment. Because tumor destruction by other therapies promotes the release of tumor antigens into the tumor microenvironment, injection of CpG-ODN into the site where tumor antigen is released has a greater effect on DC activation and antigen presentation to elicit a tumor-specific T-cell response (76–78).

Based on the positive results of preclinical studies showing that TLR9 activation can induce adjuvant effects to promote T-cell activation and reduce tumor burden, CpG-ODNs have been investigated in clinical trials as therapeutic antitumor agents (10, 35, 137, 138). The most widely investigated CpG-ODN is the B class agent PF-3512676 (also known as CpG-2006, CpG-7909, Agatolimod). Monotherapy with PF-3512676 has been investigated for treating basal cell carcinoma, renal cell cancer, melanoma, and cutaneous T-cell lymphoma via different routes, including subcutaneous, intravenous, and intratumoral injection. In patients, this CpG-ODN elicits cytokine production and antitumor T-cell responses with minimal toxicity beyond the local injection site reaction; however, its efficacy in reducing tumor growth is relatively low (139–142). Therefore, the efficacy of combination therapies using CpG-ODN with existing cancer therapeutics were investigated. In a phase II randomized trial with 184 stage IIIb/c or stage IV melanoma patients, the effect of subcutaneous PF-3512676 in monotherapy and combination therapy with intravenous dacarbazine (DITC) was investigated. Patients received either 10 mg of PF-3512676, 40 mg of PF-3512676, 40 mg of PF-3512676 plus DITC, or DITC alone as a control. The object response rate (ORR) was greatest in patients treated with 40 mg of PF-3512676 plus DITC. Nevertheless, no significant difference in overall survival (OS) or median time to progression was observed between treatment groups. Thus, the phase III portion of this study was not continued (143). Another randomized phase II trial evaluated the activity of subcutaneous PF-3512676 in combination with first-line taxine/platinum chemotherapy in 111 patients with non-small-cell lung cancer. The ORR (confirmed and unconfirmed) was 38% in the PF-3512676 arm ($n = 74$) and 19% in the chemotherapy-alone arm ($n = 37$). The median survival was 12.3 months in the PF-3512676 arm and 6.8 months in the chemotherapy-alone arm, with one-year survival of 50 and 33%, respectively (144). The combination of PF-3512676 with standard chemotherapy was further evaluated as a first-line treatment for advanced non–small-cell lung cancer in phase III trials. In one trial with 828 patients, the combination of subcutaneous PF-3512676 with intravenous paclitaxel/carplatin was compared with paclitaxel/carplatin alone. No significance improvement in OS or progression-free survival (PFS) was observed for PF-3512676 combination therapy. In another trials, comparison of PF-3512676 combined with gemcitabine/cisplatin and gemcitabine/cisplatin alone revealed a similar median OS and PFS in these two treatments (145, 146). To date, no CpG-ODN has been approved for cancer treatment, but a

TABLE 1 | FDA-approved antibodies targeting immune checkpoints for treating different type of cancers.

Inhibitor	Target	Approved	Tumor type	References
Ipilimumab (Yervoy®)	CTLA-4	2011	Advanced melanoma	(1)
		2018	Metastatic RCC (in combination of nivolumab), and CRC	(161, 162)
Pembrolizumab (Keytruda®)	PD-1	2014	Advanced melanoma	(163)
		2015	Metastatic NSCLC	(164)
		2016	Head and neck cancer	(165)
		2017	Classical Hodgkin lymphoma, urothelial carcinoma, any solid tumor with a specific genetic feature, and advanced gastric and gastroesophageal junction adenocarcinoma	(166–169)
		2018	Advanced cervical cancer, and HCC	(170, 171)
		2019	Advanced RCC (in combination of axitinib)	(172)
Nivolumab (Opdivo®)	PD-1	2014	Advanced melanoma	(173)
		2015	Lung cancer, and metastatic RCC	(174, 175)
		2016	Hodgkin lymphoma, and head and neck cancer	(175, 176)
		2017	Advanced urothelial carcinoma, CRC, and HCC (previously treated with sorafenib)	(177, 178)
Atezolizumab (Tecentriq®)	PD-L1	2016	Advanced urothelial carcinoma, and NSCLC progressed in platinum-containing therapy	(175, 179)
		2018	Advanced bladder cancer	(180)
		2019	PD-L1 positive TNBC (in combination of abraxane), and SCLC (in combination of carboplatin and etoposide)	(181, 182)
Avelumab (Bavencio®)	PD-L1	2017	Merkel cell carcinoma, and urothelial cancer	(167, 183)
		2019	Genitourinary cancer	(172)
Durvalumab (Imfinzi®)	PD-L1	2017	Advanced urothelial cancer	(167)
		2018	NSCLC	(184)
Cemiplimab-rwlc (Libtayo®)	PD-1	2018	Advanced cutaneous squamous cell carcinoma	(185)

RCC, renal cell carcinoma; CRC, colorectal cancer; NSCLC, non-small cell lung cancer; HCC, hepatocellular carcinoma; TNBC, triple-negative breast cancer; SCLC, small cell lung cancer.

wide variety of clinical studies exploring the potential of CpG-ODNs including in combinational use with immune checkpoint inhibitors for cancer therapy are still ongoing (138, 147).

COMBINATION THERAPY WITH CpG-ODNS AND IMMUNE CHECKPOINT INHIBITORS

Immune checkpoints are regulators of the immune system that maintain the immune response in a normal physiologic range and prevent inflammatory or autoimmune disorders resulting from over-activation of immune system. CTLA-4 and PD-1 are the two best-characterized immune checkpoint regulators (148–151). The expression of CTLA-4 is upregulated immediately following engagement of the T-cell receptor. This protein competes with the costimulatory receptor CD28 for its B7 ligands, CD80 (B7-1) and CD86 (B7-2), thereby interfering with the activation of CD28-mediated costimulatory signaling by these two ligands and attenuating T-cell activation. Because the negative regulatory function of CTLA-4 involves the expression of B7 ligands and CD28 signaling, CTLA-4 limits the early immune responses of T cells in lymphoid tissue (13, 152–154). In addition, CTLA-4 attenuates T-cell activation in peripheral tissues, as B7 ligands are constitutively expressed at differing levels in APCs and activated T cells. These observations suggest that CTLA-4 plays a central role in the regulation of T-cell activation and is critical for immune tolerance (14, 15, 149). In contrast to CTLA-4, PD-1 is expressed in activated and also exhausted T cells, B cells, and myeloid cells (16, 155, 156). Two ligands of PD-1, PD-L1 and PD-L2 are identified. Of them, PD-L2 induces IL-12 production in DCs. Given that IL-12 is important for T-cell differentiation into Th1-type cells, PD-L1 is a better target for inhibition to elicit antitumor immune response than is PD-L2 (18, 19, 157). PD-1 mainly regulates the late immune response of T cells in peripheral tissues, as its ligands are widely expressed in non-lymphoid tissues. Engagement of PD-1 with its ligand negatively regulates T-cell activation by activating the tyrosine phosphatase SHP2, which dephosphorylates and inactivates molecules involved in TCR signaling. SHP2 was

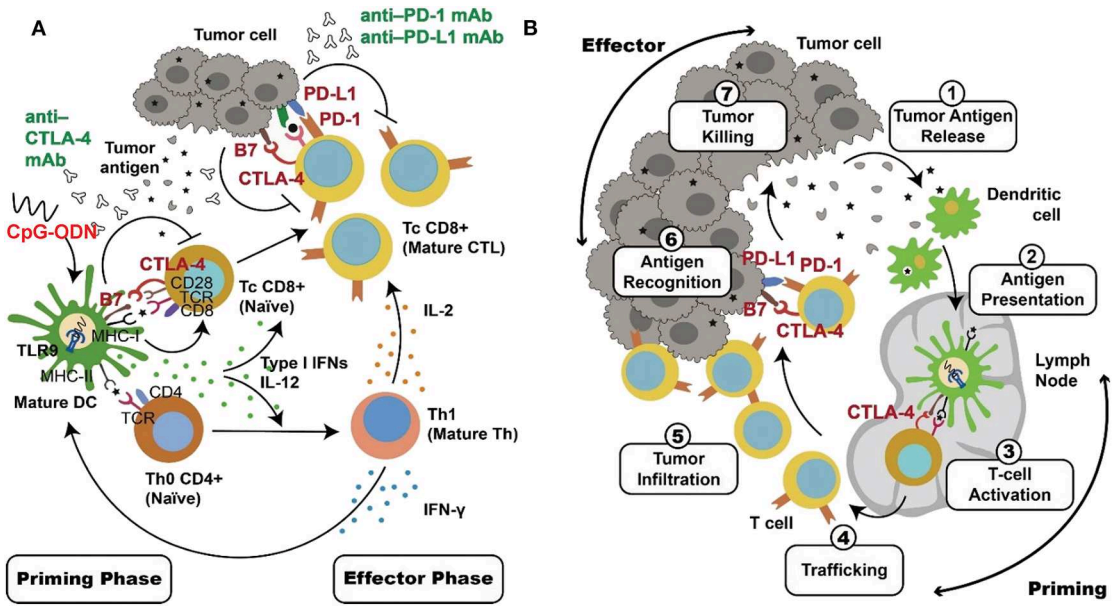

FIGURE 2 | Complementary mechanisms of TLR9 activation and checkpoint blockade in combinational cancer immunotherapy. DCs and T cells play key roles in the antitumor immune response. These two types of cells are major target for TLR9 agonists and immune checkpoint inhibitors, respectively. **(A)** Activation of TLR9 by CpG-ODN triggers innate immune responses, including cytokine production and the uptake and presentation of tumor antigen in DCs. These adjuvant effects, particularly the production of IL-12 and type I IFNs, facilitate a Th1 response of T cells and expansion of tumor-specific T cells during the priming phase. Immune checkpoint blockade by anti-PD-1/anti-PD-L1 antibody release inhibition of $CD8^+$ cytotoxic T-cell activation during the effector phase. In contrast, anti-CTLA-4 inhibition activates T cells during both of the priming and effector phases. These events lead to a more effective and more specific adaptive immune response for tumor-cell killing. **(B)** DCs and T cells involved in the antitumor immune response serve different immunological functions in different locations, as illustrated.

also shown to regulate CD28 signaling through its phosphatase activity (17, 149, 158–160). These observations suggest that CTLA-4 and PD-1 regulate T-cell activation by distinct but somewhat overlapping molecular mechanisms (148–151).

Because CTLA-4 and PD-1 act through ligand-receptor interactions, their activity can be blocked by specific monoclonal antibodies. Indeed, a variety of CTLA-4 and PD-1/PD-L1 monoclonal antibodies have been developed for immune checkpoint blockade. The anti-CTLA-4 antibody ipilimumab was approved by the FDA in 2011 for treating metastatic melanoma. Since then, six additional antibodies targeting PD-1 or PD-L1 have been approved for immunotherapy of different cancer types (**Table 1**). These checkpoint blockade therapies demonstrate notable efficacy for cancer treatment, nevertheless a large fraction of patients still fails to response to this treatment, indicating a tremendous need to improve the efficacy of therapies employing immune checkpoint inhibitors (149, 186, 187). The resistance of patients to immune checkpoint therapies may be caused by deficiencies in various aspects of T-cell activation for the antitumor response. Possibilities include poor immunogenicity of the tumor resulting from insufficient formation of tumor antigen and antigen presentation, inadequate T-cell activation and killing activity, and altered T-cell trafficking. Therefore, combining an immune checkpoint inhibitor with other treatment may increase the efficacy of such therapies (188–192).

A process of T-cell mediated antitumor response includes a priming phase which mainly involves with innate immune responses and an effector phase of an adaptive immunological tumor killing by T cells as shown in **Figure 2**. In the priming phase, activated APCs, such as dendritic cells, produce IL-12 and type 1 IFNs to facilitate a CD4+ T-cell-mediated Th 1 response. In addition, the dendritic cells produce costimulatory molecules and present antigen from a patient's cancer cells to promote proliferation of tumor-specific cytotoxic CD8+ T cells. These T cells then migrate to tumor sites, displaying their tumor-killing effects during the effector phase (29–31, 193–195). According to this mechanism, combination therapy including a TLR9 agonist and immune inhibitor is promising because these two agents use different and complementary mechanisms to up-regulate the T-cell-mediated antitumor response (138, 189–192). Activation of TLR9 in dendritic cells by CpG-ODN initiates the immune response via production of the costimulatory molecules CD80 and CD86 and cytokines TNF-α, IL-6, IL-12, and type I IFNs. Moreover, injection of CpG-ODN into the tumor site can induce local tumor-cell death, releasing more tumor antigens into the tumor microenvironment and activating antigen uptake and presentation by dendritic cells. These events promote effective generation of tumor-specific cytotoxic $CD8^+$ T cells during the priming phase (86, 87, 120, 122). In contrast, the immune checkpoint inhibitors release the inhibition of T-cell activity to promote tumor-cell killing during the effector phase (148–151). Thus, cancer therapy using a combination of TLR9 activation and immune checkpoint blockade can result in more robust and more specific tumor killing (**Figure 2**).

TABLE 2 | Current clinical trials of combination cancer immunotherapies using a TLR9 activation agonist and a checkpoint blockade agent.

TLR9 agonist	Class	Phase	Treatment	In combination with	Tumor type	References
CMP-001	A	II	I.V.	Nivolumab	Melanoma and lymph node cancer	NCT03618641
		I	I.T.	Pembrolizumab	Melanoma	NCT02680184
		I	S.C.	Ipilimumab, and nivolumab	Metastatic CRC	NCT03507699
		I/II	I.T.	Avelumab	SCCHN	NCT02554812
		I	IT/SC	Atezolizumab	NSCLC	NCT03438318
IMO-2125 (Tilsotolimod)		III	I.T.	Ipilimumab	Anti-PD-1 refractory melanoma	NCT03445533
		II	I.T.	Ipilimumab, and nivolumab	Solid tumors	NCT03865082
		I/II	I.T.	Ipilimumab, or pembrolizumab	Metastatic melanoma	NCT02644967
MGN1703 (Lefitolimod)		I	S.C.	Ipilimumab	Advanced cancers	NCT02668770
SD-101 (Dynavax)	C	II	I.T.	Pembrolizumab	Prostate cancer	NCT03007732
		I	I.T.	Nivolumab	Chemotherapy-refractory metastatic pancreatic adenocarcinoma	NCT04050085
		I/II	I.T.	Pembrolizumab	Metastatic melanoma or recurrent or metastatic HNSCC	NCT02521870
AST-008		I/II	I.T.	Pembrolizumab	Advanced solid tumors	NCT03684785
DV281	C	I	Inhaled	Nivolumab	Advanced NSCLC	NCT03326752

I.T., Intratumoural; I.V., Intravenous; S.C., Subcutaneous; CRC, colorectal cancer; SCCHN, squamous cell carcinoma of head and neck; NSCLC, non-small cell lung cancer; HNSCC, head and neck squamous cell carcinoma.

Studies using melanoma mouse models have shown that a synergistic effect on tumor regression results from combining CpG-ODN-mediated activation of APCs with immune checkpoint inhibitor-mediated T-cell activation (136, 196). Similar synergy resulting in longer survival was also observed in murine bladder cancer when CpG-ODN was combined with CTLA-4 or PD-1 inhibitors (197). Another studies revealed that CpG-ODN can revert resistence to PD-1 blockade therapy by expending CD8+ T cells in colon cancer animal model, enhances the efficacy of anti-PD-1 in head and neck cancer animal model (198, 199). CpG-ODN modulates tumor microenvironment, turns "cold" tumor into "hot" tumor, enhances the anti-tumor effect of immune checkpoint blockade in colon cancer animal model (200). Moreover, CpG-ODN delivered by inhalation is capable of priming T-cell responses against a poorly immunogenic lung tumor (201). The encouraging results in these animal studies provided the rationale for combined clinical regimens using CpG-ODNs and immune checkpoint inhibitors simultaneously. Several clinical investigations of such combination therapy are presently underway. CMP-001 is a class A CpG-ODN encapsulated into virus-like particles to render it stable. In a study of 69 patients with advanced melanoma and resistance to pembrolizumab therapy, CMP-001 and pembrolizumab were directly injected into the accessible lesions. The response rate was 21.7%, and an abscopal effect was observed, with shrinkage occurring in non-injected cutaneous, nodal, hepatic, and splenic metastases (202). In a study of SD-101/pembrolizumab combination therapy in 9 advanced melanoma patients naïve to anti-PD-1 therapy, a response rate of 78% was observed (203). Similar to the study of CMP-001, the SD101 exerted an abscopal effect, with tumor shrinkage observed in both the injected and non-injected lesions (202, 203). A clinical study of IMO-212 (Tilsotolimod), another TLR9 agonist, was conducted in a cohort of 26 patients with PD-1-inhibitor– refractory advanced melanoma. Combined therapy with IMO-2125 and ipilimumab resulted in an ORR of 38.1%, an increase over the 13% reported in a previous study of ipilimumab treatment alone. The disease control rate was 71.4% for the combination therapy, and an abscopal effect was observed with no synergistic toxicity. A global phase III randomized study comparing IMO-2125 plus ipilimumab to ipilimumab alone for treating PD-1-inhibitor refractory cancer is underway (204, 205). Combination therapy using TLR9 agonists and different immune checkpoint inhibitor are under clinical investigation for treating melanoma and other types of tumors (**Table 2**).

CONCLUSION AND PERSPECTIVES

The field of cancer immunotherapy has progressed significantly since the approval of ipilimumab in 2011. Therapy with immune checkpoint blockade has revealed benefits to cancer patients, improving their survival and quality of life. Despite breakthroughs in the field, the pool of patients benefiting from this therapy is relatively small. Thus, investigating combinations of immune checkpoint inhibitors with other currently available or novel cancer therapeutics is needed to maximize the benefits

of this cancer immunotherapy. Activation of TLR9 by CpG-ODN elicits the antitumor immune response. A wide variety of clinical trials are presently investigating the use of CpG-ODNs in antitumor therapy. Although no CpG-ODN has been approved for use as a cancer therapeutic agent, one such agent (CpG-1018) is used as an adjuvant in a Hepatitis B vaccine (HEPLISAV-B) approved by the FDA in 2018. This vaccine is proven more effective against Hepatitis B than those using aluminum salt as the adjuvant (206, 207). This observation suggests that CpG-ODN is a potent adjuvant and that is safe for therapeutic use. The mechanism of action of CpG-ODNs in activating the antitumor immune response is distinct and complementary to that underlying immune checkpoint blockade. Thus, the rationale for combining these agents for cancer therapy is sound. A number of clinical trials of therapies combining these two agents are presently underway for a variety of cancer types. The results will reveal whether combining these agents improves the efficacy of cancer immunotherapy using immune checkpoint inhibitors. Worth to note, although this review is focused on the antitumor effect of TLR9 activation, agonists of other TLRs were also shown to have antitumor activities. Imiquimod, a TLR7 agonist had FDA approved for treatment of superficial basal cell carcinoma in 2004 (3–5). Others including CADI-05 (TLR2 agonist), BO-112 (TLR3 agonist) and G100 (TLR4 agonist) were investigated in clinical trials for their antitumor effects (208–210). Whether these TLR agonists can improve the efficacy of immune checkpoint inhibitors in combinational therapies is also received attention.

AUTHOR CONTRIBUTIONS

All authors were involved in researching data for this article, contributed to discussion of the content, preparing, and writing the manuscript.

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REFERENCES

1. Dobosz P, Dzieciatkowski T. The Intriguing History of Cancer Immunotherapy. *Front Immunol.* (2019) 10:2965. doi: 10.3389/fimmu.2019.02965
2. Yousefi H, Yuan J, Keshavarz-Fathi M, Murphy JF, Rezaei N. Immunotherapy of cancers comes of age. *Expert Rev Clin Immunol.* (2017) 13:1001–15. doi: 10.1080/1744666X.2017.1366315
3. Kamath P, Darwin E, Arora H, Nouri K. A review on imiquimod therapy and discussion on optimal management of basal cell carcinomas. *Clin Drug Investig.* (2018) 38:883–99. doi: 10.1007/s40261-018-0681-x
4. Hanna E, Abadi R, Abbas O. Imiquimod in dermatology: an overview. *Int J Dermatol.* (2016) 55:831–44. doi: 10.1111/ijd.13235
5. Kobold S, Wiedemann G, Rothenfusser S, Endres S. Modes of action of TLR7 agonists in cancer therapy. *Immunotherapy.* (2014) 6:1085–95. doi: 10.2217/imt.14.75
6. Pettenati C, Ingersoll MA. Mechanisms of BCG immunotherapy and its outlook for bladder cancer. *Nat Rev Urol.* (2018) 15:615–25. doi: 10.1038/s41585-018-0055-4
7. Handy CE, Antonarakis ES. Sipuleucel-T for the treatment of prostate cancer: novel insights and future directions. *Future Oncol.* (2018) 14:907–17. doi: 10.2217/fon-2017-0531
8. Conry RM, Westbrook B, McKee S, Norwood TG. Talimogene laherparepvec: first in class oncolytic virotherapy. *Hum Vaccin Immunother.* (2018) 14:839–46. doi: 10.1080/21645515.2017.1412896
9. Cerullo V, Capasso C, Vaha-Koskela M, Hemminki O, Hemminki A. Cancer-targeted oncolytic adenoviruses for modulation of the immune system. *Curr Cancer Drug Targets.* (2018) 18:124–38. doi: 10.2174/1568009617666170502152352
10. Smith M, Garcia-Martinez E, Pitter MR, Fucikova J, Spisek R, Zitvogel L, et al. Trial watch: toll-like receptor agonists in cancer immunotherapy. *Oncoimmunology.* (2018) 7:e1526250. doi: 10.1080/2162402X.2018.1526250
11. Rohaan MW, Wilgenhof S, Haanen J. Adoptive cellular therapies: the current landscape. *Virchows Arch.* (2019) 474:449–461. doi: 10.1007/s00428-018-2484-0
12. Hammerl D, Rieder D, Martens JWM, Trajanoski Z, Debets R. Adoptive T cell therapy: new avenues leading to safe targets and powerful allies. *Trends Immunol.* (2018) 39:921–36. doi: 10.1016/j.it.2018.09.004
13. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity.* (1994) 1:405–13. doi: 10.1016/1074-7613(94)90071-X
14. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science.* (1995) 270:985–8. doi: 10.1126/science.270.5238.985
15. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity.* (1995) 3:541–7. doi: 10.1016/1074-7613(95)90125-6
16. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol.* (1996) 8:765–72. doi: 10.1093/intimm/8.5.765
17. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity.* (1999) 11:141–51. doi: 10.1016/S1074-7613(00)80089-8
18. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med.* (1999) 5:1365–9. doi: 10.1038/70932
19. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol.* (2001) 2:261–8. doi: 10.1038/85330
20. Cameron F, Whiteside G, Perry C. Ipilimumab: first global approval. *Drugs.* (2011) 71:1093–104. doi: 10.2165/11594010-000000000-00000
21. Diesendruck Y, Benhar I. Novel immune check point inhibiting antibodies in cancer therapy-Opportunities and challenges. *Drug Resist Updat.* (2017) 30:39–47. doi: 10.1016/j.drup.2017.02.001
22. Marhelava K, Pilch Z, Bajor M, Graczyk-Jarzynka A, Zagozdzon R. Targeting negative and positive immune checkpoints with monoclonal antibodies in therapy of cancer. *Cancers.* (2019) 11:1756. doi: 10.3390/cancers11111756
23. Madden DL. From a patient advocate's perspective: does cancer immunotherapy represent a paradigm shift? *Curr Oncol Rep.* (2018) 20:8. doi: 10.1007/s11912-018-0662-5
24. Haslam A, Prasad V. Estimation of the percentage of US patients with cancer who are eligible for and respond to checkpoint inhibitor immunotherapy drugs. *JAMA Netw Open.* (2019) 2:e192535. doi: 10.1001/jamanetworkopen.2019.2535

25. Das S, Johnson DB. Immune-related adverse events and anti-tumor efficacy of immune checkpoint inhibitors. *J Immunother Cancer.* (2019) 7:306. doi: 10.1186/s40425-019-0805-8
26. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science.* (2015) 348:69–74. doi: 10.1126/science.aaa4971
27. Cerezo-Wallis D, Soengas MS. Understanding tumor-antigen presentation in the new era of cancer immunotherapy. *Curr Pharm Des.* (2016) 22:6234–50. doi: 10.2174/1381612822666160826111041
28. Rossi JF, Ceballos P, Lu ZY. Immune precision medicine for cancer: a novel insight based on the efficiency of immune effector cells. *Cancer Commun.* (2019) 39:34. doi: 10.1186/s40880-019-0379-3
29. Borghaei H, Smith MR, Campbell KS. Immunotherapy of cancer. *Eur J Pharmacol.* (2009) 625:41–54. doi: 10.1016/j.ejphar.2009.09.067
30. Uher O, Caisova V, Hansen P, Kopecky J, Chmelar J, Zhuang Z, et al. Coley's immunotherapy revived: innate immunity as a link in priming cancer cells for an attack by adaptive immunity. *Semin Oncol.* (2019) 46:385–92. doi: 10.1053/j.seminoncol.2019.10.004
31. Wilkinson RW, Leishman AJ. Further advances in cancer immunotherapy: going beyond checkpoint blockade. *Front Immunol.* (2018) 9:1082. doi: 10.3389/fimmu.2018.01082
32. Medzhitov R, Janeway C Jr. The Toll receptor family and microbial recognition. *Trends Microbiol.* (2000) 8:452–6. doi: 10.1016/S0966-842X(00)01845-X
33. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol.* (2001) 2:675–80. doi: 10.1038/90609
34. Beutler B, Jiang Z, Georgel P, Crozat K, Croker B, Rutschmann S, et al. Genetic analysis of host resistance: toll-like receptor signaling and immunity at large. *Annu Rev Immunol.* (2006) 24:353–89. doi: 10.1146/annurev.immunol.24.021605.090552
35. Iribarren K, Bloy N, Buque A, Cremer I, Eggermont A, Fridman WH, et al. Trial watch: immunostimulation with toll-like receptor agonists in cancer therapy. *Oncoimmunology.* (2016) 5:e1088631. doi: 10.1080/2162402X.2015.1088631
36. Tartey S, Takeuchi O. Pathogen recognition and toll-like receptor targeted therapeutics in innate immune cells. *Int Rev Immunol.* (2017) 36:57–73. doi: 10.1080/08830185.2016.1261318
37. Patra MC, Shah M, Choi S. Toll-like receptor-induced cytokines as immunotherapeutic targets in cancers and autoimmune diseases. *Semin Cancer Biol.* (2019). doi: 10.1016/j.semcancer.2019.05.002. [Epub ahead of print].
38. Kabelitz D, Medzhitov R. Innate immunity–cross-talk with adaptive immunity through pattern recognition receptors and cytokines. *Curr Opin Immunol.* (2007) 19:1–3. doi: 10.1016/j.coi.2006.11.018
39. Suresh R, Mosser DM. Pattern recognition receptors in innate immunity, host defense, and immunopathology. *Adv Physiol Educ.* (2013) 37:284–91. doi: 10.1152/advan.00058.2013
40. Cao X. Self-regulation and cross-regulation of pattern-recognition receptor signalling in health and disease. *Nat Rev Immunol.* (2016) 16:35–50. doi: 10.1038/nri.2015.8
41. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci USA.* (1998) 95:588–93. doi: 10.1073/pnas.95.2.588
42. Takeuchi O, Kawai T, Sanjo H, Copeland NG, Gilbert DJ, Jenkins NA, et al. TLR6: a novel member of an expanding toll-like receptor family. *Gene.* (1999) 231:59–65. doi: 10.1016/S0378-1119(99)00098-0
43. Chuang TH, Ulevitch RJ. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur. Cytokine Netw.* (2000) 11:372–378.
44. Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. *Eur Cytokine Netw.* (2000) 11:362–71.
45. Chuang TH, Ulevitch RJ. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. *Biochim Biophys Acta.* (2001) 1518:157–61. doi: 10.1016/S0167-4781(00)00289-X
46. Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, et al. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science.* (1999) 285:736–9. doi: 10.1126/science.285.5428.736
47. Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, et al. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature.* (1999) 401:811–5. doi: 10.1038/44605
48. Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int Immunol.* (2001) 13:933–40. doi: 10.1093/intimm/13.7.933
49. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol.* (2002) 169:10–4. doi: 10.4049/jimmunol.169.1.10
50. Oliveira-Nascimento L, Massari P, Wetzler LM. The Role of TLR2 in Infection and Immunity. *Front Immunol.* (2012) 3:79. doi: 10.3389/fimmu.2012.00079
51. van Bergenhenegouwen J, Plantinga TS, Joosten LA, Netea MG, Folkerts G, Kraneveld AD, et al. TLR2 & Co: a critical analysis of the complex interactions between TLR2 and coreceptors. *J Leukoc Biol.* (2013) 94:885–902. doi: 10.1189/jlb.0113003
52. Lee SM, Yip TF, Yan S, Jin DY, Wei HL, Guo RT, et al. Recognition of double-stranded RNA and regulation of interferon pathway by toll-like receptor 10. *Front Immunol.* (2018) 9:516. doi: 10.3389/fimmu.2018.00516
53. Poltorak A, He X, Smirnova I, Liu MY, Van HC, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science.* (1998) 282:2085–88. doi: 10.1126/science.282.5396.2085
54. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature.* (2001) 410:1099–103. doi: 10.1038/35074106
55. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by toll-like receptor 3. *Nature.* (2001) 413:732–8. doi: 10.1038/35099560
56. Diebold SS, Kaisho T, Hemmi H, Akira S, Sousa Re. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science.* (2004) 303:1529–31. doi: 10.1126/science.1093616
57. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science.* (2004) 303:1526–9. doi: 10.1126/science.1093620
58. Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, Akira S, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci USA.* (2001) 98:9237–42. doi: 10.1073/pnas.161293498
59. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. *Nature.* (2000) 408:740–5. doi: 10.1038/35047123
60. Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. *Mediators Inflamm.* (2010) 2010: 672395. doi: 10.1155/2010/672395
61. Yu L, Wang L, Chen S. Endogenous toll-like receptor ligands and their biological significance. *J Cell Mol Med.* (2010) 14:2592–603. doi: 10.1111/j.1582-4934.2010.01127.x
62. Muller T, Hamm S, Bauer S. TLR9-mediated recognition of DNA. *Handb Exp Pharmacol.* (2008) 51–70. doi: 10.1007/978-3-540-72167-3_3
63. Kumagai Y, Takeuchi O, Akira S. TLR9 as a key receptor for the recognition of DNA. *Adv Drug Deliv Rev.* (2008) 60:795–804. doi: 10.1016/j.addr.2007.12.004
64. Trivedi S, Greidinger EL. Endosomal Toll-like receptors in autoimmunity: mechanisms for clinical diversity. *Therapy.* (2009) 6:433–42. doi: 10.2217/thy.09.2
65. Averett DR, Fletcher SP, Li W, Webber SE, Appleman JR. The pharmacology of endosomal TLR agonists in viral disease. *Biochem Soc Trans.* (2007) 35:1468–72. doi: 10.1042/BST0351468
66. Lai CY, Su YW, Lin KI, Hsu LC, Chuang TH. Natural modulators of endosomal toll-like receptor-mediated psoriatic skin inflammation. *J Immunol Res.* (2017) 2017:7807313. doi: 10.1155/2017/7807313
67. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol.* (2004) 5:190–8. doi: 10.1038/ni1028
68. McGettrick AF, O'Neill LA. Localisation and trafficking of toll-like receptors: an important mode of regulation. *Curr Opin Immunol.* (2010) 22:20–7. doi: 10.1016/j.coi.2009.12.002

69. Marongiu L, Gornati L, Artuso I, Zanoni I, Granucci F. Below the surface: the inner lives of TLR4 and TLR9. *J Leukoc Biol.* (2019) 106:147–60. doi: 10.1002/JLB.3MIR1218-483RR
70. Hacker H, Vabulas RM, Takeuchi O, Hoshino K, Akira S, Wagner H. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. *J Exp Med.* (2000) 192:595–600. doi: 10.1084/jem.192.4.595
71. Kawagoe T, Sato S, Jung A, Yamamoto M, Matsui K, Kato H, et al. Essential role of IRAK-4 protein and its kinase activity in toll-like receptor-mediated immune responses but not in TCR signaling. *J Exp Med.* (2007) 204:1013–24. doi: 10.1084/jem.20061523
72. Deng L, Wang C, Spencer E, Yang L, Braun A, You J, et al. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell.* (2000) 103:351–61. doi: 10.1016/S0092-8674(00)00126-4
73. Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature.* (2001) 412:346–51. doi: 10.1038/35085597
74. Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, et al. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat Immunol.* (2005) 6:1087–95. doi: 10.1038/ni1255
75. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature.* (2005) 434:243–9. doi: 10.1038/nature03308
76. Krieg AM. Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. *Oncogene.* (2008) 27:161–7. doi: 10.1038/sj.onc.1210911
77. Vollmer J, Krieg AM. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Adv Drug Deliv Rev.* (2009) 61:195–204. doi: 10.1016/j.addr.2008.12.008
78. Holtick U, Scheulen ME, von Bergwelt-Baildon MS, Weihrauch MR. Toll-like receptor 9 agonists as cancer therapeutics. *Expert Opin Investig Drugs.* (2011) 20:361–72. doi: 10.1517/13543784.2011.553187
79. Kawai T, Sato S, Ishii KJ, Coban C, Hemmi H, Yamamoto M, et al. Interferon-α induction through toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol.* (2004) 5:1061–8. doi: 10.1038/ni1118
80. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature.* (2005) 434:772–7. doi: 10.1038/nature03464
81. Shinohara ML, Lu L, Bu J, Werneck MB, Kobayashi KS, Glimcher LH, et al. Osteopontin expression is essential for interferon-alpha production by plasmacytoid dendritic cells. *Nat Immunol.* (2006) 7:498–506. doi: 10.1038/ni1327
82. Zhao K, Zhang M, Zhang L, Wang P, Song G, Liu B, et al. Intracellular osteopontin stabilizes TRAF3 to positively regulate innate antiviral response. *Sci Rep.* (2016) 6:23771. doi: 10.1038/srep23771
83. Sasai M, Linehan MM, Iwasaki A. Bifurcation of Toll-like receptor 9 signaling by adaptor protein 3. *Science.* (2010) 329:1530–4. doi: 10.1126/science.1187029
84. Hayashi K, Taura M, Iwasaki A. The interaction between IKKα and LC3 promotes type I interferon production through the TLR9-containing LAPosome. *Sci Signal.* (2018) 11:eaan4144. doi: 10.1126/scisignal.aan4144
85. Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, et al. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature.* (2005) 434:1035–40. doi: 10.1038/nature03547
86. Guiducci C, Ott G, Chan JH, Damon E, Calacsan C, Matray T, et al. Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. *J Exp Med.* (2006) 203:1999–2008. doi: 10.1084/jem.20060401
87. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol.* (2008) 8:594–606. doi: 10.1038/nri2358
88. Kuznik A, Bencina M, Svajger U, Jeras M, Rozman B, Jerala R. Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. *J Immunol.* (2011) 186:4794–804. doi: 10.4049/jimmunol.1000702
89. Tokunaga T, Yamamoto H, Shimada S, Abe H, Fukuda T, Fujisawa Y, et al. Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J Natl Cancer Inst.* (1984) 72:955–62.
90. Shimada S, Yano O, Inoue H, Kuramoto E, Fukuda T, Yamamoto H, et al. Antitumor activity of the DNA fraction from Mycobacterium bovis BCG. II. Effects on various syngeneic mouse tumors. *J Natl Cancer Inst.* (1985) 74:681–8.
91. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature.* (1995) 374:546–9. doi: 10.1038/374546a0
92. Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med.* (1997) 3:849–54. doi: 10.1038/nm0897-849
93. Chuang TH, Lee J, Kline L, Mathison JC, Ulevitch RJ. Toll-like receptor 9 mediates CpG-DNA signaling. *J Leukoc Biol.* (2002) 71:538–44.
94. Razin A, Friedman J. DNA methylation and its possible biological roles. *Prog Nucleic Acid Res Mol Biol.* (1981) 25:33–52. doi: 10.1016/S0079-6603(08)60482-1
95. Wagner H. Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv Immunol.* (1999) 73:329–68. doi: 10.1016/S0065-2776(08)60790-7
96. Klinman DM. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol.* (2004) 4:249–58. doi: 10.1038/nri1329
97. Jurk M, Vollmer J. Therapeutic applications of synthetic CpG oligodeoxynucleotides as TLR9 agonists for immune modulation. *BioDrugs.* (2007) 21:387–401. doi: 10.2165/00063030-200721060-00006
98. Shaw JP, Kent K, Bird J, Fishback J, Froehler B. Modified deoxyoligonucleotides stable to exonuclease degradation in serum. *Nucleic Acids Res.* (1991) 19:747–50. doi: 10.1093/nar/19.4.747
99. Stein CA, Subasinghe C, Shinozuka K, Cohen JS. Physicochemical properties of phosphorothioate oligodeoxynucleotides. *Nucleic Acids Res.* (1988) 16:3209–21. doi: 10.1093/nar/16.8.3209
100. Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol.* (2002) 20:709–60. doi: 10.1146/annurev.immunol.20.100301.064842
101. Gursel M, Verthelyi D, Gursel I, Ishii KJ, Klinman DM. Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. *J Leukoc Biol.* (2002) 71:813–20.
102. Vollmer J, Weeratna R, Payette P, Jurk M, Schetter C, Laucht M, et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur J Immunol.* (2004) 34:251–62. doi: 10.1002/eji.200324032
103. Samulowitz U, Weber M, Weeratna R, Uhlmann E, Noll B, Krieg AM, et al. A novel class of immune-stimulatory CpG oligodeoxynucleotides unifies high potency in type I interferon induction with preferred structural properties. *Oligonucleotides.* (2010) 20:93–101. doi: 10.1089/oli.2009.0210
104. Agrawal S, Kandimalla ER. Synthetic agonists of toll-like receptors 7, 8 and 9. *Biochem Soc Trans.* (2007) 35:1461–7. doi: 10.1042/BST0351461
105. Schmidt M, Hagner N, Marco A, Konig-Merediz SA, Schroff M, Wittig B. Design and structural requirements of the potent and safe TLR-9 agonistic immunomodulator MGN1703. *Nucleic Acid Ther.* (2015) 25:130–40. doi: 10.1089/nat.2015.0533
106. Kortylewski M, Kuo YH. Push and release: TLR9 activation plus STAT3 blockade for systemic antitumor immunity. *Oncoimmunology.* (2014) 3:e27441. doi: 10.4161/onci.27441
107. Zhang Q, Hossain DM, Duttagupta P, Moreira D, Zhao X, Won H, et al. Serum-resistant CpG-STAT3 decoy for targeting survival and immune checkpoint signaling in acute myeloid leukemia. *Blood.* (2016) 127:1687–700. doi: 10.1182/blood-2015-08-665604
108. Kerkmann M, Costa LT, Richter C, Rothenfusser S, Battiany J, Hornung V, et al. Spontaneous formation of nucleic acid-based nanoparticles is responsible for high interferon-alpha induction by CpG-A in plasmacytoid dendritic cells. *J Biol Chem.* (2005) 280:8086–93. doi: 10.1074/jbc.M410868200
109. Kerkmann M, Lochmann D, Weyermann J, Marschner A, Poeck H, Wagner M, et al. Immunostimulatory properties of CpG-oligonucleotides are enhanced by the use of protamine nanoparticles. *Oligonucleotides.* (2006) 16:313–22. doi: 10.1089/oli.2006.16.313

110. Pisetsky DS. Mechanisms of immune stimulation by bacterial DNA. *Springer Semin Immunopathol.* (2000) 22:21–33. doi: 10.1007/s002810000021
111. Yamamoto S, Yamamoto T, Tokunaga T. The discovery of immunostimulatory DNA sequence. *Springer Semin Immunopathol.* (2000) 22:11–9. doi: 10.1007/s002810000019
112. Rankin R, Pontarollo R, Ioannou X, Krieg AM, Hecker R, Babiuk LA, et al. CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. *Antisense Nucleic Acid Drug Dev.* (2001) 11:333–40. doi: 10.1089/108729001753231713
113. Chuang TH, Lai CY, Tseng PH, Yuan CJ, Hsu LC. Development of CpG-oligodeoxynucleotides for effective activation of rabbit TLR9 mediated immune responses. *PLoS ONE.* (2014) 9:e108808. doi: 10.1371/journal.pone.0108808
114. Krieg AM. Development of TLR9 agonists for cancer therapy. *J Clin Invest.* (2007) 117:1184–94. doi: 10.1172/JCI31414
115. Hanagata N. Structure-dependent immunostimulatory effect of CpG oligodeoxynucleotides and their delivery system. *Int J Nanomedicine.* (2012) 7:2181–95. doi: 10.2147/IJN.S30197
116. Veglia F, Gabrilovich DI. Dendritic cells in cancer: the role revisited. *Curr Opin Immunol.* (2017) 45:43–51. doi: 10.1016/j.coi.2017.01.002
117. Bol KF, Schreibelt G, Rabold K, Wculek SK, Schwarze JK, Dzionek A, et al. The clinical application of cancer immunotherapy based on naturally circulating dendritic cells. *J Immunother Cancer.* (2019) 7:109. doi: 10.1186/s40425-019-0580-6
118. Sprooten J, Ceusters J, Coosemans A, Agostinis P, De Vleeschouwer S, Zitvogel L, et al. Trial watch: dendritic cell vaccination for cancer immunotherapy. *Oncoimmunology.* (2019) 8:e1638212. doi: 10.1080/2162402X.2019.1638212
119. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci USA*. (1996) 93:2879–83. doi: 10.1073/pnas.93.7.2879
120. Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc Natl Acad Sci USA*. (1999) 96:9305–10. doi: 10.1073/pnas.96.16.9305
121. Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J Immunol.* (1996) 157:1840–5.
122. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol.* (2005) 6:769–76. doi: 10.1038/ni1223
123. Jung J, Yi AK, Zhang X, Choe J, Li L, Choi YS. Distinct response of human B cell subpopulations in recognition of an innate immune signal, CpG DNA. *J Immunol.* (2002) 169:2368–73. doi: 10.4049/jimmunol.169.5.2368
124. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science.* (2002) 298:2199–202. doi: 10.1126/science.1076071
125. Rothenfusser S, Hornung V, Ayyoub M, Britsch S, Towarowski A, Krug A, et al. CpG-A and CpG-B oligonucleotides differentially enhance human peptide-specific primary and memory CD8+ T-cell responses in vitro. *Blood.* (2004) 103:2162–9. doi: 10.1182/blood-2003-04-1091
126. Cho HJ, Hayashi T, Datta SK, Takabayashi K, Van Uden JH, Horner A, et al. IFN-αβ promote priming of antigen-specific CD8+ and CD4+ T lymphocytes by immunostimulatory DNA-based vaccines. *J Immunol.* (2002) 168:4907–13. doi: 10.4049/jimmunol.168.10.4907
127. Miconnet I, Koenig S, Speiser D, Krieg A, Guillaume P, Cerottini JC, et al. CpG are efficient adjuvants for specific CTL induction against tumor antigen-derived peptide. *J Immunol.* (2002) 168:1212–8. doi: 10.4049/jimmunol.168.3.1212
128. Baines J, Celis E. Immune-mediated tumor regression induced by CpG-containing oligodeoxynucleotides. *Clin Cancer Res.* (2003) 9:2693–700.
129. Lonsdorf AS, Kuekrek H, Stern BV, Boehm BO, Lehmann PV, Tary-Lehmann M. Intratumor CpG-oligodeoxynucleotide injection induces protective antitumor T cell immunity. *J Immunol.* (2003) 171:3941–6. doi: 10.4049/jimmunol.171.8.3941
130. Milas L, Mason KA, Ariga H, Hunter N, Neal R, Valdecanas D, et al. CpG oligodeoxynucleotide enhances tumor response to radiation. *Cancer Res.* (2004) 64:5074–7. doi: 10.1158/0008-5472.CAN-04-0926
131. Chamoto K, Takeshima T, Wakita D, Ohkuri T, Ashino S, Omatsu T, et al. Combination immunotherapy with radiation and CpG-based tumor vaccination for the eradication of radio- and immuno-resistant lung carcinoma cells. *Cancer Sci.* (2009) 100:934–9. doi: 10.1111/j.1349-7006.2009.01114.x
132. Balsari A, Tortoreto M, Besusso D, Petrangolini G, Sfondrini L, Maggi R, et al. Combination of a CpG-oligodeoxynucleotide and a topoisomerase I inhibitor in the therapy of human tumour xenografts. *Eur J Cancer.* (2004) 40:1275–81. doi: 10.1016/j.ejca.2004.01.023
133. Pratesi G, Petrangolini G, Tortoreto M, Addis A, Belluco S, Rossini A, et al. Therapeutic synergism of gemcitabine and CpG-oligodeoxynucleotides in an orthotopic human pancreatic carcinoma xenograft. *Cancer Res.* (2005) 65:6388–93. doi: 10.1158/0008-5472.CAN-05-0602
134. Wang XS, Sheng Z, Ruan YB, Guang Y, Yang ML. CpG oligodeoxynucleotides inhibit tumor growth and reverse the immunosuppression caused by the therapy with 5-fluorouracil in murine hepatoma. *World J Gastroenterol.* (2005) 11:1220–4. doi: 10.3748/wjg.v11.i8.1220
135. Wooldridge JE, Ballas Z, Krieg AM, Weiner GJ. Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. *Blood.* (1997) 89:2994–8. doi: 10.1182/blood.V89.8.2994
136. Davila E, Kennedy R, Celis E. Generation of antitumor immunity by cytotoxic T lymphocyte epitope peptide vaccination, CpG-oligodeoxynucleotide adjuvant, and CTLA-4 blockade. *Cancer Res.* (2003) 63:3281–8.
137. Melisi D, Frizziero M, Tamburrino A, Zanotto M, Carbone C, Piro G, et al. Toll-like receptor 9 agonists for cancer therapy. *Biomedicines.* (2014) 2:211–28. doi: 10.3390/biomedicines2030211
138. Adamus T, Kortylewski M. The revival of CpG oligonucleotide-based cancer immunotherapies. *Contemp Oncol.* (2018) 22:56–60. doi: 10.5114/wo.2018.73887
139. Pashenkov M, Goess G, Wagner C, Hormann M, Jandl T, Moser A, et al. Phase II trial of a toll-like receptor 9-activating oligonucleotide in patients with metastatic melanoma. *J Clin Oncol.* (2006) 24:5716–24. doi: 10.1200/JCO.2006.07.9129
140. Hofmann MA, Kors C, Audring H, Walden P, Sterry W, Trefzer U. Phase 1 evaluation of intralesionally injected TLR9-agonist PF-3512676 in patients with basal cell carcinoma or metastatic melanoma. *J Immunother.* (2008) 31:520–7. doi: 10.1097/CJI.0b013e318174a4df
141. Thompson JA, Kuzel T, Drucker BJ, Urba WJ, Bukowski RM. Safety and efficacy of PF-3512676 for the treatment of stage IV renal cell carcinoma: an open-label, multicenter phase I/II study. *Clin Genitourin Cancer.* (2009) 7:E58–65. doi: 10.3816/CGC.2009.n.025
142. Kim YH, Girardi M, Duvic M, Kuzel T, Link BK, Pinter-Brown L, et al. Phase I trial of a Toll-like receptor 9 agonist, PF-3512676 (CPG 7909), in patients with treatment-refractory, cutaneous T-cell lymphoma. *J Am Acad Dermatol.* (2010) 63:975–83. doi: 10.1016/j.jaad.2009.12.052
143. Weber JS, Zarour H, Redman B, Trefzer U, O'Day S, van den Eertwegh AJ, et al. Randomized phase 2/3 trial of CpG oligodeoxynucleotide PF-3512676 alone or with dacarbazine for patients with unresectable stage III and IV melanoma. *Cancer.* (2009) 115:3944–54. doi: 10.1002/cncr.24473
144. Manegold C, Gravenor D, Woytowitz D, Mezger J, Hirsh V, Albert G, et al. Randomized phase II trial of a toll-like receptor 9 agonist oligodeoxynucleotide, PF-3512676, in combination with first-line taxane plus platinum chemotherapy for advanced-stage non-small-cell lung cancer. *J Clin Oncol.* (2008) 26:3979–86. doi: 10.1200/JCO.2007.12.5807
145. Hirsh V, Paz-Ares L, Boyer M, Rosell R, Middleton G, Eberhardt WE, et al. Randomized phase III trial of paclitaxel/carboplatin with or without PF-3512676 (Toll-like receptor 9 agonist) as first-line treatment for advanced non-small-cell lung cancer. *J Clin Oncol.* (2011) 29:2667–74. doi: 10.1200/JCO.2010.32.8971
146. Manegold C, van Zandwijk N, Szczesna A, Zatloukal P, Au JS, Blasinska-Morawiec M, et al. A phase III randomized study of gemcitabine and cisplatin with or without PF-3512676 (TLR9 agonist) as first-line

treatment of advanced non-small-cell lung cancer. *Ann Oncol.* (2012) 23:72–7. doi: 10.1093/annonc/mdr030
147. Suek N, Campesato LF, Merghoub T, Khalil DN. Targeted APC activation in cancer immunotherapy to enhance the abscopal effect. *Front Immunol.* (2019) 10:604. doi: 10.3389/fimmu.2019.00604
148. Egen JG, Kuhns MS, Allison JP. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol.* (2002) 3:611–8. doi: 10.1038/ni0702-611
149. Wei SC, Duffy CR, Allison JP. Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discov.* (2018) 8:1069–86. doi: 10.1158/2159-8290.CD-18-0367
150. Chamoto K, Al-Habsi M, Honjo T. Role of PD-1 in Immunity and Diseases. *Curr Top Microbiol Immunol.* (2017) 410:75–97. doi: 10.1007/82_2017_67
151. Iwai Y, Hamanishi J, Chamoto K, Honjo T. Cancer immunotherapies targeting the PD-1 signaling pathway. *J Biomed Sci.* (2017) 24:26. doi: 10.1186/s12929-017-0329-9
152. Brunner MC, Chambers CA, Chan FK, Hanke J, Winoto A, Allison JP. CTLA-4-Mediated inhibition of early events of T cell proliferation. *J Immunol.* (1999) 162:5813–20.
153. Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. Human B7–1 (CD80) and B7–2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity.* (1994) 1:793–801. doi: 10.1016/S1074-7613(94)80021-9
154. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. CTLA-4 is a second receptor for the B cell activation antigen B7. *J Exp Med.* (1991) 174:561–9. doi: 10.1084/jem.174.3.561
155. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* (1992) 11:3887–95. doi: 10.1002/j.1460-2075.1992.tb05481.x
156. Iwai Y, Okazaki T, Nishimura H, Kawasaki A, Yagita H, Honjo T. Microanatomical localization of PD-1 in human tonsils. *Immunol Lett.* (2002) 83:215–20. doi: 10.1016/S0165-2478(02)00088-3
157. Tseng SY, Otsuji M, Gorski K, Huang X, Slansky JE, Pai SI, et al. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med.* (2001) 193:839–46. doi: 10.1084/jem.193.7.839
158. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science.* (2001) 291:319–22. doi: 10.1126/science.291.5502.319
159. Okazaki T, Maeda A, Nishimura H, Kurosaki T, Honjo T. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci USA.* (2001) 98:13866–71. doi: 10.1073/pnas.231486598
160. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med.* (2012) 209:1201–17. doi: 10.1084/jem.20112741
161. Gao X, McDermott DF. Ipilimumab in combination with nivolumab for the treatment of renal cell carcinoma. *Expert Opin Biol Ther.* (2018) 18:947–57. doi: 10.1080/14712598.2018.1513485
162. Franke AJ, Skelton WP, Starr JS, Parekh H, Lee JJ, Overman MJ, et al. Immunotherapy for colorectal cancer: a review of current and novel therapeutic approaches. *J Natl Cancer Inst.* (2019) 111:1131–41. doi: 10.1093/jnci/djz093
163. Poole RM. Pembrolizumab: first global approval. *Drugs.* (2014) 74:1973–81. doi: 10.1007/s40265-014-0314-5
164. Sul J, Blumenthal GM, Jiang X, He K, Keegan P, Pazdur R. FDA Approval summary: pembrolizumab for the treatment of patients with metastatic non-small cell lung cancer whose tumors express programmed death-ligand 1. *Oncologist.* (2016) 21:643–50. doi: 10.1634/theoncologist.2015-0498
165. Larkins E, Blumenthal GM, Yuan W, He K, Sridhara R, Subramaniam S, et al. FDA approval summary: pembrolizumab for the treatment of recurrent or metastatic head and neck squamous cell carcinoma with disease progression on or after platinum-containing chemotherapy. *Oncologist.* (2017) 22:873–8. doi: 10.1634/theoncologist.2016-0496
166. Colwell J. Pembrolizumab approved for hodgkin lymphoma. *Cancer Discov.* (2017) 7:OF1. doi: 10.1158/2159-8290.CD-NB2017-044
167. Schultz D. Three drugs approved for urothelial carcinoma by FDA. *Cancer Discov.* (2017) 7:659–60. doi: 10.1158/2159-8290.CD-NB2017-071
168. Prasad V, Kaestner V, Mailankody S. Cancer drugs approved based on biomarkers and not tumor type-FDA approval of pembrolizumab for mismatch repair-deficient solid cancers. *JAMA Oncol.* (2018) 4:157–8. doi: 10.1001/jamaoncol.2017.4182
169. Joshi SS, Maron SB, Catenacci DV. Pembrolizumab for treatment of advanced gastric and gastroesophageal junction adenocarcinoma. *Future Oncol.* (2018) 14:417–30. doi: 10.2217/fon-2017-0436
170. Caruso C. Pembrolizumab OK'd for cervical cancer. *Cancer Discov.* (2018) 8:904. doi: 10.1158/2159-8290.CD-NB2018-086
171. Bteich F, Di Bisceglie AM. Current and future systemic therapies for hepatocellular carcinoma. *Gastroenterol Hepatol.* (2019) 15:266–72.
172. Zarrabi K, Paroya A, Wu S. Emerging therapeutic agents for genitourinary cancers. *J Hematol Oncol.* (2019) 12:89. doi: 10.1186/s13045-019-0780-z
173. Homet Moreno B, Ribas A. Anti-programmed cell death protein-1/ligand-1 therapy in different cancers. *Br J Cancer.* (2015) 112:1421–7. doi: 10.1038/bjc.2015.124
174. Brahmer J. Nivolumab approved for lung cancer. *Cancer Discov.* (2015) 5:OF1. doi: 10.1158/2159-8290.CD-NB2015-042
175. Smuga-Otto K. Nods for Atezolizumab and Nivolumab from FDA. *Cancer Discov.* (2016) 6:811. doi: 10.1158/2159-8290.CD-NB2016-080
176. Prasad V, Kaestner V. Nivolumab and pembrolizumab: monoclonal antibodies against programmed cell death-1 (PD-1) that are interchangeable. *Semin Oncol.* (2017) 44:132–5. doi: 10.1053/j.seminoncol.2017.06.007
177. Mehrvarz Sarshekeh A, Overman MJ, Kopetz S. Nivolumab in the treatment of microsatellite instability high metastatic colorectal cancer. *Future Oncol.* (2018) 14:1869–74. doi: 10.2217/fon-2017-0696
178. Contratto M, Wu J. Targeted therapy or immunotherapy? Optimal treatment in hepatocellular carcinoma. *World J Gastrointest Oncol.* (2018) 10:108–14. doi: 10.4251/wjgo.v10.i5.108
179. Rose S. First Anti-PD-L1 drug approved for NSCLC. *Cancer Discov.* (2016) 6:OF1. doi: 10.1158/2159-8290.CD-NB2016-143
180. Colwell J. Nod for atezolizumab in advanced bladder cancer. *Cancer Discov.* (2017) 7:OF4. doi: 10.1158/2159-8290.CD-NB2017-064
181. Dolgin E. Atezolizumab combo approved for PD-L1-positive TNBC. *Cancer Discov.* (2019) 9:OF2. doi: 10.1158/2159-8290.CD-NB2019-038
182. Caruso C. First-Line Atezolizumab OK'd for SCLC. *Cancer Discov.* (2019) 9:568–9. doi: 10.1158/2159-8290.CD-NB2019-042
183. Rose S. Avelumab impresses in merkel cell carcinoma. *Cancer Discov.* (2017) 7:OF5. doi: 10.1158/2159-8290.CD-NB2017-062
184. Alvarez-Argote J, Dasanu CA. Durvalumab in cancer medicine: a comprehensive review. *Expert Opin Biol Ther.* (2019) 19:927–35. doi: 10.1080/14712598.2019.1635115
185. Harper K. Cemiplimab approved for treatment of CSCC. *Cancer Discov.* (2018) 8:OF2. doi: 10.1158/2159-8290.CD-NB2018-140
186. Alsaab HO, Sau S, Alzhrani R, Tatiparti K, Bhise K, Kashaw SK, et al. PD-1 and PD-L1 checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. *Front Pharmacol.* (2017) 8:561. doi: 10.3389/fphar.2017.00561
187. Constantinidou A, Alifieris C, Trafalis DT. Targeting programmed cell death−1 (PD-1) and ligand (PD-L1): a new era in cancer active immunotherapy. *Pharmacol Ther.* (2019) 194:84–106. doi: 10.1016/j.pharmthera.2018.09.008
188. Chamoto K, Hatae R, Honjo T. Current issues and perspectives in PD-1 blockade cancer immunotherapy. *Int J Clin Oncol.* (2020) 1–11. doi: 10.1007/s10147-019-01588-7
189. Fan L, Li Y, Chen JY, Zheng YF, Xu XM. Immune checkpoint modulators in cancer immunotherapy: recent advances and combination rationales. *Cancer Lett.* (2019) 456:23–8. doi: 10.1016/j.canlet.2019.03.050
190. Patel SA, Minn AJ. Combination cancer therapy with immune checkpoint blockade: mechanisms and strategies. *Immunity.* (2018) 48:417–33. doi: 10.1016/j.immuni.2018.03.007
191. Zappasodi R, Merghoub T, Wolchok JD. Emerging concepts for immune checkpoint blockade-based combination therapies. *Cancer Cell.* (2018) 33:581–98. doi: 10.1016/j.ccell.2018.03.005

192. Kleponis J, Skelton R, Zheng L. Fueling the engine and releasing the break: combinational therapy of cancer vaccines and immune checkpoint inhibitors. *Cancer Biol Med.* (2015) 12:201–8. doi: 10.7497/j.issn.2095-3941.2015.0046
193. Steer HJ, Lake RA, Nowak AK, Robinson BW. Harnessing the immune response to treat cancer. *Oncogene.* (2010) 29:6301–13. doi: 10.1038/onc.2010.437
194. Passarelli A, Mannavola F, Stucci LS, Tucci M, Silvestris F. Immune system and melanoma biology: a balance between immunosurveillance and immune escape. *Oncotarget.* (2017) 8:106132–42. doi: 10.18632/oncotarget.22190
195. Seya T, Takeda Y, Takashima K, Yoshida S, Azuma M, Matsumoto M. Adjuvant immunotherapy for cancer: both dendritic cell-priming and checkpoint inhibitor blockade are required for immunotherapy. *Proc Jpn Acad Ser B Phys Biol Sci.* (2018) 94:153–60. doi: 10.2183/pjab.94.011
196. Reilley MJ, Morrow B, Ager CR, Liu A, Hong DS, Curran MA. TLR9 activation cooperates with T cell checkpoint blockade to regress poorly immunogenic melanoma. *J Immunother Cancer.* (2019) 7:323. doi: 10.1186/s40425-019-0811-x
197. Mangsbo SM, Sandin LC, Anger K, Korman AJ, Loskog A, Totterman TH. Enhanced tumor eradication by combining CTLA-4 or PD-1 blockade with CpG therapy. *J Immunother.* (2010) 33:225–35. doi: 10.1097/CJI.0b013e3181c01fcb
198. Wang S, Campos J, Gallotta M, Gong M, Crain C, Naik E, et al. Intratumoral injection of a CpG oligonucleotide reverts resistance to PD-1 blockade by expanding multifunctional CD8+ T cells. *Proc Natl Acad Sci USA.* (2016) 113:E7240–49. doi: 10.1073/pnas.1608555113
199. Sato-Kaneko F, Yao S, Ahmadi A, Zhang SS, Hosoya T, Kaneda MM, et al. Combination immunotherapy with TLR agonists and checkpoint inhibitors suppresses head and neck cancer. *JCI Insight.* (2017) 2:e93397. doi: 10.1172/jci.insight.93397
200. Kapp K, Volz B, Oswald D, Wittig B, Baumann M, Schmidt M. Beneficial modulation of the tumor microenvironment and generation of anti-tumor responses by TLR9 agonist lefitolimod alone and in combination with checkpoint inhibitors. *Oncoimmunology.* (2019) 8:e1659096. doi: 10.1080/2162402X.2019.1659096
201. Gallotta M, Assi H, Degagné É, Kannan SK, Coffman RL, Guiducci C. Inhaled TLR9 agonist renders lung tumors permissive to PD-1 blockade by promoting optimal CD4+ and CD8+ T-cell interplay. *Cancer Res.* (2018) 78:4943–56. doi: 10.1158/0008-5472.CAN-18-0729
202. Poh A. Warming "Cold" Melanoma with TLR9 Agonists. *Cancer Discov.* (2018) 8:670. doi: 10.1158/2159-8290.CD-ND2018-004
203. Ribas A, Medina T, Kummar S, Amin A, Kalbasi A, Drabick JJ, et al. SD-101 in Combination with pembrolizumab in advanced melanoma: results of a Phase Ib, multicenter study. *Cancer Discov.* (2018) 8:1250–7. doi: 10.1158/2159-8290.CD-18-0280
204. Diab A, Rahimian S, Haymaker CL, Bernatchez C, Andtbacka RHI, James M, et al. A phase 2 study to evaluate the safety and efficacy of Intratumoral (IT) injection of the TLR9 agonist IMO-2125 (IMO) in combination with ipilimumab (ipi) in PD-1 inhibitor refractory melanoma. *J Clin Oncol.* (2018) 36:(Suppl. 15):9515. doi: 10.1200/JCO.2018.36.15_suppl.9515
205. Butler MO, Robert C, Negrier S, In GK, Walker JWT, Krajsova I, et al. ILLUMINATE 301: a randomized phase 3 study of tilsotolimod in combination with ipilimumab compared with ipilimumab alone in patients with advanced melanoma following progression on or after anti-PD-1 therapy. *J Clin Oncol.* (2019) 37:(Suppl. 15):TPS9599. doi: 10.1200/JCO.2019.37.15_suppl.TPS9599
206. Campbell JD. Development of the CpG Adjuvant 1018: a case study. *Methods Mol Biol.* (2017) 1494:15–27. doi: 10.1007/978-1-4939-6445-1_2
207. Hyer RN, Janssen RS. Immunogenicity and safety of a 2-dose hepatitis B vaccine, HBsAg/CpG 1018, in persons with diabetes mellitus aged 60–70years. *Vaccine.* (2019) 37:5854–61. doi: 10.1016/j.vaccine.2019.08.005
208. Belani CP, Chakraborty BC, Modi RI, Khamar BM. A randomized trial of TLR-2 agonist CADI-05 targeting desmocollin-3 for advanced non-small-cell lung cancer. *Ann Oncol.* (2017) 28:298–304. doi: 10.1093/annonc/mdw608
209. Aznar MA, Planelles L, Perez-Olivares M, Molina C, Garasa S, Etxeberria I, et al. Immunotherapeutic effects of intratumoral nanoplexed poly I:C. *J Immunother Cancer.* (2019) 7:116. doi: 10.1186/s40425-019-0568-2
210. Bhatia S, Miller NJ, Lu H, Longino NV, Ibrani D, Shinohara MM, et al. Intratumoral G100, a TLR4 agonist, induces antitumor immune responses and tumor regression in patients with merkel cell carcinoma. *Clin Cancer Res.* (2019) 25:1185–95. doi: 10.1158/1078-0432.CCR-18-0469

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CASE REPORT
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Durable Response and Good Tolerance to the Triple Combination of Toripalimab, Gemcitabine, and Nab-Paclitaxel in a Patient With Metastatic Pancreatic Ductal Adenocarcinoma

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Background: The performance of immune checkpoint inhibitor (ICI) monotherapy was proved to be disappointing in pancreatic ductal adenocarcinoma (PDAC). Increasing evidence has shown the promising efficacy of ICIs combined with systemic therapy in the first-line treatment in solid tumors.

Case presentation: We reported a case of a metastatic PDAC patient who had a long-term partial response and good tolerance to the combined approach of toripalimab (a novel PD-1 inhibitor) and gemcitabine plus nab-paclitaxel (GA). PD-L1 positive expression was detected in his liver metastases. Besides, we described a phenomenon of pseudo-progression of this patient during the course of therapy.

Conclusion: As the first-line treatment of metastatic PDAC patients, GA plus toripalimab may provide a novel combined approach with favorable response and manageable toxicity. Further clinical trials are needed to confirm the results. Pseudo-progression requires special attention and to be differentiated with true progression in patients undergoing immunotherapy.

Keywords: PD-1 inhibitor, chemotherapy, combination therapy, metastatic pancreatic ductal adenocarcinoma, durable response, good tolerance, case report

BACKGROUND

Metastatic pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal diseases with increasing incidence and mortality. Between 2009 and 2016, the 5-year survival rate for PDAC fluctuated <9% (1). Insufficient selections are efficacious in this refractory disease due to its poor response. Since the MPACT trial indicated prolonged overall survival in first-line treatment of gemcitabine plus nab-paclitaxel (GA) compared to gemcitabine alone (2, 3), GA has substituted gemcitabine as the standard of care at the expense of the high possibility of side effects (4). Therefore, GA was recommended as the first choice to metastatic PDAC patients with Eastern

Cooperative Oncology Group performance status (ECOG PS) 0 to 1, as well as on the condition of patients' preference and available support system (5). Despite some attempts of novel regimen, significant improvement in clinical outcomes of PDAC patients has remained absent.

Recently, immune checkpoint inhibitors (ICIs) have been approved in patients with mismatch repair-deficient (dMMR) (6) or microsatellite instability-high (MSI-H), irrespective of which types of tumor (7). Unfortunately, the success of ICIs has not been replicated in PDAC: no objective response was observed in either anti-PD-1/PD-L1 antibody or anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) monotherapy in any research (8, 9). Plausible explanations contributing to poor efficacy of ICIs in PDAC mainly involve the tumor cell-intrinsic characteristics, including the low immunogenicity, such as low mutational burden and fewer neoantigens, as well as the prominent desmoplastic stroma surrounding PDAC tumors, which may impede the ability of $CD8^+$ T effector cells to infiltrate into the tumor to exert their killing effect.

Herein, we report a case of a metastatic PDAC patient with high PD-L1 expression who had a partial response and good tolerance to combination of toripalimab, a novel PD-1 blockade, and GA chemotherapy. We also review relevant literature about combination therapy of ICIs and chemotherapy in PDAC.

CASE PRESENTATION

A 58-year-old man was found with some liver lumps by abdominal ultrasonography in his regular physical check-up in May 2019. Without any symptoms before, he went to the hospital for further examination. A test of tumor markers showed that serum CA125 was 1,898 U/ml and the CA199 level was out of the upper limit of detection (>1,000 U/ml). A computed tomography (CT) scan and magnetic resonance imaging (MRI) of the abdomen both indicated multiple liver lesions and a pancreatic tail mass at a size of 3.9 × 2.6 cm. He was referred to the Department of General Surgery and underwent a laparoscopic liver biopsy. Intraoperative findings showed multiple scattered nodules on the surface of the liver, whose diameters were <2 cm. Pathology showed metastatic ductal adenocarcinoma. Given these findings, his final diagnosis was pancreatic adenocarcinoma with multiple liver metastases ($cT_2N_+M_1$, stage IV). The next-generation sequencing of his tumor showed an intermediate tumor mutation burden with 5.65 mutations/megabase and microsatellite stable (MSS) status. The immunohistochemistry (IHC) data of the tumor tissue of this patient indicated the positive expression of PD-L1 protein (30%), and the tumor proportion score (TPS) was 20% and combined positive score (CPS) was 30 (**Figure 1**). Additionally, deleterious alterations occurred in CDKN2A, KRAS, TP53, and VEGFA genes. There were not any applicable targeted drugs for these gene mutations.

With his content, he was eligible for a clinical trial about the combination of doublet chemotherapy (gemcitabine 1,000 mg/m^2 and nab-paclitaxel 125 mg/m^2) and toripalimab (a novel PD-1 inhibitor, 240 mg) for the first-line treatment of metastatic PDAC conducted by our department. Therefore, he received gemcitabine 1,700 mg and nab-paclitaxel (Abraxane) 200 mg at

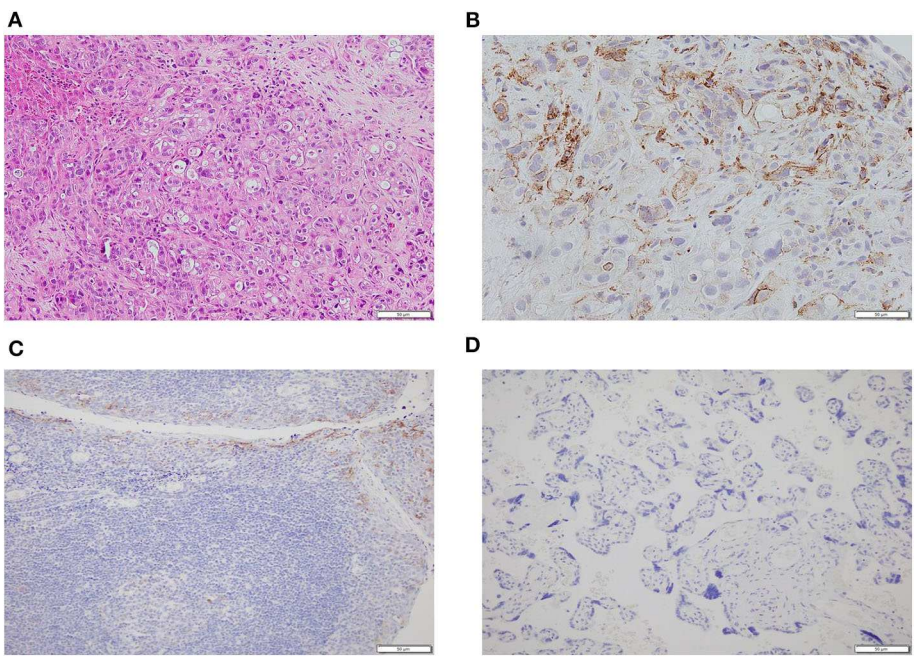

FIGURE 1 | The histopathology and immunohistochemistry (IHC) of metastatic tumor tissues of this patient. **(A)** The H&E staining in the microscopic observation (100×). **(B)** Immunohistochemical staining for PD-L1 expression (400×) showed that the tumor cells were positive for PD-L1. **(C)** The positive control of the IHC of PD-L1 expression (200×). **(D)** The negative control of the IHC of PD-L1 expression (200×).

day 1 and day 8, along with toripalimab 240 mg at day 1 every 3 weeks. After 2 cycles of the combination therapy, his metastatic liver lesions almost disappeared with an evaluation of partial response (PR) by Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (**Figure 2**). Surprisingly, he did not suffer any serious side effects except mild nausea and loss of appetite (grade 1, CTCAE 3.0), which was self-cured at the interstitial period of therapy. The treatment continued and repeated CT scans after four cycles showed shrinkage of the primary lesion but an increase in the number of the liver metastases. However, the CA199 level plummeted from 8,015 to 553.7 U/ml after the first four cycles of treatment (**Figure 2**). He was still asymptomatic but had grade 1 myelosuppression, which was successfully treated with a recombinant human interleukin-11. Through multidisciplinary therapy (MDT) and communication with the patient, we thought that it was highly possible for him to have radiological pseudo-progression and suggested he continue the therapy regimen. As we expected, the subsequent two-cycle treatment brought new clinical benefits to this patient, which in turn confirmed the previous diagnosis of pseudo-progression. The patient's continuous PR is still ongoing at the time of this report (eight cycles after the initial of the combination therapy). Primary and metastatic lesions were significantly decreased or shrank to nearly invisible status as the last evaluation showed, and the level of CA 199 has maintained within the normal for a long period but a little increase at the last test (**Figure 2**). All treatment-related adverse events (TRAEs) of this patient throughout the clinical course were listed in **Table 1**. The most serious TRAEs he had was grade 2 leukocytopenia, which was recovered under drug intervention before the next cycle treatment. Overall, he did not suffer any grade 3 or higher toxicities and maintained good tolerance. With a history of hypertension and type II diabetes, the patient also kept his blood pressure and blood glucose under good control.

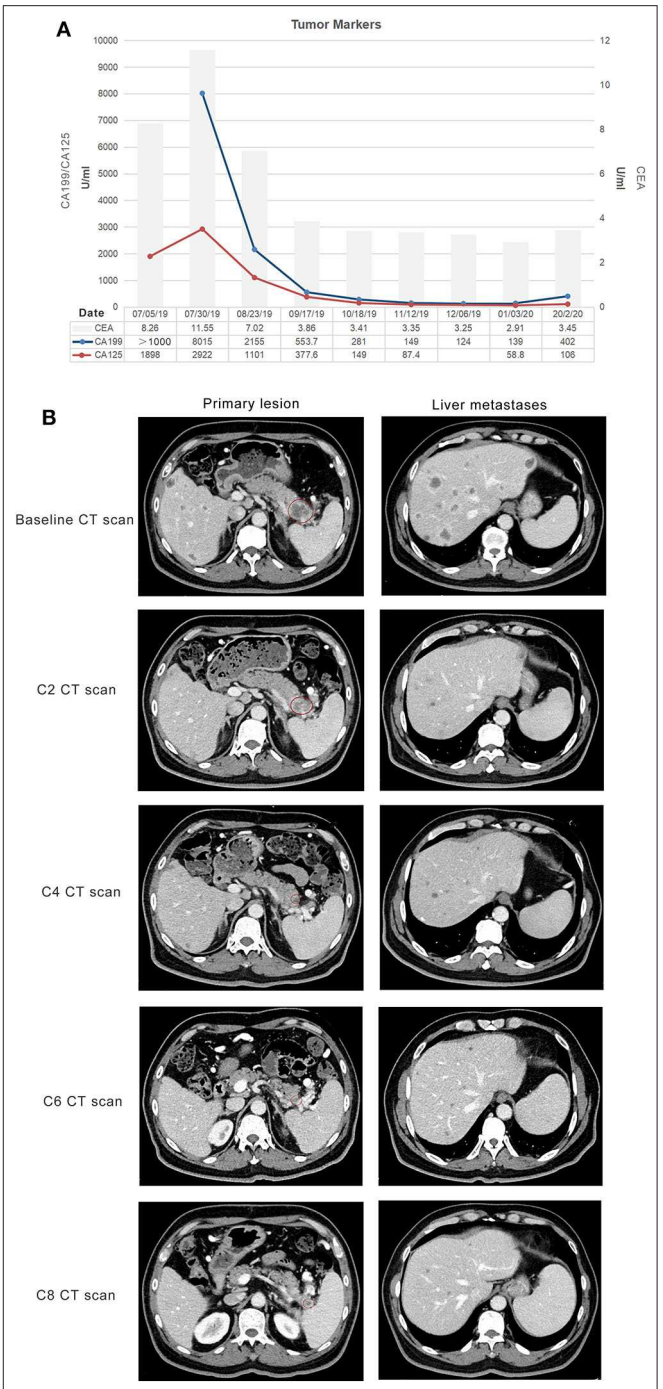

FIGURE 2 | Response evaluation during the clinical course. **(A)** Trends in the level of tumor markers, including CA 199, CA 125 (left *Y*-axis), and CEA (right *Y*-axis) corresponding to the treatment timeline. *X*-axis showing the date of the disease course. The loss of the first value of CA 199 was due to the out of range of detection. **(B)** Representative images of the CT scan showed that both primary and metastatic lesions were shrunk and decreased after two cycles of gemcitabine plus nab-paclitaxel combined with a PD-1 antibody (toripalimab). Red circles indicate the primary pancreatic lesions.

DISCUSSION

Many cases of exceptional or durable responses to ICIs have been reported. To our knowledge, however, this is the first report showing the striking long-term response and safety of doublet chemotherapy combined with toripalimab in the first-line treatment of PDAC.

Toripalimab is the first recombinant humanized anti-PD-1 monoclonal antibody which was independently developed by Chinese companies. It was approved by the National Medical Products Administration (NMPA) of China in December 2018 for locally advanced or metastatic melanoma after systemic treatment failure. It has a high binding affinity, which enables it to bind its specific antigen PD-1 receptor more firmly and compete better with PD-L1 and PD-L2 binding on tumor cells. After binding, it can induce strong endocytosis of PD-1 receptor, thus reducing the expression of PD-1 on the cell membrane surface. A study revealed the different binding orientation of toripalimab compared to other PD-1 blockade, which binds PD-1 mainly on a loop that contributes multiple interactions with PD-L1 (10). The distinct biomolecular characteristics of toripalimab might result in different properties. Recently, increasing studies about various malignancies has proven the potential superiority of toripalimab, especially good tolerability, which may provide

TABLE 1 | Hematologic and non-hematologic adverse events in the therapeutic course presented in this patient, which were graded using CTCAE 3.0.

Hematologic adverse events	Baseline	Maximum grade during treatment	Non-hematologic adverse events	Baseline	Maximum grade during treatment
CTCAE grades					
Leukocytopenia	0	II	Nausea	0	I
Thrombocytopenia	I	I	Pruritus	0	I
Hypohemia	0	I	Poor appetite	0	I

CTCAE, Common Terminology Criteria for Adverse Events.

TABLE 2 | Efficacy and safety of combined therapeutic approaches of immune checkpoint inhibitors and chemotherapy in pancreatic cancer.

References	Phase	No. of patients	Disease	Treatment	Response	Adverse events
Weiss et al. (18)	Ib/II	17	Metastatic 1st line	Gemcitabine + Nab-Paclitaxel + Pembrolizumab	25% PR; 67% SD	Any grade of TRAEs: all (100%); Grade 3 or 4 TRAEs: 12 patients (70.6%)
Renouf et al. (21)	II	11	Metastatic 1st line	Durvalumab + tremelimumab + gemcitabine + nab-paclitaxel	PR 8/11 (73%); DCR (100%); Median PFS 7.9 months	Grade 3 or greater TRAEs: fatigue (27%), anemia (36%), abnormal WBC (27%), hyponatremia (27%), hypoalbuminemia (45%), abnormal lipase (45%). colitis (9.1%)
Wainberg et al. (22)	I	50	Locally advanced/ Metastatic 1st line	Nivolumab + nab-paclitaxel + gemcitabine	2% CR; 16% PR; 46% SD	Grade 3 or 4 TRAEs: 48 patients (96%)
Borazanci et al. (23)	II, pilot	11	Metastatic 1st line	Nivolumab + nab-paclitaxel + cisplatin + gemcitabine + paricalcitol	80% PR; 100% DCR	Grade 3 or 4 TRAEs: thrombocytopenia 76%, anemia 44%, colitis 12%
Aglietta et al. (24)	Ib	34	Metastatic 1st line	Tremelimumab + gemcitabine	PR 2/19 (10.5%)	Any grade TRAEs: 12 pts (35.3%); grade 3 or 4 TRAEs: 2 pts (5.9%)
Kamath et al. (25)	Ib	21	Gemcitabine-naïve	Ipilimumab + gemcitabine	ORR 14%; PR 2/16 (12.5%)	Grade 3 or higher TRAEs: 16 pts (76%)
Wainberg et al. (26)	I	17	1st and 2nd line	Arm A: Nivolumab + nab-paclitaxel (2nd line) Arm B: Same as arm A with gemcitabine (1st line)	Arm A: PR 2/9 (22.2%) SD 4/9; Arm B: PR 3/6 (50%) SD 3/6	Grade 3/4 TRAEs: Arm A: 2/11 pts (18%); Arm B: 2/6 pts (33%)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; TRAEs, treatment-related adverse events; Pts, patients.

an opportunity to use concurrently with other anti-tumor drugs (11). In a phase II study, toripalimab combined with capecitabine and oxaliplatin (CapeOX) as the first-line treatment to treat patients with advanced gastric cancer, and the overall response rate (CR and PR) was 66.7% and the disease control rate (CR, PR, and SD) was 88.9%. Besides, nearly 38.9% of patients experienced grade 3 or 4 TRAEs (12). Compared to the ATTRACTION-4 trial, the encouraging efficacy of toripalimab was not inferior to Nivolumab with an ORR of 76.5%, but much more grade 3 or greater TRAEs occurred in 66.7% of patients with Nivolumab plus CapeOX (13).

This patient may not be sensitive to PD-1 blockade according to ASCO clinical practice guideline, which approved PD-1 blockade for patients with dMMR (6) or MSI-H (14). Given the predictive role of PD-L1 overexpression in PDAC was still controversial, our case suggested that PD-L1 overexpression may have the potential to select population. Moreover, emerging evidence supported combining systemic therapy on an ICIs backbone to overcome resistance due to the superior safety of ICIs (15). Theoretically, systemic chemotherapy was regarded as an immunogenic approach by stimulating anti-cancer immune effectors or inhibiting immunosuppressive factors (16). It may increase the expression or presentation of tumor-associated antigens on the surface of cancer cells, inducing signal emission to trigger immune response. As a method for priming the quiescent tumor microenvironment, chemotherapy has the

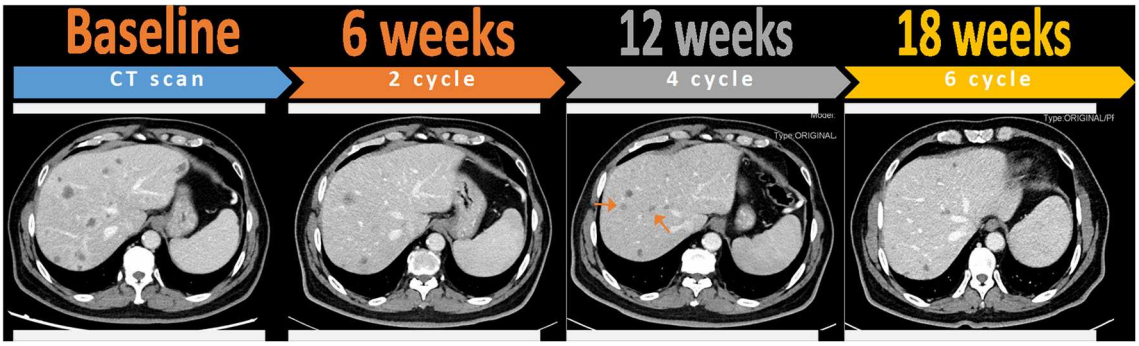

FIGURE 3 | Pseudo-progression of liver metastases after four cycles of toripalimab combined with gemcitabine and nab-paclitaxel, which was confirmed by favorable outcomes at the next assessment of six cycles. Red arrows indicate the appearance of new lesions that were invisible at previous CT evaluation and then disappeared after the continuation of therapy.

potential to potentiate immunogenicity and antigenicity of tumors, thus enhancing the likelihood of recognition and killing of tumor cells by immune effector (17). For example, gemcitabine may upregulate the expression of class I human leukocyte antigen and promote the cross-presentation of tumor antigen, therefore selectively eliminating myeloid-derived suppressor cells (MDSCs) to overcome the immunosuppression. Paclitaxel was proved to stimulate antigen-presenting cells and improve the release of granzyme B by effector cells (18). Some phase I/II studies have confirmed the synergetic effects of cytotoxic chemotherapy with ICIs in other types of cancer (18–20). There are limited data about the safety and efficacy of combination of ICIs and chemotherapy in metastatic PDAC (**Table 2**). Results from a phase Ib/II study conducted in metastatic PDAC suggested that the efficacy of combined chemo-immunotherapy appears to be slightly improved over conventional standard chemotherapy (27). Others have highlighted the importance of combination therapy in the first-line treatment to obtain initial remission. The impressive results of this case need to be further confirmed by a large-scale randomized controlled study.

Interestingly, the patient presented rare pseudo-progression on the CT scan in the treatment course. Pseudo-progression is defined as temporarily enlarging lesions or the appearance of new lesions detected by imaging tests undergoing cancer immunotherapy (28). As the term suggests, pseudo-progression is not a real progression of the disease, whereas it may be linked with a durable response to immunotherapy (29). Presentation of pseudo-progression may be explained as edema and necrosis of tumor tissues caused by the infiltration of immune cells (30), resulting in morphologically similar mass around the original lesions in the imaging. Besides, with the characteristics of a late response, immunotherapy may not induce tumor regression until CT evaluation after the next few cycles of treatment. Instead of treatment failure, this kind of transient tumor growth before the onset of immune response needs to be distinguished with the real progression. Exaggerating the occurrence of pseudo-progression is not advisable because over-treatment may damage life quality, especially for metastatic cancer patients whose main purpose is to alleviate their symptoms. In this case, the patient was found to have new liver lesions after four cycles of treatment (**Figure 3**). Given he was not accompanied by clinical deterioration and the continuously falling CA-199 level, we inferred that the emergence of new lesions may result from the pseudo enlargement of small lesions that were invisible on the baseline CT scan. As we expected, the newly presented lesions disappeared and original lesions shrank after the continuation of this therapy, which verified our diagnosis of pseudo-progression.

Besides impressive efficacy, he also had a good tolerance to these triple anti-tumor drugs, especially PD-1 inhibitors whose immune-related adverse events need special attention. After the first 2 cycles of therapy, he encountered grade 1 myelosuppression, which was successfully treated with a recombinant human interleukin-11, along with a self-cured gastrointestinal tract reaction. Subsequently, he experienced no more overt toxicities and was well-tolerated to a total of 8 cycles of combination therapy. Taking concurrent anti-hypertensive drugs and metformin with this highly intensive anti-tumor regimen, his liver and renal function were still within the normal range.

In summary, combined therapy of toripalimab and standard chemotherapy is potentially effective and well-tolerated as the first-line treatment in metastatic PDAC. Although the data are limited to conclude, we presented a patient who had a striking response to this combination as well as a manageable safety profile. The favorable clinical outcome may be attributed to safer toripalimab or the synergistic function of chemotherapy and PD-1 blockade. Furthermore, this case also displayed the possibility of the phenomenon of pseudo-progression under this regimen, which needed to be taken into consideration in the design and process of clinical trials.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

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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REFERENCES

1. Siegel RL, Miller KD. Cancer statistics, 2020. (2020) 70:7–30. doi: 10.3322/caac.21590
2. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *New Engl J Med*. (2013) 369:1691–703. doi: 10.1056/NEJMoa1304369
3. Goldstein D, El-Maraghi RH, Hammel P, Heinemann V, Kunzmann V, Sastre J, et al. nab-Paclitaxel plus gemcitabine for metastatic pancreatic cancer: long-term survival from a phase III trial. *J Natl Cancer Inst*. (2015) 107:dju413. doi: 10.1093/jnci/dju413
4. Chin V, Nagrial A, Sjoquist K, O'Connor CA, Chantrill L, Biankin AV, et al. Chemotherapy and radiotherapy for advanced pancreatic cancer. *Cochrane Database Syst Rev*. (2018) 3:Cd011044. doi: 10.1002/14651858.CD011044.pub2
5. Sohal DP, Mangu PB, Khorana AA, Shah MA, Philip PA, O'Reilly EM, et al. Metastatic pancreatic cancer: American Society of Clinical Oncology clinical practice guideline. *J Clin Oncol*. (2016) 34:2784–96. doi: 10.1200/JCO.2016.67.1412
6. Le DT, Durham JN. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science*. (2017) 357:409–13. doi: 10.1126/science.aan6733
7. Lemery S, Keegan P, Pazdur R. First FDA approval agnostic of cancer site - when a biomarker defines the indication. *N Engl J Med*. (2017) 377:1409–12. doi: 10.1056/NEJMp1709968
8. Patnaik A, Kang SP, Rasco D, Papadopoulos KP, Elassaiss-Schaap J, Beeram M, et al. Phase I study of pembrolizumab (MK-3475; Anti-PD-1 monoclonal antibody) in patients with advanced solid tumors. *Clin Cancer Res*. (2015) 21:4286–93. doi: 10.1158/1078-0432.CCR-14-2607
9. Royal RE, Levy C, Turner K, Mathur A, Hughes M, Kammula US, et al. Phase 2 trial of single agent Ipilimumab (anti-CTLA-4) for locally advanced or metastatic pancreatic adenocarcinoma. *J Immunother*. (2010) 33:828–33. doi: 10.1097/CJI.0b013e3181eec14c
10. Liu H, Guo L, Zhang J, Zhou Y, Zhou J, Yao J. Glycosylation-independent binding of monoclonal antibody toripalimab to FG loop of PD-1 for tumor immune checkpoint therapy. *MAbs*. (2019) 11:681–90. doi: 10.1080/19420862.2019.1596513
11. Sheng X, Yan X, Chi Z, Si L, Cui C, Tang B, et al. Axitinib in combination with toripalimab, a humanized immunoglobulin G4 monoclonal antibody against programmed cell death-1, in patients with metastatic mucosal melanoma: an open-label phase IB trial. *J Clin Oncol*. (2019) 37:2987–99. doi: 10.1200/JCO.19.00210
12. Wang F, Wei XL, Wang FH, Xu N, Shen L, Dai GH, et al. Safety, efficacy and tumor mutational burden as a biomarker of overall survival benefit in chemo-refractory gastric cancer treated with toripalimab, a PD-1 antibody in phase Ib/II clinical trial NCT02915432. *Ann Oncol*. (2019) 30:1479–86. doi: 10.1093/annonc/mdz197
13. Boku N, Ryu MH, Kato K, Chung HC, Minashi K, Lee KW, et al. Safety and efficacy of nivolumab in combination with S-1/capecitabine plus oxaliplatin in patients with previously untreated, unresectable, advanced, or recurrent gastric/gastroesophageal junction cancer: interim results of a randomized, phase II trial (ATTRACTION-4). *Ann Oncol*. (2019) 30:250–8. doi: 10.1093/annonc/mdy540
14. Sohal DPS, Kennedy EB, Khorana A, Copur MS, Crane CH, Garrido-Laguna I, et al. Metastatic pancreatic cancer: ASCO clinical practice guideline update. *J Clin Oncol*. (2018) 36:2545–56. doi: 10.1200/JCO.2018.78.9636
15. Gong J, Hendifar A, Tuli R, Chuang J, Cho M, Chung V, et al. Combination systemic therapies with immune checkpoint inhibitors in pancreatic cancer: overcoming resistance to single-agent checkpoint blockade. *Clin Transl Med*. (2018) 7:32. doi: 10.1186/s40169-018-0210-9
16. Zitvogel L, Kepp O, Kroemer G. Immune parameters affecting the efficacy of chemotherapeutic regimens. *Nat Rev Clin Oncol*. (2011) 8:151–60. doi: 10.1038/nrclinonc.2010.223
17. Zitvogel L, Galluzzi L, Smyth MJ, Kroemer G. Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance. *Immunity*. (2013) 39:74–88. doi: 10.1016/j.immuni.2013.06.014
18. Weiss GJ, Waypa J, Blaydorn L, Coats J, McGahey K, Sangal A, et al. A phase Ib study of pembrolizumab plus chemotherapy in patients with advanced cancer (PembroPlus). *Br J Cancer*. (2017) 117:33–40. doi: 10.1038/bjc.2017.145
19. Langer CJ, Gadgeel SM, Borghaei H, Papadimitrakopoulou VA, Patnaik A, Powell SF, et al. Carboplatin and pemetrexed with or without pembrolizumab for advanced, non-squamous non-small-cell lung cancer: a randomised, phase 2 cohort of the open-label KEYNOTE-021 study. *Lancet Oncol*. (2016) 17:1497–508. doi: 10.1016/S1470-2045(16)30498-3
20. Rizvi NA, Hellmann MD, Brahmer JR, Juergens RA, Borghaei H, Gettinger S, et al. Nivolumab in combination with platinum-based doublet chemotherapy for first-line treatment of advanced non-small-cell lung cancer. *J Clin Oncol*. (2016) 34:2969–79. doi: 10.1200/JCO.2016.66.9861
21. Renouf DJ, Dhani NC, Kavan P, Jonker DJ, Wei AC-C, Hsu T, et al. The canadian cancer trials group PA.7 trial: results from the safety run in of a randomized phase II study of gemcitabine (GEM) and nab-paclitaxel (Nab-P) versus GEM, nab-P, durvalumab (D), and tremelimumab (T) as frst-line therapy in metastatic pancreatic ductal adenocarcinoma (mPDAC). *J Clin Oncol*. (2018) 36:349. doi: 10.1200/JCO.2018.36.4_suppl.349
22. Wainberg ZA, Hochster HS, Kim E, George B, Kalyan A, Chiorean EG, et al. Phase I study of nivolumab (nivo) + nab-paclitaxel (nab-P) + gemcitabine (Gem) in advanced pancreatic cancer (APC). *J Clin Oncol*. (2019) 37(Suppl. 4):298. doi: 10.1200/JCO.2019.37.4_suppl.298
23. Borazanci EH, Jameson GS, Borad MJ, Ramanathan RK, Korn RL, Caldwell L, et al. A phase II pilot trial of nivolumab (N) + albumin bound paclitaxel (AP) + paricalcitol (P) + cisplatin (C) + gemcitabine (G) (NAPPCG) in patients with previously untreated metastatic pancreatic ductal adenocarcinoma (PDAC). *J Clin Oncol*. (2018) 36:358. doi: 10.1200/JCO.2018.36.4_suppl.358
24. Aglietta M, Barone C, Sawyer MB, Moore MJ, Miller WH, Jr., et al. A phase I dose escalation trial of tremelimumab (CP-675,206) in combination with gemcitabine in chemotherapy-naive patients with metastatic pancreatic cancer. *Ann Oncol*. (2014) 25:1750–5. doi: 10.1093/annonc/mdu205
25. Kamath SD, Kalyan A, Kircher S, Nimeiri H, Fought AJ, Benson A, et al. Ipilimumab and gemcitabine for advanced pancreatic cancer: a phase Ib study. *Oncologist*. (2019) 25:e808–15. doi: 10.1634/theoncologist.2019-0473
26. Wainberg ZA, Hochster HS, George B, Gutierrez M, Johns ME, Chiorean EG, et al. Phase I study of nivolumab (nivo) + nab-paclitaxel (nab-P) ± gemcitabine (Gem) in solid tumors: interim results from the pancreatic cancer (PC) cohorts [abstract]. *J Clin Oncol*. (2017) 35:412. doi: 10.1200/JCO.2017.35.4_suppl.412
27. Weiss GJ, Blaydorn L, Beck J, Bornemann-Kolatzki K, Urnovitz H, Schutz E, et al. Phase Ib/II study of gemcitabine, nab-paclitaxel, and pembrolizumab in metastatic pancreatic adenocarcinoma. *Invest New Drugs*. (2018) 36:96–102. doi: 10.1007/s10637-017-0525-1
28. Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C, et al. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res*. (2009) 15:7412–20. doi: 10.1158/1078-0432.CCR-09-1624

29. Seymour L, Bogaerts J, Perrone A, Ford R, Schwartz LH, Mandrekar S, et al. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. *Lancet Oncol*. (2017) 18:e143–52. doi: 10.1016/S1470-2045(17)30074-8
30. Kazandjian D, Keegan P, Suzman DL, Pazdur R, Blumenthal GM. Characterization of outcomes in patients with metastatic non-small cell lung cancer treated with programmed cell death protein 1 inhibitors past RECIST version 1.1-defined disease progression in clinical trials. *Semin Oncol*. (2017) 44:3–7. doi: 10.1053/j.seminoncol.2017.01.001

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REVIEW
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Clinical and Recent Patents Applications of PD-1/PD-L1 Targeting Immunotherapy in Cancer Treatment—Current Progress, Strategy, and Future Perspective

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Targeting PD-L1 and PD-1 interactions is a relatively new therapeutic strategy used to treat cancer. Inhibitors of PD-1/PD-L1 include peptides, small molecule chemical compounds, and antibodies. Several approved antibodies targeting PD-1 or PD-L1 have been patented with good curative effect in various cancer types in clinical practices. While the current antibody therapy is facing development bottleneck, some companies have tried to develop PD-L1 companion tests to select patients with better diagnosis potential. Meanwhile, many companies have recently synthesized small molecule inhibitors of PD-1/PD-L1 interactions and focused on searching for novel biomarker to predict the efficacy of anti-PD-1/PD-L1 drugs. This review summarized clinical studies and patent applications related to PD-1/PD-L1 targeted therapy and also discussed progress in inhibitors of PD-1/PD-L1.

Keywords: patent, PD-1, PD-L1, immunotherapy, clinical trial

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INTRODUCTION

Programmed cell death protein 1, also referred to as cluster of differentiation 279 (CD279), is a surface protein that can regulate the immune system by inhibiting T-cell activity. PD-1 is constitutively expressed on activated T-cells, B cells, natural killer (NK) cells, macrophages, and dendritic cells (DCs) (1). Programmed death-ligand 1 (PD-L1), also referred to as B7-H1 or CD274, is constitutively expressed on antigen-presenting cells, lymphoid, endothelial, and epithelial cells (2). Interferon gamma (IFN-γ) and tumor necrosis factor (TNF-α) secreted by activated T-cells can also induce PD-L1 expression on tumor cells and antigen-presenting cells (APCs) (3). **Figure 1** shows that naïve T-cells are activated through binding between T cell receptors (TCR) and the peptide-MHC complex presented by (APC); T-cell activation can lead to transient upregulation of PD-1, which is the receptor of PD-L1. Binding between PD-1 and PD-L1 negatively regulates downstream signaling mediated by co-activation of TCR and CD28 (4). When PD-L1 interacts with PD-1, the immunoreceptor tyrosine-based inhibitory motifs (ITIM) and immunoreceptor tyrosine-based switch motifs (ITSM), which are on the intracellular domain of PD-1, can be phosphorylated. The Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) and Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-2) are then recruited and bind to ITIM to further inhibit the signaling downstream of the TCR (5). After inhibiting

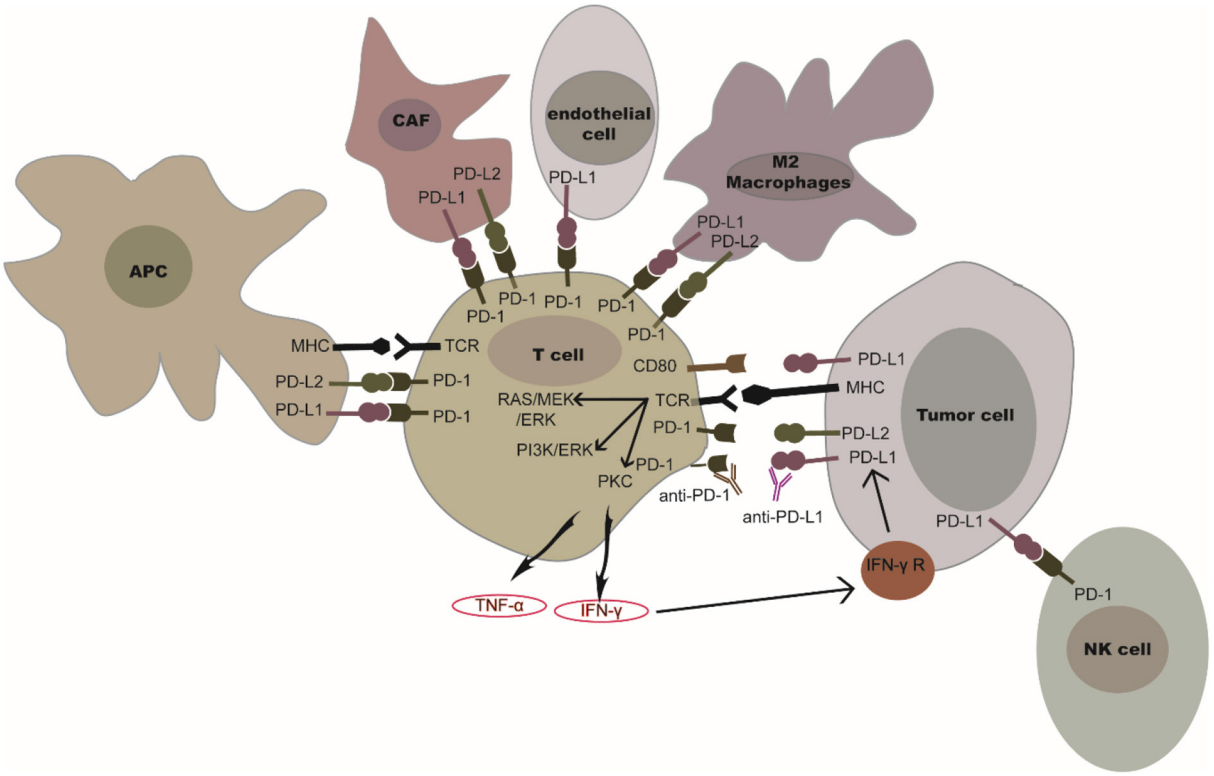

FIGURE 1 | PD-1/PD-L1 or PD-1/PD-L2 in the tumor microenvironment. PD-1 is expressed on T-cells and NK cells. PD-L1 is expressed in tumor cells, antigen presenting cells, cancer associated fibroblasts, and in several immune cells (myeloid cells, endothelial cells, M2 macrophages). The binding of PD-L1 or PD-L2 to PD-1 could inhibit the functioning of T-cells and NK cells. IFN-γ secreted by activated T-cells mediates the up-regulation of tumor PD-L1. The blockade of PD-1/PD-L1 or PD-1/PD-L2 interaction by PD-1 or PD-L1 inhibitors could restore T-cell or NK cell activation.

the TCR-mediated signaling pathway, PD-1 prevents the activation of the pathway mediated by PI3K/Akt or Ras/MEK/Erk. This further inhibits the function of CD8+ T-cells (6). Programmed cell death 1 ligand 2 (also known as PD-L2, B7-DC), which is the second ligand of PD-1, is expressed on tumor cells, APCs, cancer associated fibroblasts, and macrophages (7–9). PD-L2 plays an inhibitory role on the functioning of T-cells, which is similar to that of PD-L1. Meanwhile, PD-L1 also interacts with the surface protein CD80 (B7-1) expressed on activated T-cells. Interacting with PD-L1, CD80 could induce increased expression of Bim, which contributes to the apoptosis of CD8+ T-cells (10). As a result, the PD-1/PD-L1 signaling pathway promotes tumor cells escaping immune surveillance by inhibiting cell survival and activation of T-cells.

Targeting PD-L1 and PD-1 interactions is a novel therapeutic strategy used for cancer treatment. Antibodies targeting PD-1 or PD-L1 have marked a breakthrough in cancer immunotherapy and have become a hot topic in cancer therapy. Many companies have therefore begun studies on cancer immunotherapy and applied a series of related patents and patent applications in this field. To date, there have been about 5,000 patents published, and the number of patents continues to increase (**Figure 2**). In this review, we demonstrate the development of PD-1/PD-L1 directed immunotherapy and progress in inhibitors disrupting PD-1/PD-L1 binding. Moreover, patents or patent applications related to PD-1/PD-L1 signaling pathway and its inhibitors will also be discussed in this review, which will provide an update on PD-1/PD-L1 targeted cancer therapy.

HISTORICAL OVERVIEW OF RELEVANT PATENTS OF PD-1/PD-L1 TARGETING CANCER IMMUNOTHERAPY

The PD-1 protein was discovered by Tasuku Honjo in 1992, and he was awarded the Nobel Prize in physiology and medicine in 2018. The patent published in 1995 by Honjo firstly proposed the sequence of PD-1 protein and gene encoded PD-1 (11). Honjo's discovery also showed that PD-1 is a protein that negatively regulates the immune system (12). Later, Gordon Freeman identified B7–4 as one of the ligands to PD-1 (13). Meanwhile, Dr. Lieping Chen and his team independently discovered B7-H1. The sequence of B7-H1 protein and gene encoded B7-H1 was published in 1999 by Dong et al. (14). However, they did not mention the correlation between B7-H1 and PD-1. Based on his own findings of B7-H1, Chen et al. applied a series of patents related to B7-H1 protein. Meanwhile, in 2000, Freeman

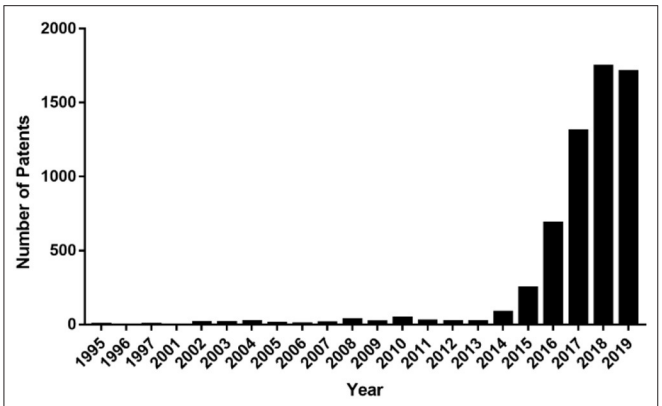

FIGURE 2 | Numbers of international patent applications published per year containing the word "PD-1" or "PD-L1" in the title, claim, or abstract.

TABLE 1 | Patents and patent applications naming Honjo, Freeman, and Dr. Chen as inventors that are related to PD-1 and PD-L1.

Patent number	Inventors	Details
US5698520A (11)	Honjo et al.	The sequence of nucleic acid and amino acid of PD-1
US7563869B2 (16)		The antibodies specifically binding to human PD-1 and the use of these antibodies.
US7038013B2 (17)	Freeman et al.	The nucleic acid sequence and amino acid sequence of PD-1 polypeptide and anti-B7-4 antibodies.
US7101550B2 (18)		PD-1 was recognized as a receptor for B7-4.
US8652465B2 (19)		A method of reducing viral titer by an anti PD-L1 antibody
US6808710B1 (20)		A method for down modulating an immune response by PD-1 antibody
US9062112B2 (21)	Chen et al.	The nucleic acid sequence can encode a B7-H1 polypeptide
US8981063B2 (22)		An isolated antibody that specifically binds to B7-H1
US7892540B2 (23)		A method for treating cancer with B7-H1 antibody

et al. published a paper mentioning that B7-4 was renamed to PD-L1 and is the same as B7-H1 protein discovered by Freeman et al. (15). Freeman also mentioned that PD-L1 is one of the members of the CD28/B7 immunoglobulin superfamily that could inhibit the T-cell function through PD-1/PD-L1 interactions (15). **Table 1** shows patents and patent applications for the finding of PD-1 and PD-L1 proteins and the development of PD-1/PD-L1 blockade therapy.

Honjo's studies suggested that suppression of the PD-1 protein could be effective in cancer treatment (12). Studies have shown that expression of PD-1 and PD-L1 was enhanced in cancer cells and was related to defective immune responses (24). These studies suggested that two immune checkpoint molecules may be important therapeutic targets for cancer and infectious disease treatment. Thus, the blockade of PD-1/PD-L1 interactions using inhibitors may be a novel and effective strategy for immunotherapy. Additionally, a previous study showed that blockade of the PD-1/PD-L1 pathway using PD-L1 antibody could inhibit T-cell apoptosis (25). This study also showed that PD-L1 antibody affected the survival of tumor cells *in vivo* (25). These results proved that PD-L1 antibodies can enhance T-cell growth to further inhibit tumor growth—this suggests that inhibition of the PD-1/PD-L1 interaction could be a new method of cancer treatment.

Honjo cooperated with Ono Pharmaceutical Co. and Medarex to develop an anti-cancer medication targeting PD-1, named nivolumab. Two studies of nivolumab conducted in Phase III trials showed impressive efficacy for this antibody in advanced melanoma (26, 27). The results of a phase III trial showed that the overall survival rate at 1 year was significantly different between the nivolumab group (72.9%) and dacarbazine group (42.1%) of previously untreated patients who had advanced melanoma without a BRAF mutation (26). In addition, nivolumab showed higher response rates and lower toxicity rates than ipilimumab and chemotherapy (27). Following the results of these two clinical trials, the Food and Drug Administration (FDA) approved nivolumab for the treatment of advanced melanoma in 2014. The discovery of the PD-1/PD-L1 signaling pathway attracted researchers' attention on developing antibodies against this pathway. The PD-1 protein has led to breakthroughs in cancer immunotherapies in the past decades. Many companies have filed patents related to antibodies during these past 20 years. **Table 2** shows the core patents related to FDA-approved antibodies while **Table 3** shows patents related to antibodies.

STRUCTURE ANALYSIS OF ANTIBODIES TARGETING PD-1 AND PD-L1

Several structures and classes of antibodies inhibiting the PD-1/PD-L1 interaction have been published recently. Most of these anti-PD-1 antibodies are fully human immunoglobulin G4 (IgG4) antibodies with the S228P mutation, including nivolumab, pembrolizumab, cemiplimab, dostarlimab, MEDI-0680, and SSI-361. These antibodies have similar binding properties to the natural IgG4, which reduce ADCC function and eliminate CDC function, but they still retain function in binding to FcγRI and FcγRIIb. Spartalizumab is a humanized IgG4κ monoclonal antibody with S228P mutations and K447 deletion (44). Tislelizumab was generated via the introduction of several mutations (including S228P, E233P, F234V, L235A, D265A, and R409K) in IgG4 antibodies (45). AMP-224 is an anti-PD-1 recombinant fusion protein that contains the extracellular domain of PD-L2 and Fc domain of human IgG1 (46).

Moreover, the crystal structures of PD-1/Anti-PD-1 antibodies have also been explored. The N-terminal extension, BC-loops, and FG-loops are crucial for binding of nivolumab and PD-1. The VL chain of nivolumab and PD-L1 residues shared an overlapping binding surface on the FG loop (47). The C'D loop of PD-1 mainly contributes to the interaction with pembrolizumab (48). Anti-PD-1 antibodies inhibit the

TABLE 2 | The key patents related to FDA-approved anti-PD-1/L1 antibodies.

Target	Drug	Company	Patent number	Inventor	Antibody class
PD-1	Nivolumab	BMS/Ono	US7595048	Honjo et al. (28)	IgG4
	Pembrolizumab	Merck&Co	US8952136	Carven et al. (29)	IgG4
PD-L1	Avelumab	MerckSerono	US2014341917	Nastri et al. (30)	IgG1
	Atezolizumab	Roche	US8217149	Irving et al. (31)	IgG1
	Durvalumab	AstraZeneca	US8779108	Queva et al. (32)	IgG1

TABLE 3 | The patents related to currently developed anti-PD-1/L1 antibodies.

Target	Drug	Company	Patent number	Inventor	Antibody class
PD-1	Spartalizumab (PDR-001)	Novartis	US9683048B2	Freeman et al. (33)	IgG4κ
	Cemiplimab (Libtayo)	Regeneron Pharmaceuticals	US20150203579	Papadopoulos et al. (34)	IgG4
	Camrelizumab (SHR-1210)	Incyte Biosciences and Jiangsu Hengrui Medicine	US20160376367A1	Yuan et al. (35)	IgG4
	Tislelizumab (BGB-A317)	BeiGene	US8735553B1	Li et al. (36)	IgG4
	Dostarlimab (TSR-042)	Tesaro/AnaptysBio	US9815897B2	King et al. (37)	IgG4
	MEDI-0680 (AMP-514)	MedImmune LLC	US8609089B2	Langermann et al. (38)	IgG4
	SSI-361	Lyvgen	US20180346569A1	Wang et al. (39)	IgG4
	AMP-224	Amplimmune Inc	US20130017199	Langermann et al. (40)	PD-L2 IgG2a fusion protein
PD-L1	CX-072	CytomX	US20160311903A1	West et al. (41)	protease activatable prodrug
	BMS-936559 (MDX 1105)	Medarex Inc	US7943743	Korman et al. (42)	IgG4
	KN035	Jiangsu Alphamab Biopharmaceuticals Co., Ltd.	US20180327494A1	Xu et al. (43)	fusion protein of humanized anti-PD-L1 single domain antibody and human IgG1 Fc

PD-1/PD-L1 interaction by competing with PD-L1 while binding to PD-1. The epitopes of these antibodies directly occupy the partial binding site of the PD-L1 protein. In addition, the binding of PD-1 and its antibodies induces optimal conformational changes in the PD-1 protein, which blocks PD-1/PD-L1 interactions, because PD-1 also interacts with PD-L1 in distinct conformations. Tislelizumab interacts with an IgV-like domain of PD-1 and is different from pembrolizumab and nivolumab, as shown by its unique binding epitopes, including Gln75, Thr76, Asp77, and Arg86 (45). Although SHR-1210 was reported to have unspecific interactions with some human receptors driving angiogenesis, the optimization of complementary determining region (CDR) domains successfully eliminated off-target binding (49). Meanwhile, the binding properties of SHR-1210 have not been reported.

Unlike anti-PD-1 antibodies, three approved anti-PD-L1 antibodies include human IgG1 antibodies. Atezolizumab and durvalumab are antibodies of eliminated FcγR-binding and effector functions while avelumab was designed to retain intact Fc functions (50). BMS-936559 is differentiated from three approved PD-L1 antibodies and is an IgG4 mAb with S228P mutations (50). KN035 is a fusion protein containing a single domain of the humanized anti-PD-L1 antibody and the Fc of an IgG1 (51). CX-072 is a human PD-L1 specific protease-activatable antibody prodrug. CX-072 was designed by linking the masking peptide links to the targeted antibody (52).

Recently, the crystal structures of the PD-L1/avelumab complex revealed that avelumab/atezolizumab/BMS-936559 binds to the IgV domain of PD-L1 through its heavy chain (VH) and light chain (VL). These are dominated by the VH chain (53). A comparison of the PD-L1/antibody and human PD-1/PD-L1 complexes demonstrates that antibodies directly occupy the partial binding site of the PD-1 protein. In contrast, the PD-L1/durvalumab Fab complex demonstrated that the binding sites of the antibody are in the N-terminal region of the PD-L1 protein (53). The KN035/PD-L1 complex showed a different pattern. The paratope of KN035 is limited to only two complementary determining regions (CDRs)—one of which contributes to binding with high-affinity (54). This narrow binding area provides an opportunity for rationally designing peptides or small-molecule inhibitors that imitate the nanobody/PD-L1 interface.

CLINICAL APPLICATION OF PD-1/PD-L1 TARGETING CANCER IMMUNOTHERAPY

There have been more than 2,000 clinical trials of anti-PD-1 antibodies and over 1,000 clinical trials of anti-PD-L1 antibodies (**Figure 3**). Based on the data from several clinical trials, some of these drugs have been approved by the FDA, the National Medical Products Administration (NMPA), and the European Medicines Agency (EMA) for

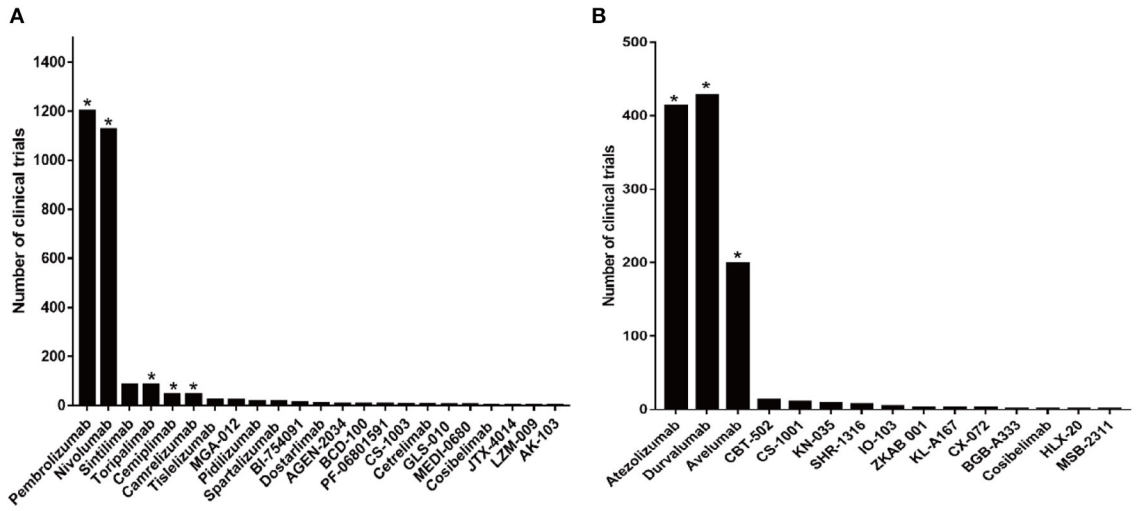

FIGURE 3 | Clinical trials related to anti-PD-1/PD-L1 antibodies. **(A)** Numbers of clinical trials of anti-PD-1 antibodies. **(B)** Numbers of clinical trials of anti-PD-L1 antibodies. Antibodies that obtained approval for cancer therapy are indicated by an asterisk.

use in the treatment of various cancers. Nivolumab and pembrolizumab, two anti-PD-1 antibodies, obtained approval for cancer therapy in 2014. After that, more PD-1 and PD-L1 drugs got FDA approval following positive results from clinical trials. There are currently several FDA-approved antibodies, including nivolumab (trade name: Opdivo), pembrolizumab (trade name: Keytruda), cemiplimab (trade name: Libtayo), atezolizumab (trade name: Tecentriq), durvalumab (trade name: Imfinzi), and avelumab (trade name: Bavencio) (**Table 4**) (50). In addition, camrelizumab and toripalimab were approved by NMPA for marketing.

Clinical trials of most antibodies have just started, and the results require further updating. **Table 5** summarizes several clinical trials of anti-PD-1/PD-L1 antibodies that are currently being developed with the latest data. The data from clinical trials revealed that newly developed antibodies also showed a durable response. **Table 5** also demonstrates that anti-PD-1/PD-L1 antibodies can cause treatment-related adverse effects (TRAEs) and immune-related adverse effects (IRAEs). In some patients, these AEs led to treatment discontinuation and treatment interruption. The Objective response rate (ORR) is 47% among the 75 patients with metastatic CSCC who received cemiplimab-rwlc. Complete response was achieved in 4% of patients (81). Among patients with relapsed/refractory cHL (NCT02961101 and NCT03250962), the response duration rate at 6 months was 76% in patients treated with camrelizumab monotherapy ($n = 19$) compared to 100% in those treated with decitabine plus camrelizumab ($n = 42$) (95). Among the 127 patients with advanced melanoma (NCT03013101), the ORR is 17.3% in overall population after treatment with toripalimab. The disease control rate (DCR) was 57.5% and median progression free survival (PFS) was 3.6 months (96). Based on the clinical results shown above, cemiplimab, camrelizumab, and toripalimab were approved for clinical use.

THE CURRENT OPTIMIZATION OF ANTI-PD-1/PD-L1 TREATMENT STRATEGY

Several clinical trials using antibodies targeting the interaction of PD-1 and PD-L1 for cancer treatment have shown promising abilities in prolonging survival, but not all patients respond to PD-1/PD-L1 inhibitors (97). In addition, clinical results have also shown that anti-PD-1 or anti-PD-L1 treatment caused TRAEs and IRAEs, although anti-PD-1/PD-L1 drugs have shown lower toxicity than standard chemotherapy (98). Most seriously, AEs caused by these antibodies sometimes could lead to treatment discontinuation and treatment interruption (98). Due to the limited success and disadvantages of anti-PD-1/PD-L1 antibodies, effective strategies are needed to improve the efficacy of PD-1/PD-L1 targeted immunotherapy. Detecting PD-L1 expression in tumor cells and tumor infiltrated T-cells would be useful for targeting patients with a big likelihood of responding to PD-1/PD-L1 treatment. Meanwhile, it is also crucial to search for potential biomarkers that could selectively reflect the efficacy and feasibility of anti-PD1/PD-L1 therapy. Furthermore, small molecule inhibitors targeting PD-1 and PD-L1 are emerging as their potential advantages are realized vs. monoclonal antibodies.

The Application of PD-L1 Immunohistochemistry (IHC) Assays

Some clinical trials have shown that more than half of patients had no response to anti-PD-1 drugs, and some responders even experience tumor relapse within 2 years after treatment of anti-PD-1 drugs (26, 99). Studies suggest that clinical efficacy of PD-1/PD-L1 targeted immunotherapies may be predicted by PD-L1 expression on tumor cells and tumor-infiltrating immune cells (100). Developing PD-L1 IHC test compounds have begun to attract scientists' attention during the past 5 years. Several companies have designed commercially available PD-L1 IHC

TABLE 4 | Drugs approved by FDA, NMPA, and EMA for cancer immunotherapy.

Target	Drug	Indication	Related clinical trials no	Phase	Remark
PD-1	Nivolumab	Deficiency mismatch repair (dMMR) or MSI-H metastatic colorectal cancer	NCT02060188 (55)	II	First line
		Melanoma	NCT01721746 (56)	III	First line
		Metastatic squamous Non-small-cell lung carcinoma (NSCLC)	NCT01673867 (57)	III	First line
		Metastatic non-squamous NSCLC	NCT01673867 (58)	III	Second line
		Locally advanced or metastatic urothelial carcinoma (UC)	NCT02387996 (59)	II	Second line
		Advanced Renal cell carcinoma	NCT01668784 (60)	III	Second line
		Hematologic malignancy	NCT01592370 (61); NCT02181738 (62)	I; II	Second line
		Advanced hepatocellular Carcinoma	NCT01658878 (63)	I&II	First line
		Recurrent/Metastatic Head and neck squamous cell carcinoma (HNSCC)	NCT02105636 (64)	III	First line
	Pembrolizumab	Advanced or unresectable melanoma	NCT01295827 (65, 66)	I	First line
		Advanced or metastatic PD-L1-positive NSCLC	NCT01295827 (67)	I	First line
		Locally advanced or metastatic UC	NCT02335424 (68); NCT02256436 (69)	II; III	First line
		Recurrent or metastatic HNSCC	NCT01848834 (70)	Ib	First line
		Hematologic malignancy	NCT02181738 (62)	II	third line therapy or greater
		Microsatellite instability or mismatch repair deficient cancers	NCT01876511 (71)	II	Second line
		Advanced gastroesophageal Cancer	NCT02335411 (72)	II	First line
		Metastatic Cervical Cancer	NCT02628067 (73)	II	First line
		Locally advanced or metastatic, esophagus squamous cell carcinoma (ESCC)	NCT02559687, NCT02564263	II	First line
	Cemiplimab	Advanced cutaneous squamous cell carcinoma (CSCC)	NCT02383212, NCT02760498	I&II	First line
	Camrelizumab	Classical Hodgkin lymphoma (cHL)	CTR20170500/NCT03155425/ SHR-1210-II-204	II	Second-line therapy or greater
	Toripalimab	Malignant melanoma	NCT03013101	II	First line
PD-L1	Avelumab	Locally advanced or metastatic UC	NCT01772004 (74)	Ib	Second line
		Metastatic Merkel cell carcinoma	NCT02155647 (75)	II	Second line
	Atezolizumab	Previously treated metastatic NSCLC	NCT01903993 (76); NCT02008227 (77)	II; III	Second line
		Locally advanced and metastatic UC	NCT02108652 (78)	II	First line
	Durvalumab	Locally advanced, unresectable NSCLC	NCT02125461 (79)	III	First or second line
		Locally advanced or metastatic UC	NCT01693562 (80)	I&II	Second line

tests, including 22C3, 28-8, SP263, SP142, E1L3N, and 73-10 assays. Merck developed a PD-L1 IHC test using 22C3 antibody and also applied for a patent (US9709568B2), which claimed the use of the 22C3 antibody for diagnostic purposes (101). In addition, BMS designed a different companion assay for PD-L1 expression using its 28-8 antibody and had a patent application (WO2013173223A1) that described a method of detecting PD-L1 expression using the clone 28-8 antibody (102). The SP142 assay was developed by Ventana and was described in patent application WO2015181343A2 (103).

These PD-L1 IHC assays are currently being tested in clinical trials, and some of them have been approved by the FDA as companion diagnostics for PD-1/PD-L1 targeted immunotherapies. **Table 6** shows that PD-L1 expression was first reported to be associated with higher response rates to pembrolizumab/atezolizumab and was approved by the FDA to guide the selection of patients for anti-PD-1/PD-L1 treatment. For example, the DAKO 22C3 IHC assay is approved for use as a companion diagnostic with pembrolizumab immunotherapy in NSCLC, gastric cancer, cervical cancer, HNSCC, and ESCC (68, 104–108). In addition, the Ventana PD-L1 (SP142) assay has also been approved as a companion diagnostics test for atezolizumab in UC and TNBC (109, 110). IHC 28–8 and SP263 (nivolumab and durvalumab, respectively) are complementary diagnostics and have not been approved by the FDA. Recent studies (e.g., shown in the meta-analysis) have also confirmed

TABLE 5 | Results of clinical evaluation of selected anti-PD-1 or anti-PD-L1 antibodies.

Target	Antibody	Pivotal indications	Most advanced phase	Most recent result	Most common adverse effects (AEs)
PD-1	Cemiplimab (Libtayo)	Squamous cell cancer	Phase IIII	Metastatic CSCC (81): ORR: 47% (95% CI, 34–61); Median follow-up months: 7.9	The most common AEs were diarrhea (27%). 4 patients (7%) had AEs leading to discontinuation.
	Pidilizumab (CT-011)	Relapsed Follicular Lymphoma	Phase II	Pidilizumab + rituximab (82): ORR: 66% Complete response (CR): 52% partial response (PR): 14% Median follow-up months: 18.8 (95% CI: 14.7 months to not reached)	Anemia (14/29), Fatigue (13/29).
	Spartalizumab (PDR-001)	BRAF V600–mutant unresectable or metastatic melanoma.	Phase III	Spartalizumab (S) + dabrafenib (D) + trametinib (T) (83): ORR: 75% CR: 33% Median follow-up months: 12 (95% CI, 47–79%)	27 (75%) had grade ≥ 3 AEs. 6 patients (17%) had AEs leading to discontinuation.
	Camrelizumab (SHR-1210)	Nasopharyngeal cancer	Phase III	Camrelizumab monotherapy (84): ORR: 34%; 95% CI 24–44 Median follow-up months: 9.9	15 (16%) patients had AEs of grade 3 or 4
	Tislelizumab (BGB-A317)	Nasopharyngeal cancer	Phase III	Tislelizumab (85): PR: 15% Stable disease (SD): 45% Median follow-up months: 5.5	Hypothyroidism (3/20). No AEs led to discontinuation.
	Toripalimab (TAB001, JS001)	Advanced melanoma	Phase III	Toripalimab (86): ORR: 20.7% PR: 19.8% SD: 39.6%	Proteinuria (25%), ALT increase (25%)
	Dostarlimab (TSR-042)	Advanced NSCLC and microsatellite instability-high (MSI-H) Endometrial cancer (EC)	Phase III	TSR-042 (87): NSCLC group: PR: 33.3% SD: 28.6% MSI-H EC group: PR: 36.4% SD: 18.2%	Diarrhea (22.4%) Nausea (22.4%)
	AGEN-2034	Cervical cancer; Solid tumors	Phase I&II	AGEN2034 (88): PR: 12% SD: 52%	2 patients (6%) had AEs leading to discontinuation.
	Sintilimab (IBI-308)	Relapsed/refractory classical Hodgkin's Lymphoma (HL)	Phase III	Sintilimab (89): ORR: 80.4%; 95% CI 70.9–88.0 Median follow-up: 10.5 (9.2–1) months; Six-month PFS: 77.6% (66.6–85.4)	93% patients had treatment-related adverse events. The most common AEs were pyrexia (3%).
	BCD-100	Malignant melanoma	Phase III	BCD-100 1 mg/kg (90): ORR: 34% CR: 6.7% PR: 27.1% DCR: 68%. BCD-100 3 mg/kg: ORR: 29% CR: 3.6% PR: 25.4% DCR: 55%.	BCD-100 1 mg/kg: TRAEs (48%); IRAEs (29%). BCD-100 3 mg/kg: TRAEs (48%); IRAEs (30%).
	GLS-010	Hodgkin's disease	Phase II	GLS-010 (91): ORR: 88.3% CR: 23.5% PR: 64.7% SD: 5.9%	The most common treatment related AEs were Neutrophil (31.25%),

(Continued)

TABLE 5 | Continued

Target	Antibody	Pivotal indications	Most advanced phase	Most recent result	Most common adverse effects (AEs)
PD-L1	CX-072	Solid tumors	Phase II	CX-072 (92): PR: 8% SD: 43% PD: 47%	2 patients had AEs leading to discontinuation.
	WBP-3155 (CS1001)	Advanced solid tumors or lymphomas	Phase III	CS1001 (93): PR: 24% SD: 28%	Anemia (48%). 2 patients had AEs leading to discontinuation.
	Cosibelimab (CK-301)	Cancer	Phase I	Cosibelimab (94): NSCLC group: ORR: 42% DCR: 83% CSCC group: ORR: 43%, DCR: 86%. In melanoma and HL group: ORR: 14% DCR: 71% Colorectal cancer group: ORR: 10% DCR: 60%	Most common AEs were rash (14%)

that efficacy of PD-1/PD-L1 inhibitors was more sensitive in PD-L1 positive patients compared to negative groups (111). Each PD-L1 IHC assay, performed in different IHC staining platforms, is independently developed for a specific anti-PD-1 or anti-PD-L1 drug. As is shown in **Table 7**, differences between six commonly used PD-L1 IHC assay were shown by detection system, staining platform, and antibody epitope. Thus, each assay potentially displayed different staining sensitivities. Different PD-L1 IHC assays and different PD-L1 tumor expression cut-off points are used in clinical trials, which raises concerns about whether the tests can be used interchangeably. The Blueprint PD-L1 IHC Assay Comparison Project was founded to enable a better understanding of the similarities and differences between these four PD-L1 IHC systems. This project is an industrial-academic partnership seeking to harmonize IHC PD-L1 testing. The result from phase I of the Blueprint project showed that the 22C3, 28-8, and SP263 assays displayed comparable sensitivity and the SP142 assay showed significantly less sensitivity (112). The phase I of the Blueprint project detect PD-L1 expression on TCs using four PD-L1 IHC assays performed in different staining platforms, and the result of staining was evaluated independently by three pathologists (112). Phase 2 of the Blueprint project compares 73-10 assay with four other PD-L1 IHC assay (including 22C3, 28-8, SP263). The results from phase 2 showed highly comparable sensitivity between 22C3, 28-8, and SP263 assays, less sensitivity with SP142 assay, and higher sensitivity with 73-10 assay when detecting PD-L1 expression on TC (113). The high concordance was observed between scorings by glass slide and scorings by digital image (113). Most importantly, a recent study has investigated the cause of distinct immunohistochemical staining generated by SP142 assay. The results suggested that discordances are more likely caused by differences of staining platform rather than antibody epitope (114).

The Current Potential Biomarkers Used to Evaluate the Feasibility of Anti-PD-1/PD-L1 Therapy

PD-1/PD-L1 inhibitors represent a breakthrough in cancer therapy. However, the response rates of PD-1/PD-L1 inhibitors in patients is, overall, unsatisfactory and results in limited applications in clinical practice. Therefore, searching for biomarkers predicting the efficacy of PD-1/PD-L1 inhibitors is crucial for patient selection. There are several biomarkers associated with the response to anti-PD-1/anti-PD-L1 therapy (**Table 8**) including PD-L1 expression, lactate dehydrogenase (LDH), mismatch-repair (MMR) deficiency, gene alteration, tumor mutational burden, etc. A clinical study conducted by Diem showed that patients with an elevated baseline LDH showed a significantly shorter OS ($P = 0.0292$) and lower response rate compared with patients with normal LDH at baseline and during treatment. This suggests that LDH could predict early response or progression in advanced melanoma patients with anti-PD-1 therapy (115, 116). In addition, patients who achieved clinical benefit after treatment of anti–PD-1 therapy were detected with a higher percentage of $Bim^{+}PD\text{-}1^{+}CD8^{+}$ T-cells in the peripheral blood (117). The levels of Bim in $PD\text{-}1^{+}CD11a^{hi}CD8^{+}$ T-cells (also indicated tumor reactive T cell) could be a predictive factor of clinical benefit in patients with metastatic melanoma treated with anti–PD-1 therapy (117). High pretreatment lymphocyte count (LC) and relative eosinophil count (REC) were associated with improved overall survival of melanoma patients with pembrolizumab treatment (118). Patients with T-cells expressing SRY-Box 2 (SOX-2) experienced disease regression following the treatment of nivolumab, suggesting that SOX-2 is associated with a clinical response upon immunotherapy with anti-PD-1 monoclonal antibodies (119). A retrospective study showed that the median

TABLE 6 | Summary of studies on the PD-L1 IHC assay.

Study information	Population	Cut-off value of PD-L1 expression	Response
PD-L1 IHC assay: DAKO 22C3 IHC assay Drug: Pembrolizumab	NSCLC (104)	Tumor proportion score (TPS)>1%	TPS<1%: 8.3% (ORR) 1%≤TPS≤49%: 17.3% (ORR) TPS>50%: 51.9% (ORR)
	Gastric or gastroesophageal junction adenocarcinoma (105)	Combined proportion score (CPS)≥1	CPS≥1: 16% (ORR) CPS<1: 6% (ORR)
	Cervical cancer (106)	CPS≥1	CPS≥1: 14.3% (ORR) CPS<1: 0 (ORR)
	UC (68)	CPS>10	CPS>10: 39% (ORR) 1%≤CPS≤10%: 20% (ORR) CPS<1: 11% (ORR)
	HNSCC (107)	CPS≥1	Median overall survival: Pembrolizumab vs. cetuximab plus chemotherapy: 12.3:10.3 (HR 0.78; 95% CI: 0.64, 0.96; $p = 0.0086$)
	ESCC (108)	CPS≥10	Median OS: Pembrolizumab vs. chemotherapy: 10.3:6.7 (HR 0.64; 95% CI: 0.46, 0.90); ORR: Pembrolizumab vs. chemotherapy: 22%: 7%
PD-L1 IHC assay: Ventana SP142 IHC assay Drug: Atezolizumab	UC (109)	PD-L1 tumor infiltrating immune cell (IC) expression ≥5%	IC≥5%: 26% (ORR) IC<5%: 9.5% (ORR)
	Triple-negative breast cancer (TNBC) (110)	PD-L1 IC expression ≥1%	IC≥1%: 12% (ORR); 15% (DCR) IC<1%: 0% (ORR); 5% (DCR)

TABLE 7 | The comparison of commonly used PD-L1 IHC assay.

Antibody clone	Manufacturer	Detection systems	Staining platform	Species	Heat-induced epitope retrieval	Binding sites of antibody
22C3,28-8,73-10	Dako	EnVision FLEX visualization system	Dako Autostainer Link 48	Rabbit	EnVision FLEX	extracellular domain of PD-L1
SP142, SP263	Ventana/Roche	OptiView detection kit	Ventana BenchMark ULTRA	Rabbit	CC1 Cell conditioning	the cytoplasmic domain at the extreme C-terminus of PD-L1
E1L3N	Cell Signaling Technology	Laboratory detection system	Laboratory detection system	Rabbit	Laboratory detection system	cytoplasmic domain of PD-L1

PFS of patients with a neutrophil-lymphocyte ratio (NLR) of ≥3 was shorter than that in patients with a NLR of < 3 (2.0 vs. 5.3 months, $p = 0.00515$) at 4 weeks after treatment (120). The clinical data suggested that the NLR ratio might be an indicator of a poor prognosis in patients with advanced NSCLC receiving nivolumab (120). Patients with a 1.5-fold increase in circulating soluble PD-L1 (sPD-L1) concentrations were more likely to achieve partial responses to anti–PD-1 antibodies after 5 months upon anti-PD-1 therapy. This shows the predictive effect of sPD-L1 on clinical response to anti-PD-1 therapy (121). Among 36 EGFR-mutated metastatic NSCLC patients, compared with patients detecting decreased levels of sPD-1, patients with an increased or stable sPD-1 level achieved longer PFS ($p = 0.004$) and OS ($p = 0.002$) after two cycles of nivolumab (122). In melanoma, the pre-treatment tumors in responding patients were detected with higher expressions of IFN-γ and IFN-γ-inducible genes, including indoleamine 2,3-dioxygenase 1 (IDO1) and C-X-C motif Chemokine Ligand 9 (CXCL9) (123). These associations were also found in NSCLC or renal cell carcinoma patients (123). In addition, genetic aberrations within tumors were also found to be associated with clinical efficacy in anti-PD-1/PD-L1 therapy. For example, among 155 patients, six patients with MDM2/MDM4 amplification and seven of eight patients with Epidermal Growth Factor Receptor (EGFR) alterations were found to have time-to-treatment failure (TTF) <2 months (124). Meanwhile, hyper-progressors harbored MDM2/4 amplifications or EGFR alterations (124). A retrospective analysis showed that EGFR-mutant and ALK-positive NSCLC patients receiving anti-PD-1/PD-L1 therapy showed lower ORR ($P = 0.053$) (125). Immunotherapeutic

TABLE 8 | Current investigational biomarkers for PD-1/PD-L1 targeting therapy.

Biomarkers	Population	Drug	End point result	References
LDH	Melanoma	Ipilimumab Pembrolizumab	LDH level: Elevated group vs. Normal group: Median: 9.7 vs. not reached; 6-month OS: 60.8% vs. 81.6%; 12-month OS: 44.2% vs. 71.5%; $P = 0.0292$	(115)
	Melanoma	Pembrolizumab Nivolumab	LDH level: Elevated group: 22.3, 95% CI (17.1–28.1) Normal group 42.0, 95% CI (36.6–47.5)	(116)
Bim levels in circulating T cells	melanoma	Pembrolizumab	In patients with 4 cycles of anti–PD-1 therapy with clinical benefit, higher percentage of $Bim^{+}PD\text{-}1^{+}CD8^{+}$ T cells in the peripheral blood was detected.	(117)
REC, LC	Melanoma	Pembrolizumab	High REC and absolute LC were negatively related with OS. $P < 0.001$	(118)
SOX-2 reactive T-cells	NSCLC	Nivolumab	Patients who responded to therapy (partial response, PR; $n = 5$) showed significantly greater immune response against SOX2 as compared non-responder ($p = 0.02$).	(119)
NLR	NSCLC	Nivolumab	NLR of <3 vs. NLR of ≥3: 2 weeks after treatment Median PFS: 5.3 vs. 2.1 months ($P = 0.00528$) 4 weeks after treatment Median PFS: 5.3 vs. 2.0 months ($P = 0.00515$)	(120)
sPD-L1	Melanoma	Pembrolizumab	Eight patients with ≥1.5-fold increases in $sPD\text{-}L1^{all}$ after 5 months of treatment experienced partial responses (Fisher exact test $P = 0.007$), and four patients with ≥1.5-fold increases in $sPD\text{-}L1^{L}$ after 5 months of treatment experienced partial responses (Fisher exact test, $P = 0.103$)	(121)
sPD-1	NSCLC	Nivolumab	After two cycles of nivolumab, an increased or stable sPD-1 level independently correlated with longer PFS (HR: 0.49, $p = 0.004$) and OS (HR: 0.39, $p = 0.002$).	(122)
IFN-γ,IDO1, CXCL9	Melanoma, NSCLC, RCC	Atezolizumab	Higher expression of IFN-γ and IDO1 as well as CXCL9 were detected in pretreatment tumors in responding patients. $P = 0.024$	(123)
Mutation of EGFR, MDM2, MDM4	Adenocarcinoma of lung Bladder carcinoma Breast cancer endometrial stromal sarcoma	Pembrolizumab Nivolumab Atezolizumab	Alteration of EGFR and MDM2/4 showed significance for correlation with TTF <2 months ($p = 0.02$).	(124)
ALK, EGFR	NSCLC	PD-1/PD-L1 inhibitors (Pembrolizumab, Nivolumab, Atezolizumab, Durvalumab, other)	Objective response (OR): EGFR-mutant or ALK-positive patients: 1/28 (3.6%); EGFR wild-type and ALK-negative/unknown patients: 7/30 (23.3) $P = 0.053$	(125)
KRAS/TP53	NSCLC	Pembrolizumab Nivolumab	Median PFS: TP53-mutant vs. KRAS-mutant vs. wild-type: 14.5 vs. 14.7 vs. 3.5 months; $P = 0.012$	(126)
STK11	KRAS mutant -LUAC	PD-1/PD-L1 inhibitors (Pembrolizumab, Nivolumab, Atezolizumab)	KRAS-mutant LUAC: Objective response rates: KL vs. KP vs. K-only: 7.4% vs. 35.7 vs. 28.6%, $P < 0.001$; Patients treated with nivolumab: KL vs. KP vs. K-only: 0 vs. 57.1 vs. 18.2%; $P = 0.047$.	(127)
MMR deficiency	12 different tumor types	Pembrolizumab	Objective radiographic responses were noted in 53% of patients (95% CI, 42–64%). Disease control was achieved in 77% of patients (95% CI, 66–85%). complete radiographic response was achieved in 21%.	(71)

(Continued)

TABLE 8 | Continued

Biomarkers	Population	Drug	End point result	References
PBRM1	ccRCC	Nivolumab Atezolizumab	PBRM1 were enriched in tumors from patients in the CB vs. NCB group (9/11 vs. 3/13; Fisher's exact $p = 0.012$, $q = 0.086$)	(128)
DDR gene	Advanced urothelial cancers	Nivolumab Atezolizumab	ORR: known or likely deleterious DDR alterations vs. unknown significant DDR alterations vs. wildtype DDR: 67.9 vs. 80 vs. 19%, $P < 0.001$	(129)
Single nucleotide polymorphisms (SNPs) of tumor microenvironment-related genes	NSCLC HNSCC Melanoma	PD-1/PD-L1 inhibitors (Pembrolizumab, Nivolumab, Atezolizumab, Durvalumab, other)	Objective response rate (complete or partial response) was significantly correlated to tumor microenvironment-related SNPs concerning *CCL2, NOS3, IL1RN, IL12B, CXCR3, and IL6R* genes.	(130)
rs17388568	Metastatic Melanoma	Nivolumab Pembrolizumab	rs17388568 was associated with increased anti-PD-1 response (OR 0.26; 95% CI 0.12–0.53; $p = 0.0002$).	(131)
CD8-, PD-1-and PD-L1-expressing cells	Metastatic Melanoma	Pembrolizumab	Compared to the progression group, the response group was detected with significantly higher numbers of CD8+, PD-1+, and PD-L1+ cells. (CD8, $P = 0.0001$; PD-1, $P = 0.0002$; PD-L1, $P = 0.006$)	(132)
PD-L2	HNSCC	Pembrolizumab	PD-L2–positive patients showed an ORR of 26.5% and PD-L2–negative patients showed an ORR of 16.7%, PD-L2 status was also significantly associated with OS ($P = 0.030$) and PFS ($P = 0.005$)	(133)

analysis and prospective observation suggested that patients harboring TP53 or KRAS mutations—especially co-mutations of TP53/KRAS—showed significantly better clinical responses to anti-PD-1 therapy (126). Among the 174 lung adenocarcinoma (LUAC) patients with KRAS mutations, patients harboring (Serine/Threonine Kinase 11) STK11 alterations showed lower ORR to PD-1 inhibitors vs. LUAC patients with mutant KRAS and wildtype STK11 ($P < 0.001$) (127). Another study evaluated the clinical efficacy of PD-1 inhibitors in patients with MMR-deficient tumors across 12 tumor types. ORR was achieved in 53% of patients, disease control was achieved in 77% of patients, and complete responses were achieved in 21% of patients (71). The MMR deficiency was defined by the presence of either MSI-H or by loss of MutL Homolog 1 (MLH1), MutS Homolog 2 (MSH2), MutS Homolog 6 (MSH6), or PMS1 Homolog 2 (PMS2) protein expression. Among the 35 patients with clear cell renal cell carcinoma (ccRCC), a clinical benefit was associated with loss-of-function mutations in the Polybromo 1 (PBRM1) gene ($p = 0.012$) after treatment of pembrolizumab and nivolumab (128). The presence of DNA damage response gene (DDR) alteration was associated with a higher response rate ($P < 0.001$) (129). The most commonly altered genes were ATM ($n = 7$), DNA Polymerase Epsilon (POLE) ($n = 3$), and BRCA2, ERCC2, FA Complementation Group A (FANCA), and MutS Homolog 6 (MSH6) ($n = 2$) (129). Gene variations that occur in at least 1% of the population used to be called polymorphism. Single nucleotide polymorphisms (SNPs) of tumor microenvironment-related genes (including *CCL2, NOS3, IL1RN, IL12B, CXCR3, and IL6R*) were significantly associated with ORR of patients treated with anti-PD-1/PD-L1 therapies (130). And safety of anti-PD-1/PD- L1 targeted therapies was significantly associated with gene SNPs including *UNG, IFNW1, CTLA4, PD-L1, and IFNL4 genes* (130). Besides that, rs17388568, which maps to a locus of IL2 gene and IL21 gene, was correlated with a higher response to anti-PD-1 targeting therapy (131). CD8, PD-1, and PD-L1 expression in the tumor and at the invasive margin significantly correlated with treatment outcome ($P = 0.001$) (132). Versus the progression group, the response group had significantly higher numbers of $CD8^+$, $PD\text{-}1^+$, and $PD\text{-}L1^+$ cells (CD8, $P = 0.0001$; PD-1, $P = 0.0002$; PD-L1, $P = 0.006$) (132). Among HNSCC patients treated with pembrolizumab, PD-L2-positive patients showed higher ORR compared with PD-L2-negative patients (133). And longer PFS and OS were observed in PD-L2–positive patients (133).

Except for the biomarkers mentioned above, the tumor mutation burden/load (TMB) also served as a predictive or prognostic factor for response to anti-PD-1/PD-L1 immunotherapy. TMB is an estimate of somatic mutations by accessing the data from whole exome sequencing (WES) or sequencing a select panel of genes. Foundation Medicine has developed clinical testing platforms to measure TMB using hybrid capture-based next generation sequencing. FDA has approved FoundationOne CDx to be used as a companion diagnostic for therapy selection. Several studies have shown that TMB is associated with a clinical response to anti-PD-1/PD-L1 treatment in melanoma and NSCLC (**Table 9**). Recently, a novel blood-based TMB (bTMB) assay was developed for cell-free DNA by researchers from Foundation Medicine. A retrospective analysis using bTMB assay showed that bTMB is correlated with significant PFS benefit ($P = 0.013$) and TMB (Spearman rank correlation = 0.64) in patients with NSCLC treated with atezolizumab (139). Neoantigens derived from mutated genes are tumor-specific and show significant correlation with the clinical response to anti-PD-1/PD-L1 treatment. A significantly higher candidate neoantigen burden was detected in patients with CB vs. those with NCB and associated with improved PFS (median 14.5 vs. 3.5 months, log-rank $P = 0.002$) (134). The

TABLE 9 | Studies on the predictive effect of TMB on anti-PD-1/PD-L1 immunotherapy.

Approach for detecting TMB	TMB	Population	Drug	Cut-off value	Result	References
WES	Non-synonymous mutation burden	NSCLC	Pembrolizumab	High: > 200; Low: < 200.	High non-synonymous burden vs. low non-synonymous burden ORR: 63 vs. 0%; Median PFS: 14.5 vs. 3.7 months $P = 0.03$	(134)
	Non-synonymous mutations in genes on the foundation medicine panel (FM-CGP) and institutional panel (HSLCGP)	Melanoma NSCLC Melanoma	Pembrolizumab	FM-CGP: High: ≥7; Low: <7 HSL-GCP: High: ≥13; Low: <13	CGP-mutational load was significantly associated with progression-free survival (PFS) (FM-CGP $P = 0.005$; HSL-CGP $P = 0.008$). and durable clinical benefit (FM-CGP $P = 0.03$, HSL-CGP $P = 0.01$) in patients treated with PD-1 blockade.	(135)
	Total number of somatic missense mutations	Small cell lung cancer (SCLC)	Nivolumab	Low: 0–<143 mutations; Medium: 143–247 mutations; High: ≥248 mutations.	ORR:High vs. medium vs. low:21.3 vs. 6.8 vs. 4.8% P = not reported	(136)
Hybrid capture-based NGS—FoundationOne assay	Hybrid capture NGS panel (315 gene)	Melanoma	Anti PD-1/PD-L1 antibodies (Pembrolizumab, Nivolumab, Atezolizumab)	Low: <3.3 mutations/MB Medium: 0.3–23.1 mutations/MB High: > 23.1 mutations/MB	Mutation load: Initial cohort: Responders vs. non-responders: median 45.6 vs. 3.9 mutations/MB; $P = 0.003$ Validation cohort: Responders vs. non-responders: median 37.1 vs. 12.8 mutations/MB; $P = 0.002$	(137)
	Hybrid-capture-based NGS (182, 236, or 315 genes, depending on the time period)	NSCLC, Melanoma, Other tumors	Anti-PD-1/PD-L1	Low: 1–5 mutations/MB; Medium: 6–19 mutations/MB; High: ≥20 mutations/MB.	High vs. low to medium: RR:58 vs. 20%, $P = 0.001$; PFS:12.8 vs. 3.3 months $P < 0.0001$	(138)

PFS in patients with a higher non-synonymous burden were higher than those with low non-synonymous burden (median PFS 14.5 vs. 3.7 months, log-rank $P = 0.01$) (134). These data suggested that higher non-synonymous mutation or candidate neoantigen burden in tumors were associated with improved PFS of anti-PD-1-treated NSCLC patients. A recent study has shown that a minority of somatic mutations in tumors could lead to neoantigens and TMB could be used to estimate tumor neoantigen load (140).

Discovery of Small Molecule Compounds Inhibiting PD-1/PD-L1 Interactions

The limited success and disadvantage of antibodies prompted researchers to search for more effective strategies for PD-1/PD-L1 targeted therapy and improve the efficacy of cancer immunotherapy. Thus, studies on the discovery of low-molecular-weight compounds inhibiting PD-1/PD-L1 interaction have begun to attract scientist's attention. During the past 5 years, many companies, such as Arising International Inc, Chemocentryx Inc, Institute of Materia Medica, Guangzhou Maxinovel Pharmaceuticals Co, Incyte Corporation, Bristol Myers Squibb (BMS), and Aurigene, have discovered a series of small molecule chemical compounds and peptides.

Meanwhile, these companies have applied for a series of patents related to inhibitors (**Table 10**). Most of these patents presented not only the structure of PD-1/PD-L1 inhibitors, but also the method of compound synthesis and the use of inhibitors as immunomodulators. In addition, the patents showed verified inhibitory effects of these inhibitors. Some of these inhibitors could only block PD-L1/PD-1 interactions. Other inhibitors, such as the peptides discovered by BMS company, could inhibit interactions of PD-L1 with PD-1 or CD80. All inhibitors discovered by Aurigene, including small molecule chemical compounds and peptides, showed an inhibitory effect on the PD-1 signaling pathway.

BMS has published biphenyl derivatives as immunomodulators, and these are the first reported small compounds inhibiting PD-1/PD-L1 interaction. Interestingly, most of the inhibitory compounds showed IC_{50} values of 1 μM or even 0.018 μM as measured by the PD-1/PD-L1 homogenous time-resolved fluorescence (HTRF) binding assay (141). Further modification of the BMS compounds, such as hydrophobic modifications, enhanced the potency of compounds (lowest $IC_{50} = 0.48$ nM) (143). Moreover, the introduction of symmetric biaryl scaffolds could also improve binding affinities (lowest $IC_{50} = 0.04$ nM)(144). Arising International LLC published symmetric or semi-symmetric compounds as immunomodulators (IC_{50} values from 0.1 to 25 μM) (146, 147). ChemoCentryx reported 4-phenyl-2,3-dihydro-1H-inden-1-ol derivatives as inhibitors of the PD-1/PD-L1 interaction (147). The Institute of Materia Medica at the Chinese Academy of Medical Sciences has also discovered a series of bromo benzyl ether derivative and phenylate derivative blocking PD-1/PD-L1 interaction (IC_{50}: 1×10^{-4} nM−1 nM) (149–151).

TABLE 10 | Patents and patent applications of small molecule inhibitors of PD-1 and PD-L1.

Type	Target		Patent number	Inventor
Small molecules	PD-1/PD-L1 interaction	Bristol-Myers Squibb Company	WO2015034820A1	Chupak et al. (141)
	Interaction of PD-L1 with PD-1/CD80	Bristol-Myers Squibb Company	WO2015160641A2	Chupak et al. (142)
			WO2018009505A1	Yeung et al. (143)
			WO2017066227A1	Yeung et al. (144)
			WO2018044963A1	Yeung et al. (145)
		Arising International, LLC	WO2018026971A1	Wang et al. (146)
			WO2018045142A1	Webber et al. (147)
		Chemocentryx, Inc.	WO2018005374A1	Lange et al. (148)
	PD-1/PD-L1 interaction	Institute of Materia Medica, Chinese Academy of Medical Sciences.	WO2017202275A1	Feng et al. (149)
			WO2017202273A1	Feng et al. (150)
			WO2017202276A1	Feng et al. (151)
		Guangzhou Maxinovel Pharmaceuticals Co., Ltd	WO2018006795A1	Wang et al. (152)
	PD-1 signaling pathway.	Aurigene Discovery Technologies Limited.	WO2016142852A1	Sasikumar et al. (153)
			WO2016142894A1	Sasikumar et al. (154)
			WO2015033301A1	Sasikumar et al. (155)
			WO2015033299A1	Sasikumar et al. (156)
			WO2016142886A2	Sasikumar et al. (157)
			WO2016142833A1	Sasikumar et al. (158)
			WO2018051255A1	Sasikumar et al. (159)
			WO2018051254A1	Sasikumar et al. (160)
	PD-1/PD-L1 interaction	Incyte Corporation	WO2017205464A1	Lu et al. (161)
			US20170107216A1	Wu et al. (162)
			WO2017070089A1	Wu et al. (163)
			WO2017106634A1	Wu et al. (164)
			US20170174679A1	Lajkiewicz et al. (165)
			US20180057486A1	Wu et al. (166)
			WO2018013789A1	Yu et al. (167)
			US20170362253A1	Xiao et al. (168)
			WO2017192961A1	Li et al. (169)
		Rijksuniversiteit Groningen	WO2017118762A1	Alexander et al. (170)
Peptides	PD-1 signaling pathway.	Aurigene Discovery Technologies Limited	US9096642B2	Sasikumar et al. (171)
			WO2015036927A1	Sasikumar et al. (172)
			WO2015044900A1	Sasikumar et al. (173)
			US9422339B2	Sasikumar et al. (174)
			WO2015033303A1	Sasikumar et al. (175)
			WO2016142835A1	Sasikumar et al. (176)
	Interaction of PD-L1 with PD-1/CD80	Bristol-Myers Squibb Company	US9308236B2	Miller et al. (177)
			US9879046B2	Miller et al. (178)
			WO2016039749A1	Miller et al. (179)
			WO2017176608A1	Miller et al. (180)
			WO2016077518A1	Gillman et al. (181)
			WO2016100608A1	Sun et al. (182)
			US20170252432A1	Allen et al. (183)
			WO2016126646A1	Miller et al. (184)

Guangzhou Maxinovel Pharmaceuticals Co., Ltd reported that aromatic acetylene or aromatic ethylene compounds had a significant inhibitory effect on PD-1 and PD-L1 (152). A series of oxadiazole- and thiadiazole- compounds have been developed to inhibit the PD-1/PD-L1 pathway by Aurigene Discovery Technologies Limited (153–160). Incyte Corporation

identified a series of heterocyclic compounds as inhibitors for PD-1/PD-L1 protein/protein interaction (IC_{50} values range from the nanomolar to micromolar) (161–169). Meanwhile, Aurigene Discovery Technologies Limited has designed a series of tripeptide peptidomimetics and developed cyclopeptides and macrocyclic-peptides based on peptidomimetics (171–176). Furthermore, BMS developed a series of macrocyclic peptides against the PD-1/PD-L1 pathway (177–184).

However, the discovery of PD-L1/PD-1 inhibitors has only just started. Nearly all inhibitors are still being investigated in preclinical studies. Only CA-170, a PD-L1 inhibitor discovered by Aurigene and Curis, has entered Phase I clinical trial (No: NCT02812875). This has shown acceptable safety of CA-170 (185). The phase II study of CA-170 showed a positive response in two patients with Hodgkin's lymphoma, and the clinical benefit rate is 68.18% (186). Due to its short half-life (6–8 h) vs. other long-lasting antibodies, CA-170 showed less sequalae after being permanently discontinued (186). In addition, preclinical data of the compound CCX4503, published by ChemoCentryx, markedly reduced tumor growth in a human melanoma/peripheral blood mononuclear cell co-implantation model. This preclinical result suggested that the small molecule inhibitors may offer effective anti-tumor therapy (187).

DISCUSSION AND PERSPECTIVE

Anti-PD-1/PD-L1 antibodies have achieved success in the field of cancer immunotherapy during the past decade and mark a breakthrough in oncology. Eight antibodies blocking PD-1 and PD-L1 interactions have been approved for several indications. Despite the promising results reported in some clinical trials, limited drug efficacy caused by IRAEs has been observed and durable responses have been found in only a limited number of patients. In addition, immune-related adverse events caused by anti-PD-1 drugs have been reported in several clinical trials. Due to the limited successes and disadvantages of anti-PD-1/PD-L1 antibodies, more attention has been given to developing more effective strategies to improve clinical response rates. However, using PD-L1 expression as a biomarker of response is important in identifying patients who could obtain a positive clinical response from PD-1/PD-L1 targeted immunotherapy. The use of a single PD-L1 IHC assay with immunotherapy using a specific anti-PD-1/PD-L1 antibody would be one strategy for improving clinical trial outcomes. However, responses were also seen in patients with negative or low PD-L1 expression. For example, in three trials (CheckMate 017, CheckMate 025, and OAK), favorable long-term outcomes were achieved in PD-L1-negative patients (26, 188, 189). The CheckMate 227 trial among NSCLC patients with a high tumor mutational burden showed that progression-free survival was significantly longer with first line nivolumab plus ipilimumab than with chemotherapy, regardless of PD-L1 status (190). These studies also suggested that a higher mutation or neoantigen load could potentially result in a higher likelihood of response to PD-1 or PD-L1 inhibitors. Apart from TMB, there are several other biomarkers including LDH, MMR-deficiency, gene alteration, and IFN-γ related gene. These are useful biomarkers for the response to anti-PD-1/PD-L1 cancer therapy in solid tumors. Some studies have shown dynamic PD-L1 expression in the tumor cells further limits the feasibility of PD-L1 IHC (191). PD-L1 expression could be regulated through extrinsic and intrinsic signaling pathways such as mitogen-activated protein kinase (MAPK) signaling pathway, Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway, miRNA-related pathway, as well as IFN-γ and TNF-α (192–194). An understanding of the mechanism of regulation of dynamic PD-L1 expression may be useful for developing novel strategies to improve the efficacy of anti-PD-1/PD-L1 drugs. On the other hand, small molecules are expected to reduce immune-related adverse events and promote higher efficacy. Studies on small molecule PD-1/PD-L1 inhibitors have just begun within the preclinical stage. CA-170 is the first PD-1/PD-L1 inhibitor successfully entering clinical trial, and it is potentially a small molecule PD-1/PD-L1 inhibitor in cancer therapy. Future clinical trial results of CA-170 would be important for developing small molecule inhibitors.

AUTHOR'S NOTE

This review has made a summary about clinical studies and patent application of PD-1/PD-L1 targeted therapies. The paper has also shown the promising result of anti-PD-1/PD-L1 drug in various cancer types and several kinds of strategies improving efficacy of anti-PD-1/PD-L1 drug have been mentioned in the paper, including developing companion PD-L1 test, searching for biomarkers, and discovering small molecule PD-1/PD-L1 inhibitors. The paper has shown the development of anti-PD-1/PD-L1 therapies and provided broad knowledge of PD-1/PD-L1 targeted therapies.

AUTHOR CONTRIBUTIONS

LG, RW, and HK contributed conception and design of the review article. LG organized the database collection. LG and RW wrote the first draft of the manuscript. HK wrote and revised sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REFERENCES

1. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol.* (1996) 8:765–72. doi: 10.1093/intimm/8.5.765
2. Gibbons JR, Dong H. Functional expression of programmed death-ligand 1 (B7-H1) by immune cells and tumor cells. *Front Immunol.* (2017) 8:961. doi: 10.3389/fimmu.2017.00961
3. Ni L, Lu J. Interferon gamma in cancer immunotherapy. *Cancer Med.* (2018) 7:4509–16. doi: 10.1002/cam4.1700
4. Granier C, De Guillebon E, Blanc C, Roussel H, Badoual C, Colin E, et al. Mechanisms of action and rationale for the use of checkpoint inhibitors in cancer. *Esmo Open.* (2017) 2:e000213. doi: 10.1136/esmoopen-2017-000213
5. Riley JL. PD-1 signaling in primary T cells. *Immunol Rev.* (2009) 229:114–25. doi: 10.1111/j.1600-065X.2009.00767.x
6. Patsoukis N, Sari D, Boussiotis VA. PD-1 inhibits T cell proliferation by upregulating p27 and p15 and suppressing Cdc25A. *Cell Cycle.* (2012) 11:4305–9. doi: 10.4161/cc.22135
7. Yang H, Zhang Q, Xu M, Wang L, Chen X, Feng Y, et al. CCL2-CCR2 axis recruits tumor associated macrophages to induce immune evasion through PD-1 signaling in esophageal carcinogenesis. *Mol Cancer.* (2020) 19:41. doi: 10.1186/s12943-020-01165-x
8. Nazareth MR, Broderick L, Simpson-Abelson MR, Kelleher RJJ, Yokota SJ, Bankert RB. Characterization of human lung tumor-associated fibroblasts and their ability to modulate the activation of tumor-associated T cells. *J Immunol.* (2007) 178:5552–62. doi: 10.4049/jimmunol.178.9.5552
9. Rozali EN, Hato SV, Robinson BW, Lake RA, Lesterhuis WJ. Programmed death ligand 2 in cancer-induced immune suppression. *Clin Dev Immunol.* (2012) 2012:656340. doi: 10.1155/2012/656340
10. Rollins MR, Gibbons Johnson RM. CD80 Expressed by CD8(+) T cells contributes to PD-L1-induced apoptosis of activated CD8(+) T cells. *J Immunol Res.* (2017) 2017:7659462. doi: 10.1155/2017/7659462
11. Honjo T, Ishida Y, Shinohara T. *Peptide Related to Human Programmed Cell Death and DNA Encoding the Same.* US5698520A. Alexandria, VA: USPTO (1997).
12. Okazaki T, Honjo T. PD-1 and PD-1 ligands: from discovery to clinical application. *Int Immunol.* (2007) 19:813–24. doi: 10.1093/intimm/dxm057
13. Seung E, Dudek TE, Allen TM, Freeman GJ, Luster AD, Tager AM. PD-1 Blockade in chronically HIV-1-infected humanized mice suppresses viral loads. *PLoS ONE.* (2013) 8:e77780. doi: 10.1371/journal.pone.0077780
14. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med.* (1999) 5:1365–9. doi: 10.1038/70932
15. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med.* (2000) 192:1027–34. doi: 10.1084/jem.192.7.1027
16. Honjo T, Shibayama S, Takeda K, Matsuo M, Yoshida T, Miyamoto M. *Substance Specific to Human PD-1.* US7563869B2. Alexandria, VA: USPTO (2009).
17. Freeman G, Boussiotis V, Chernova T, Malenkovich N. *B7-4 Polypeptides and Uses Therefor.* US7038013B2. Alexandria, VA: USPTO (2006).
18. Wood C, Freeman GJ. *PD-1, a Receptor for B7-4, and Uses Therefor.* US7101550B2. Alexandria, VA: USPTO (2006).
19. Freeman G, Sharpe A, Dorfman DM, Ahmed R, Barber D, Wherry EJ. *Methods and Compositions for the Treatment of Persistent Infections.* US8652465B2. Alexandria, VA: USPTO (2014).
20. Wood C, Freeman GJ. *Downmodulating an Immune Response With Multivalent Antibodies to PD-1.* US6808710B1. Alexandria, VA: USPTO (2004).
21. Chen L. *B7-H1, a Novel Immunoregulatory Molecule.* US9062112B2. Alexandria, VA: USPTO (2015).
22. Chen L. B7-H1 *Antibodies*. US8981063B2. Alexandria, VA: USPTO (2015).
23. Chen L, Strome SE, Kwon ED. *B7-H1 and Methods of Diagnosis, Prognosis, and Treatment of Cancer.* US7892540B2. Alexandria, VA: USPTO (2011).
24. Lyford-Pike S, Peng S, Young GD, Taube JM, Westra WH, Akpeng B, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. *Cancer Res.* (2013) 73:1733–41. doi: 10.1158/0008-5472.CAN-12-2384
25. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med.* (2002) 8:793–800. doi: 10.1038/nm730
26. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, et al. Nivolumab in previously untreated melanoma without BRAF mutation. *New Engl J Med.* (2015) 372:320–30. doi: 10.1056/NEJMoa1412082
27. Schadendorf D, Hodi FS, Robert C, Weber JS, Margolin K, Hamid O, et al. Pooled analysis of long-term survival data from phase II and Phase III trials of ipilimumab in unresectable or metastatic melanoma. *J Clin Oncol.* (2015) 33:1889–94. doi: 10.1200/JCO.2014.56.2736
28. Honjo T, Minato N, Iwai Y, Shibayama S. *Method for Treatment of Cancer by Inhibiting the Immunosuppressive Signal Induced by PD-1.* US7595048B2. Alexandria, VA: USPTO (2004).
29. Carven GJ, van Eenennaam H, Dulos GJ. *Antibodies to Human Programmed Death Receptor PD-1.* US8952136B2. Alexandria, VA: USPTO (2013).
30. Nastri HG, Iffland C, Leger O, An Q, Cartwright M, McKenna SD, et al. *Anti-pd-l1 Antibodies and Uses Thereof.* US20140341917A1. Alexandria, VA: USPTO (2014).
31. Irving B, Chiu H, Maecker H, Mariathasan S, Lehar SM, Wu Y, et al. *Anti-PD-L1 Antibodies, Compositions and Articles of Manufacture.* US8217149B2. Alexandria, VA: USPTO (2010).
32. Queva C, Morrow M, Hammond S, Alimzhanov M, Babcook J, Foltz IN, et al. *Targeted Binding Agents Against B7-H1.* US8779108B2. Alexandria, VA: USPTO (2014).
33. Freeman GJ, Sharpe AH, Blattler WA, Mataraza JM, Sabatos-peyton CA, Chang HW, et al. *Antibody Molecules to PD-1 and Uses Thereof.* WO2015112900. Geneva: WIPO (2015).
34. Papadopoulos NJ, Murphy AJ, Thurston G, Burova EI. *Human Antibodies to PD-1.* US20150203579A1. Alexandria, VA: USPTO (2015).
35. Yuan J, Qu X, Lin J, Ye X, Cao G, Tao W, et al. *Pd-1 Antibody, Antigen-Binding Fragment Thereof, and Medical Application Thereof.* US20160376367A1. Alexandria, VA: USPTO (2016).
36. Li K, Zhang T, Song J, Xu L, Liu Q, Peng H. *Anti-PD1 Antibodies and Their Use as Therapeutics and Diagnostics.* US20150079109A1. Alexandria, VA: USPTO (2015).
37. King DJ, Kehry M. *Antibodies Directed Against Programmed Death-1 (PD-1).* US9815897B2. Alexandria, VA: USPTO (2017).
38. Langermann S, Liu L. *Compositions of PD-1 Antagonists And Methods of Use.* US8609089B2. Alexandria, VA: USPTO (2013).
39. Wang J, Dong L, Wang Y. *Anti-pd-1 Antibodies and Therapeutic Uses Thereof.* US20180346569A1. Alexandria, VA: USPTO (2018).
40. Langermann S. *Simultaneous Inhibition of pd-l1/pd-l2.* US20130017199A1. Alexandria, VA: USPTO (2013).
41. West JW, Mei L, Moore SJ, Nguyen MTL, Hostetter DR, Vasiljeva O, et al. *Anti-pdl1 Antibodies, Activatable Anti-pdl1 Antibodies, and Methods of Use Thereof.* US20160311903A1. Alexandria, VA: USPTO (2016).
42. Korman AJ, Selby MJ, Wang C, Srinivasan M, Passmore DB, Huang H, et al. *Human Monoclonal Antibodies to Programmed Death Ligand 1 (PD-L1).* US7943743B2. Alexandria, VA: USPTO (2011).
43. Xu T, Dong Y, Wang P, Chen T. *Single Domain Antibody and Derivative Proteins Thereof Against Programmed Death-Ligand (pdl1).* US20180327494A1. Alexandria, VA: USPTO (2018).
44. Dumet C, Pottier J, Gouilleux-Gruart V, Watier H. Insights into the IgG heavy chain engineering patent landscape as applied to IgG4 antibody development. *Mabs-Austin.* (2019) 11:1341–50. doi: 10.1080/19420862.2019.1664365
45. Qin SK, Finn RS, Kudo M, Meyer T, Vogel A, Ducreux M, et al. RATIONALE 301 study: tislelizumab versus sorafenib as first-line treatment for unresectable hepatocellular carcinoma. *Future Oncol.* (2019) 15:1811–22. doi: 10.2217/fon-2019-0097
46. Floudas CS, Brar G, Mabry-Hrones D, Duffy AG, Wood B, Levy E, et al. A pilot study of the PD-1 targeting agent AMP-224 used with low-dose cyclophosphamide and stereotactic body radiation therapy in patients with metastatic colorectal cancer. *Clin Colorectal Canc.* (2019) 18:e349–60. doi: 10.1016/j.clcc.2019.06.004
47. Tan S, Zhang H, Chai Y, Song H, Tong Z, Wang Q, et al. An unexpected N-terminal loop in PD-1 dominates binding by nivolumab. *Nat Commun.* (2017) 8:14369. doi: 10.1038/ncomms14369
48. Na Z, Yeo SP, Bharath SR, Bowler MW, Balikci E, Wang CI, et al. Structural basis for blocking PD-1-mediated immune suppression

by therapeutic antibody pembrolizumab. *Cell Res.* (2017) 27:147–50. doi: 10.1038/cr.2016.77

49. Finlay WJ, Coleman JE, Edwards JS, Johnson KS. Anti-PD1 'SHR-1210' aberrantly targets pro-angiogenic receptors and this polyspecificity can be ablated by paratope refinement. In: *Proceedings of Mabs-Austin.* (2019) 11:26–44. doi: 10.1080/19420862.2018.1550321
50. Chen X, Song X, Li K, Zhang TJF. FcγR-binding is an important functional attribute for immune checkpoint antibodies in cancer immunotherapy. *Front Immunol.* (2019) 10:292. doi: 10.3389/fimmu.2019.00292
51. Zhang F, Wei H, Wang X, Bai Y, Wang P, Wu J, et al. Structural basis of a novel PD-L1 nanobody for immune checkpoint blockade. *Cell Discov.* (2017) 3:17004. doi: 10.1038/celldisc.2017.4
52. Autio KA, Boni V, Humphrey RW, Naing A. Probody therapeutics: an emerging class of therapies designed to enhance on-target effects with reduced off-tumor toxicity for use in immuno-oncology. *Clin Canc. Res.* (2019) 26:984–89. doi: 10.1158/1078-0432.CCR-19-1457
53. Tan S, Liu K, Chai Y, Zhang CW, Gao S, Gao GF, et al. Distinct PD-L1 binding characteristics of therapeutic monoclonal antibody durvalumab. *Protein Cell.* (2018) 9:135–9. doi: 10.1007/s13238-017-0412-8
54. Zak KM, Grudnik P, Magiera K, Domling A, Dubin G, Holak TA. Structural biology of the immune checkpoint receptor PD-1 and its ligands PD-L1/PD-L2. *Structure.* (2017) 25:1163–74. doi: 10.1016/j.str.2017.06.011
55. Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. *Lancet Oncol.* (2017) 18:1182–91.
56. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. *Lancet Oncol.* (2015) 16:375–84. doi: 10.1016/S1470-2045(15)70076-8
57. Brahmer J, Reckamp KL, Baas P, Crino L, Eberhardt WEE, Poddubskaya E, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *New Engl J Med.* (2015) 373:123–35. doi: 10.1056/NEJMoa1504627
58. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *New Engl J Med.* (2015) 373:1627–39. doi: 10.1056/NEJMoa1507643
59. Sharma P, Retz M, Siefker-Radtke A, Baron A, Necchi A, Bedke J, et al. Nivolumab in metastatic urothelial carcinoma after platinum therapy (CheckMate 275): a multicentre, single-arm, phase 2 trial. *Lancet Oncol.* (2017) 18:312–22. doi: 10.1016/S1470-2045(17)30065-7
60. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al. Nivolumab versus everolimus in advanced renal-cell carcinoma. *New Engl J Med.* (2015) 373:1803–13. doi: 10.1056/NEJMoa1510665
61. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med.* (2015) 372:311–9. doi: 10.1056/NEJMoa1411087
62. Younes A, Santoro A, Shipp M, Zinzani PL, Timmerman JM, Ansell S, et al. Nivolumab for classical Hodgkin's lymphoma after failure of both autologous stem-cell transplantation and brentuximab vedotin: a multicentre, multicohort, single-arm phase 2 trial. *Lancet Oncol.* (2016) 17:1283–94. doi: 10.1016/S1470-2045(16)30167-X
63. El-Khoueiry AB, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet.* (2017) 389:2492–502. doi: 10.1016/S0140-6736(17)31046-2
64. Ferris RL, Blumenschein G, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. *New Engl J Med.* (2016) 375:1856–67. doi: 10.1056/NEJMoa1602252
65. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, et al. Safety and tumor responses with lambrolizumab (Anti-PD-1) in melanoma. *New Engl J Med.* (2013) 369:134–44. doi: 10.1056/NEJMoa1305133
66. Robert C, Ribas A, Wolchok JD, Hodi FS, Hamid O, Kefford R, et al. Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. *Lancet.* (2014) 384:1109–17. doi: 10.1016/S0140-6736(14)60958-2
67. Sul J, Blumenthal GM, Jiang X, He K, Keegan P, Pazdur R. FDA approval summary: pembrolizumab for the treatment of patients with metastatic non-small cell lung cancer whose tumors express programmed death-ligand 1. *Oncologist.* (2016) 21:643–50. doi: 10.1634/theoncologist.2015-0498
68. Balar AV, Castellano D, O'Donnell PH, Grivas P, Vuky J, Powles T, et al. First-line pembrolizumab in cisplatin-ineligible patients with locally advanced and unresectable or metastatic urothelial cancer (KEYNOTE-052): a multicentre, single-arm, phase 2 study. *Lancet Oncol.* (2017) 18:1483–92. doi: 10.1016/S1470-2045(17)30616-2
69. Bellmunt J, de Wit R, Vaughn DJ, Fradet Y, Lee JL, Fong L, et al. Pembrolizumab as second-line therapy for advanced urothelial carcinoma. *New Engl J Med.* (2017) 376:1015–26. doi: 10.1056/NEJMoa1613683
70. Seiwert TY, Burtness B, Mehra R, Weiss J, Berger R, Eder JP, et al. Safety and clinical activity of pembrolizumab for treatment of recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): an open-label, multicentre, phase 1b trial. *Lancet Oncol.* (2016) 17:956–65. doi: 10.1016/S1470-2045(16)30066-3
71. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science.* (2017) 357:409–13. doi: 10.1126/science.aan6733
72. Fuchs CS, Doi T, Jang RWJ, Muro K, Satoh T, Machado M, et al. KEYNOTE-059 cohort 1: efficacy and safety of pembrolizumab (pembro) monotherapy in patients with previously treated advanced gastric cancer. *J Clin Oncol.* (2017) 35:4003. doi: 10.1200/JCO.2017.35.15_suppl.4003
73. Chung HC, Schellens JHM, Delord JP, Perets R, Italiano A, Shapira-Frommer R. Pembrolizumab treatment of advanced cervical cancer: updated results from the phase 2 KEYNOTE-158 study. *J Clin Oncol.* (2018) 36:5522. doi: 10.1200/JCO.2018.36.15_suppl.5522
74. Apolo AB, Infante JR, Balmanoukian A, Patel MR, Wang D, Kelly K, et al. Avelumab, an anti-programmed death-ligand 1 antibody, in patients with refractory metastatic urothelial carcinoma: results from a multicenter, phase Ib study. *J Clin Oncol.* (2017) 35:2117–24. doi: 10.1200/JCO.2016.71.6795
75. Kaufman HL, Russell J, Hamid O, Bhatia S, Terheyden P, D'Angelo SP, et al. Avelumab in patients with chemotherapy-refractory metastatic Merkel cell carcinoma: a multicentre, single-group, open-label, phase 2 trial. *Lancet Oncol.* (2016) 17:1374–85. doi: 10.1016/S1470-2045(16)30364-3
76. Fehrenbacher L, Spira A, Ballinger M, Kowanetz M, Vansteenkiste J, Mazieres J, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. *Lancet.* (2016) 387:1837–46. doi: 10.1016/S0140-6736(16)00587-0
77. Rittmeyer A, Barlesi F, Waterkamp D, Park K, Ciardiello F, von Pawel J, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. *Lancet.* (2017) 389:255–65. doi: 10.1016/S0140-6736(16)32517-X
78. Rosenberg JE, Hoffman-Censits J, Powles T, van der Heijden MS, Balar AV, Necchi A, et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *Lancet.* (2016) 387:1909–20. doi: 10.1016/S0140-6736(16)00561-4
79. Antonia SJ, Villegas A, Daniel D, Vicente D, Murakami S, Hui R, et al. Durvalumab after chemoradiotherapy in stage III non-small-cell lung cancer. *N Engl J Med.* (2017) 377:1919–29. doi: 10.1056/NEJMoa1709937
80. Powles T, O'Donnell PH, Massard C, Arkenau HT, Friedlander TW, Hoimes CJ, et al. Efficacy and safety of durvalumab in locally advanced or metastatic urothelial carcinoma: updated results from a phase 1/2 open-label study. *JAMA Oncol.* (2017) 3:e172411. doi: 10.1001/jamaoncol.2017.2411
81. Migden MR, Rischin D, Schmults CD, Guminski A, Hauschild A, Lewis KD, et al. PD-1 blockade with cemiplimab in advanced cutaneous squamous-cell carcinoma. *N Engl J Med.* (2018) 379:341–51. doi: 10.1056/NEJMoa1805131
82. Westin JR, Chu F, Zhang M, Fayad LE, Kwak LW, Fowler N, et al. Safety and activity of PD1 blockade by pidilizumab in combination with rituximab in

patients with relapsed follicular lymphoma: a single group, open-label, phase 2 trial. *Lancet Oncol.* (2014) 15:69–77. doi: 10.1016/S1470-2045(13)70551-5
83. Long GV, Lebbe C, Atkinson V, Mandalà M, Nathan PD, Arance Fernandez AM, et al. The anti–PD-1 antibody spartalizumab (S) in combination with dabrafenib (D) and trametinib (T) in previously untreated patients (pts) with advanced BRAF V600–mutant melanoma: Updated efficacy and safety from parts 1 and 2 of COMBI-i. In: *Proceedings of 2019 ASCO Annual Meeting,* Chicago, IL (2019).
84. Fang W, Yang Y, Ma Y, Hong S, Lin L, He X, et al. Camrelizumab (SHR-1210) alone or in combination with gemcitabine plus cisplatin for nasopharyngeal carcinoma: results from two single-arm, phase 1 trials. *Lancet Oncol.* (2018) 19:1338–50. doi: 10.1016/S1470-2045(18)30495-9
85. Wang S, Huang X, Bai Y-X, Yuan Y, Li J, Wang Z, et al. Preliminary results with tislelizumab, an investigational anti-PD-1 antibody, in Chinese patients with nasopharyngeal cancer (NPC). In: *Proceedings of 2019 ASCO Annual Meeting,* Chicago, IL (2019).
86. Chi Z, Tang B, Sheng X, Si L, Cui C, Kong Y, et al. A phase II study of JS001, a humanized PD-1 mAb, in patients with advanced melanoma in China. In: *Proceedings of 2018 ASCO Annual Meeting.* Chicago, IL (2018) 1–5.
87. Moreno V, Barretina-Ginesta M-P, Guo W, Lu S, Jenkins D, McEachern K, et al. Abstract CT053: preliminary safety, efficacy, and PK/PD characterization from GARNET, a phase 1 clinical trial of the anti-PD-1 monoclonal antibody, TSR-042, in patients with recurrent or advanced NSCLC and MSI-H endometrial cancer. In: *Proceedings of AACR Annual Meeting 2018,* Chicago, IL (2018).
88. Moore KN, Dresher C, Liu J, O'Malley DM, Wang EW, Wang JS-Z, et al. Phase 1/2 open-label, multiple ascending dose trial of AGEN2034, an anti-PD-1 monoclonal antibody, in advanced solid malignancies: results of dose escalation. In: *Proceedings of 2018 ASCO Annual Meeting*, Chicago, IL (2018).
89. Shi Y, Su H, Song Y, Jiang W, Sun X, Qian W, et al. Safety and activity of sintilimab in patients with relapsed or refractory classical Hodgkin lymphoma (ORIENT-1): a multicentre, single-arm, phase 2 trial. *Lancet Haematol.* (2019) 6:e12–9. doi: 10.1016/S2352-3026(18)30192-3
90. Fogt S, Shustova M, Demidov LV, Moiseyenko V, Tjulandin S, Semiglazova T, et al. Phase II trial (BCD-100-2/MIRACULUM) of the novel PD-1 inhibitor (BCD-100) in patients with advanced melanoma. In: *Proceedings of 2019 ASCO Annual Meeting*, Chicago, IL (2019).
91. Song Y, Zhu J, Lin N, Zhang C, Zhang M, Bai H, et al. GLS-010, a novel anti-PD-1 mAb in Chinese patients with relapsed or refractory classical Hodgkin's lymphoma: preliminary result of a phase II clinical trial. In: *Proceedings of 2019 ASCO Annual Meeting*, Chicago, IL (2019).
92. Boni V, Garcia-Corbacho J, Ott PA, Cho D, Autio KA, Uboha N, et al. Preliminary results of PROCLAIM-CX-072: the first-in-human, dose-finding trial of PD-L1 probody therapeutic CX-072 as monotherapy in patients (pts) with advanced solid tumors. In: *Proceedings of ESMO 2018 Congress,* Munich (2018).
93. Shen L, Cao J, Li J, Pan H, Xu N, Zhang Y, et al. A phase Ia/Ib trial of the anti-PD-L1 human monoclonal antibody (mAb), CS1001, in patients (pts) with advanced solid tumors or lymphomas. In: *Proceedings of 2019 ASCO Annual Meeting,* Chicago, IL (2019).
94. *Checkpoint Therapeutics Announces Positive Interim Clinical Results of Anti-PD-L1 Antibody Cosibelimab*. Availabe online at: https://www.globenewswire.com/news-release/2019/05/01/1813426/0/en/Checkpoint-Therapeutics-Announces-Positive-Interim-Clinical-Results-of-Anti-PD-L1-Antibody-Cosibelimab.html (accessed May 01, 2019).
95. Nie J, Wang C, Liu Y, Yang Q, Mei Q, Dong L, et al. Addition of low-dose decitabine to anti–PD-1 antibody camrelizumab in relapsed/refractory classical hodgkin lymphoma. *J Clin Oncol.* (2019) 37:1479–89. doi: 10.1200/JCO.18.02151
96. Keam SJ. Toripalimab: first global approval. *Drugs.* (2019) 79:573–8. doi: 10.1007/s40265-019-01076-2
97. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med.* (2012) 366:2443–54. doi: 10.1056/NEJMoa1200690
98. Man J, Ritchie G, Links M, Lord S, Lee CK. Treatment-related toxicities of immune checkpoint inhibitors in advanced cancers: a meta-analysis. *Asia Pac J Clin Oncol.* (2018) 14:141–52. doi: 10.1111/ajco.12838
99. Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. *J Clin Oncol.* (2015) 33:1974–82. doi: 10.1200/JCO.2014.59.4358
100. Cree IA, Booton R, Cane P, Gosney J, Ibrahim M, Kerr K, et al. PD-L1 testing for lung cancer in the UK: recognizing the challenges for implementation. *Histopathology.* (2016) 69:177–86. doi: 10.1111/his.12996
101. Pierce RH, Bourne P, Liang L, Bigler M. *Antibodies That Bind to Human Programmed Death Ligand 1 (PD-L1)*. US9709568B2. Alexandria, VA: USPTO (2017).
102. Cogswell JP, Goldberg SM, Gupta AK, Jure-Kunkel M, Wang XT, Wigginton JM. *Cancer Immunotherapy by Disrupting pd-1/pd-l1 Signaling*. WO2013173223A1. Geneva: WIPO (2013).
103. Nitta H, Vennapusa B, Dennis E. *Multiplex Assay for Improved Scoring of Tumor Tissues Stained for pd-l1*. WO2015181343A2. Geneva: WIPO (2015).
104. Hui R, Garon E, Goldman J, Leighl N, Hellmann M, Patnaik A, et al. Pembrolizumab as first-line therapy for patients with PD-L1-positive advanced non-small cell lung cancer: a phase 1 trial. *Ann Oncol.* (2017) 28:874–81. doi: 10.1093/annonc/mdx008
105. Wainberg Z, Jalal S, Muro K, Yoon H, Garrido M, Golan T, et al. LBA28_PRKEYNOTE-059 update: efficacy and safety of pembrolizumab alone or in combination with chemotherapy in patients with advanced gastric or gastroesophageal (G/GEJ) cancer. *Ann Oncol.* (2017) 28:616–17. doi: 10.1093/annonc/mdx440.020
106. Chung HC, Ros W, Delord J-P, Perets R, Italiano A, Shapira-Frommer R, et al. Efficacy and safety of pembrolizumab in previously treated advanced cervical cancer: results from the phase II KEYNOTE-158 study. *J Clin Oncol.* (2019) 37:1470–8. doi: 10.1200/JCO.18.01265
107. Burtness B, Harrington K, Greil R, Soulières D, Tahara M, De Castro G Jr, et al. LBA8_PR KEYNOTE-048: phase III study of first-line pembrolizumab (P) for recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC). *ESMO Meeting.* (2018) 29:mdy424. 045. doi: 10.1093/annonc/mdy424.045
108. Kojima T, Muro K, Francois E, Hsu C-H, Moriwaki T, Kim S-B, et al. Pembrolizumab versus chemotherapy as second-line therapy for advanced esophageal cancer: phase III KEYNOTE-181 study. In: *Proceedings of 2019 Gastrointestinal Cancers Symposium*, San Francisco, CA (2019).
109. Aggen DH, Drake CG. Biomarkers for immunotherapy in bladder cancer: a moving target. *J Immunother Cancer.* (2017) 5:94. doi: 10.1186/s40425-017-0299-1
110. Emens LA, Cruz C, Eder JP, Braiteh F, Chung C, Tolaney SM, et al. Long-term clinical outcomes and biomarker analyses of atezolizumab therapy for patients with metastatic triple-negative breast cancer: a phase 1 study. *JAMA Oncol.* (2019) 5:74–82. doi: 10.1001/jamaoncol.2018.4224
111. Shen X, Zhao B. Efficacy of PD-1 or PD-L1 inhibitors and PD-L1 expression status in cancer: meta-analysis. *BMJ.* (2018) 362:k3529. doi: 10.1136/bmj.k3529
112. Hirsch FR, McElhinny A, Stanforth D, Ranger-Moore J, Jansson M, Kulangara K, et al. PD-L1 immunohistochemistry assays for lung cancer: results from phase 1 of the blueprint PD-L1 IHC assay comparison project. *J Thorac Oncol.* (2017) 12:208–22. doi: 10.1016/j.jtho.2016.11.2228
113. Tsao MS, Kerr KM, Kockx M, Beasley MB, Borczuk AC, Botling J, et al. PD-L1 immunohistochemistry comparability study in real-life clinical samples: results of blueprint phase 2 project. *J Thorac Oncol.* (2018) 13:1302–11. doi: 10.1016/j.jtho.2018.05.013
114. Lawson NL, Dix CI, Scorer PW, Stubbs CJ, Wong EM, Hutchinson L, et al. Mapping the binding sites of antibodies utilized in programmed cell death ligand-1 predictive immunohistochemical assays for use with immuno-oncology therapies. *Modern Pathol.* (2019) 33:758–8. doi: 10.1038/s41379-019-0379-5
115. Diem S, Kasenda B, Spain L, Martin-Liberal J, Marconcini R, Gore M, et al. Serum lactate dehydrogenase as an early marker for outcome in patients treated with anti-PD-1 therapy in metastatic melanoma. *Br J Cancer.* (2016) 114:256–61. doi: 10.1038/bjc.2015.467
116. Ribas A, Hamid O, Daud A, Hodi FS, Wolchok JD, Kefford R, et al. Association of pembrolizumab with tumor response and survival among patients with advanced melanoma. *JAMA.* (2016) 315:1600–9. doi: 10.1001/jama.2016.4059

117. Dronca RS, Liu X, Harrington SM, Chen L, Cao S, Kottschade LA, et al. T cell Bim levels reflect responses to anti–PD-1 cancer therapy. *JCI Insight.* (2016) 1:e86014. doi: 10.1172/jci.insight.86014
118. Weide B, Martens A, Hassel JC, Berking C, Postow MA, Bisschop K, et al. Baseline biomarkers for outcome of melanoma patients treated with pembrolizumab. *Clin Cancer Res.* (2016) 22:5487–96. doi: 10.1158/1078-0432.CCR-16-0127
119. Dhodapkar KM, Gettinger SN, Das R, Zebroski H, Dhodapkar MVJO. SOX2-specific adaptive immunity and response to immunotherapy in non-small cell lung cancer. *Oncoimmunology.* (2013) 2:e25205. doi: 10.4161/onci.25205
120. Nakaya A, Kurata T, Yoshioka H, Takeyasu Y, Niki M, Kibata K, et al. Neutrophil-to-lymphocyte ratio as an early marker of outcomes in patients with advanced non-small-cell lung cancer treated with nivolumab. *Int J Clin Oncol.* (2018) 23:634–40. doi: 10.1007/s10147-018-1250-2
121. Zhou J, Mahoney KM, Giobbie-Hurder A, Zhao F, Lee S, Liao X, et al. Soluble PD-L1 as a biomarker in malignant melanoma treated with checkpoint blockade. *Cancer Immunol Res.* (2017) 5:480–92. doi: 10.1158/2326-6066.CIR-16-0329
122. Tiako Meyo M, Jouinot A, Giroux-Leprieur E, Fabre E, Wislez M, Alifano M, et al. Predictive value of soluble PD-1, PD-L1, VEGFA, CD40 ligand and CD44 for nivolumab therapy in advanced non-small cell lung cancer: a case-control study. *Cancers.* (2020) 12:473. doi: 10.3390/cancers12020473
123. Herbst RS, Soria J-C, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature.* (2014) 515:563–7. doi: 10.1038/nature14011
124. Kato S, Goodman A, Walavalkar V, Barkauskas DA, Sharabi A, Kurzrock RJCCR. Hyperprogressors after immunotherapy: analysis of genomic alterations associated with accelerated growth rate. *Clin Cancer Res.* (2017) 23:4242–50. doi: 10.1158/1078-0432.CCR-16-3133
125. Gainor JF, Shaw AT, Sequist LV, Fu X, Azzoli CG, Piotrowska Z, et al. EGFR mutations and ALK rearrangements are associated with low response rates to PD-1 pathway blockade in non–small cell lung cancer: a retrospective analysis. *Clin Cancer Res.* (2016) 22:4585–93. doi: 10.1158/1078-0432.CCR-15-3101
126. Dong Z, Zhong W, Zhang X, Su J, Xie Z, Liu S, et al. Potential predictive value of TP53 and KRAS mutation status for response to PD-1 blockade immunotherapy in lung adenocarcinoma. *Clin Cancer Res.* (2017) 23:3012–24. doi: 10.1016/j.jtho.2016.11.504
127. Skoulidis F, Goldberg ME, Greenawalt DM, Hellmann MD, Awad MM, Gainor JF, et al. STK11/LKB1 mutations and PD-1 inhibitor resistance in KRAS-mutant lung adenocarcinoma. *Cancer Discov.* (2018) 8:822–35. doi: 10.1158/2159-8290.CD-18-0099
128. Miao D, Margolis CA, Gao W, Voss MH, Li W, Martini DJ, et al. Genomic correlates of response to immune checkpoint therapies in clear cell renal cell carcinoma. *Science.* (2018) 359:801–6. doi: 10.1126/science.aan5951
129. Teo MY, Seier K, Ostrovnaya I, Regazzi AM, Kania BE, Moran MM, et al. Alterations in DNA damage response and repair genes as potential marker of clinical benefit from PD-1/PD-L1 blockade in advanced urothelial cancers. *J CLin Oncol.* (2018) 36:1685–94. doi: 10.1200/JCO.2017.75.7740
130. Refae S, Gal J, Ebran N, Otto J, Borchiellini D, Peyrade F, et al. Germinal Immunogenetics predict treatment outcome for PD-1/PD-L1 checkpoint inhibitors. *Invest New Drugs.* (2020) 38:160–71. doi: 10.1007/s10637-019-00845-w
131. Chat V, Ferguson R, Simpson D, Kazlow E, Lax R, Moran U, et al. Autoimmune genetic risk variants as germline biomarkers of response to melanoma immune-checkpoint inhibition. *Cancer Immunol Immun.* (2019) 68:897–905. doi: 10.1007/s00262-019-02318-8
132. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature.* (2014) 515:568–71. doi: 10.1038/nature13954
133. Yearley JH, Gibson C, Yu N, Moon C, Murphy E, Juco J, et al. PD-L2 expression in human tumors: relevance to anti-PD-1 therapy in cancer. *Clin Cancer Res.* (2017) 23:3158–67. doi: 10.1158/1078-0432.CCR-16-1761
134. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. *Science.* (2015) 348:124–8. doi: 10.1126/science.aaa1348
135. Campesato LF, Barroso-Sousa R, Jimenez L, Correa BR, Sabbaga J, Hoff PM, et al. Comprehensive cancer-gene panels can be used to estimate mutational load and predict clinical benefit to PD-1 blockade in clinical practice. *Oncotarget.* (2015) 6:34221. doi: 10.18632/oncotarget.5950
136. Hellmann MD, Callahan MK, Awad MM, Calvo E, Ascierto PA, Atmaca A, et al. Tumor mutational burden and efficacy of nivolumab monotherapy and in combination with ipilimumab in small-cell lung cancer. *Cancer Cell.* (2018) 33:853–61.e854. doi: 10.1016/j.ccell.2018.04.001
137. Johnson DB, Frampton GM, Rioth MJ, Yusko E, Xu Y, Guo X, et al. Targeted next generation sequencing identifies markers of response to PD-1 blockade. *Cancer Immunol Res.* (2016) 4:959–67. doi: 10.1158/2326-6066.CIR-16-0143
138. Goodman AM, Kato S, Bazhenova L, Patel SP, Frampton GM, Miller V, et al. Tumor mutational burden as an independent predictor of response to immunotherapy in diverse cancers. *Mol Cancer Ther.* (2017) 16:2598–608. doi: 10.1158/1535-7163.MCT-17-0386
139. Gandara DR, Paul SM, Kowanetz M, Schleifman E, Zou W, Li Y, et al. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. *Nat Med.* (2018) 24:1441–8. doi: 10.1038/s41591-018-0134-3
140. Chan TA, Yarchoan M, Jaffee E, Swanton C, Quezada SA, Stenzinger A, et al. Development of tumor mutation burden as an immunotherapy biomarker: utility for the oncology clinic. *Ann Oncol.* (2019) 30:44–56. doi: 10.1093/annonc/mdy495
141. Chupak LS, Zheng X. *Compounds Useful as Immunomodulators*. WO2015034820Al. Geneva: WIPO (2015).
142. Chupak LS, Ding M, Martin SW, Zheng X, Hewawasam P, Connolly TP, et al. *Compounds Useful as Immunomodulators*. WO2015160641A2. Geneva: WIPO (2015).
143. Yeung K-S, Grant-Young KA, Zhu J, Saulnier MG, Frennesson DB, Meng Z, et al. *1,3-Dihydroxy-Phenyl Derivatives Useful as Immunomodulators*. WO2018009505A1. Geneva: WIPO (2018).
144. Yeung K-S, Connolly TP, Frennesson DB, Grant-Young KA, Hewawasam P, Langley DR, et al. *Compounds Useful as Immunomodulators*. WO2017066227A1. Geneva: WIPO (2017).
145. Yeung K-S, Grant-Young KA, Zhu J, Saulnier MG, Frennesson DB, Langley DR, et al. *Biaryl Compounds Useful as Immunomodulators*. WO2018044963A1. Geneva: WIPO (2018).
146. Wang M. *Symmetric or Semi-Symmetric Compounds Useful as Immunomodulators*. WO2018026971A1. Geneva: WIPO (2018).
147. Webber SE, Almassy RJ. *Immune Checkpoint Inhibitors, Compositions and Methods Thereof*. WO2018045142A1. Geneva: WIPO (2018).
148. Lange C, Malathong V, Mcmurtrie DJ, Punna S, Singh R, Yang J, et al. *Immunomodulator Compounds*. WO2018005374A1. Geneva: WIPO (2018).
149. Feng Z, Chen X, Zhang L, Yang Y, Lai F, Ji M, et al. *Bromo Benzyl Ether Derivative, Preparation Method Therefor, and Pharmaceutical Composition and Uses Thereof*. WO2017202275A1. Geneva: WIPO (2017).
150. Feng Z, Chen X, Yang Y, Lai F, Ji M, Zhang L, et al. *Benzyl Phenyl Ether Derivative, Preparation Method Therefor, and Pharmaceutical Composition and Uses Thereof*. WO2017202273A1. Geneva: WIPO (2017).
151. Feng Z, Chen X, Yang Y, Zhou C, Lai F, Jin X, et al. *Phenylate Derivative, Preparation Method Therefor, and Pharmaceutical Composition and Uses Thereof*. WO2017202276A1. Geneva: WIPO (2017).
152. Wang Y, Xu Z, Wu T, He M, Zhang N. *Aromatic Acetylene or Aromatic Ethylene Compound, Intermediate, Preparation Method, Pharmaceutical Composition and Use Thereof*. WO2018006795A1. Geneva: WIPO (2018).
153. Sasikumar PN, Ramachandra M, Naremaddepalli SS. *1,3,4-Oxadiazole and Thiadiazole Compounds as Immunomodulators*. WO2016142852A1. Geneva: WIPO (2016).
154. Sasikumar PN, Ramachandra M, Prasad A, Naremaddepalli SS. *3-Substituted 1,3,4-Oxadiazole and Thiadiazole Compounds as Immunomodulators*. WO2016142894A1. Geneva: WIPO (2016).
155. Sasikumar PN, Ramachandra M, Naremaddepalli SS. *1,3,4-Oxadiazole and 1,3,4-Thiadiazole Derivatives as Immunomodulators*. WO2015033301A1. Geneva: WIPO (2015).
156. Sasikumar PN, Ramachandra M, Naremaddepalli SS. *1,2,4-Oxadiazole Derivatives as Immunomodulators*. WO2015033299A1. Geneva: WIPO (2015).

157. Sasikumar PN, Ramachandra M, Prasad A, Naremaddepalli SS. *3-Substituted-1,2,4-Oxadiazole and Thiadiazole Compounds as Immunomodulators*. WO2016142886A2. Geneva: WIPO (2016).
158. Sasikumar PN, Ramachandra M, Naremaddepalli SS. *1,2,4-Oxadiazole and Thiadiazole Compounds as Immunomodulators*. WO2016142833A1. Geneva: WIPO (2016).
159. Sasikumar PN, Ramachandra M, Prasad A, Naremaddepalli SS. *Cyclic Substituted-1,3,4-Oxadiazole and Thiadiazole Compounds as Immunomodulators*. WO2018051255A1. Geneva: WIPO (2018).
160. Sasikumar PN, Prasad A, Naremaddepalli SS, Ramachandra M. *Cyclic Substituted-1,2,4-Oxadiazole Compounds as Immunomodulators*. WO2018051254A1. Geneva: WIPO (2018).
161. Lu L, Qian D, Wu L, Yao W. *Heterocyclic Compounds as Immunomodulators*. WO2017205464A1. Geneva: WIPO (2017).
162. Wu L, Shen B, Li J, Li Z, Liu K, Zhang F. *Heterocyclic Compounds as Immunomodulators*. US20170107216A1. Alexandria, VA: USPTO (2017).
163. Wu L, Shen B, Li J, Li Z, Liu K, Zhang F, et al. *Heterocyclic Compounds as Immunomodulators*. WO2017070089A1. Geneva: WIPO (2017).
164. Wu L, Yu Z, Zhang F, Yao W. *N-Phenyl-Pyridine-2-Carboxamide Derivatives and Their Use as pd-1/pd-l1 Protein/Protein Interaction Modulators*. WO2017106634A1. Geneva: WIPO (2017).
165. Lajkiewicz N, Wu L, Yao W. *Heterocyclic Compounds as Immunomodulators*. US20170174679A1. Alexandria, VA: USPTO (2017).
166. Wu L, Zhang F, Mei S, Yao W. *Heterocyclic Compounds as Immunomodulators*. US20180057486A1. Alexandria, VA: USPTO (2018).
167. Yu Z, Wu L, Yao W. *Heterocyclic Compounds as Immunomodulators*. WO2018013789A1. Geneva: WIPO (2018).
168. Xiao K, Zhang F, Wu L, Yao W. *Heterocyclic Compounds as Immunomodulators*. US20170362253A1. Alexandria, VA: USPTO (2017).
169. Li Z, Wu L, Yao W. *Heterocyclic Compounds as Immunomodulators*. WO2017192961A1. Geneva: WIPO (2017).
170. Alexander D. *Inhibitors of the pd-1/pd-l1 Protein/Protein Interaction*. WO2017118762A1. Geneva: WIPO (2017).
171. Sasikumar PN, Ramachandra M, Vadlamani SK, Shrimali KR, Subbarao K. *Therapeutic Compounds for Immunomodulation*. US9096642B2. Alexandria, VA: USPTO (2014).
172. Sasikumar PN, Ramachandra M, Naremaddepalli SS. *Immunomodulating Peptidomimetic Derivatives*. WO2015036927A1. Geneva: WIPO (2015).
173. Sasikumar PN, Ramachandra M, Naremaddepalli SS. *Therapeutic Immunomodulating Compounds*. WO2015044900A1. Geneva: WIPO (2015).
174. Sasikumar PN, Ramachandra M. *Immunomodulating Cyclic Compounds*. US9422339B2. Alexandria, VA: USPTO (2016).
175. Sasikumar PN, Ramachandra M, Naremaddepalli SS. *Cyclic Peptidomimetic Compounds as Immunomodulators*. WO2015033303A1. Geneva: WIPO (2015).
176. Sasikumar PN, Ramachandra M, Naremaddepalli SS. *Therapeutic Cyclic Compounds as Immunomodulators*. WO2016142835A1. Geneva: WIPO (2016).
177. Miller MM, Mapelli C, Allen MP, Bowsher MS, Boy KM, Gillis EP, et al. *Macrocyclic Inhibitors of the pd-1/pd-l1 and cd80(b7-1)/pd-l1 Protein/Protein Interactions*. US9308236B2. Alexandria, VA: USPTO (2016).
178. Miller MM, Allen MP, Bowsher MS, Boy KM, Gillis EP, Langley DR, et al. *Macrocyclic Inhibitors of the PD-1/PD-L1 and CD80(B7-1)/PD-L1 Protein/Protein Interactions*. US9879046B2. Alexandria, VA: USPTO (2018).
179. Miller MM, Mapelli C, Allen MP, Bowsher MS, Gillis EP, Langley DR, et al. *Macrocyclic Inhibitors of the pd-1/pd-l1 and cd80 (b7-1)/pd-li Protein/Protein Interactions*. WO2016039749A1. Geneva: WIPO (2016).
180. Miller MM, Allen MP, Li L, Bowsher MS, Gillis EP, Mull E, et al. *Macrocyclic Inhibitors of the pd-1/pd-l1 and cd80/pd-l1 Protein/Protein Interactions*. WO2017176608A1. Geneva: WIPO (2017).
181. Gillman KW, Goodrich J, Boy KM, Zhang Y, Mapelli C, Poss MA, et al. *Macrocyclic Peptides Useful as Immunomodulators*. WO2016077518A1. Geneva: WIPO (2016).
182. Sun L, Zhao Q, Gillis EP, Miller MM, Allen MP, Mull E, et al. *Immunomodulators*. WO2016100608A1. Geneva: WIPO (2016).
183. Allen MP, Gillis EP, Langley DR, Miller MM, Mull E, Sun L, et al. *Immunomodulators*. US20170252432A1. Alexandria, VA: USPTO (2017).
184. Miller MM, Allen MP, Li L, Mapelli C, Poirier MA, Sun L, et al. *Immunomodulators*. WO2016126646A1. Geneva: WIPO (2016).
185. Lee JJ, Powderly JD, Patel MR, Brody J, Hamilton EP, Infante JR, et al. Phase 1 trial of CA-170, a novel oral small molecule dual inhibitor of immune checkpoints PD-1 and VISTA, in patients (pts) with advanced solid tumor or lymphomas. *J Clin Oncol.* (2017) 35:3099. doi: 10.1200/JCO.2017.35.15_suppl.TPS3099
186. Radhakrishnan VS, Bakhshi S, Prabhash K, Deshmukh C, Nag S, Lakshmaiah K, et al. Phase 2 trial of CA-170, a novel oral small molecule dual inhibitor of immune checkpoints VISTA and PD-1, in patients (pts) with advanced solid tumor and Hodgkin lymphoma. In: *Proceedings of 33rd Annual Meeting & Pre-Conference Programs of the Society for Immunotherapy of Cancer (SITC 2018)*, Washington, DC (2018).
187. Vilalta Colomer M, Punna S, Li S, Malathong V, Lange C, McMurtrie D, et al. A small molecule human PD-1/PD-L1 inhibitor promotes T cell immune activation and reduces tumor growth in a preclinical model. In: *Proceedings of ESMO Immuno-Oncology Congress 2018,* Geneva. (2018).
188. Horn L, Spigel DR, Vokes EE, Holgado E, Ready N, Steins M, et al. Nivolumab versus docetaxel in previously treated patients with advanced non-small-cell lung cancer: two-year outcomes from two randomized, open-label, phase III trials (checkMate 017 and CheckMate 057). *J Clin Oncol.* (2017) 35:3924–33. doi: 10.1200/JCO.2017.74.3062
189. Tomita Y, Fukasawa S, Shinohara N, Kitamura H, Oya M, Eto M, et al. Nivolumab versus everolimus in advanced renal cell carcinoma: Japanese subgroup analysis from the CheckMate 025 study. *Jpn J Clin Oncol.* (2017) 47:639–46. doi: 10.1093/jjco/hyx049
190. Hellmann MD, Ciuleanu TE, Pluzanski A, Lee JS, Otterson GA, Audigier-Valette C, et al. Nivolumab plus ipilimumab in lung cancer with a high tumor mutational burden. *N Engl J Med.* (2018) 378:2093–104. doi: 10.1056/NEJMoa1801946
191. Yue C, Jiang Y, Li P, Wang Y, Xue J, Li N, et al. Dynamic change of PD-L1 expression on circulating tumor cells in advanced solid tumor patients undergoing PD-1 blockade therapy. *Oncoimmunology.* (2018) 7:e1438111. doi: 10.1080/2162402X.2018.1438111
192. Wei R, Guo L, Wang Q, Miao J, Kwok HF, Lin Y. Targeting PD-L1 protein: translation, modification and transport. *Curr Protein Pept Sci.* (2019) 20:82–91. doi: 10.2174/1389203719666180928105632
193. Wang Q, Lin W, Tang X, Li S, Guo L, Lin Y, et al. The roles of microRNAs in regulating the expression of PD-1/PD-L1 immune checkpoint. *Int J Mol Sci.* (2017) 18:2540. doi: 10.3390/ijms18122540
194. Guo L, Lin Y, Kwok HF. The function and regulation of PD-L1 in immunotherapy. *ADMET & DMPK.* (2017) 5:159–72. doi: 10.5599/admet.5.3.442

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frontiers in Oncology

CASE REPORT
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Somatostatin Receptors in Merkel-Cell Carcinoma: A Therapeutic Opportunity Using Somatostatin Analog Alone or in Association With Checkpoint Inhibitors Immunotherapy. A Case Report

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Background: Merkel-cell carcinoma (MCC) is a rare, highly aggressive skin cancer typically involving elderly people. Surgery is usually the first treatment for primary tumor. In adjuvant setting, radiotherapy is effective in reducing local recurrence and in improving overall survival. Regarding advanced disease, systemic chemotherapy ended up disappointing results whereas antiPD1/antiPD-L1 immunotherapy recently gave relevant clinical benefits. Interestingly, about the half of MCC patients expresses high somatostatin receptors (SRs) to possibly represent a target for the therapeutic use of somatostatin analogs (SSAs). Nevertheless, SSAs have been little studied in MCC and cases treated with SSAs in association with checkpoint inhibitor immunotherapy have not been published yet.

Case Report: We report the case of a 73-year-old man affected by metastatic MCC of right arm previously treated with surgery and adjuvant radio and chemotherapy. Three years later the patient presented loco-regional relapse involving lateral-cervical, mediastinal, and submandibular lymph nodes with high value of chromogranin A and neuron specific enolase. Due to the high expression of SRs at octreoscan and immunoistochemistry, patient started octreotide 30 mg i.m. every 28 days with a good control of disease for about 2 years. A widespread progression of disease was reported afterwards. The patient started the antiPD-L1 avelumab immunotherapy, only recently available in Italy, while still taking SSA. The patient showed an impressive regression of the disease after only four cycles of avelumab until complete remission.

Conclusions: SSA could be a valid therapeutic option in patients with MCC with high SR expression. When combined with PD-1/PD-L1 immune-checkpoint inhibition, SSA is likely to enhance antiproliferative activity. Our case report provides the rationale to conduct a prospective trial and translational research to verify the efficacy and safety of combined SSA and checkpoint inhibitors for advanced MCC.

Keywords: immunothearpy, somatostatin analog, Merkel cell carcinoma (MCC), Merkel carcinoma, somatostatin—receptor

INTRODUCTION

Merkel-cell carcinoma (MCC) is a rare but highly aggressive skin cancer typically involving elderly people, although it has been also described in young adult and exceptionally in childhood (1).

Factors involved in the pathogenesis of MCC included age over 65 years, ultraviolet radiation exposure, immunosuppression, and infection by Merkel cell polyomavirus (MCPV) which is detected in almost 80% of MCC cases. Other primary cancers seem to increase the risk of MCC incidence, especially prior multiple myeloma, chronic lymphocytic leukemia, and malignant melanoma (2). Ultraviolet light exposure has been reported to induce an extremely high genome mutation rate, whereas MCPV-infected Merkel carcinoma cells show low rates of genome mutation (3).

MCC typically tends to grow locally, but soon it spreads to the locoregional lymph nodes and than through the blood circulation to distant organs, particularly to liver, lung, brain, and to bone (4).

Therapeutic management of MCC is controversial. Early diagnosis and adequate treatment of primary MCC are important prognostic factors. Surgery and radiotherapy are usually chosen in localized forms. Adjuvant radiotherapy showed effective in reducing the local recurrence and in increasing the overall survival (5).

Systemic chemotherapy has been used to treat advanced disease with disappointing results. First-line chemotherapy with platinum-based regimens produced high response rates of about 50–60%. The main therapeutic regimens included cis-platinum or carboplatin in association with etoposide or ifosfamide or anthracyclines. CAV regimen (cyclophosphamide + Adriamycin

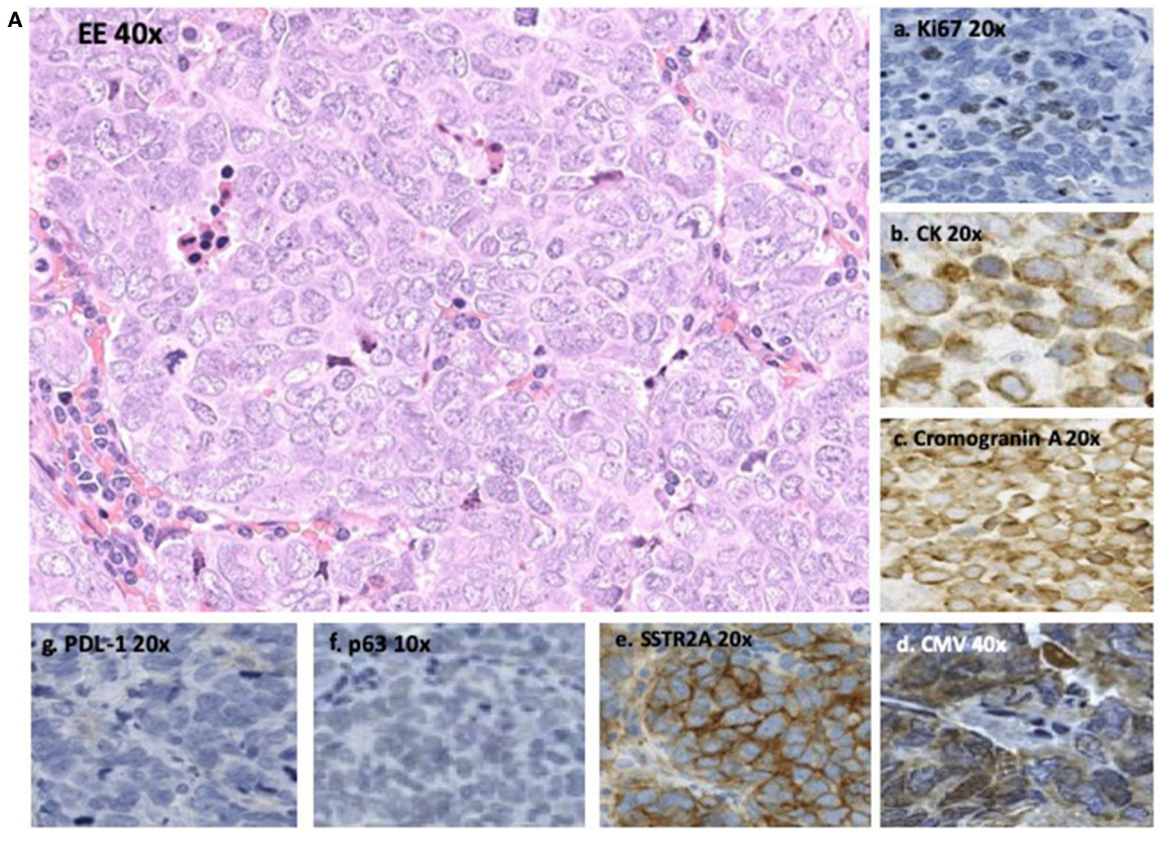

FIGURE 1 | Continued

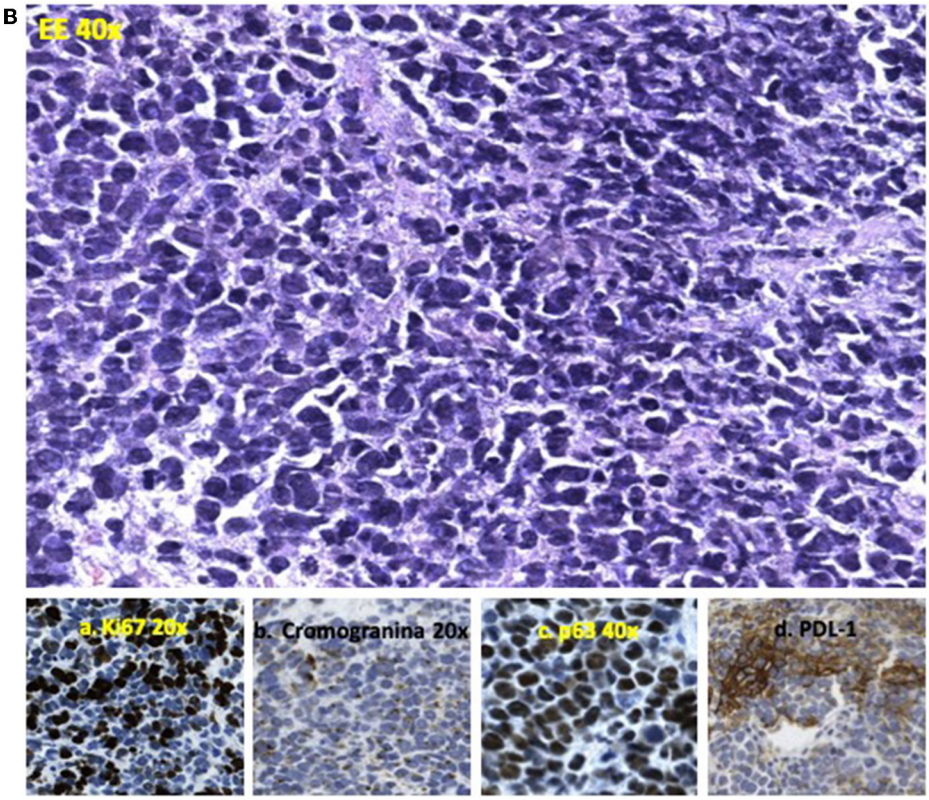

FIGURE 1 | (A) Sample of lymph node metastasis performed before SSA therapy showing an epithelioid feature of cells with "salt and pepper" nuclei (EE40x). Immunohistochemistry showed (a) positivity for Ki67 in 25% of cells (20×); (b) positivity for CK20 with nuclear dot (20×); (c) positivity for chromogranin A with nuclear dot (20×); (d) nuclear positivity for poliomavirus Merkel cells carcinoma associated/CMV (40×); (e) diffuse and strong membranous positivity for somatostatin receptor 2A (SSTR2A) (20×); (f) negativity for p63 (10×); and (g) for PDL-1 (20×). **(B)** Sample of lymph node metastasis performed after disease progression to SSA therapy showing cells having smaller size than those of pre-treatment and a round shape with dark nuclei (40×). Immunohistochemistry showed (a) Ki67 positivity in 85% of cells (20×); (b) reduction of positivity for chromogranin (20×); (c) diffuse nuclear positivity for p63 (40×); (d) strong positivity for PDL-1 in 35% of neoplastic cells (10×).

+ Vincristine) was used in patients unfit for platinum-based regimens. Unfortunately, response duration was only a few months with PFS of 3–4 months. Moreover, treatment was associated to significant toxicity (6, 7).

Checkpoints inhibitors antiPD1/antiPD-L1 (Programmed Death Ligand1) immunotherapy have been recently investigated in the metastatic setting and positive results were reported (8–10). The antiPD-L1 avelumab was first tested in a multicentre phase 2 trial involving 88 patients with stage IV chemotherapy-refractory MCC. The response rate was 31.8%, including eight complete responses and 20 partial responses (8). Based on these results, the U.S. Food and Drug Administration granted accelerated approval of the antiPD-L1 avelumab to treat adults and children above 12 years with metastatic MCC.

Other two antiPD-1 antibodies have also been investigated in advanced MCC. Pembrolizumab was tested as first-line treatment in advanced MCC (9) whereas nivolumab was proposed as neoadjuvant therapy in patients with resectable MCC (10). In both studies, an objective response rate over 50% was reported. Of note, responses were observed in both patients with virus-positive tumors and those with virus-negative tumors (9–11).

Due to these new therapeutic options, chemotherapy is now indicated just for patients who are not candidates for immunotherapy or after immunotherapy failure.

About half of MCC expresses highly somatostatin receptors (SRs) that could represent a potential target for both imaging and treatment purposes (12). Somatostatin analogs (SSAs) have been used in the past with palliative intent for functioning neuro-endocrine tumors and remarkable results were reported. More recently, direct anti-proliferative effects of SSAs have also been demonstrated in neuroendocrine neoplasms (13). The use of SSA in MCC has been little studied (14) and cases of MCC treated with SSA in combination with checkpoint inhibitor immunotherapy have not been published yet.

We report for the first time the case of a metastatic MCC successfully treated first with SSA and then, when disease progressed, with SSA plus anti PD-L1 avelumab.

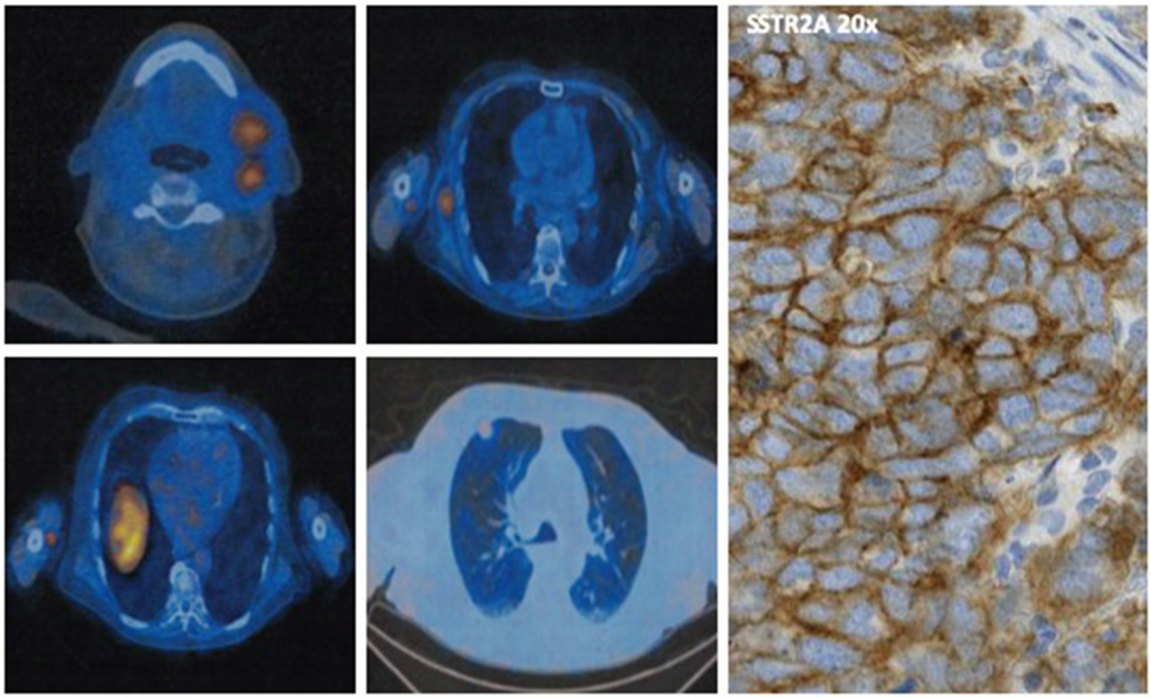

FIGURE 2 | Octreoscan showing high uptake of 99mTc-EDDA/HYNIC-TOC in the right axillary and thoracic wall, in the left sub-mandibular and latero-cervical region, and at right superior pulmonary lobe in the sub-pleurical region. On the right is shown the membranous diffuse expression of SSTR2A on immunohistochemistry.

CASE REPORT

A 73-year-old man affected by metastatic MCC on the right arm treated with surgery and adjuvant radio and chemotherapy came to our observation in December 2015. At the diagnosis, immunohistochemistry reported a Ki67 of 25% and a positivity for synaptophysin, chromogranin A, CK20, nuclear polyomavirus marker, and SSTR2A, whereas SSTR5A, PD-L1, and p63 (a transcription factor often associated with worse prognosis) were negative (**Figure 1A**). The tumor infiltrating lymphocytes (TILs) in the tumor microenvironment were moderate as was the infiltration of intra-tumor lymphocytes.

Three years later the patient presented relapse involving loco-regional lymph nodes and the right lung with high value of chromogranin A (CgA) and neuron specific enolase (NSE).

Due to the high expression of SRs at octreoscan with 99mTc-EDDA/HYNIC-TOC (**Figure 2**) and at immunohistochemistry (**Figure 1A**), the patient started the SSA octreotide at the dose of 30 mg intramuscularly every 28 days obtaining a progressive decrease of serum markers and a partial regression of disease lasting over 2 years. After this long period, the patient presented disease progression in all known sites and new lesions at lung middle lobe and left adrenal gland. At this time, circulating CgA remained under normal value whereas NSE increased rapidly (**Figure 4**). A new lymph node biopsy was performed revealing a profound changing in the morphology of the neoplastic cells. Immunohistochemistry also showed a Ki67 of 85% compared to 25% of the first biopsy, whereas p63 and PD-L1 became strongly positive (**Figure 1B**). At this time, peritumoral TILs were very scarce and completely absent the intra-tumor lymphocytes component.

Considering the disease progression and the recent availability in Italy of the antiPD-L1 avelumab, the patient started this drug at the dose of 10 mg/kg intravenously every 2 weeks continuing octreotide at the same dose and timing. After four administrations of avelumab, an impressive response was observed until achieving a complete remission (**Figures 3A,B**). Also circulating NSE level progressively decreased until normal value (**Figure 4**).

Peripheral lymphocytes remained quite under normal value during all SSA therapy period without significant variations. Nevertheless, when avelumab was started, a sudden and rapid increase just after the first administration of avelumab was noted reaching the maximum value at the second month of therapy. Then, lymphocytes progressively decreased until pre-treatment value (**Figure 4**).

Treatment was well tolerated by the patient with only mild hyposthenia reported.

At present, after 17 months of therapy, the patient is in good clinical status with complete remission of disease. He is continuing avelumab in combination with octreotide without remarkable side effects.

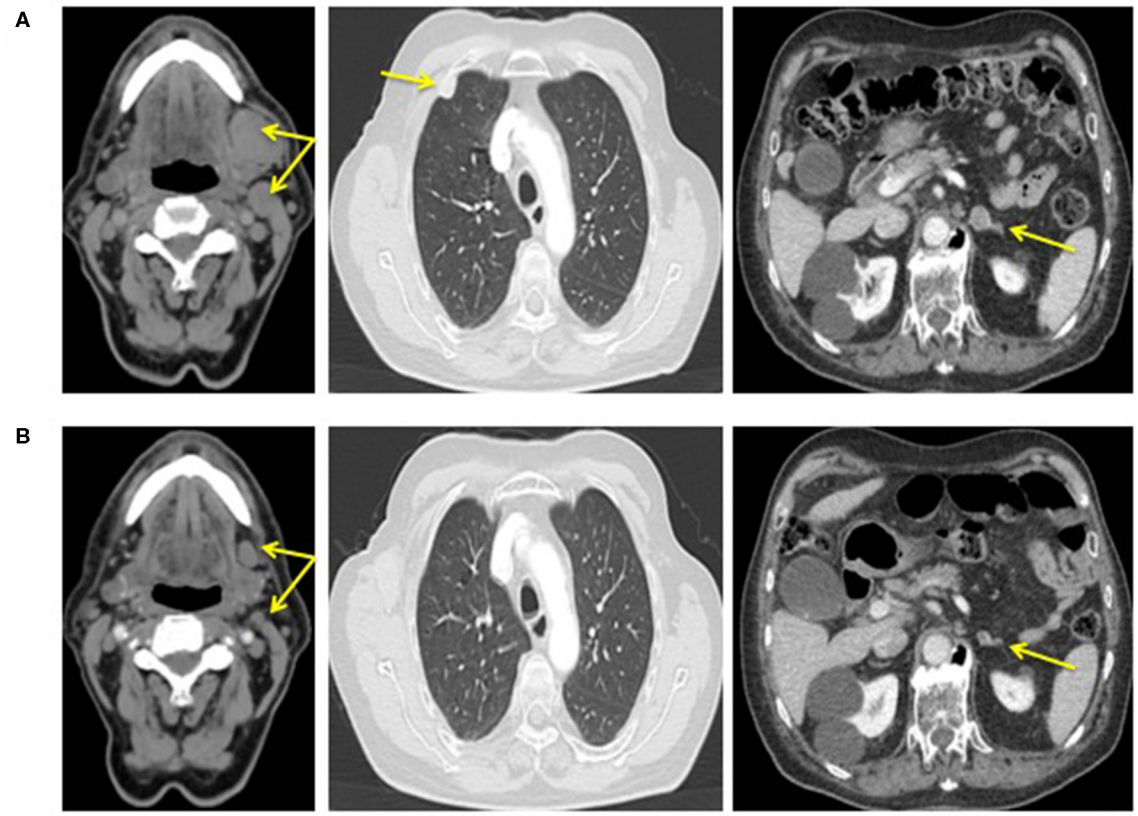

FIGURE 3 | CT scan performed before therapy with the association octreotide plus avelumab **(A)** and after 10 months of therapy **(B)**.

DISCUSSION

Avelumab was the first-ever drug approved in advanced MCC after showing meaningful efficacy. In the second line setting after chemotherapy, the response rate reaches 33% with responses occurring on average after about 6 weeks of therapy. Early objective response to avelumab was associated with significant improved overall survival. Moreover, in patients who responded, about two-third remained relapse free after 2 years with half of patients and one-third of patients still alive at 1 and 2 years, respectively. When used in a first-line setting, PD-1/PD-L1 inhibitors including avelumab, pembrolizumab, and nivolumab are even more promising as objective responses were observed in ∼50–70% of patients with a 2-year survival rate of about 70%. Due to these results, PD-1/PD-L1 inhibitors are considered the standard of care in advanced MCC and are currently being investigated in the adjuvant and neoadjuvant settings (9–11).

Somatostatin is a hormone with multiple actions such as endocrine, paracrine, and antiproliferative. The direct antiproliferative effects of somatostatin are mediated by its high affinity for somatostatin specific receptors (SRs) present on neuroendocrine neoplasms (15–17). Five sub-types of specific membrane receptors have been found in a lot of normal tissues as brain and leptomeningeal tissue, anterior pituitary gland, endocrine and exocrine pancreas, gastrointestinal mucosa, immune system cells (16, 17). Of note is that each type of tumor shows different sub-types of SRs and the amplitude of the antiproliferative activity of somatostatin analogs (SSAs) seems to correlate with the number of receptors present on the cellular surface (16, 17).

SSTR2A and 5 have been recently reported to be expressed in MCC in about 60 and 45%, respectively (12). Overall, at least one SR was expressed in 76.5% of cases and no association was found either with the severity of the disease, nor with clinical features, proliferative index of Ki 67, relapse-free survival, and overall survival. SRs expression between the primary skin tumor and the corresponding metastases was consistent in 43 and 86% for SRs 2A and SRs 5, respectively. The expression of SSTR2A but not SRs 5 was finally associated with MCPyV status (12).

Considering the expression of these receptors, this tumor may be candidate for SSAs therapy. To our knowledge, the use of SSAs in MCC has been little investigated with only three cases published in English literature and disappointing results were reported (14). No cases treated with SSAs plus checkpoints inhibitors have been published until now.

We obtained a good control of the disease for about 2 years using octreotide alone. Of note, when the patient presented a widespread progression of the disease, a radical change in terms of cell morphology and higher proliferative index were noted, whereas p63 and PD-L1 became strongly positive (**Figure 1B**).

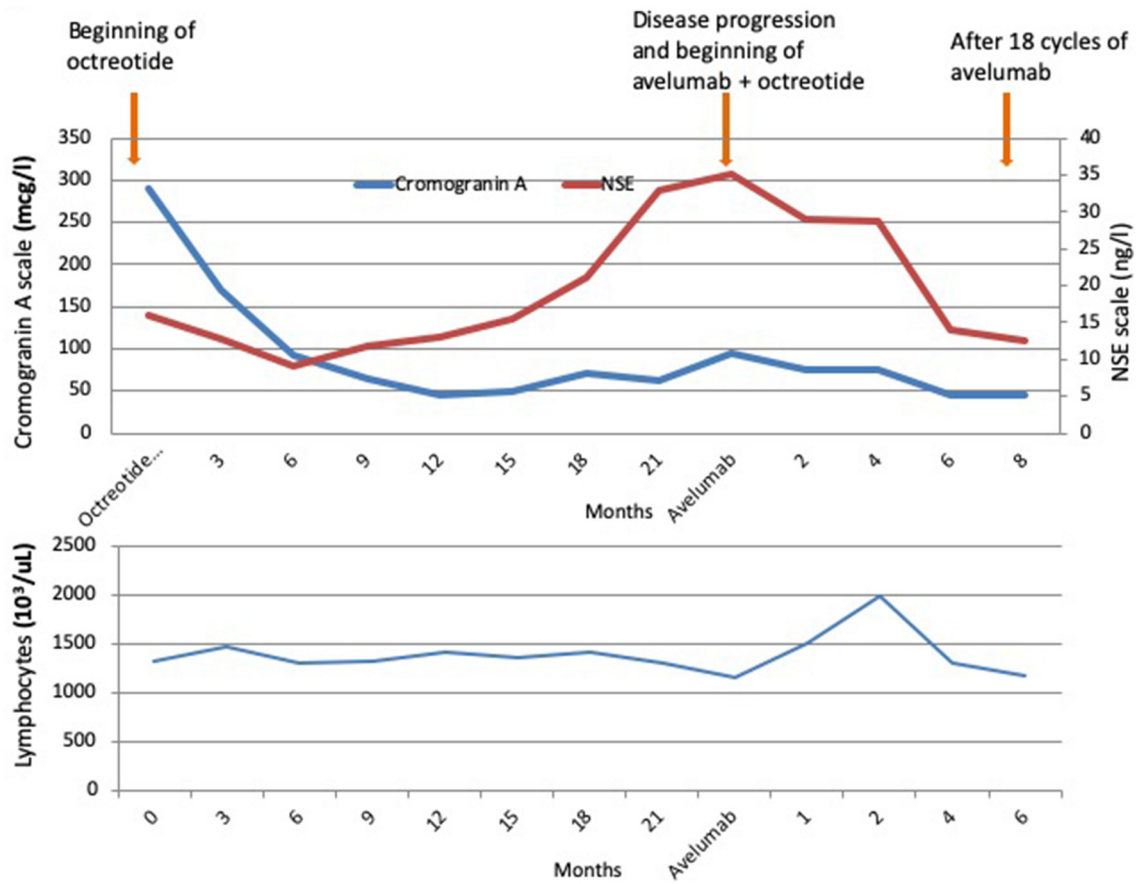

FIGURE 4 | Behavior of chromogranin A (CgA), Neuron-Specific Enolase (NSE), and peripheral lymphocytes count during octreotide and then during the association of octreotide plus avelumab.

The behaviors of tumor markers also confirmed the biological switch of disease with CgA remaining within normal values and NSE slowly increased (**Figure 4**).

We obtained an impressive regression of the disease with avelumab in association with octreotide until complete remission lasting over 10 months without relevant side effects with only mild hyposthenia reported by our patient. Data from literature regarding the toxic profile of avelumab reported that this drug is associated with a very good toxicity profile. No treatment-related grade 4 adverse events or treatment-related deaths were reported. Moreover, grade 3 treatment-related adverse events occurred in only 6% of patients with lymphopenia, blood creatine phosphokinase increase, aminotransferase increase, and blood cholesterol increase being more common side effects. Finally, serious treatment-related adverse events included enterocolitis, infusion-related reaction, aminotransferases increased, chondrocalcinosis, synovitis, and interstitial nephritis. All side effects were effectively addressed with prompt recognition and appropriate management (8).

Interestingly, peripheral lymphocytes which showed no significant variation during SSA therapy, increased significantly after the first administrations of avelumab with the maximum value at the second month of therapy and then progressively decreased.

Although considerable therapeutic progress has been made in the MCC with the arrival of checkpoint inhibitors, about 50% of patients with advanced disease do not respond to immunotherapy. Unfortunately, no clear predictors of response are available yet. Regarding PD-L1, its prognostic value is controversial as well as its predictive role of response to checkpoint inhibitors. Although more remarkable responses have been reported in cancer overexpressing PD-L1 levels regardless of histology, as the case of our patient, even patients with low PD-L1 levels can have an important clinical benefit with immunotherapy. Our patient showed high expression of PD-L1 on tumoral cells before starting therapy with avelumab. Therefore, using PD-L1 as predictive factor is questionable for its weak and unreliable capability to discriminate responder from no-responder patients. Thus, PD-L1 could constitute one of the partners when associated with other finer and more specific parameters such as mutational load, tumor neoantigen burden, cytotoxic activity, and IFN gamma signature. It is urgent to identify new predictive factors of response to immunotherapy. It is thought that better understanding of

tumor microenvironment and use of combined biomarkers are necessary to better identify patients who will benefit from PD-1/PD-L1 checkpoint blockade therapy. Moreover, new immunotherapeutic strategies are now being investigated both alone or in combinations to enhance the immune antitumoral response against (18, 19).

Our case demonstrates that SSA represents a valid therapeutic option in MCC patients expressing high SRs levels. When combined with check point inhibitors immunotherapy, SSA can safely enhance the anti-proliferative activity of immunotherapy with final strengthen results. Thus, it provides a rationale to conduct a prospective trial with an adequately powered sample to test the efficacy and safety of this combination and to optimize the schedule and timing of the two drugs. Furthermore, translational research is also recommended to better characterize the potential immune activation properties and the synergistic activity of SSA and to verify the predictive value of SRs to response to this therapeutic combination.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

Written informed consent was obtained from the patient for the publication of this case report.

AUTHOR CONTRIBUTIONS

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

REFERENCES

1. Heath M, Jaimes N, Lemos B, Mostaghimi A, Wang LC, Peñas PF, et al. Clinical characteristics of Merkel cell carcinoma at diagnosis in 195 patients: the AEIOU features. *J Am Acad Dermatol.* (2008) 58:375–81. doi: 10.1016/j.jaad.2007.11.020
2. Howard RA, Dores GM, Curtis RE, Anderson WF, Travis LB. Merkel cell carcinoma and multiple primary cancers. *Cancer Epidemiol Biomarkers Prev.* (2006) 15:1545–9. doi: 10.1158/1055-9965.EPI-05-0895
3. Wong SQ, Waldeck K, Vergara IA, Schröder J, Madore J, Wilmott JS, et al. UV-associated mutations underlie the etiology of MCV-negative Merkel cell carcinomas. *Cancer Res.* (2015) 75:5228–34. doi: 10.1158/0008-5472.CAN-15-1877
4. Emge DA, Cardones AR. Updates on Merkel cell carcinoma. *Dermatol Clin.* (2019) 37:489–503. doi: 10.1016/j.det.2019.06.002
5. Hasan S, Liu L, Triplet J, Li Z, Mansur D. The role of postoperative radiation and chemoradiation in merkel cell carcinoma: a systematic review of the literature. *Front Oncol.* (2013) 3:276. doi: 10.3389/fonc.2013.00276
6. Tello TL, Coggshall K, Yom SS, Yu SS. Merkel cell carcinoma: an update and review: current and future therapy. *J Am Acad Dermatol.* (2018) 78:445–54. doi: 10.1016/j.jaad.2017.12.004
7. Nghiem P, Kaufman HL, Bharmal M, Mahnke L, Phatak H, Becker JC. Systematic literature review of efficacy, safety and tolerability outcomes of chemotherapy regimens in patients with metastatic Merkel cell carcinoma. *Future Oncol.* (2017) 13:1263–79. doi: 10.2217/fon-2017-0072
8. Kaufman HL, Russell J, Hamid O, Bhatia S, Terheyden P, D'Angelo SP, et al. Avelumab in patients with chemotherapy-refractory metastatic Merkel cell carcinoma: a multicentre, single-group, open-label, phase 2 trial. *Lancet Oncol.* (2016) 17:1374–85. doi: 10.1016/S1470-2045(16)30364-3
9. Nghiem P, Bhatia S, Lipson EJ, Sharfman WH, Kudchadkar RR, Brohl AS, et al. Durable tumor regression and overall survival in patients with advanced Merkel Cell Carcinoma receiving pembrolizumab as First-Line Therapy. *J Clin Oncol.* (2019) 37:693–702. doi: 10.1200/JCO.18.01896
10. Topalian SL, Bhatia S, Kudchadkar RR. Nivolumab as neoadjuvant therapy in patients with resectable Merkel cell carcinoma in CheckMate 358 [Abstract]. *J Clin Oncol.* (2018) 36:9505. doi: 10.1200/JCO.2018.36.15_suppl.9505
11. Samimi M. Immune checkpoint inhibitors and beyond: an overview of immune-based therapies in Merkel cell carcinoma. *Am J Clin Dermat.* (2019) 20:(3):391–407. doi: 10.1007/s40257-019-00427-9
12. Gardair C, Samimi M, Touzé A, Coursaget P, Lorette G, Caille A, et al. Somatostatin receptors 2A and 5 are expressed in Merkel Cell Carcinoma with no association with disease severity. *Neuroendocrinology.* (2015) 101:223–35. doi: 10.1159/000381062
13. Rinke A, Wittenberg M, Schade-Brittinger C, Aminossadati B, Ronicke E, Gress TM, et al. Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors (PROMID): results of long-term survival. *Neuroendocrinology.* (2017) 104:26–32. doi: 10.1159/000443612
14. Slovacek L. Merckel cell carcinoma of the skin treated with somatostatin analogue and mTOR inhibitor exhausted after primary surgery, adjuvant radiotherapy and palliative chemotherapy. *Bratisl Lek Listy.* (2014) 115:663–5. doi: 10.4149/BLL_2014_128
15. Lamberts SW, Van der Lely AJ, de Herder WW, Hofland LJ. Octreotide. *NEJM.* (1996) 334:246–54. doi: 10.1056/NEJM199601253340408
16. Culler MD, Oberg K, Arnold R, Krenning EP, Sevilla I, Díaz JA. Somatostatin analogs for the treatment of neuroendocrine tumors. *Cancer Metastasis Rev.* (2011) 30(Suppl 1):9–17. doi: 10.1007/s10555-011-9293-0
17. Hofland LJ, van Koetsveld PM, Wouters N, Waaijers M, Reubi JC, Lamberts SW. Dissociation of antiproliferative and antihormonal effects of the somatostatin analog octreotide on 7315b pituitary tumor cells. *Endocrinology.* (1992) 131:571–7. doi: 10.1210/endo.131.2.1322274
18. Wang X, Teng F, Kong L, Yu J. PD-L1 expression in human cancers and its association with clinical outcomes. *Onco Targets Ther.* (2016) 9:5023–39. doi: 10.2147/OTT.S105862
19. Tétu P, Baroudjian B, Madelaine I, Delyon J, Lebbé C. Update in treatment for Merkel cell carcinoma and clinical practice guide. *Bull Cancer.* (2019) 106:64–72. doi: 10.1016/j.bulcan.2018.11.009

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Re-education of the Tumor Microenvironment With Targeted Therapies and Immunotherapies

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The clinical success of cancer immunotherapies targeting PD-1 and CTLA-4 has ignited a substantial research effort to improve our understanding of tumor immunity. Recent studies have revealed that the immune contexture of a tumor influences therapeutic response and survival benefit for cancer patients. Identifying treatment modalities that limit immunosuppression, relieve T cell exhaustion, and potentiate effector functions in the tumor microenvironment (TME) is of much interest. In particular, combinatorial therapeutic approaches that re-educate the TME by limiting the accumulation of immunosuppressive immune cells, such as Foxp3 regulatory T cells (Tregs) and tumor-associated macrophages (TAMs), while promoting $CD8^+$ and $CD4^+$ effector T cell activity is critical. Here, we review key approaches to target these immunosuppressive immune cell subsets and signaling molecules and define the impact of these changes to the tumor milieu. We will highlight the preclinical and clinical evidence for their ability to improve anti-tumor immune responses as well as strategies and challenges for their implementation. Together, this review will provide understanding of therapeutic approaches to efficiently shape the TME and reinvigorate the immune response against cancer.

Keywords: tumor-associated myeloid cells, regulatory T cells (Tregs), natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, adenosine, transforming growth factor (TGF)β, prostaglandin, immune toxicity

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INTRODUCTION

The clinical validation of key conceptual developments in the field of tumor immunology has engendered much interest in strategies to initiate immune cell function within the tumor microenvironment (TME). Central to effective anti-tumor immunity induced by cancer immunotherapies is the ability to re-educate and re-activate immune effector and cytotoxic T cells to eliminate cancer cells. As such, immunotherapies targeting T cell immune checkpoint receptors cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and/or programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) have ascended to first-line therapies for a number of solid malignancies (1). Combinatorial anti-tumor efficacy of ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1) in advanced stage melanoma and renal cell carcinoma (RCC) highlights

the importance of targeting multiple immune pathways to unleash a more robust anti-tumor immune response (2, 3). In addition, FDA-approval of pembrolizumab (anti-PD-1) for the treatment of microsatellite instability-hi (MSI-h) and deficient DNA mismatch repair (dMMR) tumors, the first cancer-site agnostic treatment approval, as well as the correlation between tumor mutational burden and survival outcome sheds light on the significance of tumor genetics in initiating an immune response (4–6). Similarly, PD-L1 status has been shown to impact therapeutic outcome to PD-1/PD-L1 targeting immunotherapies (7). This highlights that a better understanding of the immune contexture and its interaction with surrounding tumor, stroma, and their derivatives (e.g., chemokines and other soluble factors) is crucial to developing novel therapeutic targets to efficiently shape and re-condition the TME, reinvigorating the immune response against cancer.

Functional anti-tumor immunity relies on both the quality (effector and cytotoxic function) and quantity (numbers and localization) of tumor-infiltrating lymphocytes (TILs) in the TME. Targeting CTLA-4 and PD-1 non-redundantly mobilizes and activates alternate T cell components, with CTLA-4 shown to inhibit priming and generation of antigen-specific T cells in the lymph nodes whereas PD-1 limits $CD8^+$ T cell numbers in the tissue, for superior anti-tumor outcomes (8, 9). The concept of targeting two or more non-redundant immune regulatory pathways for enhanced anti-tumor immunity is not limited to adaptive immunity. A combination of anti-DR5, anti-CD40, and anti-CD137 agonistic antibodies aiming to induce apoptosis in tumor cells, activate antigen presenting cells (innate immunity), and co-stimulate $CD8^+$ T cells (adaptive immunity), respectively, has been shown to eradicate both established transplantable and spontaneous tumors (10). Similarly, it has been shown that a combination of recombinant interleukin (IL)-2, anti-PD-1, a tumor-antigen targeting antibody, and an additional vaccine targeting three individual tumor antigens is able to eradicate a poorly immunogenic murine melanoma, via the activation of both innate and adaptive immunity (11). Here, we review key approaches to target pathways alternative to mainstream T cell checkpoint receptors to re-educate the TME and alleviate immune suppression and highlight challenges for therapy selection and implementation in the clinic.

Abbreviations: A2AR, A2A adenosine receptor; APCs, antigen presenting cells; CSF1, colony stimulating factor 1; CSF1R, colony stimulating factor 1 receptor; COX, cyclooxygenase; CTLA-4, cytotoxic T-lymphocyte associated protein 4; dMMR, deficient DNA mismatch repair; EZH2, enhancer of zeste homolog 2; FLT3L, FMS-like tyrosine kinase 3 ligand; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; irAEs, immune-related adverse events; IL, interleukin; MAIT, mucosal-associated invariant T; MSI-h, microsatellite instability-hi; NKT, natural killer T; NRP1, neuropilin-1; NSCLC, non-small cell lung cancer; PDAC, pancreatic ductal adenocarcinoma; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cells; PTSG2, prostaglandin-endoperoxide synthase 2; RCC, renal cell carcinoma; Tregs, regulatory T cells; TAMs, tumor-associated macrophages; TGF, transforming growth factor; TILs, tumor-infiltrating lymphocytes; TME, tumor microenvironment; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

IMPROVING TUMOR CONTROL WITH MYELOID CELLS IN THE TME

Myeloid cells predominate the TME and in many cases evolve to display an immunosuppressive phenotype and ineffective antigen presenting cells (APCs) due to the inflammatory milieu (**Figure 1**). Tumor-associated macrophages (TAMs) are innate immune cells of heterogeneous origins that have been shown to accumulate in the TME as tumors progress (12–14). The presence of immunosuppressive TAMs can interfere with T cell-mediated anti-tumor immune responses (15, 16). Given the absence of a universal definition for TAMs, we have listed relevant markers used in individual studies. It has been reported that an accumulation of monocyte-derived TAMs ($CD11b^{lo}$ MHC-$II^{-/lo}$) positively correlates with the proportion of tumor-infiltrating exhausted PD-1^+ $CD8^+$ T cells in a mouse model of mammary cancer (12), illustrating a potential mechanism by which TAMs promote tumor escape by modulating the $CD8^+$ T cell response. More recently, it has been shown that TAMs ($CD11b^+$ MHC-II^+) are capable of stripping anti-PD-1 bound to PD-1^+ T cells by binding to the antibody Fc domain, abrogating the anti-tumor activity of this immune checkpoint inhibitor (17). It remains unclear if a similar resistance mechanism also exists in the context of anti-PD-L1 therapy. However, in preclinical mouse models Fc engagement is critical for anti-PD-L1 (clone 10F.9G2) therapeutic efficacy by enabling depletion of immunosuppressive TAMs ($CD11b^+$ F4/80^+) (18). Therefore, defining the functionality of an antibodies Fc region for optimal therapeutic activity in the context of both T cells and myeloid cells is an important consideration. Additionally, modulating TAMs via targeted depletion, inhibiting active migration, and promoting activation and differentiation, as a means to re-educate the TME may increase permissiveness to immune checkpoint inhibitor therapy.

The colony stimulating factor 1 (CSF1)/CSF1 receptor (CSF1R) axis is crucial for TAM differentiation (19). Selective depletion of TAMs by targeting CSF1R using monoclonal antibodies or small molecule inhibitors has been shown to restrict $CSF1R^+$ TAM accumulation in the TME, leading to reduced tumor growth in a number of mouse models (20–23). Depletion of $CSF1R^+$ TAMs demonstrated efficacy in improving a wide range of existing cancer therapies, including chemotherapy, oncogene-targeted therapy, and immunotherapy (21, 23, 24). In preclinical BRAF-mutant melanoma, co-administration of PLX3397 (CSF1R inhibitor) together with PLX4720 (mutant BRAF inhibitor) effectively sensitizes a PD-1-resistant tumor model to anti-PD-1/PD-L1 therapies (23). However, CSF1R inhibition induces the expansion of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC) that may abrogate the efficacy of combination CSF1R inhibition and anti-PD-1 treatment (23, 25). Evidence that targeting CSF1R and CXCR2 signaling to inhibit TAM and PMN-MDSC expansion, respectively, alongside anti-PD-1 facilitates improved anti-tumor immune responses than either doublet combination (25). Alternatively, targeting the CCL2/CCR2 axis, a key chemokine pathway involved in macrophage migration to inflammatory sites, to limit their entry into the TME

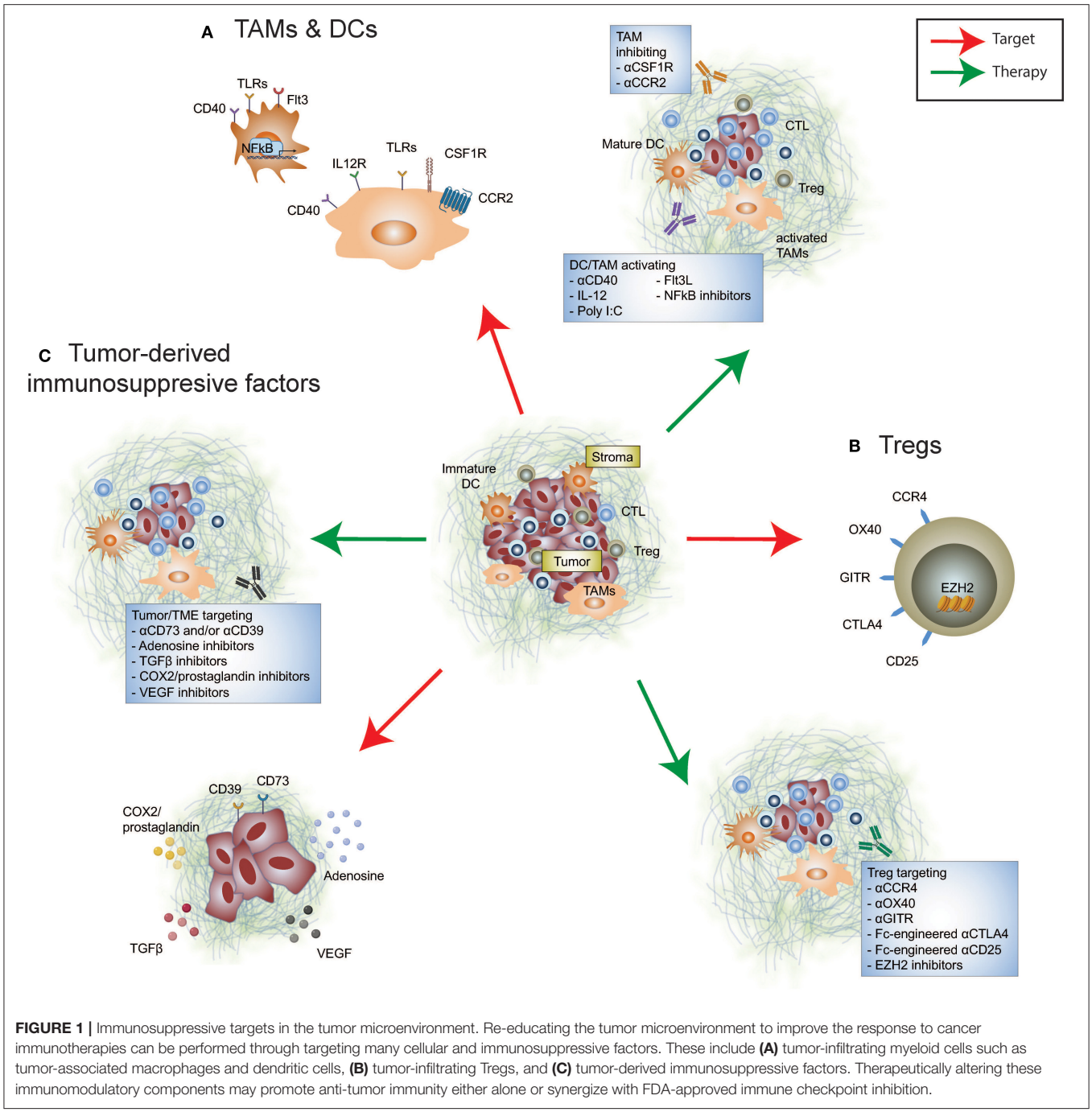

FIGURE 1 | Immunosuppressive targets in the tumor microenvironment. Re-educating the tumor microenvironment to improve the response to cancer immunotherapies can be performed through targeting many cellular and immunosuppressive factors. These include **(A)** tumor-infiltrating myeloid cells such as tumor-associated macrophages and dendritic cells, **(B)** tumor-infiltrating Tregs, and **(C)** tumor-derived immunosuppressive factors. Therapeutically altering these immunomodulatory components may promote anti-tumor immunity either alone or synergize with FDA-approved immune checkpoint inhibition.

enabled numerical and functional improvement of intratumoral lymphocyte infiltrate (26–29). Wu et al. demonstrated improved survival outcomes in cutaneous T-cell lymphoma-bearing mice treated with a CCR2 inhibitor and anti-PD-1 (28). Collectively, these studies highlight that inhibition of pro-tumor TAMs in the TME reinvigorates the anti-PD-1-driven T cell response. Of note, a phase I/II clinical trial accessing the combinatorial effect of nivolumab, GVAX (a cancer vaccine expressing GM-CSF) and BMS-813160 (a CCR2/CCR5 dual antagonist) in pancreatic ductal adenocarcinoma (PDAC) is currently underway (NCT03767582).

It is worth highlighting that not all tumor-infiltrating myeloid cells promote tumor growth. The production of CXCL9 and CXCL10, predominantly by TAMs ($CD11b^{+}$ $Ly6C^{int}$ $CD11c^{+}$ $F4/80^{+}$), enhances $CD8^{+}$ T cell infiltration and tumor control in response to combination anti-PD-1 and anti-CTLA-4 in a mouse model of mammary adenocarcinoma (30). High production of these chemokines within the TME is associated with better

survival outcomes in melanoma patients receiving combination treatment (30). In light of these findings, reprogramming these innate immune cell subsets may be beneficial due to their antigen presenting properties promoting infiltration of an effective anti-tumor T cell response. In KPC (LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre) PDAC TAMs (CD11b^{+} Gr1^{-} F4/80^{+}) enable T cell exclusion and consequently, resistance to immune checkpoint therapy is likely driven by the absence of effector T cells that can be modulated (31, 32). Independent studies of mouse pancreatic models have demonstrated the remodeling activity of the agonistic CD40 antibody by overcoming T cell exclusion in the TME, leading to improved therapeutic response to anti-CTLA-4, and/or anti-PD-1 (33–35). Using a T cell-rich but anti-PD-1 resistant mammary carcinoma model, we have recently demonstrated that IL-12 induced by an agonistic CD40 antibody could render terminally exhausted PD-1^{hi} tumor-infiltrating CD8^{+} T cells into their PD-1^{int} progenitor state (36), leading to improved anti-tumor immunity in response to anti-PD-1 following sensitization by anti-CD40 agonism.

An additional therapeutic approach to re-educate the TME and bolster the efficacy of immune checkpoint therapy is combination treatment with FMS-like tyrosine kinase 3 ligand (FLT3L) and poly I:C treatment, to expand and enhance maturation of anti-tumor CD103^{+} dendritic cells (DCs) resulting in a dramatic increase of intratumoral T cells (37). Notably, T cell-activating IL-12 producing CD103^{+} DCs diminish over time (37–41), suggesting that they may facilitate tumor control during tumor initiation. Beavis et al. also reported a role for anti-PD-1/CTLA-4 activated CD4^{+} Foxp3^{-} cells in enhancing IL-12 production by CD103^{+} DCs, which in turn promoted T cell-mediated anti-tumor immunity in mice (42). The persistence of intratumoral stimulatory DCs (CD103^{+} BDCA-3^{+}) defined by gene expression profiles corelated with improved overall survival outcomes and was associated with higher TIL measurements in metastatic melanoma (43). Mediating the abundance of intratumoral stimulatory DCs was the presence of tumor-infiltrating natural killer cells and expression of FLT3L, together these components may assist in determining anti-PD-1 therapeutic response and identify therapeutic strategies to potentiate efficacy (43). More recently, a cluster of DCs named mregDCs (mature DCs enriched in immunomodulatory molecules) co-expressing immunoregulatory genes (*Cd274*, *Pdcd1lg2*, and *Cd200*) and maturation genes (*Cd40*, *Ccr7*, and *Il12b*) was found in single cell analysis of mouse and human non-small cell lung cancer (NSCLC) DC infiltrate (44). Of note, neutralizing IL-4 was shown to enhance mregDC IL-12 production, repressing lung adenocarcinoma in mice (44). With advances in high-throughput single-cell analysis to provide fine-detail of immune infiltrate in tumors, it is likely to facilitate an expansion of our repertoire of novel targets that will assist to re-educate DCs and other myeloid cells specifically in the TME.

RE-EDUCATING SUPPRESSIVE AND UNCONVENTIONAL T CELLS IN THE TME

Regulatory T cells (Tregs) serve as a barrier to limit inflammation, however, their enrichment in the TME of established cancer correlates with poor prognosis and a dampened anti-tumor immune response (**Figure 1**). Clinical studies have resolved that a higher effector/Treg ratio is associated with favorable outcomes in multiple solid cancers (45, 46). In addition to Treg-induced suppression of effector T cells by manipulating their migration, activation, functionality and/or survival (47, 48), Tregs are able to form an immunosuppressive barrier capable of limiting the trafficking of activated antigen-specific CD8^{+} T cells into the TME (49). Importantly, Foxp3^{+} Tregs promote effector CD4^{+} and CD8^{+} TIL dysfunction, with improved cytokine-producing capacity upon Treg depletion and reinvigorated T cell responses to immune checkpoint blockade (50–52). However, systemic Treg depletion introduced transiently can still increase susceptibility of mice to autoimmunity (53), indicative that identifying an appropriate target to specifically remove intratumoral Foxp3^{+} Tregs will be advantageous for maintaining therapeutic safety.

Intratumoral Foxp3^{+} Tregs are highly suppressive, with an activated phenotype marked by the expression of several classes of immune receptors [ENTPD1 (CD39), CTLA-4, OX40, and GITR], and chemokine receptors (CCR4). Studies using preclinical mouse models showed that anti-CTLA-4 (clone 9H10, 9D9, and H11; antagonist), anti-OX40 (clone OX86; agonist) and anti-GITR (clone DTA-1; agonist) exhibited varying levels of intratumoral Treg depleting activity *in vivo* that was critical to their efficacy (54–59). Anti-CTLA-4 (clone 9D9, mouse IgG2a) and anti-OX40 were shown to specifically deplete intratumoral Tregs but not peripheral Tregs (56, 57). Tumor-infiltrating Treg depletion by anti-CTLA-4 enhanced anti-PD-1 sensitivity to the previously resistant AT3 mouse mammary carcinoma (36). In the clinic, mogamulizumab (an anti-human CCR4 antibody, engineered for ADCC activity) was developed to specifically deplete CCR4^{+} suppressive Tregs found in the TME (60), and is undergoing testing in combination with T cell checkpoint targets in Phase I/II clinical trials (NCT02301130, NCT02705105, NCT02476123, and NCT02946671). While it remains unclear whether combining anti-CCR4 and anti-PD-1 provides favorable survival benefits, an increase in the proportion of CD8^{+} T cells and a reduction in activated Foxp3^{hi} Tregs was observed in TILs from patients, along with an acceptable safety profile [(61); NCT02476123]. With advances in antibody engineering, we should expect refinement of antibodies for both existing and novel targets to modulate TME-specific Tregs to enhance anti-tumor immunity (51, 62, 63). Revisiting targeting CD25-expressing Tregs, Vargas et al. found that by altering the IgG backbone (from rat IgG1 to mouse IgG2a) greater specificity was afforded toward intratumoral Treg depleting activity by an Fc-optimized version of CD25 antibody (64). Anti-CD25-mediated intratumoral Treg depletion synergized with PD-1 blockade therapy in a number of mouse cancer models (64), highlighting the importance of remodeling the Treg dynamics within the TME to enhance checkpoint blockade therapy. Translation of this combination needs to be thoroughly examined, to limit the depletion of alternate CD25-expressing cell types including effector T cells and NK cells.

Given the critical role of Tregs in maintaining immune homeostasis, attenuating intratumoral Treg suppressive function may be a safer approach to remodel the TME while minimizing the risk of systemic autoimmunity. Studies from a series of experimental modeling showed that the disruption of Foxp3, the critical transcription factor to maintain Treg lineage, altered their suppressor function (65–67). This also resulted in the generation of pathogenic effector T cells (67, 68). However, disruption of intratumoural Treg suppressive function has been shown without the loss of its Foxp3$^+$ Treg identity. Neuropilin-1 (NRP1) appears crucial to maintain intratumoural Treg stability without aberrant loss of Foxp3 identity, and anti-NRP1 displayed therapeutic efficacy in suppressing tumor growth (69). Notably, using a co-transfer model of NRP1-intact and NRP1-deficient Tregs, interferon (IFN)-γ produced by NRP1-deficient Tregs is capable of causing fragility to the suppressive capacity of NRP1-intact Tregs, resulting in improved host anti-tumor immunity (70). Similar to the role of NRP1 to maintain Treg stability, the histone H3K27 methyltransferase enhancer of zeste homolog 2 (EZH2) has been recently shown to be critical for the maintenance of activated Foxp3$^+$ Tregs (71). EZH2 inhibition destabilizes Foxp3 expression and inhibits tumor growth *in vivo* (72, 73). While targeting these pathways may be able to provide an opportunity to dismantle Treg suppression within the TME, these therapies still lack specificity to this cell type. Understanding the role of these molecules in multiple cell types and disease settings is likely to dictate their applicability for utility in cancer immunity.

Besides Tregs, unconventional T cells have also received considerable interest in tumor immunology for their immunoregulatory role. In contrast to CD8$^+$ and CD4$^+$ T cells that interact with MHC class I and II molecules, unconventional T cells such as natural killer T (NKT) cells interact with non-classical MHC CD1d molecules (74). α-GalCer (a glycoplipid molecule derived from a marine sponge extract) is a known ligand for NKT cells, and has been widely used to experimentally modulate NKT cells (75). α-GalCer-activated NKT cells are capable of producing high levels of cytokines (including IFN-γ and IL-21), anti-tumor effector and cytotoxic molecules (perforin and granzymes), and elicit direct tumor lysing properties (76–78), which assists to alleviate immunosuppression and enhances DC maturation, leading to improved anti-tumor T cell immunity (79–81). Song et al. demonstrated that NKT cells specifically kill monocytes pulsed with neuroblastoma cell lysate and reduce tumor-infiltrating monocytes in a non-classical MHC-dependent manner (82), highlighting a role for NKT cells in shaping the immune infiltrate in the TME. Studies in mice reported superior anti-tumor activity when α-GalCer therapy to drive NKT cell activity was combined with anti-PD-1 (83, 84). However, most clinical trials assessing the anti-tumor effect of α-GalCer-related compounds have not yet yielded promising outcomes (74). Discoveries of novel NKT cell agonists (β-mannosylceramide) and improved α-GalCer analogs (α-C-GalCer) (74, 85), as well as greater understanding of tumors where this cell type is prominent may assist in harnessing the potential of NKT cells to improve T cell checkpoint therapy.

Mucosal-associated invariant T (MAIT) cells are another class of unconventional T cell that have gained much attention, given their relative abundance in humans and their association to a number of inflammatory diseases (74). MAIT cells primarily recognize a number of microbial vitamin B metabolites (such as riboflavin metabolized to 5-OP-RU) (86–89) presented by the unconventional non-polymorphic MHC I-like molecule, MR1 (90). Additional MR1-independent IL-12/18-induced activation has been reported (91, 92). Upon activation, MAIT cells are capable of producing cytokines [(IL-17, IL-2, IFN-γ, and tumor necrosis factor (TNF)], proliferate and gain cytotoxic function (93–95). In the absence of defined tumor antigens binding to tumor-derived MR1, it is reasonable to speculate that MAIT cells may be regulated by microbial antigens and may be more frequent in tumors with a microbial presence. Circulating levels of MAIT cells were reduced in patients with mucosal-originated cancers (gastric, colon, and lung), but appeared normal in patients with breast, liver, or thyroid cancer (96). In colon cancer patients, MAIT cells were shown to be preferentially enriched in the TME in comparison to unaffected tissue (96–99). Poor survival prognosis has been associated to increased levels of tumor-infiltrating MAIT cells in colon cancer patients (98). In contrast, MAIT cells did not show a correlation to patient survival in esophageal adenocarcinoma (100). In concordance with the activation of MAIT cells (TCR-MR1 or IL-12/18 cytokine), they likely elicit direct (MAIT cell to tumor cell) and indirect (MAIT cell to non-MAIT cell or IL-12/18 cytokine competition) effects, regulating host anti-tumor immunity in a TME-specific manner (74, 101). Yan et al. recently reported MR1-deficient mice (which lack MAIT cells) showed improved anti-tumor immunity when assessed using models of experimental lung metastasis, subcutaneous tumor growth, and *de novo* carcinogenesis (102). In light of these findings, a further assessment of MAIT cells in the cancer setting and their relationship to prognosis and therapeutic outcome should be determined. In addition, given a great interest in microbial modification of the TME (103–105) determining whether microbes can be used to initiate metabolic functions that promote anti-tumor immunity is also of interest.

LIMITING IMMUNOSUPPRESSIVE FACTORS IN THE TME

As well as initiation of immunosuppression by immune cell subsets, the tumor itself produces a range of molecules to enable tumor progression and facilitate immune escape (**Figure 1**). Many of these are soluble factors that prevent overzealous inflammation during tissue damage and infection, however also mediate tumor immune evasion. Transforming growth factor (TGF)β plays an essential role in mediating immune homeostasis, however, in the context of tumor, TGFβ has been shown to both directly promote tumor progression and initiate a broad range of immune responses. These include enhancing suppressive myeloid cell infiltrate (106, 107), disabling NK cell function,

and promoting transition to group 1 innate lymphoid cells in the TME (108), as well as altering the functionality of effector T cell populations while promoting Treg immune suppression (109–111). SMAD-3, which acts downstream of TGFβ1 signaling, directly induces PD-1 expression in adoptively transferred tumor antigen-specific $CD8^+$ T cells isolated from the TME (112). This suggests that PD-1-upregulation that facilitates tumor immune escape may in part be dependent on TGFβ. In addition, TGFβ is associated with excluding $CD8^+$ T cell entry into the tumor core, which is known to diminish immunotherapy efficacy (113). Correspondingly, *TGFβ1* gene expression is significantly higher in patients that show stable or progressive disease, compared to those with complete or partial responses (113). Targeting TGFβ and therapies directed toward PD-1/PD-L1 or CTLA-4 amplifies tumor control by enabling a robust T cell response with improved $CD4^+$ and $CD8^+$ T cell activation and $CD8^+$ T cell cytokine and cell killing capacity (113–116). By inhibiting TGFβ in combination with PD-1/PD-L1 blockade, $CD8^+$ T cells also display enhanced capacity to infiltrate the tumor periphery and core, promoting T cell inflammation, and resolving T cell exclusion (113, 117). This highlights that targeting TGFβ may be most effective in tumor types in which TGFβ signaling mediates immune exclusion from the TME.

Impeding the clinical utility of pan-TGFβ inhibitors (blocking isoforms TGFβ1,−2, and−3) is the potential for significant toxicity, particularly pertaining to cardiac function. Development of galunisertib (LY2157299), a TGFβRI inhibitor, has shown promise for both its anti-tumor activity and ability to modulate the TME to provide improved anti-tumor control to immunotherapies (118). However, due to toxicity concerns, intermittent administration of galunisertib has been performed in clinical trials [(119) NCT01246986]. Whether intermittent drug exposure provides selective pressure for tumor escape is unclear. As TGFβ1 appears to be the predominantly enriched in human cancers, targeting this isoform specifically may prove advantageous. Development of TGFβ1-specific inhibitors that promote synergistic anti-tumor immune responses when combined with anti-PD-1, but lack cardiovascular pathologies have been identified and may lead to greater clinical utility (120). Downstream targets of tumor-derived TGFβ activation may also be more desirable and in some cases are already being assessed for therapeutic potential alongside immune checkpoint inhibitors and other immunomodulatory compounds in the clinic.

Angiogenesis in the TME is an important component to enable nutrient accessibility and maintain tumor growth, this is driven in part by TGFβ induction of vascular endothelial growth factor (VEGF). While VEGF inhibitors have been approved for a number of indications in both oncology and vascular-related diseases, in the cancer setting interest in both anti-angiogenic and immunomodulatory properties for this target are increasing. Notably, VEGF-A promotes inhibitory checkpoint expression and transcriptional reprogramming relating to exhaustion in $CD8^+$ T cells (121, 122). TOX, the transcription factor that mediates $CD8^+$ T cell exhaustion, was shown to be tightly regulated by VEGF-A (122). In addition, both VEGF-A and TOX expression levels were significantly reduced in MSI-h colon cancer patients compared to patients with microsatellite stability (122). MSI-h patients have better survival outcomes in response to cancer immunotherapies, which is predominantly attributed to higher tumor mutational burden (123, 124), but may also be in part be due to reduced angiogenic factors and relieved T cell exhaustion. By combining VEGF-targeted therapies and anti-PD-1, improved anti-tumor immune response was achieved (121, 122). However, TGFβ and VEGF are not completely redundant, and co-targeting these molecules together either therapeutically or through tumor-specific genetic ablation provides additional therapeutic benefit to overcome immune tolerance in the TME (125). As clinical cohorts involving combination VEGF/TGFβ and immune checkpoint blockade treatment mature, determining which patients respond to this therapy, but also which patients are refractory and the mechanism that initiates tumor escape is of importance.

Generation of immunosuppressive adenosine limits anti-tumor immunity (126). Both CD39, which catabolizes ATP to AMP, and CD73, the enzyme that generates adenosine from ATP, are expressed by and relate to poor prognosis in a number of cancer types (127–130). Regulation of tumor-derived CD73 remains complex with multiple mediators identified, including TGFβ (131, 132). Additional evidence that CD73 expression is driven by adenosine-sensing through host-A2A adenosine receptor (A2AR) expression (133, 134), TNF (135), and hypoxia within the TME (136, 137). In melanoma, CD73 levels have been linked to low MITF expression and highly invasive tumors (135). CD73 expression appears to increase with adaptive resistance in anti-PD-1-treated melanoma patients as well as MART-1 adoptive T cell therapy (135), suggesting that tumor expression may facilitate therapeutic resistance in response to active anti-tumor immunity. In melanoma patients with innate therapeutic resistance, CD73 is not present or induced with exposure to anti-PD-1 treatment (135), likely due to a lack of inflammatory stimuli in the TME. Therapies targeting the adenosinergic pathway have been shown preclinically to potentiate the response of chemotherapies (127), immune checkpoint inhibitors (138, 139), chimeric antigen receptor T cells (140) and oncogenic BRAF inhibitors (141). This can also be through indirectly targeting the adenosine pathway, with both systemic oxygenation which relieves hypoxia-driven adenosine production, and blockade of an alternate mechanism of adenosine-production by inhibiting CD38, with both shown to potentiate the therapeutic efficacy of immune checkpoint blockade (137, 142, 143). Similarly, targeting upstream CD39 has been shown preclinically to promote therapeutic activity of immune checkpoint inhibitors (144), chemotherapies (145), and even can potentiate CD73 blockade in suboptimal concentrations (146). This highlights the complex regulatory network and the multi-faceted combination strategies involving adenosine-related molecules that may add benefit to patient care.

Targeting adenosine production and signaling have both moved forward to early phase clinical trials with promising results (NCT02403193, NCT02503774, NCT03454451). Notably, citforadenant (an A2AR inhibitor) in combination with atezolizumab (anti-PD-L1) initiated therapeutic response in

both patients naïve to immunotherapies and those refractory to prior immunotherapy exposure, highlighting the potential for this combination to reinvigorate anti-tumor immunity (147). Analysis of tumor biopsies from renal cell carcinoma (RCC) preceding therapeutic intervention revealed an adenosine signature that may predict patients who will benefit from adenosine-related therapies (147). The adenosine signature was consistent with myeloid inflammation and reduced angiogenesis, both of which have been defined as poor prognostically for atezolizumab and sunitinib (tyrosine kinase inhibitor) treatment. This identifies a patient group for which adenosine may be most applicable, that inadvertently are less responsive to current clinically approved therapies for RCC. In addition, an extended disease control rate in response to citforadenant, with or without atezolizumab, was linked to improved $CD8^+$ T cell infiltration (147). Adenosine has previously been shown to limit the proliferation and maturation of lymphocytic immune cell subsets (126, 134), and increased immune infiltrate in to the tumor core has been observed in response to co-targeting CD73 and A2AR in preclinical models (133). Understanding the regulation of the adenosinergic pathway in particular tumor types and in response to cancer therapies, including immunotherapy, may identify patient populations where adenosine-related therapies may be implemented with greatest success.

With increasing examination of the TME it is clear that a number of therapeutic regimens may be successfully repurposed in the treatment of cancer. For instance, targeting adenosine has been utilized previously in the setting of neurodegeneration, but has increasingly shown merit for initiating anti-tumor immunity. Additionally, aspirin may provide a combinatorial approach to overcome therapeutic resistance to immune checkpoint inhibitors. Increasing evidence demonstrates that cyclooxygenase (COX)-driven production of prostaglandins mediates anti-PD-1 resistance and limits the proinflammatory tumor milieu (148). COX enzymatic activity is disrupted by high-dose aspirin, which valuably may be repurposed to the cancer setting alongside immunotherapies to promote anti-tumor immunity. Regular aspirin users with colorectal cancer patients displaying low tumor PD-L1 expression are also afforded significantly improved survival outcomes (149). This survival advantage was not identified in PD-L1 high tumors, suggesting that engagement of the PD-1/PD-L1 axis in the TME may abrogate aspirin-mediated anti-tumor benefit and the potential utility of combination treatment. Genetic ablation of prostaglandin-endoperoxide synthase 2 (*PTSG2)*, which encodes COX-2, has been shown to promote $CD8^+$ T cells and decrease the frequency of Tregs within the TME (150), both of which are predictive markers of good prognosis. Induction of COX-2 may be in part regulated by TGFβ, highlighting the complex nature of direct and indirect regulatory pathways that the tumor elicits to subdue the anti-tumor immune response. Clinical trials to develop an understanding of prostaglandin/COX-2 inhibition and immune checkpoint blockade therapeutic responses are underway in multiple tumor types [(151) NCT03396952, NCT03638297, NCT03864575, NCT03926338].

IMPLEMENTING COMBINATION THERAPEUTIC REGIMENS

While preclinical studies have identified a number of clinically relevant therapeutic strategies to reinvigorate the immune response against cancer, their successful clinical utility has been difficult to implement (**Figure 2**). Combining indoleamine 2,3-dioxygenase (IDO) inhibition, an enzyme upregulated in human cancers that initiates the breakdown of tryptophan leading to multi-faceted immunosuppression within the TME (152), alongside immune checkpoint inhibitors showed promise for enhancing anti-tumor immunity in mice (153, 154). However, in a phase 3 clinical trial assessing the survival benefit for stage III/IV unresectable melanoma patients treated with epacadostat (selective IDO1 inhibitor) and pembrolizumab (anti-PD-1), this combination failed to provide additive therapeutic potential compared to pembrolizumab alone (155). Improved understanding of the TME is necessary to assist rational selection of immunotherapies required for optimal treatment outcomes. Of course, this remains a challenge even for clinically approved agents, where aside from tumor PD-L1 expression and genetic stablility of the tumor, no biomarkers for efficacy or toxicity of immune checkpoint inhibitors are approved for clinical use. Significant investment to establish biomarkers to denote responders and non-responders should be performed in early phase clinical trials to identify subgroups for which combination therapies may show greatest activity, an important first-step to facilitate response, mitigate toxicity, and minimize unnecessary cost.

In the same vein, examining optimal timing for immunotherapeutic combinations is often not well-defined. In most cases, therapeutic benefit for novel clinical agents are tested either alone or alongside concurrent anti-PD-1/PD-L1 treatment, often in cancer patients refractory to previous immune checkpoint inhibitor exposure. Preclinical evidence suggests synchronous administration of multiple immunotherapies can in some circumstances be detrimental. Two independent studies identified that concurrent administration of anti-OX40 to anti-PD-1 therapy either with or without a tumor vaccine diminished the anti-tumor immune response compared to addition of anti-OX40 alone in preclinical mouse models (156, 157). Notably, staggering the timing of these therapies where anti-OX40 preceded anti-PD-1 treatment facilitated greatest tumor control (156). Using a preclinical PDAC model, the use of gemcitabine and nab-paclitaxel, a standard chemotherapy combination for PDAC, impaired the efficacy of anti-CD40, anti-PD-1, and anti-CTLA-4 (34). Additionally, transient treatments preceding immune checkpoint inhibition can also significantly re-educate the immune response to promote anti-tumor immunity. In an anti-PD-1-resistant mouse model, a single-dose of agonistic anti-CD40 sensitized the TME to anti-PD-1 treatment in a synergistic manner (36). Strongly activating or agonistic therapies may provide greatest benefit as sensitizing agents to remodel the TME and promote entry of anti-tumor immune cells that are then targetable by immune checkpoint blockade. Alternating timing of treatments or reducing the therapeutic window for

FIGURE 2 | Growing challenges in the implementation of immunotherapeutic strategies. Decision-making for the most efficacious immunotherapeutic combination for cancer patients presents multiple layers of challenges. These include those surrounding the patient's tumor microenvironment, the potential risk for initiation of immunotoxicities, and the sequence for appropriate timing of therapeutic interventions. Surrounding each of these challenges are multidimensional considerations that relate to improving anti-tumor immunity and targets that influence the selection of immunomodulatory agents.

largely inflammatory combinations may also assist to potentiate therapeutic response and minimize immunotherapy-induced immune-related adverse events (irAEs).

CHALLENGES FOR RE-EDUCATING THE TME

With the advent of high-throughput screening to delineate critical components that prevent immune infiltrate or disable active anti-tumor immunity, a growing understanding of rational targets to re-ignite a therapeutic response is becoming increasingly available (158–161). This aims to equip patients who develop adaptive or acquired resistance with greater tools to re-engage the immune response against cancer and for patients with innate resistance to enable visibility of tumors (162, 163). New subgroups of cancer patients that present distinct challenges to the efficacy of immunotherapies are emerging. Of growing interest, is the relationship between metastatic site and therapeutic outcome, in which liver metastasis appears to be a major obstacle even for combination anti-PD-1 and anti-CTLA-4 treatment (164, 165). Most prominently, melanoma patients bearing liver metastasis have reduced CTLA-4 and PD-1 co-expression in $CD8^+$ T cells (164), which has been shown to stratify therapeutic response to immune checkpoint inhibitor treatment (166). Efforts to provide mechanistic insight

as to whether therapeutic resistance is due to myeloid cell dysfunction, Treg suppression, and immunosuppressive factors accumulating in the TME are essential, highlighting the need for tailored immunotherapies.

As the number and type of combination immunotherapies expands, the risk for increasing irAEs may also become more prevalent. Combination nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4) clearly exhibits greater levels of severe grade 3-4 immunotoxicities than either therapy alone (3, 167). Surprising levels of irAEs have been observed with other rational combinations. For instance, targeted therapies (such as BRAF inhibitors and MEK inhibitors) have been shown to potentiate immune checkpoint inhibitor activity in preclinical models (168), but when used together in melanoma patients severe irAEs were observed forcing closure of the study (169, 170). This highlights a need for improved preclinical models that emulate clinical conditions and allow for simultaneous assessment of tumor control and development of irAEs (171). Developing tumor models in autoimmune-prone mice or lowering the threshold for self-tolerance in mice with available syngeneic tumors that are resistant to autoimmune responses may facilitate improved therapeutic modeling (53, 171, 172). With the expanding use of cancer immunotherapies in more diverse populations of cancer patients, including those with pre-existing autoimmune diseases or previous immunotherapy-induced irAEs (173), or under persistent immunosuppression due to chronic viral infections (174) and allogeneic transplantation (175), developing preclinical models that incorporate multiple elements relating to tumor origin, patient history, and environment that assist in providing a more informed understanding of the clinical impact of therapeutic combinations will be essential.

As the number of immunotherapeutic targets expands, initiating smarter multi-modal strategies, to provide greater efficacy with lower toxicity will be appealing. Advancement in engineering therapies to have delayed release or greater tissue and cellular specificity have great promise. As mentioned, depletion of intratumoral Tregs without impacting peripheral Tregs would be advantageous for inducing TME-specific modulation of the $CD8^+$ to Treg ratio, while avoiding toxicity induced by systemic depletion. Notably, the development of a dual variable domain anti-CTLA-4 antibody, which exhibits an outer tumor antigen-binding site that hides the CTLA-4 binding region of the antibody until reaching the TME has been shown to reduce immunotherapy-induced toxicity without impeding anti-tumor immunity (176). Similarly, bispecific or trispecific antibodies that target multiple markers upregulated in the TME on both immune cell subsets and the tumor may also lead to greater efficacy (177, 178). One such example, is the bispecific antibody combination targeting OX40 and CTLA-4, which significantly enhanced $CD8^+$ T cells and reduced Tregs specifically within the TME, leading to better tumor control than either therapy alone (178). In addition to modifying the target antigen, antibodies may also be conjugated to biomaterials or nanoparticles, to ensure sustained, local release (179). Refinement made to antibodies by factoring TME properties, such as pH activation and PD-1 glycosylation (180–182), should also significantly improve the specificity and potency of immunotherapies and limit unwanted toxicity.

FUTURE DIRECTIONS

While determining the optimal immunotherapy for an individual TME remains a challenge, encouraging is the range of available strategies to re-educate the immune response against tumor. Refinement of therapeutic targets against cellular and immunomodulatory molecules within the TME are increasing, and their promise for clinical utility is growing. Importantly, greater effort in defining the therapeutic setting where each may be applicable will be essential for clinical success. Since anti-PD-1/PD-L1 has shown value in multiple modalities, examining tumor types where anti-PD-1 activity is limited may yield greatest therapeutic breakthroughs in identifying strategies to remodel inert immune circumstances in the tumor. In deciding on the use of these therapies, a cost-benefit analysis relating to the purported immunotoxicity and likelihood for the immunotherapy strategy to enhance anti-tumor immunity is necessary. This emphasizes a need for rational and selective combination immunotherapies to be utilized within a defined TME in order to re-educate the anti-tumor immune response.

AUTHOR CONTRIBUTIONS

SN and AY contributed equally to the writing and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Khalil DN, Smith EL, Brentjens RJ, Wolchok JD. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nat Rev Clin Oncol.* (2016) 13:394. doi: 10.1038/nrclinonc.2016.65
2. Hammers HJ, Plimack ER, Infante JR, Rini BI, McDermott DF, Lewis LD, et al. Safety and efficacy of nivolumab in combination with ipilimumab in metastatic renal cell carcinoma: the checkmate 016 study. *J Clin Oncol.* (2017) 35:3851–8. doi: 10.1200/JCO.2016.72.1985
3. Wolchok JD, Chiarion-Sileni V, Gonzalez R, Rutkowski P, Grob JJ, Cowey CL, et al. Overall survival with combined nivolumab and ipilimumab in advanced melanoma. *N Engl J Med.* (2017) 377:1345–56. doi: 10.1056/NEJMoa1709684
4. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Cancer immunology. mutational landscape determines sensitivity to

PD-1 blockade in non-small cell lung cancer. *Science.* (2015) 348:124–8. doi: 10.1126/science.aaa1348
5. Goodman AM, Kato S, Bazhenova L, Patel SP, Frampton GM, Miller V, et al. Tumor mutational burden as an independent predictor of response to immunotherapy in diverse cancers. *Mol Cancer Ther.* (2017) 16:2598–608. doi: 10.1158/1535-7163.MCT-17-0386
6. Yarchoan M, Hopkins A, Jaffee EM. Tumor mutational burden and response rate to PD-1 inhibition. *N Engl J Med.* (2017) 377:2500–1. doi: 10.1056/NEJMc1713444
7. Teng MW, Ngiow SF, Ribas A, Smyth MJ. Classifying cancers based on T-cell infiltration and PD-L1. *Cancer Res.* (2015) 75:2139–45. doi: 10.1158/0008-5472.CAN-15-0255
8. Twyman-Saint Victor C, Rech AJ, Maity A, Rengan R, Pauken KE, Stelekati E, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature.* (2015) 520:373–7. doi: 10.1038/nature14292
9. Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang NAS, Andrews MC, et al. Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. *Cell.* (2017) 170:1120–33 e17. doi: 10.1016/j.cell.2017.07.024
10. Uno T, Takeda K, Kojima Y, Yoshizawa H, Akiba H, Mittler RS, et al. Eradication of established tumors in mice by a combination antibody-based therapy. *Nat Med.* (2006) 12:693–8. doi: 10.1038/nm1405
11. Moynihan KD, Opel CF, Szeto GL, Tzeng A, Zhu EF, Engreitz JM, et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. *Nat Med.* (2016) 22:1402–10. doi: 10.1038/nm.4200
12. Franklin RA, Liao W, Sarkar A, Kim MV, Bivona MR, Liu K, et al. The cellular and molecular origin of tumor-associated macrophages. *Science.* (2014) 344:921–5. doi: 10.1126/science.1252510
13. Zhu Y, Herndon JM, Sojka DK, Kim KW, Knolhoff BL, Zuo C, et al. Tissue-resident macrophages in pancreatic ductal adenocarcinoma originate from embryonic hematopoiesis and promote tumor progression. *Immunity.* (2017) 47:323–38.e6. doi: 10.1016/j.immuni.2017.07.014
14. Gubin MM, Esaulova E, Ward JP, Malkova ON, Runci D, Wong P, et al. High-dimensional analysis delineates myeloid and lymphoid compartment remodeling during successful immune-checkpoint cancer therapy. *Cell.* (2018) 175:1014–30.e19. doi: 10.1016/j.cell.2018.09.030
15. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev.* (2010) 29:309–16. doi: 10.1007/s10555-010-9223-6
16. Veglia F, Perego M, Gabrilovich D. Myeloid-derived suppressor cells coming of age. *Nat Immunol.* (2018) 19:108–19. doi: 10.1038/s41590-017-0022-x
17. Arlauckas SP, Garris CS, Kohler RH, Kitaoka M, Cuccarese MF, Yang KS, et al. *In vivo* imaging reveals a tumor-associated macrophage-mediated resistance pathway in anti-PD-1 therapy. *Sci Transl Med.* (2017) 9:eaal3604. doi: 10.1126/scitranslmed.aal3604
18. Dahan R, Sega E, Engelhardt J, Selby M, Korman AJ, Ravetch JV. FcgammaRs modulate the anti-tumor activity of antibodies targeting the PD-1/PD-L1 axis. *Cancer Cell.* (2015) 28:285–95. doi: 10.1016/j.ccell.2015.08.004
19. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature.* (2013) 496:445–55. doi: 10.1038/nature12034
20. Strachan DC, Ruffell B, Oei Y, Bissell MJ, Coussens LM, Pryer N, et al. CSF1R inhibition delays cervical and mammary tumor growth in murine models by attenuating the turnover of tumor-associated macrophages and enhancing infiltration by CD8(+) T cells. *Oncoimmunology.* (2013) 2:e26968. doi: 10.4161/onci.26968
21. Zhu Y, Knolhoff BL, Meyer MA, Nywening TM, West BL, Luo J, et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Res.* (2014) 74:5057–69. doi: 10.1158/0008-5472.CAN-13-3723
22. Mok S, Koya RC, Tsui C, Xu J, Robert L, Wu L, et al. Inhibition of CSF-1 receptor improves the antitumor efficacy of adoptive cell transfer immunotherapy. *Cancer Res.* (2014) 74:153–61. doi: 10.1158/0008-5472.CAN-13-1816
23. Ngiow SF, Meeth KM, Stannard K, Barkauskas DS, Bollag G, Bosenberg M, et al. Co-inhibition of colony stimulating factor-1 receptor and BRAF oncogene in mouse models of BRAF(V600E) melanoma. *Oncoimmunology.* (2016) 5:e1089381. doi: 10.1080/2162402X.2015.1089381
24. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov.* (2011) 1:54–67. doi: 10.1158/2159-8274.CD-10-0028
25. Kumar V, Donthireddy L, Marvel D, Condamine T, Wang F, Lavilla-Alonso S, et al. Cancer-associated fibroblasts neutralize the anti-tumor effect of CSF1 receptor blockade by inducing PMN-MDSC infiltration of tumors. *Cancer Cell.* (2017) 32:654–68.e5. doi: 10.1016/j.ccell.2017.10.005
26. Teng KY, Han J, Zhang X, Hsu SH, He S, Wani NA, et al. Blocking the CCL2-CCR2 axis using CCL2-neutralizing antibody is an effective therapy for hepatocellular cancer in a mouse model. *Mol Cancer Ther.* (2017) 16:312–22. doi: 10.1158/1535-7163.MCT-16-0124
27. Mitchem JB, Brennan DJ, Knolhoff BL, Belt BA, Zhu Y, Sanford DE, et al. Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. *Cancer Res.* (2013) 73:1128–41. doi: 10.1158/0008-5472.CAN-12-2731
28. Wu X, Singh R, Hsu DK, Zhou Y, Yu S, Han D, et al. A small molecule CCR2 antagonist depletes tumor macrophages and synergizes with anti-PD-1 in a murine model of cutaneous T-cell lymphoma (CTCL). *J Invest Dermatol.* (2020) 140:1390–400.e4. doi: 10.1016/j.jid.2019.11.018
29. Grossman JG, Nywening TM, Belt BA, Panni RZ, Krasnick BA, DeNardo DG, et al. Recruitment of CCR2(+) tumor associated macrophage to sites of liver metastasis confers a poor prognosis in human colorectal cancer. *Oncoimmunology.* (2018) 7:e1470729. doi: 10.1080/2162402X.2018.1470729
30. House IG, Savas P, Lai J, Chen AXY, Oliver AJ, Teo ZL, et al. Macrophage-derived CXCL9 and CXCL10 are required for antitumor immune responses following immune checkpoint blockade. *Clin Cancer Res.* (2020) 26:487–504. doi: 10.1158/1078-0432.CCR-19-1868
31. Clark CE, Hingorani SR, Mick R, Combs C, Tuveson DA, Vonderheide RH. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. *Cancer Res.* (2007) 67:9518–27. doi: 10.1158/0008-5472.CAN-07-0175
32. Aiello NM, Bajor DL, Norgard RJ, Sahmoud A, Bhagwat N, Pham MN, et al. Metastatic progression is associated with dynamic changes in the local microenvironment. *Nat Commun.* (2016) 7:12819. doi: 10.1038/ncomms12819
33. Vonderheide RH. The immune revolution: a case for priming, not checkpoint. *Cancer Cell.* (2018) 33:563–9. doi: 10.1016/j.ccell.2018.03.008
34. Morrison AH, Diamond MS, Hay CA, Byrne KT, Vonderheide RH. Sufficiency of CD40 activation and immune checkpoint blockade for T cell priming and tumor immunity. *Proc Natl Acad Sci USA.* (2020) 117:8022–31. doi: 10.1073/pnas.1918971117
35. Luheshi NM, Coates-Ulrichsen J, Harper J, Mullins S, Sulikowski MG, Martin P, et al. Transformation of the tumour microenvironment by a CD40 agonist antibody correlates with improved responses to PD-L1 blockade in a mouse orthotopic pancreatic tumour model. *Oncotarget.* (2016) 7:18508–20. doi: 10.18632/oncotarget.7610
36. Ngiow SF, Young A, Blake SJ, Hill GR, Yagita H, Teng MW, et al. Agonistic CD40 mAb-driven IL12 reverses resistance to anti-PD1 in a T-cell-rich tumor. *Cancer Res.* (2016) 76:6266–77. doi: 10.1158/0008-5472.CAN-16-2141
37. Salmon H, Idoyaga J, Rahman A, Leboeuf M, Remark R, Jordan S, et al. Expansion and activation of CD103(+) dendritic cell progenitors at the tumor site enhances tumor responses to therapeutic PD-L1 and BRAF inhibition. *Immunity.* (2016) 44:924–38. doi: 10.1016/j.immuni.2016.03.012
38. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science.* (2008) 322:1097–100. doi: 10.1126/science.1164206
39. Broz ML, Binnewies M, Boldajipour B, Nelson AE, Pollack JL, Erle DJ, et al. Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. *Cancer Cell.* (2014) 26:638–52. doi: 10.1016/j.ccell.2014.09.007

40. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CM, Pryer N, et al. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. *Cancer Cell.* (2014) 26:623–37. doi: 10.1016/j.ccell.2014.09.006
41. Roberts EW, Broz ML, Binnewies M, Headley MB, Nelson AE, Wolf DM, et al. Critical role for CD103(+)/CD141(+) dendritic cells bearing CCR7 for tumor antigen trafficking and priming of T cell immunity in melanoma. *Cancer Cell.* (2016) 30:324–36. doi: 10.1016/j.ccell.2016.06.003
42. Beavis PA, Henderson MA, Giuffrida L, Davenport AJ, Petley EV, House IG, et al. Dual PD-1 and CTLA-4 checkpoint blockade promotes antitumor immune responses through CD4(+)Foxp3(-) cell-mediated modulation of CD103(+) dendritic cells. *Cancer Immunol Res.* (2018) 6:1069–81. doi: 10.1158/2326-6066.CIR-18-0291
43. Barry KC, Hsu J, Broz ML, Cueto FJ, Binnewies M, Combes AJ, et al. A natural killer-dendritic cell axis defines checkpoint therapy-responsive tumor microenvironments. *Nat Med.* (2018) 24:1178–91. doi: 10.1038/s41591-018-0085-8
44. Maier B, Chen ST, Tung N, Chang C, LeBerichel J, Chudnovskiy A, et al. A conserved dendritic-cell regulatory program limits antitumour immunity. *Nature.* (2020) 508:257–62. doi: 10.1038/s41586-020-2134-y
45. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer.* (2012) 12:298–306. doi: 10.1038/nrc3245
46. Shang B, Liu Y, Jiang SJ, Liu Y. Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. *Sci Rep.* (2015) 5:15179. doi: 10.1038/srep15179
47. Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Cell Res.* (2017) 27:109–18. doi: 10.1038/cr.2016.151
48. Binnewies M, Mujal AM, Pollack JL, Combes AJ, Hardison EA, Barry KC, et al. Unleashing type-2 dendritic cells to drive protective antitumor CD4(+) T cell immunity. *Cell.* (2019) 177:556–71e16. doi: 10.1016/j.cell.2019.02.005
49. Qi S, Li H, Lu L, Qi Z, Liu L, Chen L, et al. Long-term intravital imaging of the multicolor-coded tumor microenvironment during combination immunotherapy. *Elife.* (2016) 5:e14756. doi: 10.7554/eLife.14756
50. Taylor NA, Vick SC, Iglesia MD, Brickey WJ, Midkiff BR, McKinnon KP, et al. Treg depletion potentiates checkpoint inhibition in claudin-low breast cancer. *J Clin Invest.* (2017) 127:3472–83. doi: 10.1172/JCI90499
51. Sakuishi K, Ngiow SF, Sullivan JM, Teng MW, Kuchroo VK, Smyth MJ, et al. TIM3(+)FOXP3(+) regulatory T cells are tissue-specific promoters of T-cell dysfunction in cancer. *Oncoimmunology.* (2013) 2:e23849. doi: 10.4161/onci.23849
52. Penaloza-MacMaster P, Kamphorst AO, Wieland A, Araki K, Iyer SS, West EE, et al. Interplay between regulatory T cells and PD-1 in modulating T cell exhaustion and viral control during chronic LCMV infection. *J Exp Med.* (2014) 211:1905–18. doi: 10.1084/jem.20132577
53. Liu J, Blake SJ, Harjunpaa H, Fairfax KA, Yong MC, Allen S, et al. Assessing immune-related adverse events of efficacious combination immunotherapies in preclinical models of cancer. *Cancer Res.* (2016) 76:5288–301. doi: 10.1158/0008-5472.CAN-16-0194
54. Coe D, Begom S, Addey C, White M, Dyson J, Chai JG. Depletion of regulatory T cells by anti-GITR mAb as a novel mechanism for cancer immunotherapy. *Cancer Immunol Immunother.* (2010) 59:1367–77. doi: 10.1007/s00262-010-0866-5
55. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med.* (2013) 210:1695–710. doi: 10.1084/jem.20130579
56. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, et al. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer Immunol Res.* (2013) 1:32–42. doi: 10.1158/2326-6066.CIR-13-0013
57. Bulliard Y, Jolicoeur R, Zhang J, Dranoff G, Wilson NS, Brogdon JL. OX40 engagement depletes intratumoral tregs via activating FcγRs, leading to antitumor efficacy. *Immunol Cell Biol.* (2014) 92:475–80. doi: 10.1038/icb.2014.26
58. Arce Vargas F, Furness AJS, Litchfield K, Joshi K, Rosenthal R, Ghorani E, et al. Fc effector function contributes to the activity of human anti-CTLA-4 antibodies. *Cancer Cell.* (2018) 33:649–63.e4. doi: 10.1016/j.ccell.2018.02.010
59. Ingram JR, Blomberg OS, Rashidian M, Ali L, Garforth S, Fedorov E, et al. Anti-CTLA-4 therapy requires an Fc domain for efficacy. *Proc Natl Acad Sci USA.* (2018) 115:3912–7. doi: 10.1073/pnas.1801524115
60. Sugiyama D, Nishikawa H, Maeda Y, Nishioka M, Tanemura A, Katayama I, et al. Anti-CCR4 mAb selectively depletes effector-type FoxP3+CD4+ regulatory T cells, evoking antitumor immune responses in humans. *Proc Natl Acad Sci USA.* (2013) 110:17945–50. doi: 10.1073/pnas.1316796110
61. Doi T, Muro K, Ishii H, Kato T, Tsushima T, Takenoyama M, et al. A phase I study of the anti-CC chemokine receptor 4 antibody, mogamulizumab, in combination with nivolumab in patients with advanced or metastatic solid tumors. *Clin Cancer Res.* (2019) 25:6614–22. doi: 10.1158/1078-0432.CCR-19-1090
62. Kurtulus S, Sakuishi K, Ngiow SF, Joller N, Tan DJ, Teng MW, et al. TIGIT predominantly regulates the immune response via regulatory T cells. *J Clin Invest.* (2015) 125:4053–62. doi: 10.1172/JCI81187
63. Bournazos S, Wang TT, Dahan R, Maamary J, Ravetch JV. Signaling by antibodies: recent progress. *Annu Rev Immunol.* (2017) 35:285–311. doi: 10.1146/annurev-immunol-051116-052433
64. Arce Vargas F, Furness AJS, Solomon I, Joshi K, Mekkaoui L, Lesko MH, et al. Fc-optimized anti-CD25 depletes tumor-infiltrating regulatory T cells and synergizes with PD-1 blockade to eradicate established tumors. *Immunity.* (2017) 46:577–86. doi: 10.1016/j.immuni.2017.03.013
65. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol.* (2012) 30:531–64. doi: 10.1146/annurev.immunol.25.022106.141623
66. van Gool F, Nguyen MLT, Mumbach MR, Satpathy AT, Rosenthal WL, Giacometti S, et al. A mutation in the transcription factor Foxp3 drives T helper 2 effector function in regulatory T cells. *Immunity.* (2019) 50:362–77.e6. doi: 10.1016/j.immuni.2018.12.016
67. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev.* (2010) 236:219–42. doi: 10.1111/j.1600-065X.2010.00923.x
68. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature.* (2007) 445:766–70. doi: 10.1038/nature05479
69. Delgoffe GM, Woo SR, Turnis ME, Gravano DM, Guy C, Overacre AE, et al. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature.* (2013) 501:252–6. doi: 10.1038/nature12428
70. Overacre-Delgoffe AE, Chikina M, Dadey RE, Yano H, Brunazzi EA, Shayan G, et al. Interferon-gamma drives Treg fragility to promote anti-tumor immunity. *Cell.* (2017) 169:1130–41.e11. doi: 10.1016/j.cell.2017.05.005
71. DuPage M, Chopra G, Quiros J, Rosenthal WL, Morar MM, Holohan D, et al. The chromatin-modifying enzyme Ezh2 is critical for the maintenance of regulatory T cell identity after activation. *Immunity.* (2015) 42:227–38. doi: 10.1016/j.immuni.2015.01.007
72. Wang D, Quiros J, Mahuron K, Pai CC, Ranzani V, Young A, et al. Targeting EZH2 reprograms intratumoral regulatory T cells to enhance cancer immunity. *Cell Rep.* (2018) 23:3262–74. doi: 10.1016/j.celrep.2018.05.050
73. Goswami S, Apostolou I, Zhang J, Skepner J, Anandhan S, Zhang X, et al. Modulation of EZH2 expression in T cells improves efficacy of anti-CTLA-4 therapy. *J Clin Invest.* (2018) 128:3813–8. doi: 10.1172/JCI99760
74. Godfrey DI, Le Nours J, Andrews DM, Uldrich AP, Rossjohn J. Unconventional T cell targets for cancer immunotherapy. *Immunity.* (2018) 48:453–73. doi: 10.1016/j.immuni.2018.03.009
75. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science.* (1997) 278:1626–9. doi: 10.1126/science.278.5343.1626
76. Metelitsa LS, Naidenko OV, Kant A, Wu HW, Loza MJ, Perussia B, et al. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. *J Immunol.* (2001) 167:3114–22. doi: 10.4049/jimmunol.167.6.3114
77. Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc Natl Acad Sci USA.* (2008) 105:11287–92. doi: 10.1073/pnas.0801631105

78. McEwen-Smith RM, Salio M, Cerundolo V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunol Res.* (2015) 3:425–35. doi: 10.1158/2326-6066.CIR-15-0062
79. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, et al. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen *in vivo* through direct interaction with dendritic cells. *J Immunol.* (2003) 171:5140–7. doi: 10.4049/jimmunol.171.10.5140
80. Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells *in vivo* and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med.* (2003) 198:267–79. doi: 10.1084/jem.20030324
81. Silk JD, Hermans IF, Gileadi U, Chong TW, Shepherd D, Salio M, et al. Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy. *J Clin Invest.* (2004) 114:1800–11. doi: 10.1172/JCI200422046
82. Song L, Asgharzadeh S, Salo J, Engell K, Wu HW, Sposto R, et al. Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages. *J Clin Invest.* (2009) 119:1524–36. doi: 10.1172/JCI37869
83. Bae EA, Seo H, Kim BS, Choi J, Jeon I, Shin KS, et al. Activation of NKT cells in an anti-PD-1-resistant tumor model enhances antitumor immunity by reinvigorating exhausted CD8 T cells. *Cancer Res.* (2018) 78:5315–26. doi: 10.1158/0008-5472.CAN-18-0734
84. Ishii K, Shimizu M, Kogo H, Negishi Y, Tamura H, Morita R, et al. A combination of check-point blockade and alpha-galactosylceramide elicits long-lasting suppressive effects on murine hepatoma cell growth *in vivo*. *Immunobiology.* (2020) 225:151860. doi: 10.1016/j.imbio.2019.10.009
85. O'Konek JJ, Illarionov P, Khursigara DS, Ambrosino E, Izhak L, Castillo BF, et al. Mouse and human iNKT cell agonist β-mannosylceramide reveals a distinct mechanism of tumor immunity. *J Clin Invest.* (2011) 121:683–94. doi: 10.1172/JCI42314
86. Awad W, Le Nours J, Kjer-Nielsen L, McCluskey J, Rossjohn J. Mucosal-associated invariant T cell receptor recognition of small molecules presented by MR1. *Immunol Cell Biol.* (2018) 96:588–97. doi: 10.1111/imcb.12017
87. Corbett AJ, Eckle SB, Birkinshaw RW, Liu L, Patel O, Mahony J, et al. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature.* (2014) 509:361–5. doi: 10.1038/nature13160
88. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature.* (2012) 491:717–23. doi: 10.1038/nature11605
89. Mascanfroni ID, Yeste A, Vieira SM, Burns EJ, Patel B, Sloma I, et al. IL-27 acts on DCs to suppress the T cell response and autoimmunity by inducing expression of the immunoregulatory molecule CD39. *Nat Immunol.* (2013) 14:1054–63. doi: 10.1038/ni.2695
90. Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature.* (2003) 422:164–9. doi: 10.1038/nature01433
91. Loh L, Wang Z, Sant S, Koutsakos M, Jegaskanda S, Corbett AJ, et al. Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18-dependent activation. *Proc Natl Acad Sci USA.* (2016) 113:10133–8. doi: 10.1073/pnas.1610750113
92. van Wilgenburg B, Scherwitzl I, Hutchinson EC, Leng T, Kurioka A, Kulicke C, et al. MAIT cells are activated during human viral infections. *Nat Commun.* (2016) 7:11653. doi: 10.1038/ncomms11653
93. Gherardin NA, Souter MN, Koay HF, Mangas KM, Seemann T, Stinear TP, et al. Human blood MAIT cell subsets defined using MR1 tetramers. *Immunol Cell Biol.* (2018) 96:507–25. doi: 10.1111/imcb.12021
94. Le Bourhis L, Dusseaux M, Bohineust A, Bessoles S, Martin E, Premel V, et al. MAIT cells detect and efficiently lyse bacterially-infected epithelial cells. *PLoS Pathog.* (2013) 9:e1003681. doi: 10.1371/journal.ppat.1003681
95. Kurioka A, Ussher JE, Cosgrove C, Clough C, Fergusson JR, Smith K, et al. MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. *Mucosal Immunol.* (2015) 8:429–40. doi: 10.1038/mi.2014.81
96. Won EJ, Ju JK, Cho YN, Jin HM, Park KJ, Kim TJ, et al. Clinical relevance of circulating mucosal-associated invariant T cell levels and their anti-cancer activity in patients with mucosal-associated cancer. *Oncotarget.* (2016) 7:76274–90. doi: 10.18632/oncotarget.11187
97. Sundstrom P, Ahlmanner F, Akeus P, Sundquist M, Alsen S, Yrlid U, et al. Human mucosa-associated invariant T cells accumulate in colon adenocarcinomas but produce reduced amounts of IFN-gamma. *J Immunol.* (2015) 195:3472–81. doi: 10.4049/jimmunol.1500258
98. Zabijak L, Attencourt C, Guignant C, Chatelain D, Marcelo P, Marolleau JP, et al. Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients. *Cancer Immunol Immunother.* (2015) 64:1601–8. doi: 10.1007/s00262-015-1764-7
99. Ling L, Lin Y, Zheng W, Hong S, Tang X, Zhao P, et al. Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients. *Sci Rep.* (2016) 6:20358. doi: 10.1038/srep20358
100. Melo AM, O'Brien AM, Phelan JJ, Kennedy SA, Wood NAW, Veerapen N, et al. Mucosal-associated invariant T cells display diminished effector capacity in oesophageal adenocarcinoma. *Front Immunol.* (2019) 10:1580. doi: 10.3389/fimmu.2019.01580
101. Godfrey DI, Koay HF, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. *Nat Immunol.* (2019) 20:1110–28. doi: 10.1038/s41590-019-0444-8
102. Yan J, Allen S, McDonald E, Das I, Mak JYW, Liu L, et al. MAIT cells promote tumor initiation, growth, and metastases via tumor MR1. *Cancer Discov.* (2020) 10:124–41. doi: 10.1158/2159-8290.CD-19-0569
103. Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science.* (2018) 359:97–103. doi: 10.1126/science.aan4236
104. Vetizou M, Pitt JM, Daillere R, Lepage P, Waldschmitt N, Flament C, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science.* (2015) 350:1079–84. doi: 10.1126/science.aad1329
105. Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science.* (2015) 350:1084–9. doi: 10.1126/science.aac4255
106. Pang Y, Gara SK, Achyut BR, Li Z, Yan HH, Day CP, et al. TGF-β signaling in myeloid cells is required for tumor metastasis. *Cancer Discov.* (2013) 3:936–51. doi: 10.1158/2159-8290.CD-12-0527
107. Novitskiy SV, Pickup MW, Chytil A, Polosukhina D, Owens P, Moses HL. Deletion of TGF-β signaling in myeloid cells enhances their anti-tumorigenic properties. *J Leukoc Biol.* (2012) 92:641–51. doi: 10.1189/jlb.1211639
108. Gao Y, Souza-Fonseca-Guimaraes F, Bald T, Ng SS, Young A, Ngiow SF, et al. Tumor immunoevasion by the conversion of effector NK cells into type 1 innate lymphoid cells. *Nat Immunol.* (2017) 18:1004–15. doi: 10.1038/ni.3800
109. Thomas DA, Massague J. TGF-β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell.* (2005) 8:369–80. doi: 10.1016/j.ccr.2005.10.012
110. Chen ML, Pittet MJ, Gorelik L, Flavell RA, Weissleder R, von Boehmer H, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-β signals *in vivo*. *Proc Natl Acad Sci USA.* (2005) 102:419–24. doi: 10.1073/pnas.0408197102
111. Budhu S, Schaer DA, Li Y, Toledo-Crow R, Panageas K, Yang X, et al. Blockade of surface-bound TGF-β on regulatory T cells abrogates suppression of effector T cell function in the tumor microenvironment. *Sci Signal.* (2017) 10:eaak9702. doi: 10.1126/scisignal.aak9702
112. Park BV, Freeman ZT, Ghasemzadeh A, Chattergoon MA, Rutebemberwa A, Steigner J, et al. TGFβ1-mediated SMAD3 enhances PD-1 expression on antigen-specific T cells in cancer. *Cancer Discov.* (2016) 6:1366–81. doi: 10.1158/2159-8290.CD-15-1347
113. Mariathasan S, Turley SJ, Nickles D, Castiglioni A, Yuen K, Wang Y, et al. TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature.* (2018) 554:544–8. doi: 10.1038/nature25501
114. Sow HS, Ren J, Camps M, Ossendorp F, Ten Dijke P. Combined inhibition of TGF-β signaling and the PD-L1 immune checkpoint is differentially effective in tumor models. *Cells.* (2019) 8:320. doi: 10.3390/cells8040320

115. Principe DR, Park A, Dorman MJ, Kumar S, Viswakarma N, Rubin J, et al. TGFβ blockade augments PD-1 inhibition to promote T-cell-mediated regression of pancreatic cancer. *Mol Cancer Ther.* (2019) 18:613–20. doi: 10.1158/1535-7163.MCT-18-0850
116. Ravi R, Noonan KA, Pham V, Bedi R, Zhavoronkov A, Ozerov IV, et al. Bifunctional immune checkpoint-targeted antibody-ligand traps that simultaneously disable TGFβ enhance the efficacy of cancer immunotherapy. *Nat Commun.* (2018) 9:741. doi: 10.1038/s41467-017-02696-6
117. Dodagatta-Marri E, Meyer DS, Reeves MQ, Paniagua R, To MD, Binnewies M, et al. α-PD-1 therapy elevates Treg/Th balance and increases tumor cell pSmad3 that are both targeted by alpha-TGFβ antibody to promote durable rejection and immunity in squamous cell carcinomas. *J Immunother Cancer.* (2019) 7:62. doi: 10.1186/s40425-018-0493-9
118. Holmgaard RB, Schaer DA, Li Y, Castaneda SP, Murphy MY, Xu X, et al. Targeting the TGFβ pathway with galunisertib, a TGFβRI small molecule inhibitor, promotes anti-tumor immunity leading to durable, complete responses, as monotherapy and in combination with checkpoint blockade. *J Immunother Cancer.* (2018) 6:47. doi: 10.1186/s40425-018-0356-4
119. Herbertz S, Sawyer JS, Stauber AJ, Gueorguieva I, Driscoll KE, Estrem ST, et al. Clinical development of galunisertib (LY2157299 monohydrate), a small molecule inhibitor of transforming growth factor-β signaling pathway. *Drug Des Devel Ther.* (2015) 9:4479–99. doi: 10.2147/DDDT.S86621
120. Martin CJ, Datta A, Littlefield C, Kalra A, Chapron C, Wawersik S, et al. Selective inhibition of TGFβ1 activation overcomes primary resistance to checkpoint blockade therapy by altering tumor immune landscape. *Sci Transl Med.* (2020) 12:eaay8456. doi: 10.1126/scitranslmed.aay8456
121. Voron T, Colussi O, Marcheteau E, Pernot S, Nizard M, Pointet AL, et al. VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors. *J Exp Med.* (2015) 212:139–48. doi: 10.1084/jem.20140559
122. Kim CG, Jang M, Kim Y, Leem G, Kim KH, Lee H, et al. VEGF-A drives TOX-dependent T cell exhaustion in anti-PD-1-resistant microsatellite stable colorectal cancers. *Sci Immunol.* (2019) 4:eaay0555. doi: 10.1126/sciimmunol.aay0555
123. Dudley JC, Lin MT, Le DT, Eshleman JR. Microsatellite instability as a biomarker for PD-1 blockade. *Clin Cancer Res.* (2016) 22:813–20. doi: 10.1158/1078-0432.CCR-15-1678
124. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med.* (2015) 372:2509–20. doi: 10.1056/NEJMoa1500596
125. Courau T, Nehar-Belaid D, Florez L, Levacher B, Vazquez T, Brimaud F, et al. TGF-β and VEGF cooperatively control the immunotolerant tumor environment and the efficacy of cancer immunotherapies. *JCI Insight.* (2016) 1:e85974. doi: 10.1172/jci.insight.85974
126. Young A, Mittal D, Stagg J, Smyth MJ. Targeting cancer-derived adenosine: new therapeutic approaches. *Cancer Discov.* (2014) 4:879–88. doi: 10.1158/2159-8290.CD-14-0341
127. Loi S, Pommey S, Haibe-Kains B, Beavis PA, Darcy PK, Smyth MJ, et al. CD73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. *Proc Natl Acad Sci USA.* (2013) 110:11091–6. doi: 10.1073/pnas.1222251110
128. Nagate Y, Ezoe S, Fujita J, Okuzakis D, Motooka D, Ishibashi T, et al. Ectonucleotidase CD39 is highly expressed on ATLL cells and is responsible for their immunosuppressive function. *Leukemia.* (2020). doi: 10.1038/s41375-020-0788-y. [Epub ahead of print].
129. Bastid J, Regairaz A, Bonnefoy N, Dejou C, Giustiniani J, Laheurte C, et al. Inhibition of CD39 enzymatic function at the surface of tumor cells alleviates their immunosuppressive activity. *Cancer Immunol Res.* (2015) 3:254–65. doi: 10.1158/2326-6066.CIR-14-0018
130. Cai XY, Wang XF, Li J, Dong JN, Liu JQ, Li NP, et al. High expression of CD39 in gastric cancer reduces patient outcome following radical resection. *Oncol Lett.* (2016) 12:4080–6. doi: 10.3892/ol.2016.5189
131. Chen S, Fan J, Zhang M, Qin L, Dominguez D, Long A, et al. CD73 expression on effector T cells sustained by TGF-β facilitates tumor resistance to anti-4-1BB/CD137 therapy. *Nat Commun.* (2019) 10:150. doi: 10.1038/s41467-018-08123-8
132. Ryzhov SV, Pickup MW, Chytil A, Gorska AE, Zhang Q, Owens P, et al. Role of TGF-β signaling in generation of CD39+CD73+ myeloid cells in tumors. *J Immunol.* (2014) 193:3155–64. doi: 10.4049/jimmunol.1400578
133. Young A, Ngiow SF, Barkauskas DS, Sult E, Hay C, Blake SJ, et al. Co-inhibition of CD73 and A2AR adenosine signaling improves anti-tumor immune responses. *Cancer Cell.* (2016) 30:391–403. doi: 10.1016/j.ccell.2016.06.025
134. Young A, Ngiow SF, Gao Y, Patch AM, Barkauskas DS, Messaoudene M, et al. A2AR adenosine signaling suppresses natural killer cell maturation in the tumor microenvironment. *Cancer Res.* (2018) 78:1003–16. doi: 10.1158/0008-5472.CAN-17-2826
135. Reinhardt J, Landsberg J, Schmid-Burgk JL, Ramis BB, Bald T, Glodde N, et al. MAPK signaling and inflammation link melanoma phenotype switching to induction of CD73 during immunotherapy. *Cancer Res.* (2017) 77:4697–709. doi: 10.1158/0008-5472.CAN-17-0395
136. Blay J, White TD, Hoskin DW. The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. *Cancer Res.* (1997) 57:2602–5.
137. Hatfield SM, Kjaergaard J, Lukashev D, Belikoff B, Schreiber TH, Sethumadhavan S, et al. Systemic oxygenation weakens the hypoxia and hypoxia inducible factor 1alpha-dependent and extracellular adenosine-mediated tumor protection. *J Mol Med.* (2014) 92:1283–92. doi: 10.1007/s00109-014-1189-3
138. Iannone R, Miele L, Maiolino P, Pinto A, Morello S. Adenosine limits the therapeutic effectiveness of anti-CTLA4 mAb in a mouse melanoma model. *Am J Cancer Res.* (2014) 4:172–81.
139. Mittal D, Young A, Stannard K, Yong M, Teng MW, Allard B, et al. Antimetastatic effects of blocking PD-1 and the adenosine A2A receptor. *Cancer Res.* (2014) 74:3652–8. doi: 10.1158/0008-5472.CAN-14-0957
140. Beavis PA, Henderson MA, Giuffrida L, Mills JK, Sek K, Cross RS, et al. Targeting the adenosine 2A receptor enhances chimeric antigen receptor T cell efficacy. *J Clin Invest.* (2017) 127:929–41. doi: 10.1172/JCI 89455
141. Young A, Ngiow SF, Madore J, Reinhardt J, Landsberg J, Chitsazan A, et al. Targeting adenosine in BRAF-mutant melanoma reduces tumor growth and metastasis. *Cancer Res.* (2017) 77:4684–96. doi: 10.1158/0008-5472.CAN-17-0393
142. Chen L, Diao L, Yang Y, Yi X, Rodriguez BL, Li Y, et al. CD38-mediated immunosuppression as a mechanism of tumor cell escape from PD-1/PD-L1 blockade. *Cancer Discov.* (2018) 8:1156–75. doi: 10.1158/2159-8290.CD-17-1033
143. Hatfield SM, Kjaergaard J, Lukashev D, Schreiber TH, Belikoff B, Abbott R, et al. Immunological mechanisms of the antitumor effects of supplemental oxygenation. *Sci Transl Med.* (2015) 7:277ra30. doi: 10.1126/scitranslmed.aaa1260
144. Li XY, Moesta AK, Xiao C, Nakamura K, Casey M, Zhang H, et al. Targeting CD39 in cancer reveals an extracellular ATP- and inflammasome-driven tumor immunity. *Cancer Discov.* (2019) 9:1754–73. doi: 10.1158/2159-8290.CD-19-0541
145. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, et al. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. *Immunity.* (2013) 38:729–41. doi: 10.1016/j.immuni.2013.03.003
146. Perrot I, Michaud HA, Giraudon-Paoli M, Augier S, Docquier A, Gros L, et al. Blocking antibodies targeting the CD39/CD73 immunosuppressive pathway unleash immune responses in combination cancer therapies. *Cell Rep.* (2019) 27:2411–25.e9. doi: 10.1016/j.celrep.2019.04.091
147. Fong L, Hotson A, Powderly JD, Sznol M, Heist RS, Choueiri TK, et al. Adenosine 2A receptor blockade as an immunotherapy for treatment-refractory renal cell cancer. *Cancer Discov.* (2020) 10:40–53. doi: 10.1158/2159-8290.CD-19-0980
148. Zelenay S, van der Veen AG, Bottcher JP, Snelgrove KJ, Rogers N, Acton SE, et al. Cyclooxygenase-dependent tumor growth through evasion of immunity. *Cell.* (2015) 162:1257–70. doi: 10.1016/j.cell.2015.08.015
149. Hamada T, Cao Y, Qian ZR, Masugi Y, Nowak JA, Yang J, et al. Aspirin use and colorectal cancer survival according to tumor CD274 (Programmed cell death 1 ligand 1) Expression Status. *J Clin Oncol.* (2017) 35:1836–44. doi: 10.1200/JCO.2016.70.7547
150. Markosyan N, Li J, Sun YH, Richman LP, Lin JH, Yan F, et al. Tumor cell-intrinsic EPHA2 suppresses anti-tumor immunity by regulating PTGS2 (COX-2). *J Clin Invest.* (2019) 130:3594–609. doi: 10.1172/JCI127755

151. Chalabi M, Fanchi LF, Dijkstra KK, Van den Berg JG, Aalbers AG, Sikorska K, et al. Neoadjuvant immunotherapy leads to pathological responses in MMR-proficient and MMR-deficient early-stage colon cancers. *Nat Med.* (2020) 26:566–76. doi: 10.1038/s41591-020-0805-8
152. Prendergast GC, Smith C, Thomas S, Mandik-Nayak L, Laury-Kleintop L, Metz R, et al. Indoleamine 2,3-dioxygenase pathways of pathogenic inflammation and immune escape in cancer. *Cancer Immunol Immunother.* (2014) 63:721–35. doi: 10.1007/s00262-014-1549-4
153. Spranger S, Koblish HK, Horton B, Scherle PA, Newton R, Gajewski TF. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8(+) T cells directly within the tumor microenvironment. *J Immunother Cancer.* (2014) 2:3. doi: 10.1186/2051-1426-2-3
154. Holmgaard RB, Zamarin D, Munn DH, Wolchok JD, Allison JP. Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4. *J Exp Med.* (2013) 210:1389–402. doi: 10.1084/jem.20130066
155. Long GV, Dummer R, Hamid O, Gajewski TF, Caglevic C, Dalle S, et al. Epacadostat plus pembrolizumab versus placebo plus pembrolizumab in patients with unresectable or metastatic melanoma (ECHO-301/KEYNOTE-252): a phase 3, randomised, double-blind study. *Lancet Oncol.* (2019) 20:1083–97. doi: 10.1016/S1470-2045(19)30274-8
156. Messenheimer DJ, Jensen SM, Afentoulis ME, Wegmann KW, Feng Z, Friedman DJ, et al. Timing of PD-1 blockade is critical to effective combination immunotherapy with anti-OX40. *Clin Cancer Res.* (2017) 23:6165–77. doi: 10.1158/1078-0432.CCR-16-2677
157. Shrimali RK, Ahmad S, Verma V, Zeng P, Ananth S, Gaur P, et al. Concurrent PD-1 blockade negates the effects of OX40 agonist antibody in combination immunotherapy through inducing T-cell apoptosis. *Cancer Immunol Res.* (2017) 5:755–66. doi: 10.1158/2326-6066.CIR-17-0292
158. Dong MB, Wang G, Chow RD, Ye L, Zhu L, Dai X, et al. Systematic immunotherapy target discovery using genome-scale *in vivo* CRISPR screens in CD8 T cells. *Cell.* (2019) 178:1189–204.e23. doi: 10.1016/j.cell.2019.07.044
159. Lizotte PH, Hong RL, Luster TA, Cavanaugh ME, Taus LJ, Wang S, et al. A high-throughput immune-oncology screen identifies EGFR inhibitors as potent enhancers of antigen-specific cytotoxic t-lymphocyte tumor cell killing. *Cancer Immunol Res.* (2018) 6:1511–23. doi: 10.1158/1538-7445.AM2018-4935
160. Khandelwal N, Breinig M, Speck T, Michels T, Kreutzer C, Sorrentino A, et al. A high-throughput RNAi screen for detection of immune-checkpoint molecules that mediate tumor resistance to cytotoxic T lymphocytes. *EMBO Mol Med.* (2015) 7:450–63. doi: 10.15252/emmm.201404414
161. Kather JN, Charoentong P, Suarez-Carmona M, Herpel E, Klupp F, Ulrich A, et al. High-throughput screening of combinatorial immunotherapies with patient-specific in silico models of metastatic colorectal cancer. *Cancer Res.* (2018) 78:5155–63. doi: 10.1158/0008-5472.CAN-18-1126
162. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, adaptive, and acquired resistance to cancer immunotherapy. *Cell.* (2017) 168:707–23. doi: 10.1016/j.cell.2017.01.017
163. Kluger HM, Tawbi HA, Ascierto ML, Bowden M, Callahan MK, Cha E, et al. Defining tumor resistance to PD-1 pathway blockade: recommendations from the first meeting of the SITC immunotherapy resistance taskforce. *J Immunother Cancer.* (2020) 8:e000398. doi: 10.1136/jitc-2019-000398
164. Loo K, Tsai KK, Mahuron K, Liu J, Pauli ML, Sandoval PM, et al. Partially exhausted tumor-infiltrating lymphocytes predict response to combination immunotherapy. *JCI Insight.* (2017) 2:e93433. doi: 10.1172/jci.insight.93433
165. Bilen MA, Shabto JM, Martini DJ, Liu Y, Lewis C, Collins H, et al. Sites of metastasis and association with clinical outcome in advanced stage cancer patients treated with immunotherapy. *BMC Cancer.* (2019) 19:857. doi: 10.1186/s12885-019-6073-7
166. Daud AI, Loo K, Pauli ML, Sanchez-Rodriguez R, Sandoval PM, Taravati K, et al. Tumor immune profiling predicts response to anti-PD-1 therapy in human melanoma. *J Clin Invest.* (2016) 126:3447–52. doi: 10.1172/JCI87324
167. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med.* (2015) 373:23–34. doi: 10.1056/NEJMoa1504030
168. Hu-Lieskovan S, Mok S, Homet Moreno B, Tsoi J, Robert L, Goedert L, et al. Improved antitumor activity of immunotherapy with BRAF and MEK inhibitors in BRAF(V600E) melanoma. *Sci Transl Med.* (2015) 7:279ra41. doi: 10.1126/scitranslmed.aaa4691
169. Ribas A, Hodi FS, Callahan M, Konto C, Wolchok J. Hepatotoxicity with combination of vemurafenib and ipilimumab. *N Engl J Med.* (2013) 368:1365–6. doi: 10.1056/NEJMc1302338
170. Minor DR, Puzanov I, Callahan MK, Hug BA, Hoos A. Severe gastrointestinal toxicity with administration of trametinib in combination with dabrafenib and ipilimumab. *Pigment Cell Melanoma Res.* (2015) 28:611–2. doi: 10.1111/pcmr.12383
171. Young A, Quandt Z, Bluestone JA. The balancing act between cancer immunity and autoimmunity in response to immunotherapy. *Cancer Immunol Res.* (2018) 6:1445–52. doi: 10.1158/2326-6066.CIR-18-0487
172. Du X, Liu M, Su J, Zhang P, Tang F, Ye P, et al. Uncoupling therapeutic from immunotherapy-related adverse effects for safer and effective anti-CTLA-4 antibodies in CTLA4 humanized mice. *Cell Res.* (2018) 28:433–47. doi: 10.1038/s41422-018-0012-z
173. Menzies AM, Johnson DB, Ramanujam S, Atkinson VG, Wong ANM, Park JJ, et al. Anti-PD-1 therapy in patients with advanced melanoma and preexisting autoimmune disorders or major toxicity with ipilimumab. *Ann Oncol.* (2017) 28:368–76. doi: 10.1093/annonc/mdw443
174. Shah NJ, Al-Shbool G, Blackburn M, Cook M, Belouali A, Liu SV, et al. Safety and efficacy of immune checkpoint inhibitors (ICIs) in cancer patients with HIV, hepatitis B, or hepatitis C viral infection. *J Immunother Cancer.* (2019) 7:353. doi: 10.1186/s40425-019-0771-1
175. Herbaux C, Gauthier J, Brice P, Drumez E, Ysebaert L, Doyen H, et al. Efficacy and tolerability of nivolumab after allogeneic transplantation for relapsed Hodgkin lymphoma. *Blood.* (2017) 129:2471–8. doi: 10.1182/blood-2016-11-749556
176. Pai CS, Simons DM, Lu X, Evans M, Wei J, Wang YH, et al. Tumor-conditional anti-CTLA4 uncouples antitumor efficacy from immunotherapy-related toxicity. *J Clin Invest.* (2019) 129:349–63. doi: 10.1172/JCI123391
177. Wu L, Seung E, Xu L, Rao E, Lord DM, Wei RR, et al. Trispecific antibodies enhance the therapeutic efficacy of tumor-directed T cells through T cell receptor co-stimulation. *Nature Cancer.* (2020) 1:86–98. doi: 10.1038/s43018-019-0004-z
178. Kvarnhammar AM, Veitonmaki N, Hagerbrand K, Dahlman A, Smith KE, Fritzell S, et al. The CTLA-4 x OX40 bispecific antibody ATOR-1015 induces anti-tumor effects through tumor-directed immune activation. *J Immunother Cancer.* (2019) 7:103. doi: 10.1186/s40425-019-0570-8
179. Wang H, Mooney DJ. Biomaterial-assisted targeted modulation of immune cells in cancer treatment. *Nat Mater.* (2018) 17:761–72. doi: 10.1038/s41563-018-0147-9
180. Johnston RJ, Su LJ, Pinckney J, Critton D, Boyer E, Krishnakumar A, et al. VISTA is an acidic pH-selective ligand for PSGL-1. *Nature.* (2019) 574:565–70. doi: 10.1038/s41586-019-1674-5
181. Wang M, Wang J, Wang R, Jiao S, Wang S, Zhang J, et al. Identification of a monoclonal antibody that targets PD-1 in a manner requiring PD-1 Asn58 glycosylation. *Commun Biol.* (2019) 2:392. doi: 10.1038/s42003-019-0642-9
182. Sun L, Li CW, Chung EM, Yang R, Kim YS, Park AH, et al. Targeting glycosylated PD-1 induces potent anti-tumor immunity. *Cancer Res.* (2020) 80:2298–310. doi: 10.1158/0008-5472.CAN-19-3133

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REVIEW
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Specific Targeting of Notch Ligand-Receptor Interactions to Modulate Immune Responses: A Review of Clinical and Preclinical Findings

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Understanding and targeting Notch signaling effectively has long been valued in the field of cancer and other immune disorders. Here, we discuss key discoveries at the intersection of Notch signaling, cancer and immunology. While there is a plethora of Notch targeting agents tested *in vitro*, *in vivo* and in clinic, undesirable off-target effects and therapy-related toxicities have been significant obstacles. We make a case for the clinical application of ligand-derived and affinity modifying compounds as novel therapeutic agents and discuss major research findings with an emphasis on Notch ligand-specific modulation of immune responses.

Keywords: Notch signaling, Notch therapeutics, engineered Notch ligands, cancer immunotherapy, immunosurveillance, T cells, antigen presenting cells, tumor escape

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INTRODUCTION

Notch signaling plays a variety of physiological roles including, but not limited to, cell proliferation, cell fate decisions, cellular differentiation and angiogenesis (1). The role and importance of Notch signaling in hematopoietic compartment now stands undisputed. Despite the improved clinical response compared to standard chemotherapy, the efficacy of immune checkpoint inhibitors (ICI) across a variety of solid tumors is limited to a fraction of patients (2, 3). It is therefore essential to develop therapeutic agents that show promise as single agent immunomodulators or can be used in combination with ICIs to elicit antitumor immune responses.

Developing and utilizing agents that could support the induction of antitumor T cell functions while also precluding effector immune cells from immunosuppression offers great promise. Findings from murine models of solid tumors, allergic responses and autoimmune disorders

Abbreviations: CSL, CBF-1, suppressor of hairless, lag-2; DLL, Delta-like ligand; ECD, extracellular domain; GSI, γ-secretase inhibitor; HSC, hematopoietic stem cell; ICI, immune checkpoint inhibitor; JAG, Jagged ligand; LLC, Lewis lung carcinoma; MAML1, mastermind-like protein 1; NECD, Notch extracellular domain; NICD, Notch intracellular domain; NRR, negative regulatory region; NSCLC, non-small cell lung cancer; PEST, peptide sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T); RBP-Jκ, recombination signal binding protein for immunoglobulin Kappa J region; SAHM1, Stapled α-helical peptides derived from mastermind-like protein 1; T-ALL, T-cell acute lymphoblastic leukemia; TME, tumor microenvironment.

indicate great potential for the clinical application of Notch ligands and their derivatives as immunomodulatory agents for the management of malignant cancers (4, 5). Engineered Notch ligand-derived moieties could be used to induce desired immune responses and boost antitumor immunity (6, 7).

Activating mutations in Notch1 have been described in lung, breast, colorectal and pancreatic cancers to name a few. On the other hand, loss of function mutations in Notch in hepatocellular carcinomas and melanomas have established its role as a tumor suppressor (8). Notch can play a highly contextual role in tumoral, stromal and immune compartments, which adds to the signaling complexity and warrants the need to pursue its therapeutic targeting with great prudence.

In the following sections, we report findings that revealed the varied effects of Notch signaling in immune compartments driving T cell development, activation, differentiation, and regulation of effector immune responses. Non-canonical Notch signaling and its crosstalk with other signaling pathways, impact of Notch post-translational modifications on T cell differentiation, consensus and controversies and open questions in the field are discussed. We highlight how knowledge obtained by structural studies and studying the mechanisms of various steps involved in Notch activation and signal transduction offer therapeutic opportunities that enable its targeting with high specificity.

BRIEF OVERVIEW OF NOTCH SIGNALING

Canonical Notch signaling is unique in being driven by juxtracrine cell membrane bound receptor-ligand interactions (9). The mammalian Notch system is comprised of four Type I transmembrane receptors (Notch1-4) and two classes of ligands – Delta-like (DLL 1,3,4) and Jagged (JAG 1,2). Upon ligand binding, a mechanical force triggers sequential proteolytic cleavages in the intracellular portions of the receptor, ultimately releasing the Notch intracellular domain (NICD) into the cytoplasm. NICD then migrates into the nucleus where it acts along with a host of other transcriptional coactivators, including RBP-Jκ and MAML1-3 (10). To ensure tight regulation of Notch signaling, C-terminal PEST domain provides a proteolytic target for degradation of active Notch (11–13). Recent developments have indicated that Notch can also exert its functions non-canonically by interacting with members of other signaling pathways such as Wnt/β-catenin, NF-κB, TGFβ and many others (14–19).

ROLE OF NOTCH SIGNALING IN HELPER AND EFFECTOR T CELLS

Select Delta-like ligands have been shown to induce differentiation of hematopoietic stem cells (HSCs) into T cells. These findings were obtained using OP9 bone marrow stromal cells expressing DLL1 or DLL4 and similar effects were also observed using purified plate-bound ligands (20, 21). T cell differentiation of HSCs is dependent on both ligand identity and level of expression where low-level expression of the Delta-like ligands attenuates but does not eliminate the myeloid potential of HSCs. Such fine tuning of dose responses is a recurring theme in Notch signaling, faithful artificial recapitulation of which has eluded us so far.

Antigen presenting cells (APCs) expressing Delta-like ligands activate and polarize naïve $CD4^+$ T cells to a Th1 phenotype, while JAG1/2 expressing APCs lead to Treg/Th2/Th17 polarization (22–24). Convincing results in this direction showed that the intracellular domain of Notch1 is directly involved in interactions with and expression of Th1 master transcriptional factor T-bet and production of cytokine IFNγ in CD4 T cells. Notch signaling promoted the development of $CD8^+$ terminal effector T cells and suppressed memory-precursor fate in effector-memory T cell (T_{EM}) subsets (25). Activation of the Notch pathway in T_{EM} cells also suppressed memory-precursor fate. Transcription factors such as eomesodermin (EOMES) and T-bet were found to be directly regulated by Notch, further supporting the importance of Notch signaling in driving effector T cell responses (26–28).

The tumor microenvironment (TME) also plays a major role in influencing T cell responses. Notch1 and Notch2 were found to be downregulated in tumor infiltrating T cells but not in splenic T cells of tumor-bearing mice (29). This attenuation of infiltrating T cell responses was driven by Jag1/2 expressed by immunosuppressive myeloid-derived suppressor cells (MDSCs) which could be overcome by ectopic expression of Notch1 intracellular domain (N1ICD) in antigen specific T cells, indicating that the TME is programmed with mechanisms to suppress Notch signaling and evade T cell-mediated tumor cell death. This reveals another interesting aspect of Notch: the spatio-temporal regulation of Notch ligand and receptor expression.

While several studies demonstrated the involvement of Notch signaling in driving effector and helper T cell responses (summarized in **Figure 1**), the precise regulatory mechanisms behind cell surface expression of Notch ligands and receptors are only partly known. TCR stimulation has been shown to induce expression of Notch1, Notch2 and Hes1 (Notch target) in T cells (30) but T cell activation using CD28 beads alone or low-dose CD3 and CD28 stimulation induces expression of Notch ligands on T cells (31). Notch ligands were not expressed by T cells during *in vitro* activation with mature bone-marrow derived dendritic cells. The induced ligands also co-localized with Notch receptors on the surface indicating cis-inhibition. Notch ligand expression was abrogated in the presence of NF-κB inhibitor, demonstrating the combined role of Notch and NF-κB pathways in driving T cell functions downstream of TCR stimulus. The observations suggest additional regulatory mechanisms, possibly to prevent erroneous T cell activity in the absence of both TCR and co-stimulatory CD28 signals.

Notch extracellular domain (NECD) binding to cognate ligands is influenced by a variety of post-translational modifications, prominent among them being O-linked glycosylation by Fringe glycosyl transferases (32, 33). The three mammalian fringe proteins, Lunatic (Lfng), Manic (Mfng)

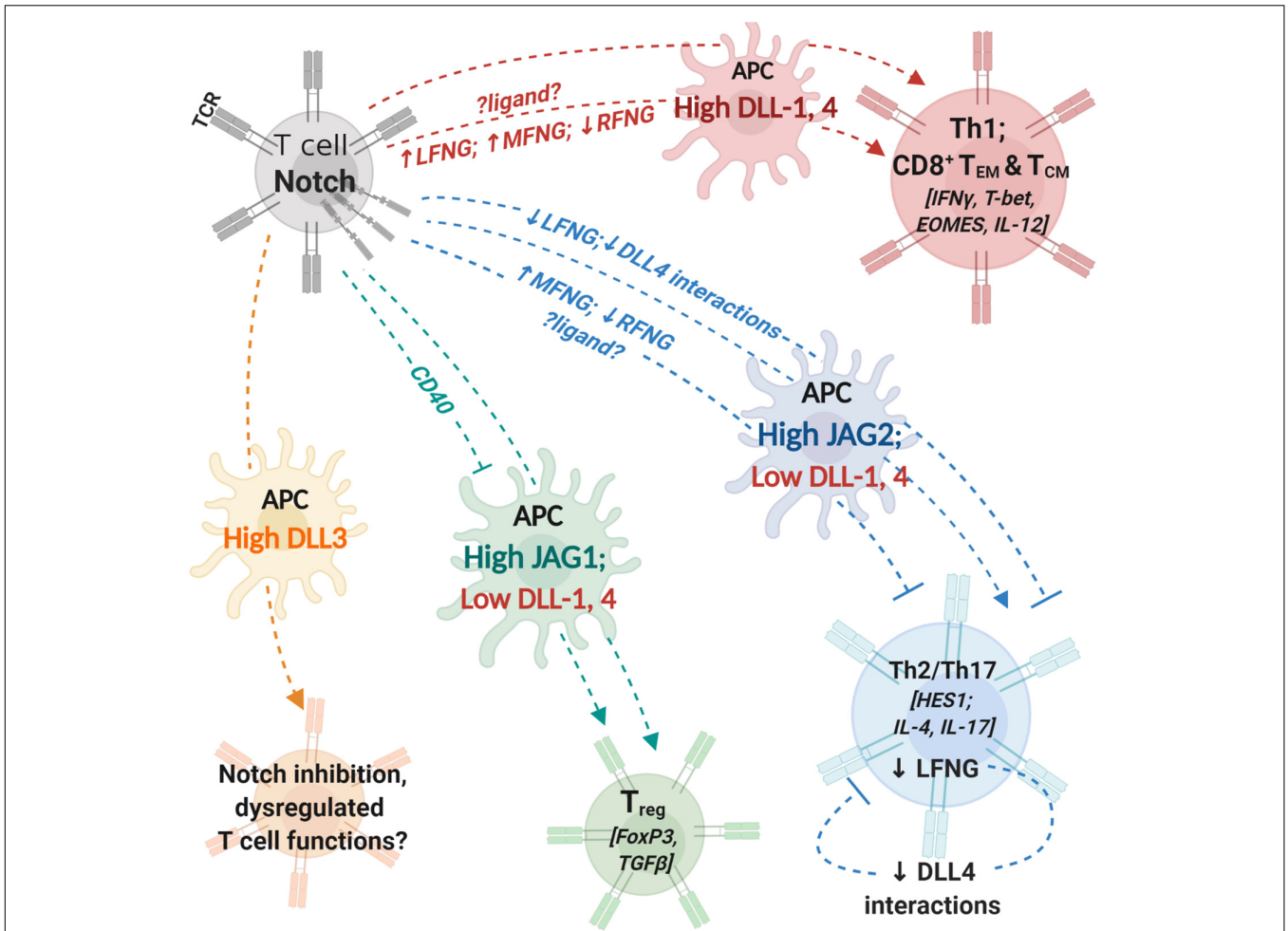

FIGURE 1 | Notch interactions between antigen-presenting cells and T cells influence helper and effector T cell activity. T cells express T cell receptor (TCR) complex and Notch receptors. Antigen-presenting cells (APCs) express costimulatory molecules and Notch ligands. During T cell activation, the identity of Notch ligand present on the cell surface of APCs can influence T cell polarization and differentiation. Changes in expression levels of fringe glycosyl transferases can influence the process by modifying Notch receptor affinity to different ligands. Notch signaling in T cells regulates expression of transcription factors and cytokines (indicated within []) involved in helper and cytotoxic T cell functions. APCs with high expression levels of DLL1 or DLL4 can polarize $CD4^+$ T cells into aTh1 phenotype and drive $CD8^+$ T cell differentiation into memory cells. Increase (↑) in LFNG and MFNG expression and downregulation/loss (↓) of RFNG expression can enhance Th1 differentiation; identity of ligands involved in fringe-mediated Th1 differentiation are yet to be investigated (represented by ?ligand?). APCs with high JAG2 and low DLL1,4 expression drive helper T cell differentiation into Th2 or Th17 phenotypes. Expression of MFNG and downregulation of RFNG can block Th2 differentiation. Loss of LFNG in uncommitted T cells as well as Th2 polarized cells inhibits Notch interactions with DLL4 and attenuates Th2 responses. APCs with high JAG1 expression can induce T cell polarization into regulatory T cells (T_{reg}). CD40 blockade together with JAG1 expression on APCs enhances immunosuppressive functions of T_{reg} cells. APC, antigen presenting cell; DLL, Delta-like ligand; JAG, Jagged ligand; LFNG, lunatic fringe; MFNG, manic fringe; MHC, major-histocompatibility complex; TCR,; Th1, T helper type 1, Th2: T helper type 2; Th17, T helper type 17; T_{reg}, T regulatory cell; T_{EM}, effector-memory T cell; T_{CM}, central-memory T cell; RFNG, radical fringe.

and Radical (Rfng) extend *O*-Fucose moieties with GlcNAc at conserved serine or tyrosine residues in EGF repeats of NECD (34, 35). Glycosylation of Notch by Lfng and Mfng enhances interactions with Delta-like ligands while suppressing interactions with Jagged ligands. On the other hand, Notch glycosylation by Rfng enhances receptor interactions with both classes of ligands.

Tumor-mediated decrease in Lfng and Mfng expression levels have been shown to promote metastasis and poor survival (36, 37). Lfng interacts cooperatively with p53 to suppress tumors and Mfng suppresses tumorigenic activity of JAG1 and Notch3 (38–40). Lfng and Mfng thus appear to have a tumor-suppressive role in solid tumors and restoring their expression levels can be pursued as a therapeutic strategy to achieve tumor regression.

Fringe-mediated changes in Notch ligand-receptor interactions lead to dysregulations in thymic and ectopic T cell development resulting in altered T/B cell population ratios (41–44). Tumor burden and tumor-derived immunosuppressive cytokines also cause abnormalities in intrathymic T cell differentiation and development (45–47). Notch glycosylation by fringes influence the differentiation of mature T cell populations as well. It was found that Lfng and Mfng were downregulated

and Rfng was upregulated in naïve $CD4^+$ T cells in asthmatic rats (48). This was associated with more active Notch signaling in asthmatic naïve $CD4^+$ T cells compared to control naïve T helper cells. Restoring Lfng and Mfng expression and silencing Rfng enhanced the number of Th1 cells while lowering Th2 cell differentiation. Lfng overexpression in naïve $CD4^+$ T cells was able to drive Th1 and Th2 differentiation in a Notch-independent and dependent manner, respectively. These findings indicate that modulating Fringe expression levels can be potential therapeutic strategies for the management of allergic diseases. Moreover, the observation that fringe expression levels vary even in naïve $CD4^+$ T cells under asthmatic conditions provide a basis for the hypothesis that there might be molecular factors that can alter T cell programs, which need to be elucidated. While Gu et al., were able to demonstrate the role of fringe glycosylation in influencing T helper cell differentiation, the source and identity of Notch ligands involved in this process were not identified.

Using a mouse airway allergic disease model, another study found that transcription of Lfng was driven by STAT5 in Th2 helper cells (49). Th2-mediated airway hyper-reactivity, mucus production and IL4 production was driven by DLL4-mediated Notch activation. Specifically, deletion of Lfng but not Mfng or Rfng in Th2 and $CD4^+$ T cells resulted in reduced Th2 responses and inflammation. While STAT5 and GATA3 were previously known to drive Th2 differentiation independent of Notch signals (50, 51), the regulation of Lfng expression by STAT5 in Th2 subsets is a novel and interesting finding. It is likely that other inflammatory factors that can influence STAT5 signaling can potentially alter fringe expression levels. Notch activity in T cells thus can be profoundly influenced by complex intracellular networks of cytokines and signaling pathways involved in fine-tuning immune responses (52, 53).

CONTRASTING OBSERVATIONS AND AN ARGUMENT FOR NON-CANONICAL NOTCH SIGNALING IN T CELLS

Differentiation of complete T cell effector program has been observed to be dictated by the identity of Notch ligand expressed on APCs, which in turn is dictated by the type of antigenic stimulus encountered (22, 23). This is in stark contrast to observations from *in vitro* T cell differentiation by polarizing cytokines even in the absence of Notch ligands (54). In some *in vitro* experiments, Notch activity was shown to confer a proliferative effect in T cells but could not drive Th1/Th2 differentiation in the absence of polarizing cytokines (55). While some studies have demonstrated that DLL1/4 ligands can promote a Th1 polarization, others have argued that the Th1 phenotype is not acquired as a consequence of Notch signaling but by suppression of the alternative Th2/17 fate (56, 57). The disease model used, type of antigenic responses, stimuli involved in DC maturation and the relative expression levels of different Notch ligands are all factors that could potentially influence T cell polarization by APCs. Most studies, however, have produced convincing data in favor of Notch1-ICD binding directly to promoters of genes and transcription factors driving Th1 and cytotoxic responses. Non-canonical Notch signaling and crosstalk with NF-κB pathway is also observed in activated T cells (58). γ-secretase inhibitors reduced IFNγ production in *in vitro* activated $CD8^+$ T cells but not in $CD4^+$ cells, which can indicate that helper and cytotoxic T cells respond differently to Notch stimuli at least *in vitro*. It is likely that DC-borne ligands could orchestrate T cell survival and proliferation within an existing cytokine milieu instead of having an instructive role in naïve T cell differentiation (59–61).

These observations prompt a question: do Notch ligands play a deterministic/instructive role or do they simply enhance pre-existing T cell programs in an unbiased manner? It could be possible that Notch serves as a costimulatory signal that can set in motion any of the numerous downstream signaling pathways (62, 63). It might also be possible that Notch signaling might have different effects before, during and after T cell activation and differentiation. Majority of the studies on the role of Notch in immune cell functions have looked at Hes/Hey/Deltex family members, which are themselves transcriptional factors effecting expression of several genes. T cell functions might be ultimately dictated by a combinatorial framework in which terminal effector molecules are further regulated by Notch targets.

UNANSWERED QUESTIONS

Notch signaling does not always appear to operate as a simple ON/OFF switch. It has been shown to be regulated by a complex system of fine-tuning and crosstalk of input signals including relative expression levels of ligands and receptors, numerous post-translational modifications and a combination of cis- and trans- interactions (64–67). While attempts are being made to target Notch in various disease settings, a large number of therapies developed so far have led to undesirable side-effects and toxicities (7). To address these shortcomings, it is important to study the mechanistic and physical aspects of ligand-receptor interactions (68) and role of post-translational modifications such as ligand glycosylation and ubiquitination (32, 33, 35). It is also necessary to understand how the physiological consequences of ectopic Notch expression are similar to and differ from ligand-specific receptor activation and how different sources of ligands can influence differences in immunological outcomes. Redundancies in receptor and ligand paralogs also need to be resolved.

THERAPEUTIC STRATEGIES TO TARGET NOTCH SIGNALING

Knowledge-based approaches on the activation mechanisms of Notch have led to the development of several Notch inhibitory agents. These include selective ligand/receptor-specific decoys, agents that block receptor cleavage, molecules that inhibit formation of Notch-CSL activator complex, antibodies, and post translational modifications influencing ligand-receptor interactions (**Table 1** and **Figure 2**). In addition to being used as single agents in various clinical and preclinical studies,

TABLE 1 | Strategies to target Notch signaling.

Class	Agent(s)	Target	Mechanism	Cancer type; *in vivo*/*in vitro* model	Treatment-related toxicities	References
GSI	PF03084014, MK0752	γ-secretase complex	Juxtamembrane cleavage and NICD dissociation	T-ALL*, breast*, lung adenocarcinomas*, thyroid*, prostate*, CNS malignancies*	Gastrointestinal toxicities, diarrhea, nausea, rash, fatigue	(140), NCT00645333, NCT01098344
	A5226A	Nicastrin	Inhibition of γ-secretase activity	Lymphoblastic leukemia^{t}, NSCLC^{t}	na	(141)
Blocking peptides	SAHM1	MAML1	Direct binding to pre-assembled Notch1–CSL/RBP-Jκ complexes and competitive inhibition of the MAML1 co-activator binding	T-ALL^{t}, murine asthma model	na	(142, 143)
Blocking antibodies	OMP-59R5, anti-NRR1, anti-NRR2	Notch1, Notch2, Notch3	Blocking receptor mediated signaling	Stage IV NSCLC*, extensive stage small-cell lung cancer*	Atrial fibrillation, diarrhea	PINNACLE (NCT01859741), (144, 145)
	OMP-21M18, REGN421	hDLL4	Humanized antibody that blocks DLL4 interactions with Notch	Breast^{t}, colon^{t}, ovarian^{t}, pancreatic^{t}, NSCLC^{t} & patients with advanced malignancies*	Hypertension, congestive heart failure	NCT01189968, NCT01189929, NCT00871559, (146, 147)
Decoys [soluble ligand or receptor forms]	$N1_{1-24}$	DLL1,4	Pan ligand blocking	Mammary, pancreatic, lung and melanoma tumor models	na	(28)
	$N1_{1-36}$	JAG1,2				
	$N1_{1-13}$	DLL1,4	Specific blocking of Delta-like ligands			
	$N1_{10-24}$	JAG1, 2	Specific blocking of Jagged ligands			
	sJ1, $sJ1_{N-E3}$	JAG1	Endogenous Jagged1	LLC		(6)
L-Fucose analogs	6-alkynyl and 6-alkenyl fucose	Notch ECD fucosylation	Substrate for POFUT-1 incorporated into Notch1 ECD, preventing binding to DLL1,4	T cell differentiation model [OP9 stromal coculture]	na	(98)
Soluble multivalent ligands	cDLL1	Notch1-4	Provides DLL1 stimulus to activate Notch receptors	Lung tumor models, *in vitro* mouse and human T cell cultures	na	(2, 5)

*Examples of Notch-targeting agents used in in vitro, pre-clinical and clinical studies. *, tested in clinical trial; t, preclinical/in vitro data.*

Notch inhibitors are also being studied in combination with current chemotherapeutic drugs. Despite being uncharacterized for the active component, some natural compounds show promising anti-proliferative effects on cancer cell lines and have traditionally been used as part of dietary modifications as chemo-preventative measures (69–71). Inhibition of the γ-secretase complex is the most widely employed method of blocking Notch signaling but has been fraught with toxicities (72, 73). There is a need to focus on Notch activators in the management of cancers like lung squamous cell carcinoma, where Notch acts as a tumor suppressor. The development of Notch modulators should be guided at every stage by the biological and physiological effects of the compounds being tested. Mechanism-based combinatorial regimens, biomarkers of response and contextual frameworks need to be developed and evaluated on a case by case basis.

While most reagents presented in **Table 1** were initially used to alter Notch signaling in stroma and the tumor microenvironment, recent focus has shifted to targeting Notch in tumor-infiltrating and circulating immune compartments. This

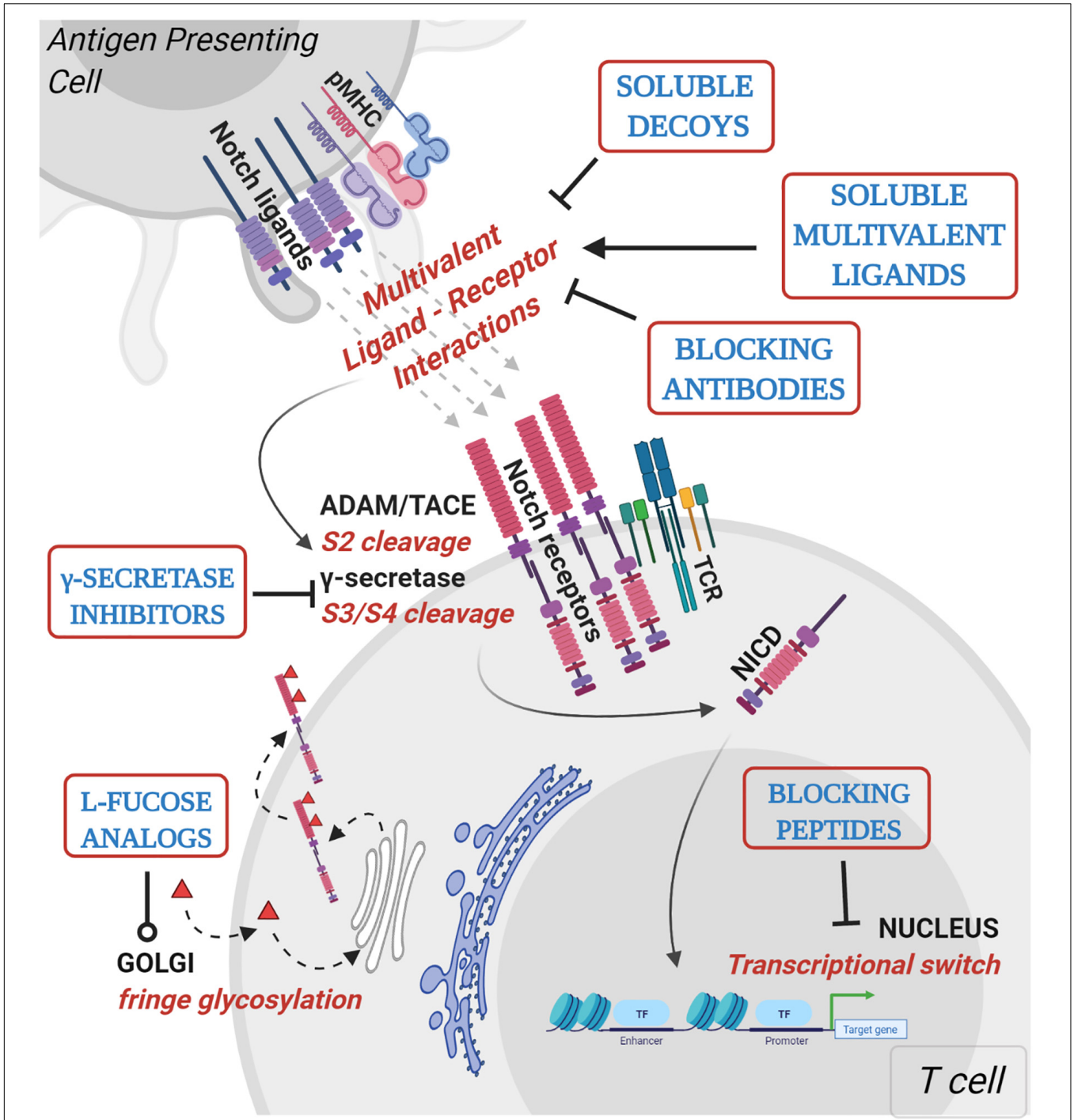

FIGURE 2 | Mechanistic basis for therapeutic targeting of ligand-specific Notch signaling. Agents targeting Notch signaling can be grouped by the step or process in the Notch signaling pathway that is being affected. Soluble decoys comprise of extracellular portions of Notch ligands or receptors that can competitively inhibit multivalent receptor-ligand interactions. Soluble multivalent ligands comprise of clustered ligands that provide and/or augment ligand-specific Notch activation. Blocking antibodies block receptor interactions with ligands and are paralog-specific antagonists with high selectivity. γ-secretase inhibitors prevent NICD release by inhibiting S3 cleavage of Notch receptors at the juxtamembrane domain. L-fucose analogs (solid red triangles) are taken up by cells from media and incorporated into receptor extracellular domains. Fucose analogs on Notch receptors alter ligand-binding affinities and can be used to block selective ligand interactions. Blocking peptides target protein-protein interface in the nuclear Notch transcriptional complex and prevent transcription of Notch target genes. *In vitro*, pre-clinical and clinical studies demonstrating Notch-modulatory activities and anti-tumor efficacy of various classes of Notch therapeutics are presented in **Table 1**.

has been done using agents directly targeting Notch receptors expressed by immune cells or in *in vitro* settings where Notch ligand-based agents are employed to activate, prime, and expand helper and effector T cell populations.

NOTCH-BASED REAGENTS FOR ADOPTIVE T CELL THERAPY

As the biology of Notch signaling in driving T cell development began to be better understood, the system was applied to generate antigen-specific T cells *in vitro*. By coculturing with DLL1-expressing bone marrow stromal cells, embryonic and hematopoietic stem cells could be differentiated into immunocompetent T lymphocytes (74). NY-ESO-1–specific and human p53–specific, HLA-A2–restricted human TCR vectors were used to transduce human umbilical cord HSCs, which were then cultured on OP9-GFP or OP9-DL1 cells and expanded (75). The T cells thus generated displayed very little endogenous TCR and had a high expression of antigen-restricted tumor-reactive TCR. They were also less differentiated than *in vitro* expanded lymphocytes that are currently employed in clinic. The differentiated and expanded HSCs in this study expressed the NK cell markers CD56 and CD16 as well as the T cell markers CD3 and CD7 but did not express IFNγ and IL-4 as NK-T cells do. Both NY-ESO1 and p53 TCR-transduced and differentiated cells exhibited antigen-specific lysis of target cells indicating T cell properties. The p53-TCR transduced HSCs, however, lysed both specific and non-specific tumor cells, indicating an NK cell-like behavior. While these cells could be useful candidates for adoptive cell transfer in both HLA-restricted and HLA-independent settings, safety evaluations and detailed characterization of the observed dual T and NK cell behavior is needed. Activated and differentiated effector T cells possess enhanced tumor reactivity *in vitro* but they demonstrate reduced tumor attenuation compared to naïve and early effector cells *in vivo* (76). This was overcome by the generation of stem cell memory T cells [T_{SCM}], a class of highly proliferative memory T cells, again using the OP9-DL1 coculture system, to generate ova-specific reactive T cells (77). Of note, the iT_{SCM} cells displayed a loss of PD1 and CTLA4 expression, which contributed in part to enhanced cytolytic activity of the adoptively transferred naïve-like stem cell memory T cells. While Notch1 activity could upregulate PD-1 expression on $CD8^+$ T cells activated with artificial APCs expressing both Delta-like and Jagged ligands (78), expression of inhibitory receptors was not seen with the OP9-DL1 coculture system. This indicates the advantage of employing ligand-specific T cell stimulation and expansion for therapeutic applications.

NOTCH AS A TARGET OF OTHER THERAPEUTIC AGENTS AND PATHWAYS

Notch signaling has the unique feature of integrating signals from several pathways. This leads to an extensive "hyper-network" situation within a cell as well as at the multicellular level (18, 79). From a therapeutic standpoint, it becomes important to identify key regulatory nodes between different pathways. This will enable the development therapeutic agents with high specificity and prevent cross-pathway side effects. Some studies have identified how Notch in immune cells can be altered by therapeutic and experimental interventions targeting molecules in other signaling pathways.

While T cells can upregulate Notch expression a few hours after TCR stimulation, the exact molecular crosstalk between the two pathways is only partially known (30, 80). PKCθ has been linked to actin regulation as well as Notch induction, leading to the discovery of a spatio-temporal link between T cell stimulation by professional APCs and Notch activity (81). p38 MAPK was shown to induce Jagged1 as well as Notch1 during the maturation of macrophages (82).

Adenosine is an immunosuppressive ATP metabolite that is increased in the extracellular space in response to hypoxia and tissue injury, which can have profound effects on both lymphoid and myeloid cells that express adenosine receptors, predominantly, A2AR by T cells. A2AR agonists have been used in the treatment of inflammatory diseases while A2AR antagonists are being developed as novel cancer immunotherapeutics (83). Notch1 was identified as a target of A2AR-mediated immunosuppression. This is believed to be orchestrated by Cbl-mediated ubiquitination of Notch1 modulated by A2AR via cAMP. $CD8^+$ T cells exposed to an A2AR agonist prior to TCR stimulation lowered Notch1 expression, heterodimer cleavage and reduced transcripts of Notch1 target genes *Hes1* and *Myc* (84).

Thus, disparate pathways could potentially converge to drive Notch expression and function. This makes it all the more important to fully understand the fundamental cellular and molecular levels at which Notch signaling is regulated.

NOVEL NOTCH MODULATORY AGENTS

Engineered Ligand-Specific Therapeutics

An important feature of Notch signaling is the stoichiometry of interactions: activation of Notch receptors requires polyvalent interactions between multiple receptors and ligands. On the other hand, interfering with even a few of the productive multivalent interactions can lead to disruption of Notch signaling. Therefore, soluble monovalent forms of Notch ligands or receptors can potentially act as efficient competitive inhibitors. In contrast, presenting Notch ligands in a multivalent form can provide or enhance ligand-specific stimulus. This mechanistic detail of Notch receptor-ligand interaction can be exploited to design ligand-based reagents that can uniquely stimulate or block Notch signaling.

Studies have demonstrated that endogenous Notch ligand-receptor interactions can be selectively blocked or enhanced to influence signaling in tumoral, stromal and immune compartments (5, 6, 85, 86). Although receptor-ligand interactions can be abrogated using blocking antibodies, this presents some limitations including high costs, low tissue

penetration, unclear mode of action *in vivo*, cytotoxicity, and affinity for inhibitory Fc receptors which reduces their overall efficacy (87). Soluble decoys that can interfere with specific ligand-receptor interactions and are small enough to achieve good biodistribution in solid tumors present more attractive options. Extracellular domains of Notch1 that uniquely interact with Delta-like or Jagged classes of ligands have been used to develop Notch decoys achieving ligand-specific inhibition (28, 86, 88). A fragment of Notch1 ECD comprised of EGF repeats 10 to 24 ($N1_{10-24}$) could selectively inhibit Notch1-JAG interactions without interfering with Notch1-DLL interactions indicating competitive binding to Jagged ligands. $N1_{10-24}$ demonstrated potent antitumor effects in various murine tumor models by reducing angiogenic sprouting and disruption of tumor endothelium, both of which are phenotypes associated with JAG1-driven Notch signaling. Similarly, the Notch1 ECD fragment comprised of EGF repeats 1 to 13 ($N1_{1-13}$) could specifically inhibit DLL4-mediated Notch signaling effects leading to hyper-sprouting and poor perfusion. On the other hand, a larger Notch1 decoy, $N1_{1-24}$, recapitulated the effects of inhibiting either JAG1 or DLL4 or both, depending on the tumor microenvironment and *in vitro* angiogenesis model used.

Soluble inhibitory receptor-derived decoys have been reported for Notch3 as well. Distinct, short peptides derived from EGF repeats 7–10 and 21–22 of Notch3 bound directly to JAG1 (89). The ligand-binding domains of Notch3 were distinct from those of Notch1 despite the high sequence similarity in conserved EGF repeats. The peptide forms as well as recombinant immunoglobulin Fc chimeras (IgG-Fc) of Notch3-derived peptides were able to induce apoptosis in tumor cells, preferentially reduced Notch3 activation and the expression of Notch3-specific target *Hey1*. Peptide-IgGFc chimeras could also suppress tumor growth in a Notch3-driven human lung cancer xenograft model.

Thus, certain regions of Notch receptor extracellular domains that uniquely interact with different ligand classes and paralogs can be used to design soluble inhibitory decoys with high specificity.

Full-length or partial extracellular domains of Notch ligands could be also be used to modulate ligand-specific Notch signaling events. Soluble monomeric fragments comprised of the DSL and first two EGF repeats of DLL1 (sDLL1) and the first five N-terminal domains of JAG1 ($sJAG1_{N-E3}$) could selectively inhibit Notch1-DLL1 and Notch1-JAG1 interactions, respectively (6). The sDLL1 fragment attenuated *in vitro* T cell proliferation in cocultures with DLL1-bearing dendritic cells, indicating its potential ability to impair T cell responses by blocking endogenous DLL1. A short synthetic peptide derived from DSL region of JAG1 spanning residues 188–204 demonstrated Notch activation driving keratinocyte differentiation *in vitro* in its soluble form (90). Portions of the DSL domain of human JAG1 as well as the complete extracellular region of human JAG1 ligands could function as activators affecting differentiation of myeloid progenitors (91). In contrast, soluble purified human Jagged1-immunoglobulin IgG1 Fc chimera protein inhibited growth of myeloid colonies and macrophage progenitors from human cord blood, indicating its inhibitory properties (92). Therefore, the exact sequence of Notch ligand extracellular domains used (partial fragment versus full length ECD, specific portions of extracellular domains) could determine whether the soluble ligand forms act as activators or inhibitors. This might be driven by the area and structural conformation of ligand-receptor interface being bound by the soluble ligands. Detailed structural and binding studies need to be done to evaluate the mechanistic aspects of soluble ligands as Notch modulators.

Ligand multivalency is commonly mimicked by immobilizing the ligands on culture plates prior to seeding cells or by pre-clustering of ligand immunoglobulin Fc fragment chimeras by anti-Fc antibodies (2, 93). While plate-bound ligands can provide multimeric ligand stimulus, their use is restricted to *in vitro* applications. For *in vivo* administration, pre-clustered ligands provide a more suitable format. Further complexing anti-Fc antibodies is possible by tagging them with biotin, FLAG or other non-immunogenic short peptides or affinity tags (94) and using anti-tag antibodies to in turn complex those. This would greatly increase the valency of ligand being provided and could be used for pharmacological stimulation of ligand-specific responses. A multiplexed reagent called clustered DLL1 (cDLL1) was developed which is comprised of three components: a chimera of full length murine or human DLL1 and Fc region of IgG_{2A}, biotinylated anti-IgG_2Fc antibody, and NeutrAvidin (a deglycosylated version of avidin with unaltered affinity to biotin) (1). This produces a tertiary complex with multiple ligand extracellular domains being available for Notch activation. cDLL1 administration to tumor-bearing mice improved antigen-specific $CD8^+$ T cell responses and attenuated tumor growth in preclinical murine models of lung cancer. Multivalent DLL1 stimulus provided by cDLL1 could enhance $CD8^+$ T effector-memory cells and reduce the number of regulatory T cells in spleen.

Apart from providing *in vitro* therapeutic agents, plate-bound, cell-expressing, and multimerized Notch ligands could thus be used in various multivalent formats for cancer treatment.

Affinity-Modifying Compounds

Carbohydrate moieties at the ligand-receptor interface in *trans* interactions can influence canonical ligand-mediated Notch receptor activation via steric effects (95–97). L-fucose analogs that could be directly incorporated into Notch EGF repeats can be exploited to manipulate Notch receptor binding to cognate ligands. Peracetylated forms of *O*-Fucose, 6-alkynyl and 6-alkenyl fucose, act as substrates by Pofut-1 and can differentially modulate ligand binding (98). Fucose analogs incorporated into Notch1 EGF repeats inhibit trans interactions with DLL1 and DLL4 whereas interactions with JAG1 remain unaffected. Mutational and structural analysis revealed that fucosylation at Notch EGF8 is the site contributing to steric clashes and subsequent ablation of interactions with Delta-like ligands. This can be explained by a higher sensitivity of Delta-like ligands to Notch post-translational modifications compared to the Jagged class of ligands.

Notch ligand-based and affinity-modifying reagents thus offer the benefit of specific targeting along with fine-tuning and

versatility to activate or inhibit Notch activity in a ligand-specific manner.

The following sections summarize evidence from preclinical and clinical studies that provided substantial evidence in favor of Notch ligand-based moieties as immunomodulatory agents driving anti-tumor T cell functions.

RESTORING DLL1-SPECIFIC NOTCH SIGNALING CAN REVERSE IMPAIRED T CELL DEVELOPMENT IN TUMOR-BEARING MICE

Tumor presence alters a number of cytokine-mediated intracellular signaling pathways, expression of chemotactic ligands and receptors by thymic populations (99–101). This results increased apoptosis of TECs, dysregulated lineage-commitment checkpoints, diminished TCR repertoire and low thymic output, all of which ultimately dampen immunosurveillance and promote tumor escape. In immature DN2 T cell subsets, CCR7 is a target of Notch1 and is important for the migration of developing T cell precursors through the thymic cortex to medulla (102–104). Reduction in expression levels of Notch1 and its targets in thymic pre-T cells of tumor-bearing mice is mediated by IL-10 produced by thymic epithelial cells (TECs) (45). This is associated with an upregulation in of Ikaros and IRF8 signaling which shunts the developing pre-T cell toward differentiating into dendritic cells. A network of interactions between Notch, Wnt, Ikaros, and IL10 (among several others) is involved in determining the balance between T and myeloid lineage commitment under normal physiologic conditions (105–108). Given the indispensable role of Notch in ensuring normal thymic T cell development, therapeutic interventions to restore Notch activity in thymic and peripheral T cells can promote antitumor immunity (109, 110).

Advanced stage cancer patients have high mean serum levels of vascular endothelial growth factor (VEGF) compared to healthy humans. Mice infused with VEGF to mimic this pathophysiology showed thymic atrophy and decreased percentage of peripheral T cells in their spleen and lymph nodes (46, 111). This effect was coupled to a significant decrease in the number of $CD4^+CD8^+$ thymic populations. The reduction in $CD4^+CD8^+$ numbers was not due to an induction of thymocyte apoptosis or inhibition of thymocyte development, as the VEGF-exposed thymic cells could develop normally in mice without tumors and in *in vitro* fetal thymic organ cultures. Administration of anti-VEGFR2 but not anti-VEGFR1 antibody restored normal hematopoiesis revealing a mechanistic link between tumor derived VEGF and impaired peripheral immunity (112). The thymic atrophy observed in tumor-bearing mice could be a consequence of a pre-thymic event such as a VEGF-mediated block in emigration of thymic progenitors from the bone marrow.

Compared to age-matched controls, tumor-bearing mice have low DLL1 and DLL4 expression levels in bone marrow cells as well as low splenic T:B cell ratios. When VEGF-infused mice were irradiated and received bone marrow progenitors overexpressing DLL1, the inhibitory effects on T cell development were reversed, indicating that DLL1 stimulus alone is sufficient to resuscitate VEGF-driven impaired antitumor immunity. In order to mimic the effects of BM transplantation with DLL1-overexpressing hematopoietic precursors, the more pharmacologically relevant multivalent DLL1 form (cDLL1 – described in the previous section) was employed (5). Administration of cDLL1 significantly lowered tumor burden in treated mice compared to untreated tumor-bearing controls. Tumor regression was T-cell mediated, as was seen with the loss of cDLL1 efficacy in tumor-bearing $Rag1^{-/-}$ mice and mice receiving anti-CD8 antibody to deplete $CD8^+$ T cells. This was associated with increased number of antigen-specific memory T cells, improved IFNγ production, and higher intracellular pSTAT1&2 in differentiated T cells. Additionally, the transcript levels of T-bet were significantly higher in $CD4^+$ T cells after cDLL1 administration, providing direct evidence of tumor attenuating Th1 responses being enhanced (112–115). Stimulation of Notch signaling in effector $CD8^+$ T cells was also able to achieve tumor regression in mutant $EGFR^{L858R}$ oncogene-driven tumor models. Patients with EGFR-driven non-small cell lung cancers treated with tyrosine kinase inhibitors (TKI) such as erlotinib eventually acquire drug resistance (116–118). The TKI treatment also shapes the tumor microenvironment leading to an upregulation of PD-L1 expression (119–121). Given the low response rate of EGFR-mutant tumors to ICI treatment (122–124), DLL1-mediated enhancement of Type I immune responses might provide therapeutic benefit when used in combination with ICI.

Different Notch ligand-receptor interactions can result in distinct downstream outcomes hence while DLL4 stimulation promotes angiogenesis, DLL1 signaling does not (125–127). Concurrently, the administration of cDLL1 to tumor-bearing mice did not result in vascular defects and, in fact, significantly decreased tumor vascularization.

In this manner, Notch ligand-based therapeutics can selectively stimulate helper and effector immune functions with high ligand and contextual specificity. Multivalent DLL1-derived Notch activators could thus potentially provide clinically relevant immunotherapeutic agents to overcome thymic atrophy and impaired T cell functions.

REGULATORY T CELL FUNCTIONS CAN BE MODULATED IN A JAG1-SPECIFIC MANNER

Interaction of T cells with APCs is necessary to induce effector T cell function and differentiation by providing TCR stimulus from cognate peptide-bound MHC (signal 1) and costimulatory CD28 (signal 2) (128–130). Several findings have revealed the additional role of interactions between specific Notch ligands presented by DCs and Notch receptors on T-cells in providing critical activation, differentiation, and polarization signals.

Adoptive transfer of antigen-pulsed, Jagged1 (JAG1)-expressing DCs inhibited established immune responses in immunized mice. This inhibition was $CD4^+$ T cell-specific and long lived (131). With JAG1-expressing DC administration,

peripheral naive $CD4^+$ T cells were found to differentiate into regulatory cells. These cells could induce antigen-specific tolerance when transferred into naïve hosts. Similar effects were seen in human peripheral naïve blood cells; stimulation of $CD45RA^+$ naïve T cells by allogeneic antigen-presenting cells overexpressing JAG1 resulted in reduced production of IFNγ, IL-2 and IL-5. The activated cells upregulated TGFβ and inhibited proliferative and cytotoxic immune responses in freshly stimulated lymphocyte cultures (132). This reveals the molecular basis of regulatory T cell induction when naïve cells are activated by JAG1-borne APCs. Further investigation revealed that the immunosuppression was antigen-specific and affected both $CD4^+$ and $CD8^+$ T cells (133).

Mouse bone marrow cells could be differentiated into tolerogenic dendritic cells by culturing them in the presence of GM-CSF (GM-BMDCs). GM-BMDCs were found to express JAG1, essential for induction of regulatory T cell phenotype in $CD4^+$ T cells (134). Abrogating JAG1-Notch interactions by using anti-JAG1 blocking antibodies suppressed T_{reg} proliferation. Similar results were obtained by shRNA-mediated knockdown of JAG1 in murine bone marrow mesenchymal stromal cells (MSC) (135). $CD4^+$ T cells cocultured with JAG1-expressing MSC differentiated into tolerogenic T_{reg} cells capable of producing anti-inflammatory cytokine, IL-10. T_{regs} thus obtained could also protect against inflammation *in vivo* in a mouse model of allergen-induced airway pathology.

Since DCs expressing JAG1 exhibit a tolerogenic potential, they could be used in a transplantation setting to inhibit immune responses and prolong allograft survival. Indeed, when JAG1-overexpressing DCs were used in along with CD40 blocking antibody, murine allograft heart transplants were better tolerated in recipient mice (136). This was achieved by induction of alloantigen-specific T cell suppression and upregulation of TGFβ and FoxP3-expressing T_{reg} numbers driven by JAG1-Notch interactions expressed by transferred DCs and host T cells, respectively. While JAG1-driven Notch activation of host T cells could attenuate Th1 responses, it did not effect Th2 differentiation. By employing JAG1-transduced DCs, this study could provide mechanistic insights into the specific source and functions of Notch ligands. It is worth noting that overexpression of JAG1 could lead to ligand being in far in excess of receptors available on T cells and can diminish Notch activation owing to reduced ligand trans-endocytosis (137, 138).

Further evidence that T_{reg} -mediated suppression of effector T cell responses was mediated by Notch came from a systematic lineage-specific deletion of Notch pathway components in T_{regs}. Targeted deletion of *Pofut1*, *Rbpj* and *Notch1* enhanced T_{reg} cell frequency and decreased $CD4^+$ and $CD8^+$ T-cell immune responses (139). On the other hand, overexpression of a constitutionally active Notch1 intracellular domain in T_{reg} cells resulted in autoimmunity, skewing to a Th1 phenotype and apoptosis of regulatory T cells. Notch inhibition appears to dictate the balance between inflammatory effector T cells and tolerant regulatory T cells.

Tissue tissue-specific genetic ablation of Jagged1 and systemic administration of soluble inhibitory JAG1 provided further proof of ligand-mediated Notch activation in T_{regs} (6). CD11c-specific ablation of JAG2 did not have any effects on IFN-γ production but significantly decreased IL-4 production by activated T cells. On the other hand, CD11c-specific deletion of DLL1 resulted in accelerated tumor growth in murine tumor models coupled to a reduction in $CD8^+$ T cell activation and reduced differentiation of antigen-specific cytotoxic T cells and memory cells. Tumor-bearing mice treated with inhibitory monomeric soluble JAG1 (sJAG1) showed a significant reduction in tumor burden concurrent with a decrease in splenic T_{reg} cell numbers. This was associated with low tumor infiltration of $CD11c^+Gr1^+$ cells, thereby providing further evidence of JAG1 as a factor mediating immunosuppressive tolerogenic responses. *In vitro* T:DC coculture experiments in the presence of sJAG1 could also downregulate the expression of PD-1 on $CD8^+$ T effector memory cells.

Taken together, these studies provide a strong evidence in favor of specifically targeting JAG1 to modulate regulatory T cell functions. The use of well designed, soluble inhibitory JAG1 decoys could provide a therapeutic edge in the context of enhancing antitumor immune responses and attenuating immunosuppression. Distinct ligand-specific effects provide a great opportunity to avoid undesirable effects associated with pan-Notch inhibition.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

From the perspective of both basic and applied immunology, study of Notch signaling in immune subsets can provide valuable insights into the management and cure of metastatic solid tumors that are recalcitrant to conventional treatments. As the field of immunology progresses, so will our understanding of the role that Notch plays in immune cell function and regulation. This can be accomplished by interdisciplinary and complementary techniques such as tissue and lineage-specific genetic ablation, biochemical and molecular modulation of ligand-receptor interactions, evaluation of antigen specific immune responses and computational analysis of large patient datasets. More fundamental approaches such as investigating ligand/receptor redundancies, effector differentiation by cytokines in combination with ligand-specific Notch activation and non-canonical Notch signaling are also needed. Outcomes from current therapeutic regimens can be improved by using Notch-ligand based reagents in combination with or prior to checkpoint blockade to prime the immune system. Preclinical studies using Notch ligand-derived selective activators and inhibitors also provide mechanistic insights into how the immune system can be modulated in a ligand-specific manner in cancer and other immunopathological conditions. Such agents constitute a novel class of immunomodulatory drugs addressing unmet medical needs.

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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REFERENCES

1. Kovall RA, Gebelein B, Sprinzak D, Kopan R. The canonical notch signaling pathway: structural and biochemical insights into shape, sugar, and force. *Dev Cell.* (2017) 41:228–41. doi: 10.1016/j.devcel.2017.04.001
2. Carbone DP, Reck M, Paz-Ares L, Creelan B, Horn L, Steins M, et al. First-line nivolumab in stage IV or Recurrent non–small-cell lung cancer. *New Engl J Med.* (2017) 376:2415–26. doi: 10.1056/NEJMoa1613493
3. Fares CM, Van Allen EM, Drake CG, Allison JP, Hu-Lieskovan S. Mechanisms of resistance to immune checkpoint blockade: why does checkpoint inhibitor immunotherapy not work for all patients? *Am Soc Clin Oncol Educ Book.* (2019) 39:147–64. doi: 10.1200/EDBK_240837
4. Huang Y, Lin L, Shanker A, Malhotra A, Yang L, Dikov MM, et al. Resuscitating cancer immunosurveillance: selective stimulation of DLL1-Notch signaling in T cells rescues T cell function and inhibits tumor growth. *Cancer Res.* (2011) 71:6122–31. doi: 10.1158/0008-5472.CAN-10-4366
5. Biktasova AK, Dudimah DF, Uzhachenko RV, Park K, Akhter A, Arasada RR, et al. Multivalent forms of the notch ligand DLL-1 enhance antitumor T-cell immunity in lung cancer and improve efficacy of EGFR-targeted therapy. *Cancer Res.* (2015) 75:4728–41. doi: 10.1158/0008-5472.CAN-14-1154
6. Tchekneva EE, Goruganthu MUL, Uzhachenko RV, Thomas PL, Antonucci A, Chekneva I, et al. Determinant roles of dendritic cell-expressed Notch Delta-like and Jagged ligands on anti-tumor T cell immunity. *J Immun Ther Cancer.* (2019) 7:95. doi: 10.1186/s40425-019-0592-2
7. Andersson ER, Lendahl U. Therapeutic modulation of Notch signalling — are we there yet? *Nat Rev Drug Discov.* (2014) 13:357–78. doi: 10.1038/nrd4252
8. Aster JC, Pear WS, Blacklow SC. The varied roles of notch in cancer. *Annu Rev Pathol.* (2017) 12:245–75. doi: 10.1146/annurev-pathol-052016-100127
9. Bray SJ. Notch signalling in context. *Nat Rev Mol Cell Biol.* (2016) 17:722–35. doi: 10.1038/nrm.2016.94
10. Kopan R, Ilagan MXG. The canonical notch signaling pathway: unfolding the activation mechanism. *Cell.* (2009) 137:216–33. doi: 10.1016/j.cell.2009.03.045
11. Fryer CJ, Lamar E, Turbachova I, Kintner C, Jones KA. Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. *Genes Dev.* (2002) 16:1397–411. doi: 10.1101/gad.991602
12. Fryer CJ, White JB, Jones KA. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol Cell.* (2004) 16:509–20. doi: 10.1016/j.molcel.2004.10.014
13. Kopan R. All good things must come to an end: how is notch signaling turned off? *Sci. Signal.* (1999) 1999:e1. doi: 10.1126/stke.1999.9.pe1
14. Heitzler P. Chapter fourteen–biodiversity and noncanonical notch signaling. In: Kopan R editor. *Current Topics in Developmental Biology*. Cambridge, MA: Academic Press (2010). p. 457–81. doi: 10.1016/S0070-2153(10)92014-0
15. Perumalsamy LR, Nagala M, Banerjee P, Sarin A. A hierarchical cascade activated by non-canonical Notch signaling and the mTOR–Rictor complex regulates neglect-induced death in mammalian cells. *Cell Death Different.* (2009) 16:879–89. doi: 10.1038/cdd.2009.20
16. Genz B, Coleman MA, Irvine KM, Kutasovic JR, Miranda M, Gratte FD, et al. Overexpression of miRNA-25-3p inhibits Notch1 signaling and TGF-β-induced collagen expression in hepatic stellate cells. *Sci Rep.* (2019) 9:8541. doi: 10.1038/s41598-019-44865-1
17. Poellinger L, Lendahl U. Modulating Notch signaling by pathway-intrinsic and pathway-extrinsic mechanisms. *Curr Opin Genet Dev Different Gene Regul.* (2008) 18:449–54. doi: 10.1016/j.gde.2008.07.013
18. Borggrefe T, Lauth M, Zwijsen A, Huylebroeck D, Oswald F, Giaimo BD. The Notch intracellular domain integrates signals from Wnt, Hedgehog, TGFβ/BMP and hypoxia pathways. *Biochim Biophys Acta (BBA) Mol Cell Res.* (2016) 1863:303–13. doi: 10.1016/j.bbamcr.2015.11.020
19. Krejčí A, Bernard F, Housden BE, Collins S, Bray SJ. Direct response to notch activation: signaling crosstalk and incoherent logic. *Sci Signal.* (2009) 2:ra1. doi: 10.1126/scisignal.2000140
20. Schmitt TM, Zúñiga-Pflücker JC. Induction of T Cell Development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity.* (2002) 17:749–56. doi: 10.1016/S1074-7613(02)00474-0
21. Cheng P, Nefedova Y, Corzo CA, Gabrilovich DI. Regulation of dendritic-cell differentiation by bone marrow stroma via different Notch ligands. *Blood.* (2007) 109:507–15. doi: 10.1182/blood-2006-05-025601
22. Rutz S, Mordmüller B, Sakano S, Scheffold A. Notch ligands Delta-like1, Delta-like4 and Jagged1 differentially regulate activation of peripheral T helper cells. *Eur J Immunol.* (2005) 35:2443–51. doi: 10.1002/eji.200526294
23. Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell.* (2004) 117:515–26. doi: 10.1016/S0092-8674(04)00451-9
24. Yamane H, Paul WE. Early signaling events that underlie fate decisions of naive CD4+ T cells toward distinct T−helper cell subsets. *Immunol Rev.* (2013) 252:12–23. doi: 10.1111/imr.12032
25. Backer RA, Helbig C, Gentek R, Kent A, Laidlaw BJ, Dominguez CX, et al. A central role for Notch in effector CD8+ T cell differentiation. *Nat Immunol.* (2014) 15:1143–51. doi: 10.1038/ni.3027
26. Amsen D, Gisbergen KPJM, van Hombrink P, van Lier RAW. Tissue-resident memory T cells at the center of immunity to solid tumors. *Nat Immunol.* (2018) 19:538. doi: 10.1038/s41590-018-0114-2
27. Intlekofer AM, Takemoto N, Wherry EJ, Longworth SA, Northrup JT, Palanivel VR, et al. Notch controls generation and function of human effector CD8+ T cells. *Blood.* (2013) 121:2638–46. doi: 10.1182/blood-2012-07-442962
28. Kangsamaksin T, Murtomaki A, Kofler NM, Cuervo H, Chaudhri RA, Tattersall IW, et al. notch decoys that selectively block DLL/Notch or JAG/Notch disrupt angiogenesis by unique mechanisms to inhibit tumor growth. *Cancer Discov.* (2015) 5:182–97. doi: 10.1158/2159-8290.CD-14-0650
29. Sierra RA, Thevenot P, Raber PL, Cui Y, Parsons C, Ochoa AC. Trillo-Tinoco, J., CD8+ T cells overcomes tumor-induced T-cell suppression and enhances immunotherapy in cancer. *Cancer Immunol Res.* (2014) 2:800–11. doi: 10.1158/2326-6066.CIR-14-0021
30. Palaga T, Miele L, Golde TE, Osborne BA. TCR-Mediated Notch signaling regulates proliferation and IFN-γ production in peripheral T cells. *J Immunol.* (2003) 171:3019–24. doi: 10.4049/jimmunol.171.6.3019
31. Mitra A, Shanthalingam S, Sherman HL, Singh K, Canakci M, Torres JA, et al. CD28 signaling drives notch ligand expression on CD4 T cells. *Front Immunol.* (2020) 11:735. doi: 10.3389/fimmu.2020.00735
32. Stahl M, Uemura K, Ge C, Shi S, Tashima Y, Stanley P. Roles of pofut1 and O-fucose in mammalian notch signaling. *J Biol Chem.* (2008) 283:13638–51. doi: 10.1074/jbc.M802027200
33. Haines N, Irvine KD. Glycosylation regulates notch signalling. *Nat Rev Mol Cell Biol.* (2003) 4:786–97. doi: 10.1038/nrm1228
34. Rampal R, Li ASY, Moloney DJ, Georgiou SA, Luther KB, Nita-Lazar A, et al. Lunatic fringe, manic fringe, and radical fringe recognize similar specificity determinants in o-fucosylated epidermal growth factor-like repeats. *J Biol Chem.* (2005) 280:42454–63. doi: 10.1074/jbc.M509552200
35. Kakuda S, Haltiwanger RS. Deciphering the fringe-mediated notch code: identification of activating and inhibiting sites allowing discrimination between ligands. *Dev Cell.* (2017) 40:193–201. doi: 10.1016/j.devcel.2016.12.013
36. Del Castillo V-HM, van der Weyden L, Nsengimana J, Speak AO, Sjöberg MK, Bishop DT, et al. Comparative genomics reveals that loss of lunatic fringe (LFNG) promotes melanoma metastasis. *Mol Oncol.* (2018) 12:239–55. doi: 10.1002/1878-0261.12161

37. Zhang S, Chung W, Wu G, Egan SE, Xu K. Tumor-suppressive activity of lunatic fringe in prostate through differential modulation of notch receptor activation. *Neoplasia.* (2014) 16:158–67. doi: 10.1593/neo.131870
38. Chung W-C, Zhang S, Challagundla L, Zhou Y, Xu K. Lunatic fringe and p53 cooperatively suppress mesenchymal stem-like breast cancer. *Neoplasia.* (2017) 19:885–95. doi: 10.1016/j.neo.2017.08.006
39. López-Arribillaga E, Rodilla V, Colomer C, Vert A, Shelton A, Cheng JH, et al. Manic Fringe deficiency imposes Jagged1 addiction to intestinal tumor cells. *Nat Commun.* (2018) 9:2992. doi: 10.1038/s41467-018-05385-0
40. Yi F, Amarasinghe B, Dang TP. Manic fringe inhibits tumor growth by suppressing Notch3 degradation in lung cancer. *Am J Cancer Res.* (2013) 3:490–9.
41. Tsukumo S, Hirose K, Maekawa Y, Kishihara K, Yasutomo K. Lunatic fringe controls T cell differentiation through modulating notch signaling. *J Immunol.* (2006) 177:8365–71. doi: 10.4049/jimmunol.177.12.8365
42. Yuan JS, Tan JB, Visan I, Matei IR, Urbanellis P, Xu K, et al. Lunatic fringe prolongs delta/notch-induced self-renewal of committed αβ T-cell progenitors. *Blood.* (2011) 117:1184–95. doi: 10.1182/blood-2010-07-296616
43. Song Y, Kumar V, Wei H-X, Qiu J, Stanley P. Lunatic, manic, and radical fringe each promote T and B cell development. *J Immunol.* (2016) 196:232–43. doi: 10.4049/jimmunol.1402421
44. Visan I, Tan JB, Yuan JS, Harper JA, Koch U, Guidos CJ. Regulation of T lymphopoiesis by Notch1 and Lunatic fringe–mediated competition for intrathymic niches. *Nat Immunol.* (2006) 7:634–43. doi: 10.1038/ni1345
45. Guha I, Bhuniya A, Shukla D, Patidar A, Nandi P, Saha A, et al. Tumor arrests DN2 to DN3 Pro T cell transition and promotes its conversion to thymic dendritic cells by reciprocally regulating notch1 and ikaros signaling. *Front Immunol.* (2020) 11:898. doi: 10.3389/fimmu.2020.00898
46. Ohm JE, Gabrilovich DI, Sempowski GD, Kisseleva E, Parman KS, Nadaf S, et al. VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. *Blood.* (2003) 101:4878–86. doi: 10.1182/blood-2002-07-1956
47. Lopez DM, Charyulu V, Adkins B. Influence of breast cancer on thymic function in mice. *J Mamm Gland Biol Neopl.* (2002) 9:191–9. doi: 10.1023/A:1020356020542
48. Gu W, Xu W, Ding T, Guo X. Fringe controls Naïve CD4+T cells differentiation through modulating notch signaling in asthmatic rat models. *PLoS One.* (2012) 7:e47288. doi: 10.1371/journal.pone.0047288
49. Mukherjee S, Rasky AJ, Lundy PA, Kittan NA, Kunkel SL, Maillard IP, et al. STAT5-induced lunatic fringe during Th2 development alters Delta-like 4-mediated Th2 cytokine production in respiratory syncytial virus-exacerbated airway allergic disease. *J Immunol.* (2014) 192:996–1003. doi: 10.4049/jimmunol.1301991
50. Zhu J, Cote-Sierra J, Guo L, Paul WE. Stat5 activation plays a critical role in Th2 differentiation. *Immunity.* (2003) 19:739–48. doi: 10.1016/S1074-7613(03)00292-9
51. Villarino AV, Kanno Y, Ferdinand JR, O'Shea JJ. Mechanisms of Jak/STAT signaling in immunity and disease. *J Immunol.* (2015) 194:21. doi: 10.4049/jimmunol.1401867
52. Delgoffe GM, Murray PJ, Vignali DA. Interpreting mixed signals: the cell's cytokine conundrum. *Curr Opin Immunol.* (2011) 23:632–8. doi: 10.1016/j.coi.2011.07.013
53. Verdeil G, Chaix J, Schmitt−Verhulst A-M, Auphan−Anezin N. Temporal cross-talk between TCR and STAT signals for CD8 T cell effector differentiation. *Eur J Immunol.* (2006) 36:3090–100. doi: 10.1002/eji.200636347
54. Saravia J, Chapman NM, Chi H. Helper T cell differentiation. *Cell Mol Immunol.* (2019) 16:634–43. doi: 10.1038/s41423-019-0220-6
55. Ong C-T, Sedy JR, Murphy KM, Kopan R. Notch and presenilin regulate cellular expansion and cytokine secretion but cannot instruct Th1/Th2 fate acquisition. *PLoS One.* (2008) 3:e2823. doi: 10.1371/journal.pone.0002823
56. Maekawa Y, Tsukumo S, Chiba S, Hirai H, Hayashi Y, Okada H, et al. Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity.* (2003) 19:549–59. doi: 10.1016/S1074-7613(03)00270-X
57. Minter LM, Turley DM, Das P, Shin HM, Joshi I, Lawlor RG, et al. Inhibitors of γ-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21. *Nat Immunol.* (2005) 6:680–8. doi: 10.1038/ni1209x
58. Shin HM, Tilahun ME, Cho OH, Chandiran K, Kuksin CA, Keerthivasan S, et al. NOTCH1 can initiate NF-κB activation via cytosolic interactions with components of the T cell signalosome. *Front Immunol.* (2014) 5:249. doi: 10.3389/fimmu.2014.00249
59. Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. *Nat Immunol.* (2000) 1:199–205. doi: 10.1038/79734
60. Grogan JL, Mohrs M, Harmon B, Lacy DA, Sedat JW, Locksley RM. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity.* (2001) 14:205–15. doi: 10.1016/S1074-7613(01)00103-0
61. Lehar SM, Bevan MJ. Immunology: polarizing a T-cell response. *Nature.* (2004) 430:150–1. doi: 10.1038/430150a
62. Laky K, Evans S, Perez-Diez A, Fowlkes BJ. Notch signaling regulates antigen sensitivity of naive CD4+ T cells by tuning co-stimulation. *Immunity.* (2015) 42:80–94. doi: 10.1016/j.immuni.2014.12.027
63. Tindemans I, Peeters MJW, Hendriks RW. Notch signaling in T helper cell subsets: instructor or unbiased amplifier? *Front Immunol.* (2017) 8:419. doi: 10.3389/fimmu.2017.00419
64. Nandagopal N, Santat LA, LeBon L, Sprinzak D, Bronner ME, Elowitz MB. Dynamic ligand discrimination in the notch signaling pathway. *Cell* (2018) 172:869–80.e19. doi: 10.1016/j.cell.2018.01.002
65. Yaron A, Sprinzak D. The cis side of juxtacrine signaling: a new role in the development of the nervous system. *Trends Neurosci.* (2012) 35:230–9. doi: 10.1016/j.tins.2011.12.003
66. LeBon L, Lee TV, Sprinzak D, Jafar-Nejad H, Elowitz MB. Fringe proteins modulate Notch-ligand cis and trans interactions to specify signaling states. *Elife.* (2014) 3:e02950. doi: 10.7554/eLife.04998
67. Boareto M, Jolly MK, Lu M, Onuchic JN, Clementi C, Ben-Jacob E. Jagged–Delta asymmetry in Notch signaling can give rise to a Sender/Receiver hybrid phenotype. *Proc Natl Acad Sci USA.* (2015) 112:E402–9. doi: 10.1073/pnas.1416287112
68. Hozumi K. Distinctive properties of the interactions between Notch and Notch ligands. *Dev Growth Different.* (2020) 62:49–58. doi: 10.1111/dgd.12641
69. Nobili S, Lippi D, Witort E, Donnini M, Bausi L, Mini E, et al. Natural compounds for cancer treatment and prevention. *Pharmacol Res.* (2009) 59:365–78. doi: 10.1016/j.phrs.2009.01.017
70. Huang C-Y, Ju D-T, Chang C-F, Muralidhar RP, Velmurugan BK. A review on the effects of current chemotherapy drugs and natural agents in treating non–small cell lung cancer. *Biomedicine (Taipei).* (2017) 7:23. doi: 10.1051/bmdcn/2017070423
71. Blagodatski A, Yatsunskaya M, Mikhailova V, Tiasto V, Kagansky A, Katanaev VL. Medicinal mushrooms as an attractive new source of natural compounds for future cancer therapy. *Oncotarget.* (2018) 9:29259–74. doi: 10.18632/oncotarget.25660
72. Kopan R, Ilagan MXG. γ-Secretase: proteasome of the membrane? *Nat Rev Mol Cell Biol.* (2004) 5:499–504. doi: 10.1038/nrm1406
73. Olsauskas-Kuprys R, Zlobin A, Osipo C. Gamma secretase inhibitors of Notch signaling. *Onco Targets Ther.* (2013) 6:943–55. doi: 10.2147/OTT.S33766
74. de Pooter RF, Zúñiga-Pflücker JC. Generation of immunocompetent T cells from embryonic stem cells. In: Fairchild PJ editor. *Immunological Tolerance: Methods and Protocols*. Totowa, NJ: Humana Press (2007). p. 73–81. doi: 10.1007/978-1-59745-395-0_5
75. Zhao Y, Parkhurst MR, Zheng Z, Cohen CJ, Riley JP, Gattinoni L, et al. Extrathymic generation of tumor-specific T cells from genetically engineered human hematopoietic stem cells via notch signaling. *Cancer Res.* (2007) 67:2425–9. doi: 10.1158/0008-5472.CAN-06-3977
76. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, et al. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest.* (2005) 115:1616–26. doi: 10.1172/JCI24480
77. Kondo T, Morita R, Okuzono Y, Nakatsukasa H, Sekiya T, Chikuma S, et al. Notch-mediated conversion of activated T cells into stem cell memory-like T cells for adoptive immunotherapy. *Nat Commun.* (2017) 8:1–14. doi: 10.1038/ncomms15338

78. Mathieu M, Cotta−Grand N, Daudelin J-F, Thébault P, Labrecque N. Notch signaling regulates PD-1 expression during CD8+ T-cell activation. *Immunol Cell Biol.* (2013) 91:82–8. doi: 10.1038/icb.2012.53
79. Hurlbut GD, Kankel MW, Lake RJ, Artavanis-Tsakonas S. Crossing paths with Notch in the hyper-network. *Curr Opin Cell Biol Cell Regulat.* (2007) 19:166–75. doi: 10.1016/j.ceb.2007.02.012
80. Steinbuck MP, Arakcheeva K, Winandy S. Novel TCR-Mediated mechanisms of notch activation and signaling. *J Immunol.* (2018) 200:997–1007. doi: 10.4049/jimmunol.1700070
81. Britton GJ, Ambler R, Clark DJ, Hill EV, Tunbridge HM, McNally KE, et al. PKCθ links proximal T cell and Notch signaling through localized regulation of the actin cytoskeleton. *ELife.* (2017) 6:e20003. doi: 10.7554/eLife.20003
82. Monsalve E, Pérez MA, Rubio A, Ruiz-Hidalgo MJ, Baladrón V, García-Ramírez JJ, et al. Notch-1 Up-regulation and signaling following macrophage activation modulates gene expression patterns known to affect antigen-presenting capacity and cytotoxic activity. *J Immunol.* (2006) 176:5362–73. doi: 10.4049/jimmunol.176.9.5362
83. Leone RD, Lo Y-C, Powell JD. A2aR antagonists: next generation checkpoint blockade for cancer immunotherapy. *Comput Struct Biotechnol J.* (2015) 13:265–72. doi: 10.1016/j.csbj.2015.03.008
84. Sorrentino C, Hossain F, Rodriguez PC, Sierra RA, Pannuti A, Hatfield S, et al. Adenosine A2A receptor stimulation inhibits TCR-induced notch1 activation in CD8+T-cells. *Front Immunol.* (2019) 10:162. doi: 10.3389/fimmu.2019.00162
85. Vas V, Szilágyi L, Pálóczi K, Uher F. Soluble Jagged-1 is able to inhibit the function of its multivalent form to induce hematopoietic stem cell self-renewal in a surrogate in vitro assay. *J Leuk Biol.* (2004) 75:714–20. doi: 10.1189/jlb.1003462
86. Funahashi Y, Hernandez SL, Das I, Ahn A, Huang J, Vorontchikhina M, et al. A Notch1 ectodomain construct inhibits endothelial notch signaling tumor growth, and angiogenesis. *Cancer Res.* (2008) 68:4727–35. doi: 10.1158/0008-5472.CAN-07-6499
87. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol.* (2009) 157:220–33. doi: 10.1111/j.1476-5381.2009.00190.x
88. Klose R, Berger C, Moll I, Adam MG, Schwarz F, Mohr K, et al. Soluble Notch ligand and receptor peptides act antagonistically during angiogenesis. *Cardiovasc Res.* (2015) 107:153–63. doi: 10.1093/cvr/cvv151
89. Lin L, Mernaugh R, Yi F, Blum D, Carbone DP, Dang TP. Targeting specific regions of the notch3 ligand-binding domain induces apoptosis and inhibits tumor growth in lung cancer. *Cancer Res.* (2010) 70:632–8. doi: 10.1158/0008-5472.CAN-09-3293
90. Nickoloff B, Qin J-Z, Chaturvedi V, Denning M, Bonish B, Miele L. Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kB and PPARg. *Cell Death Different.* (2020) 14:842–55. doi: 10.1038/sj.cdd.4401036
91. Li L, Milner LA, Deng Y, Iwata M, Banta A, Graf L, et al. The human homolog of Rat jagged1expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. *Immunity.* (1998) 8:43–55. doi: 10.1016/S1074-7613(00)80457-4
92. Masuya M, Katayama N, Hoshino N, Nishikawa H, Sakano S, Araki H, et al. The soluble notch ligand, Jagged-1, inhibits proliferation of CD34+ macrophage progenitors. *Int J Hematol.* (2002) 75:269–76. doi: 10.1007/BF02982040
93. Varnum-Finney B, Wu L, Yu M, Brashem-Stein C, Staats S, Flowers D, et al. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. *J Cell Sci.* (2000) 113(Pt 23):4313–8.
94. Terpe K. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol.* (2003) 60:523–33. doi: 10.1007/s00253-002-1158-6
95. Stanley P, Guidos CJ. Regulation of notch signaling during T- and B-cell development by O-fucose glycans. *Immunol Rev.* (2009) 230:201–15. doi: 10.1111/j.1600-065X.2009.00791.x
96. Luca VC, Jude KM, Pierce NW, Nachury MV, Fischer S, Garcia KC. Structural basis for Notch1 engagement of Delta-like 4. *Science.* (2015) 347:847–53. doi: 10.1126/science.1261093
97. Luca VC, Kim BC, Ge C, Kakuda S, Wu D, Roein-Peikar M, et al. Notch-Jagged complex structure implicates a catch bond in tuning ligand sensitivity. *Science.* (2017) 355:1320–4. doi: 10.1126/science.aaf9739
98. Schneider M, Kumar V, Nordstrøm LU, Feng L, Takeuchi H, Hao H, et al. Inhibition of Delta-induced Notch signaling using fucose analogs. *Nat Chem Biol.* (2018) 14:65–71. doi: 10.1038/nchembio.2520
99. Driss V, Quesnel B, Brinster C. Monocyte chemoattractant protein 1 (MCP-1/CCL2) contributes to thymus atrophy in acute myeloid leukemia. *Eur J Immunol.* (2015) 45:396–406. doi: 10.1002/eji.201444736
100. Carrio R, Torroella-Kouri M, Iragavarapu-Charyulu V, Lopez DM. Tumor-induced thymic atrophy: alteration in interferons and Jak/Stats signaling pathways. *Int J Oncol.* (2011) 38:547–53. doi: 10.3892/ijo.2010.870
101. Wang W, Thomas R, Sizova O, Su D-M. Thymic function associated with cancer development, relapse, and antitumor immunity – a mini-review. *Front Immunol.* (2020) 11:773. doi: 10.3389/fimmu.2020.00773
102. Nitta T, Nitta S, Lei Y, Lipp M, Takahama Y. CCR7-mediated migration of developing thymocytes to the medulla is essential for negative selection to tissue-restricted antigens. *Proc Natl Aacd Sci USA.* (2009) 106:17129–33. doi: 10.1073/pnas.0906956106
103. Ueno T, Hara K, Willis MS, Malin MA, Höpken UE, Gray DHD, et al. Role for CCR7 ligands in the emigration of newly generated T lymphocytes from the neonatal thymus. *Immunity.* (2002) 16:205–18. doi: 10.1016/S1074-7613(02)00267-4
104. Chen ELY, Thompson PK, Zúñiga-Pflücker JC. RBPJ-dependent Notch signaling initiates the T cell program in a subset of thymus-seeding progenitors. *Nat Immunol.* (2019) 20:1456–68. doi: 10.1038/s41590-019-0518-7
105. Zhou J, Cheng P, Youn J-I, Cotter MJ, Gabrilovich DI. Notch and wnt signaling cooperate in regulation of dendritic cell differentiation. *Immunity.* (2009) 30:845–59. doi: 10.1016/j.immuni.2009.03.021
106. Mastio J, Simand C, Cova G, Kastner P, Chan S, Kirstetter P. Ikaros cooperates with Notch activation and antagonizes TGFβ signaling to promote pDC development. *PLoS Genet.* (2018) 14:e1007485. doi: 10.1371/journal.pgen.1007485
107. Dumortier A, Jeannet R, Kirstetter P, Kleinmann E, Sellars M, dos Santos NR, et al. Notch activation is an early and critical event during T-cell leukemogenesis in Ikaros-deficient mice. *Mol Cell Biol.* (2006) 26:209–20. doi: 10.1128/MCB.26.1.209-220.2006
108. Kleinmann E, Geimer Le Lay A-S, Sellars M, Kastner P, Chan S. Ikaros represses the transcriptional response to notch signaling in T-cell development. *Mol Cell Biol.* (2008) 28:7465–75. doi: 10.1128/MCB.00715-08
109. Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, et al. Deficient T cell fate specification in mice with an induced inactivation of notch1. *Immunity.* (1999) 10:547–58. doi: 10.1016/S1074-7613(00)80054-0
110. Fiorini E, Merck E, Wilson A, Ferrero I, Jiang W, Koch U, et al. Dynamic regulation of notch 1 and notch 2 surface expression during T cell development and activation revealed by novel monoclonal antibodies. *J Immunol.* (2009) 183:7212–22. doi: 10.4049/jimmunol.0902432
111. Kaiserlian D, Savino W, Hassid J, Dardenne M. Studies of the thymus in mice bearing the Lewis lung carcinoma. III. Possible mechanisms of tumor-induced thymic atrophy. *Clin Immunol Immunopathol.* (1984) 32:316–25. doi: 10.1016/0090-1229(84)90275-7
112. Huang Y, Chen X, Dikov MM, Novitskiy SV, Mosse CA, Yang L, et al. Distinct roles of VEGFR-1 and VEGFR-2 in the aberrant hematopoiesis associated with elevated levels of VEGF. *Blood.* (2007) 110:624–31. doi: 10.1182/blood-2007-01-065714
113. Afkarian M, Sedy JR, Yang J, Jacobson NG, Cereb N, Yang SY, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naïve CD4 + T cells. *Nat Immunol.* (2002) 3:549–57. doi: 10.1038/ni794
114. Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH. Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. *Science.* (2002) 295:338–42. doi: 10.1126/science.1065543
115. Marshall HD, Chandele A, Jung YW, Meng H, Poholek AC, Parish IA, et al. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4+ cell properties during viral infection. *Immunity.* (2011) 35:633–46. doi: 10.1016/j.immuni.2011.08.016

116. Kibirova A, Mattes MD, Smolkin M, Ma PC. The journey of an EGFR-mutant lung adenocarcinoma through erlotinib, osimertinib and ABCP immunotherapy regimens: sensitivity and resistance. *Case Rep Oncol.* (2019) 12:765–76. doi: 10.1159/000503417
117. Santoni-Rugiu E, Melchior LC, Urbanska EM, Jakobsen JN, de Stricker K, Grauslund M, et al. Intrinsic resistance to EGFR-tyrosine kinase inhibitors in EGFR-mutant non-small cell lung cancer: differences and similarities with acquired resistance. *Cancers (Basel).* (2019) 11:923. doi: 10.3390/cancers11070923
118. Peng S, Wang R, Zhang X, Ma Y, Zhong L, Li K, et al. EGFR-TKI resistance promotes immune escape in lung cancer via increased PD-L1 expression. *Mol Cancer.* (2019) 18:165. doi: 10.1186/s12943-019-1073-4
119. Isomoto K, Haratani K, Hayashi H, Shimizu S, Tomida S, Niwa T, et al. Impact of EGFR-TKI treatment on the tumor immune microenvironment in EGFR mutation–positive non–small cell lung cancer. *Clin Cancer Res.* (2020) 26:2037–46. doi: 10.1158/1078-0432.CCR-19-2027
120. Ayeni D, Miller B, Kuhlmann A, Ho P-C, Robles-Oteiza C, Gaefele M, et al. Tumor regression mediated by oncogene withdrawal or erlotinib stimulates infiltration of inflammatory immune cells in EGFR mutant lung tumors. *J. Immunother Cancer.* (2019) 7:172. doi: 10.1186/s40425-019-0643-8
121. Akbay EA, Koyama S, Carretero J, Altabef A, Tchaicha JH, Christensen CL, et al. Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. *Cancer Discovery.* (2013) 3:1355–63. doi: 10.1158/1535-7163.TARG-13-B290
122. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non–small-cell lung cancer. *New Engl J Med.* (2015) 373:1627–39. doi: 10.1056/NEJMoa1507643
123. Hastings K, Yu HA, Wei W, Sanchez-Vega F, DeVeaux M, Choi J, et al. EGFR mutation subtypes and response to immune checkpoint blockade treatment in non-small-cell lung cancer. *Ann Oncol.* (2019) 30:1311–20. doi: 10.1093/annonc/mdz141
124. Lee CK, Man J, Lord S, Links M, Gebski V, Mok T, et al. Checkpoint inhibitors in metastatic EGFR-mutated non–small cell lung cancer—a meta-analysis. *J Thorac Oncol.* (2017) 12:403–7. doi: 10.1016/j.jtho.2016.10.007
125. Andersson ER, Sandberg R, Lendahl U. Notch signaling simplicity in design, versatility in function. *Development.* (2011) 138:3593–612. doi: 10.1242/dev.063610
126. De La Coste A, Freitas AA. Notch signaling: distinct ligands induce specific signals during lymphocyte development and maturation. *Immunol Lett.* (2006) 102:1–9. doi: 10.1016/j.imlet.2005.06.014
127. Phng L-K, Gerhardt H. Angiogenesis: a team effort coordinated by notch. *Dev Cell.* (2009) 16:196–208. doi: 10.1016/j.devcel.2009.01.015
128. Bretscher PA. A two-step, two-signal model for the primary activation of precursor helper T cells. *Proc Natl Acad Sci USA.* (1999) 96:185–90. doi: 10.1073/pnas.96.1.185
129. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol.* (2013) 13:227–42. doi: 10.1038/nri3405
130. Deborah J, Lenschow TL, Walunas JAB. CD28/B7 System Of T Cell Costimulation. *Ann Rev Immunol.* (1996) 14:233–58. doi: 10.1146/annurev.immunol.14.1.233
131. Hoyne GF, Le Roux I, Corsin-Jimenez M, Tan K, Dunne J, Forsyth LMG, et al. Serrate1-induced Notch signalling regulates the decision between immunity and tolerance made by peripheral CD4+ T cells. *Int Immunol.* (2000) 12:177–85. doi: 10.1093/intimm/12.2.177
132. Yvon ES, Vigouroux S, Rousseau RF, Biagi E, Amrolia P, Dotti G, et al. Overexpression of the Notch ligand, Jagged-1, induces alloantigen-specific human regulatory T cells. *Blood.* (2003) 102:3815–21. doi: 10.1182/blood-2002-12-3826
133. Vigouroux S, Yvon E, Wagner H-J, Biagi E, Dotti G, Sili U, et al. Induction of antigen-specific regulatory T cells following overexpression of a notch ligand by human B lymphocytes. *J Virol.* (2003) 77:10872–80. doi: 10.1128/JVI.77.20.10872-10880.2003
134. Gopisetty A, Bhattacharya P, Haddad C, Bruno JC, Vasu C, Miele L, et al. OX40L/Jagged1 cosignaling by GM-CSF–induced bone marrow-derived dendritic cells is required for the expansion of functional regulatory T cells. *J Immunol.* (2013) 190:5516–25. doi: 10.4049/jimmunol.1202298
135. Cahill EF, Tobin LM, Carty F, Mahon BP, English K. Jagged-1 is required for the expansion of CD4+ CD25+ FoxP3+ regulatory T cells and tolerogenic dendritic cells by murine mesenchymal stromal cells. *Stem Cell Res Therapy.* (2015) 6:19. doi: 10.1186/s13287-015-0021-5
136. Lin Y, Chen W, Li J, Yan G, Li C, Jin N, et al. Overexpression of Jagged-1 combined with blockade of CD40 pathway prolongs allograft survival. *Immunol Cell Biol.* (2015) 93:213–7. doi: 10.1038/icb.2014.84
137. Hansson EM, Lanner F, Das D, Mutvei A, Marklund U, Ericson J, et al. Control of Notch-ligand endocytosis by ligand-receptor interaction. *J Cell Sci.* (2010) 123:2931–42. doi: 10.1242/jcs.073239
138. Parks AL, Klueg KM, Stout JR, Muskavitch MA. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development.* (2000) 127:1373–85.
139. Charbonnier L-M, Wang S, Georgiev P, Sefik E, Chatila TA. Control of peripheral tolerance by regulatory T cell–intrinsic Notch signaling. *Nat Immunol.* (2015) 16:1162–73. doi: 10.1038/ni.3288
140. Wei P, Walls M, Qiu M, Ding R, Denlinger RH, Wong A, et al. Evaluation of selective γ-secretase inhibitor PF-03084014 for Its antitumor efficacy and gastrointestinal safety to guide optimal clinical trial design. *Mol Cancer Ther.* (2010) 9:1618–28. doi: 10.1158/1535-7163.MCT-10-0034
141. Hayashi I, Takatori S, Urano Y, Miyake Y, Takagi J, Sakata-Yanagimoto M, et al. Neutralization of the γ-secretase activity by monoclonal antibody against extracellular domain of nicastrin. *Oncogene.* (2012) 31:787–98. doi: 10.1038/onc.2011.265
142. Moellering RE, Cornejo M, Davis TN, Del Bianco C, Aster JC, Blacklow SC, et al. Direct inhibition of the Notch transcription factor complex. *Nature.* (2009) 462:182–8. doi: 10.1038/nature08543
143. KleinJan A, Tindemans I, Montgomery JE, Lukkes M, de Bruijn MJW, van Nimwegen M, et al. The Notch pathway inhibitor stapled α-helical peptide derived from mastermind-like 1 (SAHM1) abrogates the hallmarks of allergic asthma. *J Aller Clin Immunol.* (2018) 142:76–85.e8. doi: 10.1016/j.jaci.2017.08.042
144. Pietanza MC, Spira AI, Jotte RM, Gadgeel SM, Mita AC, Hart LL, et al. Final results of phase Ib of tarextumab (TRXT, OMP-59R5, anti-Notch2/3) in combination with etoposide and platinum (EP) in patients (pts) with untreated extensive-stage small-cell lung cancer (ED-SCLC). *JCO.* (2015) 33:7508–7508. doi: 10.1200/jco.2015.33.15_suppl.7508
145. Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y, et al. Therapeutic antibody targeting of individual Notch receptors. *Nature.* (2010) 464:1052–7. doi: 10.1038/nature08878
146. Smith DC, Eisenberg PD, Manikhas G, Chugh R, Gubens MA, Stagg RJ, et al. A Phase I dose escalation and expansion study of the anticancer stem cell agent demcizumab (Anti-DLL4) in patients with previously treated solid tumors. *Clin Cancer Res.* (2014) 20:6295–303. doi: 10.1158/1078-0432.CCR-14-1373
147. Chiorean EG, LoRusso P, Strother RM, Diamond JR, Younger A, Messersmith WA, et al. A Phase I first-in-human study of enoticumab (REGN421), a fully human delta-like ligand 4 (Dll4) monoclonal antibody in patients with advanced solid tumors. *Clin Cancer Res.* (2015) 21:2695–703. doi: 10.1158/1078-0432.CCR-14-2797

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Identification of an Immune Signature Predicting Prognosis Risk and Lymphocyte Infiltration in Colon Cancer

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Increasing studies have highlighted the effects of the tumor immune micro-environment (TIM) on colon cancer (CC) tumorigenesis, prognosis, and metastasis. However, there is no reliable molecular marker that can effectively estimate the immune infiltration and predict the CC relapse risk. Here, we leveraged the gene expression profile and clinical characteristics from 1430 samples, including four gene expression omnibus database (GEO) databases and the cancer genome atlas (TCGA) database, to construct an immune risk signature that could be used as a predictor of survival outcome and immune activity. A risk model consisting of 10 immune-related genes were screened out in the Lasso-Cox model and were then aggregated to generate the immune risk signature based on the regression coefficients. The signature demonstrated robust prognostic ability in discovery and validation datasets, and this association remained significant in the multivariate analysis after controlling for age, gender, clinical stage, or microsatellite instability status. Leukocyte subpopulation analysis indicated that the low-risk signature was enriched with cytotoxic cells (activated CD4/CD8$^+$ T cell and NK cell) and depleted of myeloid-derived suppressor cells (MDSC) and regulatory T cells. Further analysis indicated patients with a low-risk signature harbored higher tumor mutation loads and lower mutational frequencies in significantly mutated genes of *APC* and *FBXW7*. Together, our constructed signature could predict prognosis and represent the TIM of CC, which promotes individualized treatment and provides a promising novel molecular marker for immunotherapy.

Keywords: colon cancer, immune signature, tumor mutation load, immune infiltration, significantly mutated genes

INTRODUCTION

Colon cancer (CC) is one of the most common cancers and remains one of the leading causes of cancer death worldwide (1). Despite continuous achievements in early CC detection, treatment, and management leading to reductions in the incidence and mortality, 30–50% of patients develop recurrence or metastasis within five years of treatment (2). Therefore, in addition to the current clinical and pathological factors for determining the disease prognosis and patient survival, reliable and robust new molecular markers are urgently needed to improve the personalized therapy for CC patients.

Numerous studies have recently highlighted the effects of the immune microenvironment on cancer tumorigenesis, development, and metastasis (3–5). Indeed, assessment of the enrichment of tumor-infiltrating lymphocytes (TIL) was demonstrated to be an important complement role to the TNM staging system for relapse and mortality prediction in CC (6–8). Besides, recent immunotherapies targeting specific immune checkpoints such as PD-1/L1 have demonstrated a remarkably durable response in CC treatment (9, 10). Patients with certain histopathologic patterns, such as intratumoral infiltration by cytotoxic lymphocytes and tumor neoepitope burden, have also been reported with a better clinical prognosis (11–13). Conventional methods for detecting the tumor immune infiltrate, such as flow cytometry or immunohistochemistry (IHC), cannot comprehensively evaluate the immune effects due to the limitation of the number of immune markers. As an alternative, continuously accumulating transcriptomics data provides an ideal resource for large-scale immune landscape analysis (14). However, there has been no appropriate signature that can systematically evaluate the tumor immune micro-environment (TIM) based on immune-related genes and predict the patients' survival or response to immunotherapies of CC patients. Therefore, it's essential to develop a reliable immune signature on the basis of a comprehensive list of immune-related genes to represent the immune status of TIM and have the prognostic ability of CC.

In this study, we concentrated on constructing an immune signature with survival prediction and lymphocyte infiltration estimation ability based on the comprehensive list of immune-related genes curated from The Immunology Database and Analysis Portal (ImmPort) database (15). The microarray data from the gene expression omnibus database (GEO) database and RNA sequencing (RNA-seq) data from the cancer genome atlas (TCGA) database were used for analysis and validation. We then evaluated whether this signature was associated with survival outcomes and clinicopathological factors in the CC subgroups. Furthermore, we tried to figure out the relationship between the signature and tumor immune-related indexes including TIL and tumor mutation load (TML) in CC. And finally, we evaluated the effects of this risk signature in identifying the immune responders from immune check-point inhibitors (ICI) therapy. Findings gleaned from this study may be valuable for predicting patients' prognosis and guiding immunotherapy treatment for CC.

MATERIALS AND METHODS

Publicly Attainable Expression Datasets and Immune-Related Genes

Gene expression data and clinical features of CC samples were retrospectively collected from publicly available datasets at the NCBI GEO database[1] and at TCGA[2]. The selection criteria of CC datasets were adopted from the workflow of the Dai et.al study (16). A total of 1430 patients were enrolled for analysis, including GSE39582 (N = 557) (17), GSE17538 (N = 200) (18), GSE37892 (N = 130) (19), and GSE33113 (N = 90) (20), and TCGA (N = 453). The GSE14333 (21) dataset was excluded from this analysis owing to the fact that its probe cell intensity (CEL) files overlapped extensively with the GSE17538 series. Among these cohorts, GSE39582 was the largest set consisting of 557 CC samples, and hence, it was marked as a discovery series and used for constructing the gene signature. Considering the small sample sizes of the GSE17538, GSE33113, and GSE37892 cohorts, and the fact that they shared the same microarray sequencing platform (Affymetrix HG-U133 plus 2.0), we integrated the three datasets into a combined large cohort and regarded this as external validation. The ComBat method from the SVA R package was used to remove the batch effects among different GEO datasets (16). The clinical and survival information of the included datasets was summarized in **Supplementary Table S1**. An immunotherapeutic cohort of advanced urothelial cancer (IMvigor210 cohort) treated with atezolizumab (anti-PD-L1 McAb) were utilized to further validate the efficiency of the immune risk signature (22). The detailed clinical annotations and complete gene expression profile of the anti-PD-L1 cohort were obtained from http://research-pub.gene.com/IMvigor210CoreBiologies. The comprehensive list of immune-related genes, containing a total of 1508 genes, was downloaded from the ImmPort database[3] (15).

Tumor Mutational Load and Neoantigen Analysis

The somatic mutational profile of colon adenocarcinoma (COAD) in the Cancer Genome Atlas (TCGA) mutation annotation format (MAF) were downloaded from Genomic Data Commons (GDC)[4]. Non-synonymous mutations counts were recognized as TML and used for investigating the relationship with the immune signature. Non-synonymous mutations included splice site mutation, missense mutation, nonsense mutation, in-frame mutation, and frameshift mutation. Neoantigens of COAD were collected from previously published studies (23). The antigen peptides resulting from

[1]https://www.ncbi.nlm.nih.gov/geo/
[2]https://cancergenome.nih.gov/
[3]https://immport.niaid.nih.gov
[4]https://portal.gdc.cancer.gov/

non-synonymous mutated HLA sequences with predicted binding affinities below 500 nM are defined as neoantigens (24).

Gene Set Enrichment Analysis

The R packages limma (25) were used to evaluate differential expression of more than 21,000 genes in samples with different risk groups. To specify, the expression data were background corrected and quantile normalized and probe sets were summarized using RMA with the affy R package. Subsequently, the normalized expression data were then fed into lmFit and eBayes functions to calculate the differential statistics with the limma package. The logFC produced by limma was used as an input to perform gene set enrichment analysis (GSEA) (26) against the REACTOME reference gene set (MSigDB database v7.1). The fast GSEA algorithm implemented in the Bioconductor R package fgsea was used.

Immune Cell Infiltration Estimation With ssGSEA

The relative infiltration of 28 immune cell types in the CC tumor microenvironment were quantified by the single sample gene set enrichment analysis (ssGSEA) (27). Special feature gene panels for each immune cell subset were curated from a recent research (4, 28). The relative abundance of each immune cell type was represented by an enrichment score in the ssGSEA analysis. The ssGSEA score was normalized to unify distribution from 0 to 1 for each immune cell type. The bio-similarity of the immune cell filtration was estimated by multidimensional scaling (MDS) and a Gaussian fitting model.

Quantify the Immunotherapy Response Predictor: Immunophenoscore

The superior immune response molecular marker, Immunophenoscore (28), was used to characterize the intratumoral immune landscapes and the cancer antigenomes. The scoring scheme was created from a panel of immune-related genes belonging to the four clusters: major histocompatibility complex (MHC)-related molecules, checkpoints or immunomodulators, effector cells, and suppressor cells. For each class, a sample-wise Z score from gene expression data was extracted and calculated. The weighted averaged Z score was then calculated by averaging the Z scores within the respective category leading to four values, and the sum of the weighted averaged Z score of the four categories.

Significantly Mutated Genes

We identified significantly mutated genes (SMG) by using the MutSigCV algorithm (29). MutSigCV measures the significant enrichment of non-silent somatic mutations in a gene by addressing mutational context-specific background mutation rates. Candidate SMGs were required to meet these criteria: statistically significant ($q < 0.1$) and expressed in the human cancer cell lines Encyclopedia (CCLE) (30).

Deciphering Mutational Signature Operative in the Genome

The R package Maftools proposed by Mayakonda et al. (31) was used to extract mutational signatures from the TCGA genomic data. The ExtractSignatures function based on Bayesian variant non-negative matrix factorization, factorized the mutation portrait matrix into two non-negative matrices "***signatures***" and "***contributions***," where "***signatures***" represented mutational processes and "***contributions***" represented the corresponding mutational activities. Specifically, the number of columns of matrix "***signatures***" indicated the number of extracted signatures and the rows indicated the 96 mutational contexts (i.e., C > G, C > A, C > T, T > C, T > A, T > G and combined their 5′ and 3′ adjacent bases). The SignatureEnrichment function can automatically determine the optimal number of extracted mutational signatures and assign them to each sample based on the mutational activities. The extracted mutational portrait of CC was compared and annotated by cosine similarity analysis against the Catalogue of Somatic Mutations in Cancer (COSMIC) (32).

Statistical Analysis

The statistical analysis in this study was generated by R-3.6.1. For quantitative data, statistical significance for comparisons of two groups or more than two groups was estimated by the Wilcoxon rank-sum test or the Kruskal–Wallis H test, respectively. Fisher's exact test was employed for comparisons of qualitative variables. Logistic regression analysis was used to test the association between TML and risk signature. For the genes with prognostic ability, the Cox proportional hazards model with a Lasso penalty (iteration = 1000) was employed to find the best gene model utilizing the R package "glmnet." The immune signature was based on the linear combination of the selected mRNA expression level and weighted by their Lasso-Cox regression coefficients. The association between immune signature and prognosis were analyzed by the Cox proportional hazards model and the Kaplan-Meier survival analysis with the R survival package (Survminer 0.4.7). The receiver operating characteristic (ROC) curve was used to assess the prognosis classification performance of the immune risk signature and tumor stage, and the area under the curve (AUC) was compared by DeLong's test. All comparisons were two-sided with an alpha level of 0.05, and multiple hypothesis testing with the Benjamini-Hochberg method was used to control false discovery rate (FDR) (33).

RESULTS

Construction of Immune Risk Signature

After removing samples without necessary clinicopathological or follow-up data, a total of 1430 CC patients were included for this analysis, including GSE39582 (N = 557), GSE17538 (N = 200), GSE37892 (N = 130), and GSE33113 (N = 90), and TCGA (N = 453). Considering that the GSE39582 cohort from the Marisa et al. contained the largest sample size (N = 557) and included detailed clinical features, we therefore selected it as the discovery dataset to identify the immune risk signature

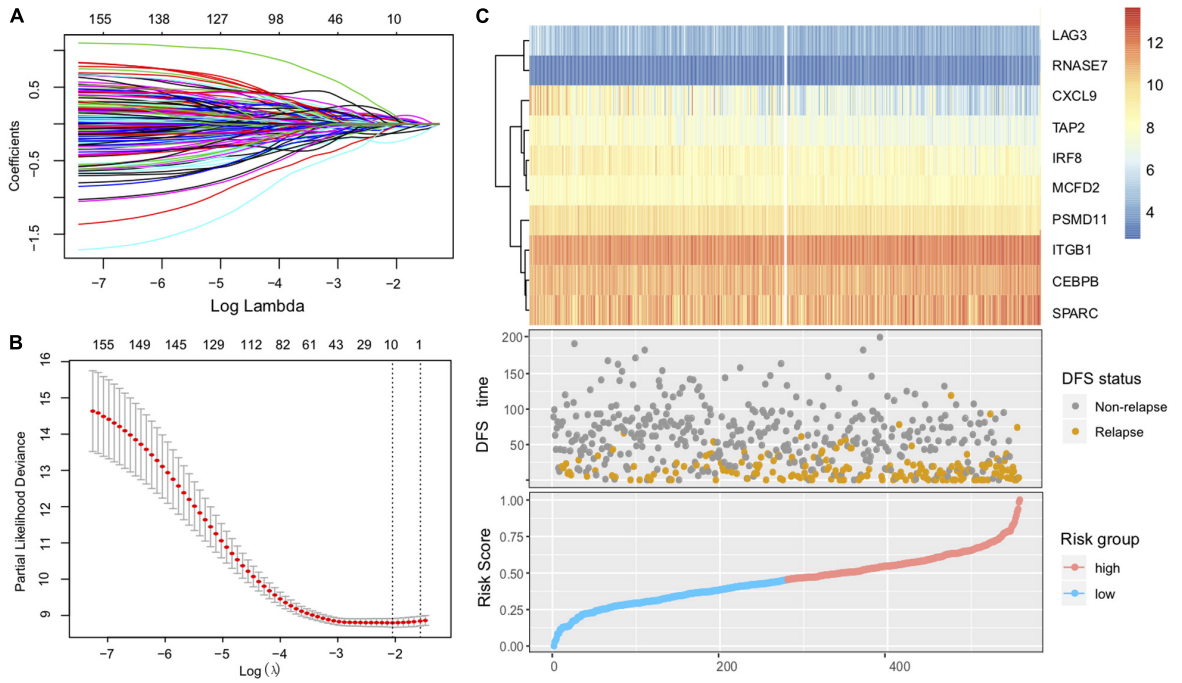

FIGURE 1 | Construction of the immune risk signature model. **(A)** Lasso coefficient profiles of the 161 prognosis-associated immune genes from the discovery (GSE39582 microarray) dataset. **(B)** Partial likelihood deviance of variables revealed by the Lasso regression model. The red dots represented the partial likelihood of deviance values, the gray lines represented the standard error (SE), the two vertical dotted lines on the left and right represented optimal values by minimum criteria and 1-SE criteria, respectively. **(C)** Heatmap of the signature consisting of 10 immune-related genes and the risk score curve based on the Lasso coefficients. Patients were divided into high-risk and low-risk groups and the median risk score was utilized as the cutoff value.

that is associated with CC patients' prognosis. The univariate analysis was performed in all of the 1508 immune-related genes for the discovery dataset (GSE39582). Through the univariate analysis and log-rank test, a total of 161 genes with prognostic ability were identified ($P < 0.05$). The 161 immune-related genes were then subjected to the Lasso-Cox proportional hazards regression and tenfold cross-validation to generate the best gene model. The Lasso coefficient profile plot was produced against the log(k) sequence, and the minimize k method resulted in 10 optimal coefficients (**Figures 1A,B**). Finally, a gene model with 10 immune-related genes reached the optimal regression efficiency to speculate the prognostic ability.

The identified immune-related genes included antigen processing and presentation related genes (*LAG3, PSMD11, TAP2*), defense response to infection (*CEBPB, CXCL9, IRF8, RNASE7*), epithelial cell migration (*ITGB1, SPARC*), and *MCFD2*. Furthermore, we constructed an immune risk signature to estimate the risk score of each patient based on the linear combination of the 10 mRNA expression levels weighted by their Lasso-Cox regression coefficients: Immune Risk Score = (0.1979) × *CEBPB* + (−0.2140) × *CXCL9* + (−0.0927) × *IRF8* + (0.5896) × *ITGB1* + (0.2108) × *LAG3* + (0.2489) × *MCFD2* + (−0.2909) × *PSMD11* + (0.5255) × *RNASE7* + (0.0881) × *SPARC* + (−0.1490) × *TAP2* (**Figure 1C**, **Supplementary Table S1**). A heatmap of the identified 10-gene expression level and the scatterplot of relapse-free survival (RFS) with corresponding risk score were illustrated in **Figure 1C**.

The Prognostic Value of 10-mRNA Immune Signature

To identify the immune signature responsible for CC survival prediction, we divided the discovery cohort samples into a low-risk group ($N = 279$) and a high-risk group ($N = 278$) by using the median risk score as a cutoff point. Patients with low-risk were significantly associated with better RFS compared with those of high-risk ($P < 0.001$, log-rank test; **Figure 2A**). This association remained markedly significant in the multivariate Cox model after controlling for age, gender, clinical stage, and mismatch repair (MMR) status (HR, 0.41 [95% CI: 0.30–0.57], $P < 0.001$; **Figure 2B**).

To confirm that the 10-mRNA-based immune signature classifier had similar prognostic value in different populations, we further corroborate this association in the TCGA dataset and another combined-GEO microarray dataset (including GSE17538, GSE33113, and GSE37892; "Materials and Methods" section). Heatmaps of the signature consisting of 10 immune-related genes and the scatterplot of RFS time with corresponding risk score in two external validation cohorts were shown in **Supplementary Figure S1**. In the TCGA and combined-GEO datasets, we also found that patients with low-risk scores demonstrated a better prognosis than those with high-risk scores (TCGA: $P = 0.003$, **Figure 2C**; Combined-GEO cohort: $P = 0.013$, **Figure 2E**; log-rank test). Multivariate Cox proportional hazards regression analysis further revealed that the

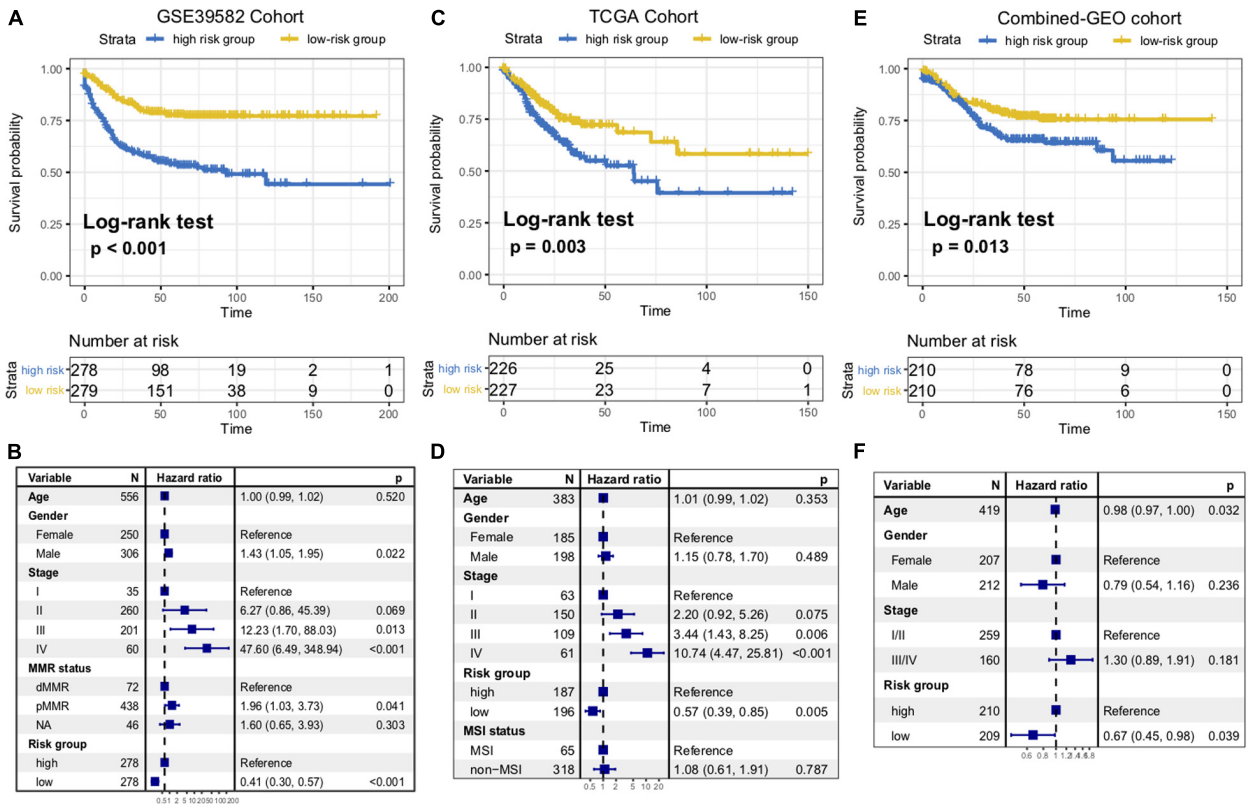

FIGURE 2 | Immune risk signature was associated with CC survival. Kaplan-Meier curves of relapse-free survival according to immune signature groups in the GSE39582 discovery cohort **(A)**, TCGA cohort **(C)**, and another combined-GEO validation cohort **(E)**. Forest plot representation of the multivariate Cox regression model delineated the association between immune risk signature and survival in the three cohorts **(B,D,F)**. Age, gender, clinical stage, or dMMR were taken into account.

signature could serve as an independent predictor of patients' survival outcome after being adjusted for clinicopathologic features in two validation cohorts (TCGA: HR, 0.57 [95%CI, 0.39–0.85], P = 0.005, **Figure 2D**; Combined-GEO cohort: HR, 0.67 [95%CI, 0.45–0.98], P = 0.039. **Figure 2F**). Further analysis confirmed that the higher immune risk signature score was associated with significantly worse tumor staging in CC cohorts (Kruskal–Wallis H test, GSE39582, and TCGA cohorts, both $P < 0.001$, **Supplementary Figures S2A,B**). Moreover, we found the risk scoring model could improve the accuracy of predictions of survival when combined with the tumor staging system (AUC of GSE39582: Stage vs Risk score + Stage, 67.44 vs 71.56, P = 0.002; Risk score vs Risk score + Stage, 67.63 vs 71.56, P = 0.071; AUC of TCGA: Stage vs Risk score + Stage, 69.27 vs 72.26, P = 0.035; Risk score vs Risk score + Stage, 64.65 vs 72.26, P = 0.004; DeLong's test, **Supplementary Figures S2C,D**).

Extracted Immune Risk Signature Associated With Leukocytes Infiltration and Tumor Immunogenicity

Since the prognosis-related risk signature was extracted from the immune-related genes database, we speculated that its status may regulate the leukocyte infiltration and gene pathways enrichment. Therefore, we composed a heatmap with ssGSEA to visualize the relative abundance of 28 immune infiltrating cell subpopulations from the discovery dataset (**Figure 3A**). Anti-tumor lymphocyte cell subpopulations, like activated $CD4^+/CD8^+$ T cells, effector memory $CD4^+/CD8^+$ T cells, and natural killer T cells were enriched in the low-risk signature group ($P < 0.05$). Nevertheless, myeloid-derived suppressor cells (MDSC), immature dendritic cells, neutrophils, and regulatory T cells, which belonged to pro-tumor leukocytes, were elevated in the high-risk signature group ($P < 0.05$). We also further characterized the immune infiltration profile in TCGA and the combined-GEO validation cohort, and a similar tendency was observed in these cohorts of such risk signature stratification (**Supplementary Figure S3**).

Furthermore, GSEA on the CC gene expression profile against REACTOME reference datasets revealed the risk signature related biological signaling pathway. Genes involved in antigen processing cross-presentation, B cell/T cell receptor and immune cytokine signaling pathways were significantly enriched in the low immune risk signature group (**Figure 3B**, **Supplementary Figure S4A**). However, chromatin organization and RNA processing and modification were enriched in the high-risk group (**Figure 3C**, **Supplementary Figure S4B**).

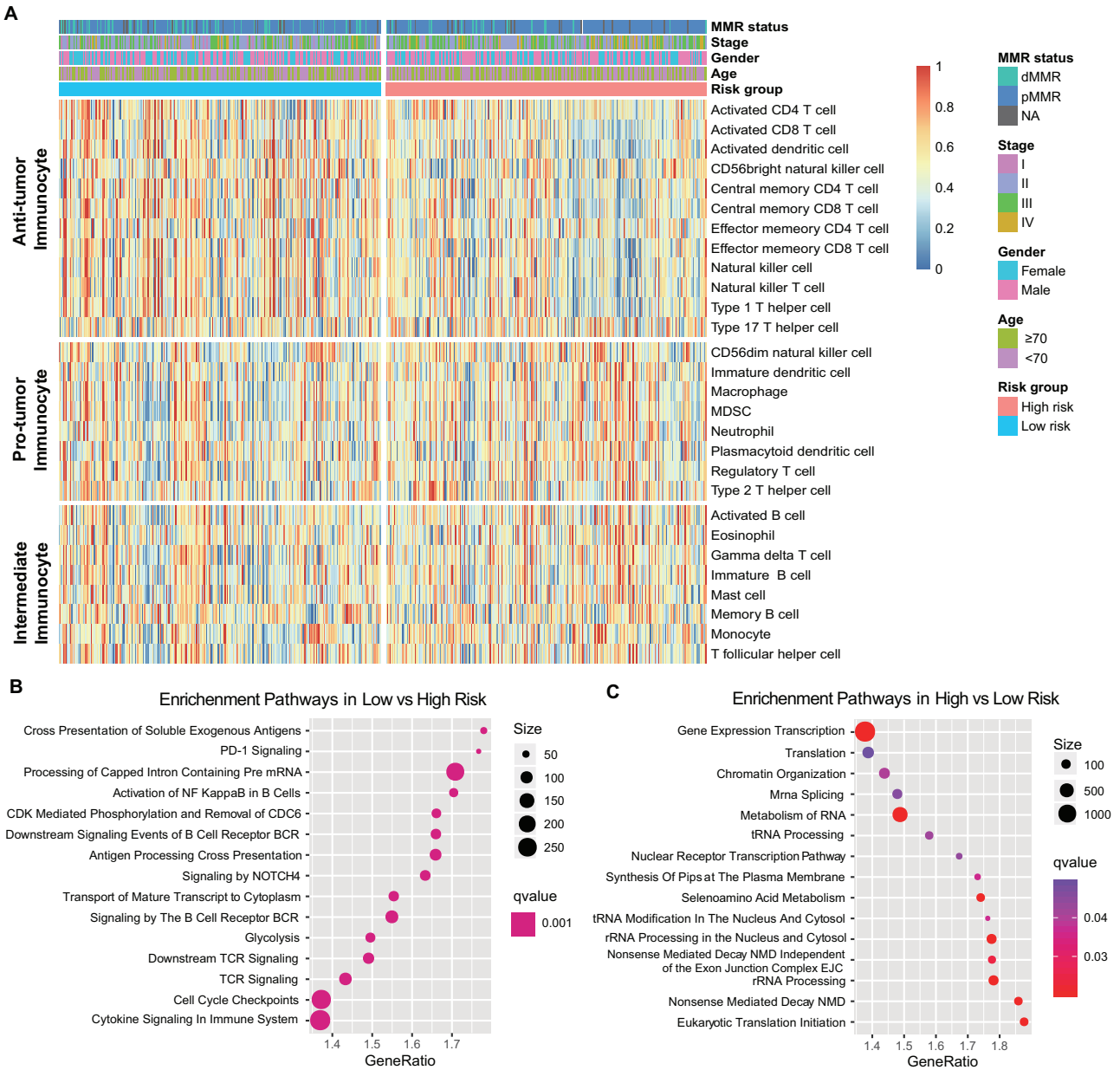

FIGURE 3 | Immune risk signature was associated with the immune infiltration. **(A)** Single-sample gene set enrichment analysis identified the relative infiltration of 28 types of immune cell subpopulations with different risk signature subgroups. The relative infiltration of each cell type was normalized into a Z score. **(B,C)** Top enriched gene pathways in distinct immune risk signature groups (low vs high, left panel; high vs low, right panel) from discovery cohort were assessed by using the GSEA algorithm.

Immunophenoscore (IPS) was known to determine the tumor immunogenicity and predict response to ICI therapy in multi-types of tumors. Here, we utilized IPS to investigate the relationship between the newly identified signature and immune response. In the discovery cohort, the low-risk signature group had a significantly higher IPS compared with the high-risk group (Wilcoxon rank-sum test, $P < 0.001$, **Figure 4A**), and this association was also verified in TCGA and the combined-GEO cohort (TCGA, $P < 0.001$, combined-GEO cohort, $P = 0.001$; **Figures 4B,C**). These findings indicated that CC patients with the immune signature may be more sensitive to ICI treatment.

Immune Signature Determined the Colon Cancer Genomic Landscape

Genomic characteristics, such as tumor non-synonymous mutation load (TML) and mutational signatures (e.g., MMR, POLE signature) have shown a strong correlation with clinical

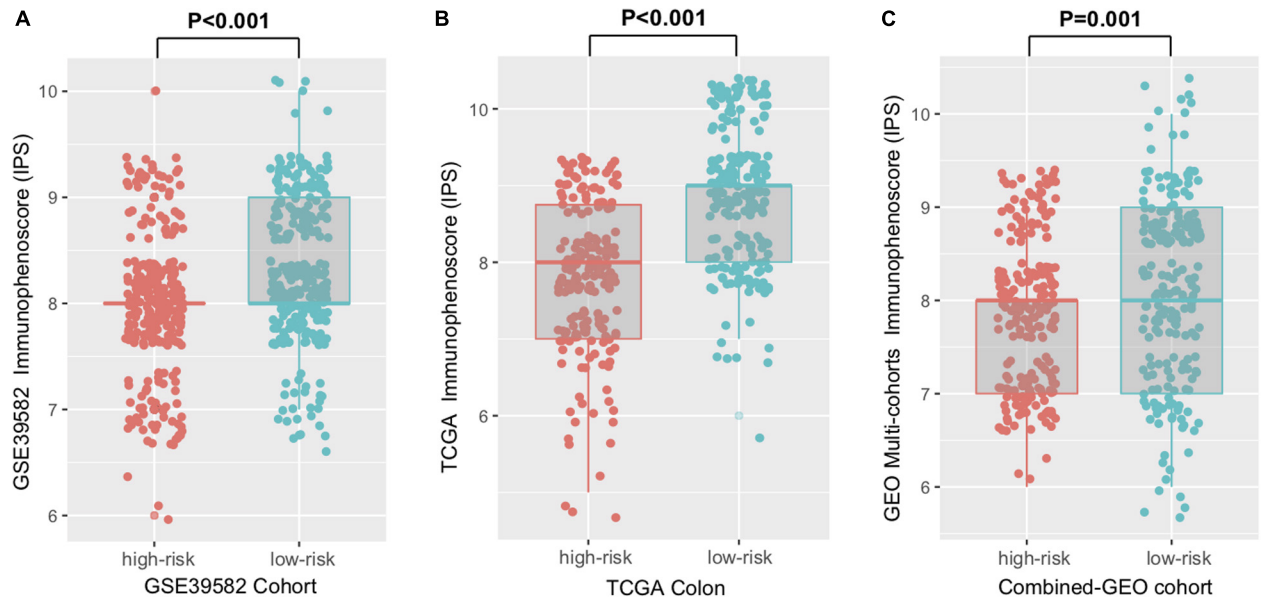

FIGURE 4 | Distribution of immunophenoscore (IPS) in high-risk versus low-risk colon cancer subtypes. Boxplot representation of IPS in the high-risk versus low-risk groups in discovery cohort **(A)**, TCGA cohort **(B)**, and another combined-GEO cohort **(C)**. *P* values as indicated (Wilcoxon rank-sum test).

response to ICI treatment (34). Therefore, we investigated the association between the immune signature and the genomic mutational landscape. Patients with a low-risk immune signature exhibited a higher mutation load than those with a high-risk signature in the TCGA dataset ($P = 0.030$, **Figure 5A**). We further compared the tumor neoantigen counts and observed similar results in the group classification ($P = 0.005$, **Figure 5B**). As high microsatellite instability (MSI-H) tumors accumulated substantial numbers of somatic mutations and significantly affected the TML, we removed the samples with MSI-H and obtained a significantly higher TML in the low-risk signature ($P < 0.001$, **Figure 5C**).

To gain further insights into the mutational processes operative in CC samples, we extracted the mutational signatures (i.e., signatures 1, 6, 10, **Supplementary Figure S5**) against the COSMIC database with varying numbers of somatic mutations from the genomic data (**Figure 5D**). The extracted mutational signatures included defects in DNA proofreading owing to recurrent somatic mutations in POLE (signature 10, 79524 of 264763 [30.0%]), clock-like accumulation of C > T at cytosine-phosphate-guanine dinucleotide (signature 1, 46,106 of 264,763 [17.4%]), and defective MMR (signature 6, 139,133 of 264,763 [52.6%]) (**Figure 5E**). Hence, mutational counts attributed to signature 6 were significantly higher than other signatures (Kruskal–Wallis H test, $P = 0.019$). To rule out the possibility that associations between immune signature and TML were affected by these confounding factors, we included all mutational signatures and clinical factors in the multivariate logistic regression model. Associations between the immune risk signature and TML remained statistically significant (OR, 0.15 [95% CI, 0.06–0.24], $P < 0.001$, **Figure 5F**).

We also performed SMG analysis for CC samples in the low-risk versus the high-risk subgroup. The SMG mutational landscapes of these two subgroups (**Figure 6**) exhibited a distinct mutation ratio in *APC* [138 of 200 (69.0%) vs 154 of 194 (79.4%); $P = 0.021$], *TP53* [111 of 200 (55.5%) vs 83 of 194 (42.8%); $P = 0.012$], *FBXW7* [19 of 200 (9.5%) vs 50 of 194 (25.7%); $P < 0.001$], and *MSH6* [24 of 200 (12.7%) vs 9 of 194 (7%); $P = 0.010$]. The mutation plot of the four SMGs with different immune signature status were shown in **Supplementary Figure S6**. Besides, we also explored the mutational rate of the aforementioned 10-immune genes, and observed *RNASE7* mutation was enriched in the high-risk subgroup [1 of 200 (0.5%) vs 3 of 194 (3.1%); $P = 0.014$].

The Immune Risk Signature in the Role of ICI Treatment

Immune checkpoint inhibitors (ICI) therapy represented by anti-PD-1/L1 agents have undoubtedly made a great breakthrough in anti-tumor therapy. Therefore, we curated the gene expression profile and clinical features from an immunotherapy cohort (Imvigor210) of urothelial cancer (UC) treated by anti-PD-L1 agent, so as to investigate the relationship between the constructed risk signature and immune response. In this anti-PD-L1 cohort, patients with a low-risk immune signature score exhibited markedly clinical benefits and a significantly prolonged survival rate (HR, 0.71 [95% CI: 0.55–0.92], $P = 0.009$, **Figure 7A**). The significant therapeutic advantages and immune response to PD-L1 blockades were observed in samples with a low-risk score compared to those with a high-risk score (Fisher extract test, $P = 0.008$, **Figure 7B**; Kruskal-Wallis H test, $P < 0.001$, **Figure 7C**). Further analysis revealed that TML and neoantigen burden were significantly elevated in tumors

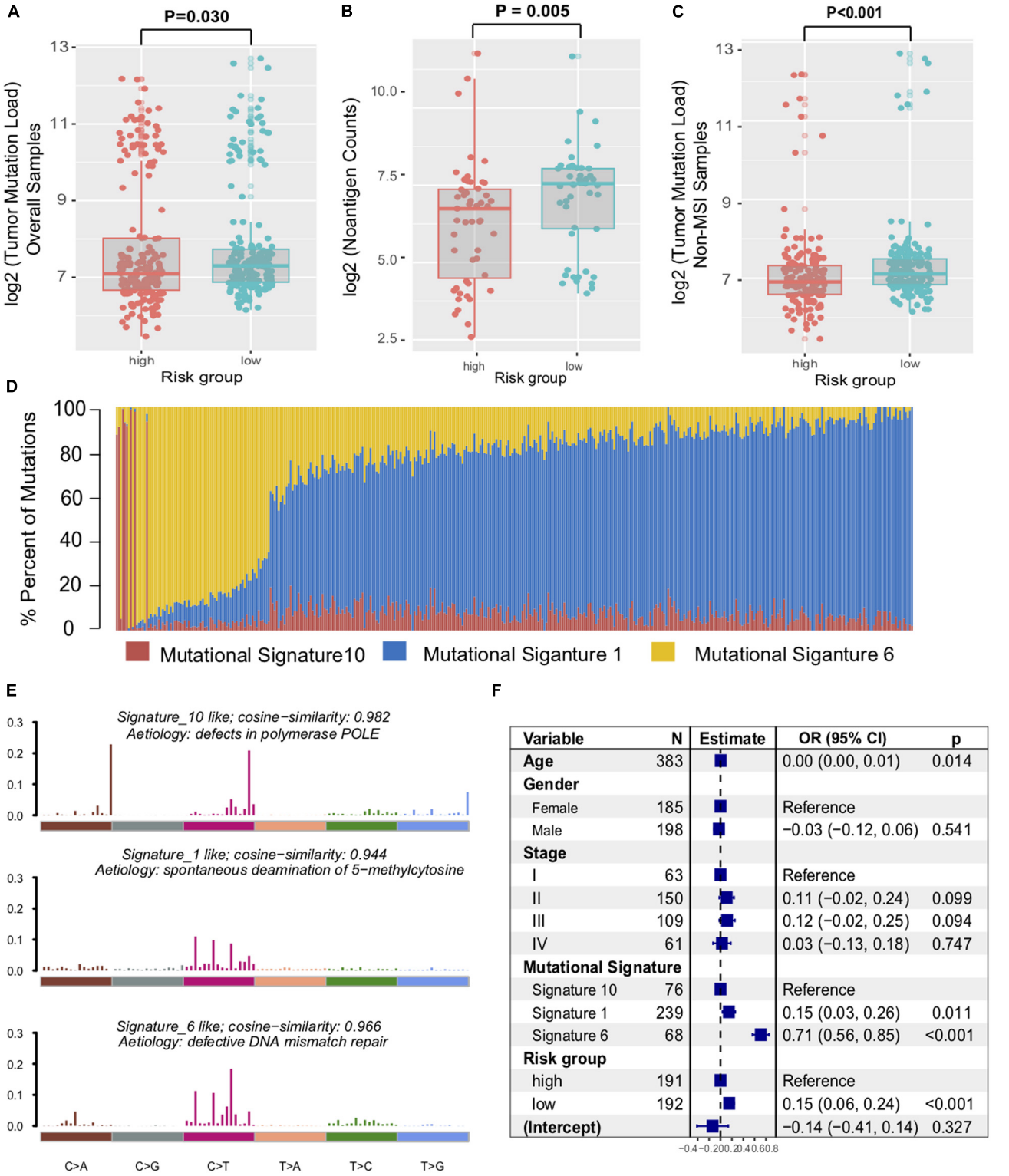

FIGURE 5 | Immune signature was associated with the TML in colon cancer. Tumor mutation load **(A)** and neoantigen counts **(B)** in colon cancer samples were compared with the immune risk signature group. **(C)** Distribution of mutational load in non-MSI samples were also assessed between high-risk and low-risk subtypes. **(D)** Mutational exposures (number of mutations) were attributed to each mutation signature. **(E)** The mutational activities of corresponding extracted mutational signatures (signature 1, 6, and 10, named as COSMIC database). **(F)** Multivariate Logistic regression analysis of TML with respect to immune signature was adjusted by taking into account age, gender, stage, and mutational signatures. *P* values as indicated (Wilcoxon rank-sum test).

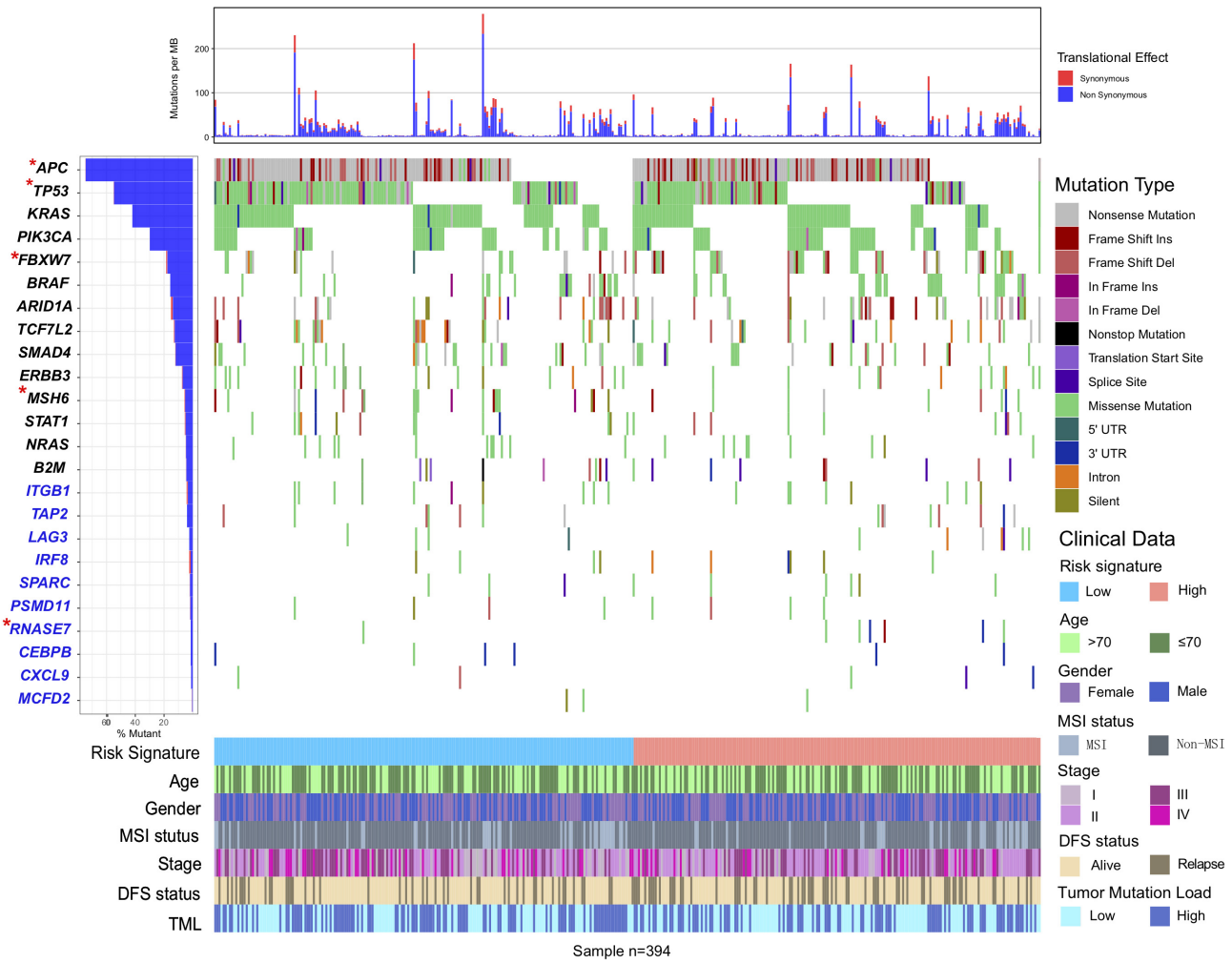

FIGURE 6 | Mutational landscape of SMGs and immune-genes in TCGA COAD cohort stratified by high-risk and low-risk signature groups. The middle panel depicts the mutation relation of SMGs across analyzed cases with mutation types color-coded differently. SMGs with significantly different mutation rates between subgroups were highlighted in upper left asterisk.

with low-risk score, which closely linked to immunotherapeutic efficacy (**Figures 7D,E**). Besides, the association between immune risk score and immunotherapy survival remained statistically significant after taking into account gender, smoking, ECOG score, immunophenotype and, TML status (HR, 0.60 [95%CI, 0.40–0.90], $P = 0.015$; **Figure 7F**).

DISCUSSION

Although it has long been recognized that immune contexture plays a vital role in tumor initiation and development (35), these insights have not formed a significant impact on routine clinical application. This highlights the important role of TIM estimation in predicting clinical development and progression of CC patients. In this investigation, we established a reliable prognostic risk signature based on 10 immune-related genes in an independent microarray dataset and proved its efficacy in the TCGA and combined GEO datasets across different platforms. This signature stratified the patients into subgroups with different immune risk, representing distinct tumor immune infiltration level and neoantigen burden. Therefore, the newly identified immune risk signature presumably represented the status of TIM for CC patients and served as a potential biomarker for prognosis estimation and clinical response prediction to immunotherapy.

This study confirmed that the immune risk signature was significantly associated with CC patients' RFS, and this association remained significant after controlling for clinical-pathological features. More importantly, our signature was based on immune-related genes and revealed a correlation with non-synonymous mutation load and neoantigen counts. Considering the importance of mutational load in predicting the response to anti-PD-1/L1 treatment (12, 36), we speculated that patients with a low-risk immune signature may be more sensitive to ICI therapy. Actually, the IPS and neoantigen load, which determined

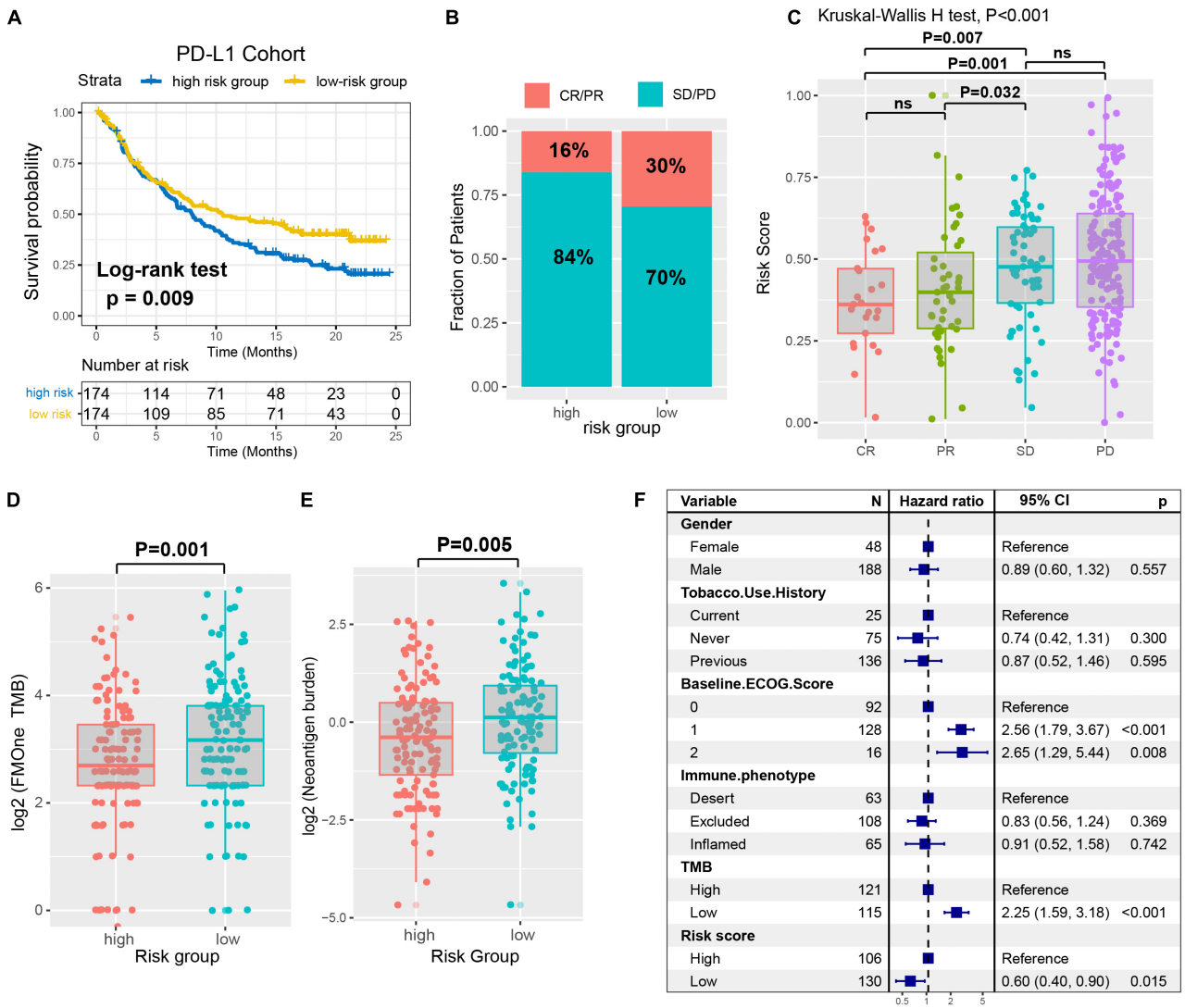

FIGURE 7 | The immune risk signature in the role of ICI treatment. **(A)** Survival analysis of the high versus low immune risk subgroup in the anti-PD-L1 cohort (IMvigor210 cohort) was created using Kaplan-Meier curves. **(B)** The proportion of immune response to anti-PD-L1 treatment in high versus low immune risk score subgroups. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. **(C)** Distribution of immune risk score in different immune response statuses. Tumor mutation load **(D)** and neoantigen burden **(E)** in the immunotherapy cohort were compared among distinct immune risk signature subgroups. **(F)** Multivariate Cox regression analysis of immune risk signature with gender, smoking, ECOG score, immunophenotype, and TML status were taken into account. *P* values as indicated (Kruskal–Wallis H test).

the tumor immunogenicity and antitumor immune response, also demonstrated a strong connection with this signature. To clarify the effects of this immune signature, we took TML and IPS as the confounding factors into the multivariate Cox regression models, and identified that the immune risk score remained statistically significant in the TCGA cohort (HR, 0.59 [95%CI, 0.39–0.87], P = 0.008; **Supplementary Figure S7**). These findings further indicated its practical implication in precision immunotherapy.

In recent years, numerous studies focused on the immune landscape have brought attention to biological and clinical cancer research. Individual immune cell markers such as $CD3^+$ and $CD8^+$ T cells have shown prognostic impacts in patients suffering from CC (6). The immune cell subpopulations estimation algorithm (e.g., ssGSEA, CIBERSORT) was frequently utilized to characterize the immune infiltration profiles and analyze the association with clinical therapy (37). Our research also leveraged the aforementioned method and demonstrated enhanced effector T-cells ($CD4/CD8^+$ T cell, NK cells), reduced suppressive regulatory T-cells, and MDSC infiltration in low immune risk signature. Meanwhile, signaling pathways involved in the antigen processing and presentation, B cell/T cell receptor and immune cytokine were significantly altered in different risk subgroups, suggested that our signature was a superior prediction determinant of tumor immune infiltration.

Comprehensive knowledge of the mutated driver genes underlying human cancers is a critical foundation for cancer diagnostics, therapeutics, and selection of rational therapies. Here, we used MutSigCV algorithms followed by further filter criteria and identified that SMGs of *APC* and *FBXW7* mutations were enriched in high-risk groups, *TP53* and *MSH6* were enriched in low-risk groups. *APC* was the most common mutational gene in colorectal cancer, and its mutation has indicated a highly significant association with immune resistance (38). *FBXW7* is a critical tumor suppressor of human cancers, missense mutations in this gene show a shorter overall survival rate when compared with wild-type patients in CC (39). *TP53* and *MSH6* mutations may lead to a higher TML owing to the dysregulation of DNA damage repair function (40). Recent research suggested that *TP53* mutations significantly induced the expression of immune checkpoint molecules and activated T-effector and interferon-γ signatures, indicating *TP53* mutation patients would be more sensitive to checkpoint blockade (41).

Nevertheless, there were several limitations in our investigation. The main limitation stemmed from using a public dataset for different cohorts which can be somewhat heterogeneous in data processing and patient population. The risk signature was identified by using retrospective datasets, therefore, the expression profiles of the 10 genes combined with clinical validation in the patients of CC prospective cohort are needed to prove its efficacy. Besides, mutational results derived from the TCGA COAD genomic landscape were not validated in independent datasets owing to the unavailability of mutation data. Finally, due to a lack of CC cohorts being treated by ICIs, we are unable to verify the association between the signature and the immunotherapeutic responsiveness and believe further research is needed.

To summarize, this study identified a new immune risk signature that can not only predict CC patients' survival outcomes but also represent the immune infiltration status. This signature can be clinically utilized for the improvement of CC patients' survival, personalize therapy methods based on the risk score, and provide new clues for enrolling CC patients in ICI treatment. However, further randomized control trials are required to validate the significance of the generated signature.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GEO – GSE39582, GSE17538, GSE37892, and GSE33113.

AUTHOR CONTRIBUTIONS

HC, WC, and XYL: conception and design. XYL and HC: development of methodology. XYL, DW, XKL, and CY: acquisition of data (provided animals, acquired and managed patients, and provided facilities). XYL, HC, and WC: analysis and interpretation of data (e.g., statistical analysis, biostatistics, and computational analysis). HC and WC: writing, reviewing, and/or revising the manuscript, administrative, technical, or material support (i.e., reporting or organizing data, and constructing databases), and study supervision. All authors contributed to the article and approved the submitted 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.2020.01678/full#supplementary-material

FIGURE S1 | Heatmap of the signature consisting of 10 immune related genes and the risk score curve in the TCGA and combined-GEO colon cancer cohort.

FIGURE S2 | Immune risk score associated with tumor stage. Distribution of immune risk score with respect to clinical tumor stage was shown in the GSE39582 **(A)** and TCGA **(B)** cohort. A receiver operating characteristic (ROC) curve was used to assess the prognosis classification performance of the immune risk signature vs tumor stage vs risk signature plus stage in the GSE39582 **(C)** and TCGA **(D)** cohort. The area under the curve (AUC) in different subgroups was calculated by DeLong's test.

FIGURE S3 | Estimation of the relative infiltration of 28 types of immune cell subpopulations with different immune signature groups in the external TCGA and combined-GEO cohort.

FIGURE S4 | GSEA enrichment plots show enriched gene sets against REACTOME datasets in low-risk vs high-risk **(A)** and high-risk vs low-risk **(B)**. NES, Normalized Enrichment Score.

FIGURE S5 | Mutational signatures extracted from the TCGA COAD genomic dataset. **(A)** The progress of automatically determining the optimal number of mutational signatures ($N = 3$). **(B)** Cosine similarity analysis of extracted mutational signatures against the 30 identified signatures in the Catalog of Somatic Mutations in Cancer (COSMIC, v2) with heatmap illustration.

FIGURE S6 | Lollipop plot showing the protein change of four novel SMGs with respect to risk signature in the COAD cohort.

FIGURE S7 | Multivariate Cox regression analysis of immune risk signature by taking into account confounding factors, such as age, gender, stage, MSI status, TML, and IPS.

TABLE S1 | Lasso Cox regression coefficients of the 10 immune signature genes.

TABLE S2 | Clinical characteristics of patients with colon cancer in five datasets.

REFERENCES

1. Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A, et al. Colorectal cancer statistics, 2017. *CA Cancer J Clin*. (2017) 67:177–93. doi: 10.3322/caac.21395
2. Schmoll HJ, Van Cutsem E, Stein A, Valentini V, Glimelius B, Haustermans K, et al. ESMO consensus guidelines for management of patients with colon and rectal cancer. A personalized approach to clinical decision making. *Ann Oncol*. (2012) 23:2479–516. doi: 10.1093/annonc/mds236
3. Mahajan UM, Langhoff E, Goni E, Costello E, Greenhalf W, Halloran C, et al. Immune cell and stromal signature associated with progression-free survival of patients with resected pancreatic ductal adenocarcinoma. *Gastroenterology*. (2018) 155:1625–39.e2. doi: 10.1053/j.gastro.2018.08.009
4. Jia Q, Wu W, Wang Y, Alexander PB, Sun C, Gong Z, et al. Local mutational diversity drives intratumoral immune heterogeneity in non-small cell lung cancer. *Nat Commun*. (2018) 9:5361. doi: 10.1038/s41467-018-07767-w
5. Souza P, Rizzardi F, Noleto G, Atanazio M, Bianchi O, Parra ER, et al. Refractory remodeling of the microenvironment by abnormal type V collagen, apoptosis, and immune response in non-small cell lung cancer. *Hum Pathol*. (2010) 41:239–48. doi: 10.1016/j.humpath.2009.07.018
6. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science*. (2006) 313:1960–4. doi: 10.1126/science.1129139
7. Mlecnik B, Tosolini M, Kirilovsky A, Berger A, Bindea G, Meatchi T, et al. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol*. (2011) 29:610–8. doi: 10.1200/JCO.2010.30.5425
8. Pages F, Mlecnik B, Marliot F, Bindea G, Ou FS, Bifulco C, et al. International validation of the consensus immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet*. (2018) 391:2128–39. doi: 10.1016/S0140-6736(18)30789-X
9. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science*. (2017) 357:409–13. doi: 10.1126/science.aan6733
10. Xiao Y, Freeman GJ. The microsatellite instable subset of colorectal cancer is a particularly good candidate for checkpoint blockade immunotherapy. *Cancer Discov*. (2015) 5:16–8. doi: 10.1158/2159-8290.CD-14-1397
11. Ruiz-Banobre J, Goel A. DNA mismatch repair deficiency and immune checkpoint inhibitors in gastrointestinal cancers. *Gastroenterology*. (2019) 156:890–903. doi: 10.1053/j.gastro.2018.11.071
12. Samstein RM, Lee CH, Shoushtari AN, Hellmann MD, Shen R, Janjigian YY, et al. Tumor mutational load predicts survival after immunotherapy across multiple cancer types. *Nat Genet*. (2019) 51:202–6. doi: 10.1038/s41588-018-0312-8
13. Llosa NJ, Cruise M, Tam A, Wicks EC, Hechenbleikner EM, Taube JM, et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov*. (2015) 5:43–51. doi: 10.1158/2159-8290.CD-14-0863
14. Finotello F, Trajanoski Z. Quantifying tumor-infiltrating immune cells from transcriptomics data. *Cancer Immunol Immunother*. (2018) 67:1031–40. doi: 10.1007/s00262-018-2150-z
15. Bhattacharya S, Andorf S, Gomes L, Dunn P, Schaefer H, Pontius J, et al. ImmPort: disseminating data to the public for the future of immunology. *Immunol Res*. (2014) 58:234–9. doi: 10.1007/s12026-014-8516-1
16. Dai W, Li Y, Mo S, Feng Y, Zhang L, Xu Y, et al. A robust gene signature for the prediction of early relapse in stage I-III colon cancer. *Mol Oncol*. (2018) 12:463–75. doi: 10.1002/1878-0261.12175
17. Marisa L, de Reynies A, Duval A, Selves J, Gaub MP, Vescovo L, et al. Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. *PLoS Med*. (2013) 10:e1001453. doi: 10.1371/journal.pmed.1001453
18. Smith JJ, Deane NG, Wu F, Merchant NB, Zhang B, Jiang A, et al. Experimentally derived metastasis gene expression profile predicts recurrence and death in patients with colon cancer. *Gastroenterology*. (2010) 138:958–68. doi: 10.1053/j.gastro.2009.11.005
19. Laibe S, Lagarde A, Ferrari A, Monges G, Birnbaum D, Olschwang S, et al. A seven-gene signature aggregates a subgroup of stage II colon cancers with stage III. *OMICS*. (2012) 16:560–5. doi: 10.1089/omi.2012.0039
20. de Sousa EMF, Colak S, Buikhuisen J, Koster J, Cameron K, de Jong JH, et al. Methylation of cancer-stem-cell-associated Wnt target genes predicts poor prognosis in colorectal cancer patients. *Cell Stem Cell*. (2011) 9:476–85. doi: 10.1016/j.stem.2011.10.008
21. Jorissen RN, Gibbs P, Christie M, Prakash S, Lipton L, Desai J, et al. Metastasis-associated gene expression changes predict poor outcomes in patients with dukes stage B and C colorectal cancer. *Clin Cancer Res*. (2009) 15:7642–51. doi: 10.1158/1078-0432.CCR-09-1431
22. Mariathasan S, Turley SJ, Nickles D, Castiglioni A, Yuen K, Wang Y, et al. TGFbeta attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature*. (2018) 554:544–8. doi: 10.1038/nature25501
23. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell*. (2015) 160:48–61. doi: 10.1016/j.cell.2014.12.033
24. Chen H, Yang M, Wang Q, Song F, Li X, Chen K. The new identified biomarkers determine sensitivity to immune check-point blockade therapies in melanoma. *Oncoimmunology*. (2019) 8:1608132. doi: 10.1080/2162402X.2019.1608132
25. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. (2015) 43:e47. doi: 10.1093/nar/gkv007
26. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. (2005) 102:15545–50. doi: 10.1073/pnas.0506580102
27. Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature*. (2009) 462:108–12. doi: 10.1038/nature08460
28. Charoentong P, Finotello F, Angelova M, Mayer C, Efremova M, Rieder D, et al. Pan-cancer immunogenomic analyses reveal genotype-immunophenotype relationships and predictors of response to checkpoint blockade. *Cell Rep*. (2017) 18:248–62. doi: 10.1016/j.celrep.2016.12.019
29. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature*. (2013) 499:214–8. doi: 10.1038/nature12213
30. Klijn C, Durinck S, Stawiski EW, Haverty PM, Jiang Z, Liu H, et al. A comprehensive transcriptional portrait of human cancer cell lines. *Nat Biotechnol*. (2015) 33:306–12. doi: 10.1038/nbt.3080
31. Mayakonda A, Lin DC, Assenov Y, Plass C, Koeffler HP. Maftools: efficient and comprehensive analysis of somatic variants in cancer. *Genome Res*. (2018) 28:1747–56. doi: 10.1101/gr.239244.118
32. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, et al. Mutational landscape and significance across 12 major cancer types. *Nature*. (2013) 502:333–9. doi: 10.1038/nature12634
33. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. (2014) 15:550. doi: 10.1186/s13059-014-0550-8
34. Mandal R, Samstein RM, Lee KW, Havel JJ, Wang H, Krishna C, et al. Genetic diversity of tumors with mismatch repair deficiency influences anti-PD-1 immunotherapy response. *Science*. (2019) 364:485–91. doi: 10.1126/science.aau0447
35. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. (2012) 12:298–306. doi: 10.1038/nrc3245
36. Chen H, Chong W, Wu Q, Yao Y, Mao M, Wang X. Association of LRP1B mutation with tumor mutation burden and outcomes in melanoma and non-small cell lung cancer patients treated with immune check-point blockades. *Front Immunol*. (2019) 10:1113. doi: 10.3389/fimmu.2019.01113
37. Zhou R, Zhang J, Zeng D, Sun H, Rong X, Shi M, et al. Immune cell infiltration as a biomarker for the diagnosis and prognosis of stage I-III colon cancer. *Cancer Immunol Immunother*. (2019) 68:433–42. doi: 10.1007/s00262-018-2289-7
38. Cristescu R, Mogg R, Ayers M, Albright A, Murphy E, Yearley J, et al. Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. *Science*. (2018) 362:eaar3593. doi: 10.1126/science.aar3593

39. Yeh CH, Bellon M, Nicot C. FBXW7: a critical tumor suppressor of human cancers. *Mol Cancer*. (2018) 17:115. doi: 10.1186/s12943-018-0857-2
40. Gelsomino F, Barbolini M, Spallanzani A, Pugliese G, Cascinu S. The evolving role of microsatellite instability in colorectal cancer: a review. *Cancer Treat Rev*. (2016) 51:19–26. doi: 10.1016/j.ctrv.2016.10.005
41. Dong ZY, Zhong WZ, Zhang XC, Su J, Xie Z, Liu SY, et al. Potential predictive value of TP53 and KRAS mutation status for response to PD-1 blockade immunotherapy in lung adenocarcinoma. *Clin Cancer Res*. (2017) 23:3012–24. doi: 10.1158/1078-0432.CCR-16-2554

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REVIEW
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Pro-tumor γδ T Cells in Human Cancer: Polarization, Mechanisms of Action, and Implications for Therapy

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The tumor immune microenvironment contributes to tumor initiation, progression and response to therapy. Among the immune cell subsets that play a role in the tumor microenvironment, innate-like T cells that express T cell receptors composed of γ and δ chains (γδ T cells) are of particular interest. Indeed, γδ T cells contribute to the immune response against many cancers, notably through their powerful effector functions that lead to the elimination of tumor cells and the recruitment of other immune cells. However, their presence in the tumor microenvironment has been associated with poor prognosis in various solid cancers (breast, colon and pancreatic cancer), suggesting that γδ T cells also display pro-tumor activities. In this review, we outline the current evidences of γδ T cell pro-tumor functions in human cancer. We also discuss the factors that favor γδ T cell polarization toward a pro-tumoral phenotype, the characteristics and functions of such cells, and the impact of pro-tumor subsets on γδ T cell-based therapies.

Keywords: γδ T cells, cancer, pro-tumor functions, immunosuppression, therapy

INTRODUCTION

Within a tumor, the malignant features of cancer cells are tightly regulated by their local environment and the reciprocal network they form with host cells (e.g., immune cells, angiogenic vascular cells, endothelial cells, and cancer-associated fibroblasts) and that define the cancer ecosystem. The tumor immune microenvironment is a critical determinant of cancer evolution and outcome. In this context, the nature and frequency of tumor-infiltrating immune cells are considered to be prognostic factors in many cancers. A better knowledge of this dynamic immune environment is required to improve prognosis, choose therapies, and evaluate the response to treatments.

Among the tumor-infiltrating immune cells, T cell sub-populations, especially CD8+ T lymphocytes, are a key anti-tumor immune component. γδ T cells, a subgroup of T cells that belong to the non-conventional or innate lymphocyte family, also are found in the tumor microenvironment and are involved in tumor surveillance. Although they share many properties with αβ T cells, such as cytotoxic activity and pro-inflammatory cytokine production, the structure of their T cell receptor (TCR; composed of γ and δ chains) is different as well as their activation mechanisms that are independent of major histocompatibility complex (MHC) molecules. Human

γδ T cells can be divided in three main populations, based on their TCR δ chain (δ1, δ2, δ3) (1, 2). Vδ2 T cells, also known as Vγ9Vδ2 T cells, are the main γδ T subtype (90%) in peripheral blood. The Vδ1 and Vδ3 subsets are mostly found in tissues and mucosa, respectively.

Vγ9Vδ2 T cells display specific properties, such as the TCR-dependent recognition of non-peptidic phosphorylated antigens, called phosphoantigens. Phosphoantigens are molecules produced by the isoprenoid synthesis pathways of prokaryotic pathogens and by infected or transformed eukaryotic cells. Although phosphoantigen recognition does not require MHC molecule presentation, several studies brought evidences of the involvement of the cell surface butyrophilin 3A (BTN3A) (3) and the requirement of butyrophilin 2A1 (BTN2A1) (4). Phosphoantigen-induced TCR activation of Vγ9Vδ2 T cells triggers their proliferation, cytokine production, and cytotoxic activity (5). Vγ9Vδ2 T cells also express natural killer (NK) receptors, such as NKG2A and NKG2D, and their activation is modulated by the presence of their ligands in the environment (6, 7).

Vδ1 T cells recognize the stress-inducible MHC class I-related chain A and B (MICA and MICB) proteins that are expressed by some tumor and virus-infected cells (8), as well as glycolipid antigens presented by the CD1c (9) and CD1d proteins (10, 11), and the algal protein phycoerythrin (12). Additionally, Vδ1 T cells can be activated independently of their TCR, via ligation of stimulatory receptors, including NKG2C, NKG2D, NKp30, toll-like receptors, and the β-glucan receptor dectin 1 (13–17). To date, little is known on the activation mechanisms of the Vδ3 T cell subset.

Although the human Vδ1, Vδ2 and Vδ3 T cell subsets display a strong reactivity against tumor cells, γδ T cell-based immunotherapies primarily target the Vδ2 subset because they are easily expanded and activated by synthetic clinical-grade phosphoantigens (e.g., bromohydrin pyrophosphate) or by pharmacological inhibitors (e.g., zoledronate) of the isoprenoid synthesis pathway that produces these metabolites (18, 19).

Many clinical trials using Vγ9Vδ2 T cells have been carried out. Although their safety have been proven, response rate was moderate and only in 10–33% of patients with hematologic and solid malignancies benefit from Vγ9Vδ2 T cell-based immunotherapies (20–25). This suggests the presence in the tumor microenvironment (TME) of suppressive mechanisms that inhibit/divert Vγ9Vδ2 T cell functions and/or their ability to infiltrate tumors. New tools to target and boost Vγ9Vδ2 T cell anti-tumor functions are currently under study (26), while other γδ T cell subtypes (e.g., Vδ1 T cells) are now tested as new therapeutic candidates (27). Although therapies using γδ T cells received a new burst of interest due to these new research axes, the existence of γδ T cell subsets with pro-tumor functions has also been suggested.

In this review, we will discuss the evidences concerning γδ T cell pro-tumor functions in human cancer, and the factors that could favor γδ T cell polarization toward a pro-tumoral phenotype, the characteristics and functions of these cells, and also the possible consequences for γδ T cell-based therapies.

EVIDENCE OF PRO-TUMORAL γδ T CELLS IN HUMAN CANCER (TABLE 1)

In line with the potent anti-tumor properties of γδ T cells, a large study of publicly available gene expression data from bulk tumors showed that the γδ T cell signature is associated with the most significant favorable prognosis in 25 malignancies (37). However, it was later demonstrated that the sorting algorithm used in this study could not accurately differentiate γδ T cells from CD8+ and NK cells due to the transcriptome overlaps in these three cell types (38). Using a refined signature for the Vγ9Vδ2 T cells subset based on sorted cells, the authors found that a high-level infiltration of γδ T cells in tumors was not always associated with a positive outcome (38). In line with these results, recent studies suggested that these cells may also have a pro-tumor role in some cancers.

In breast cancer, high Vδ1 T cell prevalence has been associated with immunosuppressive functions, such as inhibition of naive T cell proliferation and the impairment of dendritic cell (DC) maturation and function (28). Moreover, γδ T cell infiltration level in breast cancer was the most significant independent prognostic factor of disease severity, in terms of survival and relapse (29).

In colorectal cancer, CD39+ Vδ1 T cell infiltration establishes an immunosuppressive microenvironment through the adenosine pathway and the recruitment of myeloid-derived suppressive cells (MDSCs). The presence of these cells has been associated with the disease severity (31). Another study demonstrated the pro-tumor functions of IL-17-producing γδ T cells in colon cancer through their capacity to recruit MDSCs (33). Moreover, pro-inflammatory Vδ2 T cells might participate in colorectal cancer pathogenesis by supporting chronic inflammation (39). Besides breast and colon cancer, several studies have shown a potentially deleterious role of γδ T cell subsets in pancreatic, ovarian, gallbladder and renal cancer (32, 34–36).

POLARIZATION OF γδ T CELLS TOWARD A PRO-TUMOR FUNCTIONAL PHENOTYPE (FIGURE 1)

Although γδ T cells have been originally described as pro-inflammatory cells with a Th1-like phenotype, they display high plasticity and can be polarized toward different functional phenotypes, depending on their environment (40). Understanding precisely the influence of different environmental factors, such as cytokines, on γδ T cells and the limits of their plasticity is crucial to determine how the TME can skew γδ T cells toward a pro-tumor function that will directly or indirectly impair the anti-tumor immune response and support tumor growth. Although studying T cell functional plasticity within tumors is a complex endeavor, several *ex vivo* studies involving the activation of naive γδ T cells in the presence of various cytokines have brought some insights into how γδ T cells can be skewed toward a pro-tumoral activity. Specifically, it has been shown that TGF-β, IL-4 and more recently IL-21 favor the

TABLE 1 | Pro-tumoral characteristics of infiltrating γδ T cells in human cancer.

Type of cancer	γδ sub-populations	Phenotype (surface markers)	Mode of action	Pro-tumoral/suppression factors	Prognosis value	References
Breast cancer	Vδ1 (predominantly)	CD8αα+, CD25–, FoxP3– (TILs clones)	Suppression of T cells and DC	Undefined soluble factor (not TGF-β or IL-10)	Correlation with advanced tumor stages, inverse correlation with OS and RFS	(28, 29)
	Vδ1 and Vδ2	CD39+, CD73+	n/a	n/a	Associated with late stage disease	(30)
Colorectal cancer	Vδ1 (predominantly)	CD39+, CD25+, FoxP3+	Suppression of T cells	Adenosine	Correlation with malignant clinicopathological features	(31)
	Vδ1 (Vδ2 defined as anti-tumoral)	n/a	Suppression of T cells	n/a	Correlation of Vδ1 with disease T stage (negative correlation with Vδ2)	(32)
	Vδ1 (predominantly)	CD45RO+, CD161+, CCR6+, CD69+ TEM phenotype CD45RA–, CD27–	Attraction of PMN-MDSCs	IL-17A, IL-8, GM-CSF	Correlation with advanced clinicopathological features	(33)
Gallbladder cancer	γδ	n/a (CXCR3)	Angiogenesis, suspected attraction of MDSCs	IL-17A	Associated with poor survival	(34)
Ovarian cancer	Vδ1 (predominantly)	n/a	Suppression of T cells, suspected promotion of pro-tumoral myeloid cells	Suppressive factor not determined, production of IL-17A	Correlation with advanced clinicopathological features	(35)
Pancreatic ductal adeno carcinoma	Non Vγ9	TEM phenotype CD45RA–, CD27–, CD62L–	Suppression of T cells (mouse model)	PD-L1, Galectin-9	n/a	(36)

acquisition of pro-tumoral properties by human and mouse γδ T cells. Moreover, various cytokine combinations can polarize γδ T cells into Th17-like cells with pro-tumor effects.

TGF-β

TGF-β is a pleiotropic cytokine that is produced by most cells in a latent form. TGF-β1 (subsequently referred to as TGF-β), the most studied isoform, is a potent suppressor of the immune system. It can be secreted in a complex with latent TGF-beta binding proteins (LTBP) and deposited in the extracellular matrix, or tethered to the surface of cells when bound in a covalent manner to glycoprotein A repetitions predominant (GARP) or leucine-rich-repeat-containing protein 33 (LRRC33). Active TGF-β needs to be released from the latent complex through the interaction with other partners, such as integrins, to act on its target cells through binding to TGF-β receptors (41, 42). TGF-β can induce the differentiation of naive CD4+ T cells into regulatory T cells (Tregs) or Th17 cells, depending on the context, and is often enriched in tumors. Therefore, TGF-β could play a crucial role in γδ T cell polarization toward pro-tumoral cells in the TME (43, 44). *In vitro*, human peripheral blood mononuclear cells (PBMCs) can be stimulated with phosphoantigens and cultured with IL-2 to selectively expand Vγ9Vδ2 T cells. Addition of TGF-β to the culture increases FOXP3 expression in these cells. FOXP3 expression remains stable for at least 10 days. Sorted FOXP3+ Vγ9Vδ2 T cells inhibit the proliferation of TCR-stimulated PBMCs (45). Another study confirmed TGF-β role in the development of FOXP3+ Vγ9Vδ2 T cells and demonstrated that decitabin, a DNA hypomethylating agent, promotes the generation and the immunosuppressive activity of FOXP3+ Vγ9Vδ2 T cells induced by TGF-β (46). Importantly, the relevance of FOXP3 as a regulatory marker depends on the type of stimulation. Indeed, Vδ2 cell activation using anti-CD3 and anti-CD28 antibodies instead of phosphoantigens leads to transient FOXP3 expression that does not correlate with the regulatory phenotype (47, 48). Interestingly, vitamin C increases the stability of TGF-β-induced FOXP3 expression in Vδ2 cells through an epigenetic modification of the FOXP3 gene, and enhances their suppressive capacities (49). Li et al. demonstrated that upon TCR stimulation Vδ1 T cells can be polarized toward a suppressive phenotype in the presence of IL-2 and TGF-β. These Vδ1 cells express FOXP3 and suppress the proliferation

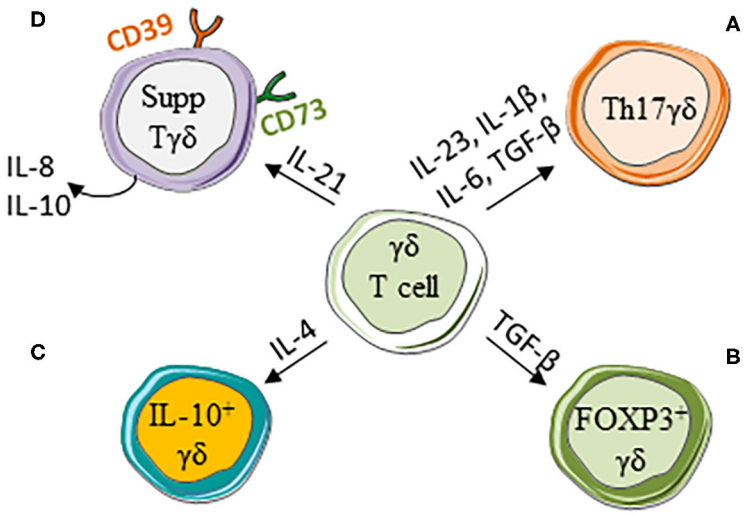

FIGURE 1 | γδ T cell polarization into pro-tumor cells. Cytokines present in the tumor microenvironment induce the differentiation of γδ T cells into pro-tumor cells: **(A)** Th17-like γδ T cells (Th17 γδ), **(B)** FOXP3+γδ T cells (FOXP3+γδ), **(C)** IL-10-producing γδ T cells (IL-10+ γδ), and **(D)** regulatory γδ T cells that express CD39 and/or CD73 (Supp γδ).

of activated CD4+ T cells (50). In human colorectal cancer, tumor-infiltrating CD39+ γδ T cells were described as regulatory γδ T cells that express FOXP3 and act mainly through the adenosine pathway (31). The authors found that TGF-B1 mRNA level is higher in the tumor than in the associated normal tissue. Moreover, CD39+ γδ T cells from normal tissue incubated with tumor supernatant acquire a potent suppressive capacity through increased adenosine production. This effect can be abrogated by incubation with an anti-TGF-β antibody, and can be reproduced by stimulating cells with recombinant TGF-β. TGF-β-induced polarization of γδ T cells toward FOXP3+ suppressive cells was also demonstrated in the mouse (51). Additionally, TGF-β is required for the polarization of Vγ9Vδ2 into IL-17-producing γδ T cells, together with IL-1β, IL-6 and IL-23, as described below (52). Overall, these results suggest that TGF-β could be one of the key factors responsible for conversion of γδ T cells into suppressive and/or IL-17-producing cells.

IL-4

IL-4 is a potent regulator of the humoral response and more generally of the adaptive immunity, particularly through the differentiation of naive T cells into Th2 cells. In cancer, IL-4 has been associated with tumor aggressiveness, and IL-4 pathway blockade is currently investigated as anti-cancer strategy (53). IL-4 is often enriched in the microenvironment of human solid tumors, notably in cancers with high γδ T cell infiltration, such as breast cancer (54). *In vitro*, human Vδ2 cells isolated from peripheral blood and activated by phosphoantigens in the presence of IL-4 produce low levels of interferon γ (IFN-γ) and high levels of IL-4, although this production is not stable over time (55). In a more recent study, Mao et al. showed that IL-4 inhibits *in vitro* the activation of blood γδ T cells induced by TCR stimulation (54). Nevertheless, IL-4 promotes the growth of activated γδ T cells and increases the levels of Vδ1 T cells, which in turn inhibit Vδ2 T-cell growth via significant IL-10 secretion (54). IL-4 inhibits γδ T cell activation when present at the moment of the stimulation, but enhances their proliferation when added later. Moreover, concanavalin A-stimulated Vδ1 T cells cultured with IL-4 retain their cytotoxic properties against tumor cells. This suggests a complex and context-dependent role of IL-4 in γδ T cell polarization (56).

IL-21

IL-21 is a potent immunomodulatory cytokine, mainly produced by activated CD4+ T cells and NKT cells. IL-21 enhances the effector functions of NK cells, helper CD4+ T cells and cytotoxic T cells (CTL), but also inhibits Tregs (57). Therefore, it is often defined as a pro-inflammatory cytokine. In colorectal cancer, IL-21 is strongly associated with chronic inflammatory colitis that precedes the malignant disease (57–59). A similar pro-inflammatory effect of IL-21 on γδ T cells was initially described. Upon *in vitro* expansion with IL-21, human Vγ9Vδ2 cells display increased levels of granzyme B and increased production of IFN-γ after activation, resulting in enhanced cytotoxic activity toward tumor cells (60). However, IL-21 modulatory role may depend on the cell type and the duration of the exposure. For example, IL-21 enhances IL-10 production by regulatory B cells and their proliferation. Similarly, our group recently found that IL-21 is implicated in the polarization of human Vγ9Vδ2 T cells and Vδ1 T cells toward a regulatory phenotype (30, 61). We isolated a subpopulation of CD73+ regulatory Vγ9Vδ2 T cells following their expansion in the presence of IL-21. We demonstrated that this subset can synthetize adenosine through CD73 enzymatic activity, and produces the suppressive cytokine IL-10 and the chemokine IL-8 (also known as CXCL8) that is involved in the recruitment of polymorphonuclear leukocytes

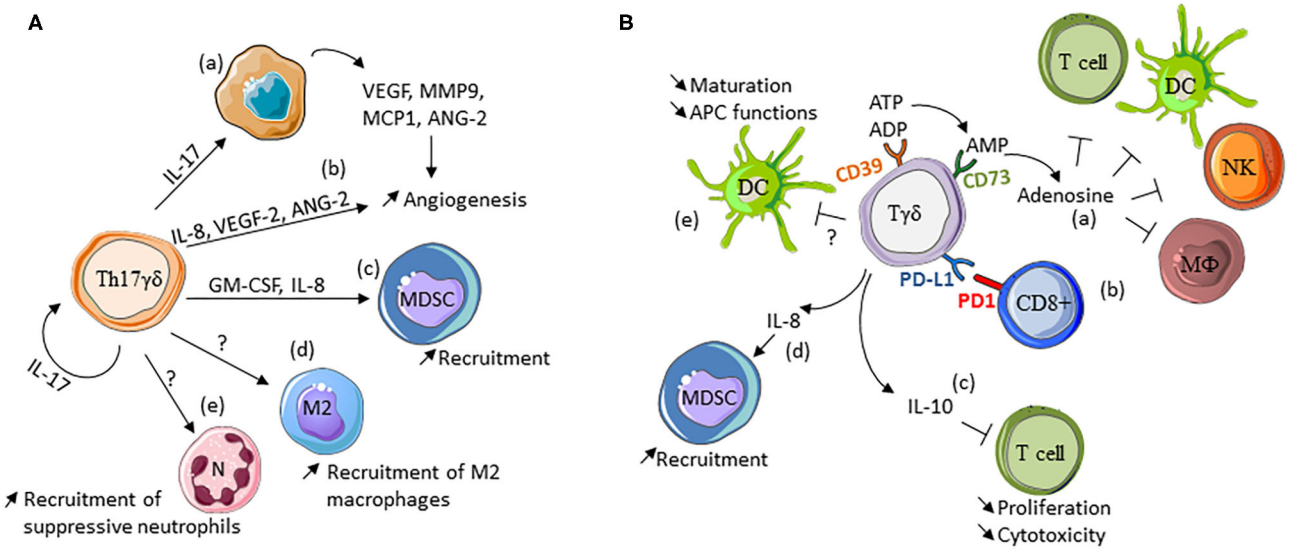

FIGURE 2 | Pro-tumor functions of γδ T cells. **(A)** Th17-like γδ T cells. Th17γδ cells promote angiogenesis indirectly by inducing the production of angiogenic factors by cancer cells (a), or directly by producing angiogenic factors (b). They also induce myeloid-derived suppressor cell (MDSC) recruitment through GM-CSF and IL-8 production (c), and recruit or polarize M2 macrophages (d) and suppressive neutrophils (e). **(B)** Pro-tumor γδ T cells. CD39+/CD73+ γδ T cells produce adenosine that inhibits αβ T cell proliferation and anti-tumor functions (a). γδ T cells can express PD-L1 and inhibit the function of PD1-expressing cells, such as CD8 + cells (b). IL-10 produced by γδ T cells inhibits CD8 + cell proliferation and cytotoxicity (c). They also favor MDSC recruitment via IL-8 (d) and inhibit dendritic cell (DC) maturation and functions through not identified mechanisms (e). NK, natural killer cells; Mϕ, macrophages, APC, antigen-presenting cell.

(PMN)-MDSCs. This CD73+ cell subpopulation can suppress the T cell immune response directly in an adenosine- and IL-10-dependent manner, and indirectly by impairing DC antigen presentation (61). We then extended these observations to Vδ1 T cells. We identified in the blood of healthy donors a Vδ1 T cell subpopulation that expresses CD73 and displays immunosuppressive phenotype and functions (i.e., production of immunosuppressive molecules, such as IL-10, adenosine and IL-8). As shown for Vγ9Vδ2 T cells, incubation with IL-21 favors the development and amplification of this Vδ1 subset. Importantly, we detected CD73+ γδ T cells in breast cancer biopsies, suggesting that they could interfere with the anti-tumor response (30). Moreover, in mouse γδ T cells, CD73 expression is increased after exposure to IL-21, suggesting that this polarization could be a common mechanism among different species (61). Interestingly, after infection with Mycobacterium bovis Bacillus Calmette-Guerin (BCG), the number of IL-17-producing γδ T cells was higher in IL-21 receptor knockout mice than wild type animals. IL-21 induces the apoptosis of these cells, suggesting the existence of a balance between IL-21-induced regulatory γδ T cells and IL-17-producing γδ T cells, at least in some contexts (62).

Polarization Into Th17-Like Cells

IL-17 production was first described in helper CD4+ cells, called Th17 cells. Th17 cell cytokine secretion, transcription regulation and effects on the immune system are now well-characterized. Their development is controlled by the transcription factors RORγt (63) and STAT3, and also by IRF4 in some cases when the differentiation is induced by cytokines (64). In mice, TGF-β, IL-6, IL-21 and IL-23 play a critical role in the differentiation or polarization of CD4+ cells into Th17 cells. In humans, IL-1 and IL-23 seem to have the most important role in Th17 cell differentiation, followed by TGF-β and IL-6 (65–67). IL-17 is produced by murine γδ T cells (68) and also by human γδ T cells (69). In both species, IL-7 strongly promotes the expansion of IL-17-producing γδ T cells (Th17 γδ T cells) (70). Moreover, several studies have shown that when cultured in the presence of various cytokine combinations, naive Vγ9Vδ2 T cells acquire an IL-17-secreting Th17-like phenotype or a mixed Th1/Th17 phenotype, and produce both IFN-γ and IL-17 (52, 71, 72). Human cord blood-derived Vγ9Vδ2 T cells stimulated with the phosphoantigen (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) require IL-6, IL-1β and TGF-β to differentiate into Th17 γδ cells, and also IL-23 for differentiation into γδ Th1/Th17 cells (71, 72). In adults, differentiation of naive γδ T cells into memory γδ Th1/Th17 T cells and Th17 γδ T cells requires IL-23, IL-1β and TGF-β, but not IL-6. γδ Th17 cells can also produce IL-22 (especially cells in the cord blood) (71, 72). The pro-tumor role of IL-17 has been well established in some contexts, and the pro-tumor role of Th17 γδ T cells will be developed in the next part.

PRO-TUMORAL FUNCTIONS OF γδ T CELLS (FIGURE 2)

Th17 γδ T Cells

IL-17 is detected in mice and human tumor (73–75), and αβ Th17 cells are not the only source of IL-17. Indeed, NK cells,

neutrophils and γδ T cells also produce IL-17. Notably, Th17 γδ T cells are the first and major source of IL-17 at sites of inflammation or infection, and also in tumors.

Although Ma et al. showed that IL-17-producing γδ T cells (Vγ4 and Vγ6) contribute to the chemotherapy-induced anti-cancer immune response (76), many studies found that Th17 γδ cells display pro-tumor functions in mouse models and human solid cancers.

In mouse models of fibrosarcoma (77), ovarian (78) and breast cancers (79), γδ T cells are the main IL-17 producers at the tumor site, and promote tumor growth. Th17 γδ T cells increase the expression of the angiogenic factors VEGF-2 and ANG-2 at the tumor sites, suggesting that tumor-infiltrating IL-17-producing γδ T cells promote tumor development by enhancing angiogenesis (77). They also participate in the establishment of an immunosuppressive TME through the recruitment, expansion and polarization of neutrophils that can suppress cytotoxic T lymphocyte (CTL) activities (79), and the recruitment of MDSCs or small peritoneal macrophages in ovarian cancer. All these cells also induce the expression of pro-tumor and pro-angiogenic factors that promote tumor growth.

In human solid cancers, Wu et al. were the first to demonstrate the pro-tumor role of IL-17-producing γδ T cells in human colorectal cancer (33). They showed that the main IL-17 producers in colon cancer are γδ T cells (up to 83% of Vδ1 T cells). In this cancer, Th17 γδ T cell differentiation and activation are triggered by IL-23 produced by activated DCs present at the tumor site. Colon cancer-infiltrating Th17 γδ T cells produce also IL-8 that participates in tumor progression through its role in angiogenesis and in MDSC recruitment. These MDSCs contribute to establishing an immunosuppressive microenvironment that favors tumor development. Interestingly, the strong and positive correlation between tumor-infiltrating Th17 γδ T cells and TNM stage (tumor size, lymphatic invasion, and metastases) strengthens the pro-tumor activities of Th17 γδ T cells in human colorectal cancer (33). Studies in patients with gallbladder cancer showed an increase of Th17 γδ T cells in the blood (compared with healthy individuals), and also of tumor-infiltrating lymphocytes in patients who did not receive any treatment. They confirmed the implication of Th17 γδ T cells in angiogenesis promotion (induction of VEGF production by gallbladder cancer cells) and tumor progression. Moreover, the presence of Th17 γδ T cells in the blood of patients is associated with poor survival compared with patients with few or without Th17 γδ T cells (34). Lo Presti et al. showed that γδ T cells are increased in the blood and at the tumor site in patients with squamous cell carcinoma. Interestingly, tumor-infiltrating γδ T cells are functionally different depending on the tumor stage (80). At early stages, γδ T cells produce mainly IFN-γ, while at late stages, they produce IL-17. Indeed, higher numbers of IL-17-producing cells (both Vδ1 and Vδ2 γδ T cell subsets) are found in advanced-stage squamous cell carcinoma compared with early stage tumors. They also showed that both Vδ1 and Vδ2 cell subsets produce high levels of IL-17 at the tumor site. Moreover, Vδ2 T cells produce IFN-γ in the blood, suggesting that Th17 γδ T polarizing factors are present in the TME (80).

Overall, many reports demonstrated the pro-tumor functions of γδ T cells with a Th17 γδ T phenotype. To date, it is not possible to say whether this Th17 γδ T cell sub-population is recruited at the tumor site or is polarized *in situ* toward IL-17-producing cells due to the presence of Th17-polarizing cytokines in the TME (e.g., IL-1β, IL-23, TGF- β, IL-6). Nevertheless, it is now well-established that Th17 γδ T cells favor tumor growth by promoting angiogenesis, metastasis development, and the recruitment of other immunosuppressive cells, such as suppressive neutrophils and MDSCs.

Production of Suppressive Cytokines

As discussed in the polarization section, upon exposure to specific stimuli γδ T cells can acquire potent regulatory functions, particularly through the production of IL-10 and TGF-β, two strongly suppressive cytokines.

IL-10 is a key anti-inflammatory cytokine that inhibits the production of pro-inflammatory cytokines and the expression of co-stimulatory molecules by Th1 and antigen-presenting cells (81). *In vitro*, IL-4-polarized Vδ1 T cells produce IL-10 and inhibit the growth of Vδ2 T cells in an IL-10-dependent manner. Similarly, Vδ1 T cells activated with anti-TCR antibodies strongly secrete IL-10 (54, 82). In the presence of IL-21, the CD73+ Vδ2 and Vδ1 T cell subsets secrete high levels of IL-10 upon activation (30, 61). In human colorectal cancer, infiltrating CD39+ γδ T cells, which are mainly Vδ1+ cells, produce more IL-10 than CD39- γδ T cells and CD39+ γδ T cells from the tumor-adjacent normal tissue. However, after several days of culture *ex vivo*, these cells do not maintain IL-10 production and lose their ability to suppress the proliferation of activated T cells (31). In mice, IL-10-producing γδ T cells have been identified in tumors. In a breast cancer model, supernatant from infiltrating γδ T cells suppresses the proliferation of anti-tumor CTLs in an IL-10-dependent manner (83). In a syngeneic model of OVA-expressing EL4 tumors (lymphoma), IL-10-producing γδ T cells suppress the CD8-dependent anti-tumor response, and their depletion significantly reduces tumor growth (84). Similarly, IL-10+ γδ T cells are observed in the spleen and tumors of mice grafted with TC1 cells (transformed lung epithelial cells) (61). IL-10-producing γδ T cells are also observed in other conditions, for instance during pregnancy (both human and mouse), and in oral tolerance and infection in the mouse (85–87). Collectively, these results suggest that Vδ1 and Vδ2 T cells can produce IL-10; however, the amount and the impact of this production in human tumors has not been clearly established yet.

TGF-β is a potent immunosuppressive factor that is tightly regulated, particularly at the post-translational level. To be active, the mature part of the protein needs to be released from the latent peptide (LAP) through interaction with the integrin αvβ6 or αvβ8, the main activating partners of TGF-β. *In vitro*, TGFβ mRNA level and LAP surface expression are increased in Vδ1 T cells sorted from PBMCs and activated with anti-CD3 and anti-CD28 antibodies (88). High TGF-β level has also been detected in the supernatant of PBMCs stimulated with an anti-TCR Vδ1 antibody (82), and in the supernatant of Vδ2 T cells stimulated with the ligand isopentenyl pyrophosphate and expanded with TGF-β and IL-15 (45). In colorectal cancer, TGF-β surface

expression is higher in γδ T cells isolated from tumors than from normal tissue (31). Interestingly, in the mouse tumor model MM2, infiltrating γδ T cells suppress the anti-MM2 CTLs through TGF-β in addition to IL-10 (83). However, it is unclear whether total or active TGF-β was measured in these studies. While total TGF-β is a measure of the whole TGF-β production by the cells, only active TGF-β quantification indicates the actual suppressive potential of such cells through TGF-β. Indeed, in these studies, γδ T cell suppressive properties were not affected by a neutralizing anti-TGF-β antibody, despite their supposed high level of TGF-β production, or the impact of TGF-β neutralization was not explored. A possible explanation for this discrepancy is that only total TGF-β was measured and not active TGF-β. This argument is supported by the reported high concentration that is more consistent with the measurement of total TGF-β. These results suggest that human γδ T cells, particularly Vδ1 T cells, can produce and present latent TGF-β at their surface in some contexts. However, because of the lack of αvβ6 or αvβ8 integrin expression, γδ T cells might not be able to produce active TGF-β on their own, unlike conventional Tregs (89, 90). Nonetheless, the presence of latent TGF-β at the γδ T cell surface is highly relevant because they represent a new source of latent TGF-β that may be activated by integrin-expressing partners within the tumor.

Besides the production of directly suppressive cytokines, γδ T cells also support the establishment of a suppressive TME through the production of other cytokines, such as IL-8 and granulocyte macrophage-colony stimulating factor (GM-CSF) that favor PMN-MDSC accumulation and expansion in colorectal cancer (33). Interestingly, IL-21, which is highly expressed in this cancer type, increases the production of IL-8 by CD73+ Vδ2 T cells and Vδ1 T cells *in vitro* (30, 61).

Involvement of the Adenosine Pathway

Extracellular ATP and adenosine are considered potent modulators of the anti-tumor immune response. Extracellular ATP, released by apoptotic cells for example, induces inflammation and promotes strong anti-tumor responses because it increases the immunogenicity of dying cancer cells (91, 92). It favors the recruitment of phagocytes, the recruitment and maturation of DC, inhibits the proliferation of tumor cells but not of healthy cells, and promotes cancer cell death (91, 93, 94). Conversely, extracellular adenosine inhibits the anti-tumor immune response and induces the establishment of an immunosuppressive microenvironment (95). The adenosine pathway involves the ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1 or CD39) that catalyzes the phosphohydrolysis of extracellular ATP into ADP and of ADP into AMP, and the ecto-5′-nucleotidase CD73 that completes AMP conversion into adenosine (92, 96, 97). It has been shown that γδ T cells express CD39 and/or CD73 during inflammation and in the TME. Their expression is associated with suppression or inhibition of the immune response (98–100). In murine pancreatic cancer, Daley et al. found that tumor-infiltrating γδ T cells upregulate CD39 expression (among other immunosuppressive molecules) and promote tumor progression by restricting αβ T cell activation (36). Hu and colleagues described in human colorectal cancer a subpopulation of regulatory γδ T cells that express CD39 (31). CD39+ γδ T cells are enriched at the tumor site and produce high levels of adenosine in the TME, compared with other regulatory cells such as conventional Tregs. Furthermore, they showed that infiltration of CD39+ γδ T cells is positively correlated with the TNM stage, suggesting that these cells participate in the establishment of an immunosuppressive TME, thus promoting tumor growth (31). *In vitro*, our group identified subpopulations of regulatory γδ T cells isolated from peripheral blood that express CD73 and can produce adenosine. These CD73+ populations (Vγ9Vδ2 or Vδ1) also express CD39 and catalyze the transformation of ATP into adenosine, thus displaying immunosuppressive functions, as revealed by their capacity to inhibit αβ T cell proliferation (30, 61). These regulatory CD73+ γδ T cells are found in human breast cancer samples, suggesting that they could interfere with the anti-tumor immune response and favor tumor progression (30). Altogether, these studies indicate that the CD39/CD73/adenosine pathway is a major component of γδ T cell regulatory/immunosuppressive functions in the TME.

Other Suppressive Mechanisms of γδ T Cells

The previously described regulatory γδ T cells can contribute to the establishment of an immunosuppressive microenvironment and to the inhibition of the anti-tumor response in different manners, for instance by producing inhibitory factors (e.g., IL-10, IL-8, TGF-β and adenosine) or by recruiting immunosuppressive cells (e.g., MDSCs and neutrophils). γδ T cells can also exert their regulatory functions by providing negative co-stimulatory signals to T cells in the TME through expression of immune checkpoint proteins. Programmed cell death 1 (PD1) and its ligand programmed cell death 1 ligand 1 (PD-L1) play a major role in the negative regulation of cell-mediated immune responses. Indeed, PD1 is expressed by T cells, and upon binding to its ligand (expressed by B cells, macrophages and cancer cells), it inhibits T cell activation, thus impairing the anti-tumor T cell response. Peters et al. showed that Vδ2 T cells obtained from the blood of healthy donors can express PD-L1 following activation (47). These cells inhibit αβ T cell proliferation in co-culture experiments, and this effect can be abrogated by PD-L1 blockade (47). This could be another mechanism by which regulatory γδ T cells exert their immunosuppressive activities and promote tumor growth. In agreement, Daley et al. showed in a pancreatic cancer mouse model that PD-L1 expression is higher in tumor-infiltrating γδ T cells than in splenic γδ T cells (36). In co-culture experiments, they found that tumor-infiltrating γδ T cells prevent αβ T cell activation and that this inhibition is reversed by an anti-PD-L1 antibody (36). Interestingly, the same regulatory phenotype is observed in pancreatic ductal adenocarcinoma (PDAC). Indeed, PD-L1 is strongly expressed in γδ T cells from the blood of patients with pancreatic cancer compared with healthy donors. Tumor-infiltrating γδ T cells also express PD-L1 in human PDAC (50% of infiltrating γδ T cells), suggesting that γδ T cells can promote tumor progression through the PD1/PD-L1 axis (36).

T-cell immunoglobulin mucin receptor 3 (TIM-3) and its ligand galectin-9 (GAL-9) are other immune checkpoint molecules that participate in T cell response inhibition. TIM-3 interaction with GAL-9 limits T cell expansion and effector function in the TME (101, 102). GAL-9 expression is upregulated on tumor-infiltrating γδ T cells in human and mouse PDAC, and γδ T cell-mediated suppression is dependent on GAL-9 (36).

Little is known about the expression of other immune checkpoint molecules, such as PD-L2, CD80/86 and CTLA-4, by γδ T cells in cancer. More studies are needed to investigate the expression of these and other suppressive molecules to fully understand the mechanisms of action of regulatory γδ T cells.

IMPLICATIONS FOR γδ T CELL-BASED TUMOR IMMUNOTHERAPY

The discovery of γδ T cell-mediated tumor immune surveillance has led to much research to understand the underlying mechanisms and to harness their potent anti-tumor properties. It is now firmly established that γδ T cells are well-equipped to recognize and eliminate malignant cells (20, 103). Thus, much effort has focused on the development of therapeutics using γδ T cells, especially the Vγ9Vδ2 subset because they can be easily obtained and expanded from the blood (104, 105). Two main strategies were first investigated: (i) *in vivo* expansion of Vγ9Vδ2 T cells by injection of phosphoantigens and low-dose IL-2 in the patient, and (ii) adoptive transfer of *ex vivo* expanded Vγ9Vδ2 T cells. Clinical trials using both strategies in patients with hematological or solid cancers confirmed the safety of this immunotherapy (well-tolerated and no toxicity), but showed moderate clinical success (106–109). Indeed, the results were not as good as expected because only few patients showed complete response to the therapy. Among the reasons of these relatively modest clinical results were the skewing of γδ T cells toward a non-reactive or even a pro-tumor phenotype. For example, Hoeres et al. showed that incubation of PBMCs from patients with leukemia with IL-2 and/or zoledronic acid, which are used to activate γδ T cells, induces PD-1 expression by γδ T cells and impairs their anti-tumor functions (110). Similarly, Castella et al. reported PD-1 expression by γδ T cells in patients with myeloma after phosphoantigen activation (111). Several *in vitro* and *in vivo* studies, summarized here, have demonstrated that γδ T cell polarization toward suppressive and/or IL-17-producing cells is a real possibility and that anti- and pro-tumor γδ T cells might co-exist in the tumor.

After these first clinical trials, new refined approaches based on recent discoveries are currently being developed. Aminobisphosphonate activation of γδ T cells in combination with chemotherapy or with FDA-approved antibodies is one of these axes. Hoeres et al. and Castella et al. showed that incubation with an anti-PD-1 antibody restores the proliferative and anti-tumor properties of Vγ9Vδ2 T cells from patients with leukemia or lymphoma (110, 111). However, Castella et al. then found that phosphoantigen stimulation of anergic PD-1+ Vγ9Vδ2 combined with PD-1 blockade increases the expression of PD-1 and of two other immune checkpoint molecules (TIM-3 and LAG-3), leading to a "super-anergic" state (112). Thus, although the combination of γδ T cell stimulation and immune checkpoint blockade is an interesting and easily feasible therapeutic alternative, it still needs to be improved, by combining for example two or more antibodies against immune checkpoint molecules. The use of bi-specific T-cell engagers (BITEs), tribodies, and engineered T cells harboring a chimeric antigen receptor (CAR) are other interesting options. For instance, the redirection of Vγ9Vδ2 T cells against tumor cells using bispecific antibodies or tribodies is efficient in HER-2-positive PDAC and ovarian cancer (113). TEGs are αβ T cells engineered to express tumor-specific Vγ9Vδ2 TCRs. In *in vitro* models and in humanized mouse cancer models, TEGs reduce colony formation of progenitor cells of primary acute myeloid leukemia blasts and inhibit leukemia growth (114). TEGs engineered from patients with myeloma can recognize and efficiently kill myeloma cells in a 3D bone marrow niche model. Phase 1 clinical trials are currently in development to test TEGs, CAR γδ T cells, and antibodies (bispecific antibodies or anti-BTN3A antibodies) to specifically "engage" γδ T cells in the anti-tumor immune response (26).

Another strategy would be to focus on Vδ1 T cells, the main subpopulation that infiltrates the TME of solid tumors. Despite their potent anti-tumor properties, Vδ1 T cells had never been tested in the clinic due to lack of suitable expansion/differentiation protocols. Recently, Silva-Santos' team developed a new and robust clinical-grade method for selective and large-scale expansion and differentiation of cytotoxic Vδ1 T cells, and showed that these cells can inhibit tumor growth and dissemination in preclinical models of chronic lymphocytic leukemia (27).

On the basis of reports demonstrating γδ T cell pro-tumor functions, regulatory γδ T cell subsets could be a thorn in the side of these newly developed therapies and need to be taken into account. Unfortunately, no clear phenotypic marker of such cells has emerged yet. Vδ1 cells have been associated with pro-tumor T cells, but when cultured in proper conditions they show very high potential for anti-tumor therapies due to their strong reactivity and cytotoxicity toward tumor cells. Adenosine pathway markers (e.g., CD39 and CD73) are interesting, but do not characterize pro-tumor γδ T cells on their own. Indeed, CD39 can be considered as an activation marker for T cells (115, 116), and CD73 is also expressed by naive γδ T cells (117, 118). More studies are needed to better characterize γδ T cell pro-tumor phenotypes and to identify markers or marker combinations that will allow the depletion of pro-tumor subsets in the whole γδ T cell population.

In the absence of such specific phenotypic markers to deplete or sort out the pro-tumor γδ T cells before cell therapy, targeting polarizing cytokines or pro-tumor cytokines produced by pro-tumor γδ T cells could be of interest. While IL-21 expression might favor the emergence of a regulatory γδ T cell population, its positive role on the cytotoxicity of other cell types, such as CTL and NK cells, might be important for the anti-tumor response. Alternatively, targeting TGF-β as a pro-tumor cytokine and a polarizing factor for γδ T cells toward both suppressive and IL-17-producing cells might be of interest. Newly developed highly

selective approaches targeting the TGF-β-anchoring protein GARP or the latent TGF-β peptide LAP could be employed in pro-tumor γδ T cell-rich tumors, such as colorectal cancer, or with γδ T cell-based therapies to avoid their polarization (119, 120). While no anti-human IL-10 antibody has been approved for cancer treatment, the production of IL-17A and adenosine could be targeted in tumors that are highly infiltrated by pro-tumor γδ T cells, such as breast and colorectal cancer.

CONCLUDING REMARKS

Although γδ T cells offer interesting perspectives for clinical applications in cell-based immunotherapy, their pro-tumor functions have to be taken into account. Indeed, environmental factors can polarize or repolarize γδ T cells, leading to loss of the anti-tumor function. Moreover, important advances in γδ T cell immunobiology have revealed a large diversity in functionality and activation modes of these cells. The new challenge is to better characterize and understand the role of the various γδ T cell subsets in function of the specific context.

AUTHOR CONTRIBUTIONS

GC, CB, and VL wrote the initial draft. GC prepared the figures and CB the table. GC, CB, VL, and NB reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Kabelitz D, Marischen L, Oberg HH, Holtmeier W, Wesch D. Epithelial defence by gamma delta T cells. *Int Arch Allergy Immunol*. (2005) 137:73–81. doi: 10.1159/000085107
2. Willcox CR, Pitard V, Netzer S, Couzi L, Salim M, Silberzahn T, et al. Cytomegalovirus and tumor stress surveillance by binding of a human gammadelta T cell antigen receptor to endothelial protein C receptor. *Nat Immunol*. (2012) 13:872–9. doi: 10.1038/ni.2394
3. Yang Y, Li L, Yuan L, Zhou X, Duan J, Xiao H, et al. A structural change in butyrophilin upon phosphoantigen binding underlies phosphoantigen-mediated Vgamma9Vdelta2 T cell activation. *Immunity*. (2019) 50:1043–53 e1045. doi: 10.1016/j.immuni.2019.02.016
4. Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, Ruan Z, et al. Butyrophilin 2A1 is essential for phosphoantigen reactivity by gammadelta T cells. *Science*. (2020) 367:eaay5516. doi: 10.1126/science.aay5516
5. Fournie JJ, Bonneville M. Stimulation of gamma delta T cells by phosphoantigens. *Res Immunol*. (1996) 147:338–47. doi: 10.1016/0923-2494(96)89648-9
6. Battistini L, Borsellino G, Sawicki G, Poccia F, Salvetti M, Ristori G, et al. Phenotypic and cytokine analysis of human peripheral blood gamma delta T cells expressing NK cell receptors. *J Immunol*. (1997) 159:3723–30.
7. De Libero G. Control of gammadelta T cells by NK receptors. *Microbes Infect*. (1999) 1:263–7. doi: 10.1016/S1286-4579(99)80043-4
8. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science*. (1998) 279:1737–40. doi: 10.1126/science.279.5357.1737
9. Spada FM, Grant EP, Peters PJ, Sugita M, Melian A, Leslie DS, et al. Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. *J Exp Med*. (2000) 191:937–48. doi: 10.1084/jem.191.6.937
10. Bai L, Picard D, Anderson B, Chaudhary V, Luoma A, Jabri B, et al. The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vdelta1 TCR. *Eur J Immunol*. (2012) 42:2505–10. doi: 10.1002/eji.201242531
11. Uldrich AP, Le Nours J, Pellicci DG, Gherardin NA, Mcpherson KG, Lim RT, et al. CD1d-lipid antigen recognition by the gammadelta TCR. *Nat Immunol*. (2013) 14:1137–45. doi: 10.1038/ni.2713
12. Zeng X, Wei YL, Huang J, Newell EW, Yu H, Kidd BA, et al. gammadelta T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific interleukin-17 response. *Immunity*. (2012) 37:524–34. doi: 10.1016/j.immuni.2012.06.011
13. Fausther-Bovendo H, Wauquier N, Cherfils-Vicini J, Cremer I, Debre P, Vieillard V. NKG2C is a major triggering receptor involved in the V[delta]1 T cell-mediated cytotoxicity against HIV-infected CD4 T cells. *AIDS*. (2008) 22:217–26. doi: 10.1097/QAD.0b013e3282f46e7c
14. Wesch D, Peters C, Oberg HH, Pietschmann K, Kabelitz D. Modulation of gammadelta T cell responses by TLR ligands. *Cell Mol Life Sci*. (2011) 68:2357–70. doi: 10.1007/s00018-011-0699-1
15. Hudspeth K, Fogli M, Correia DV, Mikulak J, Roberto A, Della Bella S, et al. Engagement of NKp30 on Vdelta1 T cells induces the production of CCL3, CCL4, and CCL5 and suppresses HIV-1 replication. *Blood*. (2012) 119:4013–6. doi: 10.1182/blood-2011-11-390153
16. Kuroda H, Saito H, Ikeguchi M. Decreased number and reduced NKG2D expression of Vdelta1 gammadelta T cells are involved in the impaired function of Vdelta1 gammadelta T cells in the tissue of gastric cancer. *Gastric Cancer*. (2012) 15:433–9. doi: 10.1007/s10120-011-0138-x
17. Maher CO, Dunne K, Comerford R, O'dea S, Loy A, Woo J, et al. *Candida albicans* stimulates IL-23 release by human dendritic cells and downstream IL-17 secretion by Vdelta1 T cells. *J Immunol*. (2015) 194:5953–60. doi: 10.4049/jimmunol.1403066
18. Bonneville M, O'brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol*. (2010) 10:467–78. doi: 10.1038/nri2781
19. Adams EJ, Gu S, Luoma AM. Human gamma delta T cells: evolution and ligand recognition. *Cell Immunol*. (2015) 296:31–40. doi: 10.1016/j.cellimm.2015.04.008
20. Gomes AQ, Martins DS, Silva-Santos B. Targeting gammadelta T lymphocytes for cancer immunotherapy: from novel mechanistic insight to clinical application. *Cancer Res*. (2010) 70:10024–7. doi: 10.1158/0008-5472.CAN-10-3236
21. Marquez-Medina D, Salla-Fortuny J, Salud-Salvia A. Role of gamma-delta T-cells in cancer: another opening door to immunotherapy. *Clin Transl Oncol*. (2012) 14:891–5. doi: 10.1007/s12094-012-0935-7
22. Buccheri S, Guggino G, Caccamo N, Li Donni P, Dieli F. Efficacy and safety of gammadeltaT cell-based tumor immunotherapy: a meta-analysis. *J Biol Regul Homeost Agents*. (2014) 28:81–90.
23. Pressey JG, Adams J, Harkins L, Kelly D, You Z, Lamb LSJr. *In vivo* expansion and activation of gammadelta T cells as immunotherapy for

refractory neuroblastoma: a phase 1 study. *Medicine*. (2016) 95:e4909. doi: 10.1097/MD.0000000000004909
24. Lo Presti E, Pizzolato G, Gulotta E, Cocorullo G, Gulotta G, Dieli F, et al. Current advances in gammadelta T cell-based tumor immunotherapy. *Front Immunol*. (2017) 8:1401. doi: 10.3389/fimmu.2017.01401
25. Pauza CD, Liou ML, Lahusen T, Xiao L, Lapidus RG, Cairo C, et al. Gamma delta T cell therapy for cancer: it is good to be local. *Front Immunol*. (2018) 9:1305. doi: 10.3389/fimmu.2018.01305
26. Garber K. gammadelta T cells bring unconventional cancer-targeting to the clinic - again. *Nat Biotechnol*. (2020) 38:389–91. doi: 10.1038/s41587-020-0487-2
27. Almeida AR, Correia DV, Fernandes-Platzgummer A, Da Silva CL, Da Silva MG, Anjos DR, et al. Delta one T cells for immunotherapy of chronic lymphocytic leukemia: clinical-grade expansion/differentiation and preclinical proof of concept. *Clin Cancer Res*. (2016) 22:5795–804. doi: 10.1158/1078-0432.CCR-16-0597
28. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang RF. Tumor-infiltrating gammadelta T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway. *Immunity*. (2007) 27:334–48. doi: 10.1016/j.immuni.2007.05.020
29. Ma C, Zhang Q, Ye J, Wang F, Zhang Y, Wevers E, et al. Tumor-infiltrating gammadelta T lymphocytes predict clinical outcome in human breast cancer. *J Immunol*. (2012) 189:5029–36. doi: 10.4049/jimmunol.1201892
30. Chabab G, Barjon C, Abdellaoui N, Salvador-Prince L, Dejou C, Michaud HA, et al. Identification of a regulatory Vdelta1 gamma delta T cell subpopulation expressing CD73 in human breast cancer. *J Leukoc Biol*. (2020) 107:1057–67. doi: 10.1002/JLB.3MA0420-278RR
31. Hu G, Wu P, Cheng P, Zhang Z, Wang Z, Yu X, et al. Tumor-infiltrating CD39(+)gammadeltaTregs are novel immunosuppressive T cells in human colorectal cancer. *Oncoimmunology*. (2017) 6:e1277305. doi: 10.1080/2162402X.2016.1277305
32. Rong L, Li K, Li R, Liu HM, Sun R, Liu XY. Analysis of tumor-infiltrating gamma delta T cells in rectal cancer. *World J Gastroenterol*. (2016) 22:3573–80. doi: 10.3748/wjg.v22.i13.3573
33. Wu P, Wu D, Ni C, Ye J, Chen W, Hu G, et al. gammadeltaT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. *Immunity*. (2014) 40:785–800. doi: 10.1016/j.immuni.2014.03.013
34. Patil RS, Shah SU, Shrikhande SV, Goel M, Dikshit RP, Chiplunkar SV. IL17 producing gammadeltaT cells induce angiogenesis and are associated with poor survival in gallbladder cancer patients. *Int J Cancer*. (2016) 139:869–81. doi: 10.1002/ijc.30134
35. Chen X, Shang W, Xu R, Wu M, Zhang X, Huang P, et al. Distribution and functions of gammadelta T cells infiltrated in the ovarian cancer microenvironment. *J Transl Med*. (2019) 17:144. doi: 10.1186/s12967-019-1897-0
36. Daley D, Zambirinis CP, Seifert L, Akkad N, Mohan N, Werba G, et al. gammadelta T cells support pancreatic oncogenesis by restraining alphabeta T cell activation. *Cell*. (2016) 166:1485–99 e1415. doi: 10.1016/j.cell.2016.07.046
37. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med*. (2015) 21:938–45. doi: 10.1038/nm.3909
38. Tosolini M, Pont F, Poupot M, Vergez F, Nicolau-Travers ML, Vermijlen D, et al. Assessment of tumor-infiltrating TCRVgamma9Vdelta2 gammadelta lymphocyte abundance by deconvolution of human cancers microarrays. *Oncoimmunology*. (2017) 6:e1284723. doi: 10.1080/2162402X.2017.1284723
39. Lo Presti E, Mocciaro F, Mitri RD, Corsale AM, Di Simone M, Vieni S, et al. Analysis of colon-infiltrating gammadelta T cells in chronic inflammatory bowel disease and in colitis-associated cancer. *J Leukoc Biol*. (2020) 108:749–60. doi: 10.1002/JLB.5MA0320-201RR
40. Lafont V, Sanchez F, Laprevotte E, Michaud HA, Gros L, Eliaou JF, et al. Plasticity of gammadelta T cells: impact on the anti-tumor response. *Front Immunol*. (2014) 5:622. doi: 10.3389/fimmu.2014.00622
41. Travis MA, Sheppard D. TGF-beta activation and function in immunity. *Annu Rev Immunol*. (2014) 32:51–82. doi: 10.1146/annurev-immunol-032713-120257
42. Qin Y, Garrison BS, Ma W, Wang R, Jiang A, Li J, et al. A milieu molecule for TGF-beta required for microglia function in the nervous system. *Cell*. (2018) 174:156–71. e116. doi: 10.1016/j.cell.2018.05.027
43. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. (2003) 198:1875–86. doi: 10.1084/jem.20030152
44. Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol*. (2008) 9:641–9. doi: 10.1038/ni.1610
45. Casetti R, Agrati C, Wallace M, Sacchi A, Martini F, Martino A, et al. Cutting edge: TGF-beta1 and IL-15 Induce FOXP3+ gammadelta regulatory T cells in the presence of antigen stimulation. *J Immunol*. (2009) 183:3574–7. doi: 10.4049/jimmunol.0901334
46. Hu Y, Cui Q, Gu Y, Sheng L, Wu K, Shi J, et al. Decitabine facilitates the generation and immunosuppressive function of regulatory gammadeltaT cells derived from human peripheral blood mononuclear cells. *Leukemia*. (2013) 27:1580–5. doi: 10.1038/leu.2012.345
47. Peters C, Oberg HH, Kabelitz D, Wesch D. Phenotype and regulation of immunosuppressive Vdelta2-expressing gammadelta T cells. *Cell Mol Life Sci*. (2014) 71:1943–60. doi: 10.1007/s00018-013-1467-1
48. Peters C, Hasler R, Wesch D, Kabelitz D. Human Vdelta2 T cells are a major source of interleukin-9. *Proc Natl Acad Sci USA*. (2016) 113:12520–5. doi: 10.1073/pnas.1607136113
49. Kouakanou L, Peters C, Sun Q, Floess S, Bhat J, Huehn J, et al. Vitamin C supports conversion of human gammadelta T cells into FOXP3-expressing regulatory cells by epigenetic regulation. *Sci Rep*. (2020) 10:6550. doi: 10.1038/s41598-020-63572-w
50. Li X, Kang N, Zhang X, Dong X, Wei W, Cui L, et al. Generation of human regulatory gammadelta T cells by TCRgammadelta stimulation in the presence of TGF-beta and their involvement in the pathogenesis of systemic lupus erythematosus. *J Immunol*. (2011) 186:6693–700. doi: 10.4049/jimmunol.1002776
51. Kang N, Tang L, Li X, Wu D, Li W, Chen X, et al. Identification and characterization of Foxp3(+) gammadelta T cells in mouse and human. *Immunol Lett*. (2009) 125:105–13. doi: 10.1016/j.imlet.2009.06.005
52. Caccamo N, La Mendola C, Orlando V, Meraviglia S, Todaro M, Stassi G, et al. Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood*. (2011) 118:129–38. doi: 10.1182/blood-2011-01-331298
53. Setrerrahmane S, Xu H. Tumor-related interleukins: old validated targets for new anti-cancer drug development. *Mol Cancer*. (2017) 16:153. doi: 10.1186/s12943-017-0721-9
54. Mao Y, Yin S, Zhang J, Hu Y, Huang B, Cui L, et al. A new effect of IL-4 on human gammadelta T cells: promoting regulatory Vdelta1 T cells via IL-10 production and inhibiting function of Vdelta2 T cells. *Cell Mol Immunol*. (2016) 13:217–28. doi: 10.1038/cmi.2015.07
55. Wesch D, Glatzel A, Kabelitz D. Differentiation of resting human peripheral blood gamma delta T cells toward Th1- or Th2-phenotype. *Cell Immunol*. (2001) 212:110–7. doi: 10.1006/cimm.2001.1850
56. Siegers GM, Ribot EJ, Keating A, Foster PJ. Extensive expansion of primary human gamma delta T cells generates cytotoxic effector memory cells that can be labeled with Feraheme for cellular MRI. *Cancer Immunol Immunother*. (2013) 62:571–83. doi: 10.1007/s00262-012-1353-y
57. Chabab G, Bonnefoy N, Lafont V. IL-21 Signaling in the Tumor Microenvironment. *Adv Exp Med Biol*. (2020) 1240:73–82. doi: 10.1007/978-3-030-38315-2_6
58. Stolfi C, Rizzo A, Franze E, Rotondi A, Fantini MC, Sarra M, et al. Involvement of interleukin-21 in the regulation of colitis-associated colon cancer. *J Exp Med*. (2011) 208:2279–90. doi: 10.1084/jem.20111106
59. De Simone V, Ronchetti G, Franze E, Colantoni A, Ortenzi A, Fantini MC, et al. Interleukin-21 sustains inflammatory signals that contribute to sporadic colon tumorigenesis. *Oncotarget*. (2015) 6:9908–23. doi: 10.18632/oncotarget.3532
60. Thedrez A, Sabourin C, Gertner J, Devilder MC, Allain-Maillet S, Fournie JJ, et al. Self/non-self discrimination by human gammadelta T cells: simple solutions for a complex issue? *Immunol Rev*. (2007) 215:123–35. doi: 10.1111/j.1600-065X.2006.00468.x

61. Barjon C, Michaud HA, Fages A, Dejou C, Zampieri A, They L, et al. IL-21 promotes the development of a CD73-positive Vgamma9Vdelta2 T cell regulatory population. *Oncoimmunology*. (2017) 7:e1379642. doi: 10.1080/2162402X.2017.1379642
62. Huang Y, Matsumura Y, Hatano S, Noguchi N, Murakami T, Iwakura Y, et al. IL-21 inhibits IL-17A-producing gammadelta T-cell response after infection with Bacillus Calmette-Guerin via induction of apoptosis. *Innate Immun*. (2016) 22:588–97. doi: 10.1177/1753425916664125
63. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity*. (2008) 28:29–39. doi: 10.1016/j.immuni.2007.11.016
64. Stockinger B, Veldhoen M. Differentiation and function of Th17 T cells. *Curr Opin Immunol*. (2007) 19:281–6. doi: 10.1016/j.coi.2007.04.005
65. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*. (2003) 421:744–8. doi: 10.1038/nature01355
66. Wilson NJ, Boniface K, Chan JR, Mckenzie BS, Blumenschein WM, Mattson JD, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol*. (2007) 8:950–7. doi: 10.1038/ni1497
67. Zhou L, Ivanov Ii, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol*. (2007) 8:967–74. doi: 10.1038/ni1488
68. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity*. (2009) 31:331–41. doi: 10.1016/j.immuni.2009.08.001
69. Deknuydt F, Scotet E, Bonneville M. Modulation of inflammation through IL-17 production by gammadelta T cells: mandatory in the mouse, dispensable in humans? *Immunol Lett*. (2009) 127:8–12. doi: 10.1016/j.imlet.2009.08.003
70. Michel ML, Pang DJ, Haque SF, Potocnik AJ, Pennington DJ, Hayday AC. Interleukin 7 (IL-7) selectively promotes mouse and human IL-17-producing gammadelta cells. *Proc Natl Acad Sci USA*. (2012) 109:17549–54. doi: 10.1073/pnas.1204327109
71. Ness-Schwickerath KJ, Jin C, Morita CT. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells. *J Immunol*. (2010) 184:7268–80. doi: 10.4049/jimmunol.1000600
72. Ness-Schwickerath KJ, Morita CT. Regulation and function of IL-17A- and IL-22-producing gammadelta T cells. *Cell Mol Life Sci*. (2011) 68:2371–90. doi: 10.1007/s00018-011-0700-z
73. Kato Y, Tanaka Y, Miyagawa F, Yamashita S, Minato N. Targeting of tumor cells for human gammadelta T cells by nonpeptide antigens. *J Immunol*. (2001) 167:5092–8. doi: 10.4049/jimmunol.167.9.5092
74. Numasaki M, Watanabe M, Suzuki T, Takahashi H, Nakamura A, Mcallister F, et al. IL-17 enhances the net angiogenic activity and *in vivo* growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. *J Immunol*. (2005) 175:6177–89. doi: 10.4049/jimmunol.175.9.6177
75. Kryczek I, Wei S, Zou L, Altuwaijri S, Szeliga W, Kolls J, et al. Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. *J Immunol*. (2007) 178:6730–3. doi: 10.4049/jimmunol.178.11.6730
76. Ma Y, Aymeric L, Locher C, Mattarollo SR, Delahaye NF, Pereira P, et al. Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy. *J Exp Med*. (2011) 208:491–503. doi: 10.1084/jem.20100269
77. Wakita D, Sumida K, Iwakura Y, Nishikawa H, Ohkuri T, Chamoto K, et al. Tumor-infiltrating IL-17-producing gammadelta T cells support the progression of tumor by promoting angiogenesis. *Eur J Immunol*. (2010) 40:1927–37. doi: 10.1002/eji.200940157
78. Rei M, Goncalves-Sousa N, Lanca T, Thompson RG, Mensurado S, Balkwill FR, et al. Murine CD27(−) Vgamma6(+) gammadelta T cells producing IL-17A promote ovarian cancer growth via mobilization of protumor small peritoneal macrophages. *Proc Natl Acad Sci USA*. (2014) 111:E3562–70. doi: 10.1073/pnas.1403424111
79. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature*. (2015) 522:345–8. doi: 10.1038/nature14282
80. Lo Presti E, Toia F, Oieni S, Buccheri S, Turdo A, Mangiapane LR, et al. Squamous cell tumors recruit gammadelta T cells producing either IL17 or IFNgamma depending on the tumor stage. *Cancer Immunol Res*. (2017) 5:397–407. doi: 10.1158/2326-6066.CIR-16-0348
81. Wei H, Li B, Sun A, Guo F. Interleukin-10 family cytokines immunobiology and structure. *Adv Exp Med Biol*. (2019) 1172:79–96. doi: 10.1007/978-981-13-9367-9_4
82. Hua F, Kang N, Gao YA, Cui LX, Ba DN, He W. Potential regulatory role of *in vitro*-expanded Vdelta1 T cells from human peripheral blood. *Immunol Res*. (2013) 56:172–80. doi: 10.1007/s12026-013-8390-2
83. Seo N, Tokura Y, Takigawa M, Egawa K. Depletion of IL-10- and TGF-beta-producing regulatory gamma delta T cells by administering a daunomycin-conjugated specific monoclonal antibody in early tumor lesions augments the activity of CTLs and NK cells. *J Immunol*. (1999) 163:242–9.
84. Ke Y, Kapp LM, Kapp JA. Inhibition of tumor rejection by gammadelta T cells and IL-10. *Cell Immunol*. (2003) 221:107–14. doi: 10.1016/S0008-8749(03)00066-2
85. Arck PC, Ferrick DA, Steele-Norwood D, Croitoru K, Clark DA. Murine T cell determination of pregnancy outcome: I. Effects of strain, alphabeta T cell receptor, gammadelta T cell receptor, and gammadelta T cell subsets. *Am J Reprod Immunol*. (1997) 37:492–502. doi: 10.1111/j.1600-0897.1997.tb00265.x
86. Rhodes KA, Andrew EM, Newton DJ, Tramonti D, Carding SR. A subset of IL-10-producing gammadelta T cells protect the liver from Listeria-elicited, CD8(+) T cell-mediated injury. *Eur J Immunol*. (2008) 38:2274–83. doi: 10.1002/eji.200838354
87. Fan DX, Duan J, Li MQ, Xu B, Li DJ, Jin LP. The decidual gamma-delta T cells up-regulate the biological functions of trophoblasts via IL-10 secretion in early human pregnancy. *Clin Immunol*. (2011) 141:284–92. doi: 10.1016/j.clim.2011.07.008
88. Kuhl AA, Pawlowski NN, Grollich K, Blessenohl M, Westermann J, Zeitz M, et al. Human peripheral gammadelta T cells possess regulatory potential. *Immunology*. (2009) 128:580–8. doi: 10.1111/j.1365-2567.2009.03162.x
89. Stockis J, Lienart S, Colau D, Collignon A, Nishimura SL, Sheppard D, et al. Blocking immunosuppression by human Tregs *in vivo* with antibodies targeting integrin alphaVbeta8. *Proc Natl Acad Sci USA*. (2017) 114:E10161–8. doi: 10.1073/pnas.1710680114
90. Siegers GM. Integral Roles for Integrins in gammadelta T Cell Function. *Front Immunol*. (2018) 9:521. doi: 10.3389/fimmu.2018.00521
91. Aymeric L, Apetoh L, Ghiringhelli F, Tesniere A, Martins I, Kroemer G, et al. Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity. *Cancer Res*. (2010) 70:855–8. doi: 10.1158/0008-5472.CAN-09-3566
92. Allard B, Longhi MS, Robson SC, Stagg J. The ectonucleotidases CD39 and CD73: novel checkpoint inhibitor targets. *Immunol Rev*. (2017) 276:121–44. doi: 10.1111/imr.12528
93. Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, et al. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature*. (2009) 461:282–6. doi: 10.1038/nature08296
94. Silva-Vilches C, Ring S, Mahnke K. ATP and its metabolite adenosine as regulators of dendritic cell activity. *Front Immunol*. (2018) 9:2581. doi: 10.3389/fimmu.2018.02581
95. La Sala A, Ferrari D, Corinti S, Cavani A, Di Virgilio F, Girolomoni G. Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate Th1 responses. *J Immunol*. (2001) 166:1611–7. doi: 10.4049/jimmunol.166.3.1611
96. Antonioli L, Pacher P, Vizi ES, Hasko G. CD39 and CD73 in immunity and inflammation. *Trends Mol Med*. (2013) 19:355–67. doi: 10.1016/j.molmed.2013.03.005
97. Bastid J, Cottalorda-Regairaz A, Alberici G, Bonnefoy N, Eliaou JF, Bensussan A. ENTPD1/CD39 is a promising therapeutic target in oncology. *Oncogene*. (2013) 32:1743–51. doi: 10.1038/onc.2012.269

98. Otsuka A, Hanakawa S, Miyachi Y, Kabashima K. CD39: a new surface marker of mouse regulatory gammadelta T cells. *J Allergy Clin Immunol*. (2013) 132:1448–51. doi: 10.1016/j.jaci.2013.05.037
99. Bastid J, Regairaz A, Bonnefoy N, Dejou C, Giustiniani J, Laheurte C, et al. Inhibition of CD39 enzymatic function at the surface of tumor cells alleviates their immunosuppressive activity. *Cancer Immunol Res*. (2015) 3:254–65. doi: 10.1158/2326-6066.CIR-14-0018
100. Liang D, Zuo A, Zhao R, Shao H, Born WK, O'brien RL, et al. CD73 Expressed on gammadelta T cells shapes their regulatory effect in experimental autoimmune uveitis. *PLoS ONE*. (2016) 11:e0150078. doi: 10.1371/journal.pone.0164502
101. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med*. (2010) 207:2187–94. doi: 10.1084/jem.20100643
102. Wherry EJ. T cell exhaustion. *Nat Immunol*. (2011) 12:492–9. doi: 10.1038/ni.2035
103. Hannani D, Ma Y, Yamazaki T, Dechanet-Merville J, Kroemer G, Zitvogel L. Harnessing gammadelta T cells in anticancer immunotherapy. *Trends Immunol*. (2012) 33:199–206. doi: 10.1016/j.it.2012.01.006
104. Bonneville M, Scotet E. Human Vgamma9Vdelta2 T cells: promising new leads for immunotherapy of infections and tumors. *Curr Opin Immunol*. (2006) 18:539–46. doi: 10.1016/j.coi.2006.07.002
105. Sugie T, Murata-Hirai K, Iwasaki M, Morita CT, Li W, Okamura H, et al. Zoledronic acid-induced expansion of gammadelta T cells from early-stage breast cancer patients: effect of IL-18 on helper NK cells. *Cancer Immunol Immunother*. (2013) 62:677–87. doi: 10.1007/s00262-012-1368-4
106. Bennouna J, Bompas E, Neidhardt EM, Rolland F, Philip I, Galea C, et al. Phase-I study of Innacell gammadelta, an autologous cell-therapy product highly enriched in gamma9delta2 T lymphocytes, in combination with IL-2, in patients with metastatic renal cell carcinoma. *Cancer Immunol Immunother*. (2008) 57:1599–609. doi: 10.1007/s00262-008-0491-8
107. Nakajima J, Murakawa T, Fukami T, Goto S, Kaneko T, Yoshida Y, et al. A phase I study of adoptive immunotherapy for recurrent non-small-cell lung cancer patients with autologous gammadelta T cells. *Eur J Cardiothorac Surg*. (2010) 37:1191–7. doi: 10.1016/j.ejcts.2009.11.051
108. Kobayashi H, Tanaka Y, Yagi J, Minato N, Tanabe K. Phase I/II study of adoptive transfer of gammadelta T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma. *Cancer Immunol Immunother*. (2011) 60:1075–84. doi: 10.1007/s00262-011-1021-7
109. Sakamoto M, Nakajima J, Murakawa T, Fukami T, Yoshida Y, Murayama T, et al. Adoptive immunotherapy for advanced non-small cell lung cancer using zoledronate-expanded gammadeltaTcells: a phase I clinical study. *J Immunother*. (2011) 34:202–11. doi: 10.1097/CJI.0b013e318207ecfb
110. Hoeres T, Holzmann E, Smetak M, Birkmann J, Wilhelm M. PD-1 signaling modulates interferon-gamma production by Gamma Delta (gammadelta) T-Cells in response to leukemia. *Oncoimmunology*. (2019) 8:1550618. doi: 10.1080/2162402X.2018.1550618
111. Castella B, Foglietta M, Sciancalepore P, Rigoni M, Coscia M, Griggio V, et al. Anergic bone marrow Vgamma9Vdelta2 T cells as early and long-lasting markers of PD-1-targetable microenvironment-induced immune suppression in human myeloma. *Oncoimmunology*. (2015) 4:e1047580. doi: 10.1080/2162402X.2015.1047580
112. Castella B, Melaccio A, Foglietta M, Riganti C, Massaia M. Vgamma9Vdelta2 T cells as strategic weapons to improve the potency of immune checkpoint blockade and immune interventions in human myeloma. *Front Oncol*. (2018) 8:508. doi: 10.3389/fonc.2018.00508
113. Oberg HH, Kellner C, Gonnermann D, Sebens S, Bauerschlag D, Gramatzki M, et al. Tribody [(HER2)2xCD16] is more effective than trastuzumab in enhancing gammadelta T cell and natural killer cell cytotoxicity against HER2-expressing cancer cells. *Front Immunol*. (2018) 9:814. doi: 10.3389/fimmu.2018.00814
114. Marcu-Malina V, Heijhuurs S, Van Buuren M, Hartkamp L, Strand S, Sebestyen Z, et al. Redirecting alphabeta T cells against cancer cells by transfer of a broadly tumor-reactive gammadeltaT-cell receptor. *Blood*. (2011) 118:50–9. doi: 10.1182/blood-2010-12-325993
115. Dombrowski KE, Ke Y, Brewer KA, Kapp JA. Ecto-ATPase: an activation marker necessary for effector cell function. *Immunol Rev*. (1998) 161:111–8. doi: 10.1111/j.1600-065X.1998.tb01575.x
116. Dwyer KM, Deaglio S, Gao W, Friedman D, Strom TB, Robson SC. CD39 and control of cellular immune responses. *Purinergic Signal*. (2007) 3:171–80. doi: 10.1007/s11302-006-9050-y
117. Regateiro FS, Cobbold SP, Waldmann H. CD73 and adenosine generation in the creation of regulatory microenvironments. *Clin Exp Immunol*. (2013) 171:1–7. doi: 10.1111/j.1365-2249.2012.04623.x
118. Bono MR, Fernandez D, Flores-Santibanez F, Rosemblatt M, Sauma D. CD73 and CD39 ectonucleotidases in T cell differentiation: beyond immunosuppression. *FEBS Lett*. (2015) 589:3454–60. doi: 10.1016/j.febslet.2015.07.027
119. Cuende J, Lienart S, Dedobbeleer O, Van Der Woning B, De Boeck G, Stockis J, et al. Monoclonal antibodies against GARP/TGF-beta1 complexes inhibit the immunosuppressive activity of human regulatory T cells *in vivo*. *Sci Transl Med*. (2015) 7:284ra256. doi: 10.1126/scitranslmed.aaa1983
120. Martin CJ, Datta A, Littlefield C, Kalra A, Chapron C, Wawersik S, et al. Selective inhibition of TGFbeta1 activation overcomes primary resistance to checkpoint blockade therapy by altering tumor immune landscape. *Sci Transl Med*. (2020) 12:aay8456. doi: 10.1126/scitranslmed.aay8456

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The Oncometabolite 5′-Deoxy-5′-Methylthioadenosine Blocks Multiple Signaling Pathways of NK Cell Activation

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Tumor cells develop various mechanisms to escape immune surveillance. In this context, oncometabolites secreted by tumor cells due to deregulated metabolic pathways, have been in the spotlight of researchers during the last years. 5′-Deoxy-5′-methylthioadenosine (MTA) phosphorylase (MTAP) deficiency in tumors results in the accumulation of MTA within the tumor microenvironment and thereby negatively influencing immune functions of various immune cells, including T and NK cells. The influence of MTA on T cell activation has been recently described in more detail, while its impact on NK cells is still largely unknown. Therefore, we aimed to illuminate the molecular mechanism of MTA-induced NK cell dysfunction. NK cell cytotoxicity against target cells was reduced in the presence of MTA in a dose-dependent manner, while NK cell viability remained unaffected. Furthermore, we revealed that MTA blocks NK cell degranulation and cytokine production upon target cell engagement as well as upon antibody stimulation. Interestingly, the immune-suppressive effect of MTA was less pronounced in healthy donors harboring an expansion of NKG2C$^+$ NK cells. Finally, we demonstrated that MTA interferes with various signaling pathways downstream of the CD16 receptor upon NK cell activation, including the PI3K/AKT/S6, MAPK/ERK, and NF-κB pathways. In summary, we revealed that MTA blocks NK cell functions like cytotoxicity and cytokine production by interfering with the signaling cascade of activating NK cell receptors. Specific targeting of MTA metabolism in MTAP-deficient tumors therefore could offer a promising new strategy to reverse immune dysfunction of NK cells within the tumor microenvironment.

Keywords: NKG2C, CD16 signaling, 5′-deoxy-5′-methylthioadenosine, NK cells, tumor escape mechanism

INTRODUCTION

Altered tumor cell metabolism is able to contribute to various tumor escape mechanisms to evade destruction by the immune system. An elevated glycolysis rate leads to increased lactic acid production, resulting in its accumulation within the tumor microenvironment (TME), where it blocks IFNγ production and reduces survival of CD8$^+$ T and NK cells *in vitro* and *in vivo* (1).

Moreover, tumor cells often favor metabolic pathways leading to the accumulation of intermediate metabolites; one example for these so-called oncometabolites is 2-hydroxyglutarate (2-HG), which is released by tumor cells harboring a gain-of-function mutation of the isocitrate dehydrogenase (IDH). Accumulation of 2-HG results in increased uptake by T cells, reprogramming their metabolism toward oxidative phosphorylation, leading to an increased frequency of regulatory T cells (Treg) and impaired polarization of T helper 17 (Th17) cells (2). Furthermore, various tumor entities have shown a reduced activity of the 5′-deoxy-5′-methylthioadenosine phosphorylase (MTAP), an important enzyme of the polyamine and methionine salvage pathway, either due to promoter hypermethylation or deletion of the chromosomal 9p21 region (3–5). MTAP is the only human enzyme that converts 5′-deoxy-5′-methylthioadenosine (MTA), a by-product of the polyamine pathway, into adenine and 5′-methylthioribose-1-phosphate. The latter one is then further metabolized to methionine within the methionine salvage pathway, which assures a sufficient production of S-adenosyl-methionine (SAM/AdoMet), the most important methyl donor within eukaryotic cells. Proper removal of MTA by MTAP is essential to guarantee an effective performance of the polyamine synthesis pathway and of methylation processes (6). We have previously demonstrated that accumulation of MTA due to MTAP deficiency is able to suppress proliferation, activation, and differentiation of human T cells (7, 8). In addition, an immune-suppressive effect of MTA has been demonstrated as well within cells of the innate immune system including macrophages (9, 10) and NK cells (11).

NK cells are innate lymphocytes, which, in contrast to T and B cells, recognize their targets through a variety of germline-encoded activating and inhibitory receptors. In this regard, tumor or virus-infected cells often down-regulate human leucocyte antigen (HLA) molecules on their surface in order to escape the adaptive immune system. However, HLA molecules like HLA-C1, C2, Bw4, or E are all ligands for inhibitory NK cell receptors like killer immunoglobulin-like receptor (KIR; HLA-C1, C2, Bw4) or NKG2A (HLA-E). Thus, down-regulation of HLA molecules with resulting predominance of activating receptors on target cells renders these cells susceptible toward NK cell cytotoxicity, a mechanism called "missing-self" (12). In addition, NK cells produce proinflammatory cytokines like interferon gamma (IFNγ) and tumor necrosis factor alpha (TNF) upon encountering a target cell, thereby inducing direct as well as indirect anti-tumor effects like the activation and differentiation of naïve T cells (13). NK cells are characterized by the lack of a TCR and its CD3 co-receptor while expressing the FcγRIII receptor CD16 and CD56; density and expression are both used for the additional division into the immature $CD56^{bright}CD16^{+/-}$ and the mature $CD56^{dim}CD16^{+}$ NK cell subsets (14). The latter one can be further divided based on the expression of NKG2A, KIR, and CD57 (15). Recently, a NK cell subset with adaptive immune features has been described in CMV-infected individuals. These cells demonstrate longevity, clonal expansion, and enhanced effector function and were transplantable into other individuals. They exhibited increased expression of the activation receptor NKG2C and of the terminal differentiation marker CD57 (16–18).

The current project aimed to explore the underlying mechanism of how MTA is blocking NK cell cytotoxicity in order to further understand this process in detail and develop new strategies to circumvent this tumor escape mechanism.

MATERIALS AND METHODS

Reagents and Cell Lines

Antibodies were purchased for CD16 biotin from BioLegend; LFA-1 open conformation isoform was from Abcam; pZAP/Syk, pS6, pSLP76, pAKT (S473), pPLCγ2, pERK1/2, and NF-κB pp65 were from BD; KIR2DL1/S1 was from Miltenyi; KIR2DL2/3/S2 was from Beckman Coulter; KIR3DL1/2 was from BioLegend; CD57 was from BioLegend; NKG2A was from Beckman Coulter; NKG2C was from Miltenyi; CD56 was from Beckman Coulter; CD16 was from BioLegend; 7AAD was from BD; dead-cell marker was from Life Technologies; and CD107a was from BioLegend. Pacific Orange and Blue Succinimidyl Ester were bought from Thermo Fisher Scientific. 5-Methylthioadenosine (MTA) and 3-deazaadenosine (3-Deaza) were purchased from Sigma-Aldrich, 5-azacytidine (5-Aza) was from Biomol/Cayman, and 2-chloroadenosine (CADO) and EPZ015666 (EZH) were from Sigma. Avidin was purchased from Sigma. K562 cell line from ATCC was cultured in RPMI 1640 media with antibiotics (penicillin/streptomycin; Invitrogen) and 10% heat-inactivated fetal calf serum (Sigma) at 37°C.

Blood Donors and PBMC Isolation

Blood from healthy volunteer donors were obtained from the Erlangen and Oslo University Hospital Blood Bank with written donor informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using density gravity centrifugation (Lymphoprep; Axis-Shield). Isolated PBMCs were frozen down in freezing media [90% fetal calf serum and 10% DMSO] at −80°C and transferred into a liquid nitrogen tank for long-term storage.

NK Cell Isolation and Culture

Frozen PBMCs were thawed and washed before they were used for NK cell isolation. NK cell isolation from fresh or frozen PBMCs was performed using a NK cell isolation kit and magnetic column separation technology (Miltenyi Biotec). Isolated NK cells were either directly used for functional assays (phospho-epitope analysis) or rested overnight in complete medium containing RPMI 1640 media with antibiotics (penicillin/streptomycin; Invitrogen) and 10% heat-inactivated fetal calf serum (Sigma) plus 100 U/ml IL-2 (Proleukin) at 37°C.

^{51}Cr Cytotoxic Assay

A total of 1×10^6 K562 cells were incubated with 100 μCi ^{51}Cr for 2 h at 37°C. Afterwards, K562 cells were intensively washed and co-incubated at a 5:1 ratio with NK cell, which has been rested overnight with 100 U/ml IL-2 and pre-incubated with different concentrations of MTA. Cells were incubated for 4 h in the presence of increasing MTA concentrations at

37°C. At the end of the culture, cells were pelleted and 100 µl of supernatant was taken away for measuring radioactivity at a Liquid Scintillation counter (Perkin Elmer). NK cell cytotoxicity was calculated as % specific lysis = [(sample cpm–spontaneous cpm)/(maximal cpm–spontaneous cpm)]*100 (19). Maximum release was achieved by incubating K562 cells in perchloric acid and spontaneous release by incubation in complete medium alone.

Annexin V Staining Assay

NK cells rested overnight with 100 U/ml IL-2 were incubated with increasing concentrations of MTA in complete medium without additional IL-2 for 4 h at 37°C. Cells were harvested, washed, and labeled in 100 µl of 1× Annexin V Binding Buffer (BD Biosciences) with 5 µl of Annexin V-FITC and 7AAD at room temperature for 15′. Afterwards, 400 µl of 1× Annexin V buffer was added and cells were analyzed at a FACSCanto II machine (BD Biosciences). FlowJo software was used for analyzing FACS data (FlowJo LLC).

Flow Cytometry-Based Functional NK Cell Assays

Isolated NK cells from thawed PBMCs were rested overnight with 100 U/ml IL-2 at 37°C and pre-incubated with various concentrations of MTA or other inhibitors for 30′ at 37°C. Afterwards, NK cells were stimulated for 4 h with either plate-bound CD16 antibodies (10 µg/ml CD16-biotin antibodies were attached to an Avidin-coated 96-flat bottom plate for 15′ at room temperature) or K562 cells at a 1:1 ratio at 37°C in complete medium. CD107a was directly pipetted into the culture medium. After 1 h, GolgiPlug™/ Stop™ (BD) were added to the culture. After 4 h, cells were harvested and stained for surface epitopes. Afterwards, cells were fixed, permeabilized, and stained for intracellular cytokines using the fixation/permeabilization solution kit (BD).

Conjugate Formation Assay

Overnight rested NK cells were labeled with the VPD450 dye (BD) for 10′. K562 cells were labeled with the PKH26 dye according to the manufacturer's instruction (Sigma-Aldrich). VPD450-labeled NK cells were pre-incubated with or without 100 µM MTA for 30′ at 37°C. Afterwards, VPD450-labeled NK cells and PKH26-labeled K562 cells were put together into a FACS tube and spin down for 1′ at 100*g*. Cells were then incubated in a water bath at 37°C for 10′ and directly fixed in 300 µl of paraformaldehyde on ice. Finally, cells were washed and analyzed at a FACSCanto II machine (BD Biosciences).

Inside-Out Signaling Assay

Isolated NK cells from thawed PBMCs were rested overnight with 100 U/ml IL-2 at 37°C and pre-incubated with or without 100 µM MTA for 30′ at 37°C. NK cells were then stimulated with K562 cells for 10′ at 37°C at a 1:1 ratio. Afterwards, cells were harvested and stained for NK cell surface markers and the LFA-1 open conformation isoform.

Fluorescent Cell Barcoding and Phospho-Epitope Staining

NK cells isolated from fresh PBMCs were pre-incubated with or without 100 µM MTA in complete medium at 37°C for 30′. NK cells were then labeled with 10 µg/ml CD16-biotin antibodies for 2′ in a water bath at 37°C. Afterwards, 50 µg/ml avidin was added to cross-link the antibodies and induce the CD16 signaling cascade. After 0, 1, 5, 10, and 30′, NK cells were fixed in 4% paraformaldehyde for 7′ at 37°C, pelleted, washed, and stored in 100% methanol at −20°C. After overnight storage, cells were thawed, washed, and labeled with different concentrations of different dilution combinations of the two fluorescent dyes pacific blue and orange (Life Technologies). Afterwards, all cells were collected, labeled with surface and different phospho-epitope specific antibodies, and analyzed at a FACSCanto II machine (BD Biosciences).

Microarray Analysis

The dataset GSE23695 containing expression profiles of $CD56^{dim}CD16^{+}CD57^{+}$ and $CD57^{-}$ NK cells was downloaded into R version 3.6.1 using the package GEOquery 2.54.1 (20). Subsequent differential expression analysis was carried out with limma 3.42.2. Gene set enrichment analysis (GSEA) was performed with GSEA v4.0.3 on gene set M10911 (KEGG_CYSTEINE_AND_METHIONINE_METABOLISM).

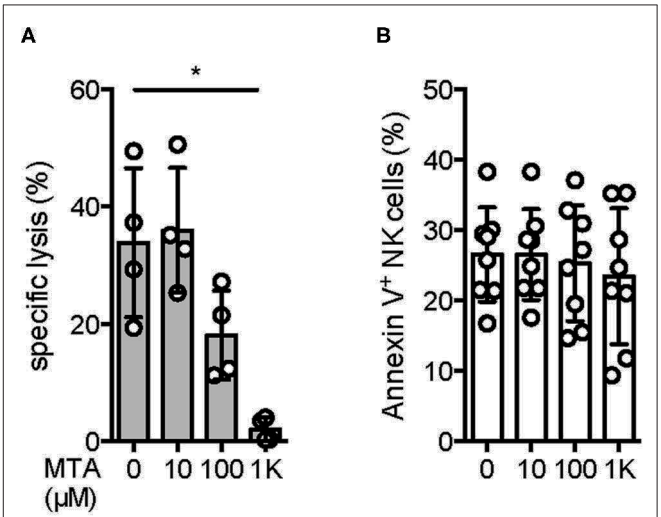

FIGURE 1 | NK cells' cytotoxic activity is reduced upon MTA co-incubation without affecting NK cell viability. Isolated NK cells were incubated overnight with 100 U/ml IL-2 and pre-incubated for 30′ with various concentrations of MTA at 37°C. NK cells were further incubated for 4 h with the prior MTA concentration in the presence **(A)** or absence **(B)** of ^{51}Cr-labeled K562 cells at a 5:1 ratio. For evaluating NK cells' cytotoxic activity against K562 cells (**A**; $n = 4$), the % specific lysis was calculated as followed: [(sample cpm–spontaneous cpm)/(maximal cpm–spontaneous cpm)]*100. To analyze the effect of MTA on NK cells' viability, they were harvested and stained for Annexin V expression (**B**; $n = 8$). Significance was calculated using a one-way ANOVA test (*p* value: * <0.05).

Statistical Analysis

For comparing single paired samples, a Wilcoxon test was used, while a Mann–Whitney test was used for single unpaired samples. For significance analysis for multiple matched groups with each other, a one- or two-way ANOVA test was done. Statistical significance: $^{*}p < 0.05$. Analysis was performed using the GraphPad Prism software.

RESULTS

MTA Blocks NK Cells' Cytotoxic Activity Without Affecting Their Viability

Since NK cells are known for their cytotoxic activity against various tumor cell lines, we were interested if MTA is capable of suppressing this effect. Using the well-established NK cell target cell line K562 in a classical ^{51}Cr release assay, we observed a reduction of NK cell cytotoxic activity at a MTA concentration of 100 μM, with no suppressive effect at 10 μM. In contrast, a nearly complete block of cytotoxicity was found at a concentration of 1 mM MTA (**Figure 1A**). To rule out a cytotoxic effect of MTA on NK cells' viability as a reason for this reduced cytotoxic activity, we stained NK cells for Annexin V and 7AAD expression after a 4 h incubation period with increasing concentrations of MTA. Interestingly and importantly, even at a MTA concentration of 1 mM, no increased Annexin V (**Figure 1B**) or 7AAD (**Supplementary Figure 1A**) expression was observed. Overall, NK cell cytotoxic activity against K562 cells is reduced with increasing MTA concentrations which is not due to a cytotoxic MTA effect on NK cells' viability however.

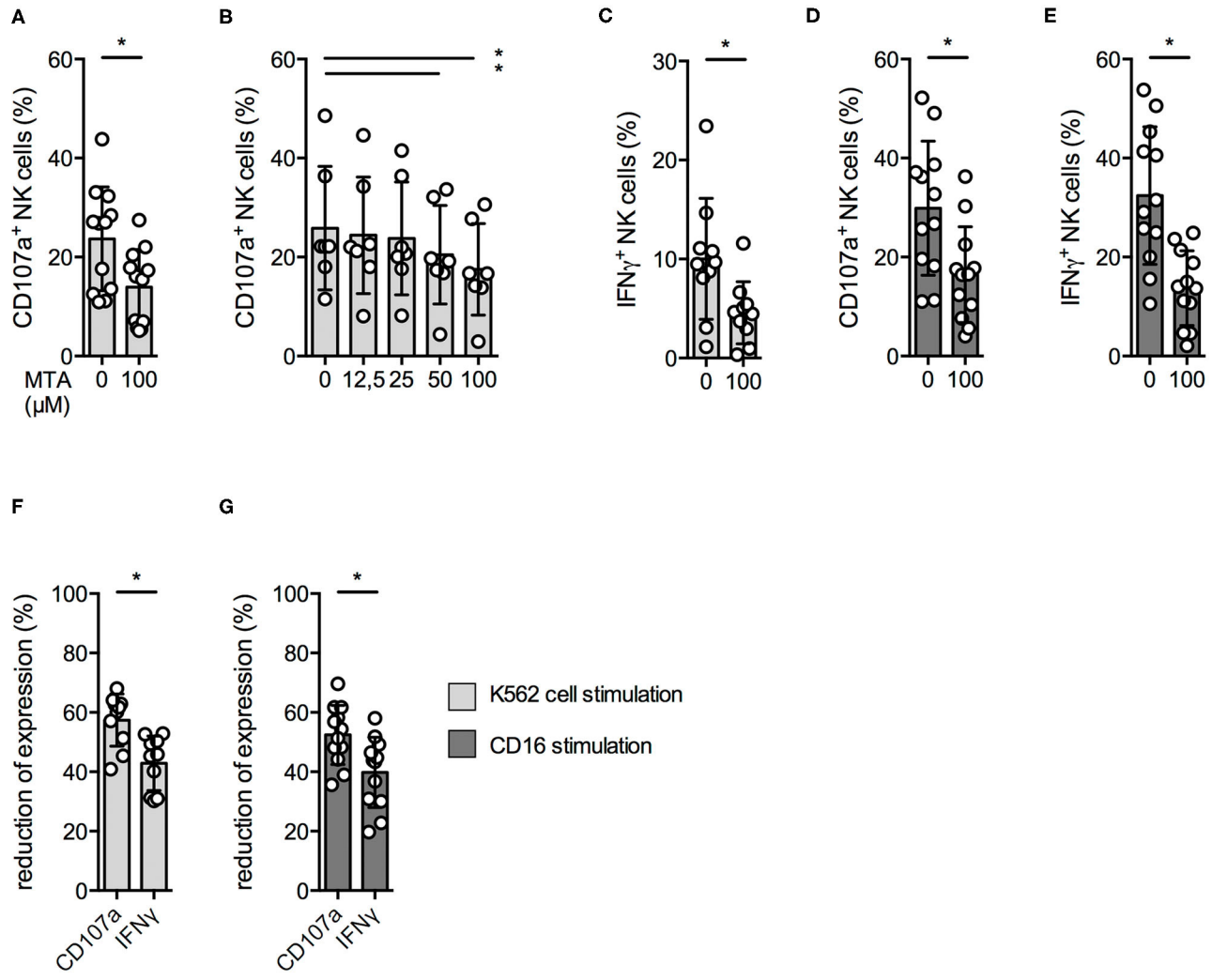

FIGURE 2 | NK cell degranulation and cytokine production are reduced by MTA. Overnight IL-2-activated NK cells were pre-incubated with different concentrations of MTA for 30′ at 37°C and then stimulated with K562 cells at a 1:1 ratio (**A–C,F**) or plate-bound CD16 (**D,E,G**) for additional 4 h at 37°C. Afterwards, the cells were harvested and CD107a or IFNγ expression was analyzed in bulk NK cells. CD107a and IFNγ expression were indicated either as absolute values **(A–E)** or as the percentage decrease **(F,G)** due to MTA co-incubation [(absolute value with MTA/absolute value without MTA)*100]. Significance was calculated using a Wilcoxon test for analyzing paired samples (**A,C–G**; $n = 12$) or a one-way ANOVA (**B**; $n = 6$) test (p value: *<0.05).

NK Cell Degranulation and Cytokine Production Are Reduced by MTA

Upon tumor cell recognition, NK cells release the content of their cytotoxic granules by a process called degranulation and produce pro-inflammatory cytokines like IFNγ. Since we observed a reduced cytotoxic activity at 100 μM MTA, we investigated if this was due to a reduced NK cell degranulation upon contact with K562 cells. Indeed, CD107a surface expression—a degranulation marker—on NK cells was significantly reduced in the presence of 100 μM MTA (**Figure 2A**); this could be observed even at lower MTA concentrations (**Figure 2B**). In accordance to our cytotoxicity experiments, NK cell degranulation in the presence of target cells was almost completely blocked at 1,000 μM MTA (**Supplementary Figure 2A**) and, in addition, IFNγ production was significantly reduced as well (**Figure 2C**).

As NK cells can also be activated by binding of antibodies to their CD16 receptor, we also investigated this stimulatory pathway. MTA was able to significantly reduce CD107a expression and IFNγ production upon CD16 stimulation (**Figures 2D,E**), while CD16 surface expression remained unchanged (**Supplementary Figure 2B**).

Of note, IFNγ production was more impaired by MTA than degranulation, independently if stimulated by either K562 cells (**Figure 2F**) or anti-CD16 (**Figure 2G**). Altogether, MTA-induced suppression of NK cells' cytotoxic activity is due to a reduced NK cell degranulation; in addition, MTA blocks IFNγ production upon various stimuli.

Adenosine Derivates and PRMT5 Inhibitor Reduce NK Cell Function

In other immune cell populations, it has been shown that MTA exerts its suppressive effect by different mechanisms. One supposed mechanism is the interaction with adenosine receptors due to the adenosine residue in MTA and another one due to its intracellular inhibition of the protein arginine methyltransferase 5 (PRMT5) (8, 21, 22).

We therefore investigated NK cell degranulation upon K562 cell stimulation in the presence of the stable adenosine analog 2-chloroadenosine (CADO) (23) and a synthetic PRMT5 inhibitor, EPZ015666 (EPZ). CD107a surface expression was significantly reduced in the presence of 100 μM CADO (**Figure 3A**) and 100 μM EPZ (**Figure 3B**). A similar effect was observed using the protein methyltransferase inhibitor 3-deazaadenosine (**Supplementary Figure 3A**), whereas no significant reduction was observed using the DNA methyltransferase inhibitor 5-azacitidine (**Supplementary Figure 3B**).

MTA Elicits Different Inhibitory Effects Within the Different NK Cell Subsets

Next, we aimed to analyze the suppressive MTA effects within the different NK cell subsets. We first analyzed the MTA-induced reduction of CD107a and IFNγ expression upon K562 cell stimulation within $CD56^{bright}$ and $CD56^{dim}$ NK cells. While the suppressive MTA effect on NK cell degranulation was similar between both subsets (**Figure 4A**), the effect on IFNγ production was significantly more pronounced within the

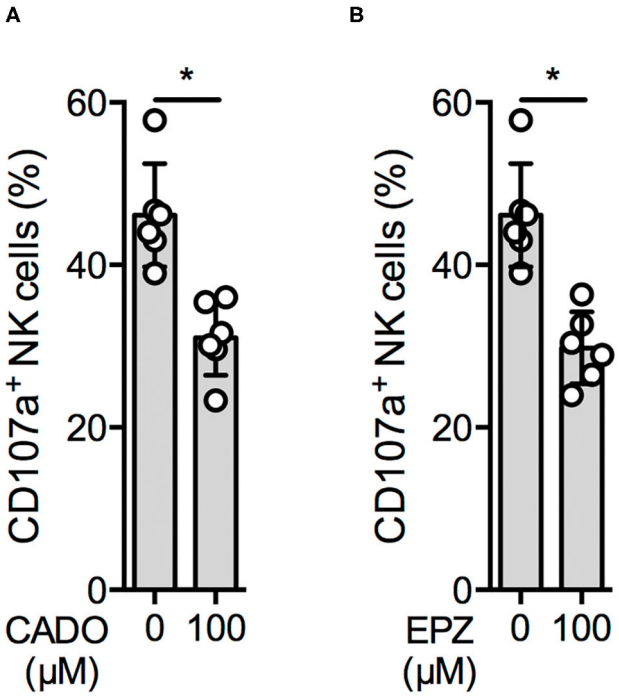

FIGURE 3 | An adenosine derivate and a PRMT5 inhibitor reduce NK cell function. Overnight IL-2-activated NK cells were pre-incubated for 30′ with 100 μM 2-chloroadenosine (CADO) or EPZ015666 (EPZ) (**A,B**; $n = 6$) and then stimulated with K562 cells at a 1:1 ratio for additional 4 h at 37°C. Afterwards, the cells were harvested and CD107a expression was analyzed in bulk NK cells. CD107a expression was indicated as absolute values **(A,B)**. Significance was calculated using a Wilcoxon test for analyzing paired samples (*p* value: * <0.05).

$CD56^{dim}$ subset (**Figure 4B**). Recently, it has been demonstrated that the $CD56^{dim}$ subset can be further divided into functional distinct subsets based on the expression of NKG2A, KIR, and CD57 (15). In terms of NK cell degranulation, the suppressive MTA effect was similar between the distinct subsets, independent if K562 cells (**Figure 4C**) or CD16 antibodies (**Figure 4E**) were used for stimulation. The same was observed for IFNγ production upon K562 cell stimulation (**Figure 4D**). In contrast, IFNγ production upon CD16 stimulation was less reduced in $CD56^{dim}$ NK cells expressing CD57 on their surface (**Figure 4F**). $CD57^{+}$ NK cells produced significantly more IFNγ upon CD16 (**Figure 4G**) or K562 stimulation (**Supplementary Figure 4B**) than $CD57^{-}$ ones, while no difference in CD107a expression was observed (**Supplementary Figures 4A,C**). Interestingly, IFNγ production upon CD16 stimulation was significantly less effected by MTA in $CD57^{+}$ than $CD57^{-}$ NK cells (**Figure 4H**), while no difference was observed upon K562 cell stimulation (**Supplementary Figure 4E**) and for CD107a expression (**Supplementary Figures 4D,F**). These differences in IFNγ production upon CD16 stimulation could be due to the significantly higher CD16 expression levels on $CD57^{+}$ NK cells resulting in a stronger activation of this NK cell subset (**Supplementary Figure 4G**). In summary, the suppressive MTA effect on IFNγ production is present in all studied

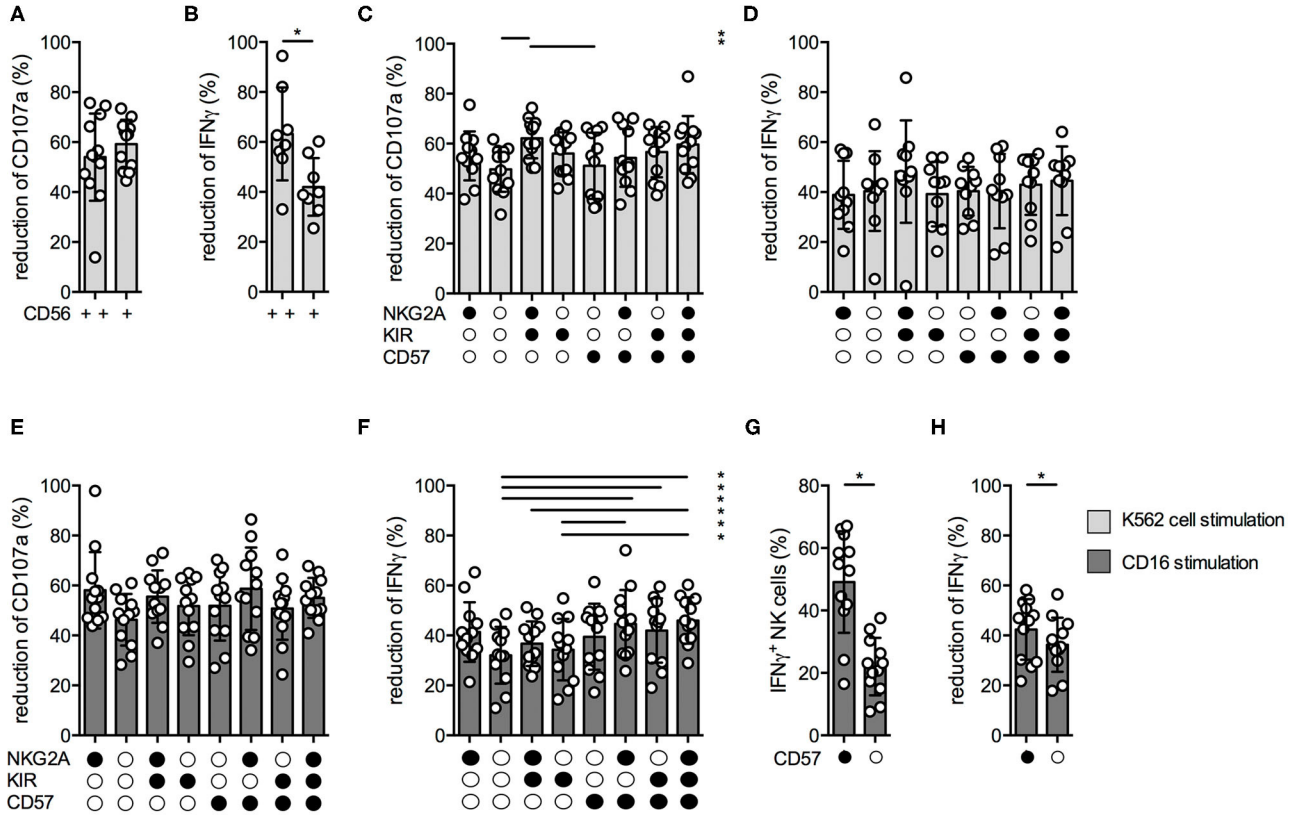

FIGURE 4 | MTA's inhibitory effect varies within the different NK cell subsets. Overnight IL-2-activated NK cells were pre-incubated with 100 μM MTA for 30′ at 37°C and then stimulated with K562 cells at a 1:1 ratio **(A–D)** or plate-bound CD16 **(E–H)** for additional 4 h at 37°C. Afterwards, the cells were harvested and CD107a or IFNγ expression were analyzed in various NK cell subsets based on their CD56, CD57, NKG2A, and KIR expression. CD107a and IFNγ expression were indicated either as absolute values **(G)** or as the percentage decrease **(A–F,H)** due to MTA co-incubation [(absolute value with MTA/absolute value without MTA)*100]. Significance was calculated using a Wilcoxon test for analyzing paired samples **(A,B,G,H)** or a one-way ANOVA **(C–F)** test (*p* value: * <0.05; $n = 12$).

NK subsets with varying extent between the distinct NK cell subsets; $CD57^+$ NK cells in particular seem less sensitive than $CD57^-$ NKs.

Gene Expression of MTA's Metabolic Pathway and Targets Is Similar in $CD57^{+/-}$ NK Cells

In order to identify potential mechanisms for the differential MTA susceptibility of the two subsets, we analyzed the publicly available microarray dataset GSE23695 of $CD56^{dim}CD16^+CD57^+$ and $CD57^-$ sorted NK cells (20). A GSEA of the methionine and cysteine pathway, including MTAP as the first degradation step of MTA, revealed no significant enrichment in any of the two subsets (**Figure 5A**). In addition, none of the four known adenosine receptors nor any of the most important methyl transferases, including PRMT5, were differentially expressed between the two groups (**Figure 5B** and **Supplementary Table 1**). All in all, $CD57^+$ and $CD57^-$ NK cells do not differ in their gene expression profile concerning known MTA targets or enzymes of the methionine (MTA) metabolic pathway.

Donors Haboring a $NKG2C^+$ NK Cell Expansion Are Less Sensitive Against MTA-Induced Suppression of IFNγ Production

Since we observed varying degrees of MTA-induced suppression of IFNγ production within the different NK cell subsets, we were interested if similar effects could be observed within the $NKG2C^+$ NK cell subset. This highly differentiated subset typically expands during CMV infection, demonstrates long-term survival, has increased capacity of IFNγ production, and is transplantable (24, 25). We identified 6 out of 12 healthy donors with an expansion of $NKGC2^+$ NK cells, defined as >10% of total NK cells. Their NKG2C surface density was significantly higher compared to those donors without a $NKG2C^+$ expansion (**Figure 6A**). In accordance with other reports (25) these $NKG2C^{hi}$ NK cells expressed significantly less NKG2A (**Figure 6B**) and higher levels of CD57 (**Figure 6C**) on their surface than their $NKG2C^-$ counterparts within the same donor, as well as compared to $NKG2C^+$ NK cells from donors without a $NKG2C^+$ expansion. In addition, $NKG2C^{hi}$ NK cells demonstrated superior IFNγ production upon CD16 stimulation (**Figure 6D**). NK cells from donors exhibiting a

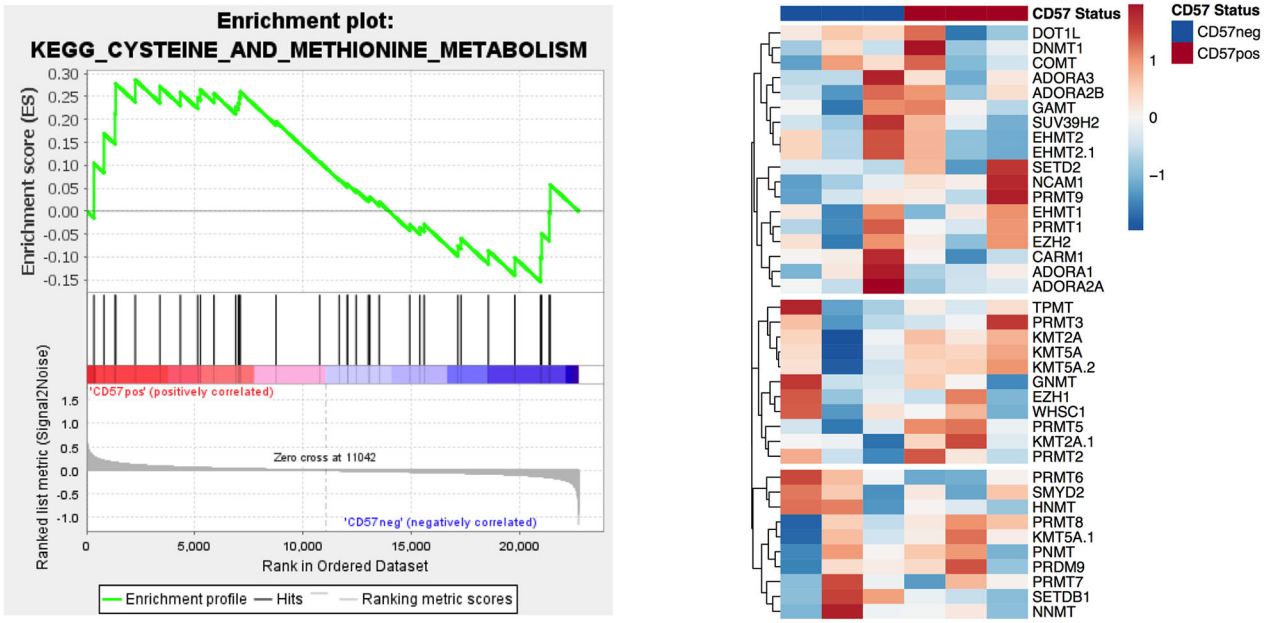

FIGURE 5 | Gene expression of MTA's metabolic pathway and targets is similar in CD57$^{+/-}$ NK cells. Analysis of publicly available microarray data (GSE23695) (20) of CD56^{dim}CD16^{+}CD57^{+} and CD57^{-} sorted NK cells performing a gene set enrichment analysis of the methionine and cysteine metabolism pathway **(A)** and a differential gene expression analysis of adenosine receptors and various methyltransferases **(B)**. For the gene set enrichment analysis, the normalized enrichment score (NES) was 0.8 and the nominal *p* value 0.6.

NKG2C^{+} expansion were less susceptible toward MTA-induced suppression of IFNγ production upon CD16 stimulation than NK cells from donors without one (**Figure 6E**). However, when comparing the suppressive MTA effect between NKG2C$^{+/-}$ NK cells in both donor groups, no significant differences were observed (**Figure 6F**). Interestingly, independent of their NKG2C expression, all NK cells from donors harboring a NKG2C^{+} expansion tend to be less susceptible toward MTA-induced suppression compared to those without an expansion. In addition, no differences between donors with or without a NKG2C^{+} expansion were observed for K562-induced IFNγ production (**Supplementary Figure 5B**) or CD107a expression independent of the used stimuli (**Supplementary Figures 5A,C**). Moreover, no significant differences in CD16 expression levels were observed between these two groups of donors (**Supplementary Figure 5D**). Overall, we observed a reduced susceptibility toward MTA-induced suppression of IFNγ production upon CD16 stimulation in donors harboring a NKG2C^{+} expansion. Strikingly, this effect was not due to a reduced susceptibility of NKG2C^{+} NK cells toward MTA compared to NKG2C^{-} ones, but rather donor dependent.

Conjugate Formation and Inside-Out Signaling Is Reduced by MTA

Our next aim was to investigate if MTA is already inhibiting early events during NK cell activation, ultimately leading to a reduced NK cell degranulation and IFNγ production. We first tested if MTA reduces the amount of conjugate formations between NK and K562 cells during their initial contact. In the presence of 100 μM MTA, NK:K562 cell conjugate formation was significantly reduced (**Figure 7A**).

Another early event during NK cell activation is the formation of the so-called inside-out signal. It is attributed to the conformation change of LFA-1, an adhesion molecule, upon NK cell activation resulting in the "open-conformation" LFA-1 isoform, which has a higher binding affinity and increases the NK cell's attachment to its target cell (26). In the presence of MTA, expression of the LFA-1 open conformation isoform upon K562 cell stimulation was significantly reduced (**Figure 7B**). Altogether, we demonstrate that MTA suppresses early events during NK cell activation, like conjugate formation and inside-out signaling.

MTA Inhibits the NF-κB, PI3K/AKT/S6, and MAPK/ERK Pathways Downstream of the CD16 Receptor

Finally, we investigated how early the suppressive MTA effect on NK cells was evident during their activation. Therefore, we analyzed the expression of various phospho-epitopes upon CD16 stimulation in the presence or absence of 100 μM MTA. Freshly isolated NK cells were stimulated for up to 60′ and subsequently analyzed using fluorescent cell barcoding and phospho-flow technologies (**Supplementary Figures 6A,B**). Interestingly, MTA had no effect on the phosphorylation of the proximal signaling molecules ZAP70/Syk upon CD16 stimulation (**Figure 8A**). Similarly, phosphorylation of SLP76

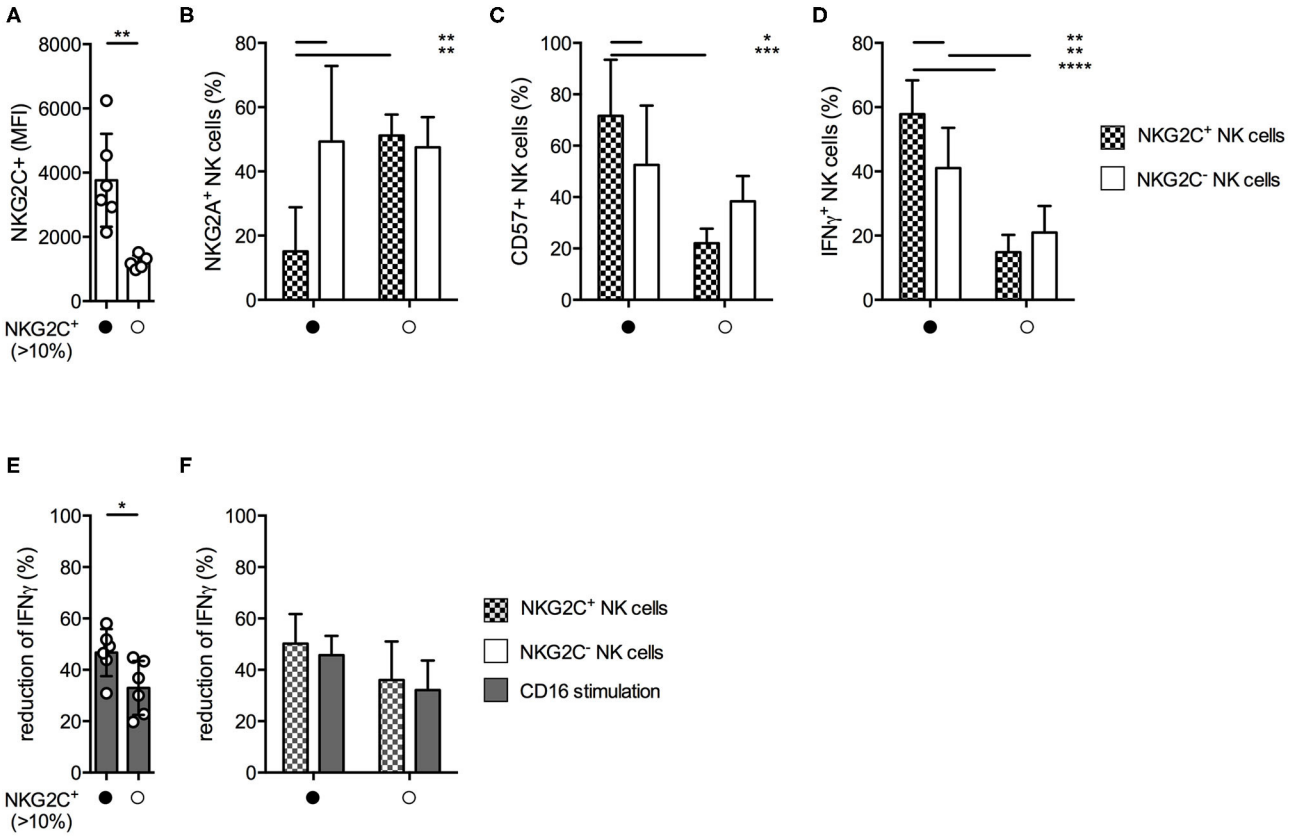

FIGURE 6 | MTA-induced suppression of IFNγ production is less pronounced in donors harboring an expansion of NKG2C$^+$ NK cells. Healthy donors were divided into two groups based on the presence of an expansion of NKG2C$^+$ expressing NK cells (>10%). Overnight IL-2-activated NK cells were stained for the surface expression of NKG2C, NKG2A, and CD57 **(A–C)** or pre-incubated with 100 μM MTA for 30′ at 37°C and then stimulated with plate-bound CD16 for additional 4 h at 37°C **(D–F)**. Afterwards, the cells were harvested and IFNγ expression was analyzed in NKG2C$^{+/-}$ NK cells. IFNγ expression was indicated either as absolute values **(D)** or as the percentage decrease **(E,F)** due to MTA co-incubation [(absolute value with MTA/absolute value without MTA)*100]. Significance was calculated using a Mann–Whitney test for analyzing unpaired samples **(A,E)** or a two-way ANOVA **(B–D,F)** test (*p* value: * <0.05, ** < 0.01, *** < 0.001, **** < 0.0001, $n = 6$).

(**Figure 8B**) and PLCγ2 (**Figure 8C**) remained mostly unaffected by MTA co-incubation. However, the NF-κ*B* (**Figure 8D**), PI3K/AKT/mTOR (**Figures 8E,F**) and MAPK/ERK (**Figure 8G**) pathways, which are involved in NK cell degranulation and cytokine production, were all negatively influenced by MTA. Additionally, we further investigated the negative MTA effect on the PI3K/AKT/mTOR pathway in CD57$^+$ and CD57$^-$ NK cells. We observed a tendency that S6 phosphorylation upon CD16 stimulation was stronger inhibited by MTA in CD57$^-$ than in CD57$^+$ NK cells (**Supplementary Figure 6C**). Together, these data demonstrate that the suppressive MTA effect on the cytotoxic activity of NK cells is due to its early inhibitory effect on various signaling pathways of activating NK cell receptors.

DISCUSSION

Loss of MTAP expression in tumor cells results in the accumulation of MTA (27). MTAP deficiency has been reported for several tumor entities, with high frequency in mesothelioma, T cell acute lymphoblastic leukemia (T-ALL), gliomas, metastatic melanoma, and non-small cell lung cancer (NSCLC) (28). It has been demonstrated that MTAP-deficient tumors exhibit a more aggressive clinical course with increased proliferation, invasiveness, and metastasis (29). In NSCLC, loss of MTAP is associated with poor overall and disease-free survival (30). In addition, MTAP deficiency is a predictive marker for the response to adjuvant interferon therapy in melanoma patients (31). However, to the best of our knowledge, no data are available on NK cell numbers and functions in these entities stratified by MTAP expression levels.

In the current study, we demonstrated that MTA suppresses several NK cell functions, which is in line with observations in other immune cells. Our group discovered that MTA blocks T cell metabolism and proliferation, thereby suppressing the induction of antigen-specific CD8 T cells and cytokine production by CD4 T cells. Interestingly, the MTA effect was reversible since T cells regained their proliferative capacity after removal of MTA from the culture medium (7, 8). Moreover, TNF production in macrophages upon toll-like receptor (TLR) stimulation, e.g.,

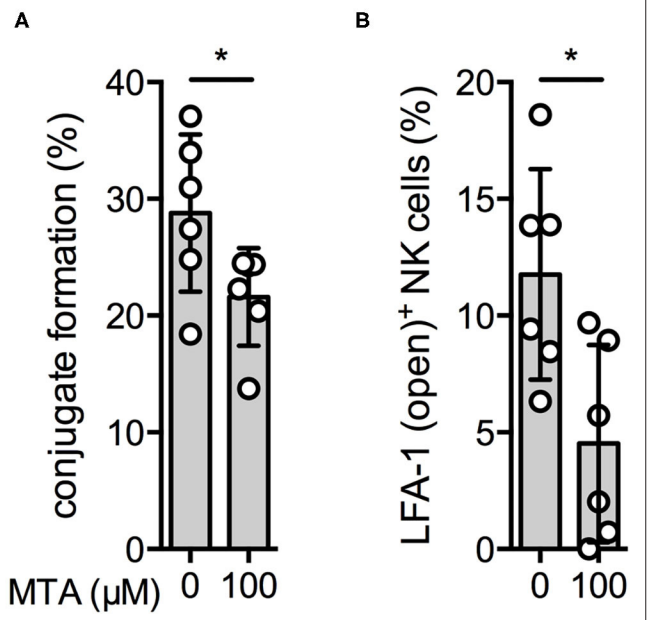

FIGURE 7 | Conjugate formation and inside-out signaling are reduced by MTA. Overnight IL-2-activated NK cells (VPD450) and K562 cells (PKH-26) were labeled with different fluorescent dyes. NK cells were pre-incubated with or without 100 μM MTA and afterwards co-incubated with K562 cells for 10′. Conjugate formation was calculated as the percentage of double positive events (VPD450/PKH-26 positive cells) of all NK cells (VPD450 positive cells) **(A)**. In addition, expression of the open conformation LFA-1 isoform on NK cells was analyzed as well **(B)**. Significance was calculated using a Wilcoxon test for analyzing unpaired samples (*p* value: * <0.05; $n = 6$).

with LPS, is sufficiently suppressed by MTA in an adenosine A2 receptor-dependent manner (9, 10). These results have been demonstrated successfully in a mouse model *in vivo* where LPS-induced lethality was completely prevented by MTA injection due to reduced TNF and increased IL-10 levels (32). Similar results have been observed in an autoimmune encephalomyelitis (EAE) and a colon cancer mouse model, in which MTA treatment prevented acute EAE and reduced the inflammation-induced tumor load in treated mice, respectively (33, 34). Overall, these results clearly demonstrate a suppressive effect of MTA on innate and adaptive immune cells.

However, an important question is whether the used MTA concentrations are physiological. MTA concentrations within MTAP-deficient human melanoma cell lines have been reported to be up to 140 nM (29) and up to 1,000 nM within the supernatant (7). However, due to the close proximity and narrow space between a tumor and NK cell during immunological synapse formation, effective *in situ* MTA concentrations are to be expected significantly higher than those measured under *in vitro* cell culture conditions. We used a MTA concentration of 100 μM in our experiments, but also observed a suppressive effect on NK cells at lower concentrations. This is in line with the MTA concentration that has been used in other publications that investigated MTA's suppressive effect on immune cells *in vivo* and *in vitro* (7, 9–11, 35).

MTA is known to execute its effect by different mechanisms. In melanoma cells, MTA activates the transcription factor AP-1, potentially leading to increased expression of metalloproteases and growth factors (29). The activation is dependent on the adenosine receptor ADORA2B, since blocking or knockdown of the receptor resulted in reduced AP-1 signaling. Interestingly, MTA did not cause an increase in cyclic adenosine monophosphate (cAMP) or intracellular calcium levels (21). This is in line with previous reports demonstrating that incubation with MTA concentrations ≥100 μM did not cause an increase of cAMP levels in human peripheral lymphocytes, whereas NK cell cytotoxicity was significantly reduced at these concentrations, confirming our observations (11).

Importantly, MTA led to an accumulation of S-adenosyl-homocysteine (AdoHcy), due to its inhibitory effect on the AdoHcy hydrolase (36, 37). AdoHcy is a by-product during methylation reactions involving S-adenosyl-methionine (AdoMet) as a methyl-group donor, and, if not properly removed, inhibits methylation reactions (38, 39). The role of MTA in interfering in protein methylation processes has been proposed for a long time (40) and, besides its indirect effect *via* inhibiting AdoHcy hydrolase, recent works demonstrated a direct inhibition of several protein arginine methyltransferases (PRMT). MTA selectively inhibits PRMT5 at low concentrations (≤10 μM), whereas other PRMTs were inhibited only at higher concentrations. MTAP-deficient tumor cells accumulate high levels of MTA leading to a reduced PRMT5 activity, which renders these cells more susceptible toward additional PRMT5 inhibition compared to MTAP-competent cells (8, 22, 41, 42). Cell signaling events are mediated by different protein–protein interactions, which are regulated through post-translational modification (PTM) of which phosphorylation is the best studied one (43). However, in recent years, modification of proteins by methylation during signal transduction has stepped into the spotlight of research. We revealed that distinct signaling pathways downstream of the CD16 receptors were suppressed by MTA. Several groups demonstrated that MTA is able to suppress the PI3K/AKT/mTOR axis by reducing AKT and S6 phosphorylation (7, 8, 44), which is in line with our observation. Interestingly, a current work demonstrated that, upon activation, AKT is methylated by the histone methyltransferase SETDB1, resulting in sustained AKT phosphorylation and signaling (45). In addition, the NF-κB pathway is known as well to be regulated by methylation of arginine and lysine residues of the p65 subunit of NF-κB (46). Upon activation with IL-1β, PRMT5 dimethylates arginine 30 (R30) of the p65 subunit, leading to increased expression of NF-κB-induced genes (47). Although we did not observe a suppressive MTA effect on SLP-76 or PLCγ2 phosphorylation, another member of this signaling pathway, Vav-1, is known to be regulated by protein methylation. Upon CD28 engagement, Vav-1 was R-methylated, which could be blocked by incubation with 300 μM MTA (35). In summary, our results indicate that MTA blocks phosphorylation and methylation of distinct signaling pathways downstream of activating NK cell receptors by its suppressive effect on

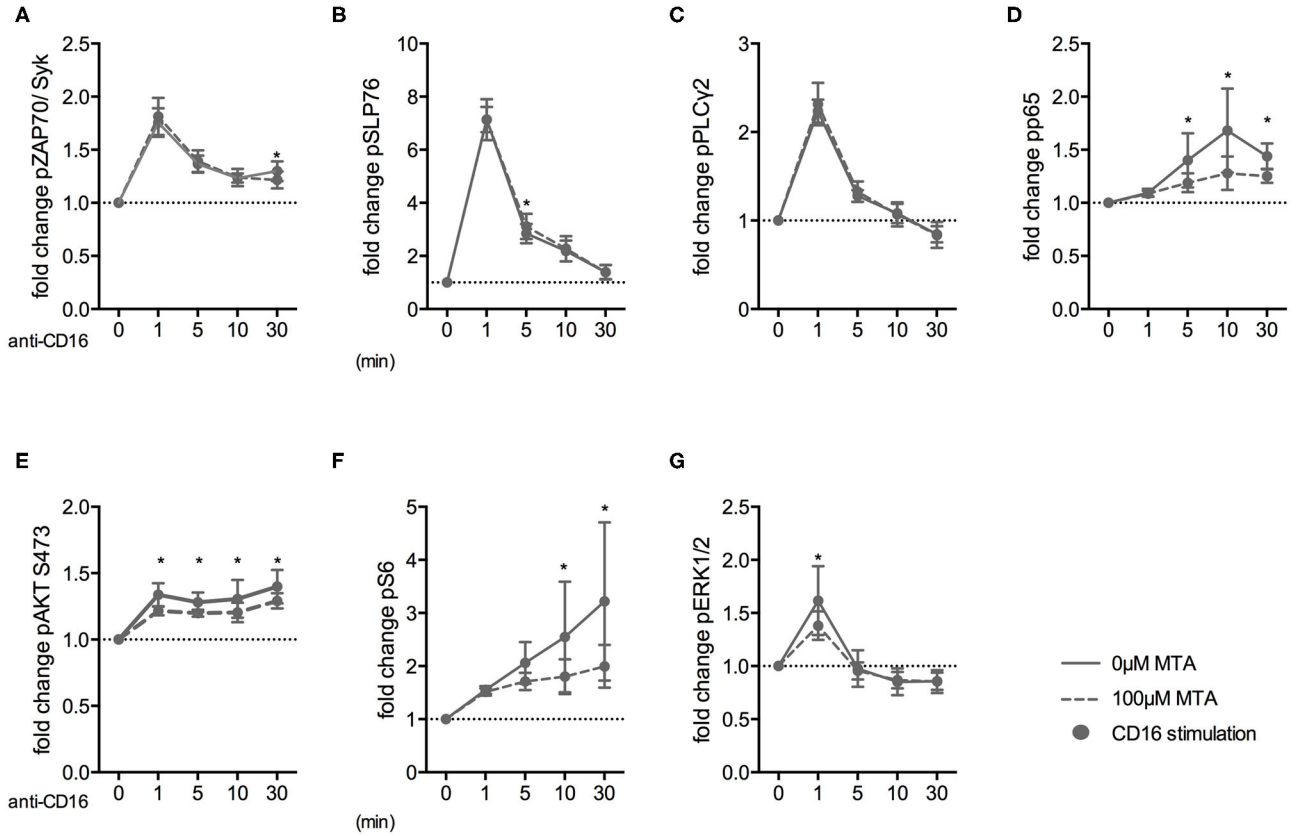

FIGURE 8 | MTA inhibits the NF-κB, PI3K/AKT/S6, and MAPK/ERK pathways downstream of the CD16 receptor. Freshly isolated NK cells were pre-incubated with or without 100 μM MTA for 30′ at 37°C and then stimulated with anti-CD16 antibodies for 0, 1, 5, 10, and 30′. Cells were labeled with a fluorescent bar cell code and stained for various phospho-epitopes of the CD16 signaling cascade. The fold increase of phospho-epitope staining was calculated for the different time points compared to baseline staining at 0′ (**A–G**; $n = 6$). Significance was calculated using a two-way ANOVA test (p value: * <0.05).

protein methylation rather than by the suppressive ability of its adenosine residue.

Here, we discovered that MTA blocks NK cell effector functions by interfering with NK activation to the end that IFNγ production was significantly stronger affected than degranulation. It has been demonstrated that signals of different strengths are needed for the individual NK cell functions including degranulation, chemokine, and cytokine production. While the signal of a single activating receptor is sufficient to induce chemokine production, NK cell degranulation needs the coordinated engagement of two activating receptors. Moreover, IFNγ production depends on the activation of several receptors, pointing to high regulatory demands for sufficient IFNγ production and therefore to a higher susceptibility toward MTA suppression (48, 49).

In addition, we observed that IFNγ production upon CD16 stimulation was significantly higher in CD57-expressing NK cells and less susceptible toward MTA suppression. This is in line with previous reports demonstrating that during terminal differentiation, which is accompanied by CD57 expression, NK cells produced higher levels of IFNγ upon activation. Mechanistically, terminal differentiated NK cells show a higher NF-κB activation upon stimulation, higher *IFNG* and *TBX21* transcripts, as well as demethylation of the *IFNG* promoter (50). Given the fact that MTA reduces NF-κB signaling in NK cells upon CD16 engagement, it is likely that the stronger NF-κB activation in $CD57^+$ NK cells upon activation is sufficient to partially overcome MTA suppression. This is further supported by our observation of a stronger MTA-induced inhibition of S6 phosphorylation upon CD16 stimulation in $CD57^-$ than in $CD57^+$ NK cells, since the PI3K/AKT/mTOR pathway contributes to IFNγ production in NK cells as well (51). Importantly, we observed differences within the CD16 expression levels between these two subsets, an indication that $CD57^+$ are probably receiving a stronger initial activation signal upon CD16 stimulation, which is sufficient to partly overcome MTA suppression. In addition, the data from the microarray analysis between $CD57^+$ and $CD57^-$ NK cells demonstrate that neither the expression of adenosine receptors, various methyltransferases (including PRMT5), nor enzymes of the methionine salvage pathway (including MTAP) differ between those two subsets.

Furthermore, IFNγ production was less affected by MTA suppression in healthy donors, harboring a NKG2C$^+$ expansion. These NKG2C$^+$ cells have been demonstrated to be major IFNγ producers upon CD16 stimulation due to epigenetic modulation of their *IFNG* locus (52). Additionally, CD16-induced phosphorylation of signaling molecules like SLP-76, ERK1/2, and S6RP was stronger in NKG2C$^+$ NK cells compared to NKG2C$^-$ ones (25).

Surprisingly, when comparing NKGC2$^{+/-}$ NK cells in donors with a NKG2C$^+$ expansion with each other, we only detected a small, non-significant difference in the susceptibility toward MTA. Moreover, NKG2C$^-$ NK cells of NKG2C$^+$ expansion donors demonstrated superior IFNγ production compared to NKG2C$^{+/-}$ NK cells in donors without an expansion. Therefore, the difference between the two donor groups might rely not only on the existence of a NKG2C$^+$ expansion but also on the whole NK cell population. One explanation could be the higher frequency of CD57-expressing NK cells within NKG2C$^+$ and NKG2C$^-$ NK cells compared to their counterparts in donors without an expansion. Another effect could be that our group of NKG2C$^+$ expansion donors are harboring a polymorphism of their CD16 receptor resulting in a higher affinity to human IgG1 and therefore superior activation upon stimulation (53). Importantly, no significant differences were observed within the CD16 expression levels of these two groups of donors. All in all, we observed differences in MTA-induced suppression of distinct NK cell functions and subsets, which could be explained due to differences in the strength of activating signaling pathways within the various subsets upon stimulation.

Overall, the current work demonstrates additional mechanisms by which tumor cells are able to escape NK cell surveillance, illustrating the necessity for new strategies to counteract these escape mechanisms. MTAP-deficient tumor cells are more susceptible toward PRMT5 inhibition than MTAP-competent ones, so using PRMT5 inhibitors is an opportunity to specifically target this tumor cell group (22, 41, 42). Although we demonstrated that PRMT5 inhibition is also able to reduce NK cell degranulation, a very high concentration of the inhibitor was needed to observe an effect. Based on our results, another opportunity to overcome MTA suppression is to increase the strength of the activation signal in NK cells. In the case of adoptive NK cell transfer treatments, a possibility would be to increase the percentage of CD57-expressing NK cells within the cell product as we demonstrated that these cells were less susceptible toward MTA suppression than CD57$^-$ ones. This could be achieved, for example, by expanding NKG2C$^+$ NK cells from donors harboring a NKG2C$^+$ expansion, as these cells express high levels of CD57. Liu et al. demonstrated that specific expansion of NKG2C$^+$ NK cells is feasible by stimulating NK cells with HLA-E, the ligand for NKG2C, and IL-15. NKG2C$^+$ NK cells expanded within 14 days, expressed high levels of CD57, and demonstrated superior effector cell functions against PHA and pediatric ALL blasts (54). In addition, genetic manipulation of these NK cells to overexpress MTAP before using them for adoptive cell transfer could prove beneficial.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local Ethics committee of the University of Erlangen and Oslo. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BJ, AM, and MA contributed to the conception and design of the study. DM advised on the topic of metabolomics. SV advised on the topic of phospho-epitope staining. K-JM advised on the topic of NK cell subsets and adaptive NK cells. BJ, SS, CS, and AS performed the experiments and the statistical analysis. BJ wrote the first draft of the manuscript. MA revised and wrote sections of the manuscript. All authors contributed to the article and approved the submitted 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.2020.02128/full#supplementary-material

Supplementary Figure 1 | NK cells' cytotoxic activity is reduced upon MTA co-incubation without affecting NK cell viability. Isolated NK cells were incubated overnight with 100 U/ml IL-2 and then incubated for 4 h with various concentrations of MTA at 37°C. NK cells were then harvested and stained for 7AAD expression (**A**; *n*: 8). Significance was calculated using a one-way ANOVA test.

Supplementary Figure 2 | NK cell degranulation and cytokine production is reduced by MTA. Overnight IL-2 activated NK cells were pre-incubated with different concentrations of MTA for 30′ at 37°C and then either stimulated with K562 cells at a 1:1 ratio (**A**; *n*: 6) or left alone (**B**; *n*: 12) with the prior MTA concentration for additional 4 h at 37°C. Afterwards the cells were harvested and CD107a **(A)** or CD16 **(B)** expression were analyzed in bulk NK cells. Significance was calculated using a Wilcoxon test for analyzing paired samples (*p*-value: * <0.05).

Supplementary Figure 3 | Adenosine derivates and PRMT5 inhibitor reduce NK cell function similar to MTA. Overnight IL-2 activated NK cells were pre-incubated for 30′ with 50μM 3-deazaadenosine (3-Deaza; **A**) or 5-azacitidine (5-Aza; **B**) and then stimulated with K562 cells at a 1:1 ratio for additional 4 h at 37°C (*n*: 6). Afterwards the cells were harvested and CD107a expression was analyzed in bulk NK cells. Significance was calculated using a Wilcoxon test for analyzing paired samples (*p*-value: * <0.05).

Supplementary Figure 4 | MTA's inhibitory effect varies within the different NK cell subsets. Overnight IL-2 activated NK cells were stimulated with K562 cells at a 1:1 ratio **(A,B,D,E)** or plate-bound CD16 **(C,F)** for 4h at 37°C with **(D–F)** or without **(A–C)** 100μM MTA. Afterwards the cells were harvested and CD107a or IFNγ expression was analyzed in NK cell subsets based on their CD57 expression. CD107a and IFNγ expression were indicated either as absolute values **(A–C)** or as the percentage decrease **(D–F)** due to MTA co-incubation [(absolute value with MTA/ absolute value without MTA)*100]. CD16 expression levels (MFI) were analyzed on CD57+/− NK cells **(G)**. Significance was calculated using a Wilcoxon test for analyzing paired samples (*p*-value: * <0.05, **** < 0, 0001; *n*: 12).

Supplementary Figure 5 | MTA-induced suppression of IFNγ production is less pronounced in donors harboring an expansion of NKG2C^{+} NK cells. Healthy donors were divided into two groups based on the presence of an expansion of NKG2C expressing NK cells (>10%). Overnight IL-2 activated NK cells were pre-incubated with 100μM MTA for 30′ at 37°C and then stimulated with K562 cells at a 1:1 ratio **(A,B)** or with plate-bound CD16 **(C)** for additional 4 h at 37°C. Afterwards the cells were harvested and CD107a and IFNγ expression were analyzed in bulk NK cells. CD107a and IFNγ expression were indicated as the percentage decrease due to MTA co-incubation [(absolute value with MTA/ absolute value without MTA)*100]. CD16 expression levels (MFI) were analyzed on CD56^{dim} NK cells within the two donor groups **(D)**. Significance was calculated using a Mann-Whitney test for analyzing unpaired samples (*n*: 6).

Supplementary Figure 6 | MTA inhibits the NF-κB, PI3K/AKT/S6 and MAPK/ERK pathways down-stream of the CD16 receptor. Freshly isolated NK cells were pre-incubated with or without 100 μM MTA for 30′ at 37°C and then stimulated with anti-CD16 antibodies for 0, 1, 5, 10, 30, and 60′. Bulk **(A,B)** or CD57+/- NK cells **(C)** were labeled with a fluorescent bar cell code **(A)** and stained for the phospho-epitope S6 (**B,C**, *n*:5).

Supplementary Table 1 | Differential expression of adenosine receptors and methyltransferases in CD57$^{+/-}$ NK cells.

REFERENCES

1. Brand A, Singer K, Koehl GE, Kolitzus M, Schoenhammer G, Thiel A, et al. LDHA-associated lactic acid production blunts tumor immunosurveillance by T and NK cells. *Cell Metab.* (2016) 24:657–71. doi: 10.1016/j.cmet.2016.08.011
2. Bottcher M, Renner K, Berger R, Mentz K, Thomas S, Cardenas-Conejo ZE, et al. D-2-hydroxyglutarate interferes with HIF-1alpha stability skewing T-cell metabolism towards oxidative phosphorylation and impairing Th17 polarization. *Oncoimmunology.* (2018) 7:e1445454. doi: 10.1080/2162402X.2018.1445454
3. Hori Y, Hori H, Yamada Y, Carrera CJ, Tomonaga M, Kamihira S, et al. The methylthioadenosine phosphorylase gene is frequently co-deleted with the p16INK4a gene in acute type adult T-cell leukemia. *Int J Cancer.* (1998) 75:51–6. doi: 10.1002/(SICI)1097-0215(19980105)75:1<51::AID-IJC9>3.0.CO;2-0
4. Behrmann I, Wallner S, Komyod W, Heinrich PC, Schuierer M, Buettner R, et al. Characterization of methylthioadenosin phosphorylase (MTAP) expression in malignant melanoma. *Am J Pathol.* (2003) 163:683–90. doi: 10.1016/S0002-9440(10)63695-4
5. Hellerbrand C, Bumes E, Bataille F, Dietmaier W, Massoumi R, Bosserhoff AK. Reduced expression of CYLD in human colon and hepatocellular carcinomas. *Carcinogenesis.* (2007) 28:21–7. doi: 10.1093/carcin/bgl081
6. Avila MA, Garcia-Trevijano ER, Lu SC, Corrales FJ, Mato JM. Methylthioadenosine. *Int J Biochem Cell Biol.* (2004) 36:2125–30. doi: 10.1016/j.biocel.2003.11.016
7. Henrich FC, Singer K, Poller K, Bernhardt L, Strobl CD, Limm K, et al. Suppressive effects of tumor cell-derived 5′-deoxy-5′-methylthioadenosine on human T cells. *Oncoimmunology.* (2016) 5:e1184802. doi: 10.1080/2162402X.2016.1184802
8. Strobl CD, Schaffer S, Haug T, Volkl S, Peter K, Singer K, et al. Selective PRMT5 inhibitors suppress human CD8(+) T cells by upregulation of p53 and impairment of the AKT pathway similar to the tumor metabolite MTA. *Mol Cancer Ther.* (2020) 19:409–19. doi: 10.1158/1535-7163.MCT-19-0189
9. Veal N, Hsieh CL, Xiong S, Mato JM, Lu S, Tsukamoto H. Inhibition of lipopolysaccharide-stimulated TNF-alpha promoter activity by S-adenosylmethionine and 5'-methylthioadenosine. *Am J Physiol Gastrointest Liver Physiol.* (2004) 287:G352–62. doi: 10.1152/ajpgi.00316.2003
10. Keyel PA, Romero M, Wu W, Kwak DH, Zhu Q, Liu X, et al. Methylthioadenosine reprograms macrophage activation through adenosine receptor stimulation. *PLoS ONE.* (2014) 9:e104210. doi: 10.1371/journal.pone.0104210
11. Fredholm BB, Jondal M, Lanefelt F, Ng J. Effect of 5'-methylthioadenosine, 3-deazaadenosine, and related compounds on human natural killer cell activity. Relation to cyclic AMP and methylation potential. *Scand J Immunol.* (1984) 20:511–8. doi: 10.1111/j.1365-3083.1984.tb01033.x
12. Ljunggren HG, Karre K. In search of the "missing self": MHC molecules and NK cell recognition. *Immunol Today.* (1990) 11:237–44. doi: 10.1016/0167-5699(90)90097-S
13. Alspach E, Lussier DM, Schreiber RD. Interferon gamma and its important roles in promoting and inhibiting spontaneous and therapeutic cancer immunity. *Cold Spring Harb Perspect Biol.* (2019) 11:a028480. doi: 10.1101/cshperspect.a028480
14. Cichocki F, Grzywacz B, Miller JS. Human NK cell development: one road or many? *Front Immunol.* (2019) 10:2078. doi: 10.3389/fimmu.2019.02078
15. Bjorkstrom NK, Riese P, Heuts F, Andersson S, Fauriat C, Ivarsson MA, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood.* (2010) 116:3853–64. doi: 10.1182/blood-2010-04-281675
16. Guma M, Angulo A, Vilches C, Gomez-Lozano N, Malats N, Lopez-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood.* (2004) 104:3664–71. doi: 10.1182/blood-2004-05-2058
17. Muntasell A, Vilches C, Angulo A, Lopez-Botet M. Adaptive reconfiguration of the human NK-cell compartment in response to cytomegalovirus: a different perspective of the host-pathogen interaction. *Eur J Immunol.* (2013) 43:1133–41. doi: 10.1002/eji.201243117
18. Cruz-Munoz ME, Valenzuela-Vazquez L, Sanchez-Herrera J, Santa-Olalla Tapia J. From the "missing self" hypothesis to adaptive NK cells: insights of NK cell-mediated effector functions in immune surveillance. *J Leukoc Biol.* (2019) 105:955–71. doi: 10.1002/JLB.MR0618-224RR
19. Kim GG, Donnenberg VS, Donnenberg AD, Gooding W, Whiteside TL. A novel multiparametric flow cytometry-based cytotoxicity assay simultaneously immunophenotypes effector cells: comparisons to a 4 h 51Cr-release assay. *J Immunol Methods.* (2007) 325:51–66. doi: 10.1016/j.jim.2007.05.013
20. Lopez-Verges S, Milush JM, Pandey S, York VA, Arakawa-Hoyt J, Pircher H, et al. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood.* (2010) 116:3865–74. doi: 10.1182/blood-2010-04-282301
21. Limm K, Wallner S, Milenkovic VM, Wetzel CH, Bosserhoff AK. The metabolite 5'-methylthioadenosine signals through the adenosine receptor A2B in melanoma. *Eur J Cancer.* (2014) 50:2714–24. doi: 10.1016/j.ejca.2014.07.005
22. Mavrakis KJ, McDonald ER III, Schlabach MR, Billy E, Hoffman GR, deWeck A, et al. Disordered methionine metabolism in MTAP/CDKN2A-deleted cancers leads to dependence on PRMT5. *Science.* (2016) 351:1208–13. doi: 10.1126/science.aad5944
23. Lokshin A, Raskovalova T, Huang X, Zacharia LC, Jackson EK, Gorelik E. Adenosine-mediated inhibition of the cytotoxic activity and cytokine production by activated natural killer cells. *Cancer Res.* (2006) 66:7758–65. doi: 10.1158/0008-5472.CAN-06-0478

24. Foley B, Cooley S, Verneris MR, Curtsinger J, Luo X, Waller EK, et al. Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand *in vivo* in response to recipient CMV antigen. *J Immunol.* (2012) 189:5082–8. doi: 10.4049/jimmunol.1201964
25. Liu LL, Landskron J, Ask EH, Enqvist M, Sohlberg E, Traherne JA, et al. Critical role of CD2 Co-stimulation in adaptive natural killer cell responses revealed in NKG2C-deficient humans. *Cell Rep.* (2016) 15:1088–99. doi: 10.1016/j.celrep.2016.04.005
26. Theorell J, Schlums H, Chiang SC, Huang TY, Tattermusch A, Wood SM, et al. Sensitive and viable quantification of inside-out signals for LFA-1 activation in human cytotoxic lymphocytes by flow cytometry. *J Immunol Methods.* (2011) 366:106–18. doi: 10.1016/j.jim.2011.01.014
27. Stevens AP, Dettmer K, Wallner S, Bosserhoff AK, Oefner PJ. Quantitative analysis of 5'-deoxy-5'-methylthioadenosine in melanoma cells by liquid chromatography-stable isotope ratio tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* (2008) 876:123–8. doi: 10.1016/j.jchromb.2008.10.038
28. Bertino JR, Waud WR, Parker WB, Lubin M. Targeting tumors that lack methylthioadenosine phosphorylase (MTAP) activity: current strategies. *Cancer Biol Ther.* (2011) 11:627–32. doi: 10.4161/cbt.11.7.14948
29. Stevens AP, Spangler B, Wallner S, Kreutz M, Dettmer K, Oefner PJ, et al. Direct and tumor microenvironment mediated influences of 5'-deoxy-5'-(methylthio)adenosine on tumor progression of malignant melanoma. *J Cell Biochem.* (2009) 106:210–9. doi: 10.1002/jcb.21984
30. Su CY, Chang YC, Chan YC, Lin TC, Huang MS, Yang CJ, et al. MTAP is an independent prognosis marker and the concordant loss of MTAP and p16 expression predicts short survival in non-small cell lung cancer patients. *Eur J Surg Oncol.* (2014) 40:1143–50. doi: 10.1016/j.ejso.2014.04.017
31. Meyer S, Wild PJ, Vogt T, Bataille F, Ehret C, Gantner S, et al. Methylthioadenosine phosphorylase represents a predictive marker for response to adjuvant interferon therapy in patients with malignant melanoma. *Exp Dermatol.* (2010) 19:e251–7. doi: 10.1111/j.1600-0625.2010.01072.x
32. Hevia H, Varela-Rey M, Corrales FJ, Berasain C, Martinez-Chantar ML, Latasa MU, et al. 5'-methylthioadenosine modulates the inflammatory response to endotoxin in mice and in rat hepatocytes. *Hepatology.* (2004) 39:1088–98. doi: 10.1002/hep.20154
33. Moreno B, Hevia H, Santamaria M, Sepulcre J, Munoz J, Garcia-Trevijano ER, et al. Methylthioadenosine reverses brain autoimmune disease. *Ann Neurol.* (2006) 60:323–34. doi: 10.1002/ana.20895
34. Li TW, Yang H, Peng H, Xia M, Mato JM, Lu SC. Effects of S-adenosylmethionine and methylthioadenosine on inflammation-induced colon cancer in mice. *Carcinogenesis.* (2012) 33:427–35. doi: 10.1093/carcin/bgr295
35. Blanchet F, Cardona A, Letimier FA, Hershfield MS, Acuto O. CD28 costimulatory signal induces protein arginine methylation in T cells. *J Exp Med.* (2005) 202:371–7. doi: 10.1084/jem.20050176
36. Ferro AJ, Vandenbark AA, MacDonald MR. Inactivation of S-adenosylhomocysteine hydrolase by 5'-deoxy-5'-methylthioadenosine. *Biochem Biophys Res Commun.* (1981) 100:523–31. doi: 10.1016/S0006-291X(81)80208-2
37. Della Ragione F, Pegg AE. Effect of analogues of 5'-methylthioadenosine on cellular metabolism. Inactivation of S-adenosylhomocysteine hydrolase by 5'-isobutylthioadenosine. *Biochem J.* (1983) 210:429–35. doi: 10.1042/bj2100429
38. Coward JK, Motola NC, Moyer JD. Polyamine biosynthesis in rat prostate. substrate and inhibitor properties of 7-deaza analogues of decarboxylated S-adenosylmethionine and 5'-methylthioadenosine. *J Med Chem.* (1977) 20:500–5. doi: 10.1021/jm00214a008
39. Borchardt RT. S-Adenosyl-L-methionine-dependent macromolecule methyltransferases: potential targets for the design of chemotherapeutic agents. *J Med Chem.* (1980) 23:347–57. doi: 10.1021/jm00178a001
40. Galletti P, Oliva A, Manna C, Della Ragione F, Carteni-Farina M. Effect of 5'-methylthioadenosine on *in vivo* methyl esterification of human erythrocyte membrane proteins. *FEBS Lett.* (1981) 126:236–40. doi: 10.1016/0014-5793(81)80250-5
41. Kryukov GV, Wilson FH, Ruth JR, Paulk J, Tsherniak A, Marlow SE, et al. MTAP deletion confers enhanced dependency on the PRMT5 arginine methyltransferase in cancer cells. *Science.* (2016) 351:1214–8. doi: 10.1126/science.aad5214
42. Marjon K, Cameron MJ, Quang P, Clasquin MF, Mandley E, Kunii K, et al. MTAP deletions in cancer create vulnerability to targeting of the MAT2A/PRMT5/RIOK1 axis. *Cell Rep.* (2016) 15:574–87. doi: 10.1016/j.celrep.2016.03.043
43. Biggar KK, Li SS. Non-histone protein methylation as a regulator of cellular signalling and function. *Nat Rev Mol Cell Biol.* (2015) 16:5–17. doi: 10.1038/nrm3915
44. Andreu-Perez P, Hernandez-Losa J, Moline T, Gil R, Grueso J, Pujol A, et al. Methylthioadenosine (MTA) inhibits melanoma cell proliferation and *in vivo* tumor growth. *BMC Cancer.* (2010) 10:265. doi: 10.1186/1471-2407-10-265
45. Guo J, Dai X, Laurent B, Zheng N, Gan W, Zhang J, et al. AKT methylation by SETDB1 promotes AKT kinase activity and oncogenic functions. *Nat Cell Biol.* (2019) 21:226–37. doi: 10.1038/s41556-018-0261-6
46. Lu T, Stark GR. NF-kappaB: regulation by methylation. *Cancer Res.* (2015) 75:3692–5. doi: 10.1158/0008-5472.CAN-15-1022
47. Wei H, Wang B, Miyagi M, She Y, Gopalan B, Huang DB, et al. PRMT5 dimethylates R30 of the p65 subunit to activate NF-kappaB. *Proc Natl Acad Sci USA.* (2013) 110:13516–21. doi: 10.1073/pnas.1311784110
48. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood.* (2006) 107:159–66. doi: 10.1182/blood-2005-04-1351
49. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood.* (2010) 115:2167–76. doi: 10.1182/blood-2009-08-238469
50. Luetke-Eversloh M, Cicek BB, Siracusa F, Thom JT, Hamann A, Frischbutter S, et al. NK cells gain higher IFN-gamma competence during terminal differentiation. *Eur J Immunol.* (2014) 44:2074–84. doi: 10.1002/eji.201344072
51. Nandagopal N, Ali AK, Komal AK, Lee SH. The Critical Role of IL-15-PI3K-mTOR pathway in natural killer cell effector functions. *Front Immunol.* (2014) 5:187. doi: 10.3389/fimmu.2014.00187
52. Luetke-Eversloh M, Hammer Q, Durek P, Nordstrom K, Gasparoni G, Pink M, et al. Human cytomegalovirus drives epigenetic imprinting of the IFNG locus in NKG2Chi natural killer cells. *PLoS Pathog.* (2014) 10:e1004441. doi: 10.1371/journal.ppat.1004441
53. Veeramani S, Wang SY, Dahle C, Blackwell S, Jacobus L, Knutson T, et al. Rituximab infusion induces NK activation in lymphoma patients with the high-affinity CD16 polymorphism. *Blood.* (2011) 118:3347–9. doi: 10.1182/blood-2011-05-351411
54. Liu LL, Beziat V, Oei VYS, Pfefferle A, Schaffer M, Lehmann S, et al. *Ex vivo* expanded adaptive NK cells effectively kill primary acute lymphoblastic leukemia cells. *Cancer Immunol Res.* (2017) 5:654–65. doi: 10.1158/2326-6066.CIR-16-0296
55. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA.* (2005) 102:15545–50. doi: 10.1073/pnas.0506580102
56. Liberzon A, Subramanian A, Pinchback R, Thorvaldsdóttir H, Tamayo P, Mesirov JP. Molecular signatures database (MSigDB) 3.0. *Bioinformatics.* (2011) 15:1739–40. doi: 10.1093/bioinformatics/btr260
57. Kanehisa M and Goto S. Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* (2000) 28:27–30. doi: 10.1093/nar/28.1.27
58. Kanehisa M, Sato Y, Furumichi M, Morishima K, Tanabe M. New approach for understanding genome variations in KEGG. *Nucleic Acids Res.* (2019) 47:D590–5. doi: 10.1093/nar/gky962
59. Kanehisa M. Toward understanding the origin and evolution of cellular organisms. *Protein Sci.* (2019) 28:1947–51. doi: 10.1002/pro.3715

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Lysophosphatidic Acid Is an Inflammatory Lipid Exploited by Cancers for Immune Evasion *via* Mechanisms Similar and Distinct From CTLA-4 and PD-1

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Immunological tolerance has evolved to curtail immune responses against self-antigens and prevent autoimmunity. One mechanism that contributes to immunological tolerance is the expression of inhibitory receptors by lymphocytes that signal to dampen immune responses during the course of an infection and to prevent immune-mediated collateral damage to the host. The understanding that tumors exploit these physiological mechanisms to avoid elimination has led to remarkable, but limited, success in the treatment of cancer through the use of biologics that interfere with the ability of cancers to suppress immune function. This therapy, based on the understanding of how T lymphocytes are normally activated and suppressed, has led to the development of therapeutic blocking antibodies, referred to as immune checkpoint blockade, which either directly or indirectly promote the activation of CD8 T cells to eradicate cancer. Here, we highlight the distinct signaling mechanisms, timing and location of inhibition used by the CTLA-4 and PD-1 inhibitory receptors compared to a novel inhibitory signaling axis comprised of the bioactive lipid, lysophosphatidic acid (LPA), signaling via the LPA5 receptor expressed by CD8 T cells. Importantly, abundant evidence indicates that an LPA-LPA5 signaling axis is also exploited by diverse cancers to suppress T cell activation and function. Clearly, a thorough molecular and biochemical understanding of how diverse T cell inhibitory receptors signal to suppress T cell antigen receptor signaling and function will be important to inform the choice of which complimentary checkpoint blockade modalities might be used for a given cancer.

Keywords: CD8 T cell, cancer, lysophophatidic acid, LPAR5, inhibitory receptor and ligand

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INTRODUCTION

Lipid biology in the context of tumor immunity remains vastly unexplored. However its role in modulating inflammation has been used for centuries (1), which has led to pharmaceutical development of nonsteroidal anti-inflammatory drugs (NSAIDs), like COX-2 inhibitors, that inhibit the generation of prostaglandin and thromboxane lipids. Interestingly, COX-2 inhibitors also lower cancer promoting inflammation and drive type I immunity, demonstrating a unique role of lipids

affecting the anti-tumor response (2). This review seeks to highlight another bioactive lipid, lysophosphatidic acid (LPA), and its role in dampening tumor immunity. Unlike COX-2, LPA suppresses the anti-tumor response in a cell intrinsic manner by signaling via an LPA receptor (LPAR) similar to checkpoint receptors. Furthermore, as LPA is found systemically, and in all tissues, we speculate mechanisms of suppression mediated by LPARs are similar to CTLA and PD-1 in inhibiting T cell antigen receptor (TCR) signaling, yet via distinct signaling pathways. Specifically, LPA signaling has been shown to suppress T cell TCR Ca^{2+} signaling which inhibits naïve T cell activation in secondary lymphoid organs. Additionally, as tumors can produce LPA at higher concentrations than adjacent tissue, this tumor-derived LPA also inhibits T cell effector function therefore representing a checkpoint in T cell function similar to that mediated by CTLA-4 and PD-1. Therefore, as a lipid, LPA/LPAR modulation of immune responses has functional similarities to other checkpoint molecules like PD-1/PD-L1 or CTLA-4/CD80 yet remains unique to other soluble or cell-associated protein-protein interactions.

CTLA-4: SUPPRESSION OF EARLY T CELL ACTIVATION

CTLA-4 is a major inhibitory receptor expressed by CD8 T cells and was an initial therapeutic target given that CTLA-4 is expressed following TCR engagement by naïve T cells and continues to rise until maximum expression at 48 h (3). Compared to the CD28 T cell costimulatory receptor, CTLA-4 displays significantly higher affinity for the CD80 and CD86 co-receptors expressed by antigen presenting cells (APCs). Thus, when expressed, CTLA-4 effectively sequesters CD80 and CD86 away from CD28 and prevents co-stimulatory signaling activity for the T cell antigen receptor (TCR) (4). Furthermore, CTLA-4 interaction with CD80/CD86 can also lead to transendocytosis, the physical capture of these ligands and removal from the surface of APCs, thereby limiting total levels of available ligands for CD28 and subsequent co-stimulation (4). The physical sequestration of CD28 ligands ultimately prevents optimal TCR signaling by CD8 T cells and is an important mechanism by which CTLA-4 suppresses T cell function (5). This process of CTLA-4-mediated CD80/CD86 transendocytosis is seen in all T cell subsets but particularly in regulatory T cells contributing to the suppressive abilities of this T cell subpopulation (5). Notably, deletion of the CTLA-4 cytoplasmic tail has been reported not to change its ability to suppress T cell proliferation indicating CTLA-4 intracellular signaling is not necessary for all T cell inhibition (6). Furthermore, agonist signaling via CTLA-4 does not induce significant changes in T cell gene expression also suggesting a mechanism of inhibition that does not rely on intracellular signaling (7). However, coimmunoprecipitation of CTLA-4 in a T cell hybridoma reveals interaction with PKC-η (8). Tregs depleted of PKC-η have compromised suppressive function both *in vitro* and *in vivo* thus suggesting a cell-intrinsic signaling role for CTLA-4 (8). The significance of immune inhibition provided by CTLA-4 is highlighted by the fact that *Ctla4*$^{-/-}$ mice survive only for the first 3–4 weeks and then die as a result of massive lymphocytic infiltration and destruction of major organs (9–11). Thus, a CTLA-4-deficiency leads to uncontrolled expansion of CD4 T cells, including autoreactive T cells that subsequently damage host organs thus indicating that CTLA-4 is required for appropriate maintenance of peripheral tolerance.

Under normal conditions, T cells that develop in the thymus and recognize self-antigens with relatively high affinity are culled from development by mechanisms of central tolerance. Given that all tumors originate from normal cells, the (non-mutated) tumor antigens they express are essentially self-antigens and central tolerance similarly deletes host CD8 T cells able to recognize tumor antigens with high affinity. Nevertheless, central tolerance is not absolute and does not remove all self-reactive T cells, especially those that display weak affinity for self-antigens. These weakly autoreactive T lymphocytes emigrate to the periphery and are further restrained by mechanisms of peripheral tolerance. Indeed, early experiments designed to block the CTLA-4 inhibitory receptor to improve the endogenous self/tumor host response against a murine 51BLim10 colon cancer resulted in immediate rejection of the tumor (12). Accordingly, anti-CTLA-4 blockade therapy has shown success in the clinic, however, this approach also presents with treatment related toxicities such as nausea and fatigue seen in 70–80% of patients or dermatitis and enterocolitis seen in 5–25% of patients (13). Recently, anti-CTLA-4 antibodies have been engineered to behave differently during the endosomal trafficking of CTLA-4 to minimize these adverse events. Specifically, ipilimumab harboring tyrosine-to-histidine mutations display increased pH sensitivity and upon entering the endosomes bound to CTLA-4, disengages from CTLA-4 in the lysosomes allowing CTLA-4 to recycle to the surface in a LRBA dependent manner which still induces tumor regression with minimal associated adverse events (14). Of note, recent data has suggested that an alternate mechanism by which anti-CTLA-4 antibodies act is via the depletion of regulatory T cells (15, 16), thus questioning its mechanism of action as a checkpoint blockade therapy and suggesting it may instead act as an antibody depleting therapy. In contrast, while treatment of patients with either ipilimumab or tremelimumab anti-CTLA-4 monoclonal antibodies has led to increased (CD4 and CD8) T cell tumor infiltration, the number of tumor-infiltrating FOXP3+ regulatory T cells was not significantly altered (17). Thus, blocking CTLA-4 represents a form of checkpoint blockade that allows for greater primary expansion of effector T cells; however, the precise mechanism by which CTLA-4 modulates CD8 T cells in the tumor microenvironment is less certain.

PD-1: CO-OPTED BY THE TUMOR MICROENVIRONMENT

As CTLA-4 expression is initiated soon after initial T cell antigen-recognition by naïve T cells, its mechanism of T cell

suppression is considered to be primarily restricted to secondary lymphoid organs where T cells typically first encounter foreign antigens. However, additional inhibitory receptors are also expressed by CD8 T cells and able to suppress cytotoxic function at various stages. PD-1 is another inhibitory receptor expressed by activated CD8 T cells and able to dampen effector function and has received abundant attention as a target of immune checkpoint blockade. Unlike CTLA-4, PD-1 harbors both ITIM and ITSM tyrosine-based motif sequences in the cytoplasmic domain (18) that facilitate inhibitory function and arguing for a mechanism of suppression dependent on receptor signaling. While it is generally considered that inhibition mediated by PD-1 is promoted through the recruitment of cytosolic phosphatases, it remains unclear precisely which stimulatory signals are the targets of inhibition. Immunoprecipitation of CD3ζ shows a ~70% reduction of phosphotyrosine when TCR and PD-1 are co-ligated in comparison to TCR ligation alone (19). Furthermore, both SHP-1 and SHP-2 are thought to mediate this suppression as both were found to immunoprecipitate with the ITSM domain of PD-1 (17). However, as PD-1 preferentially clusters with CD28 rather than the TCR, this argues that CD28 is the preferential target of PD-1 signaling (20). In fact, using a cell-free FRET-based assay, it was determined that PD-1 selectively recruited SHP-2 which in turn dephosphorylated CD28.

Despite discrepancies in the described mechanism(s) of PD-1 inhibitory action, PD-1 blockade has enjoyed major success in the clinic. This is because a major ligand for PD-1 is PD-L1, whose expression is upregulated by diverse tumors in response to IFNγ. In fact when examined, ~98% of PD-L1 expressing melanocytes were co-localized with T cells as opposed to minimal co-localization of T cells with PD-L1-negative tumor cells, suggesting tumor cells express PD-L1 in response to infiltrating T cells (21). Thus, attenuating this inhibitory signal prevents T cell suppression and leads to increased cytotoxicity from tumor-specific CD8 T cells. Indeed, the success of anti-PD-1/PD-L1 signaling is highlighted by having garnered FDA approval for the treatment of kidney cancers, melanomas, prostate cancers, lung cancers, B cells lymphomas, Hodgkin's lymphoma, urothelial carcinomas, gastric cancers, liver cancers, cervical cancers, and head and neck cancers in the last 5 years (22). Although the majority of evidence suggests the PD-1/PD-L1 signaling axis acts primarily at sites of chronic inflammation, recent data provides evidence for a role for PD-1 signaling during the early phases of T cell activation (23). Specifically, upon TCR-mediated activation, PD-1 expression by CD8 T cells is upregulated within 4 h, matching the kinetics of the CD25 activation antigen and preceding cell division, thus arguing for a physiological role for PD-1 during primary activation (23). PD-L1 blockade on day 0 and day 3 after LCMV Armstrong infection led to increased granzyme B and mTOR signaling two days later by CD8 T cells (23). Thus, PD-1 signaling appears to suppress T cell function at various stages representing another 'checkpoint' that tumors exploit to escape elimination.

Given the clinical success and limitations of these therapeutic interventions for cancer, additional effort is needed to better understand precisely how T cell function is regulated by both stimulatory and regulatory signals (24). Ongoing and new research has identified novel protein inhibitory receptors and below we further describe a lipid that signals via a cognate G-protein coupled receptor (GPCR) to deliver suppressive signals to CD8 T cells and which ultimately negatively-regulate T cell function.

LYSOPHOSPHATIDIC ACID

Lysophosphatidic acid (LPA) is a lysophospholipid structurally similar to sphingosine-1-phosphate (S1P), a lipid that has been well characterized to signal to immune cells and to orchestrate cell trafficking (25). Both lipids share a phosphate head group attached to a glycerol backbone; however, LPA differs by having a single ester linked aliphatic chain whereas S1P has a single amine linked aliphatic chain. On initial discovery both LPA and S1P were considered to be intracellular lipid metabolites and only later were characterized to function as extracellular bioactive lipids that signal to cells expressing cognate G-protein coupled receptors (GPCRs). Extracellular LPA is generated predominantly via the enzymatic activity of Autotaxin (ATX), a secreted ectoenzyme with lysophospholipase D activity that hydrolyzes the abundantly available lysophosphatidylcholine to produce LPA. Although five isoforms of ATX exist through alternative splicing of exons 12, 19, and 21, ATXβ is the form most expressed in tissue (26). Autotaxin is encoded by *ENPP2* and is highly expressed in nervous system as well as considerable expression by stromal and endothelial cells with reduced general expression in most other tissues. Structural studies have indicated that Autotaxin harbors an exposed integrin binding motif and, as a secreted enzyme, Autotoxin is thought to associate with surface-bound integrins (27, 28). Thus, current models posit that in certain microenvironments integrin-bound Autotaxin hydrolyzes lysophosphatidylcholine to produce LPA where localized concentrations are able to signal via LPARs expressed by nearby cells, including cell types not producing the Autotaxin enzyme. Extracellular LPA production also appears tightly regulated with a half-life of approximately three minutes due to its rapid hydrolysis mediated by Lipid phosphate phosphatases (LPP) 1 and LPP3. The half-life of LPA increases 4 fold when intravenously introduced into mice deficient for LPP1 (lipid phosphate phosphohydrolase type 1), an enzyme that degrades LPA, and $Lpp1^{-/-}$ mice harbor higher levels of LPA (29).

Similar to S1P, LPA also signals via cognate GPCR receptors of which 6 LPA receptors (LPA_{1-6}) have been characterized and that are variably expressed on all immune populations (**Figure 1**). Thus, given its ability to signal extracellularly and act as an intracellular second messenger (31, 32), it is not surprising that LPA has been associated with a number of physiological processes including smooth muscle contraction, platelet aggregation, and blood pressure (33–35). Systemic changes in LPA levels have been observed in pregnancy (36), aging (37), and between sexes where females have been reported to harbor significantly elevated LPA serum levels compared with males (37). Interestingly, all of these circumstances can be

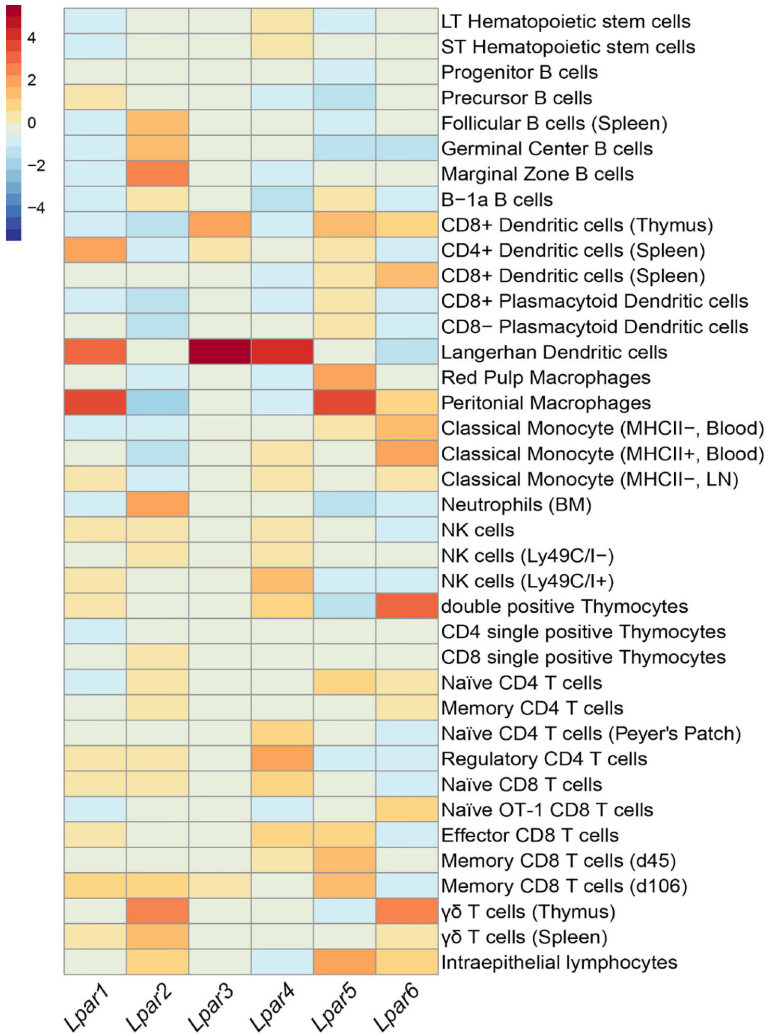

FIGURE 1 | Summary of *Lpar* expression in different leukocytes. Heatmap showing expression of LPA receptors across immune subsets. Data was compiled from Immune Genome Project microarray of sorted immune populations and scaled to columns (30). Color scale on the top left indicates level of mRNA expression. Unless specified, immune populations were sorted from 6-8 week C57BL/6 mice.

considered to require suppression of inflammation. However, its role in wound repair (38) would speak directly to the suppressive affect LPA signaling has on CD8 T cell function.

Resolution of a wound can be subdivided into 4 distinct phases: hemostasis, inflammation, proliferation, and remodeling (39) (**Figure 2**). Immediately after a physical trauma, platelets are first to arrive to help initiate the coagulation cascade and help activate fibroblasts and recruit both neutrophils and macrophages through the secretion of TGFβ (40). Interestingly, activated platelets are a potent source of LPA and soluble Autotaxin can associate with platelet integrins and produce LPA (41). Neutrophils are the initial immune cells to infiltrate wounds and help drive an inflammatory response to eliminate any microbes (42). Although short-lived cells, the infiltration of neutrophils is vital for the production of various growth factors like IL-17 and VEGF which help in the proliferation of fibroblasts, keratinocytes, and endothelial cells. Loss of early neutrophil recruitment delays epithelialization and decreases neovascularization at the site of injury (43). While LPA does not appear to have any direct effect on neutrophil migration per se, it is able to enhance the migratory response of neutrophils to suboptimal concentrations of N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP) (46) suggesting a role for LPA in aiding neutrophil migration to sites of inflammation. Monocyte recruitment, and subsequent differentiation into macrophages, occurs 5 to 6 h post injury. Anti- inflammatory macrophages are involved with the secretion of TGFβ, clearing cellular debris, helping reorganize the extracellular matrix (ECM) and contracting the wound. Macrophage-mediated degradation of the ECM leads to more endothelial proliferation and the release of angiogenesis factors such as FGF and placental growth factor (PIGF) (52). Of note, LPA is able to directly promote the conversion of monocytes to

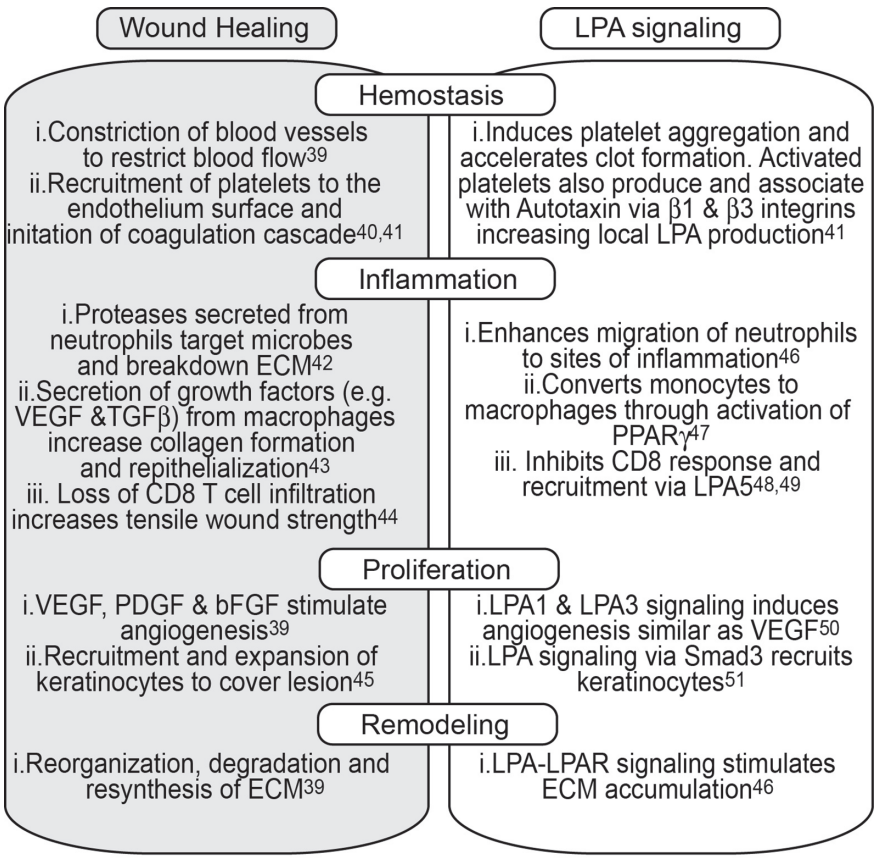

FIGURE 2 | The physiological role of LPA signaling in wound repair. A simplified version of the major biological processes that occur during each step of wound healing stratified across four major groups; Hemostasis, Inflammation, Proliferation, and Remodeling. Each group is further broken down to biological events that characterize each group (grey tile) and the role of LPA in each of those biological events (clear tile).

macrophages and is true in both humans and mice (47). In fact, culturing monocytes in media containing only LPA converts $CD11b^{+}F480^{-}$ monocytes into $CD11b^{+}F480^{+}$ macrophages more so than only M-CSF (47). While the role of T cells in wound repair remains largely unexplored, depletion of CD8 T cells increases tensile strength across lesions suggesting some inhibitory role of CD8 T cells (44). Given this overlap of LPA-mediated effects and wound healing, it is not surprising that topical application of LPA to physical wounds in rats or mice promoted accelerated healing with increased neoepithelial thickness (53, 54), an effect further seen in aged rats compared to young rats (55). As the recruitment and proliferation of keratinocytes remains critical for skin repair, LPA signaling not only induced increased migration and expansion of keratinocytes but also induced a four to eight fold increase in TGFα production (45).

LPA AS A CANCER INTRINSIC GROWTH FACTOR

Tumors have been appreciated to rely on pathways used in wound repair and have often been described as wounds that never heal (56). For example, IL-1β, IL-6, and IL-8 are cytokines that are secreted during the early inflammatory response after tissue damage and are involved with re-epithelization. However, in breast cancer, these cytokines are associated with a poor prognosis as they have been linked to tumor growth and metastasis (57–59). Similarly, LPA has been exploited by cancers to promote growth in several non-redundant ways. LPA signaling has directly been linked to hTERT upregulation as ovarian cancer lines treated with LPA have increased telomerase activity as early as 12 h after co-culture (60). This replicative advantage mediated by LPA helps explain the increased expression of the gene encoding Autotaxin, *ENPP2*, observed in ovarian cancer stem cells; a population of long lasting malignant cells that seeds cancer growth (61). In fact, autocrine LPA signaling in these cells has been shown to promote sphere forming ability and upregulation of ALDH, markers associated with cancer stem cells. This dependence on LPA signaling by ovarian cancer cells provided rationale to explore the use of LPA as a potential biomarker in ovarian cancer progression and accounts for the high levels of LPA found in ascites fluid from individuals with ovarian cancer (62). LPA signaling can also directly affect several 'hallmarks' of cancer (63), including proliferation (64), or metastasis of colorectal

cancers (65), increased angiogenesis in transformed NIH3T3 cells (66) and further demonstrated in chorio-allantoic membrane assays (50), resisting cell death by increasing insensitivity to chemotherapy in ovarian cancers (67), altered lipogenesis as LPA increases fatty acid synthase in ovarian cancers (68), and by modulating inflammation through activation of PPAR-γ in the tumor stroma (69).

LPAR SIGNALING AS A 'CHECKPOINT' IN THE ANTI-TUMOR RESPONSE

Given the mechanisms by which CTLA-4 and PD-1 suppress T cell function, the LPA signaling axis can be considered as an additional form of suppression. However unlike CTLA-4, which inhibits activating signals of antigen presenting cells to CD8 T cells, or PD-1, which inhibits interactions of effector T cells and targets cells, we find LPA signaling disrupts T cell engagement with APCs and target cells (48, 49). In initial work from our lab, we demonstrated that LPA engagement with the LPA5 receptor induces a signal that inhibits TCR-induced Ca^{2+} release from intracellular stores in naïve CD8 T cells (48). This suppressive LPA signaling is dependent on the LPA5 receptor, as intracellular Ca^{2+} levels are not depressed in LPA5-deficient CD8 T cells after TCR signaling is induced in the presence of LPA (48, 49). Evidence in B lymphocytes strongly suggests LPA inhibition of antigen receptor signaling manifests via impaired IP_3 receptor activity, thereby limiting the amount of Ca^{2+} released from ER stores after antigen receptor stimulation (70). Of note, the activity of antigen receptor proximal signaling molecules, e.g., tyrosine kinases and PLCγ, are unchanged in the presence of LPA (70). Thus, the mechanism of inhibition imposed by LPA5 on CD8 T cells differs from how PD-1 and CTLA-4 suppress CD8 T cells. We have documented that LPA5 signals via $G\alpha_{12/13}$-assoicated heterotrimeric G-proteins in lymphocytes and subsequently relies on the ARHGEF1 intracellular signaling effector molecule for antigen receptor mediated suppression of LPA (70). This is in contrast to CTLA-4 which can inhibit T cell function in the absence of intracellular signaling and PD-1 which depends on the recruitment of SHP-1 or SHP-2 for its suppressive action. Given the pleiotropic downstream effects of Ca^{2+} dependent processes that result from TCR signaling, it is perhaps not surprising that TCR-stimulated CD8 T cells fail to appropriately activate and proliferate in the presence of LPA both *in vitro* and *in vivo* (48). Moreover, *in vivo* LPA-induced suppression was not observed with CD8 T cells harboring null *Lpar5* alleles (48). Importantly, we have more recently determined that in addition to suppressing TCR-induced cytosolic calcium mobilization, LPA also inhibits TCR-driven ERK activation (49) and both calcium and ERK have been previously shown to be required for granule exocytosis (71–74). Accordingly, in the presence of an LPA5 agonist, effector CD8 T cells display impaired perforin localization to the immunological synapse upon cognate antigen stimulation (49). These data demonstrate LPA engagement of LPA5 is able to suppress cells at different stages of CD8 T cell maturation and characteristic of other checkpoint regulators. As a consequence, $Lpar5^{-/-}$ tumor-specific CD8 T cells are able to provide better control of tumor burden 8 days after adoptive transfer compared with wild type tumor-specific CD8 T cells (48, 49).

Given the negative regulation of CD8 T cells by an LPA-LPA5 axis, one might expect that a deficiency in LPA5 receptor expression or a reduction in systemic levels of LPA might lead to autoimmunity, as observed with CTLA-4-deficient mice (7–9). Interestingly, neither the $Lpar5^{-/-}$ nor $Enpp2^{+/-}$ (ATX heterozygous) mice, which harbor half the normal levels of systemic LPA, appear to present with any obvious systemic inflammatory conditions, raising questions about the role of LPA as a suppressive lipid. However, we note that PD-1-deficient mice develop significantly less severe immune pathologies compared to $Ctla4^{-/-}$ mice and neither the $Tigit^{-/-}$ nor $CD96^{-/-}$ mice present with spontaneous disease (75).

Together, these findings highlight the different functions displayed by inhibitory receptors. Furthermore, as postulated by the 'tide model' (76), the existence of multiple costimulatory and coinhibitory receptors on T cells suggest that T cell signaling is finely tuned and responds to the microenvironmental context in which TCR signaling occurs. Thus, while certain signals appear more paramount (e.g., CD28, CTLA-4, PD-1), certain contexts reveal the dominance of some (inhibitory) receptor signaling over other signals, as evident by the greater expansion of autologous CD8 T cells with DCs with anti-LAG3 blockade over anti-PD-1 blockade (77). Given that a majority of monotherapy checkpoint blockade fails to induce tumor remission, we propose that inhibition of the LPA signaling axis represents another potential 'checkpoint' to target in combinational therapy.

As all immune cells express at least one LPA receptor, it is reasonable to consider that this bioactive lipid has a role in modulating antitumor function in other tumor infiltrating leukocytes. (**Figure 3**) (78–80, 83). In fact, the suppression mediated by LPA signaling can extend beyond CD8 T cells to other cells in the adaptive arm. LPA can impair the migration of CD4 T cells and even causes chemorepulsion *in vitro* in a LPA2-dependent manner (82). Moreover, in the presence of LPA, stimulated human CD4 T cells can produce IL-13, a Th2 cytokine involved with the activation of myeloid derived suppressor cells; thus, the reduced CD4 T cells that do migrate to the tumor are still involved in maintaining a pro-tumor environment (84, 85). Unlike CD4 T cells, LPA can act as a chemoattractant to natural killer (NK) cells yet also impair effector function. LPA signaling though LPA2 can increase cAMP levels and activated protein kinase A which subsequently inhibits the release of perforin in NK cells (81). Inhibition of protein kinase A activation restores NK cell cytotoxicity in the presence of LPA, suggesting a mechanism by which tumor derived LPA can impair NK function. Similar to the of CD8 T cells, B cells signaling and function are also inhibited through LPA5 (70). Specifically, LPA signaling though an LPA5–$G\alpha_{12/13}$–Arhgef1 axis results in reduced Ca^{2+} signaling, a mechanism similar to CD8 T cells. Functionally, LPA signaling reduced humoral responses to T1-2 antigens suggesting a conserved inhibitory signal in T and B cells mediated through LPA5 (70). Functional changes mediated through LPA signaling extends further than lymphocytes as dendritic cells, macrophages, and neutrophils are

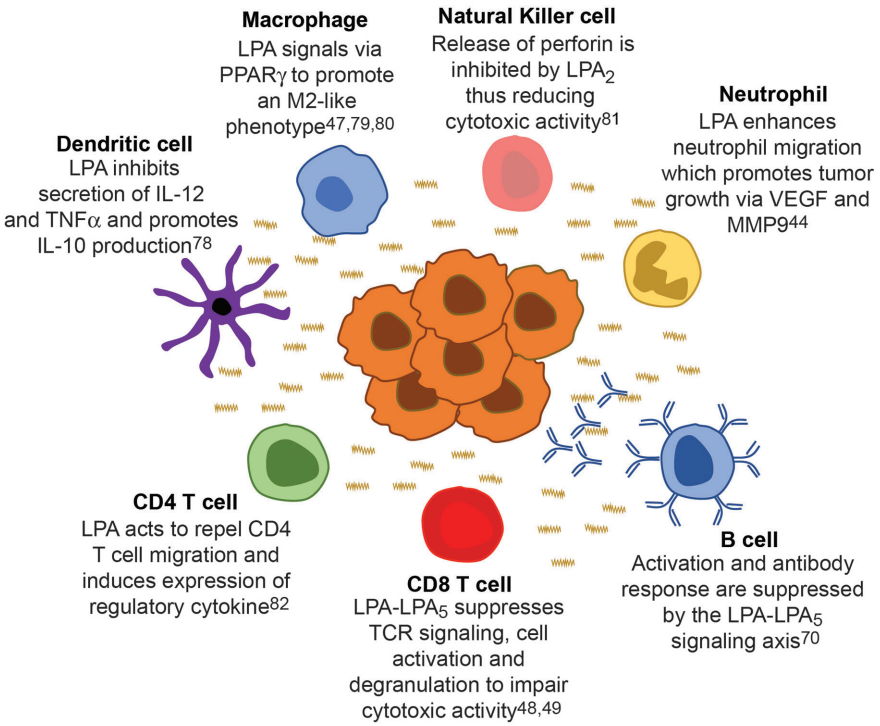

FIGURE 3 | Cancer derived LPA regulates immunity across all leukocytes. LPA (wavy line) originates from the tumor (center cellular mass) and suppresses the function of all leukocytes within the microenvironment creating a 'checkpoint' in the immune response against cancer. Each major leukocytes population is around the tumor mass with a specific example of how LPA signaling affects that immune cell, thereby generating a tumor promoting environment.

altered in the presence of this lipid. While LPA signaling does not affect endocytic function of dendritic cells, it does inhibit the secretion of IL-12 and TNFα while promoting the secretion of IL-10 (78). Thus, dendritic cells in the tumor microenvironment exposed to LPA would be poised for a tumor promoting function. Unlike CD4 T cells, LPA is a chemoattractant to neutrophils (46, 82). However this potenial influx of neutrophils has been linked to reduced overall survival in several cancers, presumably due to release of tumor promoting factors like VEGF or MMP9 (89). Futhermore, tumor infilitrating neutrophils can secrete TGFβ and have been implicated to changing the plasticity of macrophages to an M2-like state which promotes tumor growth (89). As LPA signaling though PPAR-γ can cause the differentiation of monocytes to macrophages, we speculate the LPA signaling is involved with the influx and differentiation of tumor promoting myeloid cells (47, 79, 80).

We believe LPA signaling has evolved to help with would repair to prevent an overactive immune response. Consistent with this, cancers exploit this very system to suppress immune cell function thereby representing a "checkpoint" in our endogenous antitumor response. We propose that manipulation of the LPA/LPAR axis should be considered for potentially synergizing with anti-PD1, anti-CTLA4 or other therapies to improve leukocyte function in the tumor microenvironment (51).

AUTHOR CONTRIBUTIONS

DM and RT wrote and edited the review. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Serhan CN. Treating inflammation and infection in the 21st century: new hints from decoding resolution mediators and mechanisms. *FASEB J* (2017) 31:1273–88. doi: 10.1096/fj.201601222R
2. Zelenay S, van der Veen AG, Bottcher JP, Snelgrove KJ, Rogers N, Acton SE, et al. Cyclooxygenase-Dependent Tumor Growth through Evasion of Immunity. *Cell* (2015) 162:1257–70. doi: 10.1016/j.cell.2015.08.015
3. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* (1994) 1:405–13. doi: 10.1016/1074-7613(94)90071-X
4. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* (2011) 332:600–3. doi: 10.1126/science.1202947
5. Ovcinnikovs V, Ross EM, Petersone L, Edner NM, Heuts F, Ntavli E, et al. CTLA-4-mediated transendocytosis of costimulatory molecules primarily

targets migratory dendritic cells. *Sci Immunol* (2019) 4:eaaw0902 doi: 10.1126/sciimmunol.aaw0902
6. Tai X, Van Laethem F, Pobezinsky L, Guinter T, Sharrow SO, Adams A, et al. Basis of CTLA-4 function in regulatory and conventional CD4(+) T cells. *Blood* (2012) 119:5155–63. doi: 10.1182/blood-2011-11-388918
7. Wakamatsu E, Mathis D, Benoist C. Convergent and divergent effects of costimulatory molecules in conventional and regulatory CD4+ T cells. *Proc Natl Acad Sci U S A* (2013) 110:1023–8. doi: 10.1073/pnas.1220688110
8. Kong KF, Fu G, Zhang Y, Yokosuka T, Casas J, Canonigo-Balancio AJ, et al. Protein kinase C-eta controls CTLA-4-mediated regulatory T cell function. *Nat Immunol* (2014) 15:465–72. doi: 10.1038/ni.2866
9. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* (1995) 3:541–7. doi: 10.1016/1074-7613(95)90125-6
10. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* (1995) 270:985–8. doi: 10.1126/science.270.5238.985
11. Khattri R, Auger JA, Griffin MD, Sharpe AH, Bluestone JA. Lymphoproliferative disorder in CTLA-4 knockout mice is characterized by CD28-regulated activation of Th2 responses. *J Immunol* (1999) 162:5784–91.
12. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* (1996) 271:1734–6. doi: 10.1126/science.271.5256.1734
13. Fecher LA, Agarwala SS, Hodi FS, Weber JS. Ipilimumab and its toxicities: a multidisciplinary approach. *Oncologist* (2013) 18:733–43. doi: 10.1634/theoncologist.2012-0483
14. Zhang Y, Du X, Liu M, Tang F, Zhang P, Ai C, et al. Hijacking antibody-induced CTLA-4 lysosomal degradation for safer and more effective cancer immunotherapy. *Cell Res* (2019) 29:609–27. doi: 10.1038/s41422-019-0184-1
15. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, et al. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer Immunol Res* (2013) 1:32–42. doi: 10.1158/2326-6066.CIR-13-0013
16. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med* (2013) 210:1695–710. doi: 10.1084/jem.20130579
17. Sharma A, Subudhi SK, Blando J, Vence L, Wargo J, Allison JP, et al. Anti-CTLA-4 Immunotherapy Does Not Deplete FOXP3(+) Regulatory T Cells (Tregs) in Human Cancers-Response. *Clin Cancer Res* (2019) 25:3469–70. doi: 10.1158/1078-0432.CCR-19-0402
18. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* (2004) 173:945–54. doi: 10.4049/jimmunol.173.2.945
19. Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett* (2004) 574:37–41. doi: 10.1016/j.febslet.2004.07.083
20. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* (2017) 355:1428–33. doi: 10.1126/science.aaf1292
21. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med* (2012) 4:127ra37. doi: 10.1126/scitranslmed.3003689
22. Sanmamed MF, Chen L. A Paradigm Shift in Cancer Immunotherapy: From Enhancement to Normalization. *Cell* (2018) 175:313–26. doi: 10.1016/j.cell.2018.09.035
23. Ahn E, Araki K, Hashimoto M, Li W, Riley JL, Cheung J, et al. Role of PD-1 during effector CD8 T cell differentiation. *Proc Natl Acad Sci U S A* (2018) 115:4749–54. doi: 10.1073/pnas.1718217115
24. Wei SC, Duffy CR, Allison JP. Fundamental Mechanisms of Immune Checkpoint Blockade Therapy. *Cancer Discov* (2018) 8:1069–86. doi: 10.1158/2159-8290.CD-18-0367
25. Cyster JG, Schwab SR. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. *Annu Rev Immunol* (2012) 30:69–94. doi: 10.1146/annurev-immunol-020711-075011
26. Hashimoto T, Okudaira S, Igarashi K, Hama K, Yatomi Y, Aoki J. Identification and biochemical characterization of a novel autotaxin isoform, ATXdelta, with a four-amino acid deletion. *J Biochem* (2012) 151:89–97. doi: 10.1093/jb/mvr126
27. Hausmann J, Kamtekar S, Christodoulou E, Day JE, Wu T, Fulkerson Z, et al. Structural basis of substrate discrimination and integrin binding by autotaxin. *Nat Struct Mol Biol* (2011) 18:198–204. doi: 10.1038/nsmb.1980
28. Perrakis A, Moolenaar WH. Autotaxin: structure-function and signaling. *J Lipid Res* (2014) 55:1010–8. doi: 10.1194/jlr.R046391
29. Tomsig JL, Snyder AH, Berdyshev EV, Skobeleva A, Mataya C, Natarajan V, et al. Lipid phosphate phosphohydrolase type 1 (LPP1) degrades extracellular lysophosphatidic acid in vivo. *Biochem J* (2009) 419:611–8. doi: 10.1042/BJ20081888
30. Heng TS, Painter MW, Immunological Genome Project C. The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol* (2008) 9:1091–4. doi: 10.1038/ni1008-1091
31. Bektas M, Payne SG, Liu H, Goparaju S, Milstien S, Spiegel S. A novel acylglycerol kinase that produces lysophosphatidic acid modulates cross talk with EGFR in prostate cancer cells. *J Cell Biol* (2005) 169:801–11. doi: 10.1083/jcb.200407123
32. Kalari S, Zhao Y, Spannhake EW, Berdyshev EV, Natarajan V. Role of acylglycerol kinase in LPA-induced IL-8 secretion and transactivation of epidermal growth factor-receptor in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* (2009) 296:L328–36. doi: 10.1152/ajplung.90431.2008
33. Kano K, Matsumoto H, Inoue A, Yukiura H, Kanai M, Chun J, et al. Molecular mechanism of lysophosphatidic acid-induced hypertensive response. *Sci Rep* (2019) 9:2662. doi: 10.1038/s41598-019-39041-4
34. Leblanc R, Houssin A, Peyruchaud O. Platelets, autotaxin and lysophosphatidic acid signalling: win-win factors for cancer metastasis. *Br J Pharmacol* (2018) 175:3100–10. doi: 10.1111/bph.14362
35. Smyth SS, Cheng HY, Miriyala S, Panchatcharam M, Morris AJ. Roles of lysophosphatidic acid in cardiovascular physiology and disease. *Biochim Biophys Acta* (2008) 1781:563–70. doi: 10.1016/j.bbalip.2008.05.008
36. Tokumura A, Kanaya Y, Miyake M, Yamano S, Irahara M, Fukuzawa K. Increased production of bioactive lysophosphatidic acid by serum lysophospholipase D in human pregnancy. *Biol Reprod* (2002) 67:1386–92. doi: 10.1095/biolreprod.102.004051
37. Hosogaya S, Yatomi Y, Nakamura K, Ohkawa R, Okubo S, Yokota H, et al. Measurement of plasma lysophosphatidic acid concentration in healthy subjects: strong correlation with lysophospholipase D activity. *Ann Clin Biochem* (2008) 45:364–8. doi: 10.1258/acb.2008.007242
38. Benesch MG, Zhao YY, Curtis JM, McMullen TP, Brindley DN. Regulation of autotaxin expression and secretion by lysophosphatidate and sphingosine 1-phosphate. *J Lipid Res* (2015) 56:1134–44. doi: 10.1194/jlr.M057661
39. Larouche J, Sheoran S, Maruyama K, Martino MM. Immune Regulation of Skin Wound Healing: Mechanisms and Novel Therapeutic Targets. *Adv Wound Care (New Rochelle)* (2018) 7:209–31. doi: 10.1089/wound.2017.0761
40. Rossaint J, Margraf A, Zarbock A. Role of Platelets in Leukocyte Recruitment and Resolution of Inflammation. *Front Immunol* (2018) 9:2712. doi: 10.3389/fimmu.2018.02712
41. Fulkerson Z, Wu T, Sunkara M, Kooi CV, Morris AJ, Smyth SS. Binding of autotaxin to integrins localizes lysophosphatidic acid production to platelets and mammalian cells. *J Biol Chem* (2011) 286:34654–63. doi: 10.1074/jbc.M111.276725
42. Wilgus TA, Roy S, McDaniel JC. Neutrophils and Wound Repair: Positive Actions and Negative Reactions. *Adv Wound Care (New Rochelle)* (2013) 2:379–88. doi: 10.1089/wound.2012.0383
43. Su Y, Richmond A. Chemokine Regulation of Neutrophil Infiltration of Skin Wounds. *Adv Wound Care (New Rochelle)* (2015) 4:631–40. doi: 10.1089/wound.2014.0559
44. Davis PA, Corless DJ, Aspinall R, Wastell C. Effect of CD4(+) and CD8(+) cell depletion on wound healing. *Br J Surg* (2001) 88:298–304. doi: 10.1046/j.1365-2168.2001.01665.x

45. Piazza GA, Ritter JL, Baracka CA. Lysophosphatidic acid induction of transforming growth factors alpha and beta: modulation of proliferation and differentiation in cultured human keratinocytes and mouse skin. *Exp Cell Res* (1995) 216:51–64. doi: 10.1006/excr.1995.1007
46. Gerrard JM, Clawson CC, White JG. Lysophosphatidic acids: III. Enhancement of neutrophil chemotaxis. *Am J Pathol* (1980) 100:609–18.
47. Ray R, Rai V. Lysophosphatidic acid converts monocytes into macrophages in both mice and humans. *Blood* (2017) 129:1177–83. doi: 10.1182/blood-2016-10-743757
48. Oda SK, Strauch P, Fujiwara Y, Al-Shami A, Oravecz T, Tigyi G, et al. Lysophosphatidic acid inhibits CD8 T cell activation and control of tumor progression. *Cancer Immunol Res* (2013) 1:245–55. doi: 10.1158/2326-6066.CIR-13-0043-T
49. Mathew D, Kremer KN, Strauch P, Tigyi G, Pelanda R, Torres RM. LPA5 Is an Inhibitory Receptor That Suppresses CD8 T-Cell Cytotoxic Function via Disruption of Early TCR Signaling. *Front Immunol* (2019) 10:1159. doi: 10.3389/fimmu.2019.01159
50. Rivera-Lopez CM, Tucker AL, Lynch KR. Lysophosphatidic acid (LPA) and angiogenesis. *Angiogenesis* (2008) 11:301–10. doi: 10.1007/s10456-008-9113-5
51. Sauer B, Vogler R, Zimmermann K, Fujii M, Anzano MB, Schafer-Korting M, et al. Lysophosphatidic acid interacts with transforming growth factor-beta signaling to mediate keratinocyte growth arrest and chemotaxis. *J Invest Dermatol* (2004) 123:840–9. doi: 10.1111/j.0022-202X.2004.23458.x
52. Kalucka J, Bierhansl L, Wielockx B, Carmeliet P, Eelen G. Interaction of endothelial cells with macrophages-linking molecular and metabolic signaling. *Pflugers Arch* (2017) 469:473–83. doi: 10.1007/s00424-017-1946-6
53. Balazs L, Okolicany J, Ferrebee M, Tolley B, Tigyi G. Topical application of the phospholipid growth factor lysophosphatidic acid promotes wound healing in vivo. *Am J Physiol Regul Integr Comp Physiol* (2001) 280:R466–72. doi: 10.1152/ajpregu.2001.280.2.R466
54. Demoyer JS, Skalak TC, Durieux ME. Lysophosphatidic acid enhances healing of acute cutaneous wounds in the mouse. *Wound Repair Regener* (2000) 8:530–7. doi: 10.1046/j.1524-475x.2000.00530.x
55. Rhim JH, Jang IS, Kwon ST, Song KY, Yeo EJ, Park SC. Activation of wound healing in aged rats by altering the cellular mitogenic potential. *J Gerontol A Biol Sci Med Sci* (2010) 65:704–11. doi: 10.1093/gerona/glq065
56. Dvorak HF. Tumors: wounds that do not heal-redux. *Cancer Immunol Res* (2015) 3:1–11. doi: 10.1158/2326-6066.CIR-14-0209
57. Benoy IH, Salgado R, Van Dam P, Geboers K, Van Marck E, Scharpe S, et al. Increased serum interleukin-8 in patients with early and metastatic breast cancer correlates with early dissemination and survival. *Clin Cancer Res* (2004) 10:7157–62. doi: 10.1158/1078-0432.CCR-04-0812
58. Cho YA, Sung MK, Yeon JY, Ro J, Kim J. Prognostic role of interleukin-6, interleukin-8, and leptin levels according to breast cancer subtype. *Cancer Res Treat* (2013) 45:210–9. doi: 10.4143/crt.2013.45.3.210
59. Dethlefsen C, Hojfeldt G, Hojman P. The role of intratumoral and systemic IL-6 in breast cancer. *Breast Cancer Res Treat* (2013) 138:657–64. doi: 10.1007/s10549-013-2488-z
60. Yang K, Zheng D, Deng X, Bai L, Xu Y, Cong YS. Lysophosphatidic acid activates telomerase in ovarian cancer cells through hypoxia-inducible factor-1alpha and the PI3K pathway. *J Cell Biochem* (2008) 105:1194–201. doi: 10.1002/jcb.21919
61. Seo EJ, Kwon YW, Jang IH, Kim DK, Lee SI, Choi EJ, et al. Autotaxin Regulates Maintenance of Ovarian Cancer Stem Cells through Lysophosphatidic Acid-Mediated Autocrine Mechanism. *Stem Cells* (2016) 34:551–64. doi: 10.1002/stem.2279
62. Cao L, Zhang Y, Fu Z, Dong L, Yang S, Meng W, et al. Diagnostic value of plasma lysophosphatidic acid levels in ovarian cancer patients: A case-control study and updated meta-analysis. *J Obstet Gynaecol Res* (2015) 41:1951–8. doi: 10.1111/jog.12806
63. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
64. Leve F, Peres-Moreira RJ, Binato R, Abdelhay E, Morgado-Diaz JA. LPA Induces Colon Cancer Cell Proliferation through a Cooperation between the ROCK and STAT-3 Pathways. *PloS One* (2015) 10:e0139094. doi: 10.1371/journal.pone.0139094
65. Shida D, Kitayama J, Yamaguchi H, Okaji Y, Tsuno NH, Watanabe T, et al. Lysophosphatidic acid (LPA) enhances the metastatic potential of human colon carcinoma DLD1 cells through LPA1. *Cancer Res* (2003) 63:1706–11.
66. Nam SW, Clair T, Kim YS, McMarlin A, Schiffmann E, Liotta LA, et al. Autotaxin (NPP-2), a metastasis-enhancing motogen, is an angiogenic factor. *Cancer Res* (2001) 61:6938–44.
67. Brindley DN, Lin FT, Tigyi GJ. Role of the autotaxin-lysophosphatidate axis in cancer resistance to chemotherapy and radiotherapy. *Biochim Biophys Acta* (2013) 1831:74–85. doi: 10.1016/j.bbalip.2012.08.015
68. Mukherjee A, Wu J, Barbour S, Fang X. Lysophosphatidic acid activates lipogenic pathways and de novo lipid synthesis in ovarian cancer cells. *J Biol Chem* (2012) 287:24990–5000. doi: 10.1074/jbc.M112.340083
69. Wahli W, Michalik L. PPARs at the crossroads of lipid signaling and inflammation. *Trends Endocrinol Metab* (2012) 23:351–63. doi: 10.1016/j.tem.2012.05.001
70. Hu J, Oda SK, Shotts K, Donovan EE, Strauch P, Pujanauski LM, et al. Lysophosphatidic acid receptor 5 inhibits B cell antigen receptor signaling and antibody response. *J Immunol* (2014) 193:85–95. doi: 10.4049/jimmunol.1300429
71. Lyubchenko TA, Wurth GA, Zweifach A. Role of calcium influx in cytotoxic T lymphocyte lytic granule exocytosis during target cell killing. *Immunity* (2001) 15:847–59. doi: 10.1016/S1074-7613(01)00233-3
72. Pores-Fernando AT, Zweifach A. Calcium influx and signaling in cytotoxic T-lymphocyte lytic granule exocytosis. *Immunol Rev* (2009) 231:160–73. doi: 10.1111/j.1600-065X.2009.00809.x
73. Robertson LK, Mireau LR, Ostergaard HL. A role for phosphatidylinositol 3-kinase in TCR-stimulated ERK activation leading to paxillin phosphorylation and CTL degranulation. *J Immunol* (2005) 175:8138–45. doi: 10.4049/jimmunol.175.12.8138
74. Takayama H, Sitkovsky MV. Antigen receptor-regulated exocytosis in cytotoxic T lymphocytes. *J Exp Med* (1987) 166:725–43. doi: 10.1084/jem.166.3.725
75. Blake SJ, Dougall WC, Miles JJ, Teng MW, Smyth MJ. Molecular Pathways: Targeting CD96 and TIGIT for Cancer Immunotherapy. *Clin Cancer Res* (2016) 22:5183–8. doi: 10.1158/1078-0432.CCR-16-0933
76. Zhu Y, Yao S, Chen L. Cell surface signaling molecules in the control of immune responses: a tide model. *Immunity* (2011) 34:466–78. doi: 10.1016/j.immuni.2011.04.008
77. Lichtenegger FS, Rothe M, Schnorfeil FM, Deiser K, Krupka C, Augsberger C, et al. Targeting LAG-3 and PD-1 to Enhance T Cell Activation by Antigen-Presenting Cells. *Front Immunol* (2018) 9:385. doi: 10.3389/fimmu.2018.00385
78. Panther E, Idzko M, Corinti S, Ferrari D, Herouy Y, Mockenhaupt M, et al. The influence of lysophosphatidic acid on the functions of human dendritic cells. *J Immunol* (2002) 169:4129–35. doi: 10.4049/jimmunol.169.8.4129
79. Chawla A. Control of macrophage activation and function by PPARs. *Circ Res* (2010) 106:1559–69. doi: 10.1161/CIRCRESAHA.110.216523
80. Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. *Expert Rev Mol Med* (2011) 13:e23. doi: 10.1017/S1462399411001943
83. Tazzyman S, Lewis CE, Murdoch C. Neutrophils: key mediators of tumour angiogenesis. *Int J Exp Pathol* (2009) 90:222–31. doi: 10.1111/j.1365-2613.2009.00641.x
82. Knowlden SA, Capece T, Popovic M, Chapman TJ, Rezaee F, Kim M, et al. Regulation of T cell motility in vitro and in vivo by LPA and LPA2. *PloS One* (2014) 9:e101655. doi: 10.1371/journal.pone.0101655
84. Rubenfeld J, Guo J, Sookrung N, Chen R, Chaicumpa W, Casolaro V, et al. Lysophosphatidic acid enhances interleukin-13 gene expression and promoter activity in T cells. *Am J Physiol Lung Cell Mol Physiol* (2006) 290:L66–74. doi: 10.1152/ajplung.00473.2004
85. Suzuki A, Leland P, Joshi BH, Puri RK. Targeting of IL-4 and IL-13 receptors for cancer therapy. *Cytokine* (2015) 75:79–88. doi: 10.1016/j.cyto.2015.05.026
81. Lagadari M, Truta-Feles K, Lehmann K, Berod L, Ziemer M, Idzko M, et al. Lysophosphatidic acid inhibits the cytotoxic activity of NK cells: involvement of Gs protein-mediated signaling. *Int Immunol* (2009) 21:667–77. doi: 10.1093/intimm/dxp035

86. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, von der Maase H. Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. *J Clin Oncol* (2009) 27:4709–17. doi: 10.1200/JCO.2008.18.9498
87. Jensen TO, Schmidt H, Moller HJ, Donskov F, Hoyer M, Sjoegren P, et al. Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. *Cancer* (2012) 118:2476–85. doi: 10.1002/cncr.26511
88. Zou JM, Qin J, Li YC, Wang Y, Li D, Shu Y, et al. IL-35 induces N2 phenotype of neutrophils to promote tumor growth. *Oncotarget* (2017) 8:33501–14. doi: 10.18632/oncotarget.16819
89. Zhang F, Wang H, Wang X, Jiang G, Liu H, Zhang G, et al. TGF-beta induces M2-like macrophage polarization via SNAIL-mediated suppression of a pro-inflammatory phenotype. *Oncotarget* (2016) 7:52294–306. doi: 10.18632/oncotarget.10561

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