NEW THERAPIES AND IMMUNOLOGICAL FINDINGS IN MELANOMA AND OTHER SKIN CANCERS

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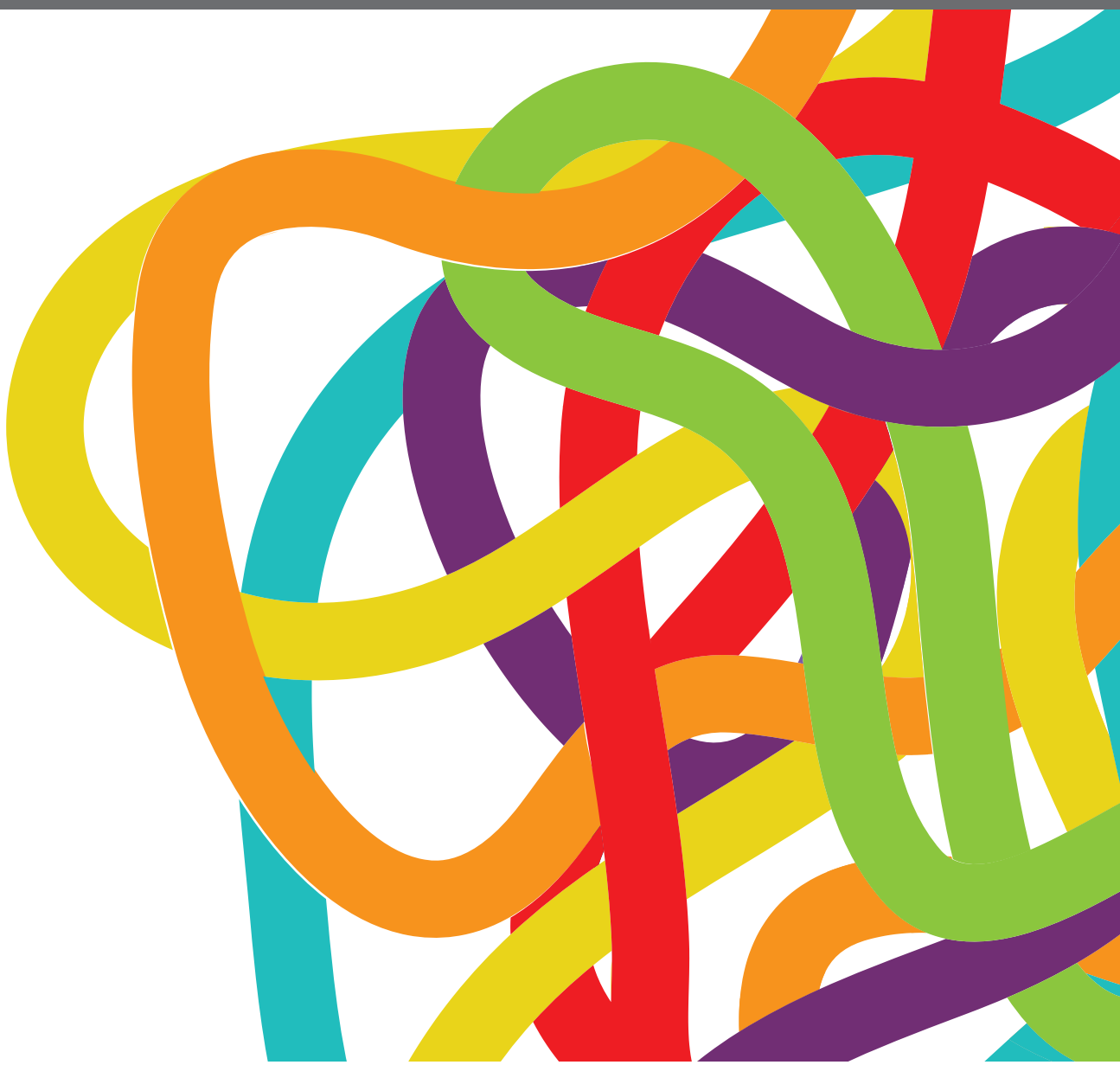

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NEW THERAPIES AND IMMUNOLOGICAL FINDINGS IN MELANOMA AND OTHER SKIN CANCERS

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REVIEW
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Biomarkers for Immune Checkpoint Inhibitors in Melanoma

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Immune checkpoint inhibitors have now become a standard therapy for malignant melanoma. However, as immunotherapies are effective in only a limited number of patients, biomarker development remains one of the most important clinical challenges. Biomarkers predicting clinical benefit facilitate appropriate selection of individualized treatments for patients and maximize clinical benefits. Many biomarkers derived from tumors and peripheral blood components have recently been reported, mainly in retrospective settings. This review summarizes the recent findings of biomarker studies for predicting the clinical benefits of immunotherapies in melanoma patients. Taking into account the complex interactions between the immune system and various cancers, it would be difficult for only one biomarker to predict clinical benefits in all patients. Many efforts to discover candidate biomarkers are currently ongoing. In the future, verification, by means of a prospective study, may allow some of these candidates to be combined into a scoring system based on bioinformatics technology.

Keywords: biomarker, immune checkpoint inhibitor, malignant melanoma, cytotoxic T-lymphocyte-associated antigen 4, programmed death-1

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INTRODUCTION

In recent years, immune checkpoint inhibitors have increasingly been applied to the clinical development of cancer immunotherapy. For malignant melanoma, ipilimumab, a humanized monoclonal antibody (mAb) that blocks cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and nivolumab, as well as pembrolizumab, a humanized mAb that blocks programmed death-1 (PD-1) on primed T cells, have been approved and are now used as standard therapies. Several clinical trials have investigated new agents, alone and in combination, for use in the treatment of advanced malignant melanoma. However, immunotherapies are effective in only a limited number of patients and severe immune-related adverse events (irAEs) develop in some patients. Biomarkers predicting clinical benefit support appropriate the selection of individualized treatments for patients and maximize clinical benefits. Thus, one of the most important tasks for advancing this form of therapy is to identify "baseline (pretreatment)" biomarkers predicting responses or toxicities. In general, biomarkers are mainly divided into two functional categories, "prognostic" and "predictive." A prognostic biomarker can be defined based on the effects of patient or tumor biology on the patient's clinical outcome. This includes patients at high risk for disease relapse who may thus derive benefit from earlier treatments. On the other hand, a predictive biomarker is defined by the effects of treatment, including tumor response and improvements in overall survival (OS), disease-free survival (DFS), and progression-free survival (PFS). Many biomarker candidates have been identified, to date, in retrospective settings. This review summarizes recent findings of biomarker studies designed to identify means of predicting the clinical benefits of immunotherapies in melanoma patients, focusing on three categories: tumor tissue, peripheral blood, and others (**Table 1**).

TABLE 1 | Biomarkers for metastatic melanoma patients treated with immune checkpoint inhibitor therapy.

Tumor (microenvironment)	Reference
Immunohistochemistry (IHC)	
Programmed death-ligand 1 (PD-L1) expression on tumor cells	(1–4)
PD-L1 expression on immune cells	(5–7)
Programmed death-1 expression on T cells	(25)
Infiltration of $CD8^+$ cells	(37–41)
Infiltration of $CD4^+$ cells	(40)
Regulatory T cells (Tregs)	(29, 30)
Myeloid-derived suppressor cells (MDSC)	(31–35)
Tumor-associated macrophages (M2)	(36)
Gene profiling (expression/mutation/amplification)	
Tumor mutation burden	(9–14)
Number of somatic mutations (non-synonymous mutations)	(10, 11, 15–19)
Activation of IFN-γ signaling	(21, 22)
Amplification of WNT/β-catenine signaling	(23)
Janus kinase (JAK) 1/JAK2 loss-of-function mutations	(25, 26)
Peripheral blood	
Number of lymphocytes	(42, 43)
Number of Tregs	(38, 44, 45)
Number of MDSCs	(46–50)
Number of proliferating $CD8^+$ T cells	(52–57)
Number of memory $CD4^+$ T cells	(58–60)
Concentrations of cytokines (e.g., IL-6, IL-8, IL-10, and TGF-β)	(62–64)
Concentration of VEGF	(66)
PD-L1 expression on circulating tumor cells	(8)
Soluble PD-L1	(67)
Others	
Microbiome	(68–70)
Fatty acids	(71)
Vitiligo and rash	(72–75)

BIOMARKERS IN TUMOR TISSUE

PD-L1 Expression on Tumor Cells

Programmed death-ligand 1 (PD-L1) expression has been investigated as a potential biomarker for PD-1 or the PD-L1 inhibitor. In phase I trials, PD-L1 expression on tumor cells correlated with the response to anti-PD-1 antibody (1). Given these promising results, several companies developed PD-L1 companion diagnostic tests for anti-PD-1/PD-L1 antibody and patients with PD-L1-positive tumors were considered to be good candidates for anti-PD-1/PD-L1 antibody treatment. In fact, the U.S. Food and Drug Administration has approved pembrolizumab, an anti-PD-1 antibody, for the treatment of PD-L1-positive non-small cell lung cancer (NSCLC) and gastric cancer. However, there are several problems while using PD-L1 expression as a biomarker for immunotherapy. First, PD-L1 expression levels show heterogeneity within tumors (2). Second, PD-L1 is a dynamic marker that can be affected by treatment and local inflammation (3). Third, the optimal threshold level of PD-L1 expression remains uncertain (4). In fact, some PD-L1 negative patients also derive benefit from treatment with an anti-PD-1/PD-L1 inhibitor.

Interestingly, PD-L1 expression on tumor infiltrating immune cells may be more predictive of responsiveness to anti-PD-1 antibody than the level of PD-L1 expression by the tumor (5). Furthermore, while PD-L1 expression on tumor cells did not tend to be related to the response rate in melanoma patients treated with anti-PD-1 antibody (nivolumab) and anti-CTLA-4-antibody (ipilimumab), there was a correlation with a good response in non-small lung cancer patients treated with these drugs (6, 7). On the other hand, Schott et al. reported that PD-L1 expression on "circulating" tumor cells might also be a potential biomarker (8). They suggested circulating tumor cells to possibly be precursors of metastatic disease, with PD-L1 expression allowing stratification according to the anticipated response to therapy. Further study is needed to determine the clinical significance of PD-LI expression.

Genes: Mutation-Burden and Gene-Expression

Melanoma is characterized by having one of the highest mutation burdens of any cancer (9, 10). These somatic mutations generate immunogenic-neoantigens recognized as tumor-antigens, possibly triggering effective anti-tumor immune responses (11–13). Genomic analysis revealed that a high mutational load at baseline may predict better survival but not treatment responses (13), and the mutation burden after PD-1 therapy was reportedly decreased in melanoma patients who responded to treatment (14).

Genes harboring significant mutations included *BRAF*, *CDKN2A*, *NRAS*, *PTEN*, and *TP53* in cutaneous melanoma, *BRAF*, *NRAS*, *NF1*, and *KIT* in acral melanoma (hands and feet), and *SF3B1* in mucosal melanoma (internal body surfaces) (15–17). The *BRAF* mutation was the most common, being detected in approximately half of metastatic melanoma patients. In the current treatment of melanoma, only *BRAF* V600 mutations are regarded as being molecular markers applicable to treatment decision-making strategies (10, 18). Several studies of CTLA-4 and PD-1 therapy have revealed that *BRAF* V600E mutations do not correlate with either the response to CTLA-4 therapy or the resulting OS, whereas the correlation with the response of melanomas to PD-1 therapy was significant (11, 19). On the other hand, inactivation of *CDKN2A* and/or *PTEN* is regarded as an important mechanism underlying resistance and/or durable responses to BRAF-inhibitor-based therapy, but is not currently taken into consideration in the clinical decision-making process (10).

Previous sequence studies, such as The Cancer Genome Atlas study, used exome and low-pass whole-genome sequencing (WGS). In 2017, Hayward et al. reported the first large, high-coverage WGS study of melanomas (cutaneous, acral, and mucosal subtypes), including analysis of the non-coding region. Their report showed that the number of mutations in the non-coding region was detected as a number equivalent to that in the coding region, and that the most common mutations in the non-coding region were in the *TERT* promoter upstream from the initiation codon (69% of all melanomas and 86% of cutaneous melanomas) (17). Moreover, Ishida et al. preliminarily reported a correlation between HLA-A*26 alleles and the response to anti-PD-1 (nivolumab) therapy in Japanese patients with metastatic melanoma (20). HLA accounts for some of the individual differences in antigen-specific immune responses, and might provide useful information for devising individualized immunotherapeutic regimens. The associations of these new findings with clinical responses to immunotherapies merit further investigation.

On the other hand, there have been several investigations of the gene expressions on tumor tissues, for their value in predicting responses to immune checkpoint inhibitors. Immunohistochemistry and gene profiling assays have suggested the presence of a "T-cell-inflamed tumor microenvironment," with an abundance of chemokines and an IFN-γ signature, to correlate with the clinical efficacy of immune checkpoint inhibitors in melanoma patients (21, 22). Numerous studies have revealed the molecular mechanisms underlying lack of T-cell infiltration and resistance of melanomas to immune checkpoint therapy, such as the melanoma-intrinsic active WNT/β-catenin-signaling pathway (23) and enrichment for mutations in *PTEN* (24), loss-of-function mutations in Janus kinase (JAK1)/JAK2 (which are involved in IFNγ signaling), and β2 microglobulin (an MHC class I subunit) (25, 26).

Tumor Infiltrating Lymphocytes (TILs)

Tumor infiltrating lymphocytes, such as T cells, macrophages, and various types of immune suppressive cells, are considered to be the most important players in the regulation of anti-tumor immune responses. Several studies have demonstrated an increase in the TIL number to correlate with good clinical responses and a higher survival rate of patients with melanoma and various other cancers (27, 28).

In melanoma patients, immune suppressive cells, such as regulatory T cells (Tregs) (29, 30), monocytic myeloid-derived suppressor cells (m-MDSCs) (31–35), and tumor-associated (activated) macrophages (TAM; M2) (36), were reportedly increased in number and thereby inhibited effector T cells, resulting in an increase in tumor growth.

In contrast, a number of investigators have reported the quantity of infiltrating $CD8^+CD45RO^+$ effector memory T cells to be clearly associated with longer DFS and OS, for many cancer types including melanoma (37–39). Recently, Wei et al. comprehensively profiled the effects of CTLA-4/PD-1-targeted immunotherapy on tumor infiltrating immune cells. Their study revealed that PD-1 blockade and CTLA-4 blockade both led to a subset of exhausted-like $CD8^+$ T cells ($CD45RO^+PD\text{-}1^+T\text{-}bet^+EOMES^+$). They also showed that CTLA-4 blockade induced the expansion of an $ICOS^+$ Th1-like CD4 effector population ($CD45RO^+PD\text{-}1^{lo}TBET^+$ and $CD69^+$) in melanoma patients. These observations suggested that these two immunotherapies target specific subsets of exhausted-like $CD8^+$ T cells, but drive different cellular mechanisms to induce tumor rejection (40). Moreover, Canale et al. described high expression of CD39 on $CD8^+$ infiltrating T cells as being increased in melanoma lesions. CD39 is the immunosuppressive enzyme termed ATP ectonucleotidase, and $CD39^{high}CD8^+$ T cells reportedly exhibit features of cellular exhaustion, such as reduced production of tumor necrosis factor and interleukin (IL)-2, as well as expressions of co-inhibitory receptors (41).

BIOMARKERS IN PERIFERAL BLOOD

Peripheral Blood Mononuclear Cells (PBMCs)

Blood biomarkers have most frequently been analyzed for correlations with clinical responses to immunotherapies. Baseline and/or post-treatment changes in absolute counts of white blood cells, lymphocytes, eosinophils, neutrophils, and monocytes, as well as ratios of neutrophils or monocytes to lymphocytes may both be promising and routinely available blood markers that have shown associations with responses to immune checkpoint inhibitors (11, 42, 43).

Recently, several studies have raised the possibility of circulating immune cells as predictive biomarkers for immune checkpoint inhibitors. The frequency of circulating Tregs is reportedly associated with disease progression and poor patient survival for many carcinomas treated with immunotherapy (38, 44, 45). Numerous studies have found that high levels of circulating m-MDSCs in various forms of cancer, including melanoma, correlate with poor survival (46–48). In patients treated with anti-PD-1 antibody, m-MDSCs were reported to be a blood cytology marker showing significant correlations with all outcome parameters (49, 50). However, human MDSCs have yet to be clearly characterized both biologically and phenotypically. A very recent study demonstrated that the frequency of $CD14^+CD16^-HLA\text{-}DR^{hi}$ monocytes predicts both PFS and OS of melanoma patients treated with anti-PD-1 antibody, based on analysis employing high-dimensional single-cell mass cytometry (51). This $CD14^+$ population including MDSCs might be useful as a predictive and/or prognostic biomarker for cancer patients receiving immunotherapy, but further investigation is needed to clarify the phenotype and biological characteristics of this diverse population of cells.

On the other hand, several studies examining circulating T cells have shown the involvement of $CD8^+$ T cells, such as the proliferating ($Ki67^+$) $CD8^+$ effector-like T cells, in NSCLC patients receiving PD-1-therapy (52), and neoantigen-specific circulating $CD8^+$ T cells in melanoma (53, 54). The latter are $CD8^+$ T cells expressing PD-1. In addition, two complementary reports showed that CD28, a member of the same family as PD-1 (including CTLA-4 and ICOS), expressed on $CD8^+$ T cells is a key molecule in PD-1-targeted therapy (55). Hui et al. showed that "CD28 is the primary target of PD-1 signaling," using a cell-free membrane reconstitution system. Their report revealed that PD-1 was phosphorylated in response to PD-L1 ligation, thereby preferentially inducing dephosphorylation of CD28 (but not the T cell receptor), resulting in the inhibition of T cell proliferation (56). On the other hand, Kamphorst et al. found that, in lung cancer patients, proliferating $Ki67^+PD\text{-}1^+CD8^+$ T cells were increased in peripheral blood, and subsequently activated ($CD38^+$, $HLA\text{-}DR^+$) and mostly expressed CD28 (57), implying that CD28 signaling is associated with rescue of the exhausted $CD8^+$ T cells in PD-1 targeted therapies. These findings are reasonable and it is interesting that CD28, belonging to the same family as PD-1, is a key molecule in PD-1-targeted therapy, although its applicability as a predictive/prognostic biomarker in melanoma patients is as yet unclear. Moreover, whether other family members, including CTLA-4 and ICOS, have similar features in immune checkpoint therapy, remains unknown. Elucidating these issues might reveal novel useful biomarkers for use alone and/or in combination with PD-1-targeted therapy. Another interesting, and potentially important, finding of these studies is that proliferating $CD8^+$

effector-like T cells were reportedly increased following PD-1-targeted therapy.

Several recent studies, focusing on circulating $CD4^+$ T cells, found that increases in central memory $CD4^+$ T cells ($CD27^+$, FAS^-, $CD45RA^-$, and $CCR7^+$) (58), and IL-9-producing $CD4^+$ T helper (Th9) cells (59), correlated with good clinical responses of melanoma patients to anti-PD-1 therapy. Moreover, in lung cancer patients treated with nivolumab, the frequencies of $CD62L^{low}CD4^+$ T cells and Tregs ($CD25^+Foxp3^+CD4^+$) in pre-treatment PBMC were reported to correlate significantly with clinical responses (60). Their ASCO presentation outlined the major differences in pre-existing immunity, among patients showing a partial response, stable disease, or progressive disease, in response to anti-PD-1 Ab, as reflected by the status of $CD4^+$ T cells, i.e., the balance between primed effector and Tregs. These recent reports raised the possibility that, in peripheral blood, not only T cell exhaustion but also activation of effector $CD8^+$ T cells and increases in memory T cells appear to be highly important, and not only phenotyping markers but also functional molecules can serve important roles as prognostic and/or predictive factors for immune checkpoint inhibitors. Although peripheral blood analysis may provide valuable insights into the responses of cancer patients to immune checkpoint inhibitors, more investigation is needed before these biomarkers can be applied in clinical settings.

OTHERS

Soluble Factors (Serum/Circulating Factors)

Lactate dehydrogenase was frequently investigated in previous studies and showed significant correlations with OS and PFS, whereas there were no correlations with responses to treatments (61). Recently, several studies have revealed that serum cytokine levels to correlate with responses to immune checkpoint inhibitors. Sanmamed et al. showed serum IL-8 levels to be highly correlated with tumor burden changes in metastatic melanoma and NSCLC patients during treatment with anti-PD-1/anti-CTLA-4 therapy (62, 63), and Yamazaki et al. reported that pretreatment serum IFN-γ, IL-6, and IL-10 levels were significantly higher in those with tumor progression among patients with advanced melanoma given nivolumab (64). In addition, in patients with metastatic melanoma receiving nivolumab, the activity of soluble CD73, which is an enzyme that hydrolyzes extracellular AMP to adenosine, in blood was shown to be significantly associated with clinical outcomes (65). Moreover, Frankhauser et al., studying metastatic melanoma patients, reported gene expression of vascular endothelial growth factor-C (VEGF-C) to correlate markedly with both CCL21 and T cell inflammation, and that serum VEGF-C concentrations were associated with both T cell activation/expansion and clinical responses to checkpoint blockade (66).

Soluble PD-L1 (sPD-L1)

Pretreatment sPD-L1 levels reportedly correlate with progression of advanced melanoma treated with anti-CTLA-4 or anti-PD-1 antibody. Although changes in circulating sPD-L1 in the early phase after starting treatment did not distinguish responders from non-responders, patients who had increased circulating sPD-L1 after 5 months of treatment tended to show partial responses (67). The biology of sPD-L1 remains unclear and merits further research.

Microbiome

A vast number of microbes colonize the human body. This colonization is associated with many diseases, including various malignancies. During the past decade, the advent of metagenomic sequencing that combines next-generation DNA sequencing technologies with computational analyses has allowed us to analyze the relationships between the microbiome and various cancers. Recent studies have suggested that the gut microbiome may affect the efficacy of immune checkpoint inhibitors and, consequently, that changing the gut microbiome of a mouse or even a human patient might make tumors more responsive to immune checkpoint inhibitors. This possibility was first evaluated using preclinical models. Vétizou et al. showed that the efficacy of anti-CTLA-4 therapy was diminished in a germ-free mouse model. In addition, the use of broad-spectrum antibiotics to eliminate gut microbiota altered the anti-tumor effect of anti-CTLA-4 therapy (68). Sivan et al. reported that *Bifidobacterium* counts decreased in parallel with the anti-tumor effects of anti-PD-L1 therapy in a mouse model (69). Furthermore, Gopalakrishnan et al. indicated that anti-PD-1 immunotherapy in melanoma patients may be modulated by the gut microbiome. These researchers reported significantly higher alpha diversity and a relative abundance of Ruminococcaceae bacteria in the gut microbiome of responders (70). These findings indicated that specific organisms comprising the gut microbiome enhanced anti-tumor responses in patients treated with immune checkpoint inhibitors. Although the gut microbiome is a potential predictive marker of immunotherapy, a larger prospective study is needed to confirm these results.

Fatty Acids

Kim et al. investigated cellular metabolome and lipidome alterations related to melanoma metastasis. Their analysis showed a progressive increase in phosphatidylinositol species with saturated and monounsaturated fatty acyl chains, as the metastatic potential of the melanoma cells rose, highlighting these lipids as possible biomarkers (71).

Vitiligo and Rash

Immune checkpoint inhibitors have a rather unique adverse event profile, generally described as irAEs, which are most commonly observed in the skin, the gastrointestinal tract, the lungs, the liver, endocrine system, and other organs. Cutaneous irAEs are much more common adverse events in patients with melanoma than in those with other solid tumors. Although vitiligo is attributed to an autoantibody to melanocytes, the etiology of vitiligo is not understood in detail. Vitiligo occurrence has long been speculated to be related to tumor shrinkage in melanoma patients (72). Vitiligo develops in 13–26% of patients treated with nivolumab (73, 74), though grade III/IV disease is rare.

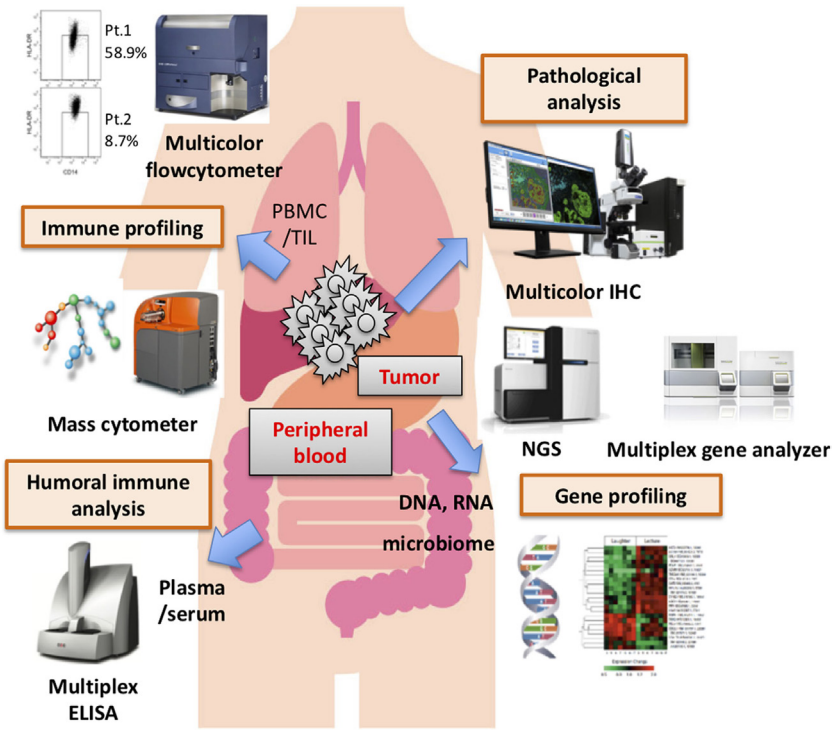

FIGURE 1 | Various assay systems for identifying biomarkers. Several biomarkers derived from the tumor microenvironment, peripheral blood biology, and other factors have been proposed as distinct biomarkers of responses to immune checkpoint blockade therapy. Recently, there have been innovative advancements in assay technology that have made it possible to comprehensively profile the biology and phenotype of the tumor-microenvironment, peripheral blood, and other factors. It would be very difficult, however, for a single biomarker to predict clinical responses and/or serve as a patient selection criterion, though multifactorial biomarkers including these and other novel findings might have great value for predicting clinical responses and/or patient prognosis.

Recent studies have shown vitiligo and rash to be associated with a significant OS improvement in metastatic melanoma patients treated with immune checkpoint inhibitors (73–75). Furthermore, Nakamura et al. suggested that the occurrence of vitiligo might not be regarded as an early marker of good clinical response because the mean time to vitiligo occurrence was approximately 5 months after starting nivolumab (73). The onset times of vitiligo vary considerably depending on the type of drug administered and patient features. Thus, when we use cutaneous irAEs as a biomarker for immune checkpoint inhibitors, we should take into consideration the characteristics of each drug.

CONCLUSION

Numerous candidate biomarkers are currently the focus of research, based mainly on retrospective analyses. Most notably, tumor mutation burden, intratumoral or immune cell expressions of PD-L1, and $CD8^+$ T cell infiltration into the tumor have been documented in several cohorts. For example, not only melanoma but also lung carcinoma, one of the carcinomas which also has a high mutation burden, shows good clinical responses to PD-1/PD-L1 therapy. In lung carcinoma, mutation burden, TIL accumulation, and/or PD-L1 expression on tumor cells correlated with good clinical responses. However, renal cell carcinoma is also reportedly responsive to PD-1 therapy, despite having a low mutation burden, while TIL accumulation and PD-L1 expression did not correlate with treatment effectiveness. These observations suggest that these factors are not always applicable to predicting clinical benefits. Taking into account the complex interactions between the immune system and malignancies *via* cell surface molecules, such as immune checkpoint molecules, humoral factors, including proteins, cytokines, and so on, it is not unreasonable to speculate that a single biomarker would not allow clinical benefits to be predicted in all patients. In the near future, by applying bioinformatics technology, several biomarkers might be combined to produce a useful scoring system, depending on the type of cancer, the stage, individual treatments, and the timing of intervention. Recent advancements in assay technology, such as mass cytometry (CyTOF), multicolor IHC, multiplex gene analyzer, and so on (**Figure 1**), have the potential to provide an abundance of biological and/or phenotypical observations in a range of environments. Now is the time to discover the candidate biomarkers which might comprise such a future scoring system. Finally, needless to say, a prospective study on a large patient population is essential.

AUTHOR CONTRIBUTIONS

SK: conception/design of the manuscript. SK, TN, and MY: writing of the manuscript.

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REVIEW
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New Therapies and Immunological Findings in Cutaneous T-Cell Lymphoma

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Primary cutaneous lymphomas comprise a group of lymphatic malignancies that occur primarily in the skin. They represent the second most common form of extranodal non-Hodgkin's lymphoma and are characterized by heterogeneous clinical, histological, immunological, and molecular features. The most common type is mycosis fungoides and its leukemic variant, Sézary syndrome. Both diseases are considered T-helper cell type 2 (Th2) diseases. Not only the tumor cells but also the tumor microenvironment can promote Th2 differentiation, which is beneficial for the tumor cells because a Th1 environment enhances antitumor immune responses. This Th2-dominant milieu also underlies the infectious susceptibility of the patients. Many components, such as tumor-associated macrophages, cancer-associated fibroblasts, and dendritic cells, as well as humoral factors, such as chemokines and cytokines, establish the tumor microenvironment and can modify tumor cell migration and proliferation. Multiagent chemotherapy often induces immunosuppression, resulting in an increased risk of serious infection and poor tolerance. Therefore, overtreatment should be avoided for these types of lymphomas. Interferons have been shown to increase the time to next treatment to a greater degree than has chemotherapy. The pathogenesis and prognosis of cutaneous T-cell lymphoma (CTCL) differ markedly among the subtypes. In some aggressive subtypes of CTCLs, such as primary cutaneous gamma/delta T-cell lymphoma and primary cutaneous $CD8^+$ aggressive epidermotropic cytotoxic T-cell lymphoma, hematopoietic stem cell transplantation should be considered, whereas overtreatment should be avoided with other, favorable subtypes. Therefore, a solid understanding of the pathogenesis and immunological background of cutaneous lymphoma is required to better treat patients who are inflicted with this disease. This review summarizes the current knowledge in the field to attempt to achieve this objective.

Keywords: cutaneous T-cell lymphoma, mycosis fungoides, Sézary syndrome, primary cutaneous $CD30^+$ T-cell lymphoproliferative disorders, adult T-cell leukemia/lymphoma

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OVERVIEW OF CUTANEOUS T-CELL LYMPHOMAS (CTCLs)

Non-Hodgkin lymphomas can occur at extranodal sites in approximately 27% of cases, with the gastrointestinal tract and skin being the first and second most common sites of extranodal involvement (1). Most nodal non-Hodgkin lymphomas are B-cell derived, which is in contrast to the approximately 75–85% of primary cutaneous lymphomas that are T-cell derived (2–6). The incidence of CTCLs has been increasing (7); consequently, 4–8 people per million currently suffer from these cancers (8, 9). CTCL represents a series of skin-based neoplasms of T-cell origin,

TABLE 1 | List of primary CTCLs.

Study group	Frequency, %			Disease-specific 5-year survival[2], %
	DACLG[2]	SEER16[5]	JSCS[6]	
Mycosis fungoides	61.5	54.1	51.7	88
Sézary syndrome	3.5	1.2	2.3	24
Primary cutaneous CD30$^+$ T-cell lymphoproliferative disorders	26.0	14.4	14.3	
Lymphomatoid papulosis	16.1		4.5	100
Primary cutaneous anaplastic large-cell lymphoma	9.9		9.4	95
Adult T-cell leukemia/lymphoma[a]		0.1	20.0	
Subcutaneous panniculitis-like T-cell lymphoma	1.2	0.8	2.3	82
Primary cutaneous gamma/delta T-cell lymphoma	0.9		0.3	NR
Primary cutaneous CD4$^+$ small/medium T-cell lymphoproliferative disorder[b]	2.7		1.7	75
Hydroa vacciniforme-like lymphoproliferative disorder[b]				
Primary cutaneous acral CD8$^+$ T-cell lymphoma[b]				
Primary cutaneous CD8$^+$ aggressive epidermotropic cytotoxic T-cell lymphoma	1.0		0.4	18
Peripheral T-cell lymphoma, NOS[a]	3.2	29.4	6.9	16
Total no. of CTCL	1,469	2,750	1,451	

NR, not reached; CTCL, cutaneous T-cell lymphoma.

[a]A portion of these diseases is considered as primary CTCL.

[b]Provisional entity in World Health Organization classification (2016).

predominantly comprised of peripheral CD4$^+$ T-cells. There are 12 distinct CTCL subtypes (**Table 1**), with mycosis fungoides (MF) being the most common (10). Primary cutaneous CD30$^+$ T-cell lymphoproliferative disorders are the second most common, except in some countries in the Pacific where adult T-cell leukemia/lymphoma (ATL) ranks second (6, 11).

MF/SÉZARY SYNDROME (SS)

Mycosis fungoides and SS constitute the most common types of primary CTCLs. MF is characterized by erythematous patches, plaques, or tumors on the skin (**Figure 1**), with the involvement of lymph nodes, blood, and viscera also possible. MF can mimic benign inflammatory skin disorders, such as atopic dermatitis or psoriasis; thus, it is not unusual for MF to remain undiagnosed for years. Although MF is typically an indolent disorder, the disease may progress toward or exhibit *de novo* more advanced forms including tumors and erythroderma (>80% of the body surface area showing patches/plaques without overt leukemia). This can lead to lymph node or organ involvement, accompanied by increased morbidity and mortality. Patients are classified as having either early-stage (patches/plaques) or advanced-stage (tumors, erythroderma, lymph node, and/or visceral involvement) (12, 13). SS is the leukemic form of the disease, in which erythroderma is accompanied by measurable levels of malignant lymphocytes with cerebriform nuclei [i.e., Sézary cells (SC)] in the blood. Typical SC counts would be ≥1,000/μL, with a CD4/CD8 ratio of ≥10 and a loss of one or more T-cell antigens (CD4$^+$CD7$^-$ > 30% or CD4$^+$CD26$^-$ > 40%). Furthermore, CD30 expression is associated with a significantly reduced disease-specific survival and is often associated with histologically detectable large cell transformation, hallmarking a more aggressive clinical course (14).

In the past, SS has been considered a leukemic and aggressive variant of MF. However, a recent study determined that MF and SS arose from distinct T-cell subsets: SS from central memory

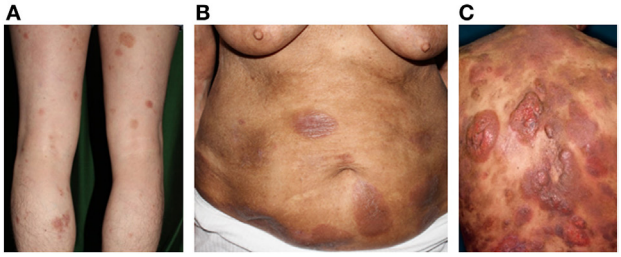

FIGURE 1 | Clinical findings of mycosis fungoides/Sézary syndrome. **(A)** Patches, **(B)** plaques, **(C)** and nodules on the plaque. Written informed consent was obtained from each patient.

T-cells and MF from skin-resident effector memory T-cells (15). CD158k/killer cell immunoglobulin-like receptor 3DL2 represents a specific marker for the evaluation of SC (16); in particular, CD4$^+$ CD158k$^+$ lymphocytes in blood from patients with SS correspond to the malignant clonal cell population (17). In addition, immunohistological finding of CD158k in affected skin is reported to distinguish SS from MF (18). Clonal malignant T-cells from the blood of patients with SS coexpress the lymph node homing molecules C–C motif chemokine receptor 7 (CCR7)/CD197 and CD62L/L-selectin, as well as the CD27 differentiation marker, a characteristic of central memory T-cells. This is consistent with the clinical presentation of peripheral blood disease, lymphadenopathy, and diffuse erythroderma of the skin. In contrast, T-cells from MF skin lesions do not express CCR7, L-selectin, and CD27, but strongly express CCR4 and cutaneous lymphocyte antigen (CLA)/CD162, characteristics of skin-resident effector memory T-cells. This difference in the putative origins between SS (central memory T-cell-derived) and MF (tissue-resident memory-derived) can explain their distinct clinical behaviors; central memory T-cells are long-lived, apoptosis-resistant cells that can be found in the peripheral blood, lymph nodes, and skin, whereas skin-resident memory T-cells remain in the skin and do not enter the general

circulation. That MF and SS are derived from different T-cell precursors is also supported by comparative genomic hybridization and gene-expression profiling, demonstrating that the CTCL genotypes are distinct (19, 20). Overall, MF is characterized by gains on chromosomes 1 and 7 and losses on chromosome 9, whereas SS is characterized by gains on chromosomes 8 and 17 and losses on chromosome 10. A multiplatform genomic analysis of patients with SS detected (1) activating *CCR4* and caspase recruitment domain-containing protein 11 (*CARD11*) mutations in nearly one-third of patients; (2) deletion of zinc finger E-box binding homeobox 1 (*ZEB1*), encoding a transcriptional repressor essential for T-cell differentiation, in over one-half of patients; and (3) overexpression of interleukin 32 (IL-32) and interleukin-2 receptor subunit gamma in nearly all patients (21).

ROLES OF CHEMOKINES IN DEVELOPMENT OF SKIN PATHOLOGY

Malignant T-cells are suggested exhibit phenotypes of mature $CD4^+$ memory T-cells, along with type 2 or 17 (Th2 or Th17 (22)) T-helper cell phenotypes, or be comprised of FOXP3 regulatory T-cells (Treg) (23, 24). Many chemokines are also reportedly expressed in the affected skin of patients with CTCL, suggesting that chemokine–receptor interactions play important roles in disease progression (25). Chemokine receptor expression on tumor cells in MF varies with disease stage. In the patch and plaque stages of MF, most infiltrating cells express CXC chemokine receptor (CXCR) 3/CD183 in the affected skin (26). CXCR3 binds three distinct ligands, namely CXC chemokine ligand (CXCL) 9/monokine induced by gamma interferon (MIG), interferon-gamma-inducible protein-10 (CXCL10/IP-10), and interferon-inducible T cell alpha chemoattractant (CXCL11/ITAC)/IP-9; all are expressed in the affected skin in the patch and plaque stages of MF (27–29). Various cell types express these chemokines including keratinocytes, dermal fibroblasts, and Langerhans cells. In the early stages of MF, expression of CXCL9, CXCL10, and CXCR3 is believed to be important for recruitment and accumulation of tumor cells in the skin (25, 28). However, later in the tumor stage, the tumor cells increase in size and tend to express CCR4 instead of CXCR3 (30). The expression levels of CXCL9 and CXCL10 also tend to be lower in the affected skin of patients with MF during the tumor stage than during the patch and plaque stages (31). Moreover, CCR6/CD196 and its ligand CCL20/macrophage inflammatory protein (MIP)-3α are upregulated in advanced CTCL (32). Tumor MF cells exhibit high levels of CCR7 (33), which is considered to be associated with loss of epidermotropism and migration to peripheral lymph nodes, which constitutively synthesize the CCR7 ligands, CCL19 and 21 (34). CCR7 is also expressed at high levels in SC (35), as mentioned in Section "MF/Sézary syndrome (SS)".

Circulating SC and skin-infiltrating cells in SS also express CCR4 (30, 35, 36). CCR4-expressing T-cells were found in CTCL lesions along with high expression of two CCR4 ligands, namely CC chemokine ligand (CCL) 17/thymus and activation-regulated chemokine and CCL22/macrophage-derived chemokine (30). CCL17 is expressed by endothelial cells and keratinocytes in the affected skin of patients with MF and SS (30, 37). During the tumor stage of MF, serum CCL17 levels are much higher than those during the patch/plaque stages (37), suggesting the importance of CCL17–CCR4 interactions in tumor cell trafficking to the skin of these patients. CCL22 is expressed by dendritic-like cells and keratinocytes (30, 37). Serum CCL22 levels are significantly higher in patients with MF than in healthy controls or patients with psoriasis vulgaris (37).

CC chemokine ligand 27/cutaneous T cell attracting chemokine is a CCR10 ligand that is constitutively produced by activated keratinocytes in various diseases (38). CCR10 is expressed on the tumor cells of MF and SS (35, 39). Strong immunostaining of CCL27 has been observed in the affected skin of patients with MF compared to that of unaffected individuals (39, 40). Serum CCL27 levels and the number of circulating $CCR10^+$ $CD4^+$ cells are both increased in patients with MF compared to that of control patients (39). Therefore, CCR10–CCL27 interactions may also contribute to the migration of lymphoma cells to the affected skin in MF and SS. In addition to CCR4 and CCR10, expression of the receptor for CXCL12/stromal cell-derived factor 1, CXCR4, is observed in SC (36). CXCL12 is a chemoattractant for CXCR4-positive cells and is strongly expressed in the affected skin of patients with MF (41) and SS (36). Therefore, CXCL12–CXCR4 interactions may also facilitate the recruitment of lymphoma cells to the skin.

Th2-DOMINANT MICROENVIRONMENT

As the microenvironment in early-stage MF consists of non-malignant Th1 cells and $CD8^+$ tumor-infiltrating T cells, MF and SS are considered Th2-type diseases, which are frequently accompanied by eosinophilia and high serum levels of IgE. In the early 1990s, peripheral blood mononuclear cells in patients with SS and non-leukemic CTCL were reported to be Th2 dominant (42, 43). In 1994, mRNA for Th2 cytokine was detected in the skin of patients with MF (44). T-cell clones from patients with SS were identified thereafter to have Th2-like properties (45). However, in the early stages of MF, affected skin and peripheral blood T-cells express a profile of Th1 cytokines (46, 47). The Th2 phenotype appears to be caused by leukemic T-cells, as culturing benign T-cells away from malignant clones reduces Th2 and enhances Th1 responses (48). The Th2-dominant microenvironment is advantageous for tumor cells, because interferon (IFN)-γ-producing Th1 cells enhance immune responses against the tumor. Indeed, IFN-γ has been shown to be effective for CTCL treatment (49, 50). Adenoviral-mediated gene therapies that increase expression of IFN-γ have also been used successfully in CTCL (51–53). CTCL cells can inhibit T-cell proliferation and suppress dendritic cell (DC) maturation by secretion of Th2 cytokines (54). Skin and nasal colonization with *Staphylococcus aureus* is common in patients with MF/SS; in particular, a Th2-dominant microenvironment may underlie this susceptibility to infection (55). Infections of *S. aureus* and sepsis also frequently occur in patients with CTCL (56). Accordingly, the major cause of death in patients with erythrodermic MF and SS is intravenous line sepsis, with *S. aureus* often being the causative microorganism (57).

In early-stage MF, signal transducers and activators of transcription (STAT) 4, the activation of which is required for Th1

differentiation, are overexpressed by IL-12 signaling *via* JAK2/TYK2 (58). In later stages, IL-2 and IL-15 signaling *via* JAK1 and JAK3 kinases activates STAT5, which increases the expression of oncogenic miR-155 (59) and subsequently inhibits STAT4 expression (60), resulting in a switch from Th1 to Th2 phenotype in malignant T cells. Downregulation of STAT4 is also induced by deficiencies in IL-12 expression (58, 61) and lack of the IL-12R β2 chain (58). During this switch, the expression of STAT6 is often upregulated in CTCL (60). STAT5 activation is seen in both early and late stages. Specifically, in the late stage, constitutive STAT5 activation is induced by cytokine-independent JAK1/JAK3 signaling (59). In the advanced stage, such constitutive STAT3 activation, which increases survival and resistance to apoptosis and promotes Th2 and Th17 phenotypes, is induced by an IL-21 autocrine signaling loop (62), the presence of IL-7 and IL-15 in the microenvironment (63), and/or constitutive cytokine-independent activation of JAK1 and JAK3 signaling (64, 65). Moreover, GATA3, a transcriptional regulator of Th2-cells, is overexpressed in SC *via* proteasome dysregulation (66).

CANCER-ASSOCIATED FIBROBLASTS

Fibroblasts are crucial components of the tumor microenvironment, promoting the growth and invasion of cancer cells through various mechanisms (67). The fibroblasts in the affected skin of patients with advanced CTCL promote a Th2-dominant microenvironment by augmenting Th2 and attenuating Th1 immune responses. Increased expression of CCL26/eotaxin-3 is observed in the dermal fibroblasts, keratinocytes, and endothelial cells of the affected skin of patients with advanced MF (68). In addition, serum CCL26 and CCL11/eotaxin-1 levels were shown to be higher in patients with CTCL than in healthy control patients, which correlated with serum soluble interleukin-2 receptor (sIL-2R) levels. However, CCR3/CD193, a receptor for CCL26 and other ligands, is not expressed on lymphoma cells in MF or SS (69). Because mRNA for CCR3 is detected in affected skin (68) and CCR3 is expressed on eosinophils and subpopulations of Th2 cells (70, 71), CCL26 and CCL11 are believed to support the Th2-dominant microenvironment in MF and SS disease lesions (25).

Periostin constitutes an extracellular matrix protein that is expressed in several cancers (72); it is prominent in the stromal area during the patch and plaque stages of MF, but decreases during the tumor stage (73). Fibroblasts are reportedly the source of periostin in MF (74). IL-4 and IL-13 can induce periostin secretion by dermal fibroblasts, periostin mediates thymic stromal protein (TSLP) production by keratinocytes, and TSLP subsequently activates immature myeloid DCs, which modulate Th2 immune responses *via* CCL17 production (75). Serum (76) and plasma (77) TSLP levels are increased in patients with CTCL, suggesting that TSLP contributes to the Th2-dominant microenvironment in MF lesions. TSLP also induces the growth of CTCL cells (74). Therefore, periostin can directly stimulate the growth of CTCL tumor cells in addition to inducing a Th2-dominant environment in CTCL tumors.

Expression of herpesvirus entry mediator (HVEM)/CD270, a member of the tumor necrosis factor-receptor superfamily, on dermal fibroblasts in the affected skin of patients with MF and SS is decreased as the disease progresses. In addition, low HVEM expression on dermal fibroblasts in the affected skin of patients with advanced CTCL attenuates the expression of Th1 chemokines, resulting in Th2-dominant microenvironments. This occurs because the interaction between HVEM and tumor necrosis factor superfamily member 14 (also termed LIGHT)/CD258 on dermal fibroblasts increases the secretion of CXCL9–11, which are chemokines that recruit CXCR3-positive Th1 cells (29).

TUMOR-ASSOCIATED MACROPHAGES (TAMs)

Macrophages constitute a major component of the leukocyte infiltrate in the tumor microenvironment (78), in which they are termed TAMs. TAMs usually comprise polarized M2 macrophages that contribute to an immune-suppressive environment and promote tumor cell growth (79). CD163 is recognized as a marker for TAMs. As with many malignancies (80), the presence of M2 macrophages in the affected skin of patients with MF has been correlated with patient prognosis (81, 82), and the presence of M2 macrophages has been correlated with lymph node staging (83); this suggests that TAMs play a significant role in MF pathogenesis. Serum sCD163 levels in patients with CTCL are significantly higher than those in normal controls and they significantly correlate with serum sIL-2R levels. TAMs are believed to play a role in the formation of CTCL by secreting various chemokines (73, 82, 84). Periostin-stimulated macrophages produce CXCL5 and CXCL10 (73), which correlates with MF tumor formation in a xenograft CTCL mouse model (84). CCL18/alternative macrophage activation-associated CC chemokine 1/MIP-4 is secreted by M2 macrophages (85) and binds to its receptor (i.e., CCR8) on Th2 cells (86). The expression of CCR8 on MF or SS tumor cells has not been reported, although the mRNA expression of CCR8 is known to be upregulated in patients with SS (21). TAMs are known to express CCL18 in the skin of patients with CTCL (87, 88). Serum CCL18 levels were significantly higher in patients with CTCL than in healthy controls, and these levels significantly correlated with modified severity-weighted assessment scores, serum sIL-2R, lactate dehydrogenase, Th2 cytokines, and chemokines (88). Furthermore, high serum levels of CCL18 were associated with poor patient prognosis (88). In the affected skin of patients with MF/SS, TAMs highly express CD30, which is the target of the anti-CD30 antibody–drug conjugate, brentuximab vedotin (89).

DENDRITIC CELLS

Dendritic cells are antigen-presenting cells with a unique capacity to induce primary immune responses (90). By secreting Th2 cytokines, CTCL cells can suppress the maturation of DCs (54). Notably, IL-10 downregulates DC functions and may promote tolerance by skin DCs, rather than immune defense (91). Immature DCs can induce tolerance by presenting antigens to T-cells without appropriate costimulation. A significant increase in various DC subsets is seen in the affected dermis of patients with MF/SS, with the majority being immature CD209/

DC-specific ICAM-3 grabbing non-integrin (DC-SIGN)-positive DCs. Increases in CD208/DC-lysosome-associated membrane glycoprotein-positive DCs (i.e., mature DCs) and CD303/blood dendritic cell antigen 2-positive DCs (i.e., plasmacytoid DCs) are also observed, but the numbers of cells expressing CD208 or CD303 are few, suggesting that many DCs in the dermis of CTCL lesions are immature. Increased number of immature DCs in CTCL lesions may be important for immunological tolerance against malignant T-cells (92). However, some CD208-positive, mature DCs may attempt to mount an immune response against the cancer cells, as mature CD208-positive DCs are elevated in the skin draining lymph nodes of patients with MF (83).

OTHER KEY PLAYERS

The keratinocytes in the affected skin of patients with MF/SS release multiple chemokines including CCL17, CCL26, CCL27, CXCL9, and CXCL10, which help to attract T-cells to the epidermis, as mentioned above. Nerve growth factor (NGF) expression is also elevated in the affected skin of patients with SS, which stimulates the sprouting of nerve fibers. NGF is associated with the severity of pruritus in atopic dermatitis (93), and serum NGF levels are elevated in patients with SS (94). The enhanced expression of NGF is supposedly associated with pruritus in SS.

Mast cells also serve as critical stimulators of the tumor microenvironment (95). Patients with CTCL have increased number of mast cells in their affected skin and this correlates with disease progression (96). Moreover, in a model of cutaneous lymphoma, tumor growth in mast cell-deficient mice was significantly decreased. Therefore, mast cells represent key players in the development of CTCL.

Th22 cells, which produce IL-22 but not IFN-γ, IL-4, or IL-17, express CCR6, CCR4, and CCR10, thus enhancing skin infiltration. IL-22 mediates host defenses against bacterial infection (97). The affected skin of patients with MF/SS expresses high levels of IL-22 and low levels of IL-17 (32). A case of SS reportedly also had high IL-22 expression that was modulated by systemic bacterial infections (98). The serum levels of IL-22 and IL-22-induced CCL20 are increased in patients with MF/SS and are associated with disease severity (32); this suggests an important role of IL-22 in establishing the tumor microenvironment in MF and SS.

Myeloid-derived suppressor cells (MDSCs) are also recognized as key players in tumor immune escape mechanisms (99). The progression from early patch/plaque lesions to tumors in MF is related to an increase in MDSCs (100). Therefore, MDSCs play a role in MF progression by decreasing antitumor immune responses.

T-cell exhaustion *via* immune checkpoints also constitutes an important factor underlying tumor survival. The expression levels of PD-1 (101, 102), PD-L1 (102), CTLA-4 (103), and ICOS (104) have been described at different stages of the disease, suggesting a role for immune checkpoint inhibitor therapies.

TREATMENT

There is currently no cure for CTCL, thus treatment is aimed primarily at improving symptoms and quality of life and maintaining remission. Therapies are tailored to the individual patient, based on age, performance status, extent of disease burden, rate of disease progression, and prior treatments (105). A typical MF progression starts at the patch and plaque stage and then advances to dermal-based tumors over many years. Effective immune control in the initial disease stages can slow disease progression. Hughes et al. reported that chemotherapy shortens the median time until the next treatment in patients with MF/SS (106). Multiagent chemotherapy often induces immunosuppression, which leads to an increased risk of infection and poor tolerance (107). Therefore, chemotherapy should be limited until all other options are exhausted. In comparison, IFN and histone deacetylase inhibitors afford greater times to next treatment than those from chemotherapy.

Both IFN-α and IFN-γ represent effective clinical treatments for CTCLs, including MF, *via* their cytotoxic and immunological effects on tumor-associated T-cells (108–110). A meta-analysis suggested that the overall response rate (ORR) to IFN-α was 70% (109). In all stages of MF, IFN-α achieves a superior time to next treatment compared to that of chemotherapy (106). IFN-γ shifts the Th2-dominant tumor microenvironment to a Th1 environment, as mentioned above. IFN-α2a and IFN-γ have been shown to decrease the expression and production of CCL17 and CCL18 and increase those of CXCL10 and CXCL11. Furthermore, subcutaneous administration of IFN-α increased the number of CXCL11-producing cells in the affected skin of patients with advanced MF (111).

Toll-like receptor (TLR) agonists induce anticancer effects by stimulating the innate immune system. Imiquimod is a topical immunomodulator that stimulates Th1 responses by activating TLR7 on plasmacytoid DCs, which leads to the production of IFN-α, IL-12, and tumor necrosis factor-α (112). The effectiveness of topical imiquimod has been reported in early-stage (113–115), folliculotropic, and tumor-stage MF (116). Resiquimod, a TLR7/8 agonist, is also effective for early-stage MF (117). TLR8 is expressed by myeloid-derived DCs, which are the most abundant DCs in human skin. Resiquimod, but not imiquimod, potently activates these cells (118).

The acetylation of histones plays a critical role in gene expression regulation (119). Histone acetylation and deacetylation control gene transcription and are mediated by histone acetyltransferases and deacetylases, respectively. Histone deacetylase inhibitors enhance the acetylation of histones and non-histone proteins and can induce apoptosis (120). Histone deacetylase inhibitors are potential therapeutic agents for the treatment of lymphoid neoplasms (121–124). Pruritus relief has also been reported with these inhibitors (121, 122, 124–126), supposedly through the reduction in the levels of IL-31-expressing T-cells (127).

Brentuximab vedotin (mentioned above) is an antibody–drug conjugate, in which an anti-CD30 monoclonal antibody is linked with the anti-tubulin agent, monomethyl auristatin E (128). Brentuximab vedotin is effective in the treatment of CD30-positive relapsed/refractory Hodgkin's lymphoma (129) and anaplastic large cell lymphoma (130). In a phase II study for MF/SS with variable CD30 expression levels, an ORR of 70% was observed with brentuximab vedotin (127). In addition, a

significant improvement in objective response was observed in a randomized, phase III clinical trial (131).

Mogamulizumab, a defucosylated humanized anti-CCR4 antibody that was first approved for relapsed ATL, as described in further detail in Section "Adult T-cell leukemia/lymphoma," is also effective for CTCL including MF/SS (132, 133), and approved for relapsed or refractory CCR4-positive CTCL. In addition, an anti-CD158k monoclonal antibody, IPH4102, has also recently been developed (134), for which clinical studies in CTCL are ongoing (135). Lenalidomide (136), bortezomib (137), and immune checkpoint blockade are also under investigation.

PRIMARY CUTANEOUS CD30$^+$ T-CELL LYMPHOPROLIFERATIVE DISORDERS

Primary cutaneous CD30$^+$ T-cell lymphoproliferative disorders (PC CD30$^+$ T-LPD) constitute the second most common form of CTCL, representing approximately 30% of all cutaneous lymphomas (2). They comprise a spectrum of diseases from lymphomatoid papulosis (LyP) to primary cutaneous anaplastic large-cell lymphoma (PCALCL) (138). The expression of CD30, a cytokine receptor belonging to the tumor necrosis factor receptor superfamily, by atypical T-cells is the common immunophenotype of this disorder.

Primary cutaneous anaplastic large-cell lymphoma is characterized by large T-cells with prominent nuclear pleomorphisms along with CD30 expression by more than 75% of the tumor cells (2). A single tumor or a group of firm nodules is seen clinically (**Figure 2**). PCALCL was established as a distinct form of ALCL because its clinical course, phenotype, and genotype are significantly different from those of systemic ALCL, including ALK-positive and ALK-negative forms (139–141). Moreover, IFN regulatory factor-4 translocations are reported to be specific for PCALCL (142). In contrast to that of systemic ALCL, the prognosis of PCALCL is reportedly excellent (143), with the exception of cases in Japan that appear to have a less favorable prognosis (144). PCALCL arising on the legs tends to produce poorer outcomes (145). The typical histology of PCALCL is a circumscribed nodular infiltrate of cohesively arranged large lymphoid cells that extends into the deep dermis or hypodermis. Neutrophil-rich and eosinophil-rich variants have been noted and appear to be associated with immunodeficiency (146). The abundant infiltration of neutrophils can be explained by the release of IL-8, a potent neutrophil chemoattractant, from the tumor cells (147).

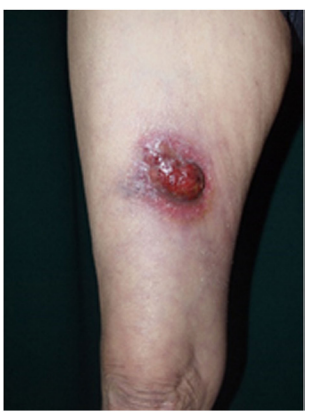

FIGURE 2 | Clinical findings of primary cutaneous CD30$^+$ T-cell lymphoma. Eroded tumor is seen on the right thigh. Written informed consent was obtained from the patient.

The tumor cells in PCALCL possess an activated T-cell phenotype and express CD2, CD4, and CD45RO, with a loss of CD2 and CD5 occurring variably. CD3 may be lacking or expressed at lower levels owing to genetic alterations in the T-cell receptor (TCR) coding regions on chromosome 1 in the tumor cells (148). Additionally, CD25/IL-2R, CD71, human leukocyte antigen–antigen D related, and CLA/CD162, as well as cytotoxic proteins, such as T-cell intracellular antigen 1 (TIA-1), granzyme B, and perforin, are expressed in half of PCALCL cases. PCALCL is often negative for epithelial membrane antigen, which differentiates it from systemic ALCL. Numerous quantities of TAMs are also present.

As opposed to MF/SS, the tumor cells of PCALCL express CCR3. CCL11, a CCR3 ligand, is also expressed by PCALCL cells and is detected in the connective tissue cells in the tumor. The CCR3$^+$ tumor cells abundantly express IL-4 but not IFN-γ (69). The expression of both CCL11 and CCR3 on the tumor cells can lead to homotypic aggregation, which can be observed as cohesive clusters of tumor cells, a characteristic finding in ALCL (149). As CCR3 is also expressed on eosinophils and subpopulations of Th2 cells (70, 71), CCR3$^+$ cells secreting CCL11 and IL-4 may produce a Th2-dominant microenvironment, which is suitable for tumor growth.

Lymphomatoid papulosis was first described by the dermatologist, Warren L. Macaulay, as a chronic recurrent, self-regressing papulonodular skin eruption with histologic features of a malignant lymphoma (138). Five histological variants (types A to E) are recognized as original variants in the updated World Health Organization classification of 2016 (10). LyP type A is the most common subtype, accounting for 75% of LyP cases (150). Type A is characterized by wedge-shaped dermal infiltrates with scattered large CD30$^+$ cells. Histiocytes, eosinophils, and neutrophils comprise the background inflammatory cells. Type B shows epidermotropic infiltrates of small to medium-sized lymphocytes with variable CD30 expression and atypical chromatin-dense nuclei. Type C shows nodular cohesive infiltrates of large CD30$^+$ pleomorphic or anaplastic lymphocytes. Type D shows epidermotropic infiltrates of atypical, small to medium-sized pleomorphic CD8$^+$ cytotoxic cells (151). Type E shows angioinvasive infiltrates of mainly medium-sized pleomorphic CD30$^+$ cells (152). Vascular occlusion by atypical lymphocytes and/or thrombi, hemorrhage, ulceration, and extensive necrosis are observed. LyP can persist for years or decades, but is not life-threatening (143, 153). However, some patients with LyP can develop secondary lymphoid neoplasms, in particular MF, Hodgkin's lymphoma, and cutaneous or nodal CD30$^+$ ALCL (140, 146, 154). Surgical excision or radiation therapy is the recommended therapy for solitary or grouped lesion(s) of PCALCL, whereas methotrexate is the most prescribed therapy for multifocal lesions (138). The brentuximab vedotin (128) has been granted breakthrough

therapy designation; in addition, bexarotene, a retinoid X receptor-specific agonist, has also been shown to be effective for both PCALCL (ORR: 50%) and LyP (ORR: 60%) in clinical trials (155). HDAC inhibitors (156), crizotinib, an ALK inhibitor (157), and anti-PD-1 are under investigation.

ADULT T-CELL LEUKEMIA/LYMPHOMA

Adult T-cell leukemia/lymphoma is a distinct T-cell malignancy caused by human T-lymphotropic virus type I (HTLV-1). HTLV-I infections are endemic in many parts of the world including southwest Japan, the Caribbean basin, and parts of central Africa and South America. Neoplastic T-cells are usually $CD4^+CD25^+CCR4^+$ (158). The general characteristics of ATL are lymphadenopathy, hepatosplenomegaly, hypercalcemia, abnormal peripheral blood lymphocytes with multilobulated nuclei, and skin lesions (**Figure 3**).

There are four clinical subtypes of ATL (159): acute, lymphoma, chronic, and smoldering, based on peripheral blood involvement, organ complications, and laboratory examinations. Patients with ATL can be stratified into two groups: aggressive, which consists of the acute, lymphoma, and unfavorable chronic types, and indolent, which consists of the favorable chronic and smoldering types. The chronic type is separated into the favorable and unfavorable subgroups according to significant prognostic factors. This stratification is important for treatment selection, with most patients with aggressive ATL being given systemic chemotherapy, whereas those with indolent ATL are given topical therapy or are placed on observation.

Cutaneous involvement is frequently observed in patients with ATL at 30–70% (160, 161), regardless of ATL subtype. Cutaneous manifestation in the smoldering type of ATL has been suggested to reflect poor prognosis (162), and cutaneous ATL was recently proposed to include the lymphoma type as an extranodal variant (163). The majority of skin lesions are caused by the direct invasion of ATL tumor cells, forming various types of eruptions (164). In addition to these primary invasive lesions, patients with ATL may present with secondary inflammatory or infectious lesions (165). Compared to those of peripheral blood tumor cells, skin-infiltrating ATL tumor cells exhibit enhanced characteristics, such as increased expression of chemokine receptors. The interaction between chemokines and chemokine receptors drives T-cell migration and activation, which plays a critical role in the pathogenesis of various neoplastic and inflammatory disorders. ATL cells produce several chemokines including CCL3/MIP-1α, CCL4/MIP-1β (166), CCL2/monocyte chemoattractant protein-1 (MCP-1) (167), and CCL1/I-309 (168), as well as several chemokine receptors, including CCR4 (158, 169), CCR7 (170), and CCR8/CDw198 (168). Overexpression of chemokine CCL1 and its receptor, CCR8, contributes to autocrine anti-apoptotic effects ATL cells (168). Increased CCR7 expression is associated with lymphoid organ infiltration (170).

Adult T-cell leukemia/lymphoma cells not only express CCR4 but also its ligands, CCL17 and CCL22 (171). Neoplastic T-cells that highly express the Th2 chemokine receptor, CCR4, are found in the peripheral blood and affected skin of patients with ATL. In CTCL, extravasation of lymphoma cells into the skin is mediated by CCL17 and CCL22 released from epidermal cells (30). In contrast, one of the major sources of CCL17 in the affected skin of patients with ATL is the tumor cell itself (171). Moreover, CCL17 and CCL22 can also attract CCR4-expressing Treg cells, which may further suppress cytotoxic T-cells and prevent tumor immunosurveillance of the ATL cells (165). As ATL cells share the $CD4^+CD25^+CCR4^+$ phenotype with Treg cells, ATL cells have been postulated as being Treg cells. In addition to CD25 and CCR4, ATL cells express CTLA-4 and FoxP3, both of which are expressed in Treg cells (172, 173). However, whether ATL cells can function as Treg cells is controversial because tumor cells possess very limited regulatory ability (174).

Th17 cells play an important role in cutaneous innate immunity. Th17-derived cytokines stimulate keratinocytes to produce antimicrobial peptides (175). ATL tumor cells can reduce the number and/or function of Th17 cells. Studies have shown that cellular immune responses are greatly impaired in patients with ATL, and ATL cells have been shown to secrete immunosuppressive cytokines such as IL-10 and transforming growth factor-β1 *in vitro*. In particular, ATL cells, as well as Treg or Th2 cells residing in the blood, produce IL-10, thereby suppressing Th17 activity (176). IL-17 enhances the synthesis of various antimicrobial peptides, such as human β-defensin 2, LL-37 (177), and S100A7, in keratinocytes. These peptides are active against fungi, such as those causing ringworm (178). More than 60% of patients with

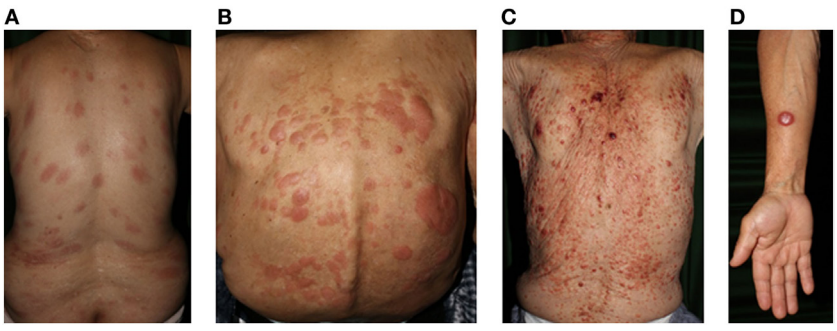

FIGURE 3 | Clinical findings of adult T-cell lymphoma/leukemia. **(A)** Patch, **(B)** plaque, **(C)** multiple nodulotumoral lesion, and **(D)** tumor. Written informed consent was obtained from each patient.

ATL have tinea pedis/unguium/corporis, candidiasis, or other cutaneous fungal infections (165). Other skin infections may occur in these patients in addition to superficial fungal infections. It has been reported that scabies is sometimes superimposed on the skin lesions of patients with ATL (179).

Programmed cell death (PD)-1/CD279 constitutes a cell surface receptor that suppresses the immune system. PD-1 expression on HTLV-1-specific cytotoxic T-cells is dramatically upregulated in HTLV-1 carriers and patients with ATL (180). PD-1 is expressed at high levels on $CD4^+$ neoplastic and non-neoplastic cells, but not on $CD8^+$ cells (181). Because normal $CD4^+$ T-cells can be infected with HTLV-1, they can sometimes express PD-1, leading to immunosuppression. Moreover, it is noteworthy that PD-L1 is expressed in ATL cells (181). Expression of both PD-1 and PD-L1 by the ATL cells suggests a self-destructive state of the tumor cells. However, it may be more important that the PD-L1 expressed by the tumor cells suppresses the function of PD-1-expressing normal $CD4^+$ T-cells, resulting in immune evasion. Of note, 25% of patients with ATL have structural variations in the 3'-region of the gene for PD-L1, which leads to marked elevations of aberrant *PDL1* transcripts (182).

The fact that the tumor cells express CCR4 provides a therapeutic strategy for ATL. The anti-CCR4 monoclonal antibody mogamulizumab markedly enhances antibody-dependent cellular cytotoxicity and has been approved for the treatment of patients with CCR4-positive ATL, peripheral T-cell lymphoma, and CTCL. In a phase II trial of patients with relapsed CCR4-positive ATL, the ORR was 50%, with a complete response rate of 30% (183). Mogamulizumab is more effective against the peripheral blood tumor cells than those in the skin and lymph nodes. Cutaneous adverse reactions (CARs) are frequently observed during treatment (183, 184) and are supposedly indicative of favorable prognoses in ATL (185); a reduction in Treg by mogamulizumab is believed to induce CARs (186, 187). Recently, pretransplantation mogamulizumab has been reported to increase the risk of severe acute graft-versus-host disease (188, 189), and non-relapse mortality is significantly higher in patients with pretransplantation mogamulizumab. Therefore, mogamulizumab should be carefully considered and monitored for patients with ATL who are eligible for allogeneic hematopoietic stem-cell transplantation.

PANNICULITIS-LIKE T-CELL LYMPHOMA

Subcutaneous panniculitis-like T-cell lymphoma (SPTCL) with α/β phenotype and SPTCL with γ/δ phenotype have been recognized as unique entities, considering their clinical, histological, and immunological characteristics (2, 190, 191). The term SPTCL is now used exclusively for cases with the α/β T-cell phenotype, whereas those of the γ/δ T-cell phenotype have been reclassified as primary cutaneous gamma/delta T-cell lymphoma (PCGD-TCL) (2). The differential diagnosis of these two diseases is important, as each has a different prognosis and therapeutic strategy. In addition, both entities should be differentiated from other types of malignant lymphoma with preferential subcutaneous involvement and from other forms of lobular panniculitis, especially lupus panniculitis (192, 193).

SUBCUTANEOUS PANNICULITIS-LIKE T-CELL LYMPHOMA

Patients with SPTCL present clinically with multiple nodules or deeply seated plaques without ulceration. The skin lesions usually involve the legs, arms, and trunk. Systemic symptoms, such as pyrexia, fatigue, and weight loss, and laboratory abnormalities, including cytopenia and elevated liver function tests, are commonly observed. Hemophagocytic syndrome (HPS) is observed in <20% of patients (194). Dissemination to extracutaneous sites rarely occurs. As many as 20% of patients have associated autoimmune disease, which is commonly systemic lupus erythematosus (194).

The histopathological findings in SPTCL are dense, nodular, or diffuse subcutaneous infiltrates with a pattern similar to lobular panniculitis. The epidermis is not typically involved. The rimming of individual fat cells by neoplastic T-cells is a curious finding, although it is not diagnostic (193). The neoplastic T-cells are interspersed with small reactive lymphocytes and many histiocytes, whereas other inflammatory cells, including neutrophils and eosinophils, as well as the plasma cells and plasmacytoid DCs that are common in lupus panniculitis (195, 196), are usually lacking (193). High-throughput sequencing of the TCR genes can assist in the diagnosis of SPTCL (192). The neoplastic cells have a mature $CD3^+CD4^-CD8^+$ T-cell phenotype and express cytotoxic proteins, such as granzyme B, TIA-1, and perforin (194). Although the exact mechanisms that neoplastic cells utilize to migrate into the hypodermis are still mostly unknown, CCR5 expression on neoplastic cells and its ligands, CCL3, CCL4, and CCL5, which can be secreted from immunologically activated adipocytes, may contribute to the pathogenesis of SPTCL (197, 198).

The differential diagnosis of SPTCL includes both PCGD-TCL and lupus panniculitis. Differentiation is critical because PCGD-TCL with panniculitis-like features generally has a poor prognosis and requires systemic chemotherapy. In contrast, SPTCL has an excellent prognosis, especially in the cases without HPS (194). Both SPTCL and PCGD-TCL have nodular skin lesions with panniculitis-like features and rimming of fat cells. In contrast to that of SPTCL, PCGD-TCL involves ulceration of the hypodermis, dermis, and/or epidermis (194). Expression of βF1, but not TCRγ/δ or CD56, is useful to differentiate between SPTCL and PCGD-TCL.

Multiagent chemotherapy is not recommended as a first-line treatment for SPTCL without HPS. Systemic corticosteroids or other immunosuppressive agents, such as cyclosporine or methotrexate, are preferred, which is also the case with relapsing disease (199–201). Oral bexarotene has also shown good response rates (202).

PRIMARY CUTANEOUS GAMMA/DELTA T-CELL LYMPHOMA

Primary cutaneous gamma/delta T-cell lymphoma is a lymphoma composed of a clonal proliferation of mature, activated γ/δ T-cells with a cytotoxic phenotype. Most patients present with deep dermal or subcutaneous plaques or tumors, either with

or without epidermal ulceration and necrosis (194, 203, 204). The skin lesions are often generalized and involve the extremities. Some patients may present with a single tumor, or scaly patches/plaques, clinically resembling early-stage MF (204). The involvement of mucosal and other extranodal sites is frequently noted, although lymph nodes, spleen, and bone marrow are rarely involved (204, 205). Most patients present with systemic symptoms including B symptoms. PCGD-TCL is frequently accompanied by HPS, particularly in patients with panniculitis-like tumors (194, 203). Chronic antigenic stimulation has been hypothesized to be involved in the pathogenesis of PCGD-TCL (206). PCGD-TCL is also associated with opportunistic infections in patients with congenital or acquired immunosuppression and autoimmunity (207–209).

The lymphoid infiltrates have a variable histological pattern and may be epidermotropic, dermal, and/or subcutaneous (203, 204). In contrast to that of SPTCL, a pure panniculitic pattern is rarely observed (204), and variable patterns can be found in skin biopsies obtained from different sites or different parts of the same biopsy (190, 203, 204). Lichenoid or vascular interface dermatitis-like patterns of epidermal infiltration may occur, which may be associated with intraepidermal vesiculation and necrosis (204). Panniculitis-like lesions may show the rimming of fat cells observed in SPTCL. Angiocentricity, angiodestruction, and tissue necrosis may be seen. Hemophagocytosis may be present, especially in cases with HPS. The tumor cells have a characteristic phenotype of TCR γ/δ$^+$, βF1$^-$, CD3$^+$, CD2$^+$, CD5$^-$, and CD56$^+$, with a strong expression of cytotoxic proteins. PCGD-TCL with subcutaneous panniculitis-like infiltrate preferentially derives from the V2 subtype (205). PCGD-TCL is resistant to multiagent chemotherapy. The effectiveness of hematopoietic stem cell transplantation has been reported in some patients with PCGD-TCL (204, 210, 211).

PRIMARY CUTANEOUS CD4$^+$ SMALL/MEDIUM T-CELL LYMPHOPROLIFERATIVE DISORDER

Primary cutaneous small/medium-sized T-cell lymphoma (PCSM-TCL) has recently been reclassified as primary cutaneous small/medium-sized T-cell lymphoproliferative disorder (PCSM-TCLPD) because of its indolent behavior and uncertain malignancy (10). PCSM-TCL was originally associated with a favorable 5-year survival rate of 60–80% (2). However, fatal outcomes have not been documented in subsequent reports (212, 213).

Primary cutaneous small/medium-sized T-cell lymphoproliferative disorder characteristically presents with a single lesion on the head, neck, or upper arms, but rarely presents as multiple papules, plaques, or tumors (212, 214). Histopathologically, PCSM-TCLPD is characterized by many small- to medium-sized CD3$^+$CD4$^+$CD8$^-$ T-cells, with a small number of large CD4$^+$ pleomorphic T-cells and variable admixtures of CD8$^+$ T-cells, B-cells, histiocytes, plasma cells, and eosinophils (2).

The few, large pleomorphic CD4$^+$ T-cells in PCSM-TCLPD express PD-1, BCL6, and CXCL13 (215), all of which are expressed on a particular germinal center T-cell subset, termed follicular helper T (TFH) cells. TFH cells are important in germinal center formation and plasma cell development. The expression of PD-1, BCL6, and CXCL13 by these large CD4$^+$ T-cells suggests that PCSM-TCLPD originates from TFH cells (215). PD-1 is typically expressed by atypical cells in PCSM-TCL and pseudo-T-cell lymphomas (216). The clinical presentation, pathological features, and immunohistochemical findings of PCSM-TCLPD are very similar to those of pseudo-T-cell lymphomas (217, 218). The demonstration of a T-cell clone and loss of pan-T-cell antigens are useful diagnostic criteria for PCSM-TCL (218). The staining pattern for nuclear factor of activated T-cells, cytoplasmic 1 is also reported to be useful for the differential diagnosis between PCSM-TCLPD and pseudo-T-cell lymphomas (219), where NFAT1c nuclear staining indicates PCSM-TCLPD and cytoplasmic staining indicates pseudo-T-cell lymphoma. The cytoplasmic staining pattern is also seen in MF, ALCL, and LyP. The clinical behavior of PCSM-TCLPD is almost always indolent, with most patients showing localized disease. Treatment with local therapies, such as excision or radiation therapy, is often curative (214, 220, 221).

HYDROA VACCINIFORME-LIKE LYMPHOPROLIFERATIVE DISORDER (HVLL)

Typical hydroa vacciniforme (HV) is characterized by light-induced herpetiform vesiculopapules on the sun-exposed areas. The eruptions form crusts and then heal to leave varicelliform scars. Systemic symptoms are absent, and the disease usually improves spontaneously in adolescence and young adulthood (222). Routine laboratory tests are normal. Since the first report in 1986 (223), peculiar HV-like eruptions have been recognized in children mainly from Asia and Central and South America. HVLL was included for the first time in the 2008 World Health Organization classification of tumors of hematopoietic and lymphoid tissues (224). HVLL is defined as an Epstein–Barr virus (EBV)-positive CTCL that occurs in children and less often in young adults (225). Unlike typical HV, HVLL eruptions become more severe with age, presenting with marked facial edema and vesiclopapules followed by ulceration and crusting. Systemic symptoms, including high-grade fever and liver damage, are usually present. Hepatosplenomegaly and lymphadenopathy are frequently observed during the acute phase. The lesions are associated with EBV infection and frequently possess monoclonal rearrangements of the TCR genes (226, 227). Although the skin lesions are not limited to sun-exposed areas, there is an increased occurrence during the summer. Most cases have a CD8$^+$ T-cell phenotype (228), whereas a small number of cases have been reported to have a natural killer-cell phenotype (229, 230). Regardless of cell-type derivation, the lymphoid cells are positive for cytotoxic markers, such as granzyme B and TIA-1 (231).

SEVERE MOSQUITO BITE ALLERGY

An associated cutaneous disorder is a severe allergy/hypersensitivity to mosquito bites (232). It is defined as an EBV$^+$ NK-cell

lymphoproliferation that is characterized by high fever, ulcers, skin necrosis, and deep scarring, with the potential to progress into overt NK/T-cell lymphoma or aggressive NK-cell leukemia in the protracted clinical course (233). Severe mosquito bite allergy was included for the first time in the 2017 World Health Organization classification of tumors of hematopoietic and lymphoid tissues (234).

PRIMARY CUTANEOUS ACRAL CD8$^+$ T-CELL LYMPHOMA

Primary cutaneous acral CD8$^+$ T-cell lymphoma is characterized as a solitary, slow-growing nodule without prior patches or plaques (235), but with precedence of bilateral, symmetrical disease and recurrent disease (236). Most cases appear on the ear, although other peripheral locations, such as the nose, hands, and feet, have been noted (237).

Primary cutaneous acral CD8$^+$ T-cell lymphoma and PCSM-TCLPD are often indistinguishable morphologically. Moreover, the overt clinical features of both diseases are similar, such as targeting adults, a preference for the face and neck, solitary tumors without ulceration, and an indolent behavior. However, T follicular markers, such as CD10, Bcl-6, PD-1, and CXCL13, which are expressed on neoplastic cells of PCSM-TCLPD, are negative in primary cutaneous acral CD8$^+$ T-cell lymphoma (236). Granzyme B expression is also typically negative in the latter (238). The clinical course for primary cutaneous acral CD8$^+$ T-cell lymphoma is invariably indolent; cutaneous relapse may occur, but there have been no reports of progression to extracutaneous sites, and overtreatment should be avoided (238). Localized therapy, such as topical steroids, radiotherapy, and surgical excision, or careful monitoring, is preferred. IFN, psoralen-ultraviolet A phototherapy, and methotrexate have been used for patients with multifocal cutaneous disease (238).

PRIMARY CUTANEOUS CD8$^+$ AGGRESSIVE EPIDERMOTROPIC CYTOTOXIC T-CELL LYMPHOMA

Primary cutaneous CD8$^+$ aggressive epidermotropic cytotoxic T-cell lymphoma (PCAETCL) is characterized by disseminated, rapidly developing papules, plaques, and nodules with central ulceration or necrosis. PCAETCL may spread to other visceral organs including the lungs, testes, central nervous system, and oral mucosa (239–241); it carries an overall poor prognosis. However, the lymph nodes are rarely involved. Histological findings demonstrate prominent epidermotropism, with necrotic keratinocytes and ulceration (240). Dermal infiltrates consist of atypical lymphocytes, often extending into the deep dermis and subcutaneous fat. Adnexal invasion is frequently observed (242). Blistering, angiocentricity, angioinvasion, riming of adipocytes, and destruction of adnexal structures may be seen (240). Cells invariably demonstrate CD8$^+$CD4$^-$ phenotypes and usually express CD3, β-F1, and TIA-1. CD45RA is expressed in the majority of cases (239). T-cell clonality is usually demonstrated. Conventional therapies for CTCL are ineffective and multiagent chemotherapies have unsatisfactory outcomes (240). Hematopoietic stem cell transplantation is a reasonable treatment choice for PCAETCL (243).

AUTHOR CONTRIBUTIONS

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

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Melanoma Immunotherapy: Next-Generation Biomarkers

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The recent emergence of cancer immunotherapies initiated a significant shift in the clinical management of metastatic melanoma. Prior to 2011, melanoma patients only had palliative treatment solutions which offered little to no survival benefit. In 2018, with immunotherapy, melanoma patients can now contemplate durable or even complete remission. Treatment with novel immune checkpoint inhibitors, anti-cytotoxic T-lymphocyte protein 4 and anti-programmed cell death protein 1, clearly result in superior median and long-term survivals compared to standard chemotherapy; however, more than half of the patients do not respond to immune checkpoint blockade. Currently, clinicians do not have any effective way to stratify melanoma patients for immunotherapies. Research is now focusing on identifying biomarkers which could predict a patient's response prior treatment initiation (or very early during treatment course), in order to maximize therapeutic efficacy, avoid unnecessary costs, and undesirable heavy side effects for the patient. Given the rapid developments in this field and the translational potential for some of the biomarkers, we will summarize the current state of biomarker research for immunotherapy in melanoma, with an emphasis on omics technologies such as next-generation sequencing and mass cytometry (CyTOF).

Keywords: melanoma, immunotherapy, biomarkers, next-generation sequencing, review literature as topic

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Immunotherapy has revolutionized the management of metastatic melanoma. Prior to 2011, the median survival for metastatic melanoma was 9 months, compared to greater than 18 months in 2017 (1). Patients now benefit from novel immune checkpoint inhibitors (ICIs), anti-cytotoxic T-lymphocyte protein 4 (CTLA-4) and anti-programmed cell death protein 1 (PD-1). From the latest survival data of the Checkmate 067 trial, progression-free survival (PFS) for ipilimumab is 2.9 months, for nivolumab 6.9 months, and for the combination of nivolumab and ipilimumab 11.5 months. Overall survival (OS) of the ipilimumab group was 19.9 and 37.6 months for the nivolumab group. Median OS was not reached in the combination nivolumab and ipilimumab group with a minimum follow-up time of 36 months (2–6). Although OS is extended, not all patients benefit from immunotherapy. Response rates for ipilimumab range from 11% to 19% (4, 5) and for pembrolizumab or nivolumab from 33% to 44% (2, 6, 7). These new ICIs clearly show superior median and long-term survivals compared to standard chemotherapy; however, more than half of the patients do not respond to immune checkpoint blockade. Currently, there are no clinically approved biomarkers to aid in patient selection in melanoma. In this review, we seek to delineate the current state of biomarker research for immunotherapy in melanoma, with an emphasis on omics technologies such as next-generation sequencing (NGS) and mass cytometry (CyTOF). Given the urgent clinical need for such biomarkers, we decided to focus on human studies only, which we think are more clinically relevant.

IMMUNE CHECKPOINTS

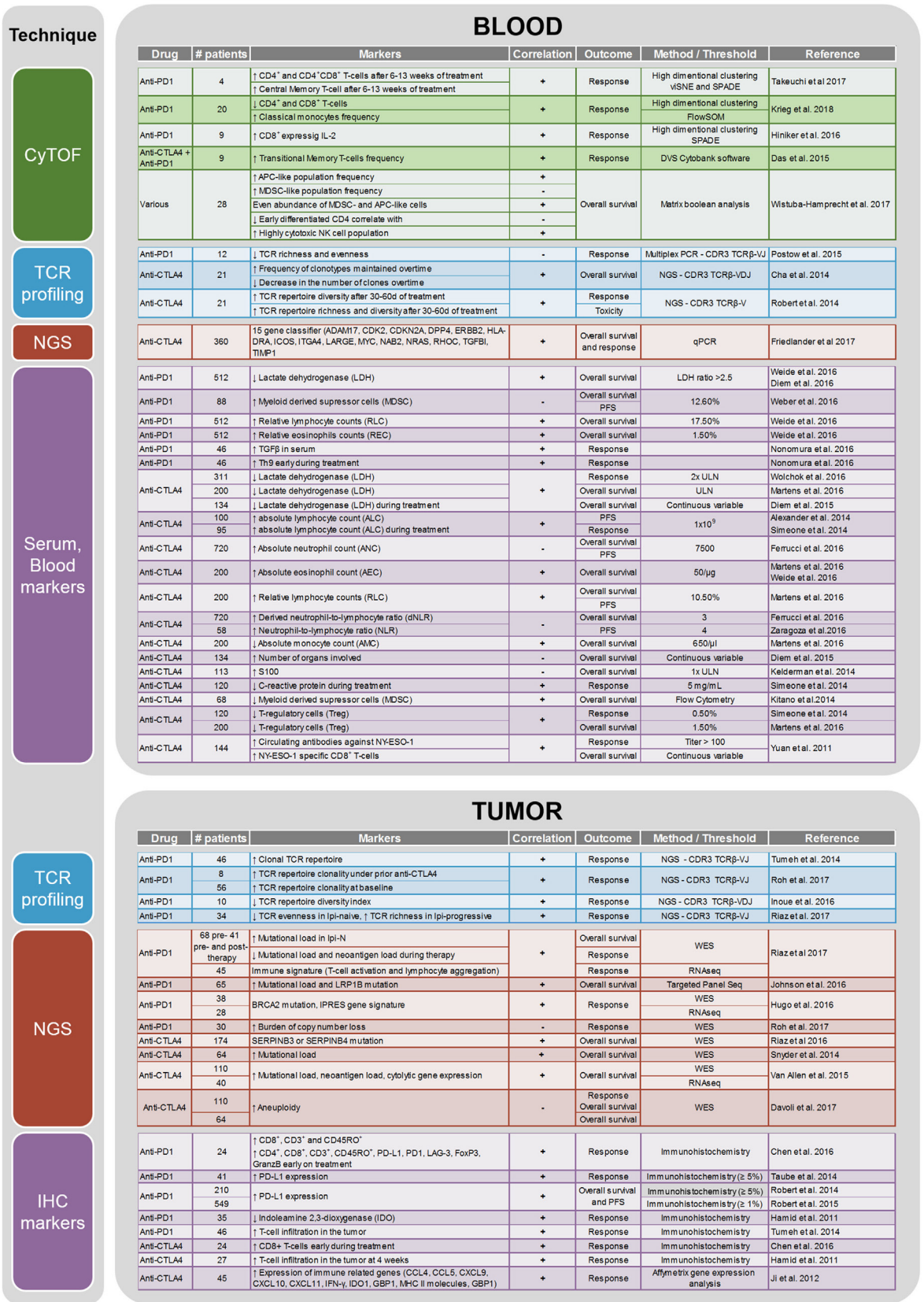
CTLA-4 and PD-1 are two immune checkpoints regulating immune homeostasis. CTLA-4 is a negative regulator of T-cell priming that acts to control naïve T-cell activation by competing with the co-stimulatory molecule CD28 for binding to shared ligands CD80 and CD86 on antigen-presenting cells (APCs) in the lymph node (8). Ipilimumab, a monoclonal antibody against CTLA-4, was the first agent approved for the treatment of unresectable or metastatic melanoma that showed an OS benefit in a randomized phase III trial (4). PD-1 is a T-cell exhaustion marker which is upregulated by T-cells upon activation during priming or expansion and binds to one of two ligands: programmed cell death 1-ligand 1 (PD-L1) and -ligand 2 (PD-L2) (9–11). Pembrolizumab and nivolumab are monoclonal antibodies against PD-1 that have both shown OS benefit in randomized phase III trials and are approved for the treatment of metastatic melanoma (2, 7). Furthermore, nivolumab and pembrolizumab have both improved OS compared with ipilimumab in metastatic melanoma patients that are naïve to both agents. Combination therapy with ipilimumab and nivolumab has demonstrated additional clinical activity with objective response rates ranging from 50% to 60% and improved OS compared to ipilimumab alone. Although ipilimumab, nivolumab, and pembrolizumab have significantly improved the survival of melanoma patients, there are major toxicities associated with the use of these drugs [reviewed in Ref. (12)]. Grade 3 and higher adverse events are seen in about 20% of patients treated with ipilimumab, in 15% of patients treated with nivolumab, and in 50% of patients treated with the combination of both drugs (6). As these therapies result in objective responses for only a subset of patients, there is a crucial need to identify biomarkers that can potentially predict the efficacy of anti-CTLA-4 or anti-PD-1 treatment or identify a specific subset of patients who may benefit from immunotherapy. A summary of current potential biomarkers for immunotherapies in metastatic melanoma patients is listed in **Figure 1**.

CLINICAL BIOMARKERS

Approved markers for melanoma monitoring have not substantially evolved over the past decade. Clinicians have mainly used the TNM staging system as a diagnostic and prognostic indicator. In 2009, lactate dehydrogenase (LDH) was shown to be an independent predictor of survival in melanoma and was therefore added to the AJCC guidelines (13). Accelerated metabolism in cancer cells requires increased glycolysis that creates a high amount of LDH as a byproduct, which is therefore a robust proxy to assess tumor burden (14). It is the only accepted serum biomarker with prognostic value for OS in melanoma (15). In the context of immunotherapies, elevated LDH is a negative prognostic marker for patients treated with ipilimumab (16) and with pembrolizumab (17, 18). However, subgroup analysis of anti-PD-1 treated cohorts recently pointed out that LDH level is not correlated with the duration of response (KEYNOTE-006). Indeed, once patients show response to the treatment, the LDH level is not associated with the duration of the remission period. As described by Diem et al. in a study specifically assessing the role of LDH as a marker for anti-PD-1 therapy, LDH is nevertheless a useful marker to monitor disease progression and help treatment decisions (19).

Another well-known marker to monitor melanoma is S100, which is a good indicator of advanced clinical disease stage (20). S100 was shown to be predictive of response to anti-CTLA-4 (16). However, similar to LDH, S100 seems to mainly be a proxy of disease stage, able to highlight very ill patients who are more unlikely to respond to the treatment due to the high tumor burden of the disease, but not actually able to predict response to immunotherapies. The same is true for the number of organs involved, which was another potential marker, proposed by Diem et al., to stratify patients prior to anti-CTLA-4 therapy (21).

C-reactive protein (CRP) was described as a negative prognostic factor for anti-CTLA-4 treatment (22). Unlike LDH and S100, CRP is directly related to immune response. However, it is a general marker of inflammation and is not specific to melanoma, ergo, an increase in CRP levels may also be the result of any other ongoing infection (23). For anti-PD-1 therapy, intra-tumoral PD-L1 expression, evaluated by immunohistochemistry, has been assessed as a predictive biomarker. The results have been inconclusive due to a lack of standards for PD-L1 "positivity". Different antibodies and different evaluation criteria have been used for PD-L1 expression in clinical trials. Some studies have used a >5% cutoff (Checkmate-066 and Checkmate-067), whereas others have used >1% cutoff (KEYNOTE-006). In the Checkmate-066 trial, both PD-L1 negative and positive patients had better outcomes than chemotherapy-treated patients, suggesting that PD-L1 status was not a relevant stratification marker (2). More research will be needed to standardize the assessment of PD-L1 expression for it to become a biomarker for anti-PD-1 therapy in melanoma. Blood markers which hold the most potential toward predicting response to immunotherapies are immune cell populations. Indeed, they are either themselves part of or directly influencing the immune response against the tumor. The different findings related to blood cytology as a biomarker for immunotherapy in melanoma are summarized in **Figure 1**. Briefly, for anti-CTLA-4 treatment, absolute neutrophil count, absolute lymphocyte count, neutrophil to lymphocyte ratio, absolute eosinophil count, relative lymphocyte count (RLC), absolute monocyte count, antibodies against NY-ESO1, T-regulatory cell count, and myeloid-derived suppressor cell (MDSC) count have been described as predictive biomarkers. In anti-PD-1-treated patients, RLC, relative eosinophil count (REC), and MDSC count seem to hold some predictive potential prior to treatment initiation. In addition, increased serum levels of TGFβ and increased frequency of Th9 cells in the peripheral blood were detected in responders to nivolumab prior to therapy initiation (24). Unfortunately, most of the studies were performed on small cohorts and the results have not been verified in larger prospective trials (25). Although guidelines have been published about how to best perform biomarker studies (26), most research groups have different evaluation criteria. In this review, we sought to document the most relevant biomarkers associated with immunotherapy outcome in melanoma patients. For a systematic review of clinical biomarkers, see Ref. (18).



BLOOD

Drug	# patients	Markers	Correlation	Outcome	Method / Threshold	Reference
Anti-PD1	4	↑ $CD4^+$ and $CD4^+CD8^+$ T-cells after 6-13 weeks of treatment ↑ Central Memory T-cell after 6-13 weeks of treatment	+	Response	High dimentional clustering viSNE and SPADE	Takeuchi et al 2017
Anti-PD1	20	↓ $CD4^+$ and $CD8^+$ T-cells ↑ Classical monocytes frequency	+	Response	High dimentional clustering FlowSOM	Krieg et al. 2018
Anti-PD1	9	↑ $CD8^+$ expressig IL-2	+	Response	High dimentional clustering SPADE	Hiniker et al. 2016
Anti-CTLA4 + Anti-PD1	9	↑ Transitional Memory T-cells frequency	+	Response	DVS Cytobank software	Das et al. 2015
Various	28	↑ APC-like population frequency ↑ MDSC-like population frequency Even abundance of MDSC- and APC-like cells ↓ Early differentiated CD4 correlate with ↑ Highly cytotoxic NK cell population	+ - + - +	Overall survival	Matrix boolean analysis	Wistuba-Hamprecht et al. 2017
Anti-PD1	12	↓ TCR richness and evenness	-	Response	Multiplex PCR - CDR3 TCRβ-VJ	Postow et al. 2015
Anti-CTLA4	21	↑ Frequency of clonotypes maintained overtime ↓ Decrease in the number of clones overtime	+	Overall survival	NGS - CDR3 TCRβ-VDJ	Cha et al. 2014
Anti-CTLA4	21	↑ TCR repertoire diversity after 30-60d of treatment ↑ TCR repertoire richness and diversity after 30-60d of treatment	+	Response Toxicity	NGS - CDR3 TCRβ-V	Robert et al. 2014
Anti-CTLA4	360	15 gene classifier (ADAM17, CDK2, CDKN2A, DPP4, ERBB2, HLA-DRA, ICOS, ITGA4, LARGE, MYC, NAB2, NRAS, RHOC, TGFBI, TIMP1	+	Overall survival and response	qPCR	Friedlander et al 2017
Anti-PD1	512	↓ Lactate dehydrogenase (LDH)	+	Overall survival	LDH ratio >2.5	Weide et al. 2016 Diem et al. 2016
Anti-PD1	88	↑ Myeloid derived supressor cells (MDSC)	-	Overall survival PFS	12.60%	Weber et al. 2016
Anti-PD1	512	↑ Relative lymphocyte counts (RLC)	+	Overall survival	17.50%	Weide et al. 2016
Anti-PD1	512	↑ Relative eosinophils counts (REC)	+	Overall survival	1.50%	Weide et al. 2016
Anti-PD1	46	↑ TGFβ in serum	+	Response		Nonomura et al. 2016
Anti-PD1	46	↑ Th9 early during treatment	+	Response		Nonomura et al. 2016
Anti-CTLA4	311	↓ Lactate dehydrogenase (LDH)	+	Response	2x ULN	Wolchok et al. 2016
	200	↓ Lactate dehydrogenase (LDH)		Overall survival	ULN	Martens et al. 2016
	134	↓ Lactate dehydrogenase (LDH) during treatment		Overall survival	Continuous variable	Diem et al. 2015
Anti-CTLA4	100	↑ absolute lymphocyte count (ALC)	+	PFS	$1x10^9$	Alexander et al. 2014
	95	↑ absolute lymphocyte count (ALC) during treatment		Response		Simeone et al. 2014
Anti-CTLA4	720	↑ Absolute neutrophil count (ANC)	-	Overall survival PFS	7500	Ferrucci et al. 2016
Anti-CTLA4	200	↑ Absolute eosinophil count (AEC)	+	Overall survival	50/µg	Martens et al. 2016 Weide et al. 2016
Anti-CTLA4	200	↑ Relative lymphocyte counts (RLC)	+	Overall survival PFS	10.50%	Martens et al. 2016
Anti-CTLA4	720	↑ Derived neutrophil-to-lymphocyte ratio (dNLR)	-	Overall survival	3	Ferrucci et al. 2016
	58	↑ Neutrophil-to-lymphocyte ratio (NLR)		PFS	4	Zaragoza et al.2016
Anti-CTLA4	200	↓ Absolute monocyte count (AMC)	+	Overall survival	650/µl	Martens et al. 2016
Anti-CTLA4	134	↑ Number of organs involved	-	Overall survival	Continuous variable	Diem et al. 2015
Anti-CTLA4	113	↑ S100	-	Overall survival	1x ULN	Kelderman et al. 2014
Anti-CTLA4	120	↓ C-reactive protein during treatment	+	Response	5 mg/mL	Simeone et al. 2014
Anti-CTLA4	68	↓ Myeloid derived supressor cells (MDSC)	+	Overall survival	Flow Cytometry	Kitano et al.2014
Anti-CTLA4	120	↓ T-regulatory cells (Treg)	+	Response	0.50%	Simeone et al. 2014
	200	↓ T-regulatory cells (Treg)		Overall survival	1.50%	Martens et al. 2016
Anti-CTLA4	144	↑ Circulating antibodies against NY-ESO-1	+	Response	Titer > 100	Yuan et al. 2011
		↑ NY-ESO-1 specific $CD8^+$ T-cells		Overall survival	Continuous variable	

TUMOR

Drug	# patients	Markers	Correlation	Outcome	Method / Threshold	Reference
Anti-PD1	46	↑ Clonal TCR repertoire	+	Response	NGS - CDR3 TCRβ-VJ	Tumeh et al. 2014
Anti-PD1	8	↑ TCR repertoire clonality under prior anti-CTLA4	+	Response	NGS - CDR3 TCRβ-VJ	Roh et al. 2017
	56	↑ TCR repertoire clonality at baseline				
Anti-PD1	10	↓ TCR repertoire diversity index	+	Response	NGS - CDR3 TCRβ-VDJ	Inoue et al. 2016
Anti-PD1	34	↓ TCR evenness in Ipi-naive, ↑ TCR richness in Ipi-progressive	+	Response	NGS - CDR3 TCRβ-VJ	Riaz et al. 2017
Anti-PD1	68 pre- 41 pre- and post-therapy	↑ Mutational load in Ipi-N	+	Overall survival	WES	Riaz et al 2017
		↓ Mutational load and neoantigen load during therapy		Response		
	45	Immune signature (T-cell activation and lymphocyte aggregation)		Response	RNAseq	
Anti-PD1	65	↑ Mutational load and LRP1B mutation	+	Overall survival	Targeted Panel Seq	Johnson et al. 2016
Anti-PD1	38	BRCA2 mutation, IPRES gene signature	+	Response	WES	Hugo et al. 2016
	28				RNAseq	
Anti-PD1	30	↑ Burden of copy number loss	-	Response	WES	Roh et al. 2017
Anti-CTLA4	174	SERPINB3 or SERPINB4 mutation	+	Overall survival	WES	Riaz et al 2016
Anti-CTLA4	64	↑ Mutational load	+	Overall survival	WES	Snyder et al. 2014
Anti-CTLA4	110	↑ Mutational load, neoantigen load, cytolytic gene expression	+	Overall survival	WES	Van Allen et al. 2015
	40				RNAseq	
Anti-CTLA4	110	↑ Aneuploidy	-	Response Overall survival	WES	Davoli et al. 2017
	64			Overall survival		
Anti-PD1	24	↑ $CD8^+$, $CD3^+$ and $CD45RO^+$ ↑ $CD4^+$, $CD8^+$, $CD3^+$, $CD45RO^+$, PD-L1, PD1, LAG-3, FoxP3, GranzB early on treatment	+	Response	Immunohistochemistry	Chen et al. 2016
Anti-PD1	41	↑ PD-L1 expression	+	Response	Immunohistochemistry (≥ 5%)	Taube et al. 2014
Anti-PD1	210	↑ PD-L1 expression	+	Overall survival and PFS	Immunohistochemistry (≥ 5%)	Robert et al. 2014
	549				Immunohistochemistry (≥ 1%)	Robert et al. 2015
Anti-PD1	35	↓ Indoleamine 2,3-dioxygenase (IDO)	+	Response	Immunohistochemistry	Hamid et al. 2011
Anti-PD1	46	↑ T-cell infiltration in the tumor	+	Response	Immunohistochemistry	Tumeh et al. 2014
Anti-CTLA4	24	↑ CD8+ T-cells early during treatment	+	Response	Immunohistochemistry	Chen et al. 2016
Anti-CTLA4	27	↑ T-cell infiltration in the tumor at 4 weeks	+	Response	Immunohistochemistry	Hamid et al. 2011
Anti-CTLA4	45	↑ Expression of immune related genes (CCL4, CCL5, CXCL9, CXCL10, CXCL11, IFN-γ, IDO1, GBP1, MHC II molecules, GBP1)	+	Response	Affymetrix gene expression analysis	Ji et al. 2012

FIGURE 1 | Summary of the biomarker studies performed on peripheral blood and tumor on anti-CTLA4 and anti-PD1 treatment.

BIOMARKERS FROM NEXT-GENERATION SEQUENCING

Whole exome (WES) and RNA sequencing (RNAseq) are powerful tools for evaluating the genomic landscape of a tumor. WES only captures the exonic gene regions, so it enriches for mutations in coding regions, while RNAseq can provide the entire transcriptome of a sample and is useful for establishing gene signatures for specific cohorts within a patient group. In terms of immunotherapy, WES has been useful in determining mutational load and discovering neoantigens in melanoma tumors (27). Melanoma has one of the highest mutation rates of all cancers (28–30) and has a high probability for neoantigen generation. Neoantigens are the result of somatic mutations which translate into a mutated protein that is detected and presented by APCs. Neoantigens are an attractive target for immunotherapy as they are only expressed by the tumor and not by the normal tissue. Many studies have utilized WES and RNAseq to evaluate the mutation profile and gene expression changes in patients treated with anti-CTLA-4 and anti-PD-1, with the aim to find biomarkers to predict response.

Snyder et al. performed WES on 64 patients treated with anti-CTLA-4 (31). This study was the first to associate mutational burden to clinical benefit and they also defined a neoantigen signature associated with clinical benefit. They concluded that, although patients with high mutation burden are more likely to respond to anti-CTLA-4, the types of neoantigen the patient expresses will ultimately determine their response. Van Allen et al. performed WES on 110 patients and RNAseq on 40 patients treated with anti-CTLA-4 (32). They could confirm that mutational load is associated with clinical benefit to anti-CTLA-4 treatment. Neoantigen load was also measured and this parameter was also significantly associated with response; however, they could not detect the neoantigen signature seen in the study performed by Snyder and colleagues. They concluded that clinically beneficial neoantigens are most likely private events (specific to each individual) and recurrent neoantigens (consistent in the general population) are quite rare. Van Allen and colleagues also analyzed the transcriptome in a subset of these patients and found that expression of cytolytic markers, such as granzyme A and perforin, were beneficial for response. Expression of CTLA-4 and PD-L2 was also associated with clinical benefit. Riaz et al. performed WES on 174 patients treated with anti-CTLA-4 therapy (33). They discovered 48 patients with mutations in SERPINB3 or SERPIN4 and observed that those patients were more likely to be responders. Patients with SERPINB3 or SERPINB4 mutations also had higher mutational loads. Friedlander et al. performed a quantitative polymerase chain reaction (PCR) study on peripheral blood from 360 patients receiving anti-CTLA-4 therapy (34). From a panel of 169 genes, they established a 15 gene signature that was predictive and prognostic for response and 1-year OS to anti-CTLA-4 treatment. The 15 genes are ITGA4, LARGE, CDK2, TIMP1, DPP4, NRAS, ERBB2, NAB2, ADAM17, RHOC, TGFB1, CDKN2A, HLADRA, MYC, and ICOS.

In order to elucidate resistance mechanisms and biomarkers of response to treatment, Hugo et al. used WES (38 patients) and RNAseq (28 patients) on a set of melanoma patients treated with anti-PD-1 (35). Mutational load did not have a significant association to response to anti-PD-1 therapy and neoantigen load was not significantly correlated with response either. Nonetheless, mutational load was associated with OS suggesting that other factors influence response to anti-PD-1 and survival. BRCA2 mutations occurred in 30% of the responders to anti-PD-1. RNAseq analysis uncovered a co-enrichment of 26 gene signatures in 9 of the 13 non-responding patients, which the authors termed innate anti-PD-1 resistance (IPRES) signature. They validated the IPRES signature on three other datasets and found over-representation in anti-PD-1 non-responding samples.

In a small study of four patients treated with anti-PD-1, Zaretsky et al. used WES on patients that developed new lesions under anti-PD-1 therapy. They discovered that the progressive tumors acquired JAK1, JAK2, or B2M loss of function mutations. JAK1 and JAK2 mutations cause insensitivity to interferon gamma-induced arrest and B2M mutations led to a loss of MHC class 1 expression (36). In a follow-up study, Shin et al. performed WES on 23 patients before anti-PD-1 treatment (37). In their cohort, mutational load had no association to response and one of the non-responders had a loss of function mutation in JAK1. This study confirms the role of JAK1 as a marker for innate and adaptive resistance to anti-PD-1, although it might be a rare occurrence.

Roh et al. performed WES on sequential biopsies of patients treated with anti-CTLA-4 and then anti-PD-1. Thirty patients had WES at baseline, 3 on anti-CTLA-4 treatment, 25 at anti-CTLA-4 progression, 18 on anti-PD-1 treatment, and 12 at anti-PD-1 progression. Overall, they found that mutation burden was not associated with response, but high copy number loss was associated with poor response (38). In the regions with recurrent copy number loss, PTEN was one of the notable tumor suppressor genes suggesting that it could be a driver of resistance mechanisms to immunotherapy. Another study also observed that PTEN loss was associated with resistance to anti-CTLA-4 therapy (39).

Johnson et al. performed targeted panel sequencing on 65 patients treated with anti-PD-1 therapy. In their cohort, mutational load was associated with response to anti-PD-1 and patients with high mutational load (>23.1 mutations/MB) had longer PFS and OS compared to the intermediate mutational load group (3.3–23.1 mutations/MB) and the low mutation load (<3.3 mutations/MB) group. They observed more frequent BRCA2 mutations in responders than in non-responders (5/32 vs. 2/33). LRP1B mutations were significantly enriched in the responder group (11/32) compared to the non-responder group (1/33). LRP1B mutated patients also had a higher mutational load compared to LRP1B wild-type patients.

Riaz et al. performed WES on 68 patients treated with anti-PD-1 that had previously progressed on anti-CTLA-4 therapy (35 patients) or were naïve to anti-CTLA-4 (33 patients). Mutational load was associated with clinical benefit in the anti-CTLA-4-naïve group, but not the anti-CTLA-4-resistant group. No single gene mutations were significantly associated with response or resistance to therapy. Decreased mutational and neoantigen load during therapy was associated with response in both anti-CTLA-4-naïve and anti-CTLA-4-resistant groups. RNAseq analysis of the pretreatment samples showed an enrichment in T-cell activation and lymphocyte aggregation pathways. These signatures

indicate an immunologically active tumor, or "hot tumor," and all patients with complete response or partial response in the anti-CTLA-4-resistant group had the "hot tumor" signature, although not all responders in the anti-CTLA-4-naïve group had the "hot tumor" signature. Riaz et al. also investigated the early effects of anti-PD-1 treatment (29 days after start) by RNAseq and uncovered a global increase in immune checkpoint genes such as PD-1, CTLA-4, CD274 (PD-L1), ICOS, and LAG3 in all samples. For responders, the significant pathways included inflammatory response and cytokine-mediate signaling pathways.

Finally, Davoli et al. investigated the role of aneuploidy in response to immunotherapy (40). They analyzed the copy number data from 5,255 tumor/normal samples, representing 12 cancer types from The Cancer Genome Atlas project, and found that for most tumors, there was a positive correlation between aneuploidy severity and mutational load. They also found that tumors with high levels of aneuploidy showed elevated expression of cell cycle and cell proliferation markers, as well as a reduced expression of markers for cytotoxic immune cell infiltrates. Aneuploidy levels were a stronger predictor of markers of cytotoxic immune cell infiltration than tumor mutational load. To correlate aneuploidy with response to immunotherapy, they used data from Snyder et al. and Van Allen et al. (31, 32) and found in both datasets that high levels of aneuploidy correlated with poorer survival.

BIOMARKERS FROM T-CELL RECEPTOR (TCR) PROFILING

Antigen detection by T-cells is by definition dependent on tumor-specific T-cell generation and clonal amplification. In the context of immunotherapies, which aim at enhancing the recognition of the cancer cell by the immune system, there is an obvious rational basis for examining the T-cell repertoire in order to shed light on the specific mode of action of the drug and find potential biomarkers of response.

The main challenge when analyzing TCR repertoire is its immense diversity. The TCR is a heterodimer comprised of two chains αβ or δγ. The β and δ chains, are generated by the random rearrangement of a variable region (V), a diversity region (D), and a joining region (J) with a constant region (C). The α and γ chains, consists of segments from the V, J, and C regions. Additional complexity is introduced by random addition or deletion of nucleotides at the junction sites of V, D, and J. The theoretical limit of the TCR repertoire is in the range of 10^{15}, which is several magnitudes higher than the total amount of T-cells in the body, approximately 4×10^{11} (41). The estimated number for the TCR repertoire is in the order of 10^6 to 10^8 (42). Most of the studies mainly assess the complementarity-determining region 3 (CDR3) from β chain, which is considered an acceptable proxy for estimating diversity since it is the most variable region of the receptor and that αβT-cells represent about 90% of all T-cells (43). Due to the advances in NGS, it is possible now to identify each individual TCR sequence in the CDR3 region (44). Multiplex PCR is one of the widely used methods to amplify the CDR3 region. Primers for the J alleles or the constant region of the TCR α and β chains are used together with a mix of primers for all known V alleles. A drawback of multiplex PCR is that it is limited to known V alleles. As a result, for TCR discovery experiments, other methods such as targeted enrichment—a technique where RNA baits capture the TCR receptor, usually the CDR3 region—are preferred. Since bait capture takes into account mismatches, it allows for discovery of new alleles and TCR receptors. In the context of immunotherapy, TCR repertoire analysis is useful for determining if the tumor-reactive clones have undergone activation and clonal expansion. In an adequate immune response, the tumor-specific T-cells will represent a significant proportion of the whole repertoire and therefore be assessable at the level of the whole TCR population.

In 2014, Robert et al. compared pre- to post-treatment peripheral blood mononuclear cell (PBMC) samples from melanoma patients treated with anti-CTLA-4 (45). The results from deep sequencing of the multiplex PCR for the TCR Vβ CDR3 region showed that 19 out of 21 patients had an increased number of unique clonotype (richness). There was no significant difference in the V or J segment usage and no difference in the total of unique sequences between responders and non-responders. The number of unique productive sequences in the top 25% of clones showed a particularly high increase in diversity after treatment. Those changes were not associated with peripheral lymphocyte count; however, $CD8^+$ tumor-infiltrating cells showed a positive correlation with the TCR repertoire diversity. Finally, they showed that patients experiencing more toxicities had more diverse sequences post treatment. Overall, this study reports that anti-CTLA-4 treatment increases TCR repertoire diversity in an unspecific manner. Subsequently, the same group performed a similar study on 9 anti-PD-1-treated patients and compared the results (46). Unlike the effect of anti-CTLA-4, anti-PD-1 therapy does not increase TCR repertoire complexity, on the contrary, 4/9 samples show a decrease >15% in the absolute number of unique sequences and only one had an increase >15%. Those results suggest that the mode of action of the two drugs is considerably different.

Cha et al. shed more light on the potential mechanism of anti-CTLA-4; their study assessed the changes in TCR repertoire between baseline and 4 weeks of treatment in PBMCs from 21 melanoma patients (47). They confirmed that anti-CTLA-4 treatment induces a significant change in the clonotypes frequency compared to healthy donors. They showed that the diversification is the result of a higher gain of new clonotypes and lower loss of existing ones. The number of therapy-induced expanding clones are not different between the responders and the non-responders, which is in line with what Robert et al. described. However, patients who survived longer exhibited less clonotypic changes overtime, they maintained the most abundant clones which were present at baseline and also had fewer clones significantly decreasing in frequency. Finally, they also demonstrated that the clones expanding in response to therapy are largely non-naïve T-cells, suggesting that patients who respond to the therapy already have pre-primed T-cells in circulation before the onset of anti-CTLA-4 treatment.

In light of these findings, Postow et al. hypothesized that the shape of the TCR repertoire prior to treatment initiation may influence the likelihood of a response to the treatment (48). Twelve baseline PBMC samples from 4 responders and 8 non-responders

to anti-CTLA-4 were analyzed for richness and evenness of the TCR repertoire. In this small cohort, they first showed that patients who responded to the treatment have more similar VJ usage among each other than compared to the VJ usages in the non-responders. Furthermore, low richness or evenness of the TCR repertoire was significantly associated with a poor response to anti-CTLA-4. That is, a TCR repertoire composed of less unique clones (less diverse) or skewed toward a few specific clones (very clonal) is predictive of a non-response to the treatment.

The detection of clonally expanded tumor-specific T-cell in the blood indicates that the immune system mounted an immune response against a foreign entity, which could be the tumor. However, it is not certain that the activated T-cells would be able to home to the tumor efficiently and be able to kill the cancer cells. Therefore, it is also important to assess the immune status at the tumor site.

Tumeh et al. performed a very elegant study exploiting tumor samples from melanoma patients prior and during anti-PD-1 treatment (49). By qualitative and quantitative immunohistochemistry, they revealed that, at baseline, patients who eventually respond to the therapy, have more $CD8^+$ T-cells at the invasive margin of the tumor compared to non-responders. This population increases and migrates toward the center of the tumor during treatment in responders. They showed that an efficient response to anti-PD-1 therapy requires pre-existing $CD8^+$ T-cells, which are most likely tumor specific. To confirm this theory, they sequenced the TCR Vβ region of tumor baseline samples and found that responders indeed had a more clonal TCR repertoire. On treatment, samples of responders showed significantly more clonal expansion than non-responders.

Johnson et al. used NGS to assess differences between baseline samples of responders and non-responders to anti-PD-1 therapy (50). They assessed mutational load as well as specific mutations differentially occurring in responders and non-responders. They also investigated the TCR repertoire clonality of 42 samples and did not find any association with response. However, it is important to mention that times of sample acquisition were not immediately before and after treatment. The timing was quite broad, the study allowed the inclusion of samples collected over 12 months before start of treatment and also after treatment initiation. When the analysis was performed only on samples obtained within 4 months of treatment initiation, the non-responder group was only represented by five samples, including one potential outlier. Nonetheless, they noticed a trend toward higher clonality at baseline in patients who eventually responded to the therapy.

Inoue et al. analyzed the TCR repertoire of 10 pre- and post-anti-PD-1 tumor samples (51). They noticed that the clonotypes with a read frequency >0.5% at baseline significantly increased after treatment in responders. The calculation of the diversity index highlighted a slight decrease in tumors of responders compared to non-responders, which suggests oligoclonal expansion of certain TCR clones.

More recently, Roh et al. published a complementary analysis on a cohort of patients for which they performed TCR sequencing (38). They analyzed tumor samples from melanoma patients treated sequentially with anti-CTLA-4 and anti-PD-1 *via* WES and TCR sequencing. The TCR clonality assay revealed that there was no significant difference between responders and non-responders, pre- or on-treatment with anti-CTLA-4. A subpopulation of the patients ($n = 8$) received anti-CTLA-4 followed by anti-PD-1 after progression. All three responders to anti-CTLA-4 followed by anti-PD-1 showed an increase in TCR clonality during anti-CTLA-4 treatment. In addition, higher TCR clonality was seen in the responders prior to treatment and on treatment with anti-PD-1.

Riaz et al. investigated the evolution of melanoma tumors and their microenvironment under anti-PD-1 therapy (52). Patients who had previously progressed on anti-CTLA-4 and were naïve to anti-CTLA-4 were included in the study. They performed TCR sequencing on 34 samples pre- and 4 weeks post- anti-PD-1 treatment. There were no statistically significant differences in the baseline samples of either group. On anti-PD-1 therapy, the anti-CTLA-4-pretreated group had increased TCR richness associated with response, whereas the anti-CTLA-4-naïve group had decreased TCR evenness associated with response. In line with Roh et al., pretreatment with anti-CTLA-4 seems to increase the expansion of tumor-specific T-cell cells, which are additionally expanded during anti-PD-1 treatment.

MASS CYTOMETRY (CyTOF)

The advent of CyTOF has allowed a more comprehensible analysis of the whole immune system and will be an important asset for immune oncology (53). The basic principle of CyTOF is similar to conventional flow cytometry. The assay quantifies multiple protein expression markers at the single-cell level. In contrast to flow cytometry, the detection is not achieved by fluorophore excitation, but by stable mass isotope quantification. The transition isotope bound to the antibodies are analyzed by a time of flight mass spectrometer. CyTOF has some advantages over flow cytometry, namely, the high purity of the metal isotopes reduces background noise, eliminating spectral spillover and cellular autofluorescence associated with conventional flow cytometry. It also enables the detection of more markers in the same experiment, theoretically up to a hundred. Multiple samples can be analyzed at the same time thanks to a barcoding strategy (up to 20), and therefore reduce inter-sample variation. CyTOF has primarily been used to analyze peripheral blood from patients undergoing immunotherapy. A better characterization of the precise mode of action of those drugs is crucial to help overcoming and predicting resistance as well as contributing to optimal development of future combination therapies.

In 2015, Das et al. analyzed peripheral blood from melanoma patients undergoing immunotherapy with anti-CTLA-4, anti-PD-1, or the combination of the two (54). Samples were collected at baseline and after 3 weeks of treatment. In this early study, CyTOF was mainly used to further characterize the cell population of interest previously identified by flow cytometry. The analysis revealed that the $Ki67^+$ cells, increasing after combination treatment, have a transitional memory T-cell-like phenotype. Additional experiments were performed using other techniques than CyTOF, which lead the authors to conclude that anti-PD-1 and anti-CTLA-4 have distinct effects on the immune system.

In the context of a clinical trial assessing the safety of combining radiotherapy and immunotherapy in melanoma patients,

Hiniker et al. analyzed baseline and follow-up PBMC samples from 9 patients (3 progressive disease, 6 complete response/partial response) (55). CyTOF analysis revealed that the level of $CD8^+$ T-cells expressing IL-2 were higher at baseline and in the follow-up samples of responding patients. The same was true for central memory $CD8^+$ T-cell levels. However, the cytokine production was not significantly different from the population seen in non-responding patients, thereby suggesting that the cells are not functionally different from the non-responders.

The first study to use CyTOF as a main technique for analyzing human melanoma patient samples was performed by Wistuba-Hamprecht et al. in early 2017 (56). The analysis consisted in performing CyTOF on 28 PBMC samples from stage IV melanoma patients who received different courses of treatments. A higher frequency in the APC-like population had a positive association with OS, whereas a higher frequency in the MDSC-like population showed negative association with OS. Overall, an equal abundance of MDSC- and APC-like cells is associated with better survival. The analysis of the T-cell compartment revealed that there was a clear interpatient heterogeneity in the $CD4^+$ and $CD8^+$ T-cell compartments compared to the other compartments which have more homogenous frequencies between patients. Only one αβT-cell population had some prognostic potential: a higher level of early differentiated $CD4^+$ T-cell was correlated with poorer OS. In the natural killer cell compartment, a highly cytotoxic cell population tends to correlate with better OS. Finally, a comprehensive analysis of immune signatures of all the melanoma-associated phenotypes identified a specific cluster with high prognostic capacity, performing even better than LDH. This cluster is significantly associated with poor OS and represented by an overall lower diversity across all the compartments, and especially in the myeloid compartment.

Takeuchi et al. investigated the effect of immunotherapy in melanoma patients by comparing PBMCs from 4 different patients receiving anti-PD-1 (2 responders and 2 non-responders) (57). The panel was composed of 35 markers and they used high-dimensional clustering to analyze the data. The main finding in this paper is that $CD4^+$ and $CD4^+CD8^+$ cell populations increase during therapy. $CD4^+CD27^+FAS^-$ central memory T-cell were shown to expand in a higher proportion in responders than in non-responders. These results were validated in a separate cohort ($n = 4$).

More recently, Krieg et al. performed a comprehensive analysis, assessing the correlation between baseline peripheral immune signature and response to anti-PD-1 in melanoma patients (58). The cohort was composed of 20 patients from whom baseline and on treatment samples were obtained. They used an optimized immune marker panel and a customized, interactive bioinformatics pipeline in order to identify potential predictive biomarkers. Three different CyTOF panels were used: one for the phenotypic characterization of lymphocytes, one to assess the T-cell functions, and the third one to characterize monocytes, which consisted of 30, 26, and 25 markers, respectively. By performing hierarchical clustering of all the samples pooled together, they identified a differential marker expression in responders compared to non-responders. Further analysis, and validation in an independent, blinded cohort by conventional flow cytometry, revealed that, at baseline, responders had a higher frequency of classical monocytes and lower frequency of lymphocytes compared to the non-responders.

OUTLOOK

The development of high-throughput technologies such as NGS and CyTOF have allowed researchers and clinicians to evaluate hundreds to tens of thousands of genes from a bulk tumor to a single-cell level. NGS is an invaluable tool for analyzing mutations and copy number profiles, gene expression changes and gene signatures, epigenetic alterations, the TCR repertoire, and single-cell gene expression changes. The recent development of CyTOF has also allowed the analysis of many markers at a single-cell level. In the context of immunotherapy, these high dimensional datasets will enhance the discovery of novel biomarkers, prognostic markers, and resistance mechanisms.

Next-generation sequencing biomarker discovery for anti-CTLA-4 treatment have uncovered that mutational load and neoantigen load are the most informative for response and OS, but they are not perfect biomarkers as some non-responders may also present with high mutational load. Aneuploidy could also help foresee response to anti-CTLA4 since it was highlighted as an independent predictor in a multivariate Cox model which included mutational load. Copy number analysis could as well be informative as loss in chromosome 10 was shown to be a poor prognostic marker in two studies. Many of these studies also analyzed tumor samples upon progression and found no recurrent genetic mutation, which could mean that resistance to anti-CTLA-4 is patient specific. In the context of anti-PD-1 treatment, mutational load is not a clear informative marker for response. As anti-PD-1 is a relatively new therapy, no large cohort studies with over 100 patients for NGS biomarker discovery have yet been performed. There are single patient examples showing that genes involved in the JAK–STAT pathway or antigen presentation could be predictive biomarkers for anti-PD-1 treatment. Loss of function mutations in JAK1, JAK2, and B2M are negative biomarkers for response and are involved in resistance to anti-PD-1 treatment in individual cases, but these mutations do not seem to be recurrent. RNAseq analysis from several studies suggest that tumors with high immune activity are more likely to respond to anti-PD-1. To better stratify patients for anti-CTLA-4 or anti-PD-1 treatment, a combinatorial approach investigating WES, copy number variation, and RNAseq would be needed.

Overall, most studies support that anti-CTLA-4 and anti-PD-1 modulate TCR repertoire clonality upon treatment. This strengthens the notion that tumor-specific T-cell populations are affected by CTLA-4 or PD-1 inhibition. In summary, most studies support that anti-CTLA-4 induces an expansion of clones in a non-specific manner and, therefore, broadens the TCR repertoire. On the other hand, anti-PD-1 seems to favor the proliferation of fewer specific clones giving rise to a more skewed repertoire, thereby suggesting that the baseline TCR repertoire of the patients plays a role in the response to the treatment. However, for the moment, those predictions arise mainly from early on-treatment evaluations that examined the evolution of the repertoire from baseline, as we are not yet able to precisely

pinpoint the tumor-specific clones that, once clonally expanded, will facilitate tumor elimination. It is also important to highlight that the mode of action of the current immunotherapies are still debated and we do not fully comprehend their overall impact on different immune cell subpopulations. As a result, it is difficult to assess the global impact of the drugs on the immune response by investigating specific mechanisms individually. This is why high-throughput techniques discussed here are powerful emerging tools, which will allow us to elucidate this problem by looking at numerous markers simultaneously. The more we increase our knowledge of exact mechanisms, the better we will be able to exploit the therapies by using them in a targeted/patient-specific manner. Interesting work by Twyman-Saint et al. combining anti-CTLA-4, anti-PD-1 and radiotherapy, underpins this assertion (59).

To our knowledge, despite the great potential held by CyTOF technology, to date, no research was published on the analysis of human melanoma tumor samples in the context of immunotherapies. One should however expect to see more forthcoming data, thanks to a novel exciting add-on technology that is starting to emerge. Indeed, a new laser system can be coupled to the CyTOF device which allows for imaging mass cytometry (60). That is, the detection of metal-labeled antibodies, as in standard CyTOF analysis, but performed on tissue sections by using multiplexed ion beam imaging. This state of the art technology will allow, not only to assess a high range of markers at the same time, but also to obtain spatial resolution and warrant a very comprehensive analysis of the cell–cell interaction in the tumor microenvironment. New developments of the system should soon facilitate the analysis of tumor samples in a similar fashion, while gaining spatial resolution to better interrogate the role of spatial interactions in immunotherapy response (with high throughput) (61).

In conclusion, the use of NGS and CyTOF has great potential to discover novel biomarkers for immunotherapy and the studies discussed above show exciting promises, but need to be further validated before clinical application. New prospective trials with large cohorts could include these technologies as a biomarker discovery platform and could validate many of these findings. In parallel, new algorithms to integrate multiple high dimensional datasets are being developed for a combinatorial biomarker approach, which could use these existing datasets as a training model. As NGS is becoming a standard service in many clinics, the development of next generation biomarkers should ultimately improve the stratification of patients for immunotherapy and thereby extend OS for these patients.

AUTHOR CONTRIBUTIONS

SH, ML, and PC conceptualized the manuscript and oversaw all aspects of its completing including writing, figure design, and literature review.

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Anti-PD-1 and Anti-CTLA-4 Therapies in Cancer: Mechanisms of Action, Efficacy, and Limitations

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Melanoma, a skin cancer associated with high mortality rates, is highly radio- and chemotherapy resistant but can also be very immunogenic. These circumstances have led to a recent surge in research into therapies aiming to boost anti-tumor immune responses in cancer patients. Among these immunotherapies, neutralizing antibodies targeting the immune checkpoints T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) are being hailed as particularly successful. These antibodies have resulted in dramatic improvements in disease outcome and are now clinically approved in many countries. However, the majority of advanced stage melanoma patients do not respond or will relapse, and the hunt for the "magic bullet" to treat the disease continues. This review examines the mechanisms of action and the limitations of anti-PD-1/PD-L1 and anti-CTLA-4 antibodies which are the two types of checkpoint inhibitors currently available to patients and further explores the future avenues of their use in melanoma and other cancers.

Keywords: immunotherapy, cancer, melanoma, side effects, biomarkers, immune checkpoint inhibitors, mode of action

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INTRODUCTION

In recent years, there has been a steep rise in the development and implementation of anti-cancer immunotherapies. The approval of anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and anti-programmed cell death protein 1 (PD-1) antibodies for human use has already resulted in significant improvements in disease outcomes for various cancers, especially melanoma. Unlike radio- and chemotherapy, which aim to directly interfere with tumor cell growth and survival, immunotherapies target the tumor indirectly by boosting the anti-tumor immune responses that spontaneously arise in many patients.

Abbreviations: ctDNA, circular tumor DNA; CTLA-4, T-lymphocyte-associated protein 4; DCs, dendritic cells; IPRES, innate anti-PD-1 resistance; LDH, lactate dehydrogenase; NK, natural killer cells; PD-1, programmed cell death protein 1; IDO, indoleamine 2,3-dioxygenase; IL-12, interleukin 12; TGF-β, tumor growth factor-β; Tregs, regulatory T cells; MDSCs, myeloid-derived suppressor cells; VISTA, V-domain Ig suppressor of T cell activation; ITIM, immunoreceptor tyrosine-based inhibition motif; IFN-γ, interferon; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; LAG-3, lymphocyte-activation protein 3; TIGIT, T-cell immunoreceptor with Ig And ITIM domains; TNF-α, tumor necrosis factor-α; ICOS, inducible co-stimulatory molecule; IFN-γ, interferon-γ; BTLA, B- And T-lymphocyte-associated protein; CSF-1R, colony stimulating factor-1 receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; Breg, regulatory B cell.

CANCERS EVADE AND INHIBIT IMMUNE RESPONSES

In order to understand the modes of action of immune checkpoint inhibitors, it is important to understand the dynamic interplay between cancers and the immune system during the course of the disease.

Cancer cells are genetically unstable, which contributes to their uncontrolled proliferation and the expression of antigens that can be recognized by the immune system. These antigens include normal proteins overexpressed by cancer cells and novel proteins that are generated by mutation and gene rearrangement (1). Cytotoxic $CD8^+$ T cells are immune cells that are particularly effective at mediating anti-tumor immune responses. These cells may learn to recognize the tumor-specific antigens presented on major histocompatibility complex (MHC) class I molecules and thereby perform targeted tumor cell killing. $CD8^+$ T cells become licensed effector cells after appropriate stimulation by antigen-presenting cells that have collected antigens at the tumor site. Apart from the antigen peptides embedded on the MHC molecules, antigen-presenting cells must provide costimulatory signals through surface receptors (such as CD28) and cytokines [such as interleukin (IL)-12] for effective T cell stimulation (2).

Tumor cells adopt a variety of mechanisms to avoid immune recognition and immunomediated destruction. Established tumors are often thought to arise through the selection of clones that are able to evade the immune system, a process known as immunoediting (3). Tumor cells may evade immune recognition directly by downregulating features that make them vulnerable such as tumor antigens or MHC class I (4–6). Alternatively, tumors may evade immune responses by taking advantage of negative feedback mechanisms that the body has evolved to prevent immunopathology. These include inhibitory cytokines such as IL-10 and tumor growth factor (TGF)-β, inhibitory cell types such as regulatory T cells (Tregs), regulatory B cells (Bregs), and myeloid-derived suppressor cells (MDSCs), metabolic modulators such as indoleamine 2,3-dioxygenase (IDO), and inhibitory receptors such as PD-1 and CTLA-4 (7, 8).

IMMUNE EXHAUSTION CONTRIBUTES TO IMMUNE DYSFUNCTION IN CANCER

Inhibitory receptors, also known as immune checkpoints, and their ligands can be found on a wide range of cell types. They are essential for central and peripheral tolerance in that they counteract simultaneous activating signaling through co-stimulatory molecules. Inhibitory receptors may act during both immune activation and ongoing immune responses. During chronic inflammation in particular, T cells are known to become exhausted and to upregulate a wide range of non-redundant inhibitory receptors that limit their effectiveness, such as PD-1, CTLA-4, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), or T-Cell immunoreceptor with Ig And ITIM domains (TIGIT) [See **Table 1** (9–11)]. Originally described in the context of chronic viral infections, where the host fails to clear the pathogen, it is now apparent that exhausted T cells can also occur in cancer (12, 13). It is believed that, under these conditions, persistent high antigenic load leads to the T cells upregulating the inhibitory receptors, whose signaling subsequently leads to a progressive loss of proliferative potential and effector functions and in some cases to their deletion (14).

Exhaustion is therefore both a physiological mechanism designed to limit immunopathology during persistent infection and a major obstacle for anti-tumor immune responses (17). It should be noted that expression of inhibitory markers is not always a sign of immune exhaustion, because the receptors may be expressed individually during conventional immune responses (18).

THE IMMUNE CHECKPOINT RECEPTOR CTLA-4

The anti-CTLA-4 blocking antibody ipilimumab was the first immune checkpoint inhibitor to be tested and approved for the treatment of cancer patients (19, 20). CTLA-4 (CD152) is a B7/CD28 family member that inhibits T cell functions. It is constitutively expressed by Tregs but can also be upregulated by

TABLE 1 | Overview of T cell surface receptors associated with immune inhibition and dysfunction.

Receptor	Expressing cells	Ligands	Ligand-expressing cells
Programmed cell death protein 1 (PD-1) (11)	CD4 (activated/exhausted, follicular), CD8 (activated/exhausted), B cells, dendritic cells (DCs), monocytes, mast cells, Langerhans cells	PD-L1, PD-L2	Antigen-presenting cells, $CD4^+$ T cells, non-lymphoid tissues, some tumors
T-lymphocyte-associated protein 4 (CTLA-4) (15)	CD4 (activated/exhausted, Tregs), CD8 (activated/exhausted), some tumors	CD80, CD86	Antigen-presenting cells
lymphocyte-activation protein 3 (LAG-3) (15)	CD4 (including Treg and exhausted), CD8 (including exhausted), natural killer cells (NK)	MHC class II, LSECtin	Antigen-presenting cells, liver, some tumors
T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) (16)	CD4 (Th1, Th17, Treg), CD8 (including exhausted and Tc1), DC, NK, monocyte, macrophages	Galectin-9, phosphatidyl serine, high mobility group protein B1, Ceacam-1	Endothelial cells, apoptotic cells, some tumors
T-cell immunoreceptor with Ig And ITIM domains (TIGIT) (16)	CD4 (including Treg, follicular helper T cells), CD8, NK	CD155 (PVR), CD122 (PVRL2, nectin-2)	APCs, T cells, some tumors

other T cell subsets, especially $CD4^+$ T cells, upon activation (21). Exhausted T cells are also often characterized by the expression of CTLA-4 among other inhibitory receptors. CTLA-4 is mostly located in intracellular vesicles and is only transiently expressed upon activation in the immunological synapse before being rapidly endocytosed (22).

CTLA-4 mediates immunosuppression by indirectly diminishing signaling through the co-stimulatory receptor CD28. Although both receptors bind CD80 and CD86, CTLA-4 does so with much higher affinity, effectively outcompeting CD28 (23). CTLA-4 may also remove CD80 and CD86 (including their cytoplasmic domains) from the cell surfaces of antigen-presenting cells *via* trans-endocytosis (24), therefore reducing the availability of these stimulatory receptors to other CD28-expressing T cells. Indeed, this process is an important mechanism by which Tregs mediate immune suppression on bystander cells (25).

By limiting CD28-mediated signaling during antigen presentation, CTLA-4 increases the activation threshold of T cells, reducing immune responses to weak antigens such as self- and tumor antigens. The central role that CTLA-4 plays in immunological tolerance is exemplified by experiments in mice that lack the CTLA-4 gene globally or specifically in the Forkhead box P3 $(FoxP3)^+$ Treg compartment. These animals develop lymphoproliferative disorders and die at a young age (25, 26). Similarly, polymorphisms within the CTLA-4 gene are associated with autoimmune diseases in humans (27). CTLA-4 signaling has been shown to dampen immune responses against infections and tumor cells (28, 29).

THE IMMUNE CHECKPOINT RECEPTOR PD-1

The surface receptor PD-1 (CD279) was first discovered on a murine T cell hybridoma and was thought to be involved in cell death (30). It has since become clear, however, that PD-1, which is homologous to CD28, is primarily involved in inhibitory immune signaling, and is an essential regulator of adaptive immune responses (31). In both humans and mice some T cell populations constitutively express PD-1; one example is follicular helper T cells (32). Although most circulating T cells do not express the receptor, they can be induced to do so upon stimulation, through the T cell receptor (TCR) complex or exposure to cytokines such as IL-2, IL-7, IL-15, IL-21, and transforming growth factor (TGF)-β (33, 34). Other cell types, such as B cells, myeloid dendritic cells, mast cells, and Langerhans cells, can also express PD-1 which may regulate their own and bystander cell functions under pathophysiological conditions (35–38). PD-1 has two ligands: PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273). Both can be found on the surface of antigen-presenting cells (such as dendritic cells, macrophages, and monocytes), but are otherwise differentially expressed on various non-lymphoid tissues (39, 40). Interferon (IFN)-γ is the main trigger known to cause PD-L1 and PD-L2 upregulation (41).

PD-1 bears an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) motif on its intracellular tail. The intracellular signaling events initiated upon PD-1 engagement are best described in T cells and are illustrated in **Figure 1**. In these cells, engagement of PD-1 causes tyrosine residues to become phosphorylated, starting an intracellular signaling cascade that mediates the dephosphorylation of TCR proximal signaling components (9, 42–44). Among these, CD28 has recently been found to be the primary target (45). In the presence of TCR stimulation, CD28 provides critical signals that are important for T cell activation. By interfering with early TCR/CD28 signaling and associated IL-2-dependent positive feedback, PD-1 signaling therefore results in reduced cytokine production [such as IL-2, IFN-γ, and tumor necrosis factor (TNF)-α], cell cycle progression, and pro-survival Bcl-xL gene expression, as well as reduced expression of the transcription factors involved in effector functions such as T-bet and Eomes (42, 43, 46, 47). PD-1 activity is therefore only relevant during simultaneous T cell activation, as its signal transduction can only come into effect during TCR-dependent signaling (39, 41, 48). Details about PD-1 signaling in other cell types that bear this receptor, such as B cells, remain to be elucidated.

Overall, PD-1 is crucial for the maintenance of peripheral tolerance and for containing immune responses to avoid immunopathology. Mice deficient in the receptor initially appear healthy, but develop autoimmune diseases such as lupus-like proliferative glomerulonephritis and arthritis with age and exacerbated inflammation during infections (18, 31, 49, 50). Humans with genetic polymorphisms in the PD-1 locus also have an increased likelihood of developing various autoimmune diseases (51, 52).

CTLA-4, PD-1, AND THEIR LIGANDS IN CANCER

CTLA-4 may be expressed in tumor lesions on infiltrating Tregs or exhausted conventional T cells as well as tumor cells themselves (53, 54). Despite the immunosuppressive role of CTLA-4, its association with disease prognosis is not clear; however, it should be noted that only a few studies have described the prognostic value of CTLA-4 levels in the tumor site. So far, the expression of CTLA-4 on tumors has been associated with decreased survival in nasopharyngeal carcinoma (54) and increased survival in non-small cell lung cancer (53).

PD-1 can be upregulated transiently during stimulation and constitutively during chronic immune activation (17). The inhibitory receptor has been detected on both circulating tumor-specific T cells and tumor-infiltrating lymphocytes, where it was associated with decreased T cell function in humans and mice (13, 29, 55–57). Other cell types may also upregulate PD-1 in tumor lesions. PD-1-positive dendritic cells, for example, have been identified in hepatocellular carcinoma where they exhibited a reduced ability to stimulate T cells (37). Another study identified a population of tumor-infiltrating PD-1-expressing regulatory B cells that

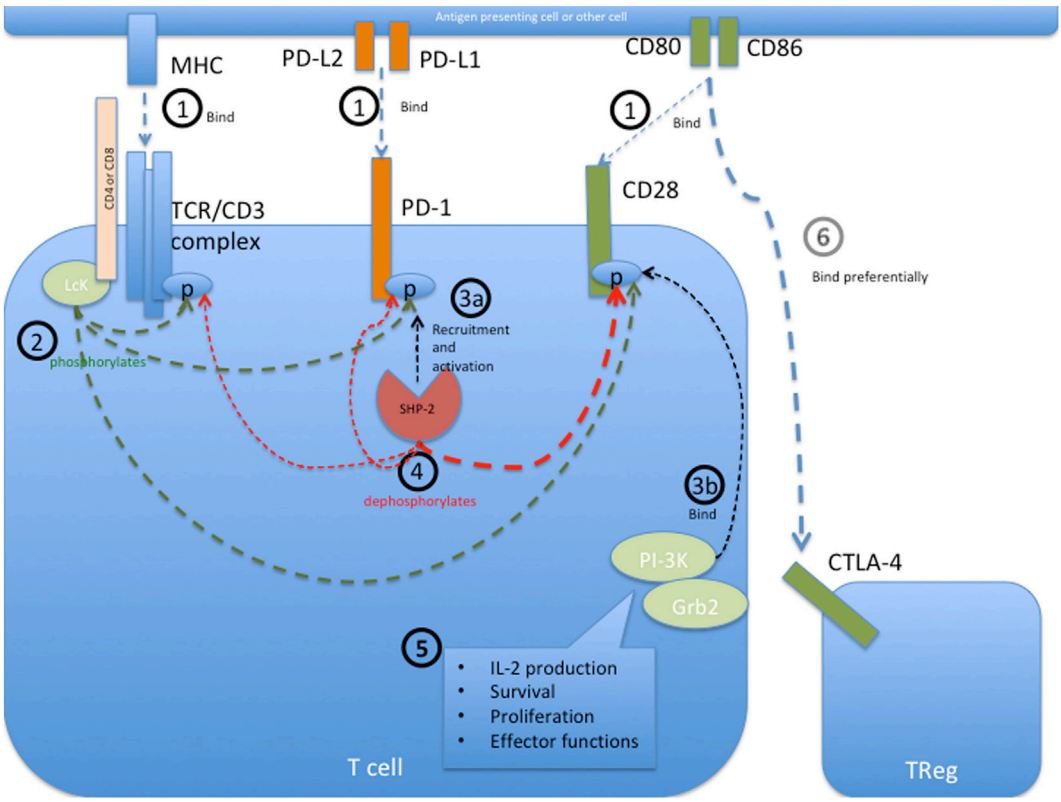

FIGURE 1 | Programmed cell death protein 1 (PD-1) mediated intracellular signaling events during T cell activation. **(1)** Upon T cell activation, the extracellular receptors PD-1, CD28, and the T cell receptor (TCR) complex (including CD4 or CD8) bind their ligands PD-L1 or PD-L2, CD80 or CD86, and major histocompatibility complex (MHC) class I or II, respectively. This brings all the receptors into close proximity with each other at the immunological synapse and allows them to interact with each other. **(2)** The Src kinase Lck (P56Lck), which is bound to the intracellular tail of CD4 and CD8, can now phosphorylate the tyrosine residues on the intracellular tails of PD-1 and CD28 as well as the CD3ζ chain of the TCR/CD3 complex. **(3a)** Phosphorylation of the immunoreceptor tyrosine-based switch motif (ITSM) motif on the intracellular tail of PD-1 allows recruitment of the Src homology region 2 domain-containing phosphatase 2 (SHP-2), resulting in the activation of SHP-2 phosphatase activity. SHP-1 may also bind PD-1 but to a lesser extent than SHP-2. **(3b)** Simultaneously, the phosphorylated tail of CD28 is now able to recruit PI-3K and Grb2 among other signaling molecules. **(4)** Through close proximity at the immunological synapse, PD-1-associated SHP-2 can dephosphorylate the cytoplasmic tail of CD28, and to a lesser extent that of the CD3ζ chain, therefore preventing the recruitment of further downstream signaling molecules associated with these molecules. SHP-2 may also dephosphorylate PD-1, causing auto-regulation of this inhibitory pathway. **(5)** CD28 provides critical signals alongside TCR stimulation, and the abrogated binding of PI3K and Grb2 to this receptor therefore leads to decreased signaling in pathways important for IL-2 production, survival, proliferation, and certain effector functions. In the absence of its ligands, PD-1 is not recruited to the immune synapse and can therefore not interfere with activation signaling. **(6)** The inhibitory receptor CTLA-4 primarily restricts CD28 signaling indirectly by reducing the availability of CD80 and CD86, to which it binds with a much higher affinity than the co-stimulatory receptor CD28. Sources (43–45).

produced IL-10; higher proportions of these cells were correlated with worse disease outcome in hepatocellular carcinoma patients (58). Tumor-associated macrophages were also recently shown to express PD-1 in both mice and humans with colorectal cancer and to impair macrophage phagocytosis (59).

Both cancer cells and tumor-infiltrating immune cells (such as macrophages) may express PD-L1 and upregulate it in response to IFN-γ (60). PD-L1 expression may therefore be indicative of active anti-tumor immune responses and may also actively contribute to local immunosuppression. The relationship between PD-1 or PD-L1 expression at the tumor site and disease outcome is thus not consistent among all tumor types and patients. High PD-1 and/or PD-L1 may correlate with poor prognosis in some cancers (including melanoma, renal cell carcinoma, esophageal, gastric, and ovarian cancers) and with improved prognosis in others (such as angiosarcoma and gastric cancer) (55, 60–65).

EFFICACY AND MODE OF ACTION OF CHECKPOINT INHIBITORS

Both CTLA-4 and PD-1 checkpoint inhibitors have resulted in increased patient survival in a number of studies, including studies on melanoma, renal cell carcinoma, squamous cell carcinoma, and non-small cell lung cancer, when compared to conventional chemotherapies (summarized in **Table 2**). In melanoma, anti-PD-1 treatment was more effective in patients with smaller tumors (66). A direct comparison between the two checkpoint inhibitors in a Phase III clinical trial found better response (44%) and survival rates (6.9 months progression-free survival) among

TABLE 2 | Treatment outcome of clinical trials for immune checkpoint inhibitors in various cancer types.

Target		Drug	Condition	Treatment regimen	Treatment in control group	Objective response rate	Complete response rates	Overall survival (months)	Progression-free survival (months)	Grade 3–5 adverse events	Participants treated (and controls)	Reference
Programmed cell death protein 1 (PD-1) signaling	PD-1	Nivolumab (IgG4a)	Melanoma (stage III/IV)	3 mg/kg/2 weeks	(*vs* combination therapy)	43.7%	8.9%	n/a	6.9	16.3%	316	(67)
			Renal cell carcinoma (metastatic)	3 mg/kg/2 weeks	10 mg/day Everolimus	25% (4% control)	1% (<1% control)	25.0 (19.6 control)	4.6 (4.4 control)	19% (27% control)	406 (397 control)	(68)
			Hodgkin's lymphoma (relapsed/refractory)	3 mg/kg/2 weeks	n/a	87%	17%	n/a	86% at 24 weeks	22%	23	(69)
			Squamous-cell carcinoma of the head and neck (recurrent)	3 mg/kg/2 weeks	Single-agent systemic therapy (methotrexate, docetaxel, or cetuximab)	13.3% (5.8% control)	2.5% (0.8% control)	36.0%/1 year (16.6% control)	19.7% at 6 months (9.9% control)	13.1% (35.1%)	240 (121 control)	(70)
			Non-small cell lung cancer	3 mg/kg/2 weeks	Docetaxel	19% (12% control)	1% (<1% control)	12.2 (9.4 control)	2.3 (4.2 control)	10% (54% control)	292 (290 control)	(71)
				3 mg/kg/2 weeks	Docetaxel	20% (9% control)	1% (0% control)	9.2 (6 control)	3.5 (2.8 control)	7% (55% control)	135 (137 control)	(72)
			Ovarian cancer (platinum-resistant)	1 or 3 mg/kg/2 weeks	n/a	15%	10%	20	3.5	40%	20	(62)
		Pembrolizumab (IgG4a)	Melanoma (stage III/IV)	10 mg/2 weeks or 3 weeks	(*vs* ipilimumab)	33.7–32.9%	5.0–6.1%	n/a	5.5–4.1	13.3–10.1%	279–277	(73)
			Merkel cell carcinoma	2 mg/kg/3 weeks	n/a	56%	16%	n/a	65% at 6 months	15%	26	(74)
			Non-small cell lung cancer	2 mg/kg/3 weeks 10 mg/kg/3 weeks 10 mg/kg/2 weeks	n/a	19.4%	n/a	12	3.7	9.5%	495	(75)
				200 mg/2 weeks (PD-L1 + patients only)	Platinum-based chemotherapy	44.8 (27.8% control)	n/a	80.2% at 6 months (72.4% control)	10.3 (6 control)	26.6% (53.3% control)	154 (154 control)	(76)
				2 or 10 mg/kg/3 weeks (PD-L1 + patients only)	Docetaxel	18/18% (9% control)	0/0% (0% control)	10.4/12.7 (8.5 control)	3.9/4.0 (4.0 control)	13/16% (35% control)	345/346 (343 control)	(77)
			Progressive metastatic colorectal cancer	10 mg/kg/every 2 weeks	n/a	40/0%	0/0%	>5 months/5	>5/2.2	41% overall	10/18	(78)
		Pidilizumab (IgG1)	B cell lymphoma (after autologous stem cell transfer)	1.5 mg/42 days	n/a	51%	34%	85% at 16 months	72% at 16 months	n/a	66	(79)

(*Continued*)

TABLE 2 | Continued

Target		Drug	Condition	Treatment regimen	Treatment in control group	Objective response rate	Complete response rates	Overall survival (months)	Progression-free survival (months)	Grade 3–5 adverse events	Participants treated (and controls)	Reference
			Follicular lymphoma (relapsed)	3 mg/kg/4 weeks (+ rituximab)	n/a	66%	52%	n/a	n/a	0%	29	(80)
	PD-L1	Atezolizumab (IgG1)	Non-small cell lung cancer (stage III–IV)	1,200 mg/3 weeks	Docetaxel	18% (16% control)	2% (<1% control)	15.7 (10.3 control)	2.8 (4 control)	15% (43% control)	425 (425 control)	(81)
			Urothelial carcinoma (locally advanced and metastatic)	1,200 mg/3 weeks	n/a	23%	9%	15.9%	2.7	16%	119	(82)
T-lymphocyte-associated protein 4 (CTLA-4) signaling	CTLA-4	Ipilimumab (IgG1)	Melanoma (stage III/IV)	10 mg/kg plus decarbazine	Decarbazine alone	15.2% (10.3% control)	1.6% (0.8% control)	11.2 (9.1 control)	n/a	56.3% (27.5%)	250 (252 control)	(83)
				3 mg/kg/3 weeks	(*vs* Pembrolizumab)	11.9%	1.4%	n/a	2.8	19.9%	278 315	(73)
				3 mg/kg/3 weeks	(*vs* combination with nivolumab)	19%	2.2%	n/a	2.9	27.3%	311	(67)
		Tremelimumab (IgG2)	Melanoma (stage III/IV)	15 mg/kg/90 days	chemotherapy (temozolomide or dacarbazine)	10.7% (9.8% control)	3% (2% control)	12.6% (10.7 control)	20.3% at 6 months (18.1% control)	52% (37% control)	328 (327 control)	(84)
Combination therapy		Nivolumab + Ipilimumab	Melanoma (stage III/IV)	3 mg/kg/2 weeks Nivolumab 3 mg/kg/3 weeks Ipilimumab	(*vs* single)	57.6%	11.5%	n/a	11.5	55%	314	(67)
			Non-small cell lung cancer	Nivo + Ipi: 1 + 3 or 3 + 1 mg/ml	Nivolumab alone	23/19% (10% control)	2/0% (0%)	7.7/6 (4.4)	2.6/1.4 (1.4 control)	30/19% (13% control)	61/54 (98 control)	(85)

n/a: not available.

Where the median values for overall or progression-free survival were not reached within the time frame of a study and the percentage of patients surviving for a given time frame are shown instead.

The anti-PD-L1 antibodies avelimumab and durvalumab are currently undergoing early-stage clinical trials and therefore no data has yet been published on their efficacy.

patients treated with the anti-PD-1 antibody nivolumab than among those treated with the anti-CTLA-4 antibody ipilimumab (19% and 2.8 months). Combined administration of both nivolumab and ipilimumab resulted in even higher response rates (58%) and survival (11.5 months) (67).

Both CTLA-4 and PD-1 act independently as brakes on CD3/CD28-dependent signaling, suggesting that underlying immune responses are required for checkpoint inhibitor treatment to take effect (66). Indeed, as mentioned in the previous section, both PD-1 and CTLA-4 blockades are more effective in tumors that are infiltrated by T cells or that have high mutation rates and are therefore more immunogenic prior to treatment (86–88).

The direct immunological consequences of anti-PD-1 and anti-CTLA-4 treatments have mostly been investigated in T cells (**Figure 2**). It is thought that the blockade of CTLA-4 most likely impacts the stage of T cell activation in the draining lymph nodes when CTLA-4 expressing Tregs remove CD80/CD86 from the surface of antigen-presenting cells, thereby reducing their ability to effectively stimulate tumor-specific T cells (24). CTLA-4 blockade may also take effect at the tumor site as exhausted CTLA-4-expressing T cells and Tregs can accumulate within the tumor microenvironment (29, 53). PD-1-expressing tumor-infiltrating T cells can be disabled by PD-L1 on the surfaces of tumor cells or other infiltrating immune cells, and blocking antibodies targeting PD-1 signaling are therefore thought to mainly affect the effector stage of the immune response (13, 55–57). Since other cell types (such as dendritic cells and B cells) can also be influenced by PD-1 signaling, inhibition of the PD-1/PD-L1 pathway may also have T cell-independent effects, whose impact on immune responses during checkpoint inhibitor therapy remain to be elucidated (36, 58).

Type I immune responses, which include IFN-γ production and cytotoxic T cell functions, are important for effective anti-tumor immune responses and are associated with better responses to anti-CTLA-4 and anti-PD-1 treatments. Indeed, mouse models have shown that local IFN-γ upregulation is essential for anti-PD-1-mediated tumor regression (89). Similarly, IFN-γ and the cytotoxic granule component granzyme B were increased in regressing lesions of melanoma patients after anti-PD-1 treatment (90). Tumors in patients treated with anti-PD-1 who initially responded and then relapsed showed mutations that caused a subsequent loss in MHC class I surface expression (to avoid cytotoxic T cell recognition) or in IFN-γ response elements (6). Th9 $CD4^+$ T cells have also been suggested to play a role according to a recent study that detected a significant increase in Th9 cell frequency in patients responding to anti-PD-1 treatment (91, 92).

It may be tempting to speculate that immune checkpoint inhibitors specifically boost the function of T cells belonging to the effector memory compartment, as these cells readily express cytotoxic molecules such as perforin and granzyme B. However, these cells lack the co-stimulatory receptor CD28 through which both PD-1 and CTLA-4 inhibit T cell function (93). Two recent studies have shown that it is indeed CD28-expressing cells rather than already terminally differentiated effector cells that respond to PD-1 blockade with a proliferative burst and differentiation (94, 95).

The characteristics of a tumor itself may also influence immune checkpoint inhibitor efficacy. The mutational burden of tumor cells may increase their antigenicity but may also enhance their ability to evade treatment-induced immune responses. Indeed, a recent study identified a melanoma gene signature associated with innate anti-PD-1 resistance, which included upregulation of genes associated with angiogenesis, wound healing, mesenchymal transitioning, cell adhesion, and extracellular matrix remodeling (96).

Commensal bacteria may also play a role in influencing the efficacy of immune checkpoint inhibitors. Anti-CTLA-4 treatment was found to be ineffective in mice reared under sterile conditions and to induce a shift in the gut flora of conventionally reared mice. Further experiments showed that the presence of certain bacterial strains, in particular *Bacteroides fragilis*, promoted Th1 polarization in the animals and was associated with an improved anti-tumor immune response (97). Importantly, antibiotic treatment was also associated with reduced responses to anti-PD-1/PD-L1 treatments in cancer patients, possibly by altering the normal gut flora. Good treatment response among patients was instead associated with the presence of the commensal *Akkermansia muciniphila*, which also improved anti-PD-1 treatment responses in mice by allowing increased recruitment of CCR9 + CXCR3 + CD4 + T lymphocytes into the tumor (98).

TREATMENT-RELATED ADVERSE EVENTS AND THEIR MANAGEMENT

PD-1 and CTLA-4 prevent autoimmunity and limit immune activation to prevent bystander damage under physiological conditions. Inhibition of these receptors through therapeutic antibodies for the treatment of cancer is therefore associated with a wide range of side effects that resemble autoimmune reactions. Rates of severe side effects vary greatly by study and treatment (see **Table 2**). Clinical trials that directly compared different types of immune checkpoint inhibitors and their combination noted that more patients experienced side effects when treated with anti-CTLA-4 (27.3%) compared to anti-PD-1 (16.3%). Even more patients were affected when treated with a combination of both (55%) (67).

Almost all patients treated with immune checkpoint inhibitors experience mild side effects such as diarrhea, fatigue, pruritus, rash, nausea and decreased appetite. Severe adverse reactions include severe diarrhea, colitis, increased alanine aminotransferase levels, inflammation pneumonitis, and interstitial nephritis (67, 73, 99). There have also been reports of patients experiencing exacerbation of pre-existing autoimmune conditions such as psoriasis (91, 92, 100) or developing new ones such as type 1 diabetes mellitus (101). Particularly severe side effects may require cessation of treatment, although these patients may still respond thereafter (102). Interestingly, certain treatment-related auto-immune reactions such as rash and vitiligo have been shown to correlate with better disease prognosis (103), suggesting an overlap between auto-immune and anti-tumor immune responses.

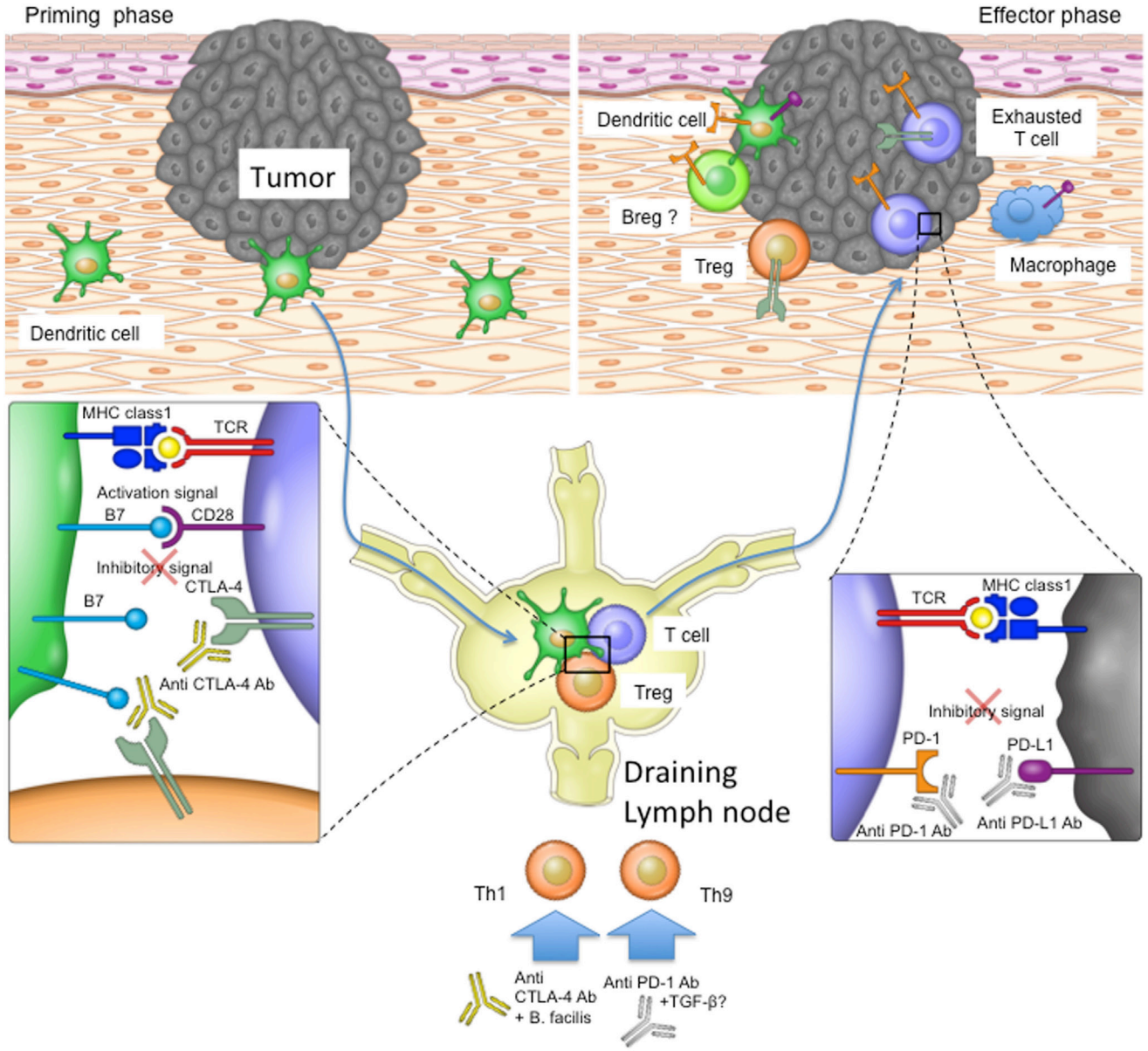

FIGURE 2 | The role of programmed cell death protein 1 (PD-1) and T-lymphocyte-associated protein 4 (CTLA-4) in the priming and effector phases of anti-tumor immune responses. For T cell priming, dendritic cells (DCs) sample antigen at the tumor site and transport it to the draining lymph nodes, where they present the antigens on their major histocompatibility complex (MHC) molecules to T cells. T cells become activated if their T cell receptors recognize and bind the antigen on MHC complexes and their CD28 costimulatory receptors bind CD80 and CD86 on DCs. CTLA-4 upregulation on T cells or bystander Tregs can interfere with the CD28 signal, as the former receptor binds CD80 and CD86 with higher affinity. Once activated, T cells migrate to the tumor site in order to kill malignant cells. Tumors or bystander cells such as macrophages may, however, upregulate PD-L1 and therefore obstruct T cell function by inducing inhibitory intracellular signaling. Anti-CTLA-4 blocking antibody may therefore restore T cell priming in the lymph nodes, and the PD-1 signaling blockade may enable T cell effector function at the tumor site. Additionally, other cell types such as Breg cells and DCs in the tumor microenvironment may express PD-1 and therefore be affected by PD-1 blockade. PD-1 and CTLA-4 blockade may also affect T helper cell profiles directly or by influencing the microbiota.

BIOMARKERS OF ANTI-PD-1/CTLA-4 TREATMENT EFFICACY

Biomarkers are needed both before and during treatment to identify the patients most or least likely to respond to immune checkpoint inhibitor treatments in order to reduce inappropriate drug exposure. Treatment response is defined as a reduction in tumor size during the course of treatment. A number of factors associated with disease prognoses in untreated patients are also linked to immune checkpoint inhibitor response rates (**Table 3**). For example, patients with smaller tumors or low serum lactate dehydrogenase (LDH) levels at baseline have a better prognosis

TABLE 3 | Biomarkers associated with favorable responses to immune checkpoint inhibitors.

	Pre-treatment	Post-treatment
Tumor	Tumor size and distribution (66) High mutation burden but no innate anti-PD-1 resistance (IPRES) gene signature (78, 86, 87, 96) PD-L1 expression on tumor cells (only confirmed by some but not all studies) (67, 108)	Reduction in tumor size
Tumor-infiltrating immune cells	Presence of CD8 + T cells inside the tumor or at the tumor margin (88) PD-L1 expression by infiltrating cells (77) Increased Th1- and CTLA-4-associated gene expression (77).	Proliferation of intratumoral CD8 + T cells (88)
Circulation	High relative lymphocyte counts (109) High relative eosinophil counts (109) High serum TGF-β levels (91, 92) Low serum LDH levels (66, 109) Low levels of ctDNA (107)	Increased levels of ICOS + T cells (110) Low neutrophil-to-lymphocyte ratio (110) High levels of Th9 cells A reduction in serum LDH levels (104) A reduction in ctDNA (107)
Host genome	Presence of HLA-A*26 allele (111)	

and are also more likely to respond to anti-PD-1 treatment (66). A reduction in LDH levels after treatment is also associated with improved response (104). Circulating tumor DNA (ctDNA), which contains melanoma-associated mutations and can be released by dead tumor cells, can be detected in the serum of some patients. CtDNA levels correlate strongly with tumor burden and progression (105, 106). A recent study in advanced stage melanoma patients treated with anti-PD-1 (alone or in combination with anti-CTLA-4) showed high treatment response rates in individuals that were ctDNA negative prior to or after treatment (107), making serum ctDNA an attractive biomarker before and during immune checkpoint treatment.

For anti-PD-1 treatments, expression of PD-L1 within the tumor microenvironment has been an obvious biomarker candidate. Although PD-L1 expression on tumor cells was correlated with treatment efficacy in melanoma patients (67, 108), it was not in patients with squamous cell carcinoma, non-small cell lung cancer and Merkel cell carcinoma (70, 72, 74). Interestingly, one study assessing the role of PD-L1 in both cancer cells and tumor-infiltrating immune cells found that only in the latter context was anti-PD-L1 treatment efficiency correlated with PD-L1 expression (77).

The presence of neoantigens on mutated tumor cells boosts anti-tumor immunogenicity and improves treatment efficacy. High genetic disparity between tumor cells and host cells is therefore an indicator of checkpoint inhibitor treatment efficacy. This was particularly noted in anti-CTLA-4-treated melanoma patients whose tumors displayed neo-antigens (87) and similarly in anti-PD-1-treated patients with colorectal cancers or non-small cell lung cancers that were mismatch-repair deficient or had high mutation rates, respectively (78, 86). Although overall mutational burden is associated with improved response to anti-PD-1 treatment, reduced responses were detected in melanoma patients whose tumors displayed the IPRES gene signature (96). Antigen presentation by the host may also play a role during anti-PD-1 treatment, as patients with the HLA-A*26 were more than twice as likely to respond than patients negative for the allele (111).

Other pre-treatment immunological factors associated with improved treatment responses include high eosinophil and lymphocyte blood counts, an abundance of $CD8^+$ T cells infiltrating the tumor or present at the tumor margin, and increased serum TGF-β levels in melanoma patients treated with anti-PD-1 (88, 91, 92, 109). Increased Th1 and CTLA-4 (but not FoxP3) gene expression levels were also noted in responder patients with various solid tumors (including melanoma) treated with anti-PD-L1 (77).

A number of post-treatment immunological observations have also been associated with improved immune-checkpoint inhibitor responses. For example, patients more likely to respond to anti-CTLA-4 treatment had increased numbers of inducible co-stimulatory molecule (ICOS) expressing T cells and lower neutrophil-to-lymphocyte ratios (110). An increase in $CD8^+$ T cell proliferation within the tumor lesion and an increased frequency of Th9 cells in the patients' circulation were also associated with treatment response (88, 91, 92).

Taken together, many of these studies indicate that immune checkpoint inhibitors are most effective in patients who already display anti-tumor immune processes prior to therapy. However, not all biomarkers listed here may be equally effective, and patients may still respond to treatment despite contrary biomarker-based predictions. Further, accessing tumor tissue may be difficult in many patients, especially after treatment, and less invasive blood-based "liquid biopsies" may therefore be more appropriate. Importantly, it has been shown that investigating several biomarkers in combination can improve treatment predictions (109). Although the recently discovered ctDNA seems to be a particularly promising biomarker candidate, more studies are needed to identify more effective biomarkers or biomarker combinations, in order to devise the most appropriate treatment strategy for each patient.

LIMITATIONS OF IMMUNE CHECKPOINT INHIBITORS

Although immune checkpoint inhibitor treatment may be effective initially, many patients will eventually relapse and develop tumor progression. A number of studies have therefore sought

to understand the mechanisms by which anti-PD-1 and anti-CTLA-4 treatments lose their efficacy.

The selection pressure caused by checkpoint inhibitor treatment may give rise to tumor cells that can evade immunomediated recognition and deletion through new pathways. Tumor cells from patients refractory to anti-PD-1 treatment, for example, were recently shown to have acquired mutations making them less susceptible to T cell-mediated killing *via* loss of IFN-γ response elements or MHC class I (6).

Anti-PD-1 or anti-CTLA-4 treatment may also cause upregulation of other inhibitory receptors. For example, patients with melanoma or prostate cancer exhibited upregulation of the inhibitory receptor V-domain Ig suppressor of T cell activation (VISTA) on various tumor-infiltrating immune cells after anti-CTLA-4 treatment (112). Another study noted the upregulation of the inhibitory receptor TIM-3 (but not VISTA) on the surface of T cells in anti-PD-1-treated mice with lung cancer as well as TIM-3 upregulation on T cells in adenocarcinoma patients refractory to PD-1 treatment (113).

Most recently, a study revealed another unexpected resistance mechanism to anti-PD-1 therapy in mice whereby tumor-associated macrophages removed the therapeutic antibody from the surface of the T cells *in vivo*, thus making them once again susceptible to inhibitory signaling through the receptor. This phenomenon could be partially overcome by administration of Fc-receptor blocking agents prior to treatment (114). A better understanding of the mechanisms limiting the effectiveness of immune checkpoint inhibitors will therefore allow improvement of future treatments.

FUTURE AVENUES: EXPANDING THE IMMUNE CHECKPOINT INHIBITOR TREATMENT REPERTOIRE

PD-1 and CTLA-4 blocking agents are not effective in all patients, and even those patients who do respond initially can relapse, highlighting the need for improved or alternative treatments. Alternative inhibitory receptors have been identified that may also be targeted for anti-tumor immune therapy. These include the TIM-3, LAG-3, TIGIT, and B- And T-Lymphocyte-Associated Protein (BTLA) receptors associated with T cell exhaustion as well as VISTA, a receptor found on tumor-infiltrating myeloid cells, whose inhibition promoted anti-tumor immune responses in murine models, and CD96, which has been shown to inhibit NK cell activity in murine cancer models (115–117).

Combinations of immune checkpoint inhibitors with each other or with other treatments are also being explored. Indeed, the combination of anti-CTLA-4 with anti-PD-1 treatments showed superior efficacy compared to individual administration, but was also associated with an increase in side effects. The tryptophan-metabolizing enzyme IDO inhibits T cell function, and combining IDO-blocking agents together with immune checkpoint inhibitors has shown promising results in mice and is also currently undergoing clinical trials in humans (105, 118). Macrophages may also interfere with anti-tumor immunity or even directly restrict therapeutic antibodies (114). Their depletion through a Colony stimulating factor-1 receptor (CSF-1R) inhibitor is therefore being explored in clinical trials together with anti-PD-1, after having shown efficacy in a glioblastoma mouse model (119). Anti-tumor T cell function induced by PD-1 blockade in mice could also be improved by a targeted increase in mitochondrial function (120).

Because immune checkpoint inhibitors work by removing brakes on the immune system rather than directly boosting immune function, patients may also benefit from combination therapies that include immunostimulatory substances. Mouse melanoma models, for example, have shown that the combination of anti-CTLA-4 with cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or with agonistic antibodies targeting costimulatory receptors such as CD40, increased tumor rejection in a synergistic manner (121, 122). The genetically modified herpes simplex virus talimogene laherparepvec is designed to replicate in tumor cells and to release GM-CSF, thus attracting immune cells into the tumor environment. The virus has been tested in recent clinical trials in combination with either CTLA-4 or PD-1 in advanced-stage melanoma patients, resulting in increased treatment response rates compared to the immune checkpoint inhibitors alone (123, 124).

Even modulation of the gut microbiome may improve immune checkpoint inhibitor-based therapies. Administration of intestinal *Bifidobacteria* alone was associated with reduced tumor growth in a murine B16 melanoma model by promoting dendritic-cell mediated $CD8^+$ T cell responses. Importantly, the administration of these bacteria also added to the therapeutic effect of anti-PD-1 treatment in these mice (125). In a similar study, administration of *B. fragilis* to sterile mice treated with anti-CTLA-4 resulted in reduced tumor growth, most likely by inducing a favorable shift toward Th1 responses (97). Studies in humans were further able to link the presence of fecal *A. muciniphila, Ruminococcaceae,* and *Faecalibacterium* to a favorable outcome to anti-PD-1 treatment (98, 126) Together, these findings suggest that human patients too may benefit from appropriate management of their intestinal flora while undergoing immune checkpoint inhibitor treatment.

A wide range of promising new avenues are therefore currently being explored, although their clinical efficacy remains to be confirmed by ongoing and future clinical trials.

CONCLUDING REMARKS

Although PD-1 and CTLA-4 targeting therapies have been able to increase average life expectancy for cancer patients, mortality remains high among advanced-stage patients, highlighting the need for further innovation in the field. Both anti-PD-1 and anti-CTLA-4 therapies appear to be more effective in patients with pre-existing anti-tumor immunity, suggesting that, in patients without such immunity, these drugs are unable to mediate anti-tumor immune responses *de novo*. However, as our understanding of the mechanisms of these drugs improves, avenues are being opened to improve their use not only by specifically targeting those patients who are most likely to respond through appropriate biomarker screening procedures, but also by pairing currently used immune checkpoint inhibitors with other complimentary

drugs to help those patients unable to respond to the current regimens.

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REVIEW
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Novel Therapeutic Targets in Cutaneous Squamous Cell Carcinoma

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Cutaneous squamous cell carcinoma (SCC) is one of the common cancers in Caucasians, accounting for 20–30% of cutaneous malignancies. The risk of metastasis is low in most patients; however, aggressive SCC is associated with very high mortality and morbidity. Although cutaneous SCC can be treated with surgical removal, radiation and chemotherapy singly or in combination, the prognosis of patients with metastatic SCC is poor. Recently, the usage of immune checkpoint blockades has come under consideration. To develop effective therapies that are less toxic than existing ones, it is crucial to achieve a detailed characterization of the molecular mechanisms that are involved in cutaneous SCC pathogenesis and to identify new drug targets. Recent studies have identified novel molecules that are associated with SCC carcinogenesis and progression. This review focuses on recent advances in molecular studies involving SCC tumor development, as well as in new therapeutics that have become available to clinicians.

Keywords: cyclin-dependent kinase, mitochondria, Drp1, PD-1 antibody, epidermal growth factor receptor

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INTRODUCTION

In light of today's demographic aging, skin cancer is becoming more prevalent. Cutaneous squamous cell carcinoma (SCC) is one of the most common cancers in Caucasian populations, and its prevalence is increasing (1). Cutaneous SCC accounts for 20–30% of cutaneous malignancies (2, 3). The risk of metastasis is low in most patients (2); however, aggressive SCC is associated with high morbidity and mortality (4). Although cutaneous SCC can be treated with surgery, radiation, and chemotherapy singly, or in combination, the prognosis of patients with metastatic SCC is almost always poor (3, 5). Today, chemotherapy with cisplatin alone or combined with 5-FU is being conducted with positive responses (6–9). However, the National Comprehensive Cancer Network Guidelines describe the evidence regarding systemic therapies for distant metastatic cutaneous SCC as limited. Recently, clinical trials on epidermal growth factor receptor (EGFR) inhibitors and immune checkpoint blockers have shown promising results as treatments for SCC (10–12). This review focuses on recent advances in molecular studies related to SCC tumor development and on new therapeutics that have become available.

RECENT PROGRESS IN CUTANEOUS SCC THERAPEUTICS

Novel Targeted Therapies

Cutaneous SCC overexpresses EGFR; thus, EGFR is a promising target for therapies. Cetuximab, an EGFR inhibitor, has been administered to cutaneous SCC patients. In some phase II studies,

there have been good responses to cetuximab in patients with locally advanced or regional SCC types (10, 13–15). However, in distant metastatic diseases, it has been reported as ineffective. Also, tyrosine kinase inhibitors have been used to disrupt EGFR pathways. Case reports on gefetinib and imatinib have described slightly positive responses in cutaneous SCC patients (16, 17). Also, a single-arm phase II clinical trial has shown gefetinib to demonstrate modest antitumor activity in metastatic or locoregionally recurrent cutaneous SCC, with limited adverse events (18). Furthermore, bortezomib, a selective inhibitor of the 26S proteasome, may have antitumor effects in cutaneous SCC, although the mechanisms have not been clarified (19).

Biological Modifiers

30 years ago, isotretinoin was reported to have efficacy as a treatment for local advanced cutaneous SCC alone or in combination. Interferons have also been used for local cutaneous SCC. A phase II study on bio-chemotherapy with interferons, retinoids, and cisplatin showed a positive response in 67% of locally advanced SCC cases (20). However, the efficacy against metastatic cases remains unclear.

Cytotoxic Chemotherapy

Regrettably, recent advances in cytotoxic chemotherapy have been limited. Capecitabine, an oral prodrug of 5-FU, has been used to treat cutaneous SCC (21). In head and neck SCC cases, intra-arterial chemotherapy has been conducted as a neoadjuvant therapy (22). To date, in cutaneous SCC, no obvious evidence for positive responses has been reported, even though some cases have been described in limited detail (23).

Immune Checkpoint Inhibitors

Recently, the US FDA approved PD-1 inhibitors (immune checkpoint inhibitors) for head and neck SCCs with continued progression during or after platinum chemotherapy (24, 25). For cutaneous SCC, several case reports have shown immune checkpoint inhibitors to have promising results. Patients with advanced cutaneous SCC responded to anti-PD-1 (nivolumab and pembrolizumab), and anti-CTLA-4 (ipilimumab) agents (26–29).

Radiation With Chemotherapies or Immunotherapies

Platinum-based chemotherapy has been combined with local radiation. Cisplatin-based chemotherapy combined with concurrent radiation showed better results than cisplatin only (13, 30). Neoadjuvant chemotherapies before radiation were also reported to have promising results (31). Recently, the abscopal effect during radiation therapy after the administration of immune checkpoint inhibitors has been spotlighted. This effect is a phenomenon in which local radiotherapy is associated with the regression of metastatic cancer at a distance from the irradiated site (32). To date, abscopal effects have been observed in melanoma patients but not in cutaneous SCC. It is not clear whether these effects will occur in cutaneous SCCs; however, combined therapies of immune checkpoint inhibitors and radiation might have a synergistic effect.

Other Candidates

Human Papilloma Virus (HPV) in Cancer Cells

Until recently, the role of HPV in cutaneous SCC was not well defined. However, a meta-analysis has found evidence that HPV is associated with cutaneous SCC (33). This systematic review indicated that cutaneous SCC harbors HPV more than normal skin does. Furthermore, an increase in HPV prevalence has been observed in SCC tumors from immunosuppressive patients. A study using an animal model showed that the interaction between UVB and HPV infection strongly promotes the development of cutaneous SCC (34). Furthermore, several targeted therapies for HPV-associated head and neck SCC have been tried (35); thus, HPV might be a promising target for cutaneous SCC as well.

MicroRNAs (miRs) in Cancer Cells

MicroRNAs are short, non-coding RNAs that suppress the expression of target genes. miRs can regulate various gene targets, and they play a crucial role in biological mechanisms (36). Certain miRs are associated with the onset and progression of cancers, suggesting that miRs could be targets for cancer therapies. In cutaneous SCC, several miRs are reported to be overexpressed or downregulated (36). miR-21 and miR-31 are upregulated in cutaneous SCC. The targets of these miRs are PDCD4/GRHL3/PTEN and RhoTBT1, respectively (36, 37). To inhibit the undesirable effects of up-regulated miRs, the administration of complementary nucleic acids might be a potential cancer therapy. By contrast, miR-1, miR-34a, and miR-124 are downregulated in cutaneous SCC (36). These miRs target important molecules of cell proliferation, which tend to be activated in cancer cells. Thus, promoting the over-expression of these miRs could be an option for cancer therapies. Furthermore, various miR delivery systems have been developed. Cheng et al. reported on pHLIP-mediated miR delivery methods, in which miRs could be transported across plasma membranes under the acidic conditions found in solid tumors (38). As such, miRs are also promising targets for cutaneous SCC as well as other tumors.

Cyclin-Dependent Kinase (Cdk) 16 in Cancer Cells

Recently, Cdk4/Cdk6 inhibitor (palbociclib) showed promising results for metastatic breast cancer (39, 40). Some Cdks are overexpressed in cutaneous SCC; thus, Cdk inhibitors may become a novel therapy option. Among Cdks, we have focused on cyclin-dependent kinase 16 (Cdk16) (also known as PCTK1, PCTAIRE1) and investigated its molecular functions. Cdk16 is a member of the Cdk family (41). Molecular functions for CDK16 are reported to vesicular transport (42) and spermatogenesis (43).

To investigate the role of Cdk16 in cancerous cells, we performed gene-knockdown experiments targeting Cdk16 (44–47). In cell lines of cutaneous SCC, prostate cancer, breast cancer, cervical cancer, and melanoma, knockdown of *Cdk16* inhibited cancer cell proliferation, and induced apoptosis over

time. But, no role for Cdk16 was observed in the proliferation of non-transformed cells (IMR-90 and HaCaT cells). To identify target molecules of Cdk16, we performed yeast two-hybrid screens with human Cdk16 protein as bait. We identified tumor suppressor p27 as a Cdk16 interactor and demonstrated that Cdk16 phosphorylates p27 at Ser10 by *in vitro* kinase assays (46). The knockdown of Cdk16 modulated p27 (Ser10) phosphorylation, leading to p27 accumulation in cancerous cells. In tumor xenografts of cutaneous SCC cells, the inducible conditional knockdown of Cdk16 suppressed tumor growth (47).

To evaluate the clinical importance of Cdk16, we also studied primary tumor samples In primary tumors from the patients with breast, prostate, cutaneous basal, or SCCs, Cdk16 was expressed more highly in cancer lesions than in normal tissues (46–48). In prostate cancers, a comparison of Cdk16 immunostaining with Gleason grade revealed lower expression levels in well-differentiated tumors than in less- differentiated tumors (46). In breast cancers, Cdk16 expression was elevated in *in situ* carcinomas and invasive cancers relative to the expression in normal mammary epithelium. The significantly higher levels of Cdk16 protein that are seen in invasive cancers are associated with higher histologic grades (46). Moreover, we showed that gene knockdown of *Cdk16* sensitizes cancer cells to TNF-family cytokines, such as Fas-ligand and TNF-related apoptosis-inducing ligand (49).

To advance *in vitro* results on Cdk16 silencing, we investigated the *in vivo* therapeutic potential by using siRNA encapsulated with lipid nanoparticles (LNP) (50). Therapy of Cdk16 siRNA was performed using colorectal cancer HCT116 cells and melanoma A2058 cells. Treatment with Cdk16 siRNA-LNP reduced tumor volume and weight significantly. TUNEL staining showed increased apoptosis of cancer cells treated with Cdk16 siRNA.

These findings show an expected role for Cdk16 in regulating p27 expression and tumor proliferation (**Figure 1**). We observed these functions for Cdk16 in various cancer cells (cutaneous SCCs; basal cell carcinomas; prostate, breast, and cervical cancers; and melanomas). This implies that the p27 regulation by Cdk16 is a common machinery in human cancers.

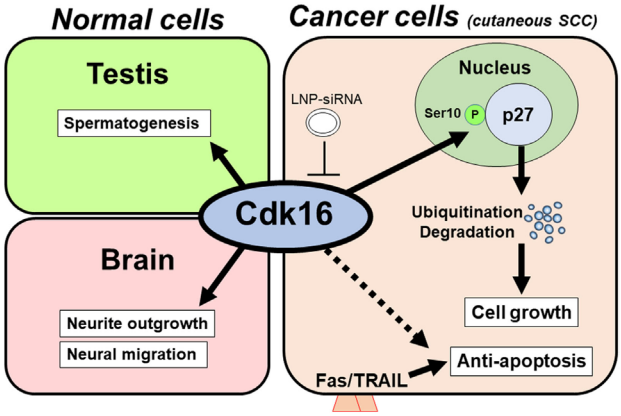

FIGURE 1 | Model of the tumorigenic role of cyclin-dependent kinase 16 (Cdk16). In normal tissue (left), Cdk16 is required for spermatogenesis and neuron differentiation. In cancer cells, including cutaneous squamous cell carcinoma (SCC) cells (right), Cdk16 phosphorylates p27 at Ser10, thereby promoting p27 ubiquitination/degradation, which leads to cell cycle progression and decreased levels of apoptosis. An unknown mechanism may also exist in the Cdk16–apoptosis pathway. Lipid nanoparticle-mediated siRNA (LNP-siRNA) therapy against Cdk16 recently succeeded in a murine xenograft model.

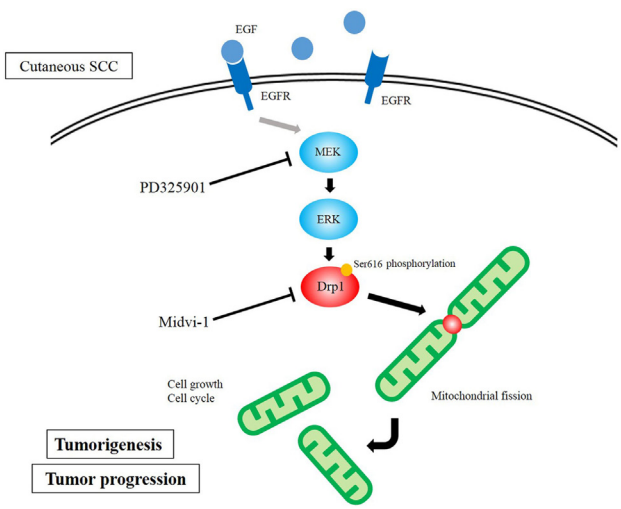

FIGURE 2 | Diagram of dynamin-related protein 1 (Drp1) function in cutaneous squamous cell carcinoma (SCC) cells. MAPK signaling activates Drp1 *via* the phosphorylation of Drp1. The overexpression of Drp1 induces mitochondrial fission, which results in cell growth and assists cell cycle.

Dynamin-Related Protein 1 (Drp1) in Cancer Cells

We have also focused on the mitochondria-associated molecule Drp1 (51). Drp1 regulates mitochondrial fission. Recently, it was found to be associated with cancer cell proliferation in melanoma and lung cancer (52, 53). Disrupted mitochondrial networks induce cell cycle arrest and apoptosis (53, 54). Also, Drp1 has been reported as a prognostic factor in several malignancies, such as lung adenocarcinomas and glioblastomas (55, 56). Based on these previous studies, we investigated the role of Drp1 in cutaneous SCCs. Drp1 gene-knockdown SCC cells showed lower cell proliferation than control cells, as assessed by cell counting and clonogenic assays. DNA content Cell Cycle analysis showed Drp1 knockdown to cause G2/M phase arrest. Morphologically, the depletion of Drp1 resulted in an elongated mitochondrial network. The MEK inhibitor, PD325901, inhibited cell proliferation, as well as inhibiting the phosphorylation of ERK1/2 and Drp1 (Ser616). PD325901 also caused the dysregulation of the mitochondrial network. In tumor xenografts of DJM1 SCC cells, the knockdown of Drp1 suppressed tumor growth *in vivo*. Clinically, the expression levels of Drp1 were higher in cutaneous SCC specimens than in normal epidermis, and those levels correlated positively with advanced clinical stages. Our data reveal a pivotal function for Drp1 in mediating tumor growth, mitochondrial fission, and cell

cycle in cutaneous SCCs (**Figure 2**), suggesting that Drp1 could be a novel target for cutaneous SCC therapies.

CONCLUDING REMARKS

In the past 10 years, novel therapeutic agents for cutaneous SCC have been developed. EGFR inhibitors and immune checkpoint inhibitors have shown particularly promising results. Furthermore, these novel treatments can be used a monotherapies or in combination with radiation; thus dermatologists and oncologists will be able to choose better treatments depending on conditions of the patient and the stage of the disease. Also, novel targeting molecules and inhibitors have been developed.

AUTHOR CONTRIBUTIONS

TY and HH designed the study. TY and SK wrote the paper. HH supervised the study.

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REVIEW
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Merkel Cell Carcinoma: An Update and Immunotherapy

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Merkel cell carcinoma (MCC) is a rare but aggressive skin cancer with frequent metastasis and death. MCC has a mortality rate of 30%, making it more lethal than malignant melanoma, and incidence of MCC has increased almost fourfold over the past 20 years in the USA. MCC has long been considered to be an immunogenic cancer because it occurs more frequently in immunosuppressed patients from organ transplant and HIV infection than in those with immunocompetent. Chronic UV light exposure and clonal integration of Merkel cell polyomavirus (MCPyV) are two major causative factors of MCC. Approximately 80% of MCC are associated with MCPyV, and T cells specific for MCPyV oncoproteins are present in the blood and tumors of patients. Several studies have shown that a subset of MCCs express PD-1 on tumor-infiltrating lymphocytes and express PD-L1 on tumor cells, which suggests an endogenous tumor-reactive immune response that might be unleashed by anti-PD-1 or anti-PD-L1 drugs.

Keywords: PD-1, PD-L1, Merkel cell carcinoma, Merkel cell polyomavirus, UV

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BACKGROUND

Merkel cell carcinoma (MCC) is a rare but highly aggressive neuroendocrine skin cancer, which was described for the first time in 1972 as trabecular carcinoma of the skin (1). Based on the ultrastructural proof of neuroendocrine granules and the expression of CK20 and CD56 (2–4), Merkel cells were considered to be the source of MCC. However, the cells of origin of MCC remain a controversial issue. Recent studies have suggested the origin of MCC may reside in epidermal/dermal stem cells in the dermis (5) or in precursor B cells (6, 7). The incidence of MCC is rising steadily and more than one-third of patients die of MCC, making it twice as lethal as malignant melanoma (8). Risk factors for MCC include fair skin, chronic sun exposure, chronic immune suppression, and advanced age (9–12). In the USA, age-adjusted incidence increased from 0.15 to 0.44 per 100,000 from 1986 to 2004 (13). Consistent with other UV-related skin cancers, incidence rate of MCC in Queensland, Australia is higher than those in the rest of the world (age-adjusted incidence of 1.6 per 100,000) (14). The incidence of MCC in Asia is thought to be low, although no population-based data are available (15, 16). The majority of MCC is associated with Merkel cell polyomavirus (MCPyV), while the remaining is triggered by UV-mediated mutations (17, 18). MCPyV DNA integrates into the host genome of approximately up to 80% of MCCs in the northern hemisphere, whereas its presence is much lower in other geographic regions such as Australia (~30%) (17, 19). Since several lines of evidence indicate the outstanding immunogenicity of MCC, irrespective of MCPyV integration, immune modulating treatment strategies are particularly attractive. Promising results from immune checkpoint inhibitor therapy in first and second line are now available, which expands the treatment armamentarium for MCC patients.

CLINICAL AND HISTOLOGICAL FEATURES

Merkel cell carcinoma presents as a firm, painless, rapidly enlarging, red-violet cutaneous nodule with a smooth surface. The most frequently affected site is the head and neck region (50%), followed

by the trunk (30%) and the limbs (10%), although MCC may arise in any body site, including the mucosae (20–22). Heath et al. developed the AEIOU acronym to define the clinical features associated with MCC: asymptomatic/lack of tenderness, expanding rapidly, immune suppression, older than age 50, and UV-exposed site on a person with fair skin. In a study of 195 patients, 89% presented with three or more of the AEIOU characteristics (23). MCC originates in the dermis and only occasionally exhibits an epidermal involvement. Histopathological characteristics of MCC include a monotonous population of tumor cells with large prominent nuclei and scant cytoplasm (24). Immunohistochemically, MCC is positive for EMA, CK20 with a perinuclear dot staining pattern, and neuroendocrine markers including synaptophysin and chromogranin (3, 25–27). Metastatic pulmonary small cell carcinoma can be excluded when the tumor cells prove negative for TTF-1 (28). Unknown primary MCC, which usually presents clinically positive nodal disease with unidentified primary tumor, are likely to have a significantly improved survival compared to those with concurrent primary tumor (29–32). Recent reports showed that unknown primary MCC had higher tumor mutational burden and lower association with MCPyV than those with known primary (33), In addition, nodal tumors from unknown primary MCC contained abundant UV-signature mutations (33), suggesting underlying immunological mechanism between regression of primary tumor and better prognosis of unknown primary MCC.

ETIOLOGY

Like Kaposi's sarcoma, immunocompromised patients with T-cell dysfunction are more likely to be affected by MCC. For example, patients with AIDS have an incidence rate that is 11–13 times greater compared with the general population (11), and solid organ transplant recipients are 5–10 times more likely to develop MCC (34, 35). Also, case reports have described spontaneous regression of MCC tumors after biopsy or an improvement in immune function, further indicating a link to the immune system (36–39). These data collectively suggested that MCC may be linked to a pathogen and in 2008, MCPyV was discovered, and it is now clear that this virus plays a key role in the majority of MCC cases (17).

Merkel cell polyomavirus is a member of the polyomavirus family comprised of non-enveloped, double-stranded circular DNA viruses. MCPyV-specific antibodies have been detected in 9% of children under 4 years of age, 35% of teenagers, and 80% of individuals 50 years or older (40), suggesting that it may be part of the cutaneous microbiome (41). Interestingly, despite this high prevalence, MCPyV has not been shown to cause any disease other than MCC (42). MCPyV-related oncogenesis requires integration of the viral genome into the host-genome and mutation of the large T (LT) antigen that is required for viral DNA replication (43). Indeed, MCPyV isolated from MCCs, in contrast with MCPyV from non-tumor sources, present mutations that are responsible for the premature truncation of the MCV LT helicase (43, 44). These mutations do not affect the Rb binding domain, but eliminate the capacity of the viral DNA to replicate. In this way, the virus loses its capability to replicate in MCC tumor cells, but continues to express motifs that may potentially lead to uncontrolled proliferation (43, 45). Prognostic significance of tumor viral status is still controversial, but the largest cohort study so far including 282 MCC cases (281 cases with available clinical data) showed that, relative to MCPyV-positive MCC patients, MCPyV-negative MCC patients had significantly increased risk of disease progression (hazard ratio = 1.77, 95% confidence interval = 1.20–2.62) and death from MCC (hazard ratio = 1.85, 95% confidence interval = 1.19–2.89) in a multivariate analysis including age, sex, and immunosuppression (46).

Merkel cell carcinoma development is also linked to exposure to UV radiation, and primary MCC lesions preferentially develop on sun-exposed skin (20, 21). The incidence of MCC was determined to be 100-fold greater in patients who underwent PUVA treatment (47). MCPyV-negative MCC is among the most mutated of all solid tumors, including melanoma (18, 48–50). These mutations are mostly UV-signature mutations, such as p53 and Rb, commonly resulting in loss of functional protein expression (18, 49). The high mutational burden in MCC correlates to frequent amino acid changes and large numbers of UV-induced neoantigens (49). Despite significant genetic differences, both MCPyV-positive and -negative MCC exhibit nuclear accumulation of oncogenic transcription factors such as NFAT, phosphorylated CREB, and phosphorylated STAT3, indicating commonly deregulated pathogenic mechanisms (50).

TREATMENT

For patients with locoregional MCC, wide excision and/or complete lymph node dissection and/or adjuvant radiation therapy is usually recommended (51). Sentinel lymph node biopsy should be considered for patients with clinically nodal negative patients, although its impact on overall survival is still unclear (51–53).

Although cytotoxic chemotherapy (carboplatin or cisplatin plus etoposide) has been commonly used to treat patients with advanced MCC, responses are rarely durable and few studies have shown a survival benefit (54–57). Early studies showed that levels of intratumoral CD8+ T cells serve as predictors of MCC-specific survival, with 100% survival reported for patients with the highest level of CD8+ infiltrate compared to 60% survival in those with little or no CD8+ infiltration (58, 59). Then MCPyV oncoprotein-specific cells were found to be present in MCC patient blood and enriched in their tumors (60), whose frequency appears to increase with tumor burden (61). Importantly, signs of dysfunction were evident in MCPyV-specific CD8+ T cells from patients, as they expressed both PD-1 and Tim3, suggesting functional exhaustion (61). MCPyV-negative MCC is also associated with high levels of T-cell infiltrates (18). Although both MCPyV-positive and -negative tumor cells express PD-L1, the expression levels of PD-L1 in virus-positive tumors seem to be higher than those in virus-negative tumors (18, 62). These findings, therefore, provide rationale for immunotherapy targeting the PD-1 pathway in advanced MCC.

A multicenter, phase 2, non-controlled clinical trial studied pembrolizumab (anti-PD-1 Ab) 2 mg/kg every 2 weeks in

TABLE 1 | Ongoing clinical trials in MCC (http://ClinicalTrials.gov).

NCT identifier	Title	Phase	Intervention
NCT03071406	Randomized Study of Nivolumab + Ipilimumab ± SBRT for Metastatic Merkel Cell Carcinoma	2	Nivolumab Ipilimumab SBRT
NCT02643303	A Phase 1/2 Study of *In Situ* Vaccination with Tremelimumab and IV Durvalumab Plus PolyICLC in Subjects with Advanced, Measurable, Biopsy-Accessible Cancers	1/2	Durvalumab Tremelimumab Poly ICLC
NCT02488759	An Investigational Immuno-therapy Study to Investigate the Safety and Effectiveness of Nivolumab, and Nivolumab Combination Therapy in Virus-Associated Tumors (CheckMate358)	1/2	Nivolumab Ipilimumab BMS-986016 Daratumumab
NCT02584829	Localized Radiation Therapy or Recombinant Interferon Beta and Avelumab with or without Cellular Adoptive Immunotherapy in Treating Patients with Metastatic Merkel Cell Carcinoma	1/2	Avelumab Merkel cell polyomavirus TAg-specific polyclonal autologous CD8-positive T cells Interferon beta, RT
NCT03271372	Adjuvant Avelumab in Merkel Cell Cancer (ADAM)	3	Avelumab
NCT02196961	Adjuvant Therapy of Completely Resected Merkel Cell Carcinoma with Immune Checkpoint Blocking Antibodies Versus Observation (ADMEC-O)	2	Ipilimumab Nivolumab

26 patients with advanced MCC who had not received prior systemic therapy. The objective response rate (ORR) to pembrolizumab among the 25 patients with at least one evaluation during treatment was 56% including a 16% complete response (CR) rate. Of the 14 responsive patients, the response duration ranged from at least 2.2 months to at least 9.7 months. Overall, the trial had an estimated progression free survival (PFS) of 67% at 6 months. Pembrolizumab was effective in both MCPyV-positive and -negative tumors (ORR 62 and 44%, respectively, not significantly different) (63). The preliminary data from this trial led to pembrolizumab being listed as a treatment option for disseminated disease in the 2017 NCCN guidelines for MCC (64).

A multicenter, international, open-label, phase 2 clinical trial studied avelumab (anti-PD-L1 Ab) in 88 patients with distant metastatic disease who had previously received at least one line of chemotherapy. This trial found an ORR of 33% with a CR rate of 11%. At 6 months, PFS was 40% and the estimated PFS at 1 year was 30%. As with pembrolizumab, avelumab was found to be effective in both MCPyV-positive and -negative tumors (ORR 26 and 35%, respectively, not significantly different) (65). Based on these results, FDA granted an accelerated approval for avelumab as first-line treatment of patients with metastatic MCC in March 2017. In the avelumab trial, a trend toward a higher response rate was observed in patients with fewer lines of prior treatment, which along with the pembrolizumab data strongly suggest that immunotherapy targeting the PD-1 pathway should be considered for first-line treatment in patients with advanced MCC.

An international, single arm, open-label trial of nivolumab (anti-PD-1 Ab) 240 mg/body every 2 weeks included both patients who had and those who had not received prior chemotherapy (36 and 64%, respectively) is ongoing (NCT02488759; CheckMate358). In this study, 15 of 22 patients (68%) had objective responses, and PFS at 3 months was 82%. Responses occurred in 10 of 14 treatment-naive patients including 3 CR, in 5 of 8 patients including 5 partial responses with 1–2 prior systemic therapies (63%) (**Table 1**). Based on the preliminary data from this trial, nivolumab was listed along with avelumab and pembrolizumab as a treatment option for disseminated disease in the 2018 NCCN guidelines for MCC (51).

CONCLUSION

Advanced MCC is generally considered to be sensitive to chemotherapy, but responses are transient, offering a median PFS of only 3 months (55). On the other hand, although no randomized trials compare chemotherapy with immunotherapy, data from treatment with immune checkpoint inhibitors are promising with responses both in MCPyV-positive and -negative MCC, although nearly half of patients do not derive durable benefit from these drugs. Now that avelumab has been approved for treatment of advanced MCC in the USA, EU, and Japan, the spectrum of current therapy for patients with MCC is changing. Several clinical trials of immune checkpoint inhibitors (anti-PD-1, PD-L1, and CTLA-4 Abs) administered as monotherapy or in combination with other agents or modalities are ongoing (**Table 1**) and may provide further treatment options for patients with advanced MCC in the near future.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Cutaneous Angiosarcoma: The Possibility of New Treatment Options Especially for Patients with Large Primary Tumor

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The most widely accepted treatment for cutaneous angiosarcoma (CAS) is wide local excision and postoperative radiation to decrease the risk of recurrence. Positive surgical margins and large tumors (T2, >5 cm) are known to be associated with poor prognosis. Moreover, T2 tumors are known to be associated with positive surgical margins. According to previous reports, the majority of CAS patients in Japan had T2 tumors, whereas less than half of the patients in the studies from western countries did so. Consequently, the reported 5-year overall survival of Japanese CAS patients without distant metastasis was only 9%, lower than that for stage-IV melanoma. For patients with T2 tumors, management of subclinical metastasis should be considered when planning the initial treatment. Several attempts to control subclinical metastasis have been reported, such as using adjuvant/neoadjuvant chemotherapy in addition to conventional surgery plus radiation. Unfortunately, those attempts did not show any clinical benefit. Besides surgery, new chemotherapeutic approaches for advanced CAS have been introduced in the past couple of decades, such as paclitaxel and docetaxel. We proposed the use of chemoradiotherapy (CRT) using taxanes instead of surgery plus radiation for patients with T2 tumors without distant metastasis and showed a high response ratio with prolonged survival. However, this prolonged survival was seen only in patients who received maintenance chemotherapy after CRT, indicating that continuous chemotherapy is mandatory to control subclinical residual tumors. With the recent development of targeted drugs for cancer, many potential drugs for CAS are now available. Given that CAS expresses a high level of vascular endothelial growth factor (VEGF) receptor, drugs that target VEGF signaling pathways such as anti-VEGF monoclonal antibody and tyrosine kinase inhibitors are also promising, and several successful treatments have been reported. Besides targeted drugs, several new cytotoxic anticancer drugs such as eribulin or trabectedin have also been shown to be effective for advanced sarcoma. However, most of the clinical trials did not include a sufficient number of CAS patients. Therefore, clinical trials focusing only on CAS should be performed to evaluate the effectiveness of these new drugs.

Keywords: cutaneous angiosarcoma, concurrent chemoradiotherapy, maintenance chemotherapy, adjuvant chemotherapy, taxanes, eribulin, pazopanib, angiosarcoma of the scalp

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BACKGROUND

According to the Surveillance, Epidemiology, and End Results Program database, the number of patients with sarcoma recorded between 2010 and 2014 was only 1/100 of the number of patients with carcinoma in the same period. Moreover, angiosarcoma accounts for only 1% of all sarcomas, so patients with angiosarcoma constitute only 1 in 10,000 of all patients with malignant neoplasms (1–3). Although the incidence of angiosarcoma has increased in the past couple of decades, it is around 0.5 per 1,000,000 persons, or fewer than 200 new patients, per year in the United States (3). Owing to this rarity, most previous publications have been case reports or small case series, making it difficult to interpret the results because of the selection bias and small number of patients included in those studies. Furthermore, because of this rarity, no randomized phase-3 study has been conducted, especially for angiosarcoma, and consequently, no clinical trial-proven standardized treatment has thus far been established. Although complete removal of the tumor was believed to be essential, as it is for other sarcomas (4, 5), some reports have suggested that wide-margin surgery will not deliver favorable results (6, 7). In this review, we will summarize the clinical features and current treatments of angiosarcoma and discuss the possibility of new therapeutic options for this rare disease.

CLINICAL PRESENTATION

Angiosarcoma develops in various soft tissues and organs, but the most commonly affected site is the skin [cutaneous angiosarcoma (CAS)] (8–10). According to an analysis of 434 cases of CAS, 62.1% of them developed in the head and neck, 24.4% in the trunk, 10.6% in the extremities, and 2.7% in other locations (11). CAS commonly occurs in the scalp and typically presents as an enlarging bruise-like purpura in the head and neck region and may be associated with ulceration and/or a tumor. Sometimes patients develop a thick blood crust. These head and neck CAS commonly develops in older men (12–14), whereas the secondary CAS, lymphedema-associated CAS [so-called Stewart-Treves syndrome (15)] and radiation-associated CAS (11, 16), usually develops within the lymphedema site and irradiated field >5 years after the surgery and radiation, respectively (12, 16).

Stewart-Treves syndrome was originally reported as lymphedema that developed after radical mastectomy and lymph node dissection (15), but in the past 15 years, we have never encountered Stewart-Treves syndrome that developed after the surgery for mammary carcinoma. Instead, in the same period, we experienced three cases of Stewart-Treves syndrome that developed in the lower limb after treatment for uterine carcinoma (17). This may be explained by the fact that the number of patients receiving conservative treatment for mammary carcinoma has increased, and as a consequence, the prevalence of Stewart-Treves syndrome in the upper extremity has decreased (18). On the other hand, the occurrence of radiation-induced CAS in the breast is likely to increase given that the prognosis for mammary carcinoma is gradually improving and radiation is more often used to treat (16).

While the incidence of Stewart-Treves syndrome is not well known, it has been reported to be about 1/10 to 1/20 of all CAS (19–22). Similarly, the cumulative incidence of radiation-associated CAS 15 years after radiotherapy for breast carcinoma was reported to be 0.9 per 1,000 patients (23), meaning less than 1 occurrence per 10,000 irradiated patients per year. In this review, considering its rarity and etiological difference, we will focus mainly on primary CAS, the narrow sense of CAS (24).

Distant metastasis could occur within a month of primary surgery, but typically it occurs on average after a year (4, 5). The most common site of metastasis is the lung, followed by the lymph nodes, bone, and liver (4, 5, 25). Interestingly, lung metastasis often presents as pneumothorax, which may require urgent medication (26, 27).

DIAGNOSIS AND STAGING

Patients with typical presenting symptoms can be diagnosed clinically, but the precise pathological diagnosis should be performed by an expert pathologist. The histologic features of angiosarcoma can vary between patients and even within the same patient. When the tissue specimens are taken from well-differentiated areas, the tumor cells usually form vessel-like structures and may be difficult to differentiate from normal vessels. However, the tumor vessels tend to form independent or separate networks with anastomoses (28). Other features such as cellular atypia, mitoses, and formation of multilayer endothelium can be helpful for diagnosis. On the other hand, in poorly differentiated areas, the tumor cells show sheet-like growth with hemorrhage and necrosis, which have fewer features than do vascular tumors. In such cases, positive staining for endothelial markers such as CD31, CD34, von Willbrand factor, and vascular endothelial growth factor (VEGF) are useful (29). Also, lymphatic endothelial markers such as D2-40 are positive for most superficial angiosarcomas (28).

The staging of CAS is based on the TNM staging system of the American Joint Committee on Cancer (AJCC) (**Table 1**). The tumor grade based on the pathologic features is included in the staging. In brief, localized disease is classified as stage I or II; nodal spread or T2 tumor with histologic grade 3, as stage III; and distant disease, as stage IV. However, because there is no standardized treatment algorithm for each stage, staging of CAS has little clinical benefit in the treatment decision.

PROGNOSIS AND FACTORS ASSOCIATED WITH SURVIVAL

Generally, soft-tissue sarcomas have a 50–60% survival rate (30), whereas the 5-year survival rate for angiosarcoma is <40% (12, 25, 31, 32). Several factors are reportedly associated with poor survival: older age (25, 32), worse performance status (33, 34), larger tumor size (5, 8, 20, 32, 35–40), positive margin status (31, 32, 38, 41, 42), higher histologic type or grade (32, 37, 41, 43, 44), scalp as the primary location (5, 36, 45), deeper location of the tumor (20, 31), and presence of distant metastasis (33, 38,

41, 46). On the other hand, the following factors were associated with favorable prognosis: surgery (20, 34), multimodal therapy (5, 39, 41) and postoperative radiotherapy (34, 36, 41, 43, 47, 48). The studies that included more than 50 patients with CAS only are summarized in **Table 2**. According to these five studies, tumor size seems to be a consistently poor prognostic factor; indeed, patients with tumors larger than 10 cm all died of the disease (35, 36).

A study by Sinnamon et al. (32) of 821 angiosarcomas included 211 cases of primary CAS in the head and neck. In their cohort, all cases of metastatic disease were excluded and all the patients received surgical treatment. They scored the following factors and classified the risk from low (total score 0–1), intermediate (total score 2–3), and high (total score 4–7): age > 70 as 1, black ethnicity as 1, histologic tumor grade 3 as 1, tumor size 3–7 cm as 1, tumor size larger than 7 cm as 2, microscopic residual tumor as 1, and macroscopic residual tumor as 2. By using this model, patients at high risk had a median overall survival of only 1.6 years with a hazard ratio of 5.65 when compared with patients at low risk.

TABLE 1 | AJCC TNM staging system for soft tissue sarcoma.

		0	1	2	
T classification			Size<=5cm	Size>5cm	
a: superficial tumor, b: deep tumor (divided by superficial fascia)					
N classification		No nodal metastasis	Nodal metastasis		
M classification		No distant metastasis	Distant metastasis		
Stage		T	N	M	Histologic grade
I	A	1a/b	0	0	1 or not assessed
	B	2a/b			
II	A	1a/b			2 or 3
	B	2a/b			2
III		2a/b			3
		Any	1		Any
IV		Any	Any	1	

Histologic grading is defined as follows: (1) Differentiation: score from 1 to 3, (2) Mitotic count: score from 1 to 3, and (3) Tumor necrosis: score from 0 to 2.
Sum (1) to (3) and determine grade as follows.
Gx: not assessed.
G1: total score of 2 or 3.
G2: total score of 4 or 5.
G3: total score of >5.

This result clearly indicates that these factors strongly correlate with poor survival.

Reports from Japan and from western countries showed differences in survival. In the study from Japan of 260 cases of CAS, the 5-year overall survival among patients who could receive surgery was <20% (49) (median overall survival: < 20 months), whereas in the studies from western countries, it was 31–51% (5, 11, 25, 31, 40). CAS patients in Japan had equivalent survival to the "high risk" group reported by Sinnamon et al. (32), with a median overall survival of 1.6 years. This difference might be explained by the fact that the tumor size in Japanese patients is generally large: in the study of 260 CAS cases, 44% of the patients had tumors of at least 10 cm (originally, described as tumors larger than 100 cm^2) (49), whereas tumors larger than 5 cm (T2) constituted only 18–38% of the patients in the studies from western countries (5, 11, 25, 40). Our multicenter study, which included only Japanese patients, was also T2 dominant: only 3 of 28 patients (11%) had a T1 tumor (19). In the meta-analysis by Hwang et al., which included 128 cases from seven studies (50), the median overall survival in the T1 group was significantly longer than that in the T2 group (31.4 months and 17.3 months, respectively: $P < 0.001$). Collectively, Japanese CAS patients have larger primary tumors than do CAS patients in western countries, and consequently, the survival of Japanese CAS patients is shorter.

TREATMENT

Current Treatment Options

Surgery

Radical surgery with no residual tumor cell on the margin (R0 resection) is generally the primary goal of sarcoma treatment. In every review or set of guidelines, surgery with R0 resection is recommended as the goal of CAS treatment (28). In a systematic review by Shin et al. (51), absence of surgery was shown to correlate with poor survival; Trofymenko et al. (52) reported similar result in a study using 764 cases of CAS extracted from the National Cancer Database in the United States. Therefore, there is little doubt that surgery is one of the best choice for the management of CAS.

TABLE 2 | Reported factors associated with poor survival determined by studies with >50 patients in CAS.

	N	Age	Tumor size	Pathological feature	Margin	Location	Others
Albores-Saavedra et al. (11)	434	>50	N.S.			Head and neck Deeper location	Lymph node metastasis Distant metastasis
Perez et al. (40)	88	N.S.	>5 cm		N.S.		
Holden et al. (36)	72	N.S.	>5 cm >10 cm	N.S.		N.S.	
Guadagnolo et al. (5)	70		>5 cm Satellitosis		N.S.		Surgery alone
Patel et al. (25)	55	>70	N.S.	N.S.	N.S.		Without multimodality or radiation therapy

N.S., not significant; PS, performance status.

Although no standardized treatment recommendation has been established, a margin of less than 1 cm is associated with poor survival (49). The depth of the resection has not been well discussed, but generally if the tumor does not extend into the deep fascia, a resection layer including the deep fascia is adequate. If the tumor directly invades into the deep fascia, removal of the underlying structures, e.g., the periosteum or even the outer shell of the skull, is required to obtain R0 resection.

Unfortunately, it is common to see positive microscopic (R1) or macroscopic (R2) margins even after a wide surgical margin from the visible tumor border has been obtained (4, 8, 31, 36, 41). Pawlik et al. (4) reported that in their series of 29 patients, 18 (62.1%) had an initial diagnosis of T1 (<5 cm) tumor, but 11 of those tumors turned out to be T2 (>5 cm) after surgical pathology evaluation of the resected tumor. The clinical margin of the tumor in CAS is difficult to determine because it often develops as a multifocal tumor and presents as a skip lesion. Moreover, when CAS develops near important structures such as the eye, surgical removal with an adequate margin is impossible. As a consequence, the rate of local recurrence after treatment is high reportedly ranging from 26 to 100% (5, 9, 25, 41). Lahat et al. (53) reported 32 of 44 cases of locally recurrent angiosarcoma treated with surgery, 70% of which achieved complete removal of the recurrent tumor, with a 5-year overall survival of 44%.

To reduce local recurrence, postoperative radiotherapy covering a wide area with a >50 Gy dose has been reported by several studies to be effective not only for local control but also for overall survival (4, 5). Currently, wide local excision followed by radiation is the most accepted treatment for CAS (28, 54, 55); however, despite such mutilating multimodal treatment, survival of patients, especially of those with large tumors, is still unsatisfactory (19, 32).

Other than radical surgery, palliative surgery might have role in patients with large tumors to reduce the tumor load. Some reports suggested the use of minimal surgery as part of the management of CAS (6, 56), such as for those cases with a diffuse lesion pattern involving vital structures, recurrent disease, or metastasis.

Chemotherapy

The chemotherapeutic options currently available for angiosarcoma are listed in **Table 3**. Chemotherapy using anthracyclines alone or in combination with ifosfamide have been used for unresectable and metastatic angiosarcoma (35, 57, 58). However, anthracyclines have cardiac toxicity which make it difficult to apply in older patients. Taxanes, which inhibit tubulin elongation, were introduced in the 1990s as a novel cytotoxic drug and have become accepted as standardized treatment options in various kinds of cancers such as those of the breast (59), lung (60), stomach (61), and uterus (62), because of their high efficacy. Although several clinical studies have shown that taxanes are of little benefit for sarcomas (63, 64), the angiosarcomas included in those clinical studies showed antitumor activity (64). Taxanes not only have a direct antitumor effect but also have been shown to exert an antiangiogenic effect (65, 66), which is thought to be suitable for the treatment of vascular tumors. Indeed, taxanes were shown to be effective for the treatment of Kaposi sarcoma (67, 68).

In 1999, Fata et al. (69) achieved a response ratio of 89% by using paclitaxel monotherapy for the treatment of head and neck CAS. Later, Penel et al. (70) conducted the first phase-2 trial for metastatic or locally advanced angiosarcoma, which included 30 patients treated with paclitaxel. In that clinical trial, the progression-free survival rate after 4 months was 45%, and the median overall survival was 8 months. Considering that the patients with distant metastasis consisted of 74% of the study population and 36% of them had had previous systemic chemotherapy, this result was encouraging. Italiano et al. (71) showed, albeit in a retrospective study, that paclitaxel achieved an equivalent outcome to that of anthracyclines in the treatment of advanced angiosarcoma despite the patients treated with paclitaxel being a decade older

TABLE 3 | Chemotherapy options and their effect for angiosarcoma.

Agent	Patients	*N*	Response/median survival (months)
Anthracyclines	Pooled analysis of 11 clinical trials for angiosarcoma from all sites Young et al. (58)	108	Response ratio: 25% for all sites PFS: 4.9, OS: 9.9
Paclitaxel	Retrospective review of angiosarcoma from all sites Italiano et al. (71)	34	Response ratio: 29.5% for all sites
		68	Response ratio: 53% for all sites Response ratio: 78% for CAS
	ANGIOTAX study: phase-2 study including angiosarcoma from all sites Penel et al. (70)	30	Response ratio: 18% for all sites Response ratio: 89% for CAS
	ANGIOTAX plus study: phase-2 study comparing paclitaxel with/without bevacizumab from all sites (showing paclitaxel arm only) Ray-Coquard et al. (74)	24	Response ratio: 45.8% for all sites PFS: 6.6, OS: 19.5
	Retrospective study for head/neck CAS Fata et al. (69)	9	Response ratio: 89% for CAS
Docetaxel	Retrospective study for CAS Nagano et al. (101)	9	Response ratio: 67% for CAS
Gemcitabine	Retrospective study with angiosarcoma from all sites Stacchiotti et al. (76)	25	Response ratio: 64% for all sites PFS: 7, OS: 17

PFS, progression-free survival; OS, overall survival; CAS, cutaneous angiosarcoma.

than those treated with anthracyclines (67.4 and 57.4 years old, respectively). Collectively, taxanes can achieve a similar level of antitumor effect to that achieved by anthracyclines, but with less toxicity, and therefore, a recent report (72) suggested using taxanes as the first-line treatment for CAS with unresectable or distant disease. Indeed, we reported (17, 73) successful treatment results using taxanes as the first-line therapy for primary CAS.

Because both taxanes have been reported to be effective, the decision about which taxane to use as the first-line might be difficult. In this review, we recommend paclitaxel as the first-line treatment since paclitaxel has been evaluated in different phase-2 studies (70, 74), whereas docetaxel has not yet been evaluated in a prospective study. However, docetaxel still has a role as a second-line therapy in patients refractory to paclitaxel (75).

Gemcitabine has been reported to be effective for sarcomas both as a single agent (76, 77) and in combination with docetaxel (78, 79). Several case series (77, 80) have been reported in which gemcitabine for the treatment of angiosarcoma was used with favorable outcomes. Moreover, albeit in a study based on a retrospective pooled analysis (76), gemcitabine showed an overall response rate of up to 68% for angiosarcoma (76). If this agent is used as monotherapy, the toxicity profile is better than that of anthracyclines but still has a significant incidence of bone marrow suppression.

Radiation

Radiation is usually delivered after surgery for better local control (28, 54, 55). However, dismal outcome have been reported when radiation was used as monotherapy (5, 38, 43). Therefore, radiation monotherapy is generally used for palliation, not for curative intent because of frequent recurrence, as high as 100% in previous studies (25, 36, 42, 43). On the other hand, Ogawa et al. (34) reported that in their cohort of 25 patients who received radiation monotherapy with curative intent, 11 of the 14 patients (79%) who received >70Gy achieved local control, whereas only 3 of the 11 patients (27%) who received <70 Gy did. A study by Scott et al. (81) of 41 patients treated with radiation recommended at least 60–65 Gy for the postoperative tumor bed and 70–75 Gy for patients who receive radiation monotherapy. Others (82) suggested that improved delivery of radiation might achieve higher efficacy. Since no prospective study has been conducted to evaluate the role of radiation as the first-line therapy, radiation monotherapy is still difficult to use as curative intent therapy for primary disease. We will discuss combination radiation and chemotherapy in the next section.

New Treatment Options

Chemoradiotherapy (CRT)

The use of chemotherapy and radiation (CRT) concurrently or concomitantly is one of the standardized treatment methods for several cancers: esophageal (83), head and neck (84), rectal (85), and cervical (86). Chemotherapeutic agents such as 5-fluorouracil (84, 85), cisplatin (87), gemcitabine (88), and taxanes (89, 90) are expected to act not only as cytotoxic but also as radiosensitizing agents. Therefore, CRT may sometimes cause higher toxicity than does monotherapy but can be justified by its high antitumor effect, and in most cases, such side effects are manageable. Besides, although many cancer treatments introduced CRT as one of the key treatments, it was an uncommon method among cutaneous malignancies. In such a situation, we started to use cisplatin and 5-fluorouracil concurrently with radiation for the management of unresectable/metastatic cutaneous squamous cell carcinoma with the same protocol used in the head and neck and reported successful treatment results (91–93).

As described previously, a Japanese retrospective study of CAS (49) revealed that the median overall survival of patients with non-metastatic localized CAS who received surgery was less than 20 months, but this finding was not surprising because we have reported a similar dismal outcome (13.5 months) (19). We suspected that increased expression of VEGF during the wound healing process (94) caused by mutilating surgery might cause progression of residual angiosarcoma because angiosarcoma has been reported to express a VEGF receptor (95–97). As discussed in the previous section, tumor size is the most common factor for poor prognosis, which is commonly related to a positive surgical margin. Therefore, it is convincing to consider that such subclinical residual tumors could be expanded by VEGF released by surgery.

In such a situation, a retrospective study (47) of use of chemotherapy (anthracyclines) and radiation for five head and neck CAS (four scalp and one lip, three of them with high-grade tumors) was reported and achieved a median overall survival of 27.0 months, which was better than the reported median survival of face and scalp CAS (<20 months) (6, 36, 45). However, there was a concern related to use of anthracyclines for older CAS patients for whom the drug might not be tolerable. On the other hand, taxanes have a better toxicity profile, and therefore, we expected that older CAS patients could tolerate it. Moreover, taxanes are known as radiosensitizers (89, 90), and therefore, possibly an ideal agent for CRT for the treatment of CAS.

The reported cases of CAS treated with CRT are described in **Table 4**. Because the study by Mark et al. (47) did not describe the timing of the chemotherapy, we could not determine whether they used chemotherapy concurrently or concomitantly with radiation. In the study by Miki et al. (98), 5 of the 12 patients who received docetaxel, the schedule was adjusted so that the drug was administered concurrently only on the first and last weeks of radiation. Another seven patients received docetaxel for 2–6 weeks during radiation in accordance with patient status. All the patients in the other two studies received chemotherapy and radiation concurrently (19, 98, 99). Most of the arms are composed of scalp CAS, which correlated with poor survival. The response to CRT was 82% (98) and 94% (19), with a statistically higher median overall survival than that of surgery followed by radiation in both studies. Representative photographs of patients who received CRT are presented in **Figures 1A–D**.

Concurrent CRT brings severe side effects than when each treatment is delivered as monotherapy. In our study, 78% of the patients who received concurrent CRT had CTCAE grade-4 neutropenia, but the neutropenia was made manageable by use of granulocyte-colony stimulating factor and no treated-related death was observed (19). In the study by Miki et al.

TABLE 4 | Study using chemotherapy and radiation therapy for CAS.

Study	*N*	Patients tumor location/size	Treatment	RT dose	Response/pattern of failure	Median OS (months)
Mark et al. (47)	5	Scalp: 4 and Face: 1 Size not described	Anthracyclines	30–76.2 Gy	Response ratio: N.D. Local: 2/5 Distant: 2/5	27.0
	4	Scalp: 1 and Face: 3 Size not described	*Surgery*	50–65 Gy	Local: 1/4 Distant: 0/4	Not reached
Rhomberg et al. (99)	1	Scalp/face: 1 T2	Razoxane Vindesine	35–66 Gy	CR Alive without failure	41
Miki et al. (98)	11	Scalp: 16 and Face: 1 T1: 1 and T2: 15	Docetaxel	70 Gy	Response ratio: 82% Local: 4 Distant: 5 Both: 4	33.7 *
	5		*Surgery/IL-2*			22.7 *
Our study (19)	16	Scalp: 14, and Extremity: 2 T1: 2 and T2: 14	Docetaxel	<65Gy:12 ≧65Gy:16	Response ratio: 94% Local: 6 Distant: 7	Not reached **
	12	Scalp: 10, extremity: 2 T1: 2 and T2: 10	*Surgery*		Only 1 patient still alive	15.0 **

*$P < 0.05$.
**$P < 0.01$.
OS, overall survival; CAS, cutaneous angiosarcoma; N.D., not described; IL-2: interleukin-2.

TABLE 5 | Maintenance chemotherapy after primary therapy.

Study	*N*	Tumor location	Primary chemotherapy	RT dose	Maintenance chemotherapy and duration (months)		Pattern of failure	Median OS (months)
Nagano et al., 2007 (101)	9	Scalp: 6, Face: 1 Neck: 1, Leg: 1	Docetaxel	–	Docetaxel	3–22 MD: 13.5	Local: 4 Distant: 0	Not described
Rhomberg et al. 2009 (99)	5	Thyroid: 4, Scalp/face: 1	Razoxane Vindesine	35–66 Gy MD: 56 Gy	Razoxane Vindesine	6 weeks-36 MD: 12	Local or distant: 2	27.0
Our study 2014	9	Scalp: 7, Limb: 2	Docetaxel	48–80 Gy MD: 70 Gy	Docetaxel	3–50 MD: 12.5	Local/LN: 3 Distant: 2	Not reached **
	7	Scalp: 7		52.5–70.4 Gy MD: 70 Gy	Not done		Local: 4 Distant: 5	21.0 **

** $P < 0.01$.
RT, radiotherapy; MD, median; OS, overall survival.

(98), all the patients developed grade 1–3 dermatitis but healed uneventfully.

Taking these finding together, CRT using taxanes could achieve satisfactory antitumor activity with good tolerability and might bring better survival than does conventional surgery followed by radiation especially for CAS of the scalp. Although the use of taxanes concurrently might bring severe side effects, we suggest concurrent CRT to gain maximum antitumor effect as long as the side effects are tolerable and manageable.

Maintenance Chemotherapy

To prevent locoregional and distant failure after response to chemotherapy, some previous report continued chemotherapy to maintain the response. Gambini et al. (100) achieved complete remission of radiation-induced angiosarcoma after treatment with paclitaxel and maintained the response for 4 years by maintenance therapy with intervals of no longer than 3 weeks. Interestingly, they had local recurrence twice when the treatment was delayed, but in both instances, a new complete remission was rapidly achieved with the same treatment and the patients remained disease-free at the time of their report. Nagano et al. (101) reported nine CAS patients treated with docetaxel, eight of whom continued docetaxel for 3–22 months (**Table 5**). None of the patients developed distant metastasis during maintenance chemotherapy. Rhomberg et al. (99) treated nine patients with angiosarcoma (five with thyroid, one with left ventricle, one with bladder, and one with scalp/face) with concurrent CRT using razoxane and vindesine. Complete remission of the tumor was obtained in six patients, five of whom received maintenance chemotherapy for 6 weeks to a year. Of those five patients, two developed recurrence but only one developed it during the maintenance chemotherapy.

In our study (19), 16 CAS patients were treated with concurrent CRT and 9 of them received maintenance chemotherapy. Locoregional relapse was seen in three of the nine patients who received maintenance chemotherapy, whereas it was seen in four of the seven patients who did not receive it. On the other hand, only two of the nine patients who received maintenance

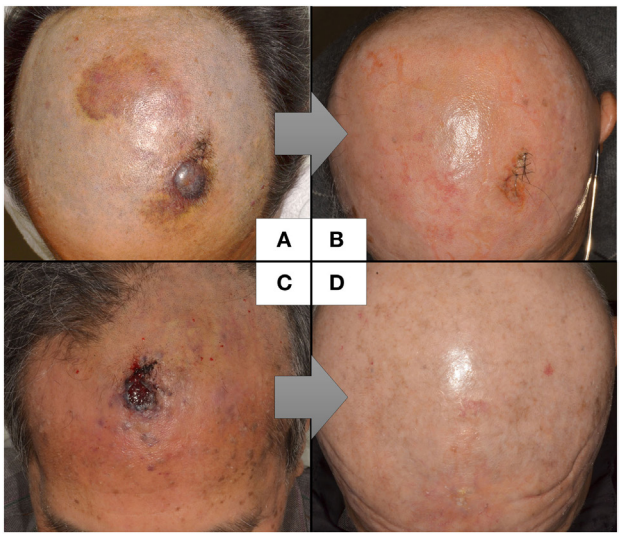

FIGURE 1 | Representative cases of CAS treated by concurrent CRT.

chemotherapy developed distant metastasis, whereas five of the seven patients who did not receive maintenance chemotherapy did develop distant metastasis ($P < 0.05$). A study by Ito et al. (75) showed that 19 patients who received maintenance chemotherapy using taxanes had significantly better survival than did 24 patients who received maintenance chemotherapy without taxanes ($P < 0.0024$) Collectively, maintenance chemotherapy after remission obtained by CRT seems to suppress tumor regrowth and development of distant metastasis. However, there is no consensus as to how long this maintenance chemotherapy should be continue. Further investigation is needed to determine the optimal length of maintenance chemotherapy.

Adjuvant/Neoadjuvant Chemotherapy

The use of adjuvant chemotherapy after complete removal of the tumor is attractive because we experience many CAS patient who develop distant metastasis even though there is no locoregional failure. However, anthracycline-based adjuvant chemotherapy did not show any survival benefit in soft tissue sarcomas (102). Indeed, we could not see any survival benefit in CAS patients by using taxanes after surgery and radiation (7). Similarly, adjuvant chemotherapy did not show a clear benefit among angiosarcoma patients treated with anthracyclines, paclitaxel, and other combinations (5, 6, 41, 44).

Some groups reported the use of chemotherapy before surgery (neoadjuvant chemotherapy) but did not show any survival benefit in face CAS (103) or in head and neck CAS (5). However, a certain percentage of patients who received neoadjuvant chemotherapy could achieve a complete response (60% in face CAS (103)) and did not require definitive surgery. Thus, the effect of neoadjuvant chemotherapy is difficult to interpret.

Since no large prospective study has been conducted to evaluate the value of adjuvant and neoadjuvant chemotherapy, those previous studies should be read with caution. However, the largest retrospective analysis of CAS including 821 patients indicated that both adjuvant and neoadjuvant therapy after surgery did not show any survival benefit on univariate and multivariate analyses (32). Further prospective study is required to evaluate the role of adjuvant/neoadjuvant chemotherapy for CAS.

New Drugs

Anti-VEGF Drugs

Angiosarcomas express VEGFR (95, 97, 104), and overexpression of VEGF converted slow-growing vascular endothelial tumors to fast-growing malignant tumors in a mouse model and formed invasive angiosarcoma in immunodeficient mice (105). Conversely, blockade of the VEGF/VEGFR pathway inhibited tumor growth *in vitro* (106). Therefore, it is reasonable for the treatment to target the VEGF/VEGFR signaling pathway. Several studies using anti-VEGF monoclonal antibody (bevacizumab) have shown antitumor activity in angiosarcomas: 4 of 30 patients treated with bevacizumab had a partial response, with a mean time to progression of 26 weeks (107), and 2 of 2 patients treated with bevacizumab and radiation had a complete response (108).

On the basis of this background, Ray-Coquard et al. (74) conducted a non-comparative, open-label, randomized phase-2 trial to explore the activity and safety of bevacizumab and paclitaxel therapy for patients with advanced angiosarcoma. Fifty patients were randomized and assigned to two arms: (1) the paclitaxel alone or (2) the paclitaxel and bevacizumab arm. From the findings, they concluded that there is no benefit from adding bevacizumab to paclitaxel (median overall survival: 19.5 versus 15.9 months).

Other than monoclonal antibody, two small-molecule multi-tyrosine kinase inhibitors that can inhibit the VEGF/VEGFR signaling pathway have been used for the treatment of angiosarcoma patients: sorafenib (109) and pazopanib (110). A phase-2 trial including 37 patients with recurrent or metastatic angiosarcoma treated with sorafenib showed a response ratio of 14% with median progression-free survival of 3.8 months (111). No clinical trial to evaluate pazopanib activity in angiosarcoma has been conducted. In a case series using pazopanib for the treatment of taxane-resistant CAS, two of five patients achieved a partial response with median progression-free survival of 94 days (112). On the other hand, a case series of eight CAS patients treated with pazopanib did not show any benefit (113). Although we do not have enough conclusive evidence, the current first-line treatment should still be taxanes and anti-VEGF pathway therapy should be considered as the second- and third-line therapy.

Eribulin Mesylate

Eribulin mesylate suppresses microtubule polymerization and sequesters tubulin into nonfunctional aggregates, which is a mechanism distinct from those of other tubulin-targeting drugs such as taxanes (114). A phase-3 study comparing dacarbazine and eribulin in patients with advanced liposarcoma or leiomyosarcoma showed improved survival in patients treated with eribulin (115). This phase-3 study did not include angiosarcoma, and therefore, we do not have any evidence on the effect of eribulin for

angiosarcoma. However, both taxanes and eribulin target microtubule polymerization, and eribulin binds to a different site of the microtubule (116), indicating that it may be effective for patients who become resistant to taxanes. Albeit in a case report, eribulin was shown to be effective for a patient who became resistant to docetaxel (117). Currently, we are conducting a prospective, observational clinical study to evaluate eribulin in patients with CAS who became resistant to taxanes (UMIN000023331); patient enrollment for this study is expected to be completed in 2018.

Checkpoint Inhibitors

Recent development of checkpoint inhibitors in melanoma treatment dramatically improved the survival of advanced melanoma. Melanoma with higher expression of programmed death receptor ligand-1 (PD-L1) correlated with a better treatment outcome when using anti-PD-1 antibody (118). This result supports the notion of a proposed immune escape mechanism by tumor cells using their PD-L1 expression on the cell surface to bind PD-1 on cytotoxic T cells and attenuate the immune response (119). Interestingly, our study group showed that CAS with PD-1 positive cell infiltration and tumor site PD-L1 expression correlated with survival (120). This result raises the possibility of using anti-PD-1 antibody for the treatment of CAS. To the best of our knowledge, there is no on-going or planned clinical trial to use checkpoint inhibitors for advanced angiosarcoma (clinicaltrials.gov).

CURRENT RECOMMENDATION AND FUTURE PERSPECTIVE

The treatment of CAS, especially T2 tumors of the scalp, is still challenging. The surgical approach seems to be difficult because such tumors usually have an unclear border and often have skip lesions that make it difficult to determine the "true" tumor border. As patients with tumors larger than 10 cm were reported to have a catastrophic prognosis (35, 36), the current standard wide-margin resection followed by wide-field radiation might be palliative rather than curative (6). Radical surgery can reduce the tumor load; however, surgery-based treatment cannot target "subclinical" metastasis, which may have already occurred by the time of diagnosis. Therefore, we strongly recommend starting systemic chemotherapy along with primary tumor therapy. CRT can achieve this task: systemic administration of taxanes can target subclinical metastases and also act as a radiosensitizer that will enhance the effect of radiation therapy against the primary tumor. Although neoadjuvant chemotherapy and adjuvant chemotherapy may also achieve this task, to the best of our knowledge, no study has shown the superiority of this strategy.

Collectively, we suggest considering concurrent CRT using taxanes when we encounter CAS of the scalp with a T2 tumor. We also recommend maintenance chemotherapy even if complete remission of the tumor has been achieved. On the other hand, for T1 CAS with a clear tumor border, the current standard surgery followed by radiation might be sufficient to obtain a successful result. However, these recommendations are based on a small number of retrospective studies. CRT and maintenance chemotherapy should be evaluated with prospective clinical studies to confirm the superiority of this strategy.

Moreover, we currently do not have many options for when the tumor becomes resistant to taxanes. We have already launched a clinical study to evaluate eribulin mesylate as the second-line treatment after taxane-failure. Several clinical studies are now ongoing or planned to evaluate the effect of multi-kinase inhibitors such as sorafenib or pazopanib (clinicaltrials.gov). We hope the treatment of CAS will be dramatically improved, as it has for melanoma, in the near future.

AUTHOR CONTRIBUTIONS

I have full responsibility of this article. YF wrote the part of the manuscript. KY, TF, YN, NO, RW, YI, and MF confirmed the manuscript for submission.

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Metastatic Extramammary Paget's Disease: Pathogenesis and Novel Therapeutic Approach

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Extramammary Paget's disease (EMPD) is a rare, slow-growing, cutaneous adenocarcinoma that usually originates in the anogenital area and axillae outside the mammary glands. EMPD mostly progresses slowly and is often diagnosed as carcinoma *in situ*; however, upon becoming invasive, it promptly and frequently metastasizes to regional lymph nodes, leading to subsequent distant metastasis. To date, several chemotherapy regimens have been used to treat metastatic EMPD; however, they present limited effect and patients with distant metastasis exhibit a poor prognosis. Recently, basic and translational investigative research has elucidated factors and molecular mechanisms underlying the promotion of metastasis, which can lead to targeted therapy-based emerging treatment strategies. Here, we aim to discuss current therapies and their limitations; advancements in illustrating mechanisms promoting invasion, migration, and proliferation of EMPD tumor cells; and future therapeutic approaches for metastatic EMPD that may enhance clinical outcomes.

Keywords: metastatic extramammary Paget's disease, HER2–PI3K/ERK signaling, lymphangiogenesis, CXCR4–stromal cell-derived factor-1 axis, CD163$^+$M2 macrophage, receptor activator of nuclear factor kappa-B ligand–RANK signaling, mismatch-repair deficient, anti-PD-1 antibody

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INVASIVE EXTRAMAMMARY PAGET'S DISEASE (EMPD) THERAPEUTIC CHALLENGE: PREVENTING AND TREATING TUMOR METASTASIS

Extramammary Paget's disease is a rare, slow-growing, cutaneous adenocarcinoma that manifests as an erythematous, eczematous plaque outside the mammary gland, occasionally accompanied by hypopigmented patches. EMPD affects apocrine gland-rich sites such as the anogenital area and axillae. Despite requiring broad local resection for the treatment of a primary lesion because of the frequent microscopic extensions and less common satellite lesions beyond the clinical tumor border (1), the prognosis of patients with EMPD is usually good because tumor cells are in the radial growth phase for a prolonged duration, and a majority of cases are treated in the stage of carcinoma *in situ* (2).

However, once EMPD invades into the dermis and becomes invasive EMPD, tumor cells gain high metastatic potential, leading to the development of lymph node (LN) metastasis even in patients with dermal microinvasion (3). Besides, over one-third of patients with LN metastasis consequently develop distant metastasis (4). To date, several chemotherapeutic regimens (**Table 1**), such as low-dose 5-fluorouracil (5-FU)/cisplatin (FP), FECOM (5-FU, epirubicin, carboplatin, vincristine, and mitomycin C), docetaxel monotherapy, S-1 monotherapy, docetaxel and S-1 combination therapy,

TABLE 1 | Current systemic therapy for metastatic extramammary Paget's disease in case studies and case reports.

Reference	No. of patients	Treatment	Type of response	Outcome (months)
Tokuda et al. (5)	22	Low-dose FP (5-FU, cisplatin)	CR (1/22), PR (12/22), SD (6/22), PD (3/22)	PFS: median 5.2, OS: median 12.0
Oashi et al. (6)	7	FECOM (5-FU, epirubicin, carboplatin, vincristine, mitomycin C)	PR (4/7), unevaluable (3/7)	PFS: median 6.5, OS: median 9.4
Yoshino et al. (7)	13	Docetaxel	PR (8/13), SD (3/13), PD (2/13)	PFS: median 7.1, OS: median 16.6
Mikoshiba et al. (8)	1	S-1	PR	PFS: 36
Kato et al. (9)	2	S-1	PR	PFS: 5, 11+
Matsushita et al. (10)	1	Docetaxel + S-1	PR	PFS: 15+
Ogata et al. (11)	1	Docetaxel + S-1	PR	PFS: 12+
Egashira et al. (12)	2	Docetaxel + S-1	CR (1/2), PR (1/2)	PFS: 12, 10, OS: 26, 23
Hirai and Funakoshi (13)	2	PET (cisplatin, epirubicin, paclitaxel)	PR (2/2)	PFS: 14+, 12+
Karam et al. (24)	1	Trastuzumab	PR	PFS: 12
Wakabayashi et al. (27)	1	Trastuzumab	PR	PFS: 12+
Barth et al. (28)	1	Trastuzumab	CR	PFS: 12+
Takahagi et al. (25)	1	Trastuzumab + paclitaxel	PR	PFS: 17, OS: 25
Hanawa et al. (26)	1	Trastuzumab + paclitaxel	PR	PFS: 13
Ichiyama et al. (29)	1	Trastuzumab + paclitaxel	PR	PFS: 24+
Yoneyama et al. (45)	1	Bicalutamide + leuprolide acetate	PR	PFS: 6, OS:14

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression-free survival; OS, overall survival; +, ongoing response; 5-FU, 5-fluorouracil.

and PET (cisplatin, epirubicin, and paclitaxel), have been used to treat metastatic EMPD (5–13); however, few patients overcome tumor recurrence despite tumors in over half of patients' initially responding to these regimens. In addition, the overall survival (OS) starts declining from 10 months after starting chemotherapy (7), and patients with EMPD with distant metastasis exhibit a poor prognosis with the median OS of 1.5 years and 5-year survival rate of 7% (4). Hence, exploring novel therapeutic strategies to prevent and treat metastatic EMPD is imperative.

Metastasis is a multistage process that comprises tumor cell invasion, venous/lymphatic intravasation, transit in the vessels, venous/lymphatic extravasation, and proliferation at a new site (14). Recently, some studies have identified that the protein expression of molecules involved in proliferation and survival, including HER2 and mTOR, in the Paget cell (tumor cell of EMPD) is associated with invasiveness, metastasis, and OS (15–17). Similarly, translational research studies have demonstrated that cytokines, chemokines, and immune cells in EMPD confer favorable microenvironment for Paget cells to invade, migrate, and proliferate, thereby promoting metastasis (18–21). The results of current study support that the involvement of both Paget cells and tumor microenvironment factors is essential for metastasis; thus, the understanding of both aspects provides opportunities for the treatment of metastatic EMPD.

HER2–PI3K/ERK SIGNALING IN EMPD

In the past, several EMPD studies have focused on investigating the HER2–PI3K/ERK signaling in EMPD because it is characterized by the presence of Paget cells, large round, vacuolated, pale-staining cells, as mammary Paget's disease (MPD) and the clinical success in targeting aberrant receptor tyrosine kinase signaling pathways in breast cancer. Both immunohistochemistry and fluorescence *in situ* hybridization studies of primary and LN metastatic lesions have revealed the HER2 overexpression (HER2 score of 3+ or 2+) in 15–58% of patients with EMPD and that all cases with a HER2 score of 3+ had amplified *ERBB2*, the gene encoding HER2 (16, 22). Of note, a HER2 score of 3+ or 2+ was significantly more common in patients with deeply invasive EMPD that those with *in situ*/superficial invasive (in which tumor invasion was limited to the papillary dermis) EMPD and was correlated with numerous LN metastases (16). Furthermore, about 90% of patients exhibited no difference in the HER2 protein overexpression and *ERBB2* gene amplification between primary tumors and corresponded LN metastasis (23), suggesting that HER2's contribution to the pathogenesis and progression in a subset of metastatic EMPD and implying the possibility that HER2 blockade disrupts the progression of both primary and metastatic lesions of this population. In corroboration with the hypothesis, six case reports determined that both primary and metastatic lesion of HER2-overexpressed EMPD responded well and attained partial or complete response for 6 months to 2 years by anti-HER2 antibody, trastuzumab alone, or trastuzumab with paclitaxel (**Table 1**) (24–29). A phase 2 study of trastuzumab with docetaxel for HER2-positive unresectable or metastatic EMPD (UMIN000021311) is ongoing based on these results.

HER2 activates several signaling pathways such as the RAS–RAF–MEK–ERK pathway and PI3K–AKT–mTOR pathway, which accelerate cell growth and increase cell survival (30). A study demonstrated that 28 and 56% of HER2-overexpressed EMPD cases presented high expression of phosphorylated ERK and phosphorylated AKT, respectively, with high Ki-67 labeling index (31). However, the high expression of phosphorylated ERK and phosphorylated AKT was also noted in 30 and 33% of EMPD patients without the HER2 overexpression, respectively, but with high Ki-67 labeling index (31). In addition, other studies have demonstrated that Ki-67, as well as mTOR, was expressed at

significantly higher levels in invasive cases than carcinoma *in situ* cases (17, 32). Furthermore, the DNA sequencing of EMPD study revealed that 19% of cases comprised mutant *RAS* or *RAF* genes and 35% of cases had mutations in *PIK3CA* (which encodes the catalytic subunit of PI3K) or *AKT1* that activate those pathways (33). In particular, cases with mutations in both *RAS*/*RAF* and *PIK3CA*/*AKT1* signaling pathways were sporadic, and a mutually exclusive pattern was observed (33). Overall, these results highlight that not only HER2 but also the activation of downstream molecules in the RAS–RAF–MEK or PI3K–AKT–mTOR pathways could contribute to the progression of EMPD. Notably, all mutations detected in *RAF* were *BRAF V600E* that can be inhibited by vemurafenib or dabrafenib, the Food and Drug Administration (FDA)-approved drugs for metastatic melanoma (33). In addition, multiple drugs targeting the RAS–RAF–MEK and PI3K–AKT–mTOR pathways have passed through clinical trials and are used for other cancers. Hence, multiple treatment options exist for developing novel therapeutics that target patients with metastatic EMPD with the aberrant RAS–RAF–MEK or PI3K–AKT–mTOR signaling (**Figure 1A**).

The HER family comprises four type 1 transmembrane tyrosine kinase receptors, HER1 (or EGFR), HER2, HER3, and HER4 (34). Of these, HER2 dimerizes with other members of the HER family, and the dimerization of HER2:HER3 dimers was proven to be the most oncogenic receptor pairing that activates the RAS–RAF–MEK and PI3K–AKT–mTOR pathways in HER2-positive breast cancer cells (35). Hence, pertuzumab, an anti-HER2 antibody that inhibits dimerization with HER1, HER3, and HER4, in combination with trastuzumab and docetaxel attained a significantly longer progression-free survival and OS in untreated HER2-positive metastatic breast cancer compared to trastuzumab plus docetaxel alone; the former regimen is now preferred as the first-line treatment of patients with HER2-positive metastatic breast cancer (36, 37). However, the expression of HER1, HER3, and HER4 has never been detected in EMPD as well as in MPD, and targeting the dimerization of HER2 is unlikely to prove clinical significance in HER2-positive metastatic EMPD (38, 39).

HORMONE RECEPTORS SIGNALING IN EMPD

Signaling through hormone receptors contributes to the tumor development and progression in some cancer types such as breast cancer. More than two-thirds of breast cancers express estrogen receptor (ER), and research has proved that ER-targeted therapy reduces relapse and improves the OS in advanced breast cancer (40). However, MPD expresses ER only in 10% of cases despite being one of the types of breast cancer. Similarly, EMPD also demonstrates a low ER-positive rate at 4% (41). In fact, both MPD and EMPD exhibit a high androgen receptor (AR)-positive rate at 54–90%. In addition, another study reported that the AR expression intensity was significantly higher in invasive EMPD than noninvasive EMPD (41–43). Furthermore, the expression intensity of 5α-reductase and 17β-hydroxysteroid dehydrogenase type 5, enzymes producing androgen, was higher in invasive EMPD than noninvasive EMPD, suggesting the possibility of androgen amplification and the association of the androgen–AR signaling with the progression of EMPD (**Figure 1A**) (43, 44). In corroboration with the hypothesis, one case report demonstrated that the combined androgen blockade (CAB) therapy by bicalutamide (an anti-androgen drug) and leuprolide acetate (LH–RH agonist), used in the treatment of prostate cancer, can significantly reduce multiple bone metastases of EMPD (45). Although the effect of CAB lasted only for 6 months, it did not cause severe bone marrow suppression; hence, androgen-deprivation therapy could be one of the potential therapeutic approaches for metastatic EMPD.

LYMPHANGIOGENESIS AND EPITHELIAL–MESENCHYMAL TRANSITION (EMT) OF PAGET CELLS IN EMPD

The clinical manifestation of erythematous, eczematous plaque of EMPD indicates the presence of vasodilation and hyperemia in the dermis of EMPD lesion. In fact, the histology of *in situ* and invasive EMPD lesions demonstrates a prominent enlargement and formation of several lymphatic and blood vessels in the dermis compared to those of healthy skin or other skin cancers such as melanoma (18). The immunohistochemical analysis established the expression of VEGF-A in Paget cells, as well as macrophages, and VEGF-C was also expressed in Paget cells (18). Both VEGF-A and VEGF-C are renowned cytokines related to angiogenesis and lymphangiogenesis, and evidence suggests that the generation of new blood and lymphatic vessels is crucial in cancer metastasis (46). Intriguingly, the immunohistochemical analysis also revealed that lymphatic endothelial cells (LECs) in a primary lesion express stromal cell-derived factor-1 (SDF-1), and N-cadherin-positive Paget cells co-expresses CXCR4, a specific ligand of SDF-1 (18). Notably, N-cadherin, vimentin, and snail are molecules that are upregulated in the EMT, a process through which epithelial cells lose their polarity, cell–cell adhesion, and E-cadherin expression, and TGF-β1 can induce EMT (47). A functional *in vitro* study using a human squamous carcinoma cell line corroborated with human pathological findings and revealed that snail increases the CXCR4 expression on tumor cells in the presence of TGF-β1 and the EMT process augments tumor cell migration through the CXCR4–SDF-1 axis (**Figure 1B**) (18). Furthermore, the CXCR4 expression of Paget cells in the primary lesion correlated with the presence of LN metastasis and reduced the disease-specific survival (18, 48). Likewise, the N-cadherin and vimentin expression of Paget cells in the primary lesion also correlated with the reduced OS (18). Hence, these findings implicate a crucial role of lymphangiogenesis and EMT of Paget cells in promoting LN metastasis of EMPD, and blocking the CXCR4–SDF-1 axis can be a novel option of adjuvant therapy for patients with CXCR4-positive invasive EMPD to prevent LN metastasis. Furthermore, the blockade of mutated PIK3CA or AKT1 might be an effective adjuvant therapy because the DNA sequencing of EMPD revealed a correlation of *PIK3CA* and *AKT1* mutations with E-cadherin hypermethylation (33).

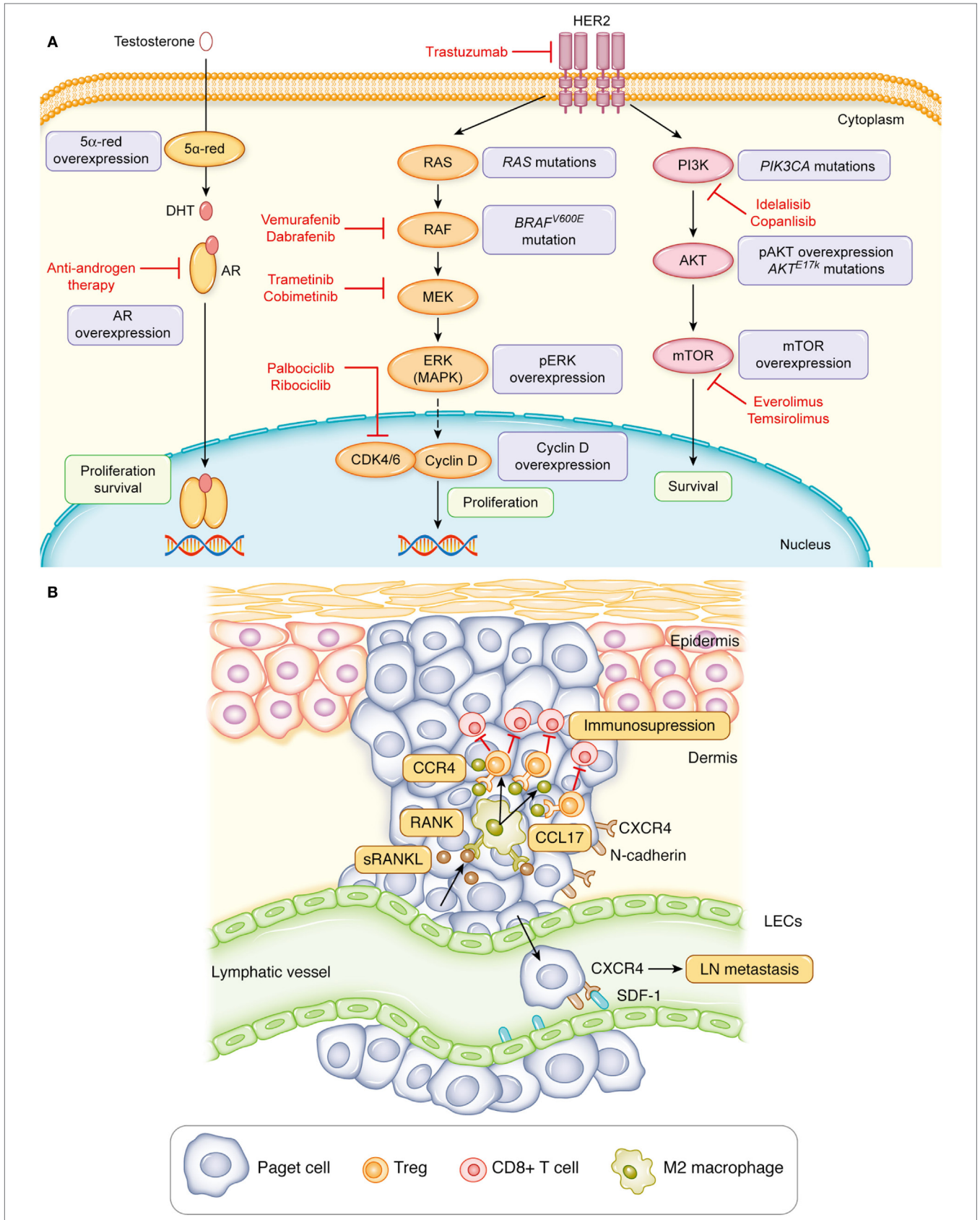

FIGURE 1 | Continued

FIGURE 1 | Signaling pathways involved in the progression of extramammary Paget's disease. **(A)** The aberrant activation of HER2, molecules involved in the RAS–RAF–MEK–ERK signaling or PI3K–AKT–mTOR signaling promote the proliferation and survival of Paget cells. Likewise, the androgen–androgen receptor (AR) signaling can induce the proliferation and survival of Paget cells. Red, Food and Drug Administration-approved drugs for other cancers that target aspects of this pathway. **(B)** The interaction of Paget cells with lymphatic endothelial cells (LECs) through the CXCR4–stromal cell-derived factor-1 (SDF-1) signaling or with $CD163^+Arg1^+$ M2 macrophages through the receptor activator of nuclear factor kappa-B ligand (RANKL)–RANK signaling facilitates metastasis of Paget cells.

THE ROLE OF RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA-B LIGAND (RANKL)–RANK INTERACTION IN THE TUMOR MICROENVIRONMENT OF EMPD

Although the aberrant activation of a signaling pathway in tumor cells and lymphangiogenesis mediates the progression of EMPD, the role of other cells, especially immune cells, in the tumor microenvironment of EMPD remains unclear. Histologically, EMPD exhibits abundant lymphocyte infiltration. The number of $CD8^+$ T cell infiltration in noninvasive and invasive EMPD is similar; however, the number of $CD8^+$ T cell-expressing granulysin and perforin is relatively lower in invasive EMPD than that of noninvasive EMPD, signifying the presence of stronger immunosuppression in the tumor microenvironment of invasive EMPD than noninvasive EMPD (49). Reportedly, invasive EMPD comprises a significantly higher number of $CD163^+Arg1^+$ M2 macrophages, an immunosuppressive macrophage, compared to noninvasive EMPD (49).

The RANKL and its receptor, RANK signaling, exerts numerous effects on immunity and promotes the survival of conventional dendritic cells and T-cell priming, thereby generating active immune responses (50). By contrast, it controls the number of regulatory T cells (Tregs) and induces tolerance against antigens (51). In EMPD lesions, Paget cells profoundly express RANKL with matrix metalloproteinase-7, which cleaves RANKL to release a soluble form (sRANKL), facilitating interaction with nearby cells expressing RANK (19). Remarkably, RANK is primarily expressed in $CD163^+Arg1^+$ M2 macrophages, and *in vitro* studies using monocyte-derived M2 macrophages have demonstrated that these cells produce CCL17 and promote the migration of CCR4-expressed $CD4^+$ T cells, which comprise effector $CD4^+$ T cells and Tregs when treated with sRANKL (20). In line with the *in vitro* experiment, CCL17 was co-expressed on the $CD163^+Arg1^+$ M2 macrophages in invasive EMPD (20). Furthermore, a study has reported that effector Tregs comprising a robust immunosuppressive effect profoundly express CCR4 (52) and that increased numbers of Tregs were related to more extensive cases of vulvar EMPD and disease recurrence (53). These studies highlight the crucial role of $CD163^+Arg1^+$ M2 macrophage and Tregs in establishing an immunosuppressive microenvironment in invasive EMPD by the RANKL–RANK interaction that promotes EMPD progression and causes poor prognosis (**Figure 1B**).

Reportedly, RANKL binds to RANK on osteoclasts and serves as a critical factor for regulating bone remodeling, and the activation of the RANKL–RANK signaling augments bone metastasis of various cancer types such as breast cancer (50). Based on this notion, a study demonstrated that denosumab, the anti-RANKL antibody, significantly delayed the appearance of skeletal-related events compared to bisphosphonates in patients with breast cancer with bone metastasis, thereby initiating its use to treat various metastatic bone tumors (54). Bone has been known as one of the most common site, where EMPD develops distant metastasis (55, 56). Based on these findings, denosumab seems not only useful for patients with metastatic EMPD and bone metastasis but also might be effective from the early stage to prevent the progression of invasive EMPD. Furthermore, since RANKL is related to immunosuppression of the EMPD microenvironment, denosumab could be a potential to enhance the efficacy of immunotherapy.

IMMUNOTHERAPY FOR METASTATIC EMPD

Although the response rate is 20–35% in solid cancers, immunotherapy with anti-PD-1 antibody is a prominent therapeutic approach for cancers. This is because it can induce a durable response in a majority of responders for more than 2 years, which is uncommon with the molecular targeted therapy (57–60). The biomarkers that define anti-PD-1 antibody responders remain unclear; however, it was recently revealed that cancer with DNA mismatch-repair (MMR) gene mutations, so called "mismatch-repair-deficient cancer," significantly responded better to anti-PD-1 antibody than MMR proficient cancer (61). In addition, it was reported that an anti PD-1 antibody induced robust antitumor immunity and attained durable disease control in heavily treated patients with colorectal cancer with MMR-deficient or microsatellite instability-high (MSI-H) status (62). Overall, these results propose that the MMR status or MSI-H is a useful biomarker to predict the clinical benefit of anti-PD-1 antibody, serving as the basis for the FDA's approval of an anti-PD-1 antibody for unresectable or metastatic MMR-deficient or MSI-H cancers, irrespective of cancer's original location.

The MMR pathway affects removal and correction of DNA base mismatches that arise either during DNA replication or caused by DNA damage (63). A mutation of genes involved in MMR, *MLH1*, *PMS1*, *MSH2*, and *MSH6* predispose to several tumorigenic conditions, such as Lynch syndrome, and cause cancer cells to display MSI-H (63). Although inactivation of MMR elevates the mutational burden, thereby promote carcinogenesis, a recent *in vivo* functional analysis revealed that it also leads to dynamic mutational profiles, resulting in the persistent renewal of mutation-associated neoantigens (MANAs) triggering durable immunosurveillance that can be further enhanced by an anti-PD-1 antibody. By contrast, MMR proficient cells exhibited stable mutational load and MANA profiles over time, which was consistent with the results of clinical trials (61, 64, 65).

Extramammary Paget's disease has long been recognized to pose a high risk of developing secondary cancer. Reportedly, 14–32% of cases have also been diagnosed with other primary cancers (66, 67). Kang et al. hypothesized that *MMR* gene mutations could be associated with the high occurrence of secondary cancer in EMPD and investigated the MMR status and the presence of gene mutation in *MLH1*, *PMS1*, *MSH2*, and *MSH6* in 20 patients with EMPD (68). Their results revealed 8 of 20 cases (40%) with germline *MMR* genes missense mutations. Of these 8 cases with *MMR* genes mutations, 1 and 4 cases exhibited MSI-high or MSI-lo, respectively, whereas none of the other 12 cases without *MMR* genes mutations exhibited MSI. Furthermore, their sequencing analysis of *MLH1* and *MSH2* gene for 172 samples identified germline and somatic mutations of *MLH1* or *MSH2* in 34.3 and 13.4% of cases, respectively. Of these, *MLH1 V384D* (15.7%) and *MLH1 R217C* (4.1%) were top two germline mutations, and these detection rates were significantly higher than healthy controls. Furthermore, the functional *in vitro* assay revealed that *MLH1 V384D* and *MLH1 R217C* mutations had 50–60% MMR efficiency than wild-type *MLH1*. Although having high tumor mutational burden does not always associate with improved survival and clinical trials should be conducted to evaluate the survival benefit, these findings suggest that there is a decent percentage of MMR-deficient EMPD, which has an impaired MMR machinery, in EMPD and this subset of patients might achieve a durable response by anti-PD-1 immunotherapy.

CONCLUSION

Metastatic EMPD is an aggressive skin adenocarcinoma with poor prognosis. Since current chemotherapeutic regimens are only moderately effective, improving clinical outcomes is imperative. The basic and translational research to date has provided an insight into the mechanisms promoting metastasis of EMPD that provide potential therapeutic targets for new drug development. Seemingly, Paget cells augment the ability of proliferation and survival by activating the RAS–RAF–MEK–ERK signaling, PI3K–AKT–mTOR signaling, or androgen–AR signaling. In addition, the interaction of Paget cells with other cells, such as LECs and $CD163^{+}Arg1^{+}$ macrophages in a tumor through the CXCR4–SDF-1 signaling and RANKL–RANK signaling, respectively, could establish a favorable tumor microenvironment to promote metastasis of Paget cells. Furthermore, recent genomic analysis of MMR has revealed that a decent percentage of EMPD comprises MMR-deficient EMPD cases that might achieve durable clinical response by an anti-PD-1 antibody. Hence, we are now beginning to understand multiple aspects involved in the pathogenesis of metastatic EMPD, and these findings will be sure to lead to better treatments for patients with metastatic EMPD in the future.

AUTHOR CONTRIBUTIONS

KF wrote the manuscript with input and guidance from TF.

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REVIEW
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Tumor-Associated Macrophages: Therapeutic Targets for Skin Cancer

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Tumor-associated macrophages (TAMs) and regulatory T cells (Tregs) are significant components of the microenvironment of solid tumors in the majority of cancers. TAMs sequentially develop from monocytes into functional macrophages. In each differentiation stage, TAMs obtain various immunosuppressive functions to maintain the tumor microenvironment (e.g., expression of immune checkpoint molecules, production of Treg-related chemokines and cytokines, production of arginase I). Although the main population of TAMs is immunosuppressive M2 macrophages, TAMs can be modulated into M1-type macrophages in each differential stage, leading to the suppression of tumor growth. Because the administration of certain drugs or stromal factors can stimulate TAMs to produce specific chemokines, leading to the recruitment of various tumor-infiltrating lymphocytes, TAMs can serve as targets for cancer immunotherapy. In this review, we discuss the differentiation, activation, and immunosuppressive function of TAMs, as well as their benefits in cancer immunotherapy.

Keywords: tumor-associated macrophages, immunosuppression, M2 polarization, chemokines, angiogenetic factors, regulatory T cells

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INTRODUCTION

Tumor-associated macrophages (TAMs) and regulatory T cells (Tregs) are significant components of the tumor microenvironment (1, 2). TAMs express immune checkpoint modulators [e.g., B7 family, B7-homolog family including programmed death ligand 1 (PD-L1)] (3) that directly suppress activated T cells. In addition, TAMs produce various chemokines that attract other immunosuppressive cells such as Tregs, myeloid-derived suppressor cells (MDSCs), and type 2 helper (Th2) T cells, which maintain the immunosuppressive factors of the tumor microenvironment (1, 2, 4). Moreover, TAMs also produce matrix metalloproteinases (MMPs), which play critical roles in tissue remodeling associated with various physiological processes such as morphogenesis, angiogenesis, tissue repair, local invasion, and metastasis (1, 5, 6). TAMs have been detected in various skin cancers such as melanoma, squamous cell carcinoma (SCC), extramammary Paget's disease (EMPD), Merkel cell carcinoma, basal cell carcinoma, and mycosis fungoides (MFs) (1, 2, 7–15) (**Table 1**). Because the stromal factor on each cancer stem cell is an important factor for TAM stimulation, leading to the induction of specific TAM phenotypes, investigating the immunomodulatory stromal cells in the tumor microenvironment is important for establishing the appropriate immunotherapy for each type of cancer (1, 8, 9, 16, 17). In addition, it may be possible to repolarize TAMs into anti-tumor macrophages, such as M1-phenotype macrophages, to suppress tumor progression by modifying the profiles of tumor-infiltrating lymphocytes (TILs) (7, 18, 19). Thus, TAMs could be a target for immunotherapy in skin cancers (1, 2). In this review, we discuss the differentiation, activation, and

TABLE 1 | Tumor-associated macrophages in skin cancer: mouse and human models.

Cancer species	Mouse (reference)	Human (reference)	Depletion	Reprogrammed	Biomarkers
Malignant melanoma	(3, 7, 13, 19, 20, 22, 39, 51, 62, 63, 64, 65)	(7, 35, 59, 60)	(13, 65)	(5, 19, 20, 22, 35, 39)	(3, 59, 60, 61)
Cutaneous squamous cell carcinoma	(23, 24, 32)	(11, 12, 34)	(23)	(24, 32)	(11, 12)
Merkel cell carcinoma	–	(14, 36)			(14, 36)
Extramammary Paget's disease	–	(8, 17)		(17)	(8)
Basal cell carcinoma	(26)	(15)	(26)		(15)
Dermatofibrosarcoma protuberans	–	(5)			(5)
Cutaneous T cell lymphoma	(25)	(9, 18, 28, 29, 30, 31, 57)	(25)	(18, 57)	(9, 28, 29, 30)

immunosuppressive function of TAMs, as well as their benefit in cancer immunotherapy.

DIFFERENTIATION AND ACTIVATION OF TAMs IN TUMORS

Tumor-associated macrophages are characterized by their heterogeneity and plasticity, as they can be functionally reprogrammed to polarized phenotypes by exposure to cancer-related factors, stromal factors, infections, or even drug interventions (1, 2, 7, 9, 11, 17, 19). Because TAMs sequentially differentiate from monocytes into functional macrophages through multiple steps, they have heterogeneity and plasticity in cancer (**Figure 1**). Monocytes recruited from the circulation differentiate into tissue macrophages by macrophage colony-stimulating factor (M-CSF), and are primed with several cytokines such as interferon gamma (IFN-γ), interleukin 4 (IL-4), and IL-13 (2). Thereafter, macrophages change their functional phenotype in response to environmental factors or even tumor-derived protein stimulation (2, 8, 17). In skin cancer, for example, targeting the M-CSF receptor with anti-CSF short interfering RNA (siCD115) in TAMs led to modulation of the TIL profile, resulting in growth suppression of B16 melanoma *in vivo* (20). In the second phase of priming, type I IFN (IFN-α, IFN-β) and type II IFN (IFN-γ) modulate the production of chemokines from TAMs, suggesting that these cytokines repolarize TAMs in several skin cancers (7, 18). Cancer stromal factors such as soluble receptor activator of nuclear factor kappa-B ligand (RANKL) derived from cancer cells could be a third mode of stimulation that activates mature M2 macrophages to produce a series of chemokines that recruit immunosuppressive cells such as Tregs and Th2, leading to maintenance of the tumor microenvironment (8, 10, 17). These reports suggest that each of these three differentiation steps could serve as a target for immunotherapies.

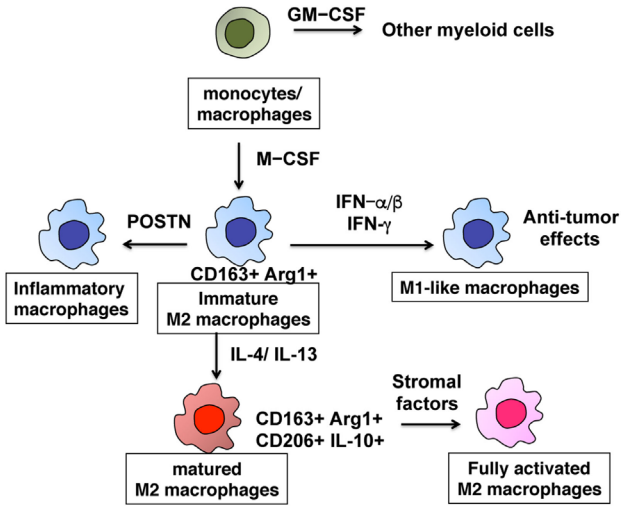

FIGURE 1 | Differentiation of M2-polarized tumor-associated macrophages. The multiple steps of the development of monocytes into fully functional macrophages.

ROLES OF TAMs IN MAINTAINING THE IMMUNOSUPPRESSIVE MICROENVIRONMENT

Chemokines from TAMs Determine the Immunological Microenvironment in Tumors

Chemokines play crucial roles in determining the profiles of TILs in the tumor microenvironment, and the profiles of chemokines from TAMs are determined by stromal factors of each skin cancer (1). For example, immune cells in the tumor microenvironment determine the aggressiveness of melanoma (21). In metastatic melanoma, periostin (POSTN) is expressed in the region surrounding melanoma cell nests in metastatic melanoma lesions that develop at the wound site (16). In addition, TAMs are prominent in the tumor stroma in melanoma (7, 19, 22), and POSTN stimulates $CD163^+$ macrophages to produce several specific cytokines including Treg-related chemokines [chemokine ligand 17 (CCL17), CCL22] (9). Because CCL17 and CCL22 from TAMs attracts Tregs to the tumor site in melanoma (7, 21, 22), repolarization of TAMs by immunomodulatory reagents such as IFN-β and imiquimod are useful for suppressing tumor growth in melanoma (7, 22). The downregulation of CCL22 production was also observed in B16F10 melanoma mouse treated with classical cytotoxic anti-melanoma drugs such as dacarbazine, nimustine hydrochloride, and vincristine, all of which have been used in the adjuvant setting for advanced melanoma for the last 30 years (19). Other reports have suggested that a series of chemokines (CCL17, CXCL10, CCL4, and IL-8) in cerebrospinal fluid may be useful for predicting brain metastasis in melanoma patients (21). Together, these reports suggest the significance of chemokines from TAMs that can be induced by POSTN in the

tumor stroma to induce melanoma-specific TILs in patients with melanoma.

Tumor-associated macrophages in non-melanoma skin cancer also secrete an array of chemokines in lesional skin to regulate the tumor microenvironment (1). In EMPD, for example, soluble RANKL released by Paget cells increases the production of CCL5, CCL17, and CXCL10 from RANK$^+$ M2 polarized TAMs (8, 10, 17), suggesting that Paget cells can determine the immunological microenvironment by the stimulation of TAMs. The results of this study led to the hypothesis that denosumab, a full human monoclonal antibody for RANKL, has therapeutic effects in invasive EMPD. In cutaneous squamous cell carcinoma (cSCC), according to its heterogeneity of differentiation of cancer cells, TAMs in cSCC heterogeneously polarized from M1 to M2 (11). Indeed, Petterson et al. (11) reported that CD163$^+$ TAMs not only express CCL18 (11), an M2 chemokine involved in remodeling of the tumor microenvironment but are also colocalized with phosphorylated signal transducer and activator of transcription 1 (11), suggesting the heterogeneous activation states of TAMs. Although the exact stimulator of cSCC is unknown, the depletion of TAMs such as antibody-mediated depletion (e.g., anti-CSF1R Ab) or bisphosphonate could be a useful therapy for unresectable cSCC (23–26).

Not only solid tumors but also hematopoietic malignancies in the skin contain CD163$^+$ TAMs (25, 27–29), which produce chemokines that direct to specific anatomic sites to form metastases (25). Indeed recently, Wu et al. (9) used a human xenograft CTCL cell model to demonstrate that chemokines from TAMs play crucial roles in tumor formation in MF lesions. In another report, it was shown that the cancer stroma of MF containing POSTN and IL-4 might stimulate TAMs to produce chemokines that correlate with tumor formation in MF (25), and that chemokines from TAMs can be modified by immunomodulatory agents such as IFN-α and IFN-γ, leading to their therapeutic effects (18). Furthermore, CCL18 produced by TAMs in MF at the invasive margin of the tumor promote the recruitment of CTCL cells, leading to cancer progression (30). These reports suggest the significance of chemokines from TAMs for the development of CTCL.

Direct Suppressive Function of TAMs

Immunomodulatory costimulatory molecules, such as B7 homologs, play representative roles in the direct cell-mediated suppressive mechanism of TAMs. Recently, several reports have suggested that the expression of PD-L1 (also known as B7H1) in TAMs is necessary for antigen-specific tolerance induction (1, 3, 31) in tumor-bearing hosts. For example, the expression of PD-L1 on TAMs is augmented by autocrine IL-10 from M2-polarized TAMs stimulated by specific antigens (31). Another report showed that the decrease of IL-10 in MDSCs led to the downregulation of PD-L1 expression in MDSC in a mouse melanoma model (3). Linde et al. (32) reported that IL-10-polarized TAMs into M2 phenotypes in the presence of IL-4 and vascular endothelial growth factor A (VEGF-A) in cSCC. These reports suggest that IL-10 upregulates PD-L1 expression on TAMs, inducing immunosuppression in the tumor microenvironment in the skin. Arginase 1 is one of the key factors for the suppressive function of TAMs. Its expression is widely detected in immature and functional M2 macrophages (1, 8, 17), leading to suppression of T cell activity by L-arginine catabolism (33). Indeed, CD163$^+$ TAMs expresses arginase 1 in several skin cancers such as EMPD and SCC (8, 34). More recently, Pico de Coaña et al. (35) reported the additional immunomodulatory effects of ipilimumab on granulocytic MDSCs, which are circulating macrophages in tumor-bearing hosts, suggesting the crosstalk between Tregs and granulocytic MDSCs through the CTLA4/B7 homolog pathway and the significance of the direct suppressive function of TAMs (35).

Angiogenetic Factors from TAMs

Tumor-associated macrophages produce angiogenetic factors such as VEGF, platelet-derived growth factor, and transforming growth factor β, or by expressing MMPs to induce neovascularization (10, 28, 32, 36–38). Linde et al. (32) reported that VEGF-A augments the recruitment of TAMs at a tumor site by promoting neovascularization in a mouse skin tumor model (32). In a human skin cancer model, Werchau et al. (36) reported that VEGF-C expressed by TAMs contributes to lymphangiogenesis and the progression of Merkel cell carcinoma (36). In angiosarcoma, TAMs express MMP9, which might be a target for amino bisphosphonate (37). Another report suggested that inhibition of the VEGF/VEGF receptor pathway inhibits M2 polarization in TAMs, leading to reduced vascular density and tumor growth in MCA205 mouse sarcoma (38). In addition, more recently, Yamada et al. (39) reported that the expression of MGF-E8 on mesenchymal stromal cells plays crucial roles in inducing M2 macrophage polarization, leading to suppression of tumor growth by the reduction of VEGF expression in TAMs in B16F10 melanoma. These reports indicate the significance of VEGF produced by M2 macrophages in tumor progression, and show that both VEGF and MMPs are key markers for M2 macrophages in skin cancers (11, 40, 41). For example, in a melanoma model, osteopontin signaling promoted macrophage recruitment by the secretion of prostaglandin E2 and MMP-9 from TAMs, leading to angiogenesis and tumor progression (41). These reports suggest that MMPs play crucial roles in tumor progression. MMPs can also be produced by TAMs upon stimulation of stromal proteins in skin cancer (9, 10). For example, the stimulation of POSTN augments the production of MMP1 and MMP12 from monocyte-derived immature M2 macrophages (9). Because POSTN is abundant in the tumor stroma of MF and dermatofibrosarcoma protuberans (DFSP) (5, 9), and because substantial numbers of CD163$^+$ TAMs have been detected in the POSTN-rich area in the lesional skin of skin tumors (5, 9), the production of MMP1 and MMP12 is prominent in the lesional skin of MF and DFSP. Notably, as reported by Livtinov et al. (42), among the MMPs, only MMP12 is a risk factor for CTCL progression, as determined by transcriptional profiling (42). RANKL is expressed in skin cancers of apocrine origin such as EMPD and apocrine carcinoma (8, 37), and is released in its soluble form. Because monocyte-derived M2 macrophages produce MMP1 and MMP25 by RANKL stimulation, TAMs in skin cancer of apocrine origin produce MMP1 and MMP25 at the tumor site (37). These reports suggest that TAMs stimulated by tumor stromal

factors play roles in the carcinogenesis of these skin cancers, and might be targets for molecular-targeted therapy in the future.

CLINICAL BENEFITS OF TAMs

The Effects of Anticancer Drug for TAMs

Because TAMs comprise the immunosuppressive microenvironment at the tumor site, they may be optimal therapeutic targets in cancer (1, 2, 4, 43–46). For example, Rogers et al. (44) reported the immunomodulatory effects of bisphosphonate on TAMs in patients with breast and prostate cancers upon the repolarization of TAMs into tumoricidal macrophages (44). More recently, several reports have also focused on the immunomodulatory effects of chemotherapeutic reagents on TAMs (19, 47, 48). For example, a non-cytotoxic dose of paclitaxel decreased MDSCs and even blocked the immunosuppressive potential of MDSCs in a mouse melanoma model (47). More recently, Fujimura et al. (19) reported the immunomodulatory effects of cytotoxic anti-melanoma drugs, dacarbazine, nimustine hydrochloride, and vincristine, on TAMs both *in vitro* and *in vivo* by inhibition of STAT3 signals (19). The authors concluded that their immunomodulatory effects could explain their antitumor effects in postoperative melanoma patients. Peplomycin administered through a superficial temporal artery using an intravascular indwelling catheter, which can cause dose-independent interstitial pneumonia (49), decreased the number of TAMs and Tregs in cSCC on the lips, leading to an increase in the number of immunoreactive cells at the tumor sites (50), and possible autoimmune-like interstitial pneumonia (49, 50). More recently, not only cytotoxic chemotherapeutic drugs but also low molecular weight compounds were reported to co-localize with TAMs at tumor sites. Indeed, Hu-Lieskovan et al. (13) reported that single-agent dabrafenib increased TAMs and Tregs in melanoma, which decreased with the addition of trametinib, leading to the synergistic effects of immune checkpoints inhibitors with dabrafenib and trametinib combination therapy. In another report, the anti-macrophage receptor with collagenous structure was reported to polarize TAMs into proinflammatory phenotypes to induce anti-melanoma immune response in B16 melanomas (51). In addition, Gordon et al. (52) reported that inhibition of PD-1/PD-L1 *in vivo* increased macrophage phagocytosis, reduced tumor growth, and prolonged the survival of macrophages. In another report, increasing expression levels of PD-L1 in TAMs, 2 months after the administration of anti-PD-1 Abs in patients with advanced melanoma, was correlated with the response to immunotherapy (53), suggesting that PD-L1 expression in TAMs could be a biomarker that predicts the effectiveness of anti-PD-1 Ab therapy. Because the anti-PD-1 Abs nivolumab and pembrolizumab are widely used to treat advanced cancer, including melanoma (53), one target of anti-PD-1 Abs in patients with advanced melanoma could be an immunomodulatory effect on TAM, which, in turn, might be correlated with both their effectiveness and the development of adverse events. TAMs produce not only chemokines that directly recruit immunosuppressive cells to the tumor microenvironment but also produce cytokines that stimulate other stromal cells such as fibroblasts to produce chemokines (54, 55). Indeed, Young et al. (54) reported that IL-1β from TAMs stimulate fibroblasts to produce CXCR2 ligand, which plays crucial roles in recruiting granulocytic MDSCs to tumor sites (55, 56). The authors concluded that CXCR2 agonists in combination with anti-CD115 Abs could suppress B16F10 melanoma *in vivo* by inhibiting the recruitment of granulocytic MDSCs and depletion of immature TAMs (56). Interestingly, the antihuman CD115 Ab, emactuzumab, decreased the number of $CD163^+$ $CD206^+$ M2 macrophages in patients with melanoma by depleting immature TAMs before the IL-4 stimulation phase (57). Together, these reports suggest that anti-CXCR2 agonists in combination with emactuzumab might induce the antimelanoma immune response by reducing the number of M2 polarized TAMs. These reports suggest the significance of assessing the effects of chemotherapeutic drugs on TAMs (13, 19, 47, 49, 50).

TAMs as a Biomarker for Disease Activity and Adverse Events

As described above, because TAMs produce tumor-specific chemokines by the stimulation of stromal factors, chemokines might serve as biomarkers that reflect disease activity. For example, TAMs produced CCL18 in the lesional skin of CTCL (26), which reflect disease severity and prognosis (58). Immunomodulatory reagents such as IFNs and imiquimod reduce CCL22 from TAMs, leading to the therapeutic effects of them in mouse B16F10 melanoma models (7, 22). CCL5, which induces Th2 cells from naive T cells (59), reflects the cancer stage and disease progression in gastric cancers (60). Another TAM-associated factor, sCD163, could be a useful biomarker for cancer treatment, as it is an activation marker for $CD163^+$ tissue macrophages that is present in the serum as a result of proteolytic shedding (61). Serum sCD163 levels increase in autoimmune diseases such as atherosclerosis, rheumatoid arthritis, moyamoya disease, pemphigus vulgaris, and bullous pemphigoid (62–64), and reflect disease activity (61). Therefore, as we previously reported, sCD163 is a possible marker for predicting immune-related adverse events caused by immune checkpoints inhibitors (64, 65). These reports suggested that the production derived from TAMs could be a biomarker for cancer treatment in the future.

CONCLUDING REMARKS

Although several studies have suggested that high numbers of TAMs in tumor-bearing individuals are associated with a poor prognosis, making them useful as prognostic markers in cancer, further studies are needed to quantify their impact in different cancers.

AUTHOR CONTRIBUTIONS

FT designed the study. FT, KY, and HT wrote the article. FT, FY, and AS supervised the study.

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Recent Successes and Future Directions in Immunotherapy of Cutaneous Melanoma

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The global health burden associated with melanoma continues to increase while treatment options for metastatic melanoma are limited. Nevertheless, in the past decade, the field of cancer immunotherapy has witnessed remarkable advances for the treatment of a number of malignancies including metastatic melanoma. Although the earliest observations of an immunological antitumor response were made nearly a century ago, it was only in the past 30 years, that immunotherapy emerged as a viable therapeutic option, in particular for cutaneous melanoma. As such, melanoma remains the focus of various preclinical and clinical studies to understand the immunobiology of cancer and to test various tumor immunotherapies. Here, we review key recent developments in the field of immune-mediated therapy of melanoma. Our primary focus is on therapies that have received regulatory approval. Thus, a brief overview of the pathophysiology of melanoma is provided. The purported functions of various tumor-infiltrating immune cell subsets are described, in particular the recently described roles of intratumoral dendritic cells. The section on immunotherapies focuses on strategies that have proved to be the most clinically successful such as immune checkpoint blockade. Prospects for novel therapeutics and the potential for combinatorial approaches are delineated. Finally, we briefly discuss nanotechnology-based platforms which can in theory, activate multiple arms of immune system to fight cancer. The promising advances in the field of immunotherapy signal the dawn of a new era in cancer treatment and warrant further investigation to understand the opportunities and barriers for future progress.

Keywords: melanoma, immunotherapy, immune checkpoint blockade, tumor microenvironment, adoptive T cell transfer, programmed cell death protein 1, tumor-infiltrating lymphocyte, tumor-infiltrating dendritic cell

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METASTATIC MELANOMA

Malignant melanoma is a highly aggressive cancer and accounts for the majority (60–80%) of deaths from skin cancer (1, 2). Non-melanoma skin cancers, including basal cell carcinomas and squamous cell carcinomas, have much lower metastatic potential and associated mortality than melanoma (3). Melanoma arises from pigment-producing cells called melanocytes that are found primarily in the skin and the eyes and to a lesser extent, in a wide range of body tissues (2, 4, 5). Melanocytes originate from the embryonic neural crest and migrate to the epidermis where they mature and produce melanin that is subsequently transferred to neighboring keratinocytes (6, 7). Melanin plays a crucial role in protecting the skin from ultraviolet (UV) solar radiation (6, 8). Neoplasia of melanocytes varies from benign melanocytic naevi to malignant melanomas (4, 5).

Malignancies can arise from any of the tissues where melanocytes are present but by far the most common type is cutaneous melanoma, comprising over 90% of all melanoma cases (5, 9). Hence, the central focus of this review will be on cutaneous melanoma. Due to the recent advances in tumor immunotherapy, a number of novel cancer treatment strategies have emerged. As such, this review will discuss the development of cancer immunotherapy in the context of melanoma and highlight potential avenues for further research.

Epidemiology

Melanoma is a fairly common cancer with an estimated global incidence rate of 3 per 100,000 (9–11). In 2015, it was reported that there were approximately 352,000 new cases of melanoma worldwide with an age-standardized incidence rate of 5 cases per 100,000 persons (12). There were nearly 60,000 deaths worldwide due to melanoma (12). The incidence rate is observed to be higher in males than in females and is associated with a younger median age (~57 years) at diagnosis than other solid tumors (~65 years) (9, 10, 12). The three regions with the highest incidence of melanoma were found to be Australasia (54%), North America (21%), and Western Europe (16%) (12). Furthermore, it is particularly concerning that the global incidence rates of melanoma continue to rise. In 2005, there were roughly 225,000 new cases of melanoma but in 2015, that number climbed to roughly 352,000 cases, representing a 56% increase (13). A large-scale cohort study from 39 countries showed that while incidence rates for melanoma are beginning to stabilize in North America and Australia, they are continuing to rise in Southern and Eastern Europe (11). Therefore, melanoma constitutes a significant burden of disease worldwide and warrants both novel treatments and prevention strategies.

Pathophysiology and Clinical Subtypes

The exact etiology of melanoma development is not well understood (4). However, there has been tremendous study on the histological and molecular profiles of the various subtypes of melanoma (14–16). Overall, it has been observed that melanomas which arise from skin that is chronically sun-damaged (CSD) occur in anatomical locations such as the head and neck. By contrast, non-CSD melanomas are found in anatomical regions that suffer only limited sun exposure such as the trunk and extremities (4). Overall, non-CSD melanomas also have lower mutational loads than CSD melanomas (4, 16). A significant number of melanomas are usually associated with benign neoplasms of melanocytes. These lesions are termed naevi (commonly called moles), and an increased presence of naevi is deemed a risk factor for melanoma (2, 4). These lesions include benign naevi, dysplastic naevi, which display atypical cellular characteristics, and non-invasive melanoma *in situ* (4, 17). Melanoma *in situ* is by definition confined to the epidermis and if resected entirely, has a 100% survival rate (17). The current staging system for melanoma is the one used by the American Joint Committee on Cancer (AJCC) and relies upon analysis of the tumor (T), the number of metastatic nodes (N), and the presence of distant metastases (M) (18, 19). These are then grouped to provide clinical stages of the cancer, ranging from 0 to stage IV (19). Stage IV melanoma is classified as metastatic melanoma due to the presence of distant metastases, while stage III is only marked by metastases in regional lymph nodes (LN) (20).

Historically, malignant melanoma was divided into four major histological subtypes but due to the complexity of the disease, a fraction of melanomas cannot be completely classified into either subtype (15, 21, 22). Moreover, as this classification system is reliant on clinical and morphological features, it yields little prognostic value but serves as a useful strategy in identifying the various histological forms of the disease (22). The four primary subtypes of melanoma are as follows: (i) superficial spreading melanoma (SSM), (ii) nodular melanoma (NM), (iii) lentigo maligna melanoma (LMM), and (iv) acral lentiginous melanoma (ALM) (14, 22). However, in recent years, a number of novel clinical subtypes have also been defined. These include desmoplastic melanoma (DM), melanoma arising from a blue naevus and persistent melanoma (22). The five common histogenic subtypes of melanoma warrant further description here. A pictorial overview of the clinical manifestation and histopathology of melanoma is presented in **Figure 1**.

Superficial Spreading Melanoma

Superficial spreading melanomas are the most common subtype representing between 50 and 70% of all cases (14, 23). They occur in relatively younger patients (~50 s) and present on anatomical regions such as the trunk, back, and extremities (22). SSM presents as a flat or a slightly elevated lesion with varying pigmentation (24). Histologically, SSM is marked by atypical melanocytes with nested or single cell upward migration (22). Malignant melanocytes display lateral spreading throughout the epidermis, poor circumscription, and increased melanization in the cytoplasm (14, 22).

Nodular Melanoma

Nodular melanomas are a fairly common subtype of melanoma (15–35%) that can present most commonly on the head and neck as a growing nodule that shows ulceration (22–24). Histologically, NMs show similarities to SSMs but differ in that they show distinct circumscription. They do not display radial growth but aggressive vertical growth evidenced by large dermal nests and sheets of atypical melanocytes (14, 22).

Lentigo Maligna Melanoma

Lentigo maligna melanomas present almost exclusively on the sun-exposed upper extremities or head and neck of elderly people (mostly octogenarians) (22). It is relatively uncommon (5–15%), and topically can be seen as patch of discolored skin showing variegated coloring (23, 24). Lentigo maligna (Hutchinson's freckle) is the term for the *in situ* melanoma phase, and a small percentage of these patients progress to invasive LMM (23). Histologically, the skin exhibits extensive solar damage resulting in an atrophic epidermis and lentiginous (back-to-back) proliferation of melanocytes, which are hyperchromatic (22). Multinucleated (starburst form)

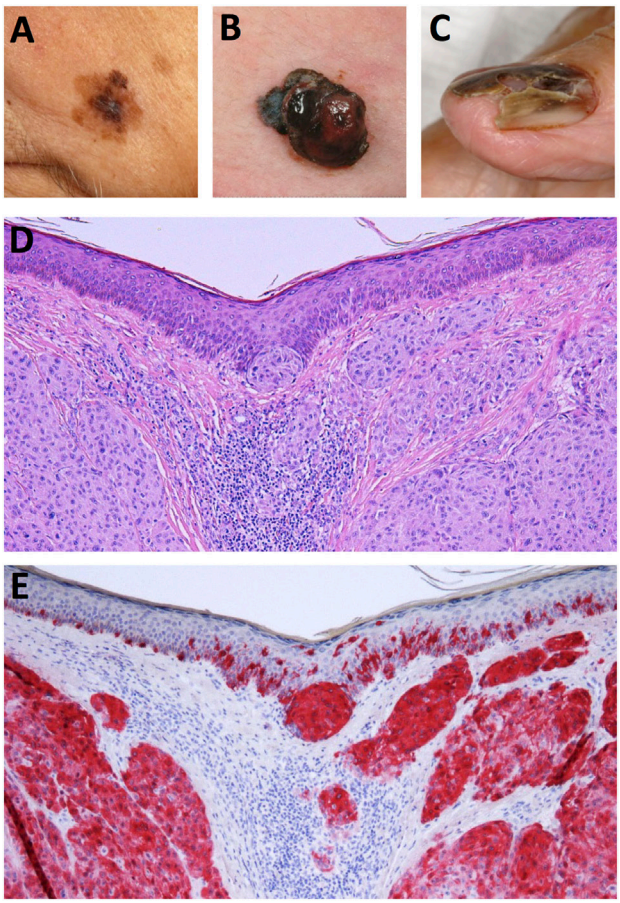

FIGURE 1 | Clinical and histological presentation of melanoma. **(A)** Superficial spreading melanoma (SSM), **(B)** nodular melanoma (NM), **(C)** acrolentiginous melanoma (ALM), **(D)** H&E stain of NM depicting asymmetrical nodular tumor infiltrates in the upper dermis. Nests of atypical cells are visible in the dermis and at the dermoepidermal junction. **(E)** Immunohistochemical staining for Melan-A reveals red stained atypical tumor cells in the dermis and epidermis (Images courtesy of RH).

melanocyte cells and solar elastosis are also hallmarks of this type of melanoma (14).

Acral Lentiginous Melanoma

Acral lentiginous melanomas are a fairly uncommon subtype (5–10%) and occur primarily in non-Caucasian populations such as people of African or Japanese descent (23). They present on acral sites such as palms, soles of the feet, or under the nails. On the skin they present as slow growing patches with variegated pigmentation (22). Histologically, this subtype displays single cells or nests of melanocytes along the dermal–epidermal junction, and the association of lymphocyte infiltrates can be used as a diagnostic marker for this subtype of melanomas (14, 22).

Desmoplastic Melanoma

Desmoplastic melanoma is a rare form of melanoma comprising 4% of primary melanomas and defined by the histological features observed in its dermal component (22, 25). It occurs primarily on the head and neck region in elderly individuals and is associated with higher probability of recurrence but a lower incidence of metastasis (25). Histologically, it is characterized by spindle-shaped melanocytes and a desmoplastic stroma, i.e., new collagen formation, and usually appears to be amelanotic (22, 25).

Risk Factors and Driver Mutations

Melanoma occurs via a complex interplay of genetic and environmental risk factors. The primary environmental risk factor of concern is UV solar radiation as well as, UV rays from tanning beds (26, 27). Individual risk factors include the increased presence of melanocytic naevi, skin complexion, and in certain cases, family history of melanoma (26, 28). Melanomas display one of the highest mutational burdens among solid tumors (25). Thus, the molecular profiles that are associated with various subtypes of melanoma are the subject of current studies. In particular, it is crucial to distinguish "driver" mutations, or mutations that confer a survival advantage, from "passenger" mutations, which have negligible or no contribution to tumor growth (29). Understanding the mutational landscapes of a cancer allows for the development of targeted therapies that can significantly improve clinical outcomes. A massive study conducted by researchers of The Cancer Genome Atlas Network, was reported in 2015, and determined the first-ever comprehensive genomic classification system for cutaneous melanomas (30). These four distinct subtypes were based on the pattern of the major significantly mutated genes, i.e., BRAF, RAS, neurofibromin 1 (NF1), and triple wild type (WT), which denotes a lack of mutations in the three aforementioned genes but is associated with higher copy number and structural rearrangement abnormalities. These subtypes do not correlate with outcome but may help delineate the genomic changes associated with melanoma thereby providing potential molecular targets (30). Of further interest was the observation that immune gene expression, and immune cellular infiltrates did correlate with patient survival (30). As the studies of the major genomic aberrations in melanoma have been extensively reviewed elsewhere, this section will describe a number of the most common driver mutations seen in cutaneous melanoma [BRAF, NRAS, NF1, microphthalmia-associated transcription factor (MITF), and PTEN] (4, 15, 25, 28, 31).

BRAF

Nearly 60% of melanoma cases have mutations in BRAF (v-raf murine sarcoma viral oncogene homolog B) (25, 32). Thus, a brief overview of BRAF signaling is warranted. *BRAF* codes for a serine/threonine protein kinase constituting part of the RAS–rapidly accelerated fibrosarcoma (RAF)–mitogen-activated protein kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) [mitogen-activated protein kinase (MAPK)] pathway, which is activated by the binding of extracellular growth factors to receptor tyrosine kinases (32). This binding leads to the activation of RAS (named for *Rat sarcoma*) family of GTPases (proteins that bind and hydrolyze guanosine triphosphate to guanosine diphosphate, i.e., GTP to GDP), which recruit and activate RAF serine/threonine protein kinases, which in turn

activate MEK resulting finally in the phosphorylation of ERK (32–35). The activation of ERK leads to downstream signaling and activation of transcription factors that mediate cell differentiation, growth, and inhibit cell death (33, 36).

BRAF is one of three mammalian RAF isoforms, and one that has the highest basal kinase activity and thus is the most common isoform mutated in human cancers that include melanoma but also hairy cell leukemia, papillary thyroid cancer and colorectal cancer (CRC) (33, 36). The missense mutation, V600E, results in a substitution from valine to glutamic acid at the 600th amino acid position and represents the majority (80%) of all BRAF activating mutations in melanoma (25, 28). Other BRAF mutations include V600K (valine–lysine) and V600R (valine–arginine). BRAF-activating mutations result in constitutively active MEK signaling leading to tumor progression. *In vitro*, the V600E mutation confers 500-fold higher activity in BRAF than normal and promotes the transformation of melanocytes to melanoma (37). $BRAF^{V600E}$ mutations are also found in benign naevi indicating that alone, these mutations may not be sufficient for tumor progression (38). The presence of these mutations has led to the development and approval of two BRAF inhibitors (BRAFi) for melanoma treatment, namely, vemurafenib (Genentech/Plexxikon) and dabrafenib (GlaxoSmithKline) as well as, a MEK inhibitor trametinib (GSK) (33, 39).

NRAS

The second most common type of driver mutations in melanomas occur in NRAS (neuroblastoma RAS viral v-ras oncogene) and are found in 15–20% of melanoma patients (28). The most common mutation in NRAS occurs at codon 61 resulting in the replacement of glutamine by lysine or arginine, thereby resulting in a constitutively active RAS (38). This leads to upregulation of both the MAPK and phosphatidylinositol 3′ kinase (PI3K) pathways and results in increased cell proliferation and invasiveness (25). NRAS mutant melanomas have increased thickness and display high rates of mitosis (25). NRAS mutations are also found in benign congenital nevi (28). NRAS and BRAF activations rarely occur in the same melanoma, albeit NRAS mutations being observed in patients with advanced BRAF tumors who had failed BRAFi therapy and which therefore may mechanistically contribute to resistance to BRAFi treatment (28). Efforts to target NRAS have focused on downstream inhibitors for the MAPK pathway and include the MEK inhibitor binimetinib, which is undergoing clinical trials (25).

Neurofibromin 1

Neurofibromin 1 encodes a large protein of more than 2,800 amino acids with multiple functional domains (40). It contains several functional domains with one domain bearing resemblance to the catalytic region of GTPase-activating protein. This is the most well-characterized domain of NF1 and acts as a negative regulator for RAS by converting the active RAS-GTP to the inactive RAS-GDP, thus playing the role of a tumor suppressor gene (40, 41). Germline mutations in NF1 lead to a genetic syndrome called neurofibromatosis type 1 (NF1), a relatively frequent genetic condition with an incidence of 1 in 3,000, resulting in a higher predisposition to multiple tumors arising from various cell types (40). The incidence of melanoma in patients with neurofibromatosis type 1 is very low. However, NF1 somatic mutations are found in a range of cancers, and it is the third common driver mutation in melanoma found in nearly 14% of tumors (25, 41). Mutations in NF1 are more commonly observed on skin with chronic UV exposure and in elderly patients (40). NF1 inactivating mutations were found in 48% of a cohort of wild-type BRAF and NRAS melanomas and are often associated with mutations in other RAS-related genes such as RAS p21 protein activator 2 (RASA2), PTPN11, and SPRED1 (25, 40). Recent studies have also shown that NF1 may be a unique driver mutation in DMs as NF1 loss-of-function in DM is more common than for other histogenic subtypes (25). Due to the crucial role of NF1 upstream of RAS/MAPK and PI3K/mTOR pathways, NF1 mutant tumors have been targeted with tyrosine kinase inhibitors (e.g., imatinib), MEK inhibitors (trametinib), and mTOR inhibitors (sirolimus), but to date, none of these agents have been reported in treatment of NF1 mutant melanomas (40).

Microphthalmia-Associated Transcription Factor

Microphthalmia-associated transcription factor is a helix-loop-helix leucine zipper transcription factor required for differentiation, proliferation, and survival of melanocytes and thus, its expression is also necessary for melanoma survival (42, 43). MITF also plays an important antiapoptotic function in melanoma cells by activating the expression of genes such as *BLC2A1*, *BCL2*, and *BIRC7* (43). MITF is observed to be amplified in 20% of metastatic melanomas and is associated with poor survival (25). MITF is regulated by the MAPK pathway and in particular, $BRAF^{V600E}$ causes induction of MITF through the transcription factor BRN2 (N-Oct-3) (25). Alternately, increased ERK signaling can also target MITF for degradation (44). Finally, MITF is also purported to contribute to BRAFi resistance through the regulation of the *BCL2A1* antiapoptotic gene (44). Although targeting of MITF directly may not be viable, the use of histone deacetylase (HDAC) inhibitors can reduce MITF expression. Hence, the HDAC inhibitor panobinostat in combination with decitabine and chemotherapy is being studied in clinical trials for metastatic melanoma treatment (25).

PTEN

Phosphatase and tensin homolog (*PTEN*) is a commonly mutated gene in melanoma and PTEN mutations were found in 14% of all melanoma samples from the TCGA genome classification study mentioned above (25, 30). *PTEN* codes for a phosphatase which targets phosphatidylinositol (3,4,5)-triphosphate and thus plays a crucial role in the aforementioned PI3K–Akt pathway (45). PTEN silencing therefore results in dysregulated apoptosis, cell cycle progression and migration, contributing to tumorigenesis (25, 45). It has been observed that *PTEN* mutations are more frequent in metastatic melanomas as opposed to early stage primary tumors (25). The loss of PTEN also interferes with genetic stability, thus

sensitizing PTEN-deficient cells to polyadenosine diphosphate ribose polymerase (PARP) inhibitors (46). Currently, there are no PARP inhibitor trials underway for the treatment of metastatic melanoma (46).

Current Treatments for Malignant Melanoma

The multiple clinical approaches to the treatment of early and advanced melanoma are reviewed elsewhere (18, 20, 47). As previously mentioned, the median survival associated with metastatic melanoma (stage IV) remains very poor, and the 10-year survival for all patients is under 10% (47). Melanoma treatments involve the use of surgery, radiation or systemic therapy (which includes immunotherapy) (18, 20). For most primary melanomas, surgical excision of the tumor remains the standard-of-care therapy. Biopsy and histological examination of the sentinel LN is an important component of melanoma staging and has been found to be a strong prognostic measure (18, 20). When surgical excision is not an option, primary lentigo maligna may also be treated with radiation or cryotherapy (20). The treatment modalities for metastatic melanoma are more complex as most single or even combination therapies are only successful in a subset of patients (18, 48). For patients with oligometastatic disease, surgery remains a primary treatment (18, 48). Melanoma is considered a relatively radiation-resistant cancer type, but radiation therapy continues to be utilized for patients with brain metastases (47, 48). Systemic therapy includes chemotherapy, targeted therapy, and immunotherapy (18, 47). Studies with various agents, including combination chemotherapy approaches, have shown that it has limited efficacy in melanoma (18, 47). The major chemotherapy drugs that have been used to treat melanoma including the alkylating agents dacarbazine, temozolomide, and nitrosoureas such as fotemustine and carmustine (47). Platinum analogs (e.g., cisplatin) and antimicrotubular agents such as vinblastine and paclitaxel have also shown modest efficacies in patients with metastatic melanoma (47). Recently, clinical studies have been performed using biochemotherapy, which combines cytotoxic drugs with immunotherapies such as interleukin-2 (IL-2) and IFNα (interferon alpha), and despite showing increased response rates these patients did not experience prolonged overall survival (OS) (18). In patients with recurrent metastatic melanoma in the limb, high doses of the cytotoxic drug melphalan and recently, tumor necrosis factor (TNF) and IFNγ are given to the patient via isolated limb perfusion to reduce systemic toxicity (48). A significant improvement in melanoma treatment was observed using targeted therapies, which pharmacologically inhibit key mutations in melanoma. These include the BRAFi drugs vemurafenib and dabrafenib, and the MEK inhibitor trametinib (39). Targeted therapies for melanoma have been expertly reviewed elsewhere (39, 49). The major clinically approved immunotherapies for melanoma include adjuvant treatments such as IL-2 and interferon alfa (18, 48). A few clinical groups have had success with adoptive T cell therapy in a subset of patients (50). Finally, immune checkpoint blockade (ICB) with antibodies targeted to cytotoxic T lymphocyte antigen-4 (CTLA-4) (ipilimumab) and programmed cell death protein 1 (PD-1) (nivolumab and pembrolizumab) has resulted in significant improvements in clinical outcomes for a proportion of melanoma patients (39). Targeting the ligand for PD-1 (i.e. PD-L1) is also being studied in clinical trials (51, 52). This review will summarize the evolution of immunotherapies in the context of melanoma and discuss novel opportunities to significantly enhance tumor immunotherapy. To assess the results of clinical studies, it is pertinent to mention some of the key measures used in clinical trials and criteria defined within the RECIST (Response Evaluation Criteria in Solid Tumors) (53). OS is defined as the time from randomization of the treatment subject to time of death due to any cause, while the more utilized progression-free survival (PFS) metric, denotes time from randomization until tumor progression or death (54). The overall objective response rate (ORR) is a measure of the percentage of patients who have had either a partial response (PR) or complete response (CR) to treatment (54). PR is defined as a decrease of at least 30% in the sum of the diameters of the target tumor lesions while CR indicates the disappearance of all target lesions (53). Finally, progressive disease (PD) is defined as at least a 20% increase in the sum of the target lesions' diameters while stable disease (SD) denotes a state where the lesions do not shrink enough to signal PR or increase sufficiently to indicate PD (53). Thus, these parameters provide an objective methodology to measure the results of a treatment (53, 54).

IMMUNOBIOLOGY OF MELANOMA

Cancer Immunoediting

Over the past decade, cancer immunotherapy has emerged as a vital new approach to cancer treatment (55, 56). The earliest evidence of the involvement of the immune response in fighting cancer was observed over a century ago. In 1893, William Coley, a surgeon in New York published a report describing tumor regression in a number of patients treated with cultures of the bacterium *Streptococcus pyogenes* (57, 58). However, the immunological basis of these results was not yet known and the approach did not gain wide acceptance in the medical field. Nevertheless, subsequent observations in murine models led to the formulation of the "cancer immunosurveillance" hypothesis by Macfarlane Burnet and Lewis Thomas in the middle of the century (59, 60). The hypothesis posited that lymphocytes played a protective role by continuous recognition and elimination of malignant cells (61). Currently, the concept of "cancer immunoediting" is forwarded as a comprehensive depiction of the continuous interplay between tumors and the immune system (62, 63). Cancer immunoediting posits the existence of three distinct phases, namely, elimination, equilibrium, and escape (63, 64). In the *elimination* phase, innate and adaptive immune mechanisms eradicate neoplastic cells before they become clinically detectable cancers (64). This phase has not been directly observed *in vivo* but the increased susceptibility to developing cancer in immunodeficient mouse models provides evidence of the existence of this stage of immunoediting (64). Further observations in humans such as the increased risks of

cancers in patients with immunodeficiencies or undergoing immunosuppression for organ transplantation, as well as cases of spontaneous tumor regression lend further proof to this paradigm (64, 65). During the *equilibrium* stage, rare cancerous cells that were not destroyed during the elimination phase, are kept in check by the immune system while influencing the immunogenicity of the tumor (62). This state results in a form of tumor dormancy and is considered to last a long time, potentially lasting the lifetime of an individual. Furthermore, this phase enacts a selective pressure on the tumor cells, allowing those with the potential to evade the immune system to escape immune control and manifest as clinical disease (62, 64). A landmark study in 2007 demonstrated the existence of the equilibrium phase *in vivo*. Using a carcinogenic compound (3′-methylcholanthrene -MCA), the authors were able to study stable tumor masses at the site of MCA injection (66). When treated with a cocktail of antibodies targeting CD4, CD8, and IFNγ, 60% of the mice developed rapidly growing tumors. Furthermore, the authors demonstrated that these rapidly growing tumors resembled "unedited" tumors from MCA-injected $RAG^{-/-}$ mice (mice lacking recombination activation gene RAG1) (66). Finally, it was shown that this equilibrium state required components of adaptive immunity (IL-12, IFNγ, $CD4^+$, and $CD8^+$ cells) but not key components of innate immunity such as NK cell recognition and effector functions (66). Thus, while the immune system is capable of controlling cancerous cells during the equilibrium phase, it also drives the selection of cells that are able to evade immune attack and develop into a progressively growing tumor. This stage is known as the *escape* phase of immunoediting. This escape is made possible due to a number of potential mechanisms which have been reviewed in detail (61, 63, 65). Briefly, the cells can evade immune detection by reducing the expression of immunogenic tumor antigens or by reducing major histocompatibility complex class I (MHC I) (62, 64). Another route of escape involves decreased susceptibility to immune-mediated cytotoxicity through upregulation of oncogenes and anti-apoptotic mediators (64). Finally, tumor cells harbor the potential to modulate the immune system by producing immunosuppressive cytokines such as transforming growth factor beta (TGFβ) and vascular endothelial growth factor (VEGF). Moreover, tumor cells can recruit regulatory immune cells [e.g., regulatory T cells (Treg)] or engage in adaptive immune resistance via the expression of immune checkpoint ligands such as programmed death-ligand 1 (PD-L1) (64). Finally, the notion of "reverse immunoediting" has been proposed as some cancers can cause the selective depletion of specific high-avidity cytotoxic T cell (CTL) clones via hitherto unknown mechanisms and thus actively shape the immune repertoire of the host (67). The pathways used by tumor cells to escape the immune system are therefore studied extensively to devise immunotherapeutic approaches for cancer treatment.

Immune Response to Melanoma

The immune response to tumor cells is currently one of the major areas of research in biomedical science. An overview of antitumor immune response is provided by the concept of the cancer-immunity cycle as described by Chen and Mellman (68). It commences with the release of tumor antigens that are presented by antigen-presenting cells (APC), primarily dendritic cells (DC), to T cells in the LN (**Figure 2**). This is followed by the trafficking of T cells including $CD8^+$ cytotoxic T lymphocytes (CTL), to the tumor where they can recognize and kill malignant cells, thereby releasing more cancer antigens (68). However, at each step, there are negative regulators that can disrupt the cancer-immunity cycle and allow progression of the tumor (68). One of the primary aims of cancer immunotherapy is therefore to ensure a sustained T cell response against the tumor (55). The complex biology of the interactions between tumor cells and the innate and adaptive immune system has been extensively reviewed elsewhere (68–72). Thus, the primary focus of this section will be to provide a basic primer to cancer immunology and in particular, to the biological and therapeutic significance of the major types of immune cells in the tumor microenvironment (TME) in melanomas. For the purposes of this review, the populations of interest are tumor-infiltrating lymphocytes (TIL), tumor-infiltrating dendritic cells (TIDC), and tumor-infiltrating natural killer (NK) cells. The cancer-specific roles of tumor-associated macrophages (TAM), NKT cells, the more recently described myeloid-derived suppressor cells (MDSC), and non-NK innate lymphoid cell subsets (ILC) have been thoroughly reviewed elsewhere (73–77).

Tumor Antigens

As tumors arise from a host's own tissue, immune recognition of these cells is hindered by the fact that a majority of potentially autoimmune cells are deleted during central (thymic) and peripheral mechanisms of self-tolerance (78). However, as early as 1943, it was observed that mice could immunologically reject chemically induced tumors (79). In the late 1970s, the ability to grow CTL cultures using IL-2 allowed for screening of tumor-derived DNA libraries to characterize tumor antigens (79). In 1988, the gene coding for a murine tumor antigen (P91A) was cloned (80). Shortly afterward, the first human tumor antigen gene was identified in melanoma, namely, *MAGEA1* (melanoma antigen family A, 1) and was found to be expressed in various types of tumors (81). Interestingly, the gene was not observed to be expressed in normal tissue except for trophoblastic cells and male germline cells (79). Since then, several tumor antigens have been discovered, and their underlying biology has been the subject of much study (82, 83). There are several types of tumor antigens, but they have been broadly classified into three major categories. The first category includes antigens that are caused by non-synonymous mutations, or are encoded by viral genes in tumors of viral etiology (83). These are labeled tumor-specific antigens (TSA) or "neoantigens" (83, 84). Alternately, tumor-associated antigens (TSA) are usually expressed at low levels in normal tissues but are found to be overexpressed in cancer cells such the surface receptor, human epidermal growth factor 2 (HER2 or ERBB2) in breast cancer, and other malignancies (85). Finally, cancer/testis antigens (CTA) such as the aforementioned MAGE family of proteins are expressed in several tumor types and only in normal germline cells such as trophoblasts, ovaries and the testes (82, 83). The advent of high-throughput next-generation sequencing technology has

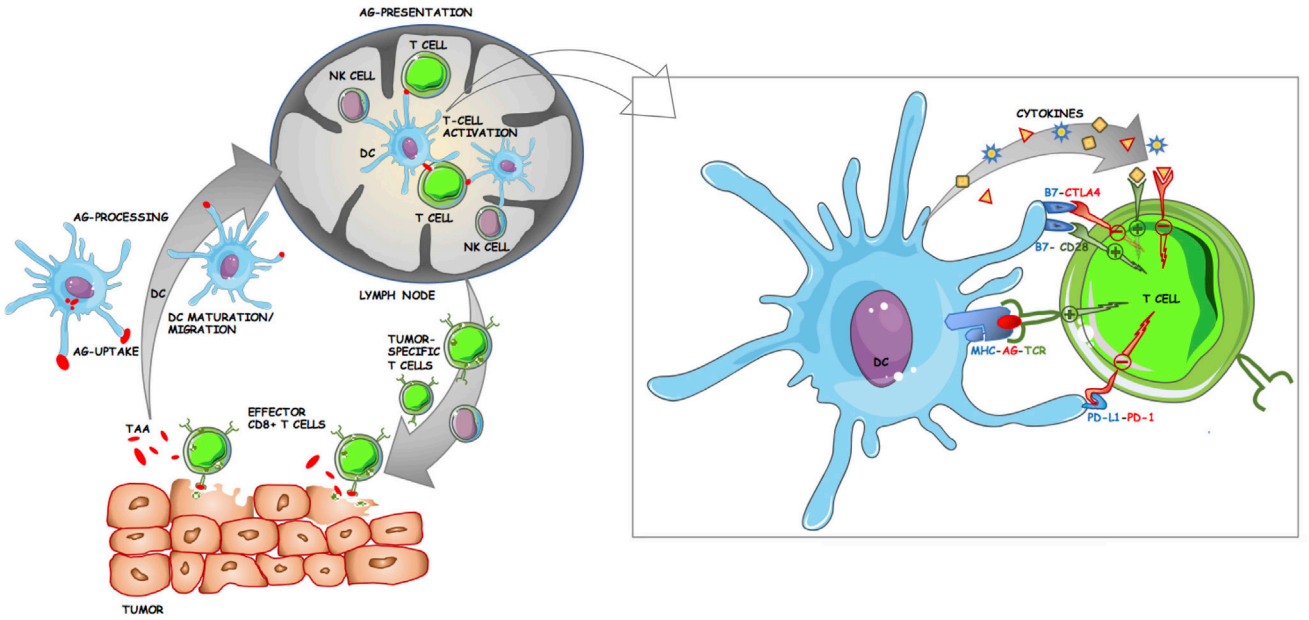

FIGURE 2 | Schematic of the roles dendritic cells (DC) play in antitumor immune response. DC take up and process tumor-associated/tumor-specific antigens (TAA/TSA) from dying tumor cells, undergo maturation, and migrate to tumor draining lymph nodes (LN) where they can present antigen to lymphocytes. Tumor-specific T cells then egress from the LN and infiltrate the tumor. Effector CD8+ cytotoxic T lymphocytes play a major role in killing tumor cells, leading to further release of TAA/TSA for DC uptake and subsequent presentation. Inset panel: Costimulatory and inhibitory interactions at the antigen-presenting cell (APC)–T cell immunological synapse. The activation of T cells by APC is tightly regulated by multiple ligand–receptor interactions. TCR binds to cognate antigen (AG) in the context of their specific MHC. Costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) on APC can either bind to CD28 on T cells resulting in downstream activation of T cell effector genes or to cytotoxic T lymphocyte antigen-4 (CTLA-4) resulting in inhibition. Further T cell activation is achieved through cytokines. Programmed cell death protein 1 (PD-1) is another immune checkpoint receptor and is expressed on activated T cells. The primary ligand for PD-1 (PD-L1) is expressed on APC and on some tumor cells, and upon binding to PD-1 acts to inhibit T cell activation.

allowed for relatively low-cost detection of somatic mutations in tumor cells. There are currently several approaches being formulated to tailor individualized immunotherapies for patients on the basis of their expression of tumor neoantigens (83). Although currently personalized approaches are highly expensive, it is posited that with the continuing reduction of sequencing costs and using combinatorial treatments, it may be possible to even target tumors that are non-responsive to immunotherapy (83). Since their discovery, tumor antigens have been used for multiple purposes in cancer treatment. They have been used as diagnostic markers, cancer vaccines, and as targets for adoptive T cell therapy (82, 86, 87). In general, most tumor antigens elicit a weak immune response against cancer and have been tested clinically in combination with adjuvants or additional treatments (87). To date, cancer vaccination or adoptive transfer targeting specific tumor antigens has not shown major survival advantages in melanoma (48, 88). The three major types of tumor antigens that have been described and used in melanoma immunotherapy are discussed below. A majority of described melanoma antigens are restricted to human leukocyte antigen A2 (HLA-A2) (89).

MAGE Family

The MAGE (melanoma antigen) family is divided into two major groups type I MAGEs and type II MAGEs. The type I MAGE subfamily consists of 25 functional genes located on the X chromosome in the regions *MAGEA*, *MAGEB*, and *MAGEC* (82, 90). These genes are classified as CTAs and are expressed in melanoma as well as other cancer types such as colon cancer, non-small cell lung cancer (NSCLC), and breast cancers (90). Conversely, type II MAGE genes are expressed in several types of normal tissue and are not X chromosome restricted. Both type I and type II MAGEs contain the MAGE homology domain (90). Due to the extensive homology between the MAGE proteins, there is a lack of antibodies that recognize specific MAGE antigens. In several cancer types, nuclear and cytoplasmic staining using widely reactive anti-MAGE antibodies have been performed and although the functions of MAGE proteins are not known, there is some evidence that they play a role in cell cycle progression and apoptosis (91). The MAGE family of proteins may serve as useful targets for immunotherapy. After encouraging results from Phase I/II studies, the DERMA phase III clinical trial aimed to assess a vaccine using MAGE-A3 protein in combination with an immunostimulant, in melanoma patients following tumor resection (92). However, in 2016 the trial was ended as it failed to show efficacy (NCT 00796445). Nevertheless, the lack of MAGE family gene expression in normal tissue and their overexpression in cancer cells is one of the key reasons they remain attractive targets for future immunotherapy treatments. Other CTAs observed in melanoma include the B-M antigen-1 (BAGE) and

G antigen (GAGE) family of proteins, and their functions are currently being studied (86).

NY-ESO-1

NY-ESO-1 (New York esophageal squamous cell carcinoma-1) is a CTA that is also located on chromosome X and is expressed in a wide range of malignancies (93). In normal cells, this antigen is primarily expressed on spermatogonia and at very low levels in pancreas, liver, and placenta (93). A homolog of NY-ESO-1, LAGE-1 has also been reported and is expressed in a wide variety of human cancer types. The biological functions of both proteins are unknown (93). NY-ESO-1 is a highly immunogenic tumor antigen and is able to elicit a detectable antibody response. In human melanoma, it is observed in a large frequency of melanoma patients (46%) and some studies indicate that its expression may be higher in metastatic lesions (93, 94). Due to its expression in a large fraction of melanomas, immunotherapy trials continue to be conducted using the NY-ESO-1 antigen as part of a tumor vaccine, or more recently using adoptively transferred lymphocytes with recombinant TCRs specific for NY-ESO-1 (95, 96). The adoptive transfer trial resulted in objective responses in 55% of treated melanoma patients but the most efficacious strategy for targeting NY-ESO-1 in melanoma immunotherapy remains to be determined.

Melanoma Differentiation Antigens

A number of TAA in melanoma that are recognized by both $CD4^+$ and $CD8^+$ T lymphocytes are on proteins specifically expressed on melanocytes and involved in melanocyte-specific functions (86, 97). These TAA are located in melanosomes, the organelles in which melanin is synthesized. Moreover, their role in oncogenesis is not known (86). These antigens include *tyrosinase*, *tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2)*, *Melan-A (MART-1)*, and *gp100 (pmel17)* (82, 97). *Tyrosinase* and *TRP-1/-2* are copper and zinc containing metalloenzymes with homology at several sequences and they play crucial roles in melanin synthesis (98). *Tyrosinase* is the key enzyme in melanin synthesis and is located on the membrane of melanosomes. It is observed in over 80% of primary and metastatic melanomas (86). The exact function of *TRP-1* (gp75) remains unclear, but it is purported to play a role in stabilizing tyrosinase (98). *TRP-2* is a DOPAchrome tautomerase and its overexpression is believed to contribute to the chemoresistance and radiotherapy resistance of metastatic melanoma (86, 97). *Melan-A* (melanoma antigen recognized by T cells-1 or MART-1) is a single domain transmembrane protein of 118 amino acids found primarily in melanosomes, endoplasmic reticulum, and trans-Golgi network (86, 99). MART-1 is crucial for the expression, trafficking, and stability of the protein gp100 (pmel17) (99). It is expressed in all melanocytic naevi, and a majority of primary and metastatic melanomas (86). It has been observed that significantly higher frequencies (100- to 1,000-fold) of naive CTL are found against a specific MART-1 peptide (Melan-$A_{26–35}$) compared to other antigens in normal (non-cancerous) individuals who express HLA-A2 (79). However, T cell recognition of MART-1 does not necessarily result in improved clinical outcomes (97). Finally, the protein gp100 (premelanosomal protein-pmel17), is a transmembrane protein that has a role in melanosome biogenesis and melanin polymerization (86). The gp100 gene was found to be widely expressed in malignant melanoma at all stages but was significantly reduced in normal melanocytes (100). HMB-45, a mouse monoclonal antibody (mAb) to gp100, is used for diagnostic purposes to distinguish non-melanocytic from melanocytic tumors (99). All of the aforementioned differentiation antigens are recognized by $CD4^+$ and $CD8^+$ T cells, while TRP-1, TRP-2, tyrosinase, and gp100 can also elicit antibody responses (97). Thus, these antigens are considered to be useful targets for melanoma immunotherapy (86). The B16 syngeneic transplant model, obtained initially from C57BL/6 mice, is one of the most widely utilized models in melanoma research (101). The most obvious advantage of this model is that it expresses murine homologs of the melanoma differentiation antigens (tyrosinase, gp100, MART-1, TRP-1, and TRP-2) (102). Melanocyte differentiation antigens continue to be used in a number of clinical studies in combination with various adjuvants and immunostimulants such as granulocyte-macrophage colony-stimulating factor (GM-CSF), but none of the studies have to date shown significant improvements in OS in melanoma patients (87, 103, 104). Due to the multiple mechanisms of tumor immune escape, it remains particularly difficult to sustain a prolonged response to cancer antigens. However, recently the use of nanoparticles (NP) containing mRNA encoding the melanoma antigens, NY-ESO-1, tyrosinase, MAGE-A3, and a novel CTA TPTE (a transmembrane phosphatase), has shown early clinical promise in a pilot study of three patients (105). To be successful, future immunotherapy trials will need to not only consider the tumor antigens to be used but also the delivery vector, the format (RNA, DNA or protein), and the appropriate adjuvants.

Tumor-Infiltrating Lymphocytes

A cardinal feature of cancer is the immunosuppressive TME (106, 107). As the disease progresses, T cells in the TME exhibit a phenotype analogous to that seen in chronic viral infection known as T cell exhaustion (108). T cell exhaustion denotes a state of hyporesponsiveness to antigen with reduced cytokine secretion and cytotoxic function (108, 109). Nevertheless, the overwhelming majority of studies in human patients have demonstrated a correlation between TIL and better disease outcomes in cancers (110, 111). An exception to this observation is that FOXP3 expression, a marker of Treg that has been shown to correlate to poor prognosis in various types of human cancer (112, 113). The term TIL was first described by Wallace Clark, who was instrumental in developing the first histological classifications for melanoma as mentioned above (114, 115). TIL have been described in primary tumors, tumor-bearing LN, and in metastases of melanoma and various other cancer types (114). The range of immune cells that infiltrate a tumor, i.e., the "immune contexture" of a tumor is heterogeneous and consists of various types of T lymphocytes, B cells, NK cells, macrophages, and DC (111, 114). In 1989, Clark published a classification of the three major patterns of lymphocyte infiltration that are commonly used today (115). The *brisk* pattern is indicated by interposed lymphocytes between tumor cells that may be diffusely present throughout the tumor nodule or along the advancing (basal) periphery of the nodule (114, 115). The *non-brisk* pattern delineates a scattered multifocal

presence of lymphocytes throughout the vertical growth phase of the nodule. Finally, an *absent* pattern is associated with a lack of lymphocytes in the tumor, or if they are present, their lack of interaction with melanoma cells (115). In recent years, various groups have attempted to further classify TIL or propose novel grading schemes, but the Clark model remains widely accepted and highly reproducible (114). In a recently published report, it was shown that melanoma tumors with *brisk* TIL patterns in primary melanoma H&E tissue, even in the absence of immunohistochemistry for specific markers, was associated with increased OS in patients versus tumors with *non-brisk* and *absent* patterns (116). The importance of TIL has been used to establish a novel classification system for cancer based on an "Immunoscore," which relies upon the quantitation of CD3 and CD8 lymphocytes with the additional marker CD45RO used to mark memory T cells. The "Immunoscore" was found to be superior to the conventional AJCC TNM system for prognosis of stage I–III colorectal cancer (CRC) (117). Similar approaches are now being tested for immunoscoring of melanoma but have not been tested in large patient cohorts (118).

An additional feature observed in cancer, and other situations of chronic inflammation is the formation of tertiary lymphoid structures (TLS—also called tertiary lymphoid organs) (119, 120). These TLS can range from loose aggregates of various immune cells to complex structures that resemble secondary lymphoid organs such as LN. They consist of T cell-rich regions containing mature DC expressing DC-LAMP (lysosomal associated membrane protein), B cells, and high endothelial venules, which play a role in immune cell extravasation and production of key chemokines (120). In 2012, Messina et al. reported that a gene expression profile consisting of 12 chemokines could accurately predict the histological presence of LN-like TLS in stage IV melanoma (primary tumors and metastases), and the TLS correlated strongly with improved overall patient survival (121). Other studies have shown that the presence of TLS is a positive prognostic indicator in melanoma and a range of other cancer types including breast carcinoma, CRC, and pancreatic cancer (120). Thus, these results suggest that lymphocyte infiltration mediates a protective immune response to cancer.

However, many tumors are not T cell inflamed, and the mechanisms underlying T cell infiltration into the tumor are poorly understood (89, 122). In the context of melanoma, a recent study compared all major classes of melanoma tumor antigens between T cell inflamed and non-T cell inflamed tumors and found that there were no differences between both groups in terms of antigen load (123). Rather it was shown that non-T cell inflamed melanomas displayed reduced gene expression associated with Batf3-dependent, $CD141^+$ DC (123). Furthermore, studies have pointed to the ability of tumors to interfere with chemokines that recruit leukocytes to tumors. Finally, the abnormal tumor vasculature may express reduced adhesion molecules required for homing and directly or indirectly suppress T cells by expression of molecules such as PD-L1, PD-L2, VEGF, and TGFβ (122). Once T cells infiltrate the TME, they are acted upon by a range of immunoregulatory mechanisms that prevent complete eradication of the tumor (72). These can be tumor-specific escape mechanisms or the recruitment of suppressive immune cells. For instance, mutations in BRAF or PTEN loss are associated with increased T cell inhibition by production of IL-1 and VEGF (72). Furthermore, conserved immunoregulatory mechanisms are also at play within the TME the production of immunosuppressive mediators [TGFβ and indoleamine 2,3 dioxygenase (IDO)], and the recruitment of regulatory myeloid and lymphoid cell populations (72). Another important consideration is that although, $CD8^+$ T cells are canonically considered the primary cytotoxic cells involved in tumor eradication, $CD4^+$ T cells can also kill tumor cells (89). However, the precise mechanisms of $CD4^+$ antitumor immunity are not well described, and the role of $CD4^+$ T cell infiltration in the TME has not been explored significantly with the exception of $FOXP3^+CD4^+$ Treg (72, 89). A recently concluded meta-analysis demonstrated that $FOXP3^+$ Treg infiltrates were predominantly associated with worse OS in a review of over 17 types of cancer (124). In most tumors, such as cervical, renal, breast cancers, and melanoma, $FOXP3^+$ Treg infiltrates correlated with shorter OS whereas they were associated with improved survival in patients with colorectal, head and neck, and esophageal cancers (124). In recent years, several studies have described the heterogeneity in FOXP3-expressing cell populations (125). In 2016, Saito et al. showed that human CRCs could be distinguished by the extent of infiltration of two distinct $FOXP3^+CD4^+$ T cell populations (126). Type A CRCs had low frequencies (<9.8%) while Type B had comparatively higher frequencies (>9.8%) of infiltrating non-suppressive $FOXP3^{lo}CD45^-$ T cells. Infiltration by these non-suppressive T cells was correlated with the presence of intestinal bacteria, in particular *Fusobacterium nucleatum* within the tumor (126). Furthermore, Type B CRCs were marked by high mRNA expression of *IL12A* and *TGFB1* compared with Type A and tumors with high expression of these mRNAs exhibited significantly longer disease-free survival versus low expressing tumors. Thus, $FOXP3^+$ T cell infiltration must be considered in combination with other immune signatures while determining the immune status of a tumor. In addition to T cells, the roles of B cells in the TME are being currently explored as they have both APC and effector lymphocyte functions (127). Studies in melanoma have demonstrated that $CD20^+$ infiltrating B cells are found in most tumors and higher levels of these infiltrates correlated with improved patient survival (127). Furthermore, B cells are known to produce IgG antibodies that can recognize tumor cells and within a murine model of organ transplantation have been observed to promote chronic allograft rejection through antigen presentation rather than their antibody secreting functions (127, 128). Finally, recent studies have also focused on the roles of putative regulatory B cells in the context of transplantation and autoimmunity, as these cells can produce potent immunosuppressive mediators such as IL-10 and TGFβ (129). The multiple immunoregulatory mechanisms that effect TIL are the targets of a majority of current immunotherapies. However, as the aforementioned observations indicate, there are several functionally redundant pathways that allow for immunological escape of tumors in immunocompetent individuals. Thus, to be successful, the field of immunotherapy must move toward combinatorial and multipronged approaches for tumor treatment. This involves investigation of the mechanisms of innate immune cells such as NK cells, TAM, and TIDC within the TME.

Tumor-Infiltrating Dendritic Cells

Despite their discovery over 40 years ago, the exact mechanisms underlying DC dysfunction in cancer remain poorly understood (107). In both mice and humans, DC are classified into two major subsets comprised of conventional or cDC, and plasmacytoid DC (pDC) (130). In non-steady state conditions such as cancer or autoimmune disease, inflammatory DC derived from monocytes have also been described in humans and in mice (130, 131). Despite the fact that nearly all DC subsets express the surface marker CD11c, there are unique transcription factors and surface proteins that characterize the major DC subsets in human and mice. These markers have been extensively reviewed in the literature, but further study is needed to accurately profile each subset (130, 132, 133). DC canonically present extracellular antigens on MHC class II while intracellular or self-antigens are presented on MHC class I (134). However, murine and human DC also possess the capacity to cross-present antigens of extracellular origin on MHC class I to activate $CD8^+$ CTL (135, 136). In humans, the primary cross-presenting DC subset is characterized by CD141 (BDCA-3) while in mice this subset is marked by surface expression of CD8α or CD103 (137). The mechanistic roles played by various DC subsets in both tumor progression and the response to treatment are a key area of research for cancer immunotherapy with little consensus as to their frequencies and functions (102, 107). In 2008, it was reported that knocking out *Batf3* in mice eliminated $CD8\alpha^+$ DC, and consequently it was demonstrated that these mice were incapable of cross-presenting antigen or rejecting highly immunogenic fibrosarcomas (138). Although pDC are purportedly not efficient at cross-presentation, studies have shown their capacity to mediate direct tumor killing and to activate NK cells via the production of type I IFN (139). Despite the key roles played by TIDC in promoting antitumor responses, generally TIDC are skewed in both phenotype and function toward an immunosuppressive role in the microenvironment (107). These alterations in TIDC have been mechanistically studied in murine models (107, 140). The TME has been reported to induce a "paralyzed" state in TIDC resembling an immature phenotype with reduced expression of costimulatory CD80 and CD86 molecules and a diminished capacity to present antigens (107). This induction is a result of various immunosuppressive factors such as VEGF, TGFβ, IDO produced by tumor cells as well as by other cells in the TME (72, 107). Furthermore, DC paralysis in mouse models has been observed to be associated with upregulation of immune checkpoint receptors such as PD-1 and T cell immunoglobulin and mucin-domain containing-3 (TIM-3), which was reported to interact with the alarmin protein high mobility group box 1 (HMGB1) resulting in reduced DC sensing of tumor-derived nucleic acids (107). TIDC with immature and paralyzed phenotypes themselves suppress immune cells in the TME through various mechanisms such as but not limited to, expression of inhibitory molecules (PD-L1), production of regulatory cytokines such as IDO and induction of Tregs (107, 141).

As previously noted, there has been significant research on TIL in melanoma. On the other hand, the mechanistic roles of TIDC in melanoma are not well studied. Melanoma is of particular interest due to the fact that skin contains multiple DC subsets. The five major DC subsets found in human skin are Langerhans cells, $CD14^+$ DC, $CD1c^+$ DC, $CD1a^+$ DC, and $CD141^+$ DC (133). The correlations between various TIDC subsets and disease outcome, their association with other cells and specific functions have not yet been fully elucidated (102). However, recently it was demonstrated that intratumoral $CD103^+$ DC in mice were crucial for trafficking of melanoma tumor antigen to LN and were dependent on surface expression of CCR7 (142). Enhanced CCR7 mRNA expression in human melanoma samples was also correlated to increased T cell infiltrates and improved patient outcomes (142). In general, it is observed that there are higher frequencies of TIDC in the peritumoral region than within the tumor (102). These peritumoral DC include arguably the most mature population of DC-$LAMP^+CD83^+fascin^+$ cells (102). In fact, DC-LAMP expression is associated with positive prognosis in not only melanoma but also lung, breast, and metastatic CRC (120). On the other hand, $CD123^+$ pDC that do in principle possess the capacity to promote antitumor responses are found to be associated with early relapse and poor prognosis in human melanoma (102, 143). It was shown in both *ex vivo* patient samples and in that a humanized melanoma mouse model that pDC in melanoma are directed toward a T_H2 promoting phenotype by induction of the molecules OX-40L (TNFSF4) and ICOSL (inducible T cell costimulator ligand), which then drive tumor progression (143). To comprehensively characterize TIDC in melanoma, it is crucial to obtain genomic data to appropriately distinguish and profile TIDC subsets. Pyfferoen et al. performed transcriptomic profiling of DC in a murine model of lung carcinoma and demonstrated that TIDC had significantly increased expression of PD-L1, acquisition of TAM surface markers and a pro-metastatic microRNA signature (144). To date, similar studies have not been performed in human melanoma. There have been several studies in murine models that have demonstrated the therapeutic reprogramming of TIDC (107). Thus, manipulation of TIDC represents a hitherto unexplored target for future melanoma immunotherapies. Many of the same agents that have been shown to induce DC activation and maturation *in vitro* have been tested for direct targeting of DC *in vivo* (133, 145). For instance, direct administration of BCG has been utilized for the treatment of bladder cancer for over 30 years although its precise mechanisms of action *in vivo* are still under study (146). Direct modulation of DC *in vivo* using DC maturation agents and mAbs is a highly desirable goal in tumor immunotherapy. This is due to the excessive costs, safety considerations, and practical limitations of using cellular products (147). As such, the identification of both targetable DC receptors and maturation stimuli continues to be an active area of research interest. In particular, targeting antigen-coupled antibodies to DC C-type lectin receptors (CLRs) such as DEC205 (CD205), Clec9A, and DC-SIGN in murine and *in vitro* studies resulted in effective $CD4^+$ and $CD8^+$ T cell responses (145, 148). Additional receptors such as XCR1 (expressed entirely on $CD141^+$ DC) are also being studied for their effects on DC function (133). Clinical trials for multiple cancer types are presently underway to investigate the efficacy of anti-DEC205 conjugated to the cancer–testis antigen NY-ESO-1, which is also used for melanoma immunotherapy (133, 149). Recently, a series of seminal papers have shown the importance of

the cytosolic DNA sensor cyclic GMP-AMP (cGAMP) synthase (cGAS) in promoting antitumor immunity (150–152). DNA introduced to the cytosol as a result of viral infections or cellular damage is a potent immune activator that leads to the production of type I IFN (153). Upon detection of DNA by cGAS, it catalyzes the production of cGAMP that binds to the adaptor protein stimulator of interferon genes (STING) ultimately resulting in the production of type I IFN (153). In 2014, Woo et al. demonstrated in a mouse model that tumor-derived DNA was responsible for inducing IFNβ production and the consequent activation of APC and $CD8^+$ T cells versus melanoma *in vivo* (150). Alternately, mice deficient in STING failed to reject these tumors highlighting the crucial role played by this pathway in the immune response to cancer (150, 151). In a more recent paper, Wang et al. showed the role of cGAMP in mediating the effects of ICB (152). It was reported that in mice lacking either cGAS or STING, PD-L1 blockade did not result in significant shrinkage of tumor volume or increase in survival compared with WT mice. Moreover, intramuscular injection of cGAMP in combination with PD-L1 significantly enhanced survival, compared with PD-L1 or cGAMP alone (152). Finally, it was also shown that cGAMP treatment of BMDC enhanced expression of DC activation markers and increased DC antigen cross-presentation. Another molecule that has recently gained interest for its effects on DC is IL-32. In 2012, Schenk et al., identified an IL-32-dependent mechanism for DC differentiation in response to nucleotide-binding oligomerization domain containing protein (NOD2) activation through its ligand muramyl dipeptide (154). DC obtained from IL-32 differentiation were found to express higher levels of MHC class I and CD86, as well as, present antigen to $CD8^+$ T cells more effectively than GM-CSF differentiated DC (154). These studies highlight the multiple pathways that may be targeted to generate effective DC *in vivo*, which is essential for antitumor immunity.

NK Cells

Natural killer cells were characterized over 40 years and are the first population of ILC to be described and studied (155, 156). NK cell defects lead to enhanced susceptibility to viruses and many forms of cancer in humans and in mouse models (156). NK cell functions are modulated by a number of surface receptors that provide either NK activating or inhibitory signals (156, 157). NK cells are broadly defined as $CD3^-CD56^+$ in humans and $CD3^-NK1.1^+$ in mice while both murine and human NK cells express the surface receptor NKp46 (CD335) (156). In humans, NK cells are further divided into $CD16^+CD56^{dim}$ which predominate in blood, and $CD16^-CD56^{bright}$ populations (156). Canonically, NK cells can recognize tumor cells that have downregulated MHC class I molecules or upregulated induced stress molecules (155, 156). NK cells can also bind to antibodies bound to tumor antigens and mediate antibody-dependent cellular cytotoxicity (156). As with $CD8^+$ CTL, NK cells mediate their cytotoxic functions through perforin and granzymes, as well as, by expressing death mediating ligands such as FasL (CD95L) and TRAIL (TNF-related apoptosis inducing ligand) (156). Activated NK cells also produce IFNγ, among other cytokines, which leads to recruitment of other immune cell populations (156).

The roles of NK cells in the TME are currently not fully described (155, 157). Several studies have indicated that NK cell infiltration is generally a positive prognostic factor in various types of cancer (155). In the context of melanoma, the roles of NK cells are an important venue of research. Analysis of several melanoma cell lines indicated that a high percentage of melanoma cells possess ligands for a NK activating receptors such as NKG2D and DNAM1, while ligands have also been identified for NK-bound NCR (natural cytotoxicity receptors) such as NKp30 (157). Melanoma cells are also known to have decreased MHC class I expression as a mechanism to escape $CD8^+$ T cells, thus making them targets for NK cells (157). Despite these observations, melanoma immunoediting leads to tumor escape from NK cells via multiple mechanisms (157). Melanoma immunoediting by NK cells increases expression of MHC I, or downregulates NK ligands supported by the decreased expression of MICA reported in metastatic versus primary melanoma (157). IDO and prostaglandin E2 (PGE2) produced by melanoma cells act directly to inhibit NK cells while increased expression of ligands to regulatory receptors such as TIGIT modulate NK cell activity (157). In light of these observations, it will be important to identify NK populations that have persistent antitumor activity and characterize their phenotypes to better understand the mechanism involved in effective NK immunity. Recently, it was reported that tumor-bearing/infiltrated LN in melanoma patients contained twice as many NK cells as ipsilateral tumor-free LN (158). These tumor-infiltrated LN also contained a population of highly cytotoxic $CD56^{dim}KIR^+CCR7^+$ NK cells that may have prognostic potential for melanoma (158). Conversely, melanoma, breast, and colon cancers were found to be infiltrated by $CD56^{bright}$ NK subsets, which are similar to decidual NK cells during pregnancy thus implying a potentially regulatory role for this subset (159). NK cells remain an important target for immunotherapy. Along with T cells, NK cells were used early on for adoptive cell transfer therapy of melanoma in the 1980s and both autologous and allogeneic NK cell adoptive transfers are being studied in clinical trials (156, 157). Currently, two antibodies for the blockade of NK checkpoints are under clinical development, namely, lirilumab (anti-KIR-studied in combination with ipilimumab) and IPH2201 (anti-NKG2A) for various types of cancers including melanoma (157). However, further study of NK cells in the melanoma TME is required to understand the several mechanisms of immune escape from NK cells and $CD8^+$ CTL and thus devise, rational combinatorial immunotherapies.

MELANOMA IMMUNOTHERAPY

In 2013, the journal *Science* hailed cancer immunotherapy as the breakthrough of the year (56). This was in recognition of the promising clinical responses that can be achieved by directing the immune system to fight cancer. Despite highly encouraging advances, current immunotherapies only result in clinical benefit for a subset of patients (160, 161). Thus, there is a significant scientific effort to understand the tumor cell-intrinsic and extrinsic mechanisms of resistance to immunotherapy (162). The three major mechanisms of resistance to immunotherapies have been conceptualized as follows. *Primary* resistance denotes a clinical

setting where the initial immunotherapy is unsuccessful. This can be due to *adaptive* resistance which defines a mechanism whereby there are initial antitumor immune responses but are inhibited by adaptation and immune escape of the tumor (162). Clinically, adaptive resistance may be seen as primary resistance, mixed responses or *acquired* resistance. Acquired resistance describes a clinical scenario where the tumor initially responded to immunotherapy but has eventually progressed and acquired resistance to the therapy (162). To overcome resistance to various forms of immunotherapy, it will be important to understand the mechanisms that allow tumor cells to escape immune attack. The clinical experience with melanoma immunotherapies has shown significant promise and there is increasing evidence that a multipronged approach may be required to ensure durable responses in a majority of patients. This section describes the major immunotherapies that have already been developed or are under clinical development for the treatment of metastatic melanoma (summarized in **Table 1**). Advances in immunotherapy for other types of cancers, as well as, the use of mAbs to specifically target tumors have been previously reviewed in detail (163–166).

Early Advances in Melanoma Immunotherapy

As previously noted, the mechanistic basis for Coley's observations remained unknown for some time and during this time, surgery, radiation treatment, and cytotoxic chemotherapy became the primary means of cancer treatment. However, in the context of melanoma, two major forms of immunotherapy witnessed encouraging breakthroughs starting in the 1980s and led to renewed interest in the entire field. These breakthroughs occurred in systemic cytokine therapy with IL-2 and adoptive cell transfer using TIL (183). In 1985, Rosenberg et al., demonstrated in C57BL/6 mice that intraperitoneal injections of recombinant IL-2 were capable of significantly attenuating pulmonary metastases from tumors generated by the MCA-105 and -106 syngeneic sarcoma and B16 syngeneic melanoma lines (184). Retrospective analyses of metastatic melanoma patients who had been treated with IL-2 demonstrated an ORR of 16% and represented a significant advance in the treatment (185). IL-2 received FDA approval in 1998 for metastatic melanoma. However, as systemic treatment of IL-2 resulted in various toxicities, several groups have shifted to intralesional administration of IL-2, which resulted in CR rates of between 41 and 76% in various trials (48). In parallel to the successes achieved with IL-2, Rosenberg and colleagues reported the first successful use of adoptive T cell transfer for the treatment of solid cancers (186). Patients were treated with IL-2 and autologous TIL expanded from surgically resected melanomas. Objective responses were observed in 60% (9/15) of treated patients (186). Subsequently, in 2002, this approach was combined with lymphodepletion prior T cell transfer and demonstrated enhanced responses in patients (50). Currently, adoptive cell therapy (ACT) using TIL remains one of the most effective therapies for metastatic melanoma (183).

Immune Checkpoint Blockade

Drugs that mediate ICB by targeting the inhibitory receptors CTLA-4 and PD-1 (**Figure 2** *inset panel*) have been shown to induce durable responses in subsets of patients with various types of cancer including melanoma, NSCLC, and renal cell cancer

TABLE 1 | Key immunotherapeutics and their primary mechanisms of action.

Treatment	Clinically tested agents	Mechanism(s) of action	Reference
Immune activating mAbs			
αCTLA-4	Ipilimumab (Yervoy®)	– Blockade of T cell checkpoint receptor – Depletion of intratumoral Treg	(160, 167)
αPD-1	Nivolumab (Opdivo®), pembrolizumab (Keytruda®)	– Blockade of T cell checkpoint receptor	(167, 168)
αPD-L1	Atezolizumab, durvalumab, avelumab	– Blockade of inhibitory checkpoint ligand expressed on immune cells and tumor cells	(167, 169)
αCD137 (4-1BB)	Urelumab	– Agonist of T cell costimulatory receptor	(170)
αKIR	Lirilumab	– Blockade of NK cell inhibitory receptor	(157, 171)
αLAG-3	BMS986016	– Blockade of T cell surface inhibitory molecule	(167)
Adoptive T cell therapy			
TIL	*Ex vivo* expanded TIL	– Infusion of pool of antitumor T cells	(50, 172)
Engineered T cells	Transgenic TCR or CAR bearing T lymphocytes	– Infusion of engineered T cells specific for tumor antigens	(50, 173)
Vaccines			
Cell-based vaccines	Tumor cells or activated DC/APC	– Induction of tumor-specific adaptive immunity	(87, 174, 175)
Peptide vaccines	Various tumor antigen peptides/lysates + adjuvant	– Induction of tumor-specific adaptive immunity	(165, 176)
Oncolytic viral vaccines	Talimogene laherparepvec (T-VEC/Imlygic™)	– Viral induction of tumor cell lysis and adjuvant mediated host immune activation	(177, 178)
Cytokines			
Interleukin-2	Aldesleukin (Proleukin®)	– Activates and expands T cells	(179, 180)
Interferon alpha	Interferon alfa 2b (Intron® A, Sylatron™)	– Activates multiple facets of immunity and has direct effects on tumor cells	(181, 182)

An overview of current immunotherapy approaches and their proposed mechanisms of action as discussed in this review.
Trade names are provided for drugs that have received clinical approval in melanoma. References provided for further description of each approach.
KIR, killer-cell immunoglobulin-like receptor; DC, dendritic cells; APC, antigen-presenting cell; TCR, T cell receptor; CAR, chimeric antigen receptor; TIL, tumor-infiltrating lymphocyte; NK, natural killer; Treg, regulatory T cells.

(RCC) (187–190). Furthermore, antibodies targeted to the PD-1 ligand, PD-L1, are undergoing clinical trials and have resulted in objective responses for multiple cancer types (51, 191). To date, the FDA has approved four mAbs for ICB therapy: (1) ipilimumab (αCTLA-4); (2) nivolumab (αPD-1); (3) pembrolizumab (αPD-1); and (4) atezolizumab (αPD-L1) (192). They have been approved for various advanced and metastatic cancers ranging from unresectable or metastatic melanoma to urothelial carcinoma (atezolizumab) (168, 192). Currently, only ipilimumab, nivolumab, and pembrolizumab have received FDA approval for melanoma (167). Due to the fact that checkpoint receptors play important roles in regulating autoimmunity, the major toxicities associated with the use of ICB drugs include a range of autoimmune symptoms labeled immune-related adverse events (IRAEs) (193). The incidence of IRAEs is quite high, ranging from 70% in patients treated with αPD-1/αPD-L1 antibodies to as high as 90% in patients treated with αCTLA-4 and require careful management in the clinic with immunosuppressive medications (193). As ICB results in objective responses for only a subset of patients, there is a crucial need to identify biomarkers that can potentially predict the efficacy of a particular ICB treatment or designate a particular subset of patients who may benefit from ICB therapy (194).

CTLA-4

Cytotoxic T lymphocyte antigen-4 (also termed cytotoxic T-lymphocyte-associated protein 4), is a crucial regulator of T cell activation and ipilimumab, a human IgG1 mAb targeted to this molecule was the first ICB drug to show clinical efficacy in advanced melanoma and a number of other cancer types (48, 195). CTLA-4 plays a key role in T cell immunity and its molecular biology has been recently reviewed elsewhere (167, 196). However, to understand the clinical role of CTLA-4 blockade, a brief summary of its mechanism of action is warranted. Naive T cells are modulated by APC through the interaction of multiple surface receptors in a region referred to as the "immunological synapse" (197). Canonically, naive T cells require 3 signals for complete activation (**Figure 2** *inset panel*) (198). The engagement of the TCR by peptide antigen presented in the context of MHC, provides the first signal of T cell activation (signal 1) (198, 199). T cells require further signaling from the binding of costimulatory molecules on T cells such as CD28, to its respective ligands CD80/86 on APC (signal 2). Finally, the complete activation requires cytokines (IL-2) binding to their cognate receptors on T cells (Signal 3) (199). As an evolutionary checkpoint to autoimmunity, activated T cells induce surface CTLA-4 expression, which binds with greater affinity to CD80/86 and mediates T cell inhibition and cell cycle arrest (195, 200). CTLA-4 is also expressed constitutively on Treg (167). The crucial role of CTLA-4 in maintaining tolerance is demonstrated by the severe multiorgan autoimmune pathologies and early mortality (3–4 weeks) observed in CTLA-4$^{-/-}$ mice (201). Humans with heterozygous germline mutations in CTLA-4 also exhibit autoantibodies, increased intra-organ lymphocyte infiltration and other symptoms of immune dysregulation (167).

In 2010, Hodi et al. demonstrated the clinical efficacy of ipilimumab in patients with stage III and IV unresectable and metastatic melanoma whose tumors were refractory to prior treatments (187). The treatment subjects received ipilimumab alone, ipilimumab plus the peptide gp100 or gp100 alone. Patients receiving ipilimumab alone or ipilimumab plus gp100 had significantly increased median OS compared with those receiving gp100 alone (roughly 10 versus 6 months) (187). Currently, ipilimumab has only received FDA approval for melanoma. However, a number of studies have shown modest responses to ipilimumab in other tumor types such as metastatic RCC and NSCLC, and it continues to be studied in clinical trials as combination therapy with PD-1/PD-L1 (discussed below) (160, 167). As mentioned previously, a number of immunological toxicities (IRAEs) are commonly observed to occur in patients treated with ipilimumab primarily in the skin, GI tract, and the endocrine system and in some rare cases result in deaths (193). The frequency of severe toxicities (grade 3 or 4) in the preliminary phase III trials of ipilimumab was demonstrated to be 20%, but this value was not significantly higher than the toxicities associated with many chemotherapy or targeted therapy drugs (163, 195). Most IRAEs can be resolved within 6–12 weeks of steroid therapy but for steroid-resistant adverse events, patients can also be treated with immunosuppressive antimetabolite drugs such as azathioprine and mycophenolate mofetil (193). Novel CTLA-4 blockade agents including modified versions of ipilimumab are also currently under study for a number of advanced solid tumors with the aim of improving safety profiles and tumor-specific delivery (202).

PD-1/PD-1 Ligand (PD-L1)

The most clinically successful agents for ICB to date target the inhibitory PD-1/PD-L1 axis (169, 195). The transmembrane receptor PD-1 (CD279) plays a crucial role in regulating antigen-specific T cell responses (169, 203). PD-1 is not only expressed on activated effector T cells but also on NK cells, B cells, macrophages, and Tregs (167, 203). Similar to the activating co-receptor CD28, PD-1 is acted upon by two distinct ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) (203). Whereas PD-L2 expression has hitherto been observed only on professional APC (including B cells), PD-L1 is expressed on various tissue types such as epithelial tissue, vascular endothelium, stromal cells as well as tumor cells and virus-infected cells (167, 203). The induction of PD-L1 expression is generally in response to pro-inflammatory cytokines such as interferons, TNF-α, and VEGF (167, 169). PD-1 does not, as its name implies, directly induce cell death. The binding of PD-1 to its ligands instead serves to attenuate T cell activation by recruiting the tyrosine phosphatase SHP-2, which interferes with signaling downstream of the TCR and leading to decreased T cell growth and reduced cytokine production (203). However, PD-1 signaling can also reduce the expression of antiapoptotic genes while upregulating proapoptotic gene expression thus impairing T cell survival (167).

PD-1-deficient mice do not display as severe a phenotype as CTLA-4$^{-/-}$ mice, developing glomerulonephritis and arthritis in a C57BL/6 background and autoantibody induced dilated cardiomyopathy in BALB/c mice as they age (204, 205). This is arguably due to the more direct inhibitory and Treg-related

functions of CTLA-4, whereas PD-1 serves to limit T cell activation indirectly and prevent peripheral autoimmunity (169). As noted previously, in certain conditions of persistent antigen exposure such as in chronic viral infections or in cancer, T cells are observed to develop a dysfunctional or "exhausted" phenotype (72, 167). Such T cells are also marked by elevated expression of PD-1 and other inhibitory receptors such as TIM-3 and LAG3 (72). Furthermore, PD-L1 and/or PD-L2 are both observed to be expressed on a number of tumor-infiltrating APC and tumor cells themselves, not only as a result of cytokines but also due to alternative factors such as gain of chromosomes carrying PD-L1 and PD-L2 or the signaling of the epidermal growth factor pathway (167). Recent studies have shown that APC and tumor cells bearing PD-L1 play additive non-redundant roles in the suppression of antitumor immunity (206). Thus, blockade of the PD-1/PD-L1 axis remains a critical area of interest in tumor immunotherapy with studies on its efficacy in nearly 20 types of solid tumors and hematological cancers (169).

In the context of melanoma, nivolumab, and pembrolizumab, both of which target PD-1 have been shown to have significant clinical efficacies (160, 169, 195). In 2012, results from a phase I study comparing various doses of nivolumab in NSCLC, prostate cancer, CRC, renal cell carcinoma, and melanoma patients were reported (188). The highest activity was demonstrated in melanoma patients where the cumulative response rate (for all doses) was 28% compared with 27% for renal carcinoma and 18% for NSCLC (188). In the same year, an αPD-L1 antibody (BMS-963559) was tested in advanced cancers ranging from melanoma to RCC and was shown to have comparatively low response rates (6–17%) (191). A number of recently concluded trials have also demonstrated the potency of pembrolizumab. The large multicenter phase II trial KEYNOTE-002 examined the efficacy and safety of pembrolizumab in patients who had progressed on ipilimumab therapy, and in patients with BRAF mutations, those who had received either BRAF or MEK inhibitor treatment (207). Patients received either two separate doses of pembrolizumab (2 or 10 mg/kg) or chemotherapy of the investigators choice (carboplatin, dacarbazine, paclitaxel, and temozolomide). The results were highly encouraging as the 6-month PFS was shown to be 38% (10 mg/kg) and 34% (2 mg/kg) in the pembrolizumab group compared with only 16% in the chemotherapy group (207). Similar efficacy over investigator choice chemotherapy (32 versus 11%) has also been reported from an open-label phase III trial of nivolumab in patients who had progressed on ipilimumab (195). Furthermore, pembrolizumab was shown to have significantly higher activity than ipilimumab in patients with advanced melanoma. Robert et al. compared two dosing schedules (every 2 or 3 weeks) of pembrolizumab to ipilimumab and reported 6-month PFS in the range of 46–47% (response rates of roughly 33%) for the pembrolizumab group versus 26.5% (RR of 11.9%) for the ipilimumab-treated patients (208). Finally, in a phase III trial of nivolumab in previously untreated advanced melanoma patients (without BRAF mutations), ICB therapy was demonstrated to have significantly higher efficacy compared with dacarbazine with a 1 year survival rate of 72.9% in the nivolumab treated group versus 42% in the dacarbazine group (189). The successes of αPD-1 in melanoma treatment have also been observed (albeit at lower rates) in a range of other cancer types (167, 169). Furthermore, the rate of grade 3 or 4 treatment related adverse events is lower in patients receiving PD-1 blockade therapy versus ipilimumab which is similar to the decreased severity of autoimmune pathologies observed in PD-1 versus CTLA-4 knockout mice (169, 193). In contrast to PD-1 blockade antibodies, the αPD-L1 agent atezolizumab (MPDL3280A) has thus far received FDA approval only for urothelial bladder cancer and lung cancer (169, 209). Recently, studies have further complicated the role of PD-L1 by demonstrating that it binds to CD80 on T cells and provides another inhibitory signal (210). Thus, further studies are warranted to determine the role of PD-L1 in T cell inhibition in tumors and investigate which tumor types may benefit most from PD-L1 versus PD-1 blockade. A large number of clinical trials are currently underway targeting PD-1/PD-L1 as well as novel combination approaches (169). As previously mentioned, further study will be required to determine biomarkers of response to ICB and further mechanistic knowledge will be necessary to design effective combinatorial immunotherapies. Four clinical biomarker profiles for ICB treatment have already been proposed based on the presence of PD-L1 and TIL (211). The tumor are characterized as type I ($PD\text{-}L1^{+}TIL^{+}$), type II ($PD\text{-}L1^{-}TIL^{-}$), type III ($PD\text{-}L1^{+}TIL^{-}$), and type IV ($PD\text{-}L1^{-}TIL^{+}$) (211). In melanoma, where the data are most complete, the majority of patients are either type I (~38%) or type II (~41%). Type I patients are deemed to be the best responders to PD-1 blockade whereas type II tumors are estimated to have very poor prognosis due to their lack of immune cell infiltrates (211). Currently, the mechanisms that regulate the immune composition of a tumor are not well understood and there is a significant interest in treatments that can convert T cell non-inflamed (non-infiltrated) tumors to T cell inflamed (infiltrated) tumors (212).

Combinatorial Checkpoint Blockade

Despite the tremendous successes of ICB, to date, only a subset of patients achieve durable clinical responses (160, 167). However, the potency of immune checkpoint therapies has ushered in a new era of cancer treatment by offering the possibility of combining these drugs with conventional cancer treatments such as radiation, chemotherapy, and targeted molecular therapy (e.g., BRAF/MEK inhibitors). The prospects for such combination treatments in melanoma and other cancer types, as well as the clinical findings to date using such approaches have been expertly reviewed this year (213–215). The primary focus of this section will be to discuss the approaches involving combination checkpoint blockade therapies for melanoma that have demonstrated efficacy thus far. Nevertheless, it is pertinent to note that currently there are no clinical data to distinguish between ICB or BRAFi/MEKi targeted therapy as first line treatment for melanoma and a clinical trial (NCT02224781) is being conducted to provide direct comparisons between clinical outcomes in patients receiving checkpoint blockade drugs following targeted therapies and vice versa (215).

The success of combined ipilimumab and nivolumab has also been recently reported in a number of clinical trials. In 2015, Postow et al. reported the results of a study where previously untreated patients with metastatic melanoma received either

ipilimumab in combination with nivolumab or with placebo preceding a subsequent treatment with nivolumab or placebo (216). The ORR was 61% in the combination treatment group versus 11% in the ipilimumab plus placebo group. Moreover, nearly 22% of patients treated with combination therapy achieved CR compared with none of the patients given ipilimumab and placebo (216). In the same year, results were published from a phase III trial in 945 patients with unresectable stage III or IV melanoma treated with nivolumab alone, nivolumab plus ipilimumab, or ipilimumab alone. The median PFS was 11.5 months for the combination group, 6.9 months for the nivolumab group, and 2.9 months for the ipilimumab group (217). However, serious (grade 3 or 5) treatment related adverse events in the combination treatment group were significantly higher reaching 55% compared with 27% for the ipilimumab group (217). These studies also indicate the superiority of combinatorial checkpoint blockade over monotherapy leading to the approval of ipilimumab and nivolumab dual therapy for melanoma in the USA, while its efficacy in other tumor types continues to be investigated (218). The successful use of combined checkpoint blockade has also sparked clinical interest in additional immune checkpoints some of which are undergoing preclinical or clinical investigation (167, 169, 218). A target of particular interest is the CD4 homolog lymphocyte activation gene-3 (LAG-3), which is expressed on Treg, effector $CD4^+$ and $CD8^+$ T cells, NK cells, B cells, and pDC and which also binds to MHC class II (167, 219). LAG-3 is an important negative regulator of $CD4^+$ and $CD8^+$ T cells and is required for Treg activity (219). The αLAG-3 antibody BMS986016 is currently being examined in a clinical trial (NCT01968109) for several advanced tumors both as a monotherapy and in combination with nivolumab (167). Another immune checkpoint that has exciting potential for tumor immunotherapy is TIGIT (T cell immunoreceptor with immunoglobulin and ITIM domain) (167). TIGIT is expressed by activated T cells, NK cells and is also expressed on highly functional subsets of Treg (219, 220). TIGIT has two ligands, namely, CD155 (poliovirus receptor, PVR) and CD112 (PVRL2) that are expressed on APC as well as on tumor cells (167). Likewise, TIGIT is reportedly expressed on TIL (219). The immunoregulatory functions of TIGIT are only recently beginning to be described (221). TIGIT can bind to CD155 on DC resulting in increased IL-10 and decreased IL-12 secretion (167). Ligation of TIGIT on Treg results in the expression of fibrinogen-like protein 2 (Fgl2), a Treg effector molecule that has broad immunosuppressive effects such as mediating Th1 and Th17 phenotype suppression in favor of Th2 (167, 222). In human melanoma, tumor-specific $CD8^+$ T cells in peripheral circulation and $CD8^+$ TIL were found to express both TIGIT and PD-1 and furthermore, TIGIT was upregulated in response to PD-1 blockade (223). Thus, the described functions of TIGIT further complicate our understanding of the immune response to αPD-1 treatment and provides further proof of the need of combinatorial approaches to overcome current barriers to ICB treatment. The positive results associated with ICB treatment have also renewed interest in a parallel treatment approach involving the development of agonistic antibodies for T cell costimulatory molecules such as CD137 (4-1BB), GITR (glucocorticoid-induced TNFR family related gene), and OX40 (CD134) many of which are currently undergoing clinical trials in combination with nivolumab (167, 169, 218). In 2016, early results were showcased for the antibody urelumab (αCD137) in combination with nivolumab (202). In melanoma, the ORR was observed to be 50% in patients who had not previously received checkpoint blockade therapy and was found to be independent of tumor PD-L1 status (202). Thus immune agonistic antibodies have revealed a plethora of novel possibilities for cancer treatment. Future studies will involve analyses of various combinations aimed at developing immunotherapies tailored to the specific tumor immune microenvironment (224).

Adoptive Cell Therapy

Adoptive cell therapy involves the use of *ex vivo* manipulated cells transferred directly to patients to mediate antitumor immunity (50, 172). Thus far, the majority of clinical research in ACT has been conducted using autologous tumor-specific T cells (TIL) harvested and cultured from resected melanoma tissue (161, 173). Other cell types such as NK cells have also been investigated since the 1980s for their use in adoptive transfer therapy but have yet to be as widely studied as T cells (156). Thus, the primary focus of this section will be on studies with T cell ACT. The benefits of this approach are that it allows for the *ex vivo* expansion of tumor-specific cells that are not modulated by the immunosuppressive TME and can be administered in sufficient numbers to induce tumor regressions (50). As mentioned previously, this field was pioneered by Rosenberg and colleagues using autologous TIL from patients with metastatic melanoma and resulted in durable antitumor responses (186). Since that time, developments in molecular biology allowed for the elucidation of various tumor antigens and the development of genetically engineered T cell products with tumor-specific TCR or chimeric antigen receptors (CARs) (50, 225). To date, successful ACT through TIL transfer has been largely limited to melanoma although it is currently being studied in metastatic HPV-associated cancer and has been demonstrated to induce potent prophylactic clinical responses in HSCT recipients against Epstein–Barr virus-associated lymphoproliferative disorders (225). Lymphodepletion before TIL therapy has been shown to significantly augment clinical response, and although its precise mechanisms of action are not well understood, it is posited to complement TIL transfer by eliminating suppressive Treg and myeloid cells (50). In patients treated with autologous TIL therapy post lymphodepletion, the group of Rosenberg and colleagues at the NCI (Bethesda, MD, USA) has reported OR rates of 55% (226). These results are similar to those observed in patients from other centers that perform ACT using TIL such as MD Anderson (Houston, TX, USA) with an ORR of 48% in their patient cohort and Ella Cancer Institute (Raman Gat, Israel) with an ORR of 40% (50, 227). Overall, TIL therapy is not reported to be associated with severe adverse events, and the major toxic side effects are associated with the lymphoablative conditioning regimens (226). The primary hematological pathologies observed are anemia and thrombocytopenia necessitating transfusion in these patients, while patients in cohorts that receive TIL and IL-2 may report to develop grade 3 and 4 non-hematological toxicities (228). Currently, the predominant clinical form of ACT for

melanoma is TIL therapy (50, 173). Nevertheless, there is also significant clinical interest in the use of highly specific T cells expressing TCRs specific to tumor antigens. These T cells can be generated through *in vitro* selection and expansion of specific antitumor clones (173). However, engineered T cells bearing conventional antitumor alpha beta TCRs or CARs have generated significant interest in the field of adoptive cell therapies (229). CARs are artificial receptors that were developed to circumvent the requirement of MHC–TCR interactions as many tumor cells downregulate MHC expression to escape the immune system (173). CARs consist of an extracellular ligand-binding domain constructed with immunoglobulin heavy and light chain variable regions fused through a transmembrane domain to intracellular CD3 zeta signaling chains in addition to CD28 or CD137 costimulatory domains for induction of complete T cell activation (50, 229). Currently, CAR T cells have demonstrated efficacy only in B cell malignancies using anti-CD19 CARs, to achieve response rates of up to 90% (173). However, a number of studies are currently underway investigating the use of CAR T cells in solid tumors (173). On the other hand, studies using transgenic tumor-specific TCRs have been tested in melanoma with the first proof-of-concept study being performed in 2006 using T cells transduced with a TCR against the melanoma differentiation antigen MART-1 (230). This early study showed evidence of clinical activity in only 2 out of 17 patients but a more recent report by Chodon et al. (231) demonstrated that MART-1 specific T cells in combination with MART-1 pulsed DC vaccine were able to induce tumor regression in 9 out of 13 studied patients (231). Thus, combining ACT with other immunotherapies may unveil potentially novel synergistic treatments that can overcome the current barriers to ACT. A number of clinical trials using ACT in conjunction with checkpoint blockade agents (nivolumab-NCT02652455) or targeted therapy (vemurafenib-NCT01659151) are being tested in patients with melanoma (173). A number of salient factors warrant consideration when discussing the merits of ACT immunotherapies for cancer. First, it is pertinent to mention that ACT requires *ex vivo* manipulation of cells, which is both expensive and labor intensive (173). Therefore ACT currently remains limited to a few specialized centers around the world (50). Furthermore, engineered T cells have the potential to induce stronger toxicities versus conventional TIL due to their clonal specificity toward a single antigen. This is a particular concern with TCRs targeted to antigens that are shared by tumor and normal tissue resulting in an immune activation versus the target but not necessarily against the tumor (on-target, off-tumor toxicity) (173). This effect has been observed in a number of trials. In a study treating patients with T cells bearing transgenic TCRs specific to MART-1 and gp100, several patients developed toxicities in the skin, ears, and eyes due to the presence of melanocytes in these organs (232). This effect has also been seen in other tumor types such as metastatic renal cancer where in a recent report, 4 out of 12 patients treated with CAR T cells specific to carbonic anhydrase IX (CAIX), developed liver toxicity due to the presence of this antigen in the bile duct (233). Thus, strategies will need to be developed to overcome such off-target effects of engineered lymphocytes and in the case of the aforementioned CAIX trial, hepatic T cell mediated toxicity was significantly lowered by treatment with blocking anti-CAIX antibodies (233). Although early studies showed that MART-1 and gp100 are among the major tumor antigens recognized by anti-melanoma TIL, recent advances in whole-exome sequencing offer the potential to reveal novel antigens (i.e. neoantigens) resulting from mutations that may be highly immunogenic but also safe due to their absence from the rest of the body (50). Another concerning immune-related toxicity observed in CAR and conventional T cell therapy is cytokine release syndrome, which presents as a systemic multisymptomatic inflammation causing fever, hypotension, and tachycardia (173). In terms of efficacy, a key concern using CAR T cells is that while they have shown remarkable results for hematological cancers, solid tumors are more difficult to treat and have a highly suppressive TME (173, 229). Nevertheless, advances in lymphocyte engineering have allowed for the conceptualization of a number of novel types of CAR T cells which can be switched on conditionally, or lack checkpoint molecules to prevent suppression. These novel CARs may have high utility for solid cancers and have been reviewed expertly elsewhere (229). Similarly, a novel type of molecule that has recently gained attention is a bispecific antibody construct that can bind to CD3 thus activating T cells as well as, a tumor antigen and is termed a bispecific T cell engager (BiTE®) (234). The anti-CD19 BiTE® blinatumomab was approved by the FDA after showing activity in acute lymphoblastic leukemia but to date, none of the tested BiTE® constructs tested in solid tumors have exhibited noteworthy antitumor responses (234). Novel developments in the field of genomic sequencing as well as T cell engineering have allowed for the conceptualization of highly personalized ACT treatment for cancer. Nevertheless, as discussed previously, without breakthroughs in *ex vivo* cell handling and automation, this therapy will remain highly costly and be limited to a few centers of excellence around the world.

Cancer Vaccines

Vaccination for infectious disease represents a landmark of human medical achievement. Cancer vaccines seek to activate the immune system, in particular the T cells, to attack the tumor with the presentation of the tumor antigen in combination with an adjuvant (176). The vaccines may be univalent incorporating a single target antigen or polyvalent, consisting of allogeneic whole cells, or autologous tumor lysates (48). To date, none of the vaccine combinations tested in established tumors have shown the same efficacy as checkpoint blockade or ACT (165, 176). A number of studies have shown modest increases in clinical activity such as the study by Schwartzentruber et al. in 2011 that showed that patients with advanced melanoma treated with IL-2 and a gp100 peptide vaccine fared better than patients treated with IL-2 alone (median OS 18 versus 11 months, respectively) (48, 235). Nevertheless, cancer vaccination for solid tumors becomes particularly challenging due to the immunosuppressive TME and a constantly evolving tumor geared toward immune escape (165). In the past 30 years, as research unveiled the crucial role of DC in antigen processing and T cell activation, DC-targeted vaccines also became a major focus of cancer vaccination research (161). DC are considered

to be ideal tools for inducing effective anticancer immunity due to their central role in antigen presentation and their ability to produce crucial effector cytokines (174, 236). The use of DC as anticancer vaccines has been comprehensively reviewed elsewhere (133, 145, 174, 237). Generally, this approach involves the generation of DC from isolated patient PBMC, which are then loaded with antigen and reinfused into the patient (161). Clinically a widely accepted DC maturation protocol involves the use of a cocktail containing TNFα, IL-1β, IL-6, and PGE2, resulting in the upregulation of MHC class I and II and costimulatory molecules (133). Other approaches in the clinic have used mixtures of prophylactic vaccines (which contain TLR agonists) containing Bacillus Calmette–Guerin (BCG)-SSI, Influvac, and Typhim (133, 238). DC maturation can also be induced by targeting the costimulatory receptor CD40 with CD40L (which is expressed by a range of immune cells but its most functionally important expression is on activated T cells *in vivo*) or anti-CD40 mAbs, resulting in the upregulation of costimulatory molecules and production of IL-12 (133, 237, 239). Currently, there is no gold standard in terms of maturation cocktails for DC and novel combinations continue to be tested both preclinically and in clinical trials (174). GVAX® (Cell Genesys, San Francisco, CA, USA) are a cell product composed of irradiated autologous or allogeneic, tumor cells engineered to produce GM-CSF (240). GVAX® vaccines were shown to elicit antitumor immune responses in a number of early clinical studies (241). However, a phase III trial using allogeneic GVAX® in prostate cancer observed that this approach was not superior to current treatments (241). In melanoma, the GVAX® approach has not shown significant clinical activity including a recent study by Lipson et al. that demonstrated that although melanoma GVAX® was safely tolerated, it did not result in markedly increased anti-melanoma responses in peripheral blood T cells (175, 241). These early and currently ongoing studies demonstrate the difficulty of using cell-based approaches for cancer vaccination. Currently, Sipuleucel-T (Provenge®) is the only cell-based vaccine to be approved by the FDA for its observed clinically significant but modest increases in the OS of patients with prostate cancer (174). No such vaccine has yet received FDA approval for melanoma (161). In 2013, Carreno et al. reported the use of an autologous CD40L/IFNγ-matured DC vaccine pulsed with gp100-derived peptides and capable of producing IL-12 (242). In six out seven patients, this treatment successfully induced immune responses with three out of the six responding patients exhibiting tumor remissions (242). Despite these encouraging results, a number of concerns with cancer vaccination still exist, in particular with the choice of target antigen as tumors continue to continuously evade the immune response while novel mutated epitopes may not be sufficient for inducing potent antitumor T cell responses (161). Thus, there has been a significant clinical interest in the use of oncolytic viral vaccines for directly inducing cell death in tumors (48, 161). This approach attempts to harness the specificity of some oncolytic viruses for tumor cells as well as the induction of tumor cytolysis as an immune activating stimulus against non-infected tumor cells (177, 161). The first viral product to receive FDA approval is talimogene laherparepvec (T-VEC) which is a construct derived from herpes simplex virus 1 with deleted ICP34.5 and ICP47 genes and coding for human GM-CSF (177). In 2015, T-VEC was the first virotherapy that showed durable antitumor responses in patients with melanoma (178). Over 400 patients were treated with intralesional T-VEC or subcutaneous GM-CSF, and median OS was demonstrably higher in the T-VEC group versus the GM-CSF group (23 versus 19 months, respectively) (178). Moreover, the durable response rates and overall response rates were also higher in the T-VEC group than in the GM-CSF group with very limited toxicities associated with T-VEC treatment (178). As a result of these findings, the field of cancer vaccine research has been energized, and currently trials are underway to examine potential combination approaches using ICB in combination with oncolytic vaccine regimens to induce a long-lasting antitumor immune response (39, 161). The major limitation of the T-VEC approach is that it was found to be more effective in patients with less advanced (stage III and locally metastatic) melanoma than in patients with visceral metastatic disease (178, 161). Thus, in patients with established and advanced tumors, cancer vaccination approaches at best provide part of the solution for complete cure. With the complex immunoregulatory pathways that are established in advanced tumors, it may be difficult to achieve continued DC stimulation and activation through vaccines. Thus, a number of studies have begun to investigate the targeting of DC *in vivo* as crucial for the success for future immunotherapies (133). The success of T cell checkpoint therapy has already demonstrated the utility of treatments that mediate *in vivo* activation of antitumor immunity. Although a number of other cell types such as NK cells and MDSC have recently gained interest as targetable populations, DC remain a primary cell of interest for *in vivo* targeted immunotherapy due to their crucial roles as APC and in cytokine production (237, 243, 244).

Nanoparticles as Multifunctional Immunotherapeutics

The past two decades have witnessed significant advances in our understanding of tumor immunology and the development of immunotherapeutic drugs (56, 163). In parallel, improvements in the field of nanomedicine provides us with a number of opportunities that can be used in combination with modern immunotherapies to enhance their antitumor efficacy (245–248). The primary advantage to NP is the supreme versatility in their design as their size, shape, constituent biomaterials, and surface modifications can be tailored for specific uses in tumor immunotherapy (**Figure 3**) (245, 247). Liposomes are self-assembling nanosized vesicles comprised of phospholipids and cholesterol arranged in one or more lipid bilayers enclosing an aqueous core (246, 249). Liposome-encapsulated drugs have been demonstrated to have reduced systemic toxicity profiles owing to improved pharmacokinetics and biodistribution (247, 249). Liposomal doxorubicin (Doxil) first received FDA approval in 1995, and even though it did not enhance OS, it is associated with improved toxicity profiles (247). This is of particular use for immunotherapy as many powerful adjuvants such as IL-2 and IFN-α have serious toxic side effects (161). In 2012, Park et al.

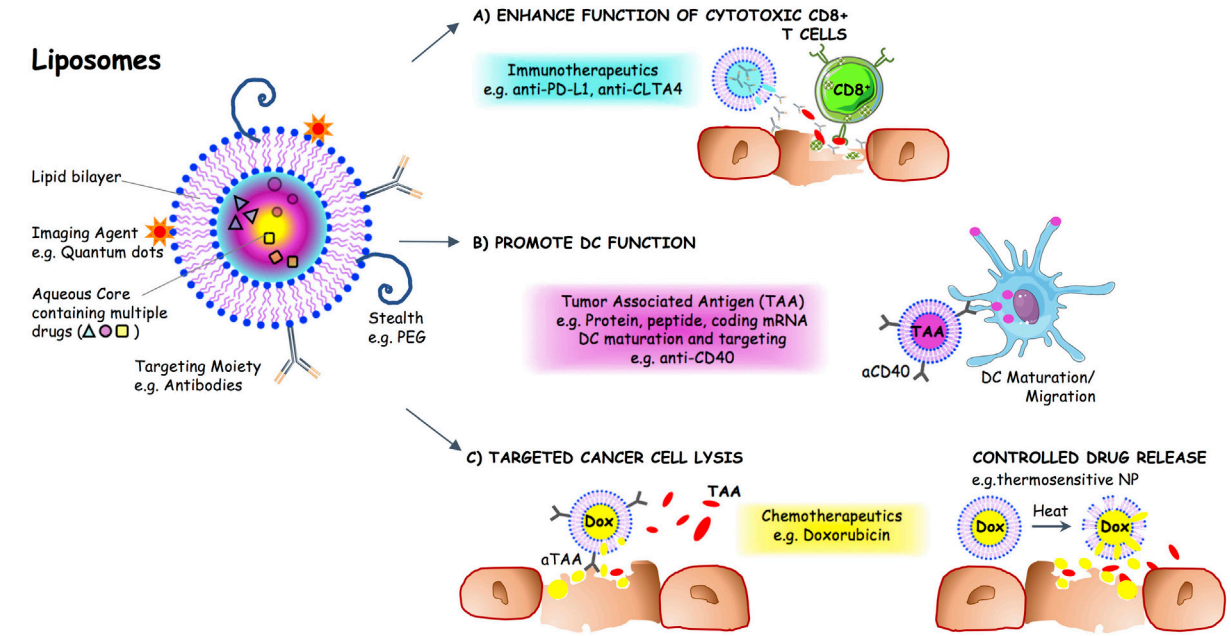

FIGURE 3 | Multifunctional nanoparticles (NP) in cancer treatment. NP can be tailored to specific applications in tumor immunotherapy using versatile designs of various sizes, constituent biomaterials, and surface modifications. The surface of NP can be functionalized with specific polymers and antibodies to increase their targeting to certain types of cells. Liposomes are self-assembling nanosized vesicles comprised of phospholipids and cholesterol arranged in one or more lipid bilayers enclosing an aqueous core. NP such as liposomes can be used as platforms for the simultaneous delivery of multiple agents, such as **(A)** immunotherapeutics, e.g., anti-PD-L1 and anti-cytotoxic T lymphocyte antigen-4 (CTLA-4), to enhance the function of tumor-specific effector T cells; **(B)** tumor-associated antigens (TAA) and adjuvant targeted to dendritic cells (DC) to promote their function; **(C)** chemotherapeutics and targeted release thereof, for instance, using thermosensitive NP, to promote cancer cell death.

demonstrated the utility of a biodegradable liposome and solid polymer hybrid gel as a dual delivery platform for IL-2 as well as an inhibitor of the immunoregulatory cytokine TGF-β (250). Treatment with this platform showed no significant toxicity in treated animals and more importantly delayed tumor growth was mediated via increased intratumoral NK and $CD8^+$ T cell infiltration (250). Thus, NP can not only deliver drugs but also serve as platforms for simultaneous delivery of multiple agents. In the context of immunotherapy, NP can deliver tumor antigens, nucleic acids, and adjuvants (246, 248). There has also been research in the field of artificial APC NP platforms that present antigen loaded MHC I in combination with antibodies to the T cell costimulatory molecule CD28 (246). Finally, the surfaces of NP can be functionalized with specific polymers and antibodies to increase their targeting to certain types of cells (245). Even without direct targeting, systemically treated NP can accumulate at tumor sites due to "leaky" tumor vasculature (247). Earlier this year, Koshy et al. reported the antitumor potency of liposome-encapsulated cGAMP (251). The authors showed that cationic liposome loaded with cGAMP resulted in passive lung-specific delivery in metastatic B16F10 melanoma lung tumors leading to pronounced antitumor activity and the formation of immune memory (251). Currently, a number of unique immunotherapeutic NP are being investigated in Phase I–III clinical trials (247). However, to date no directly DC-targeted NP formulation has reached clinical trials. As DC play central roles in priming antitumor immunity as well as directly influencing the immune infiltration of T cells into cancer (212), NP targeted to DC warrant inclusion in future combinatorial immunotherapies (252). In 2016, Kranz et al. developed a strategy to deliver RNA-NP to DC in a pilot study with three melanoma patients (105). The RNA encoded for the melanoma antigens NY-ESO-1, MAGE-A3, tyrosinase, and TPTE (transmembrane phosphatase with tensin homology) and resulted in IFNα and antigen-specific T cell responses in all three patients (105). This approach was administered systemically and was not found to be associated with any adverse effects. This study thus opens a new field of DC-targeted, highly potent immunotherapies for cancer. NP are biodegradable, relatively cost-effective (compared with *ex vivo* manipulated cells) (133) and highly multifunctional platforms for enhancing modern immunotherapies or developing independent DC-targeted treatments (247).

SUMMARY

Currently, the field of immunotherapy is one of the most promising avenues of research in the quest to develop long-term broadly acting treatments for cancer (55, 161, 253). The possibilities for synergistic combinations with radiation, chemotherapy, and small molecule targeted treatments have also unveiled countless

possibilities for tailoring individualized therapies in the drive towards "precision medicine" (213, 214, 254). However, evolutionary checkpoints against autoimmunity and the fact that cancer arises from self-tissue presents a particularly challenging landscape for developing multitargeted immunotherapies that are cost-effective, safe, and efficacious. Conceptually, there are four general facets of tumor immunity that must be achieved for successful immunotherapy (253). These are the removal of immunosuppressive cues, the induction of immunogenic cell death in tumors, improved activity of APC and increased T cell effector functions (253). In addition to a comprehensive overview of the immune contexture of a tumor, other host specific factors such as genetics and individual microbiota must be further dissected to determine their interplay with immunotherapeutic agents (255). In recent years, advances in high-throughput techniques such as next-generation sequencing and mass cytometry (CyTOF) have enabled highly detailed phenotyping of cancer (256, 257). However, there is still an unmet need for bioinformatics platforms and deep-learning algorithms that can assist biologists with mining and analyzing such massive datasets (258). Finally, due to the need to finely target various facets of tumor immunology in immunotherapy, NP technology may become indispensable as the delivery vectors and the platforms upon which these multifunctional therapeutics are designed (248).

AUTHOR CONTRIBUTIONS

MS conceptualized the manuscript and oversaw all aspects of its completing including writing, figure design, and literature review. HS and TG contributed equally to this manuscript by performing literature review and writing of the manuscript. RH provided medical expertise in the subject matter during writing of the manuscript and contributed clinical images.

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REVIEW
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Strategies to Improve the Efficacy of Dendritic Cell-Based Immunotherapy for Melanoma

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Melanoma is a highly aggressive form of skin cancer that frequently metastasizes to vital organs, where it is often difficult to treat with traditional therapies such as surgery and radiation. In such cases of metastatic disease, immunotherapy has emerged in recent years as an exciting treatment option for melanoma patients. Despite unprecedented successes with immune therapy in the clinic, many patients still experience disease relapse, and others fail to respond at all, thus highlighting the need to better understand factors that influence the efficacy of antitumor immune responses. At the heart of antitumor immunity are dendritic cells (DCs), an innate population of cells that function as critical regulators of immune tolerance and activation. As such, DCs have the potential to serve as important targets and delivery agents of cancer immunotherapies. Even immunotherapies that do not directly target or employ DCs, such as checkpoint blockade therapy and adoptive cell transfer therapy, are likely to rely on DCs that shape the quality of therapy-associated antitumor immunity. Therefore, understanding factors that regulate the function of tumor-associated DCs is critical for optimizing both current and future immunotherapeutic strategies for treating melanoma. To this end, this review focuses on advances in our understanding of DC function in the context of melanoma, with particular emphasis on (1) the role of immunogenic cell death in eliciting tumor-associated DC activation, (2) immunosuppression of DC function by melanoma-associated factors in the tumor microenvironment, (3) metabolic constraints on the activation of tumor-associated DCs, and (4) the role of the microbiome in shaping the immunogenicity of DCs and the overall quality of anti-melanoma immune responses they mediate. Additionally, this review highlights novel DC-based immunotherapies for melanoma that are emerging from recent progress in each of these areas of investigation, and it discusses current issues and questions that will need to be addressed in future studies aimed at optimizing the function of melanoma-associated DCs and the antitumor immune responses they direct against this cancer.

Keywords: dendritic cell, tumor, cancer immunotherapy, melanoma, immune suppression, immunogenic cell death, immunometabolism, microbiome

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INTRODUCTION

Melanoma is responsible for ~10,000 deaths in the United States and ~55,000 deaths worldwide each year, making it the cause of over 75% of skin cancer-related deaths (1, 2). Importantly, data collected by the SEER Program show that melanoma incidence rates have continually risen the last 40 years (3), and a recent study projects melanoma incidence to continue increasing through at least 2022

(4). In the U.S. alone, annual costs for treatment and productivity losses associated with melanoma are near $3.3 billion (5). These numbers are even more staggering when considering the U.S. ranks only third in melanoma incidence worldwide (6), thus highlighting the need to address melanoma as a global public health concern.

Although it is the least common form of skin cancer, melanoma is by far the most lethal due to its propensity to metastasize to several vital organs, including the brain, lungs, liver, and other visceral organs (7). While surgical removal of primary melanomas is highly successful in eradicating disease prior to metastasis, many melanoma patients are not diagnosed until later stages of malignant disease. In these cases, surgery is often not possible or is largely ineffective (8). Moreover, traditional therapies such as chemotherapy and radiation also exhibit limited efficacy against malignant melanoma and are characterized by variable response rates, lack of durable responses, toxicity, and minimal impact on survival (9, 10). In recent years, important insights into the basic biology of melanoma progression have led to the development of several targeted therapies that have shown promise in the treatment of metastatic melanoma patients. In particular, vemurafenib, trametinib, dabrafenib, and other inhibitors of the BRAF–MEK signaling pathway that is hyperactive in melanoma patients bearing $BRAF^{V600}$ mutations have proven superior to traditional chemotherapy in terms of both antitumor activity and clinical outcome (11–13). Unfortunately, drug resistance to BRAF or MEK inhibitors often develops within the first year of treatment and is accompanied by disease progression in many melanoma patients (14–16). While combination therapy with BRAF–MEK inhibitors delays melanoma progression and improves overall survival as compared to monotherapy, development of multi-drug resistance still leads to disease relapse in many patients (17, 18). A similar story has unfolded with regard to even the most promising immunotherapies for melanoma. Checkpoint blockade therapies with monoclonal antibodies targeting inhibitory receptors such as CTLA-4 and PD-1 on $CD8^+$ T lymphocytes have been developed to override cell intrinsic mechanisms that limit overstimulation of T cells and have dramatically improved both antitumor T cell function and clinical responses in melanoma patients. Both monotherapy and combinatorial approaches with nivolumab (anti-PD-1), pembrolizumab (anti-PD-1), and ipilimumab (anti-CTLA-4) have been promising, with reports of complete and objective responses in as high as 22 and 61% of melanoma patients, respectively (19–26). Despite these successes, though, many melanoma patients do not respond to these therapies, and others often experience disease relapse in as early as the first few months of treatment (27–29). Likewise, adoptive cell transfer (ACT) therapies that employ either naturally occurring tumor-infiltrating lymphocytes or genetically engineered T lymphocytes have produced complete tumor regression in as high as 25% of melanoma patients, though many other patients receive no clinical benefit from these regimens (30, 31). Therefore, while recent advances in the treatment of metastatic melanoma are encouraging, it is critical that we continue to explore strategies that will expand treatment options and optimize clinical outcome for patients with this disease.

Dendritic cells (DCs) have long been appreciated for their roles in the induction and maintenance of antitumor immune responses and are known to be critical regulators of both antitumor immune activation and immune tolerance. This dichotomy is highlighted by the variable outcomes of early trials employing DC-based therapies in melanoma patients. While tumor vaccines targeting host antigen (Ag)-presenting cells *in situ* or utilizing exogenous tumor Ag-loaded DC induced immunogenic responses that correlated with clinical benefits in a modest percentage of patients (32–35), many patients exhibited no clinical response to these therapies, and some immunization maneuvers even led to diminished tumor-specific T cell responses and the induction of immune tolerance, thereby potentially exacerbating disease progression (36, 37). Lessons learned from these first-generation cancer vaccines guided second-generation vaccination strategies that aimed to improve upon previous failures by (1) targeting tumor Ag to particular DC subsets *in situ* or (2) employing maturation cocktails to promote the immunostimulatory activity of exogenously generated monocyte-derived DCs. In addition to pulsing these latter DCs with recombinant synthetic peptides or tumor cell lysates, other approaches for tumor Ag loading onto exogenous DCs were also explored, including RNA/DNA electroporation and fusion of tumor cells to DCs. Details of these approaches have been described more extensively in recent reviews (38–40), and their translation to the clinic is highlighted in a recent Trial Watch (41). In brief, despite the improved immunogenicity of many of these approaches, they have unfortunately not been met with the success of checkpoint blockade and ACT therapies, and objective response rates have rarely exceeded 15%. Nevertheless, significant efforts in recent years have further improved our understanding of factors that regulate DC function in the context of cancer, and insights from this work have suggested novel strategies for improving the immunogenicity of both endogenous and exogenous DC. At the same time, advances in genetic engineering and other approaches that enable the manipulation of DC function are spearheading the translation of this basic research on DC immunobiology into novel clinical applications. Together, these findings have reinvigorated the pursuit of cutting-edge approaches that take advantage of the potential of DC as potent stimulators of robust, targeted antitumor immune responses, offering great promise for the future of DC-based cancer immunotherapies.

NEXT-GENERATION DC-BASED IMMUNOTHERAPY FOR MELANOMA

Although first- and second-generation DC vaccines, as well as other tumor Ag-based vaccines, have not yielded significant clinical benefit in a large percentage of melanoma patients to date, their relatively good safety profiles and ability to induce antitumor immune responses in some patients have encouraged the pursuit of next-generation melanoma vaccines that aim to improve upon the previous limitations of DC-based immunotherapy for this cancer. A major focus of one class of next-generation DC vaccines is the utilization of naturally occurring DC subsets, which differs from the artificial *ex vivo* generation

of monocyte-derived and $CD34^+$ precursor-derived DC that predominated both first- and second-generation DC vaccination protocols. Though large clinical trials are needed to define which DC subsets provide optimal therapeutic efficacy in particular settings, early trials with plasmacytoid DC (pDC) and $CD1c^+$ myeloid DC (mDC) have both shown promise in melanoma patients. Intranodal injection of pDC that had been activated and pulsed with melanocyte differentiation Ag-derived peptides into tumor-free lymph nodes of patients with distant metastatic melanoma-induced Ag-specific $CD8^+$ T cell responses in nearly 50% of patients, and although the sample size was too small to make definitive assessments of clinical efficacy, a comparison of clinical outcomes for these patients versus matched control patients undergoing dacarbazine chemotherapy suggest vaccination benefits for both progression-free survival and overall survival (42). Likewise, immunization of stage IIIc/IV melanoma patients with autologous, peptide-pulsed $CD1c^+$ mDC promoted Ag-specific $CD8^+$ T cell responses in 33% of tested patients and induced long-term progression-free survival (12–35 months) in nearly 30% of patients (43). Other next-generation vaccination approaches currently being explored include immunization with tumor-specific neoantigens (either alone or loaded onto DC) that promote responses against mutated tumor-specific epitopes (44–46) as well as maneuvers that induce local or systemic activation of endogenous, tumor Ag-presenting DC (47, 48). These next-generation DC-based vaccines and the ways in which they might be incorporated as part of combinatorial regimens into the current cancer immunotherapy landscape that is being dominated by checkpoint blockade and ACT therapies have recently been reviewed more thoroughly elsewhere (49). Importantly, optimization of these next-generation approaches going forward will require careful consideration of the many factors that have emerged as regulators of DC function in the context of cancer. In this regard, this review highlights recent advances in our understanding of factors that influence DC function in melanoma immunity, including the immunogenicity of tumor cell death, immunosuppressive networks within the tumor microenvironment, tumor-altered immunometabolism, and microbiome-associated regulation of DC function and DC-mediated antitumor immunity. Additionally, particular focus is given to therapeutic strategies building on this knowledge that aim to improve the quality of next-generation DC-based immunotherapies for the treatment of melanoma.

INDUCTION OF IMMUNOGENIC CELL DEATH (ICD) AS A MEANS OF PROMOTING DC-MEDIATED ANTITUMOR IMMUNITY

ICD and DC Activation

As one of the primary mediators of immune surveillance, DC function as key sentinels that aim to maintain homeostasis within the body, invoking immune tolerance in the steady state and immune activation in times of stress, such as that which occurs during a pathogenic infection. In the steady state, DCs exist as immature, inactivated cells that are highly phagocytic but tolerogenic in nature, expressing low levels of the costimulatory molecules and proinflammatory cytokines/chemokines necessary to invoke immune activation and effector cell recruitment to peripheral tissues. On the other hand, upregulation of these cell surface and soluble immunostimulatory molecules during DC maturation and activation promotes the induction of adaptive immunity capable of eliminating a particular source of Ag (50). While it was originally thought that DC maturation and activation status, and in turn the ability of DC to induce immune tolerance versus activation, was dictated solely by self/non-self discrimination (51), more recently, it has become appreciated that regardless of how self or foreign a source of Ag is, it is the microenvironmental cues within host tissues that are critical in driving the "friend or foe" decision made by DC upon Ag encounter (52). In this way, immature DC that encounter and phagocytose cells dying naturally from normal turnover can remove this cellular debris without risking aberrant autoimmune activation, while those that encounter cells dying from infection or other forms of stress (such as those ultimately imposed on at least some of the cancer cells within a growing tumor) receive "danger signals" that promote their maturation, activation, and ability to stimulate immune responses to combat the source of "danger." In the context of cancer, several of these "danger signals" have now been identified as damage-associated molecular patterns (DAMPs) (53). These include cell surface calreticulin and other endoplasmic reticulum (ER) chaperones exposed following the unfolded protein response, autophagy-mediated or conventional secretion of ATP, interleukin-1β (IL-1β) secretion as a result of inflammasome signaling, release of high-mobility group box 1 (HMGB1), and cell surface exposure/release of annexin A1, though this latter protein has been shown to promote both DC activation (54) and inhibition (55) in different settings, and its role as a DAMP is controversial. Nucleic acids released from dying tumor cells are another well-characterized DAMP that may signal through cytoplasmic sensors such as RIG-I or the TLR7/8/9-MyD88 pathway to stimulate DC. Additionally, their induction of type I IFN secretion by dying tumor cells can also lead to autocrine signals that trigger release of chemokines such as CXCL10 that promote recruitment of immune cell populations to the tumor (53, 56). Ultimately, it is the engagement of these types of DAMPs by pattern recognition receptors on DC that "alerts" these cells to an ICD and in turn promotes their stimulation of immune reactivity against "dangerous" immunogens (**Figure 1**). With this revised understanding of "danger/no danger" discrimination as the key regulator of immune activation, inducers of ICD in cancer have become a major area of investigation because of their potential to promote DC-mediated antitumor immunity.

Chemotherapy-Driven ICD and Its Potential for Activation of Endogenous Tumor-Associated DC

In recent years, a number of anticancer regimens have been investigated for their ability to induce ICD and enhance DC-based cancer immunotherapies (57–61). Interestingly, while it was once thought to be at odds with cancer immunotherapy

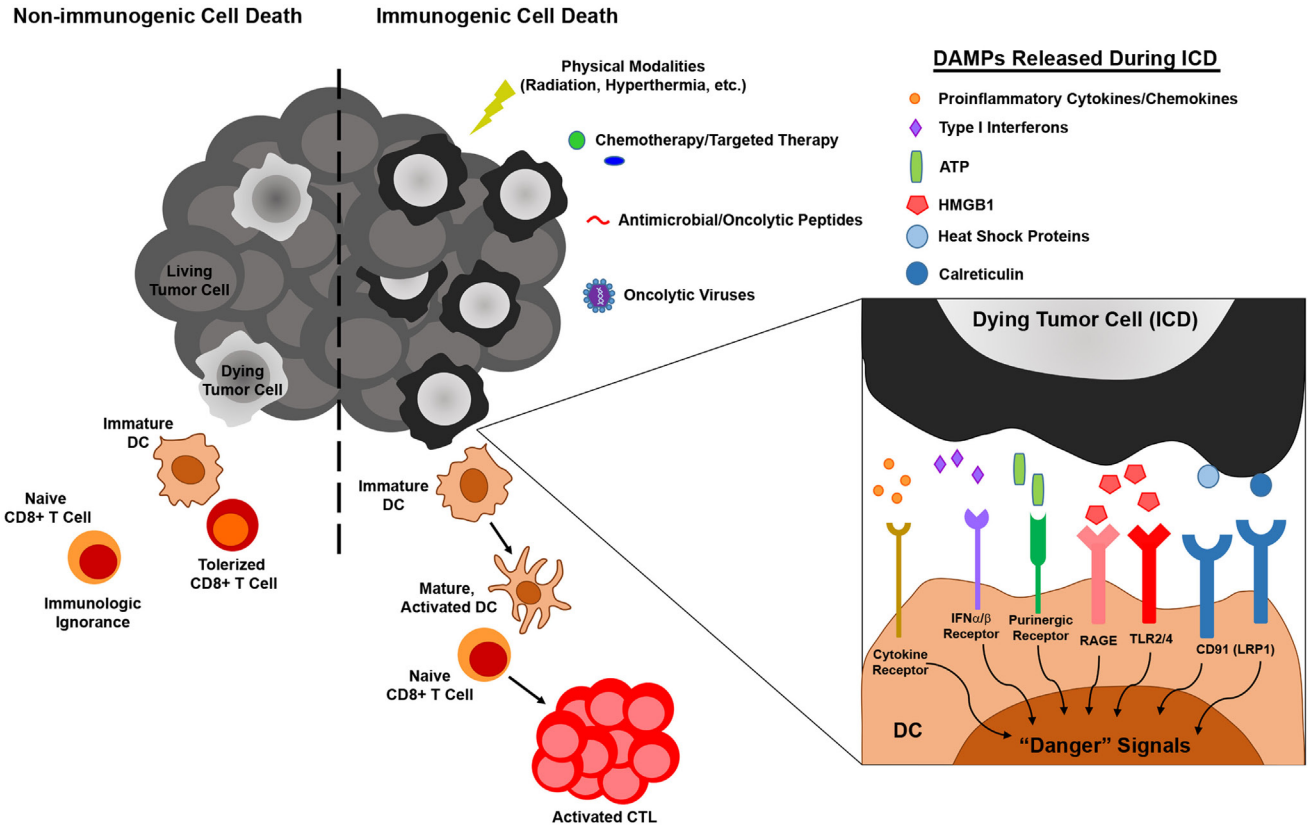

FIGURE 1 | The influence of immunogenic versus non-immunogenic tumor cell death on dendritic cell (DC) maturation/activation and DC-mediated antitumor immunity. Non-immunogenic tumor cell death does not elicit DC maturation or activation, leaving DC in an immature state in which they either (1) fail to "sense" tumor cell death and therefore do not acquire tumor antigen (Ag) for presentation to naïve T lymphocytes or (2) acquire tumor Ag through phagocytosis and induce T cell tolerance. On the other hand, immunogenic tumor cell death, which can be elicited by various physical, chemical, and biological modalities, results in the release of damage-associated molecular patterns (DAMPs) that are recognized by pattern recognition receptors on DC, resulting in the delivery of "danger" signals that promote the maturation and activation of DC capable of stimulating antitumor T cell activation.

because of its non-specific targeting of rapidly dividing cells (which could include not only tumor cells but also lymphocytes engaged in an antitumor immune response), chemotherapy has recently been revisited as a means of promoting ICD of tumor cells. Indeed, a number of chemotherapeutic agents approved for the treatment of various cancers, including doxorubicin, oxaliplatin, mitoxantrone, and others, are now known to induce ICD of some tumor cells (62). Dacarbazine is the only FDA-approved chemotherapeutic agent for the treatment of melanoma, and though its use in isolation has not produced clinical benefits of major significance (63), it has been shown to promote the efficacy of a peptide-based vaccine for melanoma patients by enhancing repertoire diversity of Melan-A-specific CTL (64, 65), suggesting that the benefit of dacarbazine as part of combinatorial therapy may be derived from its induction of melanoma ICD. Likewise, mitoxantrone has been implicated in ICD in an inducible murine model of *Braf*-driven melanoma, where the antitumor effects of this chemotherapeutic were both autophagy- and T lymphocyte-dependent (66). Studies with other chemotherapeutic agents have demonstrated either direct immunogenicity of killed melanoma cells or expression/release of ICD biomarkers by melanoma cells exposed to a particular drug. In the B16-OVA model, the immunogenicity of doxorubicin-induced cell death was shown to be dependent on DC, as depletion of these cells by diphtheria toxin treatment of mice carrying the diphtheria toxin receptor transgene under control of the CD11c promoter prevented the accumulation of OVA_{257}-specific $CD8^+$ T cells that otherwise occurred in the lymph node draining the injection site. Although the OVA Ag in this model is more akin to a completely foreign oncoviral tumor Ag, this same study demonstrated in a humanized model of the B16-F10 murine melanoma cell line that tumor cells treated with doxorubicin and then injected into HLA-A2 transgenic hosts also conferred significant protection against a subsequent challenge with live tumor cells (67). Similarly, $CD8^+$ T cell responses were also elicited against endogenous gp100 Ag in mice immunized with oxilaplatin-treated, but not live, B16-F10 cells (68). Others have also shown that lysates from oxaliplatin-treated B16-F10 melanoma cells were found to be immunogenic, conferring partial protection against subsequent challenge with live tumor cells, and this chemotherapy-driven immunogenicity was associated with markers of ICD that include cell surface calreticulin and

release of ATP and HMGB1 (69). Proinflammatory cytokines/chemokines and cell surface heat shock protein 90 (HSP90) are ICD biomarkers expressed by the human A375 melanoma cell line following treatment with melphalan, an alkylating agent whose toxicity against A375 cells promoted DC maturation *in vitro*. Similar effects in the murine B78 model were also associated with *bona fide* immunogenicity *in vivo*, as vaccination with melphalan-treated tumor cells conferred complete protection against re-challenge with live cells in 40% of mice. Interestingly, this vaccination effect was independent of HSP90 expression and could be augmented by coating of melphalan-treated tumor cells with recombinant calreticulin, which was not otherwise detectable on the cell surface (70). Together, these data highlight (1) the potential for artificial delivery of DAMPs to enhance the immunogenic nature of chemotherapy-killed tumor cells but also (2) a need to better understand the role of specific ICD markers in conferring antitumor immunogenicity. Importantly, it should also be emphasized that the immunogenic potential of many of these chemotherapeutic agents has been evaluated only in prophylactic settings, and in order to achieve clinical translatability it will be necessary going forward to determine whether the immunogenicity of these regimens confers any therapeutic benefit against established tumors.

Although the expression/release of ICD biomarkers often correlates with *bona fide* immunogenicity, as was shown to be the case in many of the aforementioned studies, detection of these markers alone is not sufficient to predict immunogenicity of dying tumor cells. For instance, although mafosfamide treatment induces HMGB1 release from both EG7 lymphoma cells and B16-F10 melanoma, this cyclophosphamide derivative promotes vaccine-verified ICD only in EG7 lymphoma (71). In fact, rather than simply failing to induce immunogenicity in melanoma, cyclophosphamide has actually been suggested to promote immune suppression. Studies in the Ret transgenic melanoma model show that although low-dose cyclophosphamide induced cell surface calreticulin on skin tumor-derived Ret cells and enhanced the *in vitro* maturation of co-cultured DC, this treatment alone did not produce any survival benefit in tumor-bearing animals and even led to an accumulation of myeloid-derived suppressor cells (MDSC) in primary tumors (72). This is in contrast to the adjuvant effect that cyclophosphamide has on a DC vaccine in the MC38 colon carcinoma model, where its contribution to tumor growth inhibition correlates with an increase in cytotoxic effector infiltration of tumors and a decrease in both regulatory T cells (Tregs) and MDSC (73). Such tumor-specific differences in responsiveness to chemotherapeutic agents remain poorly understood and underscore the need to gain new insights into factors that influence tumor cell sensitivity to chemotherapy-driven ICD. Moreover, discrepancies in ICD biomarker expression and genuine ICD following tumor cell exposure to chemotherapy drugs highlight both the importance of vaccination assays as a means of verifying *bona fide* ICD as well as the significance of future studies that are necessary to evaluate the immunologic effects of DAMPs, both individually and in combination, on DC and DC-mediated immune responses so that optimal strategies for promoting robust antitumor immunity can be realized.

Non-Chemotherapeutic Induction of ICD As a Means to Enhance Activation of Endogenous and Exogenous DC

While the aforementioned studies suggest potential utility for chemotherapy-driven ICD in promoting the immunogenicity of endogenous DC, whether this mode of ICD induction can be successful in enhancing the vaccination efficacy of exogenous DC is less clear. Combination therapy with cyclophosphamide and an autologous tumor Ag-pulsed DC vaccine has shown promise in a phase II study enrolling metastatic melanoma patients with progressive disease, but although cyclophosphamide's effect was shown not to be the result of Treg depletion, whether its adjuvant effect was the result of ICD induction is not clear (74). Another recent phase I study has demonstrated that intratumoral injection of IFNα-differentiated unloaded autologous DC 1 day following dacarbazine treatment is associated with induction of tumor-specific $CD8^+$ T cell responses and stabilization of disease in a small cohort of stage IV melanoma patients (75). Despite these hints of success, though, there is concern by many investigators that multiple cycles of chemotherapy are incongruent with the potential immunologic benefits of DC vaccination due to the lymphoablative effects of such drugs. Moreover, chemotherapeutic induction of ICD in tumor cells prior to Ag loading of DC during the production of vaccines has the potential for cytotoxicity against DC and could lead to the unintended administration of residual chemotherapeutics to vaccinated patients (49).

A number of non-chemotherapeutic interventions that overcome these limitations have been investigated for their ability to induce ICD of melanoma. Various antimicrobial/oncolytic peptides have been shown to trigger DAMP release by killed melanoma cells and promote antitumor immune responses (76, 77). Oncolytic virus therapies that take advantage of the tumoricidal potential of measles virus, vaccinia virus, and reovirus have all been shown to induce melanoma ICD as well. Specifically, studies with these oncolytic viruses have shown that infected human melanoma cells or tumor-conditioned media from these cells promote the maturation of mDC *in vitro* (78–80), and Zhang et al. have shown in a murine model that an oncolytic adenovirus co-expressing IL-12 and GM-CSF enhances the immunogenicity and antitumor efficacy of a bone marrow-derived DC (BMDC) vaccine (81). Although ICD in the context of targeted therapy for melanoma has not been thoroughly investigated, one study has shown that vemurafenib can promote cell surface exposure of calreticulin and HSP90 on various human melanoma cell lines. This same study also demonstrated that MEK inhibition could trigger exposure of these ICD biomarkers on the surface of vemurafenib-resistant melanoma cells, and tumor cells pretreated with these targeted drugs were able to promote the maturation of co-cultured DC (82). Based on these findings, it will be of interest going forward to assess how cancer immunization strategies might be coupled with targeted therapy to invoke anti-melanoma immune responses following drug-induced tumor cell death, an outcome that could result in immune-mediated eradication of tumor cells that might otherwise eventually acquire drug resistance. Finally, physical modalities that disrupt tumors, such as radiation, photodynamic therapy (PDT), high

hydrostatic pressure, and hyperthermia, have been investigated for their ability to induce ICD-mediated activation of DC. Many of these approaches have been incorporated into DC vaccination setups and are currently being assessed in clinical trials for prostate cancer, ovarian cancer, and head and neck squamous cell carcinoma (83). However, melanoma resistance to many of these modalities has made their incorporation into combinatorial DC-based therapies a particular challenge. Melanoma's relative resistance to radiotherapy is well-documented (84), and many melanomas are also resistant to PDT as a result of optical interference by melanin in pigmented tumors, the antioxidant effect of melanin, sequestration of photosensitizers in melanosomes, and other mechanisms (85). Nevertheless, interest remains in (1) exploring strategies that might sensitize melanoma cells to these physical modalities and (2) identifying particular patient populations whose melanomas might be more susceptible to these types of physical disruptions. For instance, there is evidence that depigmented melanomas are more susceptible to PDT, meaning that at least a subset of melanoma patients might benefit from PDT/DC-based combination therapies, and interventions that result in even temporary depigmentation of melanomas have the potential to increase the percentage of patients who may benefit from such combinatorial regimens (86). Along with the diverse repertoire of ICD inducers known to be effective against melanoma (**Table 1**), ongoing efforts to refine the use of physical modalities for tumor destruction will increase the array of weapons that exhibit not only direct antitumor activity but also the ability to boost immune reactivity against living melanoma cells, thus doubling the impact of therapy. Importantly, further optimization of therapeutic strategies with these and newly discovered ICD inducers in the future offers promise for enhancing not only naturally generated antitumor immune responses in melanoma patients but also DNA/RNA- and peptide/protein-based melanoma vaccines whose immunogenicity relies on endogenous DC to process and present Ag to tumor-specific T lymphocytes. Moreover, as is already being done with some of the aforementioned inducers of melanoma ICD, investigating how ICD inducers might maximize the immunogenicity of exogenous DC, either through *ex vivo* activation of these cells prior to immunization or through *in vivo* maintenance of their immunogenicity following infusion, will likely improve the quality and outcome of antitumor immune responses achieved by DC vaccines in future melanoma patients.

INTERFERING WITH IMMUNOSUPPRESSIVE NETWORKS THAT IMPAIR THE FUNCTION OF TUMOR-ASSOCIATED DC

Melanoma-Associated Suppression of DC Differentiation

A significant body of evidence now exists demonstrating that tumor cells as well as other immunosuppressive cell populations that accumulate within the tumor microenvironment produce a variety of factors that alter the function of DC (87). In the context of melanoma, such factors have been shown to interfere with the

TABLE 1 | Inducers of immunogenic cell death (ICD) in melanoma.

	Model system	ICD biomarker(s)	*Bona fide* ICD[a]	Reference
Chemotherapies				
Doxorubicin	B16-F10	Not determined	Yes	(67)
Oxilaplatin	B16-F10	Calreticulin, ATP, high-mobility group box 1 (HMGB1)	Yes	(68, 69)
Melphalan	A375	IL-8, CCL2, heat shock protein 90 (HSP90)	Not tested	(70)
	B78	HSP90	Yes	
Lidamycin	B16-F1	Calreticulin	Yes	(207)
R2016 heterocyclic quinone	B16-F10	Calreticulin, HMGB1, HSP60, HSP70, HSP90	Not tested	(208)
Ginsenoside Rg3	B16-F10	Calreticulin, HSP60, HSP70, HSP90	Not tested	(209)
Antimicrobial/oncolytic peptides				
LTX-315	B16-F1	HMGB1	Not tested	(76)
LTX-401	B16-F1	HMGB1, ATP, cytochrome c	Not tested	(77)
Oncolytic viruses				
Measles virus	Primary melanoma cells	IL-6, IL-8	Not tested	(78)
	Mel888, Mel624, MeWO, SkMel28	IL-6, IL-8, type I IFN, HMGB1		
Vaccinia virus	SK29-MEL	HMGB1, calreticulin (strain-dependent)	Not tested	(79)
Reovirus (type 3 Dearing strain)	Mel888, Mel624, MeWO, SkMel28	Proinflammatory cytokines (cell line-dependent)	Not tested	(80)
Targeted therapies				
Vemurafenib	A375, 451-LU, M1617	Calreticulin, HSP90	Not tested	(82)
U0126 (MEK inhibitor)	A375, 451-LU, M1617	Calreticulin, HSP90	Not tested	(82)
Bortezomib	A375, 451-LU, M1617	Calreticulin, HSP90	Not tested	(82)
Physical modalities				
Hyperthermia ± ionizing radiation	B16-F10	HMGB1, HSP70	Not tested	(210)

[a]*Bona fide ICD can be verified only in murine tumor models, as it is determined by vaccination assays in which tumor cells killed by a particular agent in vitro are tested for their ability to invoke protective immunity against subsequent re-challenge with live tumor cells. ICD biomarkers are indicated only if detected in a context appropriate for ICD (i.e., cell surface calreticulin and heat shock proteins, secreted ATP and HMGB1, etc.).*

development of DC from hematopoietic precursors, to suppress the maturation and activation of already-differentiated DC, and to induce the differentiation of regulatory DC with tumor-promoting functions. In terms of DC development, hyperactivation of the STAT3 and MAPK signaling pathways has been observed in progenitors that fail to differentiate into DC in the presence of melanoma-derived factors (88), and several groups have identified specific inhibitors contributing to melanoma-associated suppression of DC differentiation. Cyclooxygenase (COX)-derived prostanoids in primary melanoma-conditioned media have been shown to inhibit the differentiation of DC from both monocytes and $CD34^+$ progenitors (89). Likewise, gangliosides from human melanoma tumors impair the differentiation of DC from monocytic precursors and promote the apoptosis of monocyte-derived DC (90). A similar apoptotic effect of melanoma-derived gangliosides has also been observed on epidermal Langerhans cells (91). In addition to inhibiting the generation and viable maintenance of distinct DC subtypes, melanoma-derived factors can also skew the differentiation of DC precursors toward other myeloid populations with immunosuppressive function. For instance, TGFβ1 in B16-F10 tumor-conditioned media is capable of preventing DC differentiation from bone marrow precursors and instead drives MDSC differentiation through upregulation of the Id1 transcriptional regulator (92). COX-2-driven prostaglandin E2 (PGE_2) in supernatants of cultured human melanoma cell lines can also promote MDSC differentiation from monocytes (93). Alternatively, macrophages capable of suppressing $CD4^+$ and $CD8^+$ T cell proliferation have been differentiated from monocytes cultured in conditioned media from both metastatic and non-metastatic human melanoma cell lines (94), and IL-10, which can be secreted at high levels by melanomas (95), has been shown to promote the trans-differentiation of monocyte-derived DC into tolerogenic $CD14^+$ $BDCA3^+$ macrophage-like cells similar to those known to be enriched in melanoma metastases (96). As immunosuppressive M2-like tumor-associated macrophages often accumulate in melanoma-bearing hosts (97–99), it is interesting to speculate that these cells may arise from an influence of tumor-derived factors on the differentiation of DC *in vivo* as well. Taken together, these influences of melanoma-derived factors on DC differentiation cannot only interfere with Ag presentation and the induction of anti-melanoma immune responses, but they can also lead to active suppression of such immune responses against melanoma.

Melanoma-Associated Suppression of DC Maturation and Activation

In addition to its influence on the differentiation of DC, melanoma has also been shown to modulate the maturation/activation of already-differentiated DC as well. Importantly, although the presence of mature DC within tumors and tumor-draining lymph nodes is a positive prognostic factor in melanoma patients, immature DC are often enriched in both melanoma lesions and tumor-draining lymph nodes of hosts with progressive disease (100–104), thus highlighting the significance of DC maturation status as a key determinant of the immunologic control of melanoma progression. Immune dysfunction stemming from melanoma-associated effects on DC maturation and activation may result from defects in Ag processing and presentation (103, 105, 106) as well as diminished expression of costimulatory molecules and immunostimulatory cytokines, such as IL-12 (107–109). While an immature phenotype of tumor-associated DC may reflect a simple failure of tumor cells to support DC maturation and activation, active regulation of these processes by melanoma-derived factors has also been documented by several investigators. We have shown that tumor-conditioned media from murine melanoma cell lines suppresses costimulatory molecule expression and alters cytokine/chemokine expression profiles of multiple LPS-treated DC lines (110, 111), and our recent work has extended these observations to tissue-resident DC as well (99). This latter study has shown that the extent to which DC function is altered by melanoma-derived factors is tumor-dependent, such that LPS-induced costimulatory molecule expression on splenic DC-stimulated *ex vivo* as well as on lung tissue-resident DC in mice harboring melanoma lung metastases is suppressed by the rapidly progressing B16-F1 melanoma but not the poorly tumorigenic D5.1G4 melanoma. Moreover, we found that alterations to cytokine/chemokine expression profiles by DC in these systems also correlated with melanoma tumorigenicity and were partially driven by tumor-derived TGFβ1 and VEGF-A. Others have reported that immature tumor-infiltrating DC isolated from B16-F0 tumors are refractory to *ex vivo* stimulation with a cocktail of maturation stimuli but can be induced to undergo maturation following stimulation in the presence of an anti-IL-10R neutralizing antibody (112). Recently, Zelenay et al. employed CRISPR-Cas9 gene editing technology to demonstrate that COX-derived PGE_2 in a $BRAF^{V600E}$ melanoma cell line also suppresses costimulatory molecule expression on $CD103^+$ and CD103−, $CD11b^+$ tumor-infiltrating DC as well as IL-12p40 expression by the $CD103^+$ DC subset (113). In addition to these studies that have elucidated roles for extrinsic tumor-derived factors in the regulation of DC maturation and activation, studies from others have provided insights into dysregulated signaling pathways within tumor-associated DC that impact these processes as well. Upregulation of β-catenin, which has been reported in DC that mature but that fail to fully activate and secrete proinflammatory cytokines (114), has been observed both in DC from lymph nodes draining B16-F10 tumors and in splenic DC cultured with B16-F10-conditioned media, and its induction in tumor-associated DC suppresses their ability to cross-prime $CD8^+$ T cells (115). Similarly, impaired DC activation as measured by IL-12 secretion has been associated with hyperactivation of both the STAT3 and MAPK signaling pathways in monocyte-derived DC exposed to conditioned media or tumor lysates from human melanomas (108, 116). Most recently, upregulation of the microRNA miR148-a in tumor-associated DC was shown to impair TLR-mediated maturation by suppressing expression of the DNA methyltransferase DNMT1, which in turn led to hypomethylation of the *Socs1* gene and upregulation of the SOCS1 TLR signaling suppressor (117).

Melanoma-Associated Induction of Regulatory DC Function

Beyond limitations on the Ag processing/presentation and maturation/activation capacity of DC that can preclude induction of antitumor immunity and lead to tumor immune tolerance,

respectively, melanoma-derived factors have also been shown to trigger development of regulatory DC with various tumor-promoting functions. Such DCs have been shown to contribute to tumor angiogenesis (118), the development and recruitment of immunosuppressive Tregs (119–121), and the direct suppression of $CD4^+$ and $CD8^+$ T cells (122, 123). Importantly, several studies have now provided mechanistic insights into both the induction of regulatory DC and the tumor-supporting activities mediated by these cells. One study has reported upregulation of the PD-L1 co-inhibitor that dampens $CD8^+$ T cell effector function on tumor-infiltrating DC in the B16-F10 model (124). Another study has shown that melanoma-derived IL-10 and other unidentified factors contribute to an IL-12^{low}, IL-10^{high} phenotype in monocyte-derived DC capable of inducing $CD4^+$ $CD25^+$ $FOXP3^+$ Treg development (125), and tumor-derived IL-6, VEGF, and TGFβ1 have all been implicated in the induction of IL-12^{low}, IL-10^{high} DC in the spontaneous Ret murine melanoma model (126). Differentiation of IL-10-producing regulatory DC has also been shown to be driven by autocrine IL-6/IL-10 signaling through STAT3 in DC, which is initiated by melanoma-derived factors that activate the TLR2 signaling pathway in these cells (127). Additionally, Treg expansion in melanoma can also be driven by TGFβ1-producing regulatory DC (128). Still others have found that regulatory DCs produce enzymes that diminish the availability of metabolites crucial for T cell activation, thereby inducing metabolic suppression of anti-melanoma immunity. In particular, mDC that were imprinted by ER stress in melanoma cells suppressed $CD8^+$ T cell proliferation *via* secretion of the arginine-depleting enzyme arginase I (123), and melanoma-educated regulatory DCs have also been found to suppress $CD4^+$ T cell proliferation in an arginase-dependent manner (122). Likewise, tryptophan catabolism by indoleamine 2,3-dioxygenase (IDO)-producing regulatory pDC recovered from melanoma-draining lymph nodes is associated both with suppression of $CD8^+$ T cells (129) and with activation of $CD4^+$ Tregs (130). In addition to this IDO-mediated regulation of anti-melanoma immunity, regulatory pDC have also been shown to drive T_H2 and Treg differentiation of $CD4^+$ T cells through cell–cell interactions *via* OX40L and ICOSL, respectively (131).

Strategies to Overcome Melanoma-Associated Dysregulation of DC Function

While the previously described studies highlight diverse mechanisms by which melanoma may subvert DC-mediated antitumor immunity, insights into melanoma-altered DC function have suggested novel strategies for improving DC-based immunotherapies for this cancer (**Figure 2**). To overcome the paucity and poorly immunogenic nature of DC within melanoma lesions, strategies to increase tumor infiltration by DC and promote their activation *in situ* have shown promise in murine melanoma models. Salmon et al. recently demonstrated that systemic administration of Flt3L expanded and mobilized $CD103^+$ DC progenitors from the bone marrow and led to the accumulation of immature $CD103^+$ DC within tumor masses, and subsequent injection of polyI:C intratumorally induced local maturation of these cells and enhanced their ability to recruit and activate melanoma-specific effector $CD8^+$ T cells, leading to tumor regression (47). Similar findings were recently reported by Sánchez-Paulete et al., who demonstrated that Flt3L-mobilized Batf3-dependent DC activated by poly-ICLC synergized with anti-CD137 and anti-PD-1 monoclonal antibody therapy to promote Ag-specific $CD8^+$ T cell cross-priming and tumor control (132). Likewise, Tzeng et al. found that administration of IFNα (as well as other DC maturation stimuli) after treatment of melanoma-bearing mice with a combination therapy that mediates tumor Ag release enhanced the cross-presentation and cross-priming activities of $CD8\alpha^+$ DC in tumor-draining lymph nodes (133). Importantly, although this maneuver led to complete regression of established tumors in a large percentage of mice, minimal benefit was observed when IFNα was administered either before or concomitantly with combination therapy, as the loss of phagocytic capacity that accompanied $CD8\alpha^+$ DC maturation at these early times limited the ability of these cells to acquire tumor Ag later released as a result of therapy. These data thus highlight the importance of treatment schedule and the temporal programming of DC maturation/activation in combinatorial approaches that rely on endogenous DC to trigger therapy-associated antitumor immune responses. Early clinical studies demonstrating that it is also possible to directly manipulate the frequency and maturation status of endogenous DC in melanoma patients have also reinforced the need for optimizing strategies to maximize the immunogenicity of these cells. For instance, local administration of a mix of CpG-B and GM-CSF at the site of primary melanoma excision resulted in the maturation of both pDC and conventional DC as well as an increase in the frequency of cross-presenting $BDCA3^+$ $CD141^+$ DC in sentinel lymph nodes, and this approach enhanced the frequency of melanoma Ag-specific $CD8^+$ T cells in these nodes and reduced the frequency of lymph node metastasis (134, 135). At the same time, though, this approach also enhanced the suppressive activity of $CD4^+$ Tregs in sentinel lymph nodes, suggesting that further optimization of this regimen may enable more robust antitumor immunity and even better clinical results. The identification of optimal DC stimulation cocktails and the implementation of combinatorial regimens that offset the deleterious activities of *in situ*-stimulated DC are therefore critical areas of investigation that may drive the development of more efficacious anti-melanoma immune therapies in the future. Moreover, advances in targeted delivery of therapeutics to endogenous DC, such as those that have already been achieved with IDO siRNA-encapsulated mannosed liposomes (136) and polypeptide micelle-based nanoparticles incorporating an miRNA148-a inhibitor (117), will enable selective reprogramming of melanoma-associated DC into potent stimulators of antitumor immune responses and likely improve the outcome of immunotherapy for melanoma patients going forward.

In contrast to strategies aimed at improving the immunogenicity of endogenous melanoma-associated DC, approaches to enhance the immunostimulatory capacity of exogenous DC have also improved the efficacy of many melanoma vaccines. For example, strategies that provide immune stimulating support for exogenous DC, such as the introduction of IL-6 or IL-21 transgenes into BMDC (137, 138) or the co-administration of oncolytic adenovirus engineered to express immune stimulators

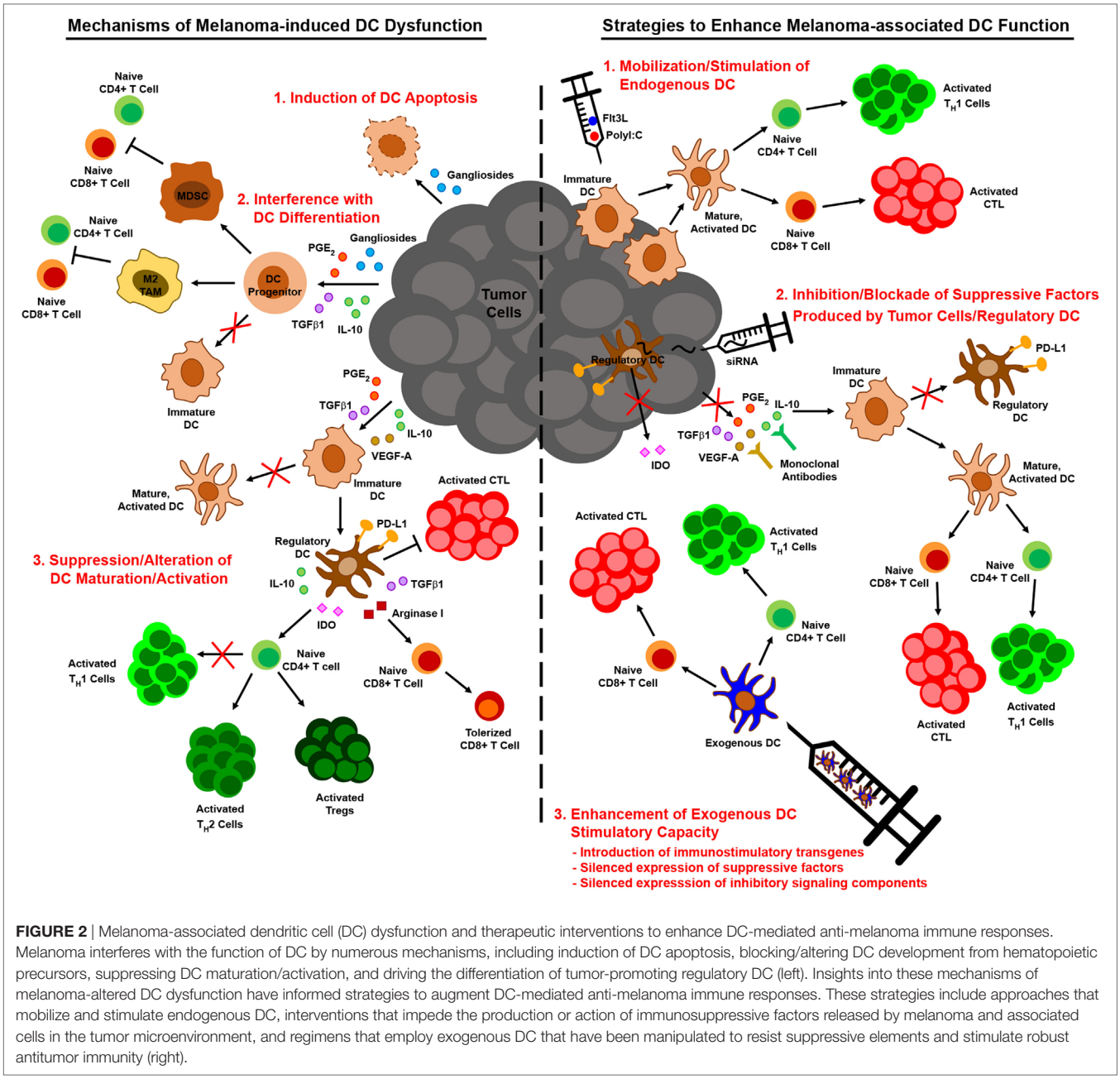

FIGURE 2 | Melanoma-associated dendritic cell (DC) dysfunction and therapeutic interventions to enhance DC-mediated anti-melanoma immune responses. Melanoma interferes with the function of DC by numerous mechanisms, including induction of DC apoptosis, blocking/altering DC development from hematopoietic precursors, suppressing DC maturation/activation, and driving the differentiation of tumor-promoting regulatory DC (left). Insights into these mechanisms of melanoma-altered DC dysfunction have informed strategies to augment DC-mediated anti-melanoma immune responses. These strategies include approaches that mobilize and stimulate endogenous DC, interventions that impede the production or action of immunosuppressive factors released by melanoma and associated cells in the tumor microenvironment, and regimens that employ exogenous DC that have been manipulated to resist suppressive elements and stimulate robust antitumor immunity (right).

such as IL-12 and GM-CSF (64, 119), have been shown to significantly improve vaccine efficacy, resulting in complete regression of established melanomas in some cases. Combinatorial approaches that aim to neutralize the effects of tumor-derived factors on exogenously administered DC, such as local siRNA-mediated silencing of TGFβ1 at the tumor site (139), have also been effective. Alternatively, manipulation of exogenous DC prior to immunization by gene-silencing approaches can promote the immunostimulatory capacity of these cells in two ways. First, silencing the expression of genes involved in signaling pathways that limit the immunostimulatory function of melanoma-associated DC can prevent their immunosuppression by tumor-derived factors. In this regard, vaccines employing SOCS1-silenced DC improve the control of established B16 melanoma (140, 141), a finding that offers exciting proof-of-principle for this approach and that suggests the silencing of other immunosuppressive signaling molecules often dysregulated in melanoma-altered DC, such as STAT3 and β-catenin, may also improve the antitumor efficacy of DC vaccines for melanoma. Second, silencing the expression of suppressive factors known to be released by melanoma-induced regulatory DC can prevent conversion of exogenous DC from immune activating cells to immunosuppressive ones. Indeed, vaccination of mice with IDO-silenced DC confers partial protection against B16 melanoma (142), and a recent case report has revealed

immunologic and clinical benefits of an IDO-silenced DC vaccine in a melanoma patient (143). Similarly, *in vitro* studies have shown that IL-10-silenced human mDC are better able to elicit CTL activation against an antigenic epitope of MART-1 (144), suggesting that immunization with such DCs might improve antitumor immunity in melanoma patients as well. Altogether, these and related strategies for improving the function of DCs in the context of melanoma offer exciting promise for DC-based immunotherapies designed to overcome melanoma-imposed limitations on these cells and the antitumor immune responses they mediate.

OVERCOMING METABOLIC CONSTRAINTS ON DC FUNCTION WITHIN THE TUMOR MICROENVIRONMENT

Metabolic Reprogramming of DC and Tumor Cells

The emerging role of immunometabolism in the regulation of DC function in recent years has revealed new mechanisms by which tumors may subvert DC-mediated antitumor immunity. Indeed, beyond the aforementioned mechanisms of tumor-associated immunosuppression of DC, metabolic suppression of DC in the tumor microenvironment is now recognized as a significant barrier to DC function, which is controlled by key metabolic pathways regulating the bioenergetic and biosynthetic needs of these cells. While immature DC in the steady state rely on fatty acid oxidation and oxidative phosphorylation (OXPHOS) as their primary modes of metabolism, TLR-stimulated DC undergo a metabolic switch to aerobic glycolysis within minutes of the maturation and activation process (145). This early switch to glycolytic metabolism provides a source of carbon for the pentose phosphate pathway and tricarboxylic acid (TCA) cycle, both of which produce intermediates for fatty acid synthesis needed to support the expansion of membrane mass for the ER and Golgi apparatus, thus allowing DC to meet the demands of protein synthesis, transport, and secretion that are associated with maturation/activation (146). Long-term commitment to glycolytic metabolism in activated DC then fuels ATP production and survival in the face of decreasing mitochondrial metabolism, which results from OXPHOS inhibition by nitric oxide in inflammatory DC (147) and from autocrine type I IFN induction of the HIF1α transcription factor that blocks mitochondrial respiration in conventional DC (148). Interestingly, metabolic suppression of tumor-associated DC is often a consequence of metabolic reprogramming in tumor cells themselves, which are driven by the activation/deactivation of oncogenes/tumor suppressor genes and harsh environmental conditions (such as hypoxia) to switch from OXPHOS to glycolysis as the primary mode of metabolism, in this case to support the energy and biosynthetic demands of rapidly proliferating cells. Indeed, even under normoxic conditions, tumor cells are reprogrammed for a primarily glycolytic-based mode of energy production (aerobic glycolysis, otherwise known as the "Warburg effect" in tumor cells), thus allowing intermediates of the glycolytic pathway to function as important metabolites for macromolecule biosynthesis by mitochondria no longer relied as heavily upon for OXPHOS (149–151). Therefore, as metabolically reprogrammed tumors grow, their increasing demand for glucose consumption contributes to an environment that is metabolically hostile to infiltrating DC and other immune cell populations, with competition for limiting nutrients and accumulation of toxic metabolic byproducts released by tumor cells into the extracellular space both impairing immune system function.

Metabolic Suppression of DC in the Context of Melanoma

In melanoma, metabolic rewiring for glycolysis may be driven by multiple signaling pathways, including BRAF-driven MAPK hyperactivation that negatively regulates OXPHOS (152) and PI3K/AKT/mTOR/HIF1α signaling that positively regulates glycolysis (153). These signaling pathways induce expression of glucose transporters as well as enzymes that favor glycolytic metabolism, such as lactate dehydrogenase A (LDHA) that converts the glycolysis end-product pyruvate into lactic acid, thus diverting pyruvate from utilization in the TCA cycle as fuel for OXPHOS (154). Importantly, depletion of glucose in the tumor microenvironment by melanomas exhibiting high glycolytic activity may impair glycolysis, and in turn ATP production, in tumor-infiltrating DC. Such effects may alter the AMP:ATP ratio in DC and lead to AMP-mediated activation of the nutrient/energy sensor AMPK (155), which is known to promote OXPHOS and suppress mTOR and HIF1α signaling (156–158), thus further contributing to the negative regulation of glycolysis in these cells. Beyond the effects of glucose deprivation in the tumor microenvironment on DC function, buildup of lactic acid in the extracellular space of glycolytically active melanomas can also suppress DC. In this regard, melanoma-derived lactic acid inhibits the differentiation of monocyte-derived DC and suppresses IL-12 production by previously differentiated monocyte-derived DC stimulated with LPS *in vitro* (159). Although the mechanism by which lactic acid influences tumor-associated DC function has yet to be elucidated, there is speculation that altered membrane transport in the lactate-rich tumor microenvironment might contribute to its suppressive effect (160, 161). Because lactate is transported passively by facilitated diffusion through monocarboxylate transporters, high levels of extracellular lactate within the tumor microenvironment might promote import of melanoma-derived lactic acid into DC while at the same time precluding export of lactic acid produced within DC also undergoing aerobic glycolysis, leading to a buildup of lactate within DC that impairs the glycolytic flux necessary to maintain an activated phenotype. Alternatively, lactate was recently shown to inhibit macrophage activation by binding to the GPR81 lactate receptor and suppressing TLR signaling (162), and it is possible that this pathway might also contribute to lactate-associated suppression of DC stimulated by tumor-derived DAMPs. Finally, evidence is emerging that suppression of glycolysis in DC is not merely a consequence of the metabolic limitations imposed by glycolytically active tumor cells, as tumor-derived immunosuppressive cytokines have also been shown to alter DC metabolism. For instance, IL-10 was found to suppress the metabolic switch

to aerobic glycolysis in LPS-stimulated DC by antagonizing TLR ligand-mediated hypophosphorylation of AMPK (145). Similarly, IL-10 is known to promote *Socs3* gene expression (163), and melanoma-associated DC have been found to exhibit SOCS3-mediated inhibition of the M2 pyruvate kinase (PKM2) that catalyzes conversion of phosphoenolpyruvate into pyruvate in the final step of glycolysis (164).

In addition to the key role played by glycolytic metabolism in the activation of DC, the metabolism of fatty acids has also been shown to be an important regulator of DC function. Although lipid synthesis is important for ER and Golgi biogenesis during DC activation, the accumulation of lipids in DC in the context of cancer is often associated with immune dysfunction. In particular, Herber et al. demonstrated that several species of triglycerides accumulate in DC cultured with various tumor explant supernatants, including that of B16-F10 melanoma, and that high lipid content in tumor-associated DC impaired tumor Ag processing and cross-presentation (165). Interestingly, DC cultured with tumor-derived supernatant also exhibited increased expression of the scavenger receptor MSR1, suggesting that the accumulation of lipids in these DCs might arise from tumor-derived factors that promote DC uptake of fatty acids in the form of lipoproteins, as triglycerides are typically not taken up by DC but can be synthesized from lipoprotein precursors within cells. Subsequent studies revealed that lipid accumulation in tumor-associated DC defective in cross-presentation resulted from an increase in polyunsaturated fatty acids, particularly linoleic acid and to a lesser extent arachidonic acid, and that DC isolated from tumor-bearing mice or exposed to tumor explant supernatants *in vitro* exhibited significantly higher levels of oxidized free fatty acids and oxidatively truncated triglycerides (166). Of note, these DC did not exhibit oxidation of phospholipids that would be a major component of ER and Golgi membranes. These data may therefore explain the apparent discrepancy between the need for DC to undergo *de novo* lipogenesis to support ER and Golgi biogenesis during activation and the dysfunction that results from lipid accumulation in the context of tumors, suggesting that it is the nature and oxidation status of the fatty acids accumulating in tumor-associated DC that is detrimental to their function. Indeed, oxidized fatty acids have been shown to inhibit DC maturation through binding and activation of the peroxisome proliferator-activated receptor PPARγ, which promotes fatty acid synthesis and storage (167). Additionally, others have reported that lipid peroxidation by reactive oxygen species within tumor-associated DC yields byproducts that upregulate the ER stress sensor XBP1, which activates genes involved in the biosynthesis and accumulation of triglycerides known to be linked with DC dysfunction (168). Altogether, these studies reveal the complex regulation of lipid metabolism that controls DC function, and they highlight how factors in the tumor microenvironment can alter this process to ultimately promote tumor immune escape.

While alterations to glycolysis and lipid metabolism impair tumor-associated DC function by influencing how major macromolecules necessary for cell survival and activation are utilized, other metabolites that frequently accumulate in the tumor microenvironment are also known to compromise the function of DC and DC-mediated immune responses. Adenosine is a particularly well-characterized metabolite that accumulates in the extracellular space of many tumors, including melanoma (169). Although ATP released from tumor cells may serve as a DAMP to promote DC activation (see Induction of Immunogenic Cell Death (ICD) as a Means of Promoting DC-Mediated Antitumor Immunity), melanoma cells often express on their surface the CD39 and CD73 ectonucleotidases that hydrolyze ATP into adenosine (170–172), thereby leading to its buildup in the tumor microenvironment. In addition to its role in the suppression of T cell signaling (173) and immunosuppressive activity of Tregs (174), adenosine has also been shown to impair DC function. *In vitro* studies with LPS-stimulated human monocyte-derived DCs have shown that adenosine promotes IL-10 secretion while suppressing IL-12 and TNFα secretion as well as the capacity of DC to promote T_H1 differentiation (175). Others have shown that DC differentiated from monocytic precursors in the presence of adenosine acquire several tumor-promoting functions that are dependent on signaling through the A_{2B} adenosine receptor. These pro-tumor functions include increased expression of angiogenic factors, immunosuppressive cytokines, and proteins that disrupt immunometabolism such VEGF, TGFβ, IDO, and arginase 2, among others (176). In the context of melanoma, *in vivo* studies in B16-F10 tumor-bearing mice have shown that adenosine signaling through the A_{2A} adenosine receptor on DC is associated with a slight decrease in MHC II and IL-12 expression and a significant increase in the expression of IL-10 (177). Interestingly, recent studies have shown that adenosine receptor signaling in DC also promotes accumulation of intracellular cAMP (178), suggesting that adenosine may ultimately suppress DC activation by influencing AMPK activity and decreasing glycolytic metabolism in these cells. Finally, whereas melanoma cells are one of the major sources of adenosine in the tumor microenvironment, immunoregulatory metabolites that compromise DC function may also be produced by other cell types known to infiltrate tumors. For instance, arginase I-producing cells such as MDSC produce ornithine as a byproduct of arginine metabolism, and ornithine decarboxylation yields polyamines that enhance IDO-1 expression in DC, thus conditioning these cells for immunosuppressive activity (179). Even melanoma-associated DC themselves can contribute immunosuppressive metabolites to the extracellular milieu of progressive tumors. Specifically, melanoma-induced activation of β-catenin signaling in DC from tumor-draining lymph nodes promotes expression of enzymes involved in vitamin A metabolism, leading to DC secretion of the vitamin A metabolite retinoic acid that in turn promotes differentiation of immunosuppressive Tregs (120). Collectively, these studies highlight the metabolically hostile nature of the tumor microenvironment that must be overcome in order for DC to elicit and maintain effective antitumor immune responses.

Metabolic Interventions to Promote DC Function in the Context of Melanoma

Just as insights into melanoma-associated immune suppression of DC have informed therapeutic strategies to enhance the immunogenicity of these cells, so too have insights into the

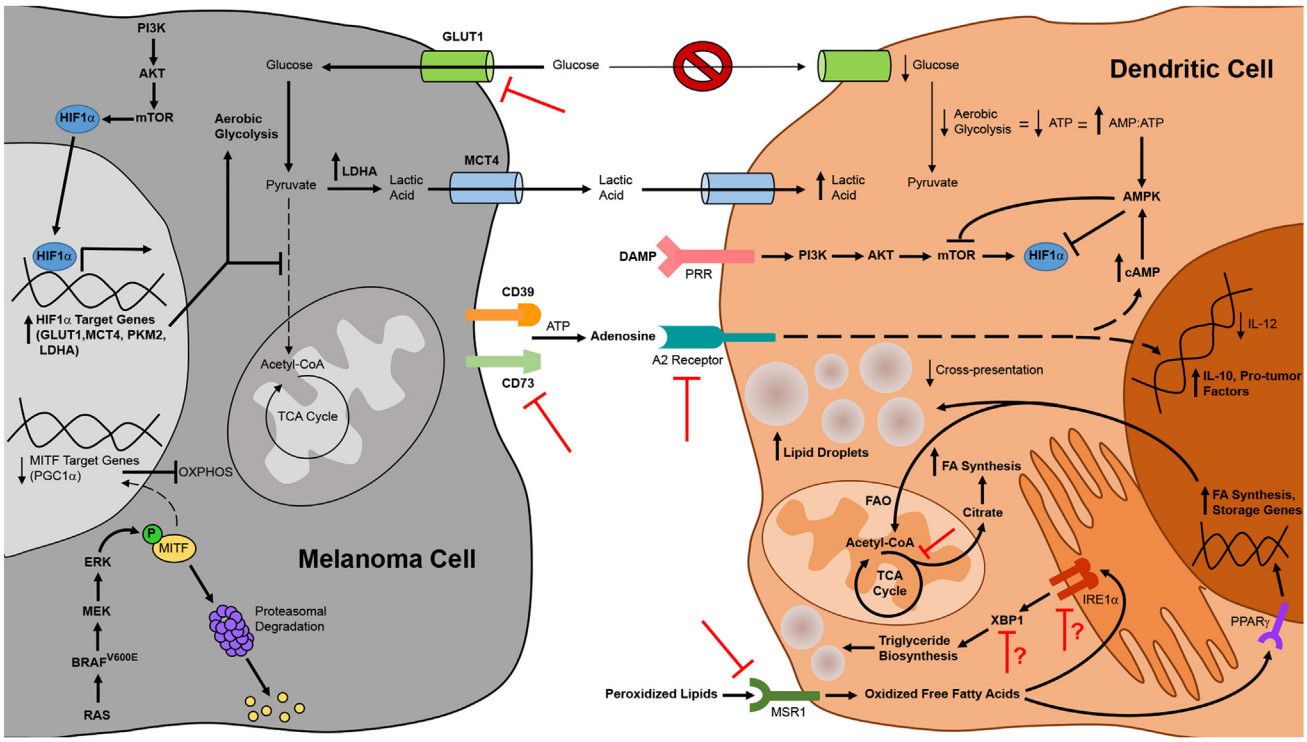

FIGURE 3 | Alterations to tumor cell and dendritic cell (DC) metabolism in the context of melanoma and therapeutic strategies to overcome metabolic suppression of melanoma-associated DC. DC function in the context of melanoma is compromised by constraints on metabolic pathways essential to DC maturation and activation. Metabolic suppression of DC in the tumor microenvironment arises from nutrient depletion and the buildup of toxic waste that results from metabolic rewiring of melanoma cells for aerobic glycolysis. Uptake of peroxidized lipids within the tumor microenvironment also promotes DC dysfunction, leading to an accumulation of oxidized lipids in DC that impairs cross-presentation of tumor antigen. Additionally, tumor cell release of immunosuppressive metabolites such as adenosine that signal through DC inhibit the antitumor function of these cells. Mechanistic insights into these phenomena have identified novel targets for therapies designed to interfere with metabolic pathways in melanoma cells or to prevent tumor-altered metabolism of melanoma-associated DC. Pharmacologic interventions or tissue-specific gene-silencing approaches that target factors upregulated by melanoma cells have direct antitumor effects and are also likely to improve DC function indirectly by creating a more hospitable metabolic microenvironment. Similarly, DC-targeted delivery of therapeutics that prevent uptake of suppressive metabolites or that block metabolic pathways associated with tolerogenicity can improve the immunostimulatory function of endogenous DC, and manipulation of exogenous DC to resist the induction of metabolic suppression can improve the efficacy of DC vaccines. Bold arrows and type designate metabolic pathways, metabolites, intermediates, and processes that are elevated in melanoma cells and tumor-altered DC. Arrows and type not in bold represent those that are downregulated in these cells. Red inhibition symbols highlight proteins and metabolic pathways that have been successfully targeted in melanoma cells and DC in preclinical studies, as described in the text. Red inhibition symbols with red question marks indicate targets that have been associated with both immune activating and immune suppressing functions in different models and whose inhibition may therefore be appropriate only in certain contexts, as is discussed in more detail in the text.

metabolic suppression of melanoma-associated DC (**Figure 3**). To overcome the immune dampening effects of retinoic acid signaling, a retinoic acid receptor α antagonist has been used to enhance the efficacy of a peptide-pulsed DC vaccine against B16 melanoma. In addition to enhancing DC production of IL-12 and lowering DC production of TGFβ and IL-10, this antagonist reduced the number of FOXP3$^+$ IL-10$^+$ Tregs that infiltrated tumors (180). Pharmacologic inhibition of the β-catenin/TCF pathway that promotes melanoma-associated DC production of retinoic acid has also been shown to reduce the expression of vitamin A-metabolizing genes in DC isolated from tumor-draining lymph nodes, and the antitumor activity associated with this inhibition correlated with reduced Treg and increased effector CD8$^+$ T cell infiltration of subcutaneous melanomas (120). Likewise, inhibition of adenosine in the tumor microenvironment may be approached in a number of ways to prevent its deleterious effects on DC function. Pharmacological antagonists of the A_{2B} receptor block the effects of adenosine on DC differentiation *in vitro*, and DC from both A_{2A} and A_{2B} receptor knockout mice are resistant to the suppressive effects of adenosine (176, 177). Therefore, neutralization of adenosine signaling in DC *via* pharmacologic agents or gene-silencing approaches that knock down expression of adenosine receptors on either endogenous or exogenous DC might improve the antitumor immunogenicity of these cells. Alternatively, strategies that interfere with the CD73 ectonucleotidase on melanoma cells have already been shown to improve antitumor immunity in preclinical models (169, 181), and this outcome is likely due to a reduction in the immunoregulatory effects of adenosine on multiple immune cell populations, including DC.

In addition to overcoming the suppressive effects of extracellular metabolites on DC in the tumor microenvironment, maneuvers that interfere with the metabolism of macromolecules in melanoma cells and/or DC may also restore metabolic and immune function in tumor-associated DC. Pharmacologic regulation of lipid levels in DC using an inhibitor of acetyl-CoA carboxylase that blocks fatty acid synthesis improved the antitumor efficacy of a peptide vaccine against B16-F10 melanoma (165). It is also possible to regulate lipid levels in DC by targeting the MSR1 scavenger receptor that promotes lipid uptake or the IRE1α/XBP1 pathway that triggers triglyceride synthesis in tumor-associated DC. To this point, immunization of tumor-bearing mice with MSR1 gene-silenced BMDC improved vaccine-induced $CD8^+$ T cell responses against multiple melanoma antigens and enhanced immunologic control of established B16 melanomas in both subcutaneous and lung metastasis models (182). Likewise, targeted delivery of nanoparticles encapsulating siRNA has been used to silence in tumor-associated DC the expression of either XBP1 or the IRE1α endoribonuclease that cleaves *Xbp1* mRNA into a form that encodes functional protein during ER stress. In a murine model of ovarian cancer, this approach reduced triglyceride levels in tumor-associated DC, augmented the activation of tumor Ag-specific T cells, and improved tumor immune control and overall survival of tumor-bearing mice (168). As triglycerides are also known to accumulate in dysfunctional melanoma-associated DC (165), silencing of IRE1α or XBP1 expression in these cells might also improve DC-mediated immune responses against this cancer in certain contexts. It is worth noting, however, that overexpression of XBP1 in BMDC actually improves DC survival, activation, and T cell stimulatory capacity, leading to enhanced immune control of established B16 melanoma following vaccination (183). Additionally, in an inducible $BRAF^{V600E}$/PTEN-driven melanoma model, a DNA vaccine that promotes XBP1 expression in endogenous DC conferred $CD8^+$ T cell-mediated immune control of small established tumors (184). While tumor microenvironment-specific differences in these ovarian cancer and melanoma models may explain differences in the impact of XBP1 on DC function, it is also possible that these discrepancies are due to differences in the particular DC under study, including the endogenous/exogenous nature of these cells and the extent of ER stress in the DC in which XBP1 is active. It is interesting to speculate that in DC which have not previously been exposed to the hostile tumor microenvironment (i.e., exogenous BMDC) or which are found in the context of early stage tumors and have not yet accumulated the types of fatty acids associated with immune dysfunction, XBP1 promotes DC immunogenicity by protecting these cells against ER stress as they increase protein synthesis during their activation. On the other hand, in endogenous DC that have incorporated significant polyunsaturated fatty acids within the microenvironment of late-stage tumors, XBP1 activation may lead to the generation of oxidized triglycerides that impair DC function. Future studies will be necessary to test this hypothesis and define the parameters under which XBP1 activation versus inactivation in DC is appropriate for optimizing the antitumor activity of these cells.

Finally, glycolytic metabolism in both melanoma cells and DC can be targeted to enhance the immunostimulatory capacity of DC. Recent studies have demonstrated that silencing of the GLUT1 glucose transporter or the CD147 gene product that regulates its expression in melanoma cell lines impairs the growth and metastasis of transplanted tumors (185, 186). In addition to having direct antitumor effects, interfering with glycolysis in melanoma cells may have pro-immune consequences as well, resulting in enhanced DC-mediated antitumor immune responses by increasing glucose availability and decreasing lactic acid concentration in the tumor microenvironment. Therefore, targeting glucose transporters and other enzymes (such as LDHA) that are involved in glycolytic metabolism in melanoma cells is a potentially attractive therapeutic option for the treatment of melanoma. While selective targeting of such therapies specifically to tumor cells might be difficult for some cancer types and could lead to compromised function of DC and other immune cell populations that also rely on glycolysis for induction and maintenance of an activated phenotype, the identification of tissue-specific genes in melanoma (such as those involved in the melanin deposition pathway) opens up the possibility of DNA-based therapies in which siRNA/shRNA expression is driven off of tissue-specific promoters active only in melanoma cells. Such a strategy would overcome issues with selective *delivery* of siRNA/shRNA to tumor cells and instead would rely on selective *activation* of a gene-silencing therapeutic specifically in melanoma cells. Alternatively, it is also possible to minimize the reliance of DC on glycolysis as the sole bioenergetic mode of metabolism during activation. Although signaling through mTOR is associated with a metabolic switch to aerobic glycolysis during DC activation as described above, this switch results less from a preference for glycolytic metabolism and more from a requirement for glycolysis as a means of generating ATP in the face of mitochondrial suppression by reactive oxygen species. Interestingly, it has been reported that inhibition of mTOR in DC does not preclude ATP synthesis in these cells and instead extends the lifespan of activated DC by reducing reactive oxygen species and preserving mitochondrial function, thus allowing flexibility in the metabolic pathways utilized by DC for bioenergetic purposes (187). Indeed, multiple groups have shown that interfering with mTOR function in BMDC enhances vaccine-induced $CD8^+$ T cell responses and immunologic control of established B16 melanomas (188, 189). Together, these data highlight how metabolic interventions may shift the profile of tumor-associated DC from tolerogenic to immunogenic, and they suggest great promise for metabolism-based therapies, either alone or in combination with immunotherapies, in the treatment of melanoma.

MODULATING THE MICROBIOME TO AUGMENT DC-MEDIATED ANTITUMOR IMMUNITY

Gut Microbiome Influences on Natural Antitumor Immunity to Melanoma

As data have emerged demonstrating that the microbiota and dysbiosis play significant roles in both cancer progression and the efficacy of anticancer therapies (190), there has been considerable interest in understanding how the microbiome regulates

the quality of antitumor immune responses. In the context of melanoma, altering the composition of the gut microbiota has been shown to impact both natural and therapy-associated antitumor immunity, and in many cases, regulation of these responses has been associated with microbial influences on DC activation. Antibiotic treatment with a mixture of ampicillin, vancomycin, and neomycin sulfate (which leads to a decreased frequency of gut bacteria belonging to the Bacteroidetes phylum and an increased frequency of gut bacteria belonging to the Firmicutes phylum) prior to B16-F10 challenge enhances tumor outgrowth and is associated with defects in natural antitumor immunity that include a decrease in the frequency of DC among tumor-infiltrating leukocytes and a reduced expression of genes associated with DC maturation and immune activation within tumor tissue (191). Addition of metronidazole to the aforementioned cocktail of antibiotics yields a different type of gut dysbiosis in treated mice (decreased frequency of both Firmicutes and Bacteroidetes phyla members and increased frequency of members of the Proteobacteria phylum), and this alteration also leads to impaired immune control of B16-F10 lung metastases (192). This latter effect results from an antibiotic-associated decrease in IL-17$^+$ γδT cells in the lungs of treated mice. Although the mechanism by which microbial dysbiosis influences γδT cell function remains to be elucidated in this model, the authors speculated that a lack of DC stimulation by PAMPs in antibiotic-treated mice could contribute to the observed decrease in gene expression in the lungs of IL-6 and IL-23, cytokines known to activate IL-17 production by γδT cells.

Gut Microbiome Influences on Therapy-Associated Immunity to Melanoma

The first study to report microbial influences on the outcome of immune therapy for cancer demonstrated that the therapeutic benefit of total body irradiation prior to adoptive T cell transfer arises in part from activation of the innate immune system following radiation-induced damage to the GI tract and subsequent translocation of gut microbiota (*Enterobacter cloacae*, *Escherichia coli*, *Lactobacillus*, and *Bifidobacterium*) to mesenteric lymph nodes (193). In addition to mobilizing the gut microbiome, total body irradiation also led to elevated serum LPS levels and an increase in the absolute number of CD86^{hi} DC in the spleen and lymph nodes, which in turn correlated with enhanced activation of adoptively transferred gp100-specific CD8$^+$ T cells and improved control of established B16-F10 tumors. Interestingly, when mice were administered the broad-spectrum antibiotic ciprofloxacin beginning two days prior to irradiation, microbial translocation to lymph nodes was not observed, nor was any elevation in serum LPS levels. Likewise, the immunologic and antitumor benefits of DC and CD8$^+$ T cell activation were also abrogated following ciprofloxacin depletion of gut microbiota. Additional experiments with the LPS-blocking antibiotic polymyxin B as well as TLR4$^{-/-}$ mice revealed that the therapeutic effect of gut microbiota translocation following total body irradiation resulted from LPS stimulation of innate immune cells that support the activation of adoptively transferred CD8$^+$ T cells. In related work, Iida et al. showed that treating mice with a cocktail of antibiotics (vancomycin, imipenem, and neomycin) abrogated the antitumor effects of combination immunotherapy with anti-IL-10 receptor antibody and intratumoral CpG-oligodeoxynucleotides (ODN) in B16-F10 tumor-bearing mice (194). Though the mechanistic basis for these findings was not further studied in the B16 melanoma model, the authors reported analogous findings in the MC38 colon adenocarcinoma model, where antibiotic treatment decreased both the frequency of TNF-producing tumor-infiltrating DC (and other leukocytes) as well as CD86 expression and IL-12p40 production by tumor-associated DC. Similar results were also observed following combination immunotherapy of germ-free MC38-bearing mice, suggesting that commensal microbes are necessary to prime DC and other myeloid cell populations for inflammatory cytokine production in response to this immune therapy.

More recently, the microbiome has been shown to influence DC function and antitumor immunity in the context of checkpoint blockade therapies for melanoma as well. In the B16-SIY melanoma model, the success of α-PD-L1 Ab therapy was shown to rely on the presence within the intestinal microbiota of *Bifidobacterium* species that enhance the antitumor effects of therapy (195). Specifically, the presence of natural *Bifidobacterium* species in C57Bl/6 mice from The Jackson Laboratory (JAX) or the introduction of *Bifidobacterium* species by oral gavage into C57Bl/6 mice from Taconic (TAC), which do not naturally harbor these bacteria, correlated with tumor-specific CD8$^+$ T cell responsiveness to α-PD-L1 Ab therapy and tumor control. Of note, the presence of intestinal *Bifidobacterium* species in these mice was also associated with an increase in the frequency of intratumoral DC expressing high levels of MHC class II, and genome-wide transcriptional profiling of these cells revealed elevated expression of several genes known to play roles in DC maturation, Ag processing and presentation, costimulation, and chemokine-mediated recruitment of immune effectors. Moreover, DC isolated from lymphoid tissues of JAX mice and *Bifidobacterium*-fed TAC mice induced higher levels of IFNγ production by CD8$^+$ T cells than did DC from untreated TAC mice that had not been exposed to *Bifidobacterium* species. In other work investigating microbial influences on checkpoint blockade therapy, pretreatment of mice with a cocktail of broad-spectrum antibiotics blocked the efficacy of α-CTLA-4 Ab therapy for established Ret murine melanomas (196). Interestingly, in mice not treated with antibiotics, CTLA-4 blockade promoted T cell-mediated destruction of intestinal epithelial cells and was associated in general with a decrease in Bacteroidales and Burkholderiales member species and an increase in Clostridiales member species in the feces, suggesting that induction of immunity to members of the Bacteroidales and Burkholderiales orders may be linked to the induction of antitumor T cell responses. In this regard, antibiotic-treated or germ-free mice that otherwise failed to exhibit any antitumor effects following α-CTLA Ab therapy were able to control tumors when fed with *Bacteroides thetaiotaomicron*, *Bacteroides fragilis*, *Burkholderia cepacia*, or a combination of *B. fragilis* and *B. cepacia* shortly after therapy, and this response was associated with enhanced maturation of intratumoral DC and T_H1 immune responses in tumor-draining lymph nodes. Moreover, fecal transplantation studies in which feces from ipilimumab-treated

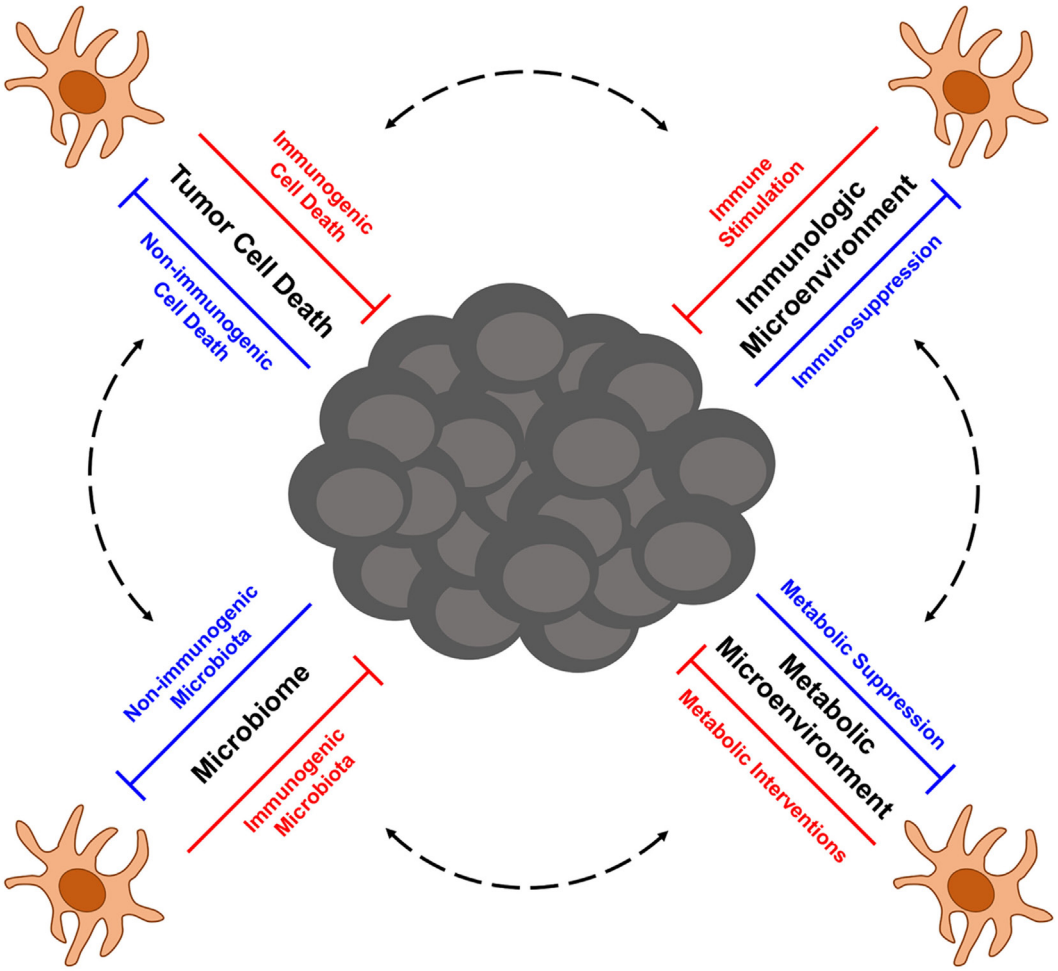

FIGURE 4 | Multifactorial influences on the function of melanoma-associated dendritic cells (DC). A variety of complex factors contribute to the immunoregulation of DC in the context of melanoma. Elements that control the immunogenicity of tumor cell death, the balance of immunostimulatory versus immunosuppressive signals in the tumor microenvironment, metabolic influences on DC function, and the microbiome all interact to dictate the immune stimulatory capacity of melanoma-associated DC. Mechanistic insights into each of these layers of DC immune regulation provide opportunities for therapeutic interventions to enhance the immunogenicity and antitumor function of melanoma-associated DC as described in more detail in the text.

metastatic melanoma patients clustered by stool microbial composition were transferred to germ-free mice two weeks prior to tumor challenge and α-CTLA-4 Ab therapy supported a role for *Bacteroides* species in promoting responsiveness to therapy. In these studies, feces from only one cluster of melanoma patients promoted colonization of immunogenic *B. thetaiotaomicron* and *B. fragilis* in mice, and these animals were the only fecal transplant recipients to mount effective antitumor responses following α-CTLA-4 Ab treatment. While these data suggest that the presence of commensal *Bacteroides* species in the gut may be a useful prognostic indicator for identifying patients most likely to benefit from checkpoint blockade therapy, it should be noted that confounding data on the influence of *Bacteroides* species on therapeutic efficacy in metastatic melanoma patients have emerged from recent clinical studies. Indeed, in a prospective study of metastatic melanoma patients receiving ipilimumab therapy, a high proportion of baseline gut *Bacteroides* actually correlated with poor clinical benefit, whereas long-term benefit (progression-free and overall survival) was associated with enrichment of *Faecalibacterium* species and other Firmicutes phylum members (unclassified *Ruminococcaceae*, *Clostridium* XIVa, and *Blautia*) (197). Similarly, Bacteroidales family members were found to be enriched in the gut microbiome of metastatic melanoma patients classified as non-responders to α-PD-1 therapy, while responders were found to exhibit greater microbial diversity in the gut and enrichment of members belonging to the Clostridiales order (198). It is possible that the differences reported in these clinical studies versus the study by Vetizou et al. (196) are due either to species-specific differences between mouse and man or to biased reconstitution of gut microbiota following fecal transplantation from humans to mice. However, it is worth noting that another clinical study comparing the baseline gut microbiota of responders versus non-responders to various checkpoint blockade regimens reported data from melanoma patients similar to that described

by Vetizou et al.—that is, that enrichment of *Bacteroides* species correlated positively with patient response to therapy (199). In this most recent study, gut microbiome diversity was not significantly different in responders versus non-responders, but metagenomic shotgun sequencing analysis of pretreatment fecal samples identified enrichment of particular species in responding patients that was unique for each therapeutic regimen under study. When comparing responders versus non-responders to all checkpoint blockade regimens under study, both *Bacteroides caccae* and *Streptococcus parasanguinis* were enriched in the gut microbiomes of responders. When analyzing patients responding to ipilimumab/nivolumab combination therapy, Firmicutes phylum members (*Faecalibacterium prausnitzii* and *Holdemania filiformis*) and the Bacteroidetes phylum member *B. thetaiotaomicron* were enriched in responders. Finally, the Firmicutes phylum member *Dorea formicigenerans* was enriched in responders to therapy with pembrolizumab. Based on these collective data, it is clear that additional studies with larger cohorts of patients are necessary to resolve these early discrepant findings and determine how particular gut microbiota regulate both natural antitumor immune responses as well as responsiveness to various tumor immunotherapies. Additionally, as evidence is accumulating that the gut microbiome also influences immunometabolism (200) as well as the metabolism and antitumor activity of chemotherapeutic drugs (201), future studies are needed to investigate how particular microbial species and their metabolites regulate chemotherapy-driven ICD and the function of DC and other immune cell populations in the context of melanoma. Together, these insights will be important for the optimization of strategies to manipulate the gut microbiome in ways that enhance antitumor immune reactivity while also minimizing adverse events such as therapy-associated colitis (202).

The Role of the Skin Microbiome in Immunity to Melanoma?

While a number of studies have been initiated to gain insights into the gut microbiome's influence on the progression of melanoma and other cancers, little is currently known about how the skin microbiome might impact immunologic protection from either the development of primary melanomas or the recurrence of melanoma in the skin or surrounding/distant tissues. To date, only one study has compared the skin microbiome of cutaneous melanomas and benign melanocytic nevi (203). While the cutaneous microbial diversity of melanomas was found to be slightly lower than that of melanocytic nevi, these differences did not reach statistical significance, and no differences were found in the relative abundance of bacterial genera between patients from these groups. However, the limited sample size of this study (15 cutaneous melanoma cases versus 17 melanocytic nevi cases) precludes any strong conclusions that the skin microbiome has no impact on melanoma progression or anti-melanoma immunity in the skin. With regard to microbial influences on cutaneous immunity, others have reported associations between the skin microbiome and patient susceptibility to inflammatory skin conditions such as atopic dermatitis (204), and dysbiosis of the skin microflora has recently been linked to autoimmune vitiligo as well (205, 206). As vitiligo results from immune-mediated destruction of melanocytes, microbial species that influence this process may be of particular relevance to melanoma. In this light, a recent study comparing bacterial communities in lesional versus non-lesional skin of vitiligo patients revealed a decrease in microbial diversity in vitiliginous lesions, and intra-community network analyses showed that Actinobacterial species predominate the microbial interaction network of non-lesional skin, while members of the Firmicutes phylum exhibit the highest degree of interactions in lesional skin (205). Future studies will be necessary to determine the cause–effect relationship of these alterations in cutaneous microbial communities during cases of vitiligo and whether such alterations might also impact immune reactivity against melanoma cells. Answers to these questions and others that address how the cutaneous microbiota might influence the maturation/activation of Langerhans cells and other skin-resident DC populations may suggest microbial interventions that support the promotion of robust, DC-mediated anti-melanoma immune responses. Coupled with an improved understanding of the gut microbiome's influence on DC-mediated immune responses against melanoma, these findings may identify appropriate dietary modifications, prebiotic/probiotic supplements, antibiotic regimens, and/or fecal transplantation strategies that can be implemented to support DC-based and other immune therapies for the treatment of melanoma.

CONCLUSION AND FUTURE DIRECTIONS

As highlighted throughout this review, DC function at the center of antitumor immunity and play major roles in determining immune activation versus tolerance against cancer. Regulation of immunity to melanoma by DC is controlled by a variety of intrinsic and extrinsic factors, and it is the collective interplay between these factors that ultimately shape the quality of DC-mediated antitumor immune responses (**Figure 4**). Advances in our understanding of the ways in which DC function is influenced by ICD, immunosuppressive networks within the tumor microenvironment, tumor-altered immunometabolism, and the microbiome have provided crucial insights into the immunoregulation of tumor-associated DC, and these insights have informed novel strategies for improving the immunogenicity of DC in the context of melanoma and other cancers. Some of these strategies have already reached patients and have improved the immunologic control of melanoma, and many others have shown great promise in murine models and in preclinical settings. It will therefore be exciting to follow the translation of these and related strategies for enhancing the immunostimulatory function of melanoma-associated DC into the clinic in the future. As we continue to build on these findings, the challenge going forward will be to dissect the complex interplay between the regulatory mechanisms discussed herein and discern how these diverse factors act in concert to control DC function. In this regard, in what ways does the microbiome impact the induction of ICD in melanoma cells? Can particular microbes provide metabolic support for DC by removing toxic byproducts from the tumor microenvironment, and how do microbe-derived metabolites themselves contribute to the metabolic milieu and its influence

on DC in the tumor microenvironment? To what extent do immunosuppressive factors in the tumor microenvironment blunt DC function through regulation of metabolic pathways in these cells, and how might altering the balance of these factors impact the abundance and diversity of the microbiota and its contribution to tumor-associated DC function? Collectively, how do these factors influence the ability of DC to maintain the immune reactivity of T cells supported by checkpoint blockade therapy, and how might DC-based therapies best be utilized in combinatorial approaches to induce antitumor T cell responses in patients who have not mounted natural responses to melanoma and are therefore currently poor candidates for treatment by checkpoint blockade? These additional insights into DC immunoregulation in the context of melanoma, coupled with ongoing technological advances that enable fine-tuned manipulation of DC function, will arm scientists with the tools necessary to devise multifaceted approaches to overcome melanoma-imposed limitations on DC immunogenicity. Based on the advances that have already been seen in recent years, there is great optimism within the field that these novel approaches will significantly improve the antitumor efficacy and clinical outcome of DC-based immunotherapies for melanoma patients in the future.

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