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Pushing Past the Blockade: Advancements in T Cell-Based Cancer Immunotherapies

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Successful cancer immunotherapies rely on a replete and functional immune compartment. Within the immune compartment, T cells are often the effector arm of immune-based strategies due to their potent cytotoxic capabilities. However, many tumors have evolved a variety of mechanisms to evade T cell-mediated killing. Thus, while many T cell-based immunotherapies, such as immune checkpoint inhibition (ICI) and chimeric antigen receptor (CAR) T cells, have achieved considerable success in some solid cancers and hematological malignancies, these therapies often fail in solid tumors due to tumor-imposed T cell dysfunctions. These dysfunctional mechanisms broadly include reduced T cell access into and identification of tumors, as well as an overall immunosuppressive tumor microenvironment that elicits T cell exhaustion. Therefore, novel, rational approaches are necessary to overcome the barriers to T cell function elicited by solid tumors. In this review, we will provide an overview of conventional immunotherapeutic strategies and the various barriers to T cell anti-tumor function encountered in solid tumors that lead to resistance. We will also explore a sampling of emerging strategies specifically aimed to bypass these tumor-imposed boundaries to T cell-based immunotherapies.

Keywords: immunotherapy, tumor-associated macrophage (TAM), CAR (chimeric antigen receptor) T cells, immune checkpoint inhibition (ICI), tumor microenvironment, immunotherapy resistance, T cell

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1 INTRODUCTION

The advent of immune checkpoint inhibition (ICI) therapies marked the beginning of the cancer immunotherapy resurgence. The remarkable efficacy of ICI therapies against a number of cancer types has prompted enthusiasm for the potential power of immunotherapies in treating both solid and hematologic malignancies. Over the past few decades, advancements in ICI, adoptive immunotherapy, and in our overall understanding of the tumor microenvironment (TME) have led to increasing treatment successes and the FDA approval of numerous immunotherapies, most of which either directly or indirectly impact T cells. Unfortunately, amidst a flood of potential targets and strategies, cancer-induced T cell exhaustion remains a notable obstacle to more widespread efficacy and applicability for T cell-directed immunotherapies.

In this review, we will provide an overview of the barriers and dysfunctions that limit effective anti-tumor T cell responses, and we will discuss emerging therapies that aim to ameliorate these

barriers, summarized in **Table 1**. An understanding of novel approaches to address T cell dysfunction will aid the development and implementation of more rationally-designed strategies to combat cancers.

Recent work has shed further light on the complexity proffered by the TME, as well as on distinct cell-cell interactions in the TME that limit immune responses to various tumors. Consequently, new strategies have emerged to target the unique immunosuppressive elements within the TME. Along with T cell directed immunotherapies, such as ICI, these strategies are expected to synergistically increase T cell effector function and patient survival.

2 CURRENT T CELL-BASED IMMUNOTHERAPEUTIC PLATFORMS

2.1 Immune Checkpoint Inhibition (ICI)

Under homeostatic conditions, the upregulation of cytotoxic T lymphocyte associated protein 4 (CTLA4), programmed cell death protein 1 (PD1), and other immune checkpoints helps to enforce peripheral tolerance by preventing immune-activation by self-antigens, thereby limiting autoimmunity (31). Under acute pathogenic conditions, the induction of immune checkpoints on the surface of activated T cells helps to resolve the immune response after pathogens are cleared, restraining collateral immunopathology. In such homeostatic and infectious circumstances, the expression of immune checkpoints and associated immune restriction is adaptive and limits damage to the host. However, in the case of cancer, the upregulation of immune checkpoints may instead maladaptively limit the anti-tumor immune response and serve an immune-evasive role on behalf of the cancer. Conversely, ICI therapies are intended to release these biological brakes on T cells and allow for prolonged and strengthened immune responses toward malignant cells (**Figure 1**).

In March 2011, the anti-CTLA4 monoclonal antibody (mAb) ipilimumab became the first ICI approved by the FDA, with an initial indication for the treatment of advanced melanoma (32). CTLA4 is expressed on recently activated T cells, where it limits further activation by competing with the costimulatory receptor CD28 for binding with CD80/CD86 on antigen presenting cells (APC). Blocking this interaction with anti-CTLA4 leads to more successful priming and activation of naïve T cells (33). In contrast to CTLA4, PD1 is found on more antigen-experienced T cells. PD1 signaling upon recognition of its ligands, PD-L1 or PD-L2, inhibits T cell receptor (TCR) activation and limits effector activity (34). In 2014, results of the KEYNOTE 001 clinical trial led to FDA approval of the anti-PD1 monoclonal antibody pembrolizumab, the first PD1 ICI for the treatment of advanced melanoma (35, 36).

Drugs targeting CTLA4 and the PD1 pathway have since been applied to a wide range of tumor types including lymphoma, lung cancer, renal cell carcinoma (RCC), head and neck squamous cell carcinoma (HNSCC), bladder cancer, liver cancer, breast cancer, and gastro-esophageal cancer (37). For melanomas, the potential for ICI to create a durable response rate has been demonstrated in multiple clinical trials. The CheckMate 067 study examining nivolumab (anti-PD1) and ipililumab (anti-CTLA4) in combination or as single agents for metastatic melanoma patients reported a 5-year overall survival rate of 52% in the combination group, as compared with historical 5-year survival rates under 20% (38, 39). The response rates for many other tumor types, however, have not been as strong, due to tumor intrinsic and extrinsic factors, such as heterogeneity and mutational burden, and the tumor microenvironment, respectively (40).

A 2020 cross sectional study estimated that approximately 38% of cancer patients in the USA were eligible for immunotherapies in 2018, and of them, less than 12% of patients responded to therapy (41, 42). Resistance to ICI therapy was evident in the initial KEYNOTE 001 study, where within the cohort of advanced melanoma patients, only 16% achieved a complete response (CR), with a median follow-up time of 2 years (35). Factors contributing to ICI therapy resistance include poor T cell access to and function within the tumor, which are often due to the presence of immunosuppressive cells and soluble factors in the TME, as well as tumor cell intrinsic factors including low tumor mutational burden and high heterogeneity (43). Therefore, novel strategies that overcome these barriers must be developed to expand the repertoire of cancers upon which ICI is effective.

TABLE 1 | Emerging therapies to overcome barriers to T cell function.

PROBLEM	SOLUTION	SOURCE
Exhaustion		
Metabolic dysfunction	4-1BB, OX40	(1, 2)
Immunosuppressive TAMs	CD40 Agonism	(3)
	CD73, CD39, A2Ar blockade	(4–6)
Alternative ICB	CD161, TIM3, LAG3 inhibition	(7–9)
Adoptive Transfer Exhaustion	Combine with ICI	(10, 11)
	Delete co-inhibitory receptors	(12–14)
	Transient rest	(15)
Tolerance		
Tregs	Depleting intratumoral Tregs	(16–19)
	Repolarizing Tregs	(20)
Infiltration		
Vascular dysfunction	VEGF/ANG2 dual blockade	(3, 21)
TAM	TAM depletion *via* CCR2 or CSF1R	(22–25)
Tumor Resistance to T cells		
Low/No Tumor MHC-I	Recombinant IFNy	(26, 27)
Antigen Heterogeneity - CAR	CAR-secreting BiTE; synNOTCH	(28–30)

2.2 CAR T Cells

Chimeric antigen receptor (CAR) T cells represent a subclass of adoptive immunotherapy. CAR T cells are most often CD8 T cells that have been genetically modified to express an extracellular antigen-targeting moiety, typically an antibody single-chain variable fragment (scFv) in tandem with intracellular T cell activating domains (44). These intracellular domains consist of the CD3ζ chain, along with CD28 and/or 4-1BB co-stimulatory components. Thus, T cells are redirected to recognize a cell-surface antigen of choice in a major histocompatibility complex (MHC)-independent manner. This MHC-independency provides CAR T cells a significant advantage over conventional TCR-bearing T cell adoptive therapies, allowing CAR T cells to bypass the low immunogenicity adapted by many tumor types.

CAR T cells have achieved success in certain hematological malignancies, including relapsed acute lymphoblastic leukemia

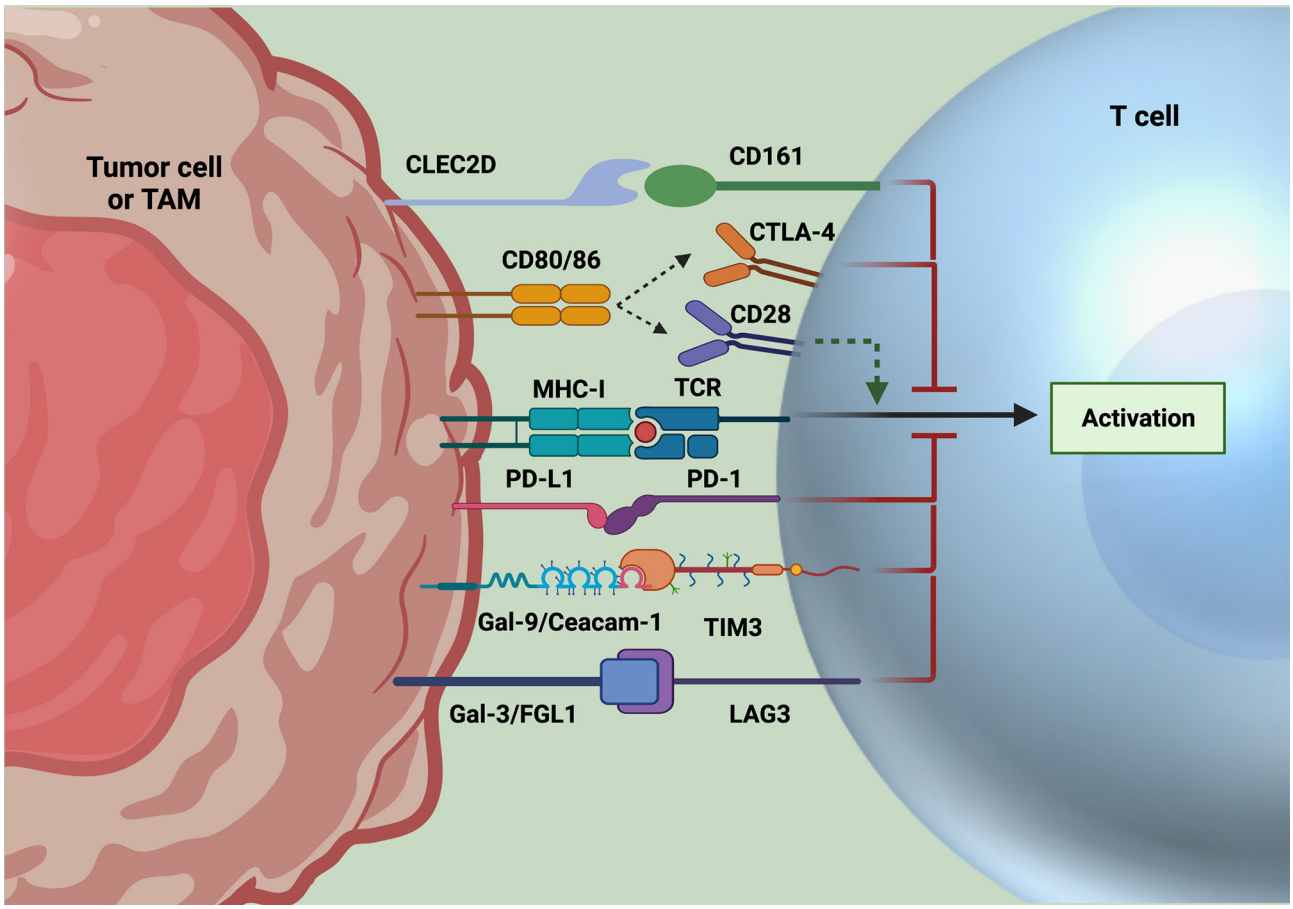

FIGURE 1 | Immune checkpoint inhibition strategies. Classically targeted immune checkpoints include PD1 and CTLA4. However, resistance to these checkpoints alone have led to the discovery of novel targets, including CD161, TIM3, and LAG3, whose ligands are expressed on tumor cells and often TAMs. After binding to their respective ligands, signaling of these immune checkpoints leads to suppression of T cell activation and effector function. Accordingly, these axes have been targeted for ICI, with promising results of increased T cell functionality to date. Created with Biorender.com.

(ALL) and refractory chronic lymphocytic leukemia (CLL), resulting in recent FDA approval of two CD19-targeting CAR products (45). However, similar success in solid tumors has yet to come to fruition. These failures are due jointly to solid tumor heterogeneity, the immunosuppressive TME, and CAR T cell exhaustion (**Figure 2**). To develop rationally-designed therapies that overcome current modes of resistance, it is necessary to first understand the factors limiting their efficacy.

3 FACTORS LIMITING THE SUCCESS OF IMMUNOTHERAPY

3.1 Lack of T Cell Access

Conventional anti-tumor cytotoxic T cells function by recognizing processed antigen in the context of MHCI on the surface of tumor cells, while CAR T cells directly recognize native antigen on the surface of tumor cells. Before recognizing tumor cells, they must gain access to the tumor, overcoming tumor-imposed boundaries.

3.1.1 Disorganized Neovasculature and Stromal Barriers

Among solid tumors, there is stark variability in TME composition and immune cell infiltration. Traditionally, "cold" and "hot" have been used to describe cancers with high and low levels of infiltration, respectively. Cold tumors, such as brain tumors and pancreatic ductal adenocarcinoma (PDAC), often consist of a high macrophage to T cell ratio (46–48). Notably, T cells in these tumors display decreased functional capacity, in both humans and mice (49, 50). In contrast, hot tumors, including skin melanoma, lung adenocarcinoma, and head and neck squamous cell carcinoma (HNSCC) have much higher T cell to macrophage ratios. This has been associated with increased responsiveness to immunotherapy (51). Factors that contribute to the degree of T cell infiltration in cancers include blood vessel permeability and organization, as well as

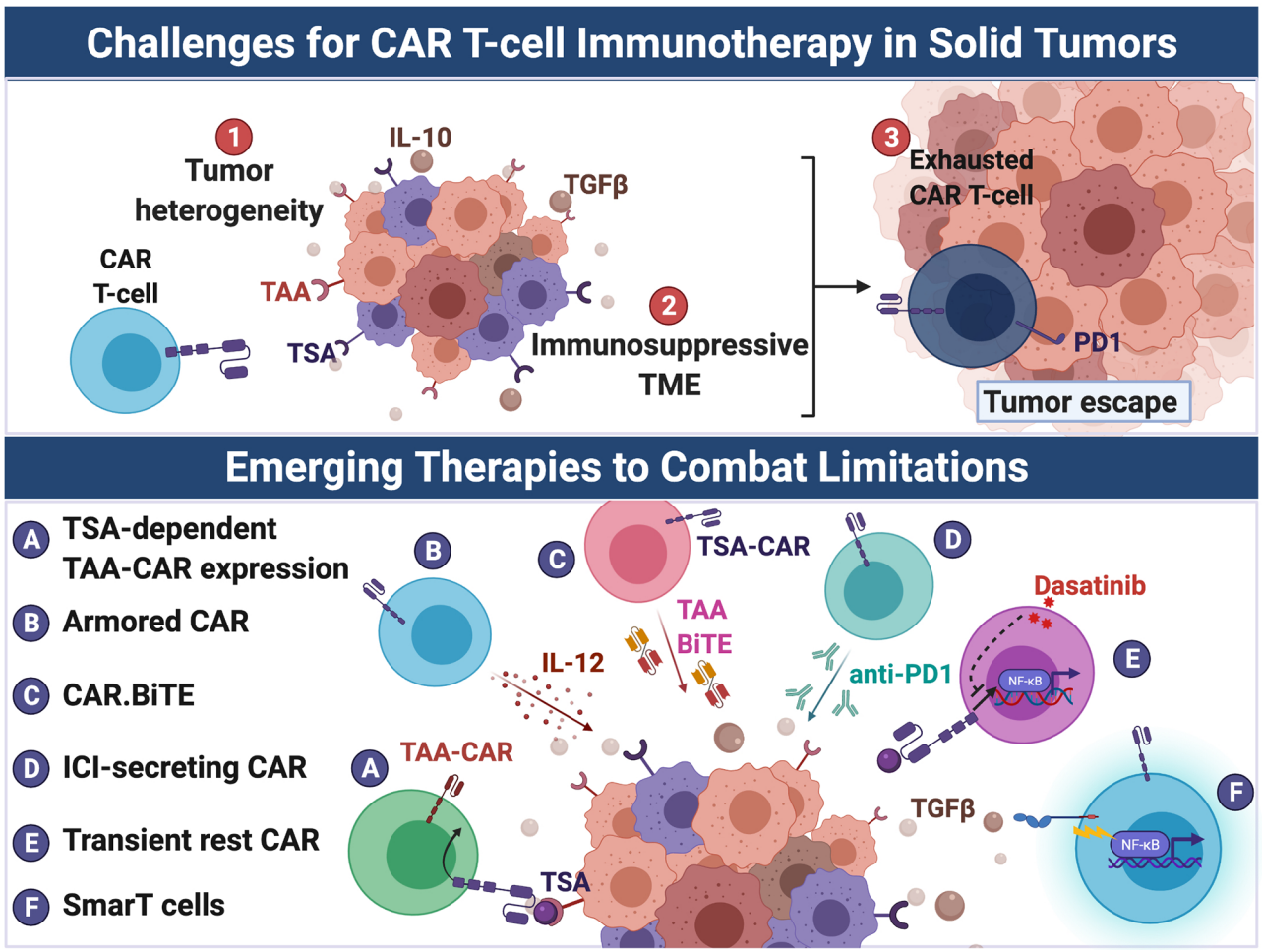

FIGURE 2 | Emerging therapies to overcome challenges to CAR-T cell immunotherapy in solid tumors. CAR T cell therapeutic efficacy has been greatly hindered in solid tumors due to (1) tumor heterogeneity, (2) the immunosuppressive TME, and (3) induction of CAR T cell exhaustion. Several strategies have been developed to overcome these barriers. Tumor heterogeneity results in antigen escape from targeted CAR therapy by antigen-negative tumor cells. To overcome the limitation of tumor heterogeneity, CAR T cells have been engineered to: **(A)** target multiple antigens including more ubuquitously-expressed TAAs but only after recognition of a TSA, and, similarly, **(B)** secrete cytokines such as IL-12 that promote endogenous anti-tumor immunity, **(C)** secrete BiTEs that target TAAs, thus resulting in intratumoral redirection of endogenous T cells to tumor cells. The immunosuppressive milieu (e.g. suppressive cytokines such as TGF-β) of the TME represses maximal CAR T cell activation. Additionally, solid tumors induce T cell exhaustion of CAR T cells through chronic antigen stimulation and tonic CAR signaling. To counter exhaustion, CAR T cells were: **(D)** engineered to secreted anti-PD-1 and **(E)** treated with dasatinib to prevent chronic CAR signaling and enhance CAR activation upon encounter of tumor. **(F)** To evade the immunosuppressive TME, SmarT CAR T cells were developed by fusing extracellular TGF-β and IL-4 receptors to intracellular co-stimulatory signaling domains, thus converting the action of TGF-β and IL-4 from inhibitory to stimulatory. Created with Biorender.com.

macrophage phenotypes and composition. Immune cell extravasation and infiltration into the tumor is highly regulated by expression of adhesion molecules and cohesiveness of endothelial cells (i.e., tight junctions). Additionally, expression of adequate adhesion molecules, and permeability are highly dependent on location (52). For example, blood vessels of the liver exhibit discontinuous endothelial cells and therefore allow for cellular infiltration. In contrast, blood vessels in the brain are characterized by tight junctions and astrocyte foot processes (known as the blood-brain barrier), which greatly restrict immune cell extravasation under normal conditions (53, 54). Tumors alter the surrounding vasculature to increase nutrient entry through the secretion of endothelial growth factors, such as vascular endothelial growth factor (VEGF) and angiopoietin 2 (ANG2). These new blood vessels are often disorganized and lack the expression of molecules required for extravasation, thereby limiting T cell trafficking (55).

Once outside of blood vessels, infiltrating T cells face additional barriers that prevent further infiltration into the tumor, such as dense extracellular matrix and tumor-associated macrophages (TAM) (46). Increased density of macrophages in the surrounding tumor stroma has been shown to limit T cell infiltration into the tumor, largely due to their roles in tissue remodeling and recruitment of cancer associated fibroblasts (56, 57). In PDAC, for example, macrophage

production of granulin promotes the accumulation of myofibroblasts (58). Furthermore, macrophage-derived transforming growth factor-beta (TGF-β) has been shown to limit T cell entry in metastatic urothelial and colon cancers (59–61). For these reasons, TAMs have become one of the foremost targets of immunotherapy in recent years. Importantly, combinatorial approaches are often successful, due to their ability to relieve more than one aspect of T cell suppression.

Still, in some cases, T cell infiltration alone does not correlate with improved prognosis or response to immunotherapy, suggesting that intratumoral entry alone is not sufficient to elicit a successful anti-tumor response (62, 63). High heterogeneity and low tumor mutational burden limit the ability of infiltrating T cells to carry out their anti-tumor functions. Furthermore, immunosuppressive cytokines, chronic antigen exposure, and inhibitory signaling can all lead to various modes and degrees of T cell dysfunction within the tumor.

3.1.2 Tumor Antigens and Mutational Burden

As mentioned previously, T cells recognize antigen on the surface of tumor cells, either in the context of MHCI for endogenous CD8 T cells or natively by CAR T cells. Tumor cells have adapted mechanisms to evade recognition by CD8 T cells by downregulating MHCI. Mutations leading to decreased or absent MHCI expression, most notably through the loss of functional beta-2 microglobulin expression, constitute a common mechanism by which tumors evade immune detection, rendering them immunologically cold (64–67).

Even with preserved MHCI expression, tumors can evade immune detection by reducing expression of tumor specific antigens (TSA), often termed neoantigens. Tumors acquire mutations as they proliferate, these mutations are reflected in the heterogenous set of antigens expressed on surface MHCI. The accumulation of non-synonymous mutations leads to the expression of altered peptide sequences on cell surface MHC-I, known as neoantigens, that are recognized as non self-antigens by immune cells (64, 68–70). Tumors with high neoantigen expression are therefore more immunogenic and postulated to be the target of CD8 T cells in response to checkpoint inhibition (70, 71). Tumors cells can also present tumor associated antigens (TAAs), which are normal self-antigens that are expressed on both healthy cells and tumor cells, although the level of expression may be higher in tumor cells (68, 72, 73). Unlike TAAs, neoantigens are not recognized as self, and therefore not subject to the same central or peripheral modes of tolerance as are TAAs (68, 70).

Higher mutational burden is associated with increased frequency of neoantigens. As such, tumors with high mutational burden, and therefore, high neoantigen expression are more responsive to T cell directed immunotherapies. Accordingly, the FDA approved indications for ICI drugs have been expanded to include tumors with high mutational burden, including tumors with evidence of high microsatellite instability (MSI-H) or mismatch repair (MMR) deficiencies (74). High neoantigen expression is not sufficient to produce an effective anti-tumor response, however, as T cells specific to the diverse array neoantigen peptides must also be present. In order to mount an effective anti-tumor CD8+ T cell response in the setting of an immunogenic tumor, TILs must be both active effector cells and have sufficient diversity of T cell receptors to provide specificity for the heterogenous antigens displayed by tumors. Several recent clinical studies on cohorts of patients with renal cell carcinoma, NSCLC, and melanoma have provided evidence for the significance of high T cell receptor diversity in predicting improved response to checkpoint blockade therapy (75–77).

3.1.3 Tumor Heterogeneity

Increased tumor mutational burden can be beneficial when it results in increased tumor neoantigen expression, however, heterogeneity of antigen expression between tumor cells can be problematic for therapies that target a specific tumor antigen, such as CAR T cells.

Tumor antigen expression is heterogeneous in that tumor cells often differ in their antigen expression profiles, both from patient to patient and within the same tumor. Thus, antigen-targeted therapies, such as CAR T cells, are typically only successful when all tumor cells within a tumor express the targeted antigen. Unfortunately, unlike their "liquid" counterparts, solid tumors do not tend to afford such universal antigenic expression. For example, EGFRvIII is one of the only and most highly expressed TSA characterized in GBM. However only approximately 30-50% of GBM tumors express EGFRvIII, and only about 30-50% of tumor cells in "EGFRvIII^{+}" tumors express the antigen (78). Our pre-clinical and clinical experiences with CAR T cells reveal that tumors possessing as few as 5-10% EGFRvIII-negative cells will easily escape EGFRvIII-targeted CARs (79). Moreover, antigen escape is documented in a variety of additional cancer types following CAR treatment, including hematologic malignancies (80, 81). The driver of intratumoral heterogeneity seems to be the genomic instability that is characteristic of tumor cells, with different individual cells acquiring different mutations (82–84). This branching evolution, as opposed to a more linear evolution, results in a tumor consisting of subclones with varied antigen profiles (16, 85, 86). Tumor heterogeneity is a major barrier to successful CAR T cell therapy in solid tumors, and strategies to overcome this hurdle will be discussed subsequently.

3.2 Suppressive TME

Once T cells have gained access to the tumor, T cells must face a complex milieu of cells and soluble factors in and around a tumor that is generally presumed to be immunosuppressive. Major contributors to such immunosuppression are tumor-recruited anti-inflammatory cell populations, most frequently TAMs and regulatory T cells (Treg). These cell populations can repress tumoricidal immune responses within the TME through tissue remodeling (creating hypoxic environments) and inhibitory interactions, hindering T cell effector capacity. Accordingly, emerging therapies often target these populations in efforts to better license T cell-based therapies (**Figure 3).**

3.2.1 Suppressive TAMs and Tregs

Macrophages represent a multifunctional class of immune cells that can operate broadly as either pro-inflammatory or anti-

inflammatory mediators. In most tumors, TAMs differentiate from monocytes that migrate to the tumor site in response to inflammatory signals secreted by tumor cells. Notably, gliomas and pancreatic cancers also contain resident macrophages of embryonic yolk-sac origins that may play differential roles in tumor progression (87–89). In murine PDAC models, monocyte-derived TAMs participated in antigen presentation and shaping the immune response, whereas resident macrophages with a pro-fibrotic transcriptional profile were more involved in producing and remodeling the extracellular matrix (89). Interestingly, microglia within a murine model of GBM were enriched for expression of inflammatory cytokines, whereas monocyte-derived TAMs were enriched for wound healing-associated chemokines and expression of Aryl-hydrocarbon receptor (Ahr), a transcription factors associated with immune suppression (87, 88, 90). Within the TME, pro-tumor TAMs dominate and can promote tumor growth through secretion of growth factors and, in part, by dampening effector T cell function through secretion of anti-inflammatory cytokines, TGF-β and interleukin (IL)-10, as well as expression of immune modulators, such as PD-L1 (87, 91, 92). Additionally, macrophages can promote angiogenesis and tumor cell metastasis (93, 94). Thus, these immunosuppressive TAMs negatively impact T cell targeting immunotherapies and must be countered to improve T cell-based therapeutic strategies.

The importance of Tregs under homeostatic conditions is illustrated by the disease Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX). Patients with IPEX have mutations in the Treg master transcription factor *FOXP3* gene, resulting in the dysfunctional development of Tregs and leading to fatal multi-organ autoimmunity within the first two years of life (95, 96). Moreover, Treg deficiencies have been identified in patients suffering from various autoimmune disorders including multiple sclerosis (MS) (97), mysasthenia gravis (98), and type 1 diabetes (T1D) (99). Unsurprisingly, many cancers have usurped the suppressive nature of Tregs to thwart effective antitumor immunity. Tregs are often found in high proportions in the tumor microenvironment of solid cancers, where they act to support an immunosuppressive tumor microenvironment (100–105). Tregs utilize a variety of mechanisms to suppress effector T cell activation. These mechanisms include modulating antigen

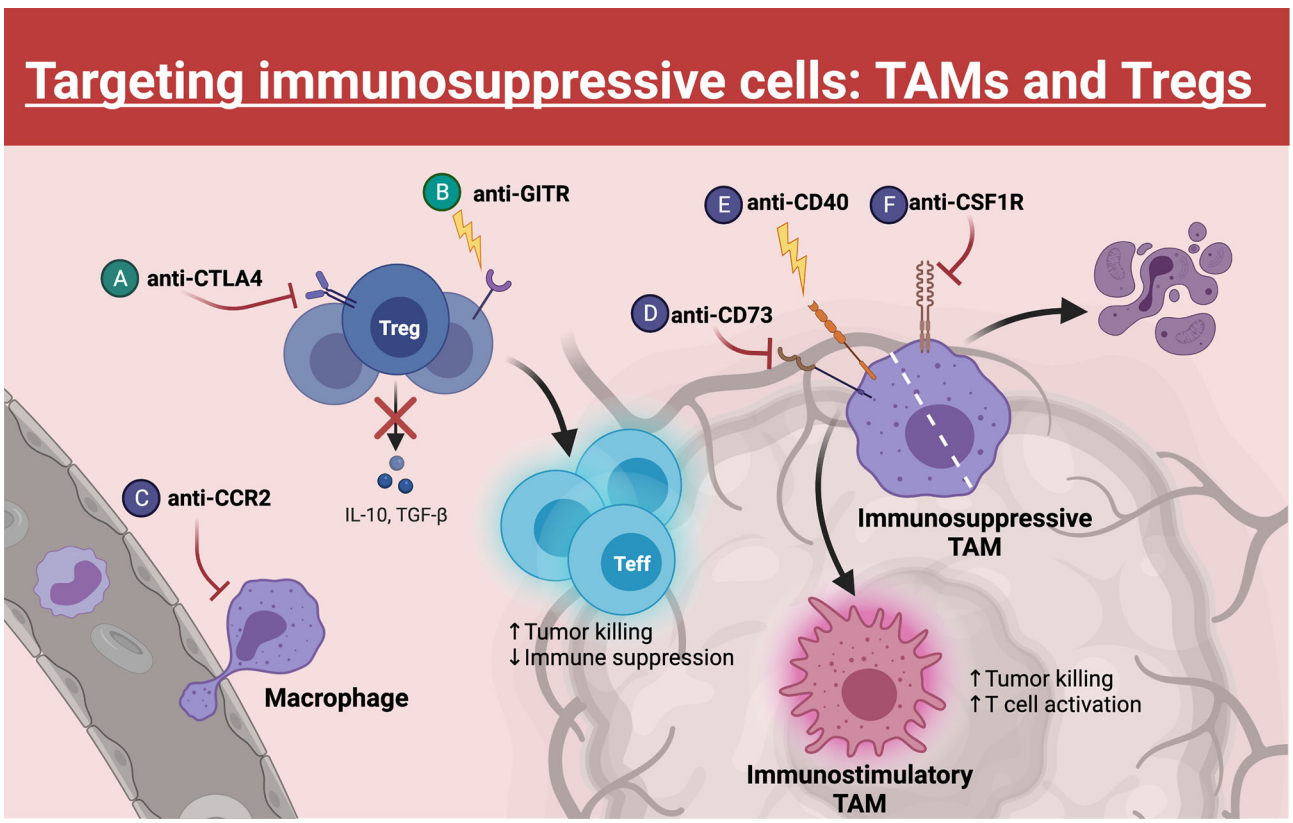

FIGURE 3 | Targeting TAMs and Tregs. Within the TME, Tregs and TAMs represent major barriers to effective anti-tumor T cell function. Many emerging therapies have been developed to target these populations, which often synergize with immune checkpoint blockade. **(A)** Blocking CTLA4 on Tregs decreases their immunosuppressive function, whereas **(B)** GITR agonism can destabilize Tregs, causing these Tregs to gain effector function. **(C)** TAM trafficking to the tumor can be inhibited through blockade of surface CCR2. **(D, E)** Repolarizing TAMs from an immunosuppressive phenotype to an immunostimulatory phenotype can be achieved through CD73 inhibition or CD40 agonism. **(F)** Another strategy to eliminate TAMs within the TME is blocking of CSF1R, a critical growth receptor on TAMs, which leads to cell death. Created with Biorender.com.

presenting cells (APCs) function through competitive blockade of CD80:CD28 costimulation with CTLA4 (106), secreting immunosuppressive soluble mediators like TGF-β and IL-10 (107, 108), and depleting local IL-2 pools due to their constitutively high CD25 (IL-2Rα) expression (109), among many others (110). Consequently, targeting Tregs to enhance antitumor immunity or to augment the efficacy of immunotherapies signifies a rational and promising strategy.

3.2.2 Hypoxia

The TME often contains hypoxic regions, resulting from locally disorganized vasculature and uncontrolled tumor cell proliferation. Hypoxia has been documented as a contributor of T cell exhaustion, due to its potential negative impacts on T cell metabolism (111, 112). Interestingly, T cells have evolutionarily acquired a mechanism to overcome this barrier through upregulation of hypoxia-responsive factors. Hypoxia inducible factor 1 subunit alpha (HIF-1α) is a transcription factor that is rapidly degraded under normoxic conditions but becomes stabilized and activated by hypoxic conditions (113, 114). When activated, HIF-1α aids in reprogramming cellular metabolism to function in hypoxic conditions by triggering a switch from oxidative to glycolytic metabolism (113, 115–117).

3.3 T Cell Exhaustion

T cell exhaustion is one of the most studied forms of T cell dysfunction due to its well-documented role in limiting the adaptive anti-tumor response (118). During many normal immune responses to chronic pathogens (especially chronic viral infection), T cell exhaustion can evolve as a programmed host-adaptive stalemate between the immune system and the host, in order to minimize immune-related collateral damage to normal tissues. Unfortunately, in the context of cancer, T cell exhaustion can be co-opted to foster tumor growth and contribute to cancer immune evasion. T cell exhaustion was initially described in the context of chronic lymphocytic choriomeningitis (LCMV) infection as a progressive and stereotyped hierarchical loss of memory and effector T cell function that is maintained in an antigen-dependent manner (119). Subsequently, years of research have led to the designation of T cell exhaustion as a separate T cell differentiation state with a unique transcriptional and epigenetic program (120, 121). The causes of T cell exhaustion are multifactorial and continue to be an active area of investigation. It was recently shown in a murine model of melanoma that metabolic function, and specifically mitochondrial fitness, is directly linked to the development of an exhausted phenotype (111).

T cell exhaustion is most often characterized by high expression of coinhibitory receptors, such as PD1 and CTLA4. However, alternative coinhibitory receptors have also been identified, including T cell immunoglobulin domain and mucin domain 3 (TIM3), and lymphocyte-activation gene 3 (LAG3). Increased expression of these molecules by T cells has been shown to limit the efficacy of immunotherapy in cancer (118). An extensive body of work now exists that details the current framework of CD8 T cell exhaustion in both chronic infection and cancer [reviewed in (122)]. Less is understood about CD4 T cell exhaustion, although CD4 T cell exhaustion is also likely important in the context of chronic infection and tumor immunology [reviewed in (123)].

Importantly, two major subsets of T cell exhaustion have recently been identified, with these termed "progenitor" and "terminal" exhaustion. Progenitor exhaustion refers to a stem-like population of intermediate PD1 expressing ($PD1^{int}$) T cells with proliferative potential and long-term self-renewal capabilities (124–126). This population has been shown to express memory-associated markers, such as TCF1, IL7R, and CXCR5 (127–129). Terminal exhaustion is characterized by high expression of PD1, TIM3 and other coinhibitory receptors, increased apoptotic signaling, and a cytotoxic program (129, 130). The importance of these subsets is underscored by their dictating differential responses to ICI. As early as 2008, it was shown that a $PD1^{int}$ population could proliferate upon PD-L1 blockade and mediate control of a chronic viral infection (131). Further analysis of this $PD1^{int}$ population demonstrated a 30-fold increase in its transition toward terminal exhaustion following PD-L1 blockade compared to the untreated controls (124).

From these studies, a model was proposed where progenitor exhausted T cells act as exhaustion "stem cells" during chronic infection that undergo slow self-renewal while also giving rise to the effector terminal exhaustion population (124, 129). Notably, this paradigm has been supported in models of melanoma, colorectal cancer, glioblastoma, and others (50, 130, 132). As an example, Miller et al. used single cell RNA sequencing (scRNAseq) to identify the stem-like progenitor exhaustion population and the more effector-like terminal exhaustion population in a murine model of melanoma. Researchers noted that the progenitor exhausted population displayed greater polyfunctionality ($TNF\alpha^{+}IFN\gamma^{+}$) and the ability to persist without antigen, whereas the terminally exhausted population had superior cytotoxicity (granzyme B $(Gzmb)^{+}$ $IFN\gamma^{+}$) and reduced long-term survival. In adoptive transfer experiments, groups with transferred progenitor exhausted cells, but not transferred terminally exhausted cells, had enhanced tumor control and retained responsiveness to PD1 blockade. Additionally, in human advanced melanoma samples, higher frequency of progenitor exhaustion correlated with improved duration of response to ICI. The authors concluded that strategies to stimulate the progenitor exhausted population more specifically are likely to improve the efficacy of ICI. Altogether, an increased understanding of T cell exhaustion will continue to guide elucidation of novel targets and newer angles on immunotherapy, which will be discussed in further detail below.

4 EMERGING ADVANCES THAT OVERCOME BARRIERS TO T CELL-BASED IMMUNOTHERAPY

The barriers described above limit the effectiveness of T cell-based immunotherapies. Novel, rational approaches that aim to overcome current limitations of immunotherapies are discussed here.

4.1 Enhancing T Cell Access

4.1.1 Increasing T Cell Infiltration

The angiogenic signal protein VEGF is upregulated by HIF-1α activation and plays an important role in the dysfunctional vasculature of the TME. At steady state, the development of new blood vessels is necessary for physiological processes, such as embryogenesis, organogenesis, and wound healing. This is a highly regulated process to ensure sufficient nutrients are reaching tissues without uncontrolled growth (133). Sustained angiogenesis has been recognized as a hallmark of cancer for decades. Early studies identified the requirement for angiogenesis for sustained tumor growth after nearby nutrients had been exhausted. These findings, coupled with the discovery of VEGF overexpression in tumors and the tumor-suppressive effects of VEGF inhibition (133), cemented the importance of angiogenesis in the growing cancer field. More recently, tumor-induced angiogenesis has been shown to produce an abnormal vasculature that may limit lymphocyte infiltration as a result of altered expression of adhesion molecules, chemokines, and cytokines (55).

Traditionally, anti-angiogenics, such as anti-VEGF or anti-ANG2, aim to prevent tumor neovascularization to limit the entry of necessary nutrients and immunosuppressive cells. Recent studies have shown that this strategy may also normalize previously abnormal blood vessels, improving the extravasation of CD8 T cells into the tumor site (3, 55). To date, anti-VEGF-A antibodies have been the most common anti-angiogenic treatment modality. These have been paired with standard of care treatments and ICI to extend PFS (134). However, many cancers demonstrate resistance to anti-VEGF-A treatment alone, including glioblastoma (21). It is hypothesized that ANG2 production mediates this resistance. Accordingly, dual blockade of VEGFA and ANG2 has demonstrated superior preclinical results through increased T cell tumor infiltration and myeloid repolarization (3). In this study, researchers observed improved perfusion and reduced leakiness of blood vessels within treated tumors. The normalized vasculature was associated with increased CD8 T cells infiltration. This was augmented by combination with anti-CD40 agonist, which further promoted intratumoral infiltration of CD8 T cells and tumor eradication in several syngeneic murine tumor models. Macrophages in this model were repolarized from an anti-inflammatory signature towards a pro-inflammatory one, highlighting potential synergy between strategies to normalize tumor vasculature and those to enhance T cell function.

While blocking VEGF-A expression is one approach, Song et al. instead demonstrated benefits to combining ICI with forced ectopic expression of VEGF-C in a syngeneic model of murine glioblastoma (135). The authors identified enhanced CD8 T cell priming following imposed increases to VEGF-C-driven lymphatic drainage of the TME. These findings reveal the importance of lymphatic drainage for guiding an effective anti-tumor T cell response, as well as challenge the dogma that inhibiting angiogenic factors is the correct strategy. Interestingly, VEGF-C is often a biomarker for metastasis in peripheral cancers (136, 137), highlighting the importance of understanding the nuanced roles of the various angiogenic factors.

4.1.2 Combatting Tumor Heterogeneity

One approach taken to combat such intratumoral heterogeneity is to attempt to elicit "bystander" immune responses and epitope spreading. An example is the recent production of IL-12-secreting CARs, often termed "Armored CARs", which are aimed at amplifying neighboring polyclonal Th1 immune responses within the TME. Local release of IL-12 in the TME is thought to promote T cell priming and activation by DCs, thereby enhancing the endogenous anti-tumor response (138). However, due to the potential toxicity of IL-12 constitutively secreted by CARs (139), CARs that only secrete IL-12 locally within the TME upon CAR engagement are currently being developed. Liu, et al. recently generated a Glypican-3-targeting CAR that contained IL-12 under the control of an NFAT-dependent promoter (140). Thus, IL-12 was only secreted upon CAR activation. In a preclinical model of hepatocellular carcinoma (HCC), these inducible IL-12 Glypican-3 CARs significantly improved survival of mice compared to non-IL-12-secreting Glypican-3 CARs. Unfortunately, this study did not measure *in vivo* efficacy of inducible IL-12 CARs in the context of antigen-heterogeneous tumors. However, the superior anti-tumor effect of inducible IL-12-secreting *versus* non-IL-12-secreting CARs provides evidence supporting pursuance of this strategy.

Multi-antigen-targeting CAR-based T cell therapies are another way to combat tumor heterogeneity. Unfortunately, many of the most highly expressed antigen targets are often only tumor-associated, not tumor-specific. If these TAAs are to be targeted, extra caution must be taken to avoid on-target, off-tumor toxicities. Choi, et al. took a creative approach to combat tumor heterogeneity in gliomas that have variable expression of EGFRvIII and wildtype (WT) EGFR (28). WT EGFR is a TAA overexpressed in many gliomas and absent on normal CNS tissue, but it is expressed on normal peripheral tissues. Therefore, a WT EGFR-targeted therapy must be delivered locally to the tumor to prevent any on-target, off-tumor toxicities. Choi, et al. generated an EGFRvIII-targeting CAR that secretes a WT EGFR-targeting bi-specific T cell engager (BiTE). These CART.BiTEs were shown to be effective when administered intracranially to mice bearing brain tumors, with the CART.BiTEs directly killing $EGFRvIII^+$ tumor cells, while also redirecting endogenous T cells to kill WT $EGFR^+$ tumor cells. Another recently reported approach utilized a synthetic Notch (synNotch) system to control surface CAR expression (29, 30). In this system, surface expression of a TAA-specific CAR construct is dependent upon signaling through a constitutively expressed TSA-specific CAR on the same T cell. Thus, the TAA CAR construct remains hidden until the CAR T cell has reached the tumor and encountered the relevant TSA. This approach was preclinically shown to be effective in models of GBM, mesothelioma, and ovarian cancer, suggesting broad applicability for this platform.

4.2 Overcoming TME-Mediated Immune Suppression

4.2.1 Targeting TAMs

To target TAMs, one strategy involves stimulating CD40 on TAMs with an anti-CD40 agonist, which can activate and

polarize them towards a pro-inflammatory phenotype (increased costimulatory and MHC molecule expression). CD40 is a tumor necrosis factor (TNF)-receptor superfamily member that is primarily expressed on APCs, such as macrophages, DCs, B cells, and monocytes (141). When bound to its ligand, CD40L, CD40 induces activation of TAMs and other APCs, leading to upregulation of costimulatory and MHC molecules. Notably, there is strong pre-clinical evidence that tumor regression can follow treatment with agonist CD40-targeting antibody (3, 141). This strategy is believed to enhance T cell priming by TAMs (and other APCs). Interestingly, chemotherapy, when followed by CD40 agonism (but not vice versa) in murine solid tumor models, has elicited effective T cell responses, tumor clearance, and T cell memory. This study suggested that CD40 agonism may lead to better priming of T cells by APCs when these APCs are loaded with tumor antigens recovered from dying tumor cells (142). These results have informed several phase I clinical trials that are testing CD40 agonism in combination with chemotherapy (NCT01103635, NCT02705196, NCT03214250).

Additionally, ongoing efforts are focused on combining the immunostimulatory effects of anti-CD40 with other therapies, such as anti-PD1 or anti-angiogenics, to capitalize on the enhanced T cell activation potential of repolarized myeloid cells. In an ongoing phase 1b clinical trial, examining CD40 agonism in combination with chemotherapy ± ICI (nivolumab), in patients with untreated PDAC demonstrated partial responses in 14/24 patients, and stable disease in 8/24 (NCT03214250) (143). Another phase 1 clinical trial demonstrated an overall response rate (ORR) of 27.3%, which included 2 complete responses and 4 partial responses, in 22 metastatic melanoma patients treated with CD40 agonism (selicrelumab) in combination with anti-CTLA4 (tremilimumab) (NCT01103635). Notably, 9 patients had long-term survival of over 3 years (144).

Another strategy targeting TAMs involves inhibiting CD73. CD73, along with CD39, are rate limiting ectonucleotidase enzymes involved in the extracellular degradation pathway of ATP to adenosine (**Figure 4**) (4, 145–147). Both ATP and adenosine serve as immunologic signaling molecules within the TME, though with opposite effects. ATP is released into the extracellular compartment by dead and dying tumor cells and serves as a pro-inflammatory signal through binding to the P2X/P2Y receptors on T cells (147). Free extracellular ATP can also be converted to adenosine monophosphate (AMP) by CD39, and AMP is subsequently converted to adenosine by CD73 (4, 145–147).

In contrast to ATP, extracellular adenosine has immunosuppressive effects. Adenosine binds to the A2A receptor on cytotoxic T cells resulting in their death (148, 149). Under homeostatic conditions, this process controls unwarranted inflammation in response to the continuous death and turnover of normal cells. In the context of cancer, apoptosis of anti-tumor effector T cells is clearly detrimental to the desired anti-tumor immune response. A major source of adenosine within the TME derives from CD73 expression on macrophages and other myeloid cell populations. This depot of intra-tumoral adenosine in turn can hinder T cell-based immunotherapies (5). Therefore, inhibiting CD73, especially on TAMs, is of great interest. Notably, CD73 was highly identified on TAMs in glioblastoma through an effort to immune profile several cancers (6). Goswami et al. identified a $CD73^{hi}$ TAM population that persisted following anti-PD1 treatment in glioblastoma patients. Furthermore, this group found an enhancement of ICI efficacy in CD73 knockout (CD73KO) mice. In these mice, intracranial tumor growth was impeded, and prolonged survival was observed. Interestingly, researchers observed no change in T cell subsets within CD73KO mice alone, but when ICB was administered, increased T cell infiltration and activation resulted. Additionally, repolarization of macrophages from anti-inflammatory ($CD206^{+}$) to pro-inflammatory ($iNOS^{+}$) phenotypes was observed. Importantly, CD73 expression is not restricted to TAMs. In fact, many tumor cells express high levels of both CD39 and CD73, usurping the immunosuppressive effects of this pathway to further inhibit antitumor immunity (146, 150). In gastric cancers, high tumor CD73 expression has been associated with poor overall survival and advanced clinical stage. These findings support the use of strategies aimed at countering immune-suppressive mechanisms employed by TAMs as a potentially effective immunotherapeutic adjunct. Phase I clinical trials are currently underway in PDAC (NCT03611556), breast cancer (NCT03742102), and many other solid tumors (NCT03454451) to evaluate the safety and efficacy of CD73 blockade in combination with PD1 axis inhibitors (anti-PD1, anti-PDL1) (151).

Strategies to remove TAMs from the TME have also been investigated. Depleting macrophages from the tumor site can be accomplished by inhibiting their trafficking or entry into the TME, or by blocking macrophage survival signals to instigate their death. Macrophage trafficking can be suppressed by targeting the CCL2/CCR2 axis. CCL2 is an important chemokine involved in monocyte recruitment. It is highly expressed by many cancer types, making it an attractive target for limiting macrophage infiltration into the TME. Overexpression of CCL2 has been documented in patients with lung adenocarcinoma, breast cancer, and hepatocellular carcinoma (152–154). Disappointingly, limited benefit has been observed in clinical trials with therapies targeting the CCL2-CCR2 axis alone. However, there is strong evidence from several preclinical tumor models supporting the combination of CCR2 inhibition and ICI (155). For instance, Flores-Toro et al. found synergy between a CCR2 antagonist and anti-PD1 treatment. In this study, median survival in a murine model of glioma was extended by 46% (35 d *vs.* 24 d), with such survival benefit correlating with elevated expression of IFN-γ by CD8 T cells (22).

In contrast to preventing macrophage tumor infiltration, efforts to deplete them entirely through colony stimulating factor 1 receptor (CSF1R) inhibition are under investigation. CSF1R is a growth factor that TAMs depend on for differentiation and survival (156). Several small molecules have been developed against CSF1R, with observed tumor regression and extended survival upon treatment in several murine models of GBM (157, 158). When tested clinically in patients with recurrent GBM, PLX3397 (pexidartinib) alone did not increase

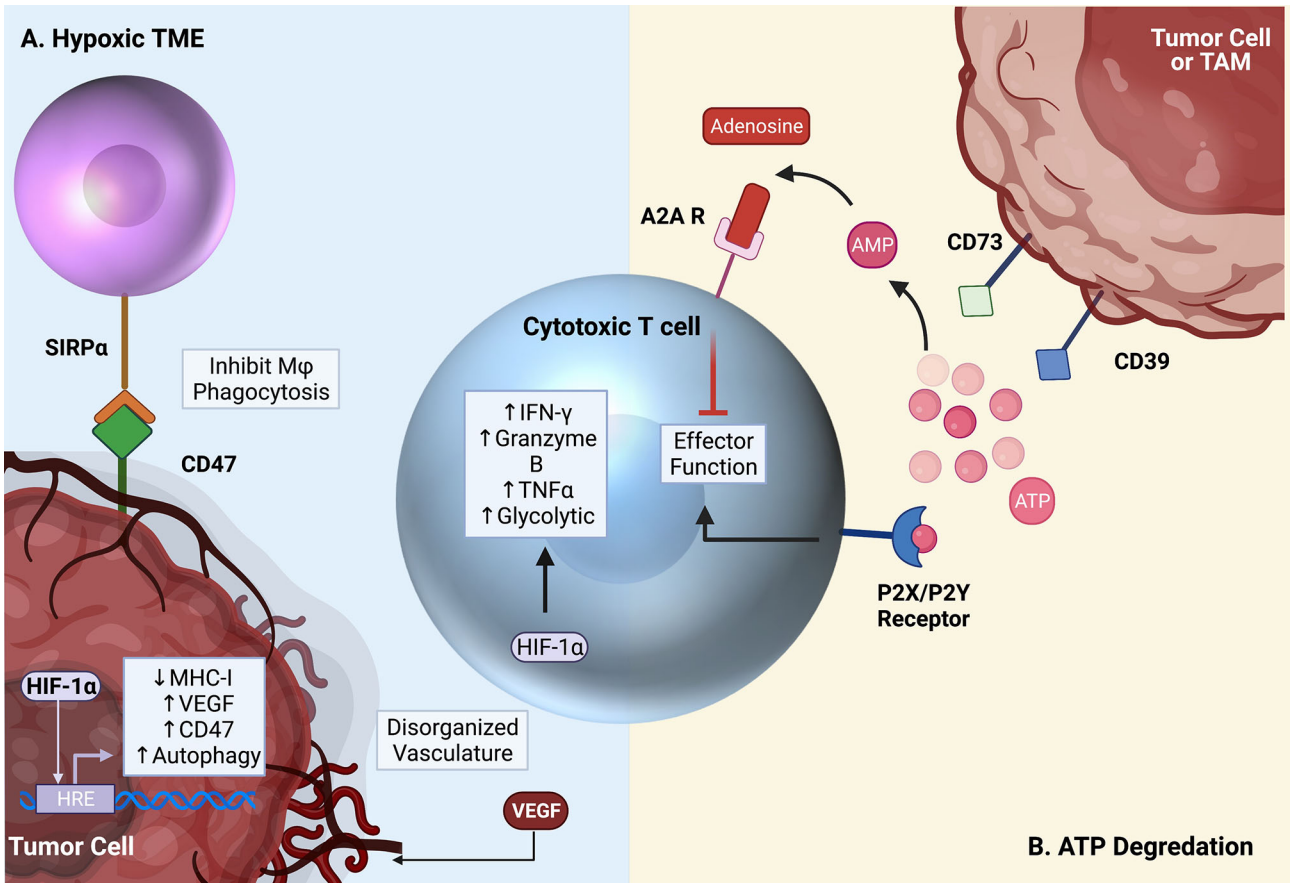

FIGURE 4 | Critical pathways targeted by emerging therapies. **(A)** The TME is characterized by hypoxia and low glucose. Increased VEGF expression by tumor cells and local immune cells leads to the creation of disorganized vasculature, ultimately worsening the metabolic environment. HIF-1α leads to the activation of several pro-tumor pathways, but also contributes to supporting T cell effector function in the hypoxic TME. **(B)** Free ATP in the TME activates T cells through the P2X/P2Y receptors. In the TME, however, free ATP is converted to immunosuppressive adenosine by surface CD39 and CD73 expression on tumor cells and TAMs. Adenosine then binds to the A2A receptor on T cells, inhibiting effector function. Created with Biorender.com.

PFS (23). Accordingly, combinations of PLX3397 with other immunotherapies, such as anti-PD1 and anti-CD40, are currently undergoing evaluation (159). MAbs that target CSF1R are also under investigation. Preclinical results with the novel anti-CSFR1 antibody RG7155 demonstrated death of $CSF1R^+$ macrophages in patients with diffuse-type giant cell tumors. Additionally, there was a reduction in $CD163^+CSF1R^+$ macrophages in tumor tissue and increased CD8:CD4 ratio in response to treatment (24). Disappointingly in a separate phase 1b study of anti-CSF1R in patients with advanced solid tumors, no objective clinical response was observed (25). As with repolarization strategies, combining macrophage targeting therapies with ICI may improve efficacy.

4.2.2 Targeting Tregs

Multiple approaches aimed at depleting Tregs have produced favorable outcomes in preclinical cancer models. CD25, the high affinity alpha chain of the IL-2 receptor, is constitutively expressed on the surface of Tregs and is often the target of choice for Treg manipulations. In one early study, Onizuka, et al. showed that depletion of Tregs in mice with the anti-CD25 antibody PC61 early after tumor implantation led to a significant regression of tumor size and an increase in survival (160). Importantly, this phenomenon was shown in several tumor models including leukemia, myeloma, and sarcoma (160) with other groups showing a similar effect in GBM (161). One major caveat of targeting Tregs through CD25 is that activated effector T cells transiently express CD25. Therefore, the true benefit of Treg depletion using anti-CD25 may be masked by concurrent depletion of anti-tumor effector T cells. Preclinical proof-of-concept studies in transgenic DEREG (DEpletion of REGulatory T cell) mice, whereby Tregs are depleted based on expression of the more faithful Treg marker Foxp3, have brought further insight. Early studies with DEREG mice showed that depleting Tregs *via* Foxp3 targeting resulted in partial regression of established B16 melanoma (162). This regression was accompanied by an increase in tumor infiltrating effector CD8 T cells. Subsequent studies using DEREG mice have confirmed

the benefit of Treg depletion in other cancer models (163–166). However, depleting Tregs based on Foxp3 in humans is currently not possible due to its intracellular/intranuclear location as a transcription factor. Thus, clinical efforts mainly rely on manipulating the IL-2/CD25 axis to achieve Treg depletion.

Nevertheless, there are several strategies that rationally aim to deplete Tregs while sparing antitumor effector T cells. The common murine Treg-depleting antibody clone PC61 efficiently depletes $CD25^{hi}$ Tregs but also blocks IL-2 signaling on remaining effector T cells thus blunting their effector activity. Solomon, et al. recently characterized a novel anti-CD25 antibody that depletes Tregs but does not block IL-2 signaling in effector T cells ($\alpha CD25^{NIB}$; NIB: Non-IL-2 Blocking) (17). This murine $\alpha CD25^{NIB}$ consists of a CD25 epitope-binding domain that is known to permit IL-2 signaling (clone 7D4) fused to the depleting murine IgG2a isotype backbone. $\alpha CD25^{NIB}$ promoted tumor rejection and prolonged survival in preclinical colorectal cancer models. Treg depletion with $\alpha CD25^{NIB}$ correlated with increased intratumoral effector T cell:Treg ratios along with increased granzyme B secretion by CD8 T cells. The anti-tumor effect was ablated when IL-2 was neutralized, signifying the dependency of IL-2 signaling for the anti-tumor activity of $\alpha CD25^{NIB}$. Importantly, these findings were translated into a human system, with similar Treg depleting, non-IL-2 blocking characteristics shown in non-human primates.

One major concern regarding systemic Treg depletion is the potential to induce autoimmunity, as Tregs are crucial for maintaining peripheral immune tolerance (167, 168). Thus, strategies designed specifically to deplete intratumoral Tregs only are emerging in an effort to assuage the risk of autoimmunity that can accompany systemic Treg depletion. One such approach involves injecting an immunotoxin-coupled anti-CD25 antibody (2E4-PE38) directly into the tumor (18). The authors elegantly showed that administering 2E4-PE38 directly into an established AB1 mesothelioma tumor led to tumor regression in both the injected tumor and a distal, non-injected tumor on the same mouse. These results indicated that local Treg depletion can potentially elicit concomitant tumor immunity. Importantly, the immunotoxin 2E4-PE38 has a relatively short half-life (<3 hours), allowing for effector cytotoxic T cells to repopulate the tumor quickly after $CD25^+$ T cell depletion.

Another approach to deplete intratumoral Tregs targets the chemokine receptor CCR8. CCR8 is up-regulated on Tregs following activation in the presence of the CCR8 ligand CCL1. Signaling through CCR8 potentiates multiple Treg-associated immunosuppressive mechanisms including up-regulation of Foxp3, CD39, and IL-10 (169). Campbell, et al. provided evidence that CCR8 was more highly expressed by tumor-infiltrating Tregs than circulating Tregs from the same patient (16). Subsequently, they showed that treatment with a CCR8-targeted depleting antibody specifically depleted intratumoral Tregs and promoted a robust anti-tumor immune response. Importantly, CCR8-mediated depletion did not affect effector T cell populations.

Another recent approach targeting intratumoral Tregs utilized near-infrared photoimmunotherapy (NIR-PIT) to locally deplete Tregs (19). Briefly, NIR-PIT involves administering a monoclonal antibody or Fab conjugated to IR700 followed by exposure to near-infrared light (690 nm) (170). Kurebayashi, et al. used an anti-CD25-IR700 conjugate to selectively and rapidly kill Tregs after tumor exposure to NIR-light. In preclinical models of MC38 colon cancer and EO771 breast cancer, intratumoral Treg depletion was accompanied by increased intratumoral infiltration of CD8 T and NK cells and a decrease in tumor size in an IFN-γ dependent manner. However, tumors began to regrow concomitantly with Treg recovery. Thus, the kinetics of Treg depletion, effector T cell activation, and Treg repopulation must all be considered when depleting Tregs to enhance antitumor immunity.

Treg depletion has also been shown in some instances to negatively impact antitumor immunity (171). In a model of pancreatic cancer, depleting Tregs actually promoted carcinogenesis in part by increasing myeloid-derived suppressor cell infiltration and by modulating fibroblast architecture. This pro-tumor effect of Treg depletion is likely cancer-type dependent, but nonetheless, suggests other approaches to Treg manipulation may be worth pursuing. Depleting Tregs is not the only Treg-focused approach that can promote tumor clearance. An alternate strategy is to reprogram Tregs from suppressive T cells to effector T cells. Tregs are inherently unstable, meaning they can lose Foxp3 expression and gain effector function under certain conditions (e.g. inflammation) (172). This instability can be detrimental in the context of autoimmunity where strategies aim to stabilize Tregs (173) but can potentially be manipulated favorably in the context of cancer. Indeed, Amoozgar, *et al.* recently showed that treatment of mice bearing GBM with an agonist antibody targeting glucocorticoid-induced TNFR-related (GITR) protein redirected intratumoral Tregs to a CD4 effector lineage (20). GITR is constitutively expressed on Tregs and transiently on activated effector T cells (174). The function of GITR is cell-context-dependent but predominantly acts as a costimulatory signal (175). Previous studies using GITR agonism showed increased effector T cells activation and decreased Treg suppressive activity with resulting autoimmunity (176). However, the utilization of GITR agonism to repolarize Tregs to effector T cells is a novel and intriguing approach. The destabilized Tregs induced by anti-GITR agonism characteristically resembled Th1 cells, secreting IFN-γ and promoting tumor cytotoxicity. Anti-GITR significantly extended mouse survival even as a monotherapy, but it showed even further benefits when coupled with anti-PD1.

4.2.3 Targeting Immunosuppressive Cytokines

Like conventional endogenous effector T cells, CAR T cells also prove susceptible to immunosuppressive mechanisms. TGF-β is a suppressive soluble factor present in high levels within various tumor types where it can prevent endogenous and CAR T cell activation. To combat this, Cadilha et al. generated a multicistronic epCAM-targeting CAR construct that encoded the chemokine receptor CCR8 and a dominant-negative TGF-β receptor (DNR) (177). CCR8 is uniquely upregulated on

intratumoral Tregs (178) and, thus, forced expression of CCR8 enhanced CAR T cell trafficking to tumor while the DNR prevented suppression by TGF-β. These epCAM CCR8$^+$ DNR CAR T cells were more efficacious at eliminating tumor and promoting survival in a preclinical model of pancreatic cancer than epCAM CAR T cells.

An interesting alternative approach to countering TGF-β (and suppressive cytokine signaling in general) involves converting the suppressive signal into a stimulatory one. Sukumaran, et al. designed and produced a prostate cancer antigen-targeting CAR that co-expressed TGF-β and IL-4 receptors in tandem with intracellular 4-1BB co-stimulatory domains and IL-7 receptor signaling domains, respectively (179). These "SmarT" cells were more potent and efficacious than normal CAR T cells in preclinical models, a benefit that was more pronounced in tumors designed to overexpress TGF-β and IL-4. Rational selection of intracellular signaling combinations to manipulate with this strategy should work to better rescue T cell activation.

4.2.4 Targeting Hypoxia

In cytotoxic T cells, HIF-1α stabilization is induced in both hypoxia-dependent and -independent fashions. For instance, TCR activation itself can lead to increased HIF-1α signaling (66, 180). The HIF-1α signaling pathway appears to be critical to T cell effector function in the setting of hypoxia, as deletion of HIF-1α in T cells lead to decreased production of IFN-γ, granzyme B, and TNFα by CD8 T cells under hypoxic conditions, facilitating tumor growth (116) (**Figure 4**).

While HIF-1α stabilization within T cells may be beneficial to T cell function and the anti-tumor response, HIF-1α signaling within tumor cells can be problematic, fostering tumor survival within its hypoxic TME. In turn, inhibition of the HIF-1α signaling pathway in tumor cells has often been a therapeutic goal. Within tumor cells, HIF-1α pathway activation aids tumor survival and combats the anti-tumor immune response by upregulating tumor expression of PD-L1 and CD47. CD47 is a cell surface ligand that binds to signal regulatory protein α (SIRPα) on macrophages, serving as a "don't eat me" signal (181, 182). HIF-1α additionally upregulates tumor autophagy, which is critical for tumor cell survival in the harsh metabolic conditions of the TME as it allows tumor cells to recycle damaged organelles and proteins into metabolic substrates for energy generation (181, 183–185). Ultimately, higher tumor expression of HIF-1α correlates with worse clinical outcome (186, 187).

HIF-1α therefore plays complex and potentially opposing roles in mediating tumor survival and the T cell antitumor response, and there are conflicting data as to whether systemic HIF-1α inhibition ultimately enhances or hinders cytotoxic T cell function and tumor growth. In a preclinical mouse study, selective deletion of HIF-1α in T cells led to increased tumor growth and decreased T cell tumor infiltration, survival, and effector function, measured by production of TNFα and IFNγ (116). On the contrary, in another study, reduced T cell HIF-1α expression was instead associated with improved CD8 memory cell formation and enhanced anti-tumor cytotoxicity of CD8 T cells (188). Clinical trials of HIF-1α inhibitors have thus far been disappointing, with many failing to produce objective responses (186, 189–191). Several recent and current studies are investigating the benefit of combination HIF-1α inhibition with the anti-VEGF drug bevacizumab (192, 193). The addition of bevacizumab appears to produce a modest clinical benefit, eliciting a 22% partial response in a phase I/II study of 22 RCC patients (192). In a phase II clinical trial of CRLX101, a nanoparticle inhibitor of HIF-1α, in recurrent ovarian cancer patients, the addition of bevacizumab improved ORR to 18% compared to 11% in CRLX101 monotherapy (193).

4.3 Combatting Exhaustion

4.3.1 Novel ICI Strategies

To overcome limitations to current therapies, research is underway to identify novel ICI targets. Common targets include alternative inhibitory receptors that are phenotypically linked to T cell exhaustion, such as TIM3 and LAG3. Additionally, a recent study identified inhibitory receptor CD161 as an attractive novel target for ICI through single cell profiling of tumor-infiltrating T cells of patients with IDH-WT and IDH-mutant gliomas (7) (**Figure 1**).

TIM3 was initially described as a cell surface molecule on activated effector CD4 and cytotoxic CD8 T cells (194). TIM3 inhibits TCR signaling and can induce T cell death by signaling through tyrosine residues in its cytoplasmic tail upon binding one of its ligands (195). Major ligands for TIM3 include galectin-9 (Gal-9), and Ceacam-1, which are expressed on macrophages and some cancers, and CD4 T cells, respectively (196–198). More recently, TIM3 expression has been identified on innate immune cells (NK cells, DCs, and monocytes) (199–201), and importantly, terminally exhausted CD8 T cells (202). Identification of TIM3 in terminally exhausted cells was initially done in models of chronic infection, where TIM3 signified virus-specific T cells with the greatest defects in pro-inflammatory cytokine production. Blockade of TIM3 restored effector function of terminally exhausted T cells in LCMV, HCV, and HBV, where co-blockade with PD1 further enhanced T cell responses (203, 204). Additionally, in several cancers, including GBM, melanoma, and NSCLC, TIM3 has been used to identify infiltrating T cells with poor anti-tumor capabilities (50, 130). The blockade of TIM3 is complicated by its expression on several immune cells, where it functions uniquely. However, initial first-in-human phase 1/2 clinical trials in several solid tumors have shown promising results (NCT02817633, NCT02608268, NCT03099109). In NSCLC cancer patients that had previously progressed with anti-PD1 treatment alone, combination anti-TIM3 (TSR-022) and anti-PD1 (TSR-042) has shown antitumor activity, as well as safety and tolerability (8).

LAG3 is a member of the Ig superfamily of proteins, originally described in activated human NK and T cells (205). It has been shown to negatively regulate T cell proliferation, activation and effector function upon binding to one of its many ligands: major histocompatibility complex II (MHC II) on APCs, galactose-binding lectin (Gal-3) on a variety of tumor cells, and more recently, fibrinogen-like protein (FGL1), which is also present on solid tumors (9, 206, 207). Targeting LAG3 on T cells with antagonistic antibodies prevents downstream inhibitory

signaling, but can also inhibit the suppressive activity of Tregs, which have constitutive expression of LAG3 (208, 209). Ongoing clinical trials with LAG3 as a monotherapy have thus far been disappointing, therefore combinatorial approaches with anti-PD1, anti-CTLA4, chemotherapies and chemoradiation are currently underway (NCT03459222, NCT04150965, NCT03978611). Of note, earlier this year, results from RELATIVITY-047 (NCT03470922), a phase 2/3 clinical trial testing the combination of anti-LAG3 (relatimab) and anti-PD1 (nivolumab) *vs.* nivolumab alone demonstrated extended progression free survival (PFS) (10.12 months *vs.* 4.63 months) in patients with advanced melanoma. Importantly, the combination was reasonably tolerated, with only 18.9% of patients experiencing grade 3-4 adverse side effects.

CD161 is encoded by killer cell lectin-like receptors subfamily B member 1 (*KLRB1*) and is expressed on human and murine T and NK cells (210). Importantly, murine CD161 was originally described as a family of seven NK receptors, known as the NKRP1 family; however, only a single human ortholog has been identified (211, 212). In 2005, C-type lectin domain family 2 member D (CLEC2D) was identified as a functional ligand for human CD161, similarly to murine NKRP1B and NKRP1D, suggesting a functional similarity to the human (213, 214). Investigators identified subsets of CD8 T cells that co-expressed common NK cell receptors, where high cytotoxicity signatures correlated with high NK cell receptor expression. Notably, CD161 was expressed on a larger fraction of infiltrating T cells than PD1 and was most highly expressed on clonally expanded CD8 T cells, effector CD4 T cells, but not Tregs.

Using tumor-specific T cells co-cultured with gliomaspheres (3-dimensional clusters of tumor cells derived from human patients), investigators showed that inactivation of *KLRB1* resulted in increased cytotoxic activity. Furthermore, in humanized mouse models, inactivation of *KLRB1* in tumor-infiltrating T cells led to enhanced anti-tumor killing, as evidenced by slower tumor progression, significantly increased survival time and increased infiltration of $PD1^{-}TIM3^{-}$ T cells compared to unaltered T cells. Importantly, using publicly available scRNAseq datasets, the *KLRB1* transcriptional program (genes that had significantly higher expression in $KLRB1^{+}$ T cells *vs.* $KLRB1^{-}$ T cells) was identified in tumor infiltrating T cells from multiple human cancers, including melanoma, NSCLC, hepatocellular carcinoma, and colorectal cancer (CRC). This highlights the CD161-CLEC2D axis for immunotherapeutic targeting more broadly. Researchers further suggested that monoclonal antibody targeting of CD161 could synergize with anti-PD1 treatment by targeting a larger group of infiltrating T cells because of the non-overlapping expression of the two targets. This study introduces the idea that inhibitory NK cell receptor expression on T cells represents a functionally relevant and targetable axis.

4.3.2 Targeting Altered T Cell Metabolism

Therapeutic targets for countering tumor-imposed T cell metabolic and effector dysfunction include TNFR superfamily members 4-1BB and OX40. 4-1BB is an inducible costimulatory receptor that is normally highly expressed on activated CD4 and CD8 T cells, while being expressed at lower levels on NK cells, DCs, monocytes, and B cells. 4-1BB ligand, in turn, is expressed by macrophages, B cells and DCs (215–218).

4-1BB agonism has shown therapeutic promise due to its ability to prolong cytotoxic T cell activation and survival *in vivo* (1, 219). Specifically, 4-1BB improves the function and biogenesis of mitochondria in T cells, helping to improve the overall metabolic fitness of T cells (1, 181). Beyond its metabolic function, 4-1BB signaling also serves a proinflammatory role, activating NK-κB and ERK (1, 117). 4-1BB signaling has proven useful as a means of improving adoptive transfer therapies. The cytoplasmic signaling domain of 4-1BB was added to third generation CAR constructs and was demonstrated to improve both CAR T cell cytotoxicity and survival *in vivo* (220–223).

As a systemic monotherapy, 4-1BB agonism has been demonstrated to increase the anti-tumor response *in vivo* and *in vitro* in pre-clinical studies, though its application in clinical trials has been limited by significant dose-dependent hepatotoxicity. The most significant therapeutic potential for 4-1BB-directed therapy appears to be when it is used in conjunction with ICB. The improvements to metabolic function that occur as a result of activation of signaling pathways downstream of 4-1BB can synergize with the functional implications of blockade of PD1 or CTLA4 signaling networks, allowing activated effector T cells to better overcome the suppressive TME (217, 224). Our group and others have demonstrated the ability of 4-1BB agonism to license PD1 checkpoint blockade *in vivo* (217, 224–226). In our murine model of GBM, 4-1BB agonism + PD1 blockade produced 50% long term survival, whereas PD1 blockade alone offered no benefit, similar to failures that have been observed clinically with these tumors (226).

OX40 is a costimulatory receptor with similar functions to 4-1BB, ultimately promoting the effector function and survival of cytotoxic CD8 T cells (227). OX40 is expressed by activated CD4 and CD8 T cells. In CD8 T cells, OX40 enhances differentiation and proliferative expansion after priming (2). The metabolic effects of OX40 signaling on CD8 T cells are not well-elucidated thus far, though similarly to 4-1BB, OX40 has been shown to increase mitochondrial mass in CD8 T cells (228). Further, the OX40 and 4-1BB pathways appear to have considerable overlap, as combination 4-1BB and OX40 agonism therapy is additive, but not synergistic (217). As with 4-1BB, the improved effector T cell function resulting from OX40 signaling is synergistic with ICB therapy and has additionally been successfully utilized in second and third generation CAR constructs (217, 224, 229). OX40 also serves a role as a suppressor of Treg development and function, in part by blocking differentiation of CD4 T cells into $Foxp3^{+}$ Tregs and by inhibiting regulatory activity of existing Tregs. Thus, OX40 agonism may produce multiple anti-tumor immune benefits (224, 227, 229).

4.3.3 Reviving Exhausted CARs

CAR T cells are also subject to T cell exhaustion. This exhaustion can either be tumor-imposed or due to tonic signaling intrinsic to the CAR construct itself. Efforts to counteract tumor-imposed exhaustion include combining checkpoint blockade with CAR T cell therapy (230, 231), deleting coinhibitory molecules from

CAR T cells (12–14), or generating CAR T cells that secrete checkpoint inhibitors (10, 11). Tonic signaling refers to ligand-independent CAR signaling that is caused by physical characteristics of the CAR construct itself (232). For example, high CAR expression on the surface of T cells can result in background activation signaling. This tonic signaling can induce T cell exhaustion that leads to suboptimal T cell activation in the presence of antigen.

Recently, Weber, et al. explored the effect of "resting" CAR T cells after viral transduction and prior to administration (15). The authors first engineered a GD2-targeting CAR with known problematic tonic signaling to contain an intracellular destabilization domain that required administration of a drug to induce surface CAR expression. CAR expression is thus restrained until tumor antigen is present, preventing CAR T cell exhaustion from tonic signaling. These CAR T cells were more efficacious *in vivo* in xenograft models. Interestingly, forcing "rest" after exhaustion had been imprinted appeared to reverse the exhaustion epigenetic profile. As an alternative approach, the Src kinase inhibitor dasatinib was used to transiently inhibit CAR T cell activation *in vitro* and *in vivo*, abrogating tonic signaling-induced exhaustion and promoting more potent antitumor immunity. Dasatinib allows for immediate applicability of this transient rest approach to a variety of CAR T cell therapies without the burden of redesigning new CAR constructs.

5 DISCUSSION

The dramatic successes that can be observed with immunotherapies have made them a mainstay in the treatment of many cancers. Currently, however, such successes remain limited to a minority of cancer patients, highlighting the need for further research and innovation in this space. Emerging therapies are beginning to take a more complete view of the TME and are uncovering complementary strategies to simultaneously rescue and stimulate immune function and generate therapeutic synergism. For instance, T cell dysfunction exists in multiple forms within the TME, can have multiple sources, and can limit immunotherapeutic efficacy. We are beginning to understand more about the TME-driven origins of such dysfunction, and this understanding is increasingly and appropriately driving therapeutic design. T cell dysfunction can arise from lack of tumor-specificity, abnormal vasculature that limits trafficking, hypoxic/nutrient deprived environments, and immunosuppressive/pro-tumor cells (Tregs and macrophages). Targeting more rationally the mechanisms underlying these barriers to T cell function and combining such strategies for T cell reinvigoration with activating platforms such as ICI, have yielded striking preclinical and clinical results. These successes underscore the importance of a rational, multi-pronged immune-based approach to tumors and their microenvironment.

AUTHOR CONTRIBUTIONS

JW, EL, and DW conceived the manuscript. JW, EL, DW, and AH-M wrote the manuscript. JW, EL, and DW edited the manuscript. PF supervised all aspects of the work. All authors contributed to the article and approved the submitted version.

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