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Regulatory T Cells in Cancer

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Abstract

The immune system has evolved complex effector mechanisms to protect the host against a diversity of pathogenic organisms and regulatory adaptations that can curtail pathological sequelae of inflammatory events, prevent autoimmunity, and assist in tissue repair. Cancers, by virtue of their local manifestations of tissue dysfunction and destruction, inflammation, and genomic instability, can evoke these protective mechanisms, which support the progression of tumors and prevent their immune eradication. Central to these processes is a subset of $CD4^+$ T cells, known as regulatory T (Treg) cells, that express the X chromosome–linked transcription factor FOXP3. In addition to their critical role in controlling autoimmunity and suppressing inflammatory responses in diverse biological settings, Treg cells are ubiquitously present in the tumor microenvironment where they promote tumor development and progression by dampening antitumor immune responses. Furthermore, Treg cells can directly support the survival of transformed cells through the elaboration of growth factors and interacting with accessory cells in tumors such as fibroblasts and endothelial cells. Current insights into the biology of tumor-associated Treg cells have opened up opportunities for their selective targeting in cancer, with the goal of alleviating their suppression of antitumor immune responses while maintaining overall immune homeostasis.

INTRODUCTION

A large body of research suggests that the tumor and immune cell interactions can affect the development of cancer and aid in its control (Dunn et al. 2004). Clinically beneficial therapeutic modulation of the immune system in cancer patients has been demonstrated by numerous modalities, including antibody blockade of inhibitory molecules, adoptive T cell transfer, and autologous cell-based vaccines. Regulatory T (Treg) cells are a subset of $CD4^+$ T cells that are requisite for control of autoimmunity, dampening excessive inflammation caused by the immune response to pathogens, and maintaining maternal-fetal tolerance (Josefowicz et al. 2012). While Treg cells are critical for maintaining peripheral tolerance, their potent immunoregulatory properties can promote the development and progression of numerous types of malignancies. Analysis of tumor-infiltrating lymphocyte (TIL) subsets has shown a clear association between the composition of the immune cell infiltrate and patient survival. TILs primarily composed of cytotoxic T cells are generally associated with a favorable prognosis, whereas an increased proportion of Treg cells among TILs is associated with poor outcomes (Fu et al. 2007, Petersen et al. 2006, Shen et al. 2010). Treg cells exert their suppressive function through several mechanisms (Josefowicz et al. 2012). CD25, a high-affinity binding subunit of the IL-2 receptor and the historical Treg cell surface marker, is constitutively and highly expressed on Treg cells but also upregulated on effector T cells (Fontenot et al. 2005). The high level of CD25 expression on Treg cells could deprive effector T cells of IL-2 and inhibit their proliferation (Chinen et al. 2016, Pandiyan et al. 2007). CTLA-4 (cytotoxic T lymphocyte antigen 4) is well known to limit responses of activated T cells and is also implicated in Treg cell–mediated suppression (Wing et al. 2008). Treg cells can produce multiple immunomodulatory proteins (see below) (von Boehmer 2005), are generally considered to be the most potent inhibitor of antitumor immunity, and are suspected to be responsible for the limited response rates to current immunotherapeutic regimens (Zou 2006). Treg cell differentiation and function are dependent on the expression of the lineage-specifying transcription factor FOXP3 (Rudensky 2011). Mice with a loss-of-function mutation in *Foxp3* lack functional Treg cells and develop lethal autoimmunity and lymphoproliferative disease at an early age (Fontenot et al. 2003, Khattri et al. 2003). Similarly, humans harboring mutations in the *FOXP3* gene suffer from immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) manifested in widespread autoimmune and inflammatory lesions (Bennett et al. 2001). The emerging modalities of Treg cell–focused therapies are context specific; in inflammatory, allergic, and autoimmune diseases the goal is to augment Treg cell numbers and functional activity while inhibiting their suppressive mechanisms or depleting them in cancer. Strategies for developing immunotherapies that target Treg cells need to take into account that they are essential for immune homeostasis. Here, we provide an overview of Treg cell biology, discuss the features and roles of Treg cells in the tumor microenvironment (TME), and review the means by which they can be targeted for cancer immunotherapy.

REGULATORY T CELL PHENOTYPE AND FUNCTION

Over a century ago Paul Ehrlich first postulated the existence of regulatory mechanisms protecting against autoimmunity: "the organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organism's own elements" (Ehrlich 1906, p. 253). Jacques Miller discovered that the thymus functions as the generation site of a major class of lymphocytes, T cells, and observed that thymectomy before day 3 of life unexpectedly results in a wasting disease (McIntire et al. 1964). Nishizuka & Sakakura (1969) further determined that the wasting disease in part was secondary to the autoimmunity. Reversal of autoimmunity induced by neonatal thymectomy by the adoptive transfer of

thymocytes or splenocytes from adult mice led to the realization that a population of cells generated in the mouse thymus after 3 days of life mediates tolerance in a dominant cell-extrinsic manner (Sakaguchi 2004). The concept of a suppressive T cell subset was fortified with the landmark findings of Sakaguchi et al. (1995), who described a population of CD25-expressing $CD4^+$ T cells capable of suppressing autoimmune responses in several experimental models. Correlative in vitro studies in humans confirmed the existence of suppressive $CD4^+CD25^+$ T cells (Ng et al. 2001). With the increasing availability of tools for phenotyping T cell subsets, the existence of a suppressive T cell subset became established, and these cells were named "regulatory" or "Treg" cells. However, there was some lingering doubt as to whether Treg cells were a distinct T cell lineage, as their characteristic features—including increased expression of CD25, CTLA-4, and the TNF receptor family member GITR (glucocorticoid-induced TNF receptor)—were also found on activated conventional $CD4^+$ T (Tconv) cells. In an effort to identify a biologically important marker specific to Treg cells, researchers conducted an intense exploration of genetic mechanisms underlying the differentiation and function of Treg cells, which resulted in the identification of FOXP3, an X chromosome–encoded forkhead transcription factor family member (Fontenot et al. 2003, Hori et al. 2003, Khattri et al. 2003) whose high level of expression is characteristic of Treg cells in experimental animals and humans. *Foxp3* was identified as the gene whose loss-of-function mutation in IPEX patients and in the spontaneously *Scurfy*-mutated mouse is responsible for fatal widespread early-onset autoimmunity (Bennett et al. 2001, Brunkow et al. 2001, Chatila et al. 2000, Wildin et al. 2001). While FOXP3 is also highly expressed in human $CD25^+CD4^+$ T cells that have a suppressor function, activated human Tconv cells also upregulate FOXP3, but only transiently and at a significantly lower level than the characteristically stable high–FOXP3 expression levels in Treg cells (Walker et al. 2003). Genetic cell fate mapping studies have shown a remarkable stability of differentiated Treg cells and a heritable maintenance of FOXP3 expression under physiologic and inflammatory conditions, strongly supporting the idea that Treg cells represent a dedicated lineage (Chen et al. 2003). A dedicated mechanism for Treg cell stability has been highlighted by the demonstration that an intronic *Foxp3 cis*-regulatory element CNS2 (conserved noncoding sequence 2) is essential for heritable maintenance of Treg cell identity during cell division when proinflammatory cytokines, promoting alternative cell fate, are present (Feng et al. 2014, Zheng et al. 2010). CNS2 [also known as TSDR (Treg cell–specific demethylated region)] contains a set of CpG residues that undergoes demethylation in fully differentiated Treg cells—a step essential for stable Foxp3 expression. Thus, CNS2 enforces the stability of the differentiated cell state in the Treg cell lineage in fluctuating environments, and this unique function of CNS2 is important in diverse chronic inflammation types including autoimmunity, chronic infection, metabolic inflammation, and cancer (Feng et al. 2014). Demethylation of CNS2 (TSDR) is presently viewed as a proxy for bona fide Treg cells with sustained functional capacity.

The neonatal thymectomy showed that the thymus is an essential source of Treg cells. The differentiation of thymic-generated Treg (tTreg) cells depends on high-avidity interactions with self-peptide/MHC class II complexes and IL-2 receptor signaling (Josefowicz & Rudensky 2009). Additionally, Treg cells could also be generated outside the thymus. These extrathymically generated or peripheral Treg (pTreg) cells develop from naïve $CD4^+$ T cells upon exposure to antigenic stimulation under tolerogenic conditions. Specifically, strong TCR (T cell receptor) signaling, suboptimal costimulation, and high amounts of TGF-β and retinoic acid favor the induction of FOXP3 in peripheral naïve CD4 T cells (Chen et al. 2003, Josefowicz et al. 2012). The differences between these Treg cell subsets suggest that tTreg cells are likely responsible for tolerance to self-antigens, whereas Treg cells restrain immune responses to so-called non-self-antigens including allergens, commensal microbiota, dietary antigens at barrier sites, and paternal alloantigens. The

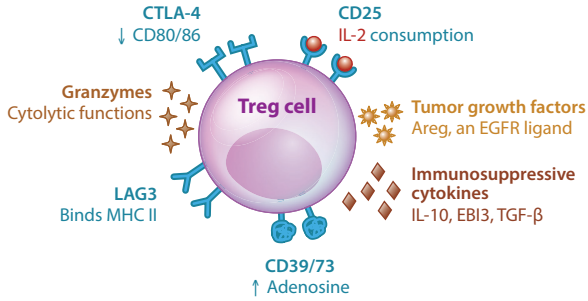

Figure 1

Mechanisms of regulatory T (Treg) cell suppression. Treg cells engage multiple mechanisms by which they suppress immune responses and support the progression of cancer. Targets of suppression include innate and adaptive immune cells, elaboration of tumor growth factors such as the epithelial growth factor receptor (EGFR) ligand amphiregulin (Areg), and modulators of metabolism.

relative contributions of tTreg and pTreg cells to progression to different cancer types remain to be established.

The suppressor function of Treg cells is essential from restraint of fatal autoimmune and inflammatory responses throughout the lifespan of an organism (Kim et al. 2007). Transcriptional profiling and flow cytometric analyses of Treg cells have revealed several candidate molecules that could potentially mediate suppression (**Figure 1**). These include CD39 ecto-nucleotidase, which is highly expressed on both human and mouse Treg cells. The latter cells also express high amounts of CD73. This pair of ecto-enzymes facilitates the conversion of extracellular ATP, which has a potent proinflammatory activity, to adenosine, which by acting through A2A receptor inhibits the proliferation of effector T cells and the activation of dendritic and myeloid cells. Treg cells also produce several secreted immunomodulatory proteins including IL-10, granzymes, and TGF-β (tumor growth factor β) (Vignali et al. 2008). IL-10 production by Treg cells is essential for keeping the immune response in check at environmental interfaces such as the colon, lungs, and likely in the TME (Rubtsov et al. 2008, Sawant et al. 2019). Furthermore, high levels of CTLA-4 expression by Treg cells may enable them to suppress costimulatory capacity of dendritic cells by restraining CD80 and CD86 expression (Wing et al. 2008). Congenital CTLA-4 deficiency limited to Treg cells is associated with a lymphoproliferative disorder. However, conditional ablation of CTLA-4 in differentiated Treg cells in adult mice does not precipitate systemic autoimmunity, suggesting the existence of compensatory regulatory mechanisms independent of CTLA-4 or a role of CTLA-4 in Treg cell differentiation (Paterson et al. 2015). Finally, IL-2 consumption by Treg cells is essential for control of $CD8^+$ T cell responses but is dispensable for control of $CD4^+$ T cell activation and expansion (Chinen et al. 2016). TCR and IL-2 receptor signaling are required for the suppressor activity of Treg cells (Chinen et al. 2016, Levine et al. 2014). However, Treg cells employ multiple mechanisms of suppression and no single mechanism can account for the entire spectrum of their suppressor function.

REGULATORY T CELLS IN CANCER

An increased frequency of Treg cells in the TME is associated with an adverse prognosis in several cancer types (Fu et al. 2007, Petersen et al. 2006, Shen et al. 2010). One notable exception is colorectal cancer, where Treg cells may suppress tumor-promoting inflammatory responses elicited by the gut microbiota (Saito et al. 2016). The frequency of Treg cells may also have implications

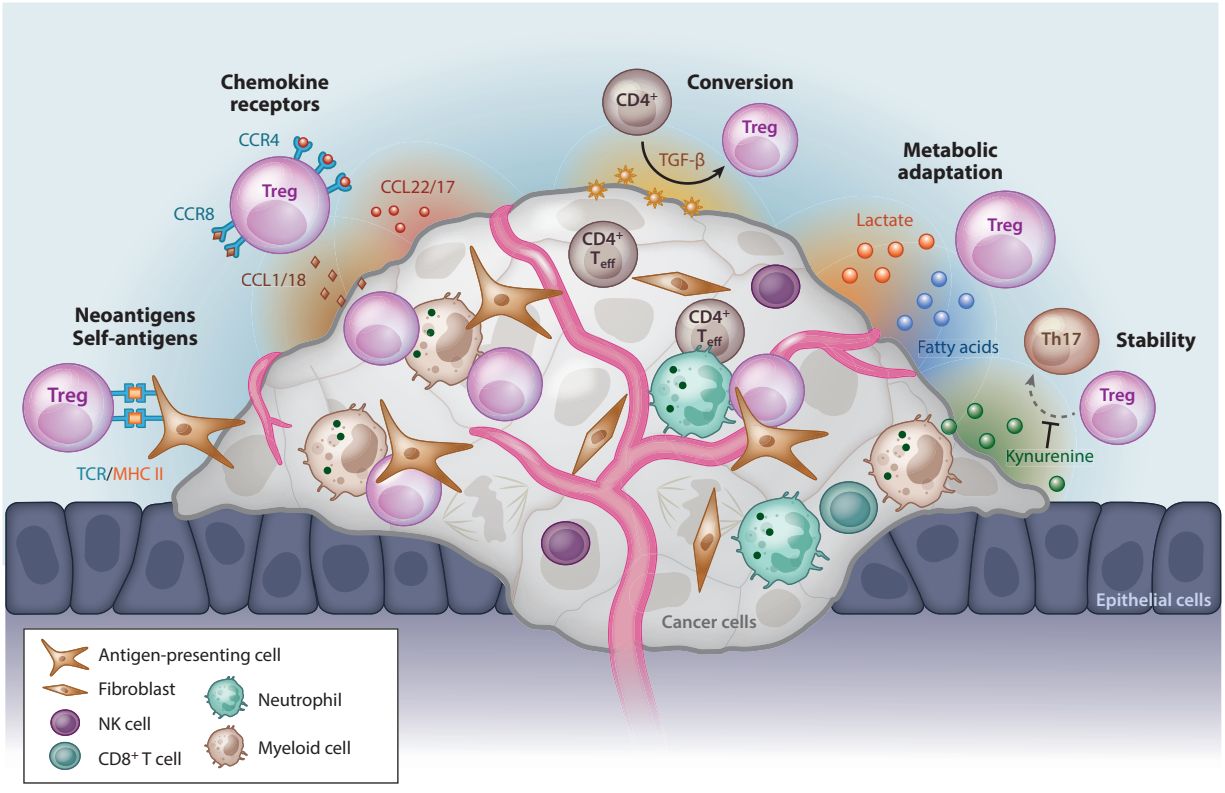

Figure 2

Recruitment and maintenance of Treg cells in the tumor microenvironment. Multiple factors contribute to the increased frequency of Treg cells in tumors over normal tissues. Treg cells express specific chemokine receptors whose ligands are produced by various cells in the tumor microenvironment. Several mechanisms exist that contribute to the survival, stability, and proliferation of Treg cells in tumors, including conversion from $CD4^+$ T cells, metabolic adaptation, and stability of FOXP3 expression. Neoantigen and self-antigen reactivity may also contribute to the prevalence of Treg cells in neoplastic tissues. Abbreviations: MHC II, major histocompatibility complex class II; NK, natural killer; TCR, T cell receptor; T_{eff}, effector T cell; Treg, regulatory T.

for predicting response to immunotherapy. Higher pretreatment levels of circulating Treg cells were associated with improved survival in melanoma patients treated with ipilimumab (Martens et al. 2016). Similarly, in non-small-cell lung cancer patients, a positive correlation has been observed between response to PD-1/PD-L1 blockade and the frequency of $PD\text{-}L1^+$ Treg cells in the TME (Wu et al. 2018). Treg cells can suppress antitumor immune responses using several different mechanisms (**Figure 2**). The high level of expression of IL-2Rα chain could deprive local environments of IL-2 and negatively impact effector T cell function and expansion (Pandiyan et al. 2007). The modulation of IL-2 levels by Treg cells also has important consequences for NK (natural killer) cell homeostasis and function (Gasteiger et al. 2013). Treg cells also regulate antigen-presenting cells as noted above, with a pronounced dendritic cell expansion and activation acutely upon Treg cell depletion (Kim et al. 2007). This suppression may in part be due to CTLA-4-dependent downregulation of CD80 and CD86 expression by a process termed trans-endocytosis (Qureshi et al. 2011). Cytolysis of target cells as a means for Treg cell–mediated suppression was first suggested by the finding that, in human Treg cells, granzyme A can be induced by a combination of CD3 and CD46 stimulation, resulting in the induction of apoptosis in

activated target cells (Grossman et al. 2004). In mice, granzyme B and perforin produced by Treg cells can lead to NK and $CD8^+$ T cell death and suppression of tumor clearance (Cao et al. 2007). CD39 and CD73 highly expressed on Treg cells can affect the TME, with its prevalent necrotic cell death associated with the release of ATP, by facilitating the conversion of ATP, the elaboration of adenosine, and the extrusion of cAMP (Deaglio et al. 2007). Adenosine signaling initiated by Treg cells not only directly inhibits the proliferation of effector T cells but also negatively regulates dendritic cells. Additionally, a high level of Treg cell surface expression of an MHC class II binding inhibitory receptor LAG-3, homologous to CD4, may facilitate the ability of Treg cells to suppress activation of dendritic cells (Liang et al. 2008).

Treg cells in tumors display a highly activated phenotype with high levels of CD45RO and correspondingly low levels of CD45RA, as well as strong expression of costimulatory (GITR, ICOS, OX40) and coinhibitory (CTLA-4, PD-1, LAG3) molecules (Azizi et al. 2018, De Simone et al. 2016, Plitas et al. 2016). The covariance of expression of these molecules contributes to the phenotypic heterogeneity of Treg cells in tumors (Azizi et al. 2018). The observed increased frequency of Treg cells among TILs is likely secondary to several factors. Several lines of evidence point to recruitment of Treg cells through chemokine-dependent migratory cues as a means of their accumulation in the TME. Chemokines are a superfamily of chemotactic secreted proteins that control leukocyte migration through G protein–coupled receptors on target cells, and they play a role both in the recruitment of immune and inflammatory cells for antitumor response and of immunomodulatory cells, including Treg cells, and in the selective homing of neoplastic B and T cells (Nagarsheth et al. 2017). For example, Treg cells can be recruited to squamous cell carcinomas and colorectal cancer tumors in a CCR5-dependent manner (Ward et al. 2015). Hypoxia-driven secretion of CCL28 from hepatocellular carcinoma cells promoted recruitment of Treg cells into liver tumors, which then promoted in turn angiogenesis through VEGF expression (Ren et al. 2016). The chemokine receptor CCR4, highly expressed by Treg cells, has been implicated in their trafficking to several nonlymphoid organs and tumors, and it has been suggested that Treg cell migration into the tumor can be inhibited through CCR4 blockade (Colombo & Piconese 2007). Another chemokine receptor, CCR8, has also been identified as preferentially expressed on tumor-resident Treg cells that exhibit chemotaxis to the CCR8 ligand CCL1 (De Simone et al. 2016, Plitas et al. 2016). CCR8 expression can be selectively induced on CCR8 Treg cells by a yet unknown soluble factor from the TME (Plitas et al. 2016).

The similarities between tumor- and normal tissue–resident Treg cells may seem to indicate that the origins of intratumoral Treg cell populations derive from their normal tissue counterparts rather than recruitment from the circulation (Plitas et al. 2016). On a functional side, the tissue repair function of normal lung–resident Treg cells in mice is recapitulated by Treg cells from lung tumors (Arpaia et al. 2015, Green et al. 2017). Lung-resident Treg cells produce the EGFR ligand amphiregulin, which promotes normal and malignant epithelial cell growth and may act on other cells both in normal lung and in the TME. However, it is much more likely that these observations reflect imprinting of the overall similar features by normal tissue and the TME and that the observed differences can be accounted for by varying the availability of oxygen and metabolites, as well as Treg cell activation. In addition, when comparing the T cell receptor repertoire of Treg cells from normal breast tissue and breast cancers in matched patients, researchers have found little overlap, suggesting that the local expansion of tissue-resident Treg cells is unlikely the origin of intratumoral Treg cells, which is consistent with the notion above that the TME imprints Treg cells with unique phenotypic and functional properties (Plitas et al. 2016). Another means by which Treg cells may accumulate in the TME is through the conversion of Tconv cells upon TCR stimulation in the presence of TGF-β in a manner dependent on the aforementioned CNS1 enhancer (Zheng et al. 2010). Immunosuppressive factors in the TME including TGF-β,

IL-10, and VEGF can promote the differentiation of pTreg cells, facilitated by tolerogenic antigen–presenting cells (Zhou & Levitsky 2007). Indoleamine 2,3-dioxygenase (IDO)-expressing APCs may promote the conversion of Treg cells through an aryl hydrocarbon receptor (Curti et al. 2007). It would be expected that if the major source of intratumoral Treg cells is from conversion of Tconv cells, there would be considerable overlap in the T cell receptor repertoire between clonally expanded effector and Treg cells. This, however, has not been observed in the human breast cancers where very limited overlap was observed between Treg and Tconv cells isolated from tumors, comparable to that between Treg and Tconv cells in normal breast tissue obtained from the same patient through contralateral mastectomy (Plitas et al. 2016). These findings have also been recapitulated in human melanoma samples where the TCR repertoires of intratumoral Treg cells displayed significant overlap with peripheral blood Treg cells but not with Tconv cells in tumor or blood (Ahmadzadeh et al. 2019). Interestingly, TCRs isolated from tumor-resident Treg cells can display reactivity against mutated neoantigens, suggesting activation and clonal expansion in the TME (Ahmadzadeh et al. 2019). Treg cells specific for the cancer-testes antigen NY-ESO-1 can be found in the peripheral blood of patients with melanoma, and their abundance correlates with an adverse prognosis (Vence et al. 2007). In patients with colorectal cancer, tumor antigen–specific Treg cells can be readily identified, and their depletion leads to an increase in effector T cell responses against the same antigens (Bonertz et al. 2009).

METABOLIC ADAPTATIONS OF REGULATORY T CELLS IN THE TUMOR MICROENVIRONMENT

Nutrient and metabolic signals modulate T cell differentiation and function (Pearce et al. 2013). TCR engagement leads to a specific metabolic program mediated by signaling through the PI3K-mTOR pathway, leading to increased amino acid and glucose uptake to fuel aerobic glycolysis. The balance of metabolic pathways can modulate the fate of T cell differentiation. For example, the relative degree to which glycolysis or fatty acid oxidation is utilized by an uncommitted precursor cell can strongly impact its Th17 (T helper 17) and Treg differentiation through differential activity of mTOR, HIF-1α, and PPAR-γ, which in turn can modulate the expression of the corresponding lineage-restricted factors RORγt and FOXP3 (Berod et al. 2014, Klotz et al. 2009). The TME supports the bioenergetic needs of cancer cells reliant on aerobic glycolysis, fatty acid synthesis, and glutaminolysis (Hanahan & Weinberg 2011). This nutrient-poor, lactate-rich, hypoxic, and acidic environment poses a considerable restrain on effector T cells, which rely primarily on glycolysis (Buck et al. 2017). To the contrary, Treg cells may have a metabolic advantage in this environment by utilizing alternative substrates and metabolic pathways for energy production, such as fatty acid oxidation and oxidative phosphorylation. FOXP3 orchestrates this distinct metabolic program by suppressing glycolysis, leading to enhanced oxidative phosphorylation and increasing nicotinamide adenine dinucleotide oxidation, potentially allowing Treg cells to resist lactate-mediated suppression of T cell function in the TME while also enabling Treg cells' use of lactate as a fuel source (Angelin et al. 2017, Fischer et al. 2007). In addition to lactate, fatty acids generated in the TME by transformed cells or tumor-associated stromal cells (Hoy et al. 2017, Rohrig & Schulze 2016) can inhibit effector T cells, while promoting the progression of carcinomas (Kleinfeld & Okada 2005, Lochner et al. 2015, Ma et al. 2016). Treg cells engage multiple mechanisms that allow for their adaptation to the increased presence of fatty acids. Tumor-resident Treg cells in murine models of cancer display intracellular lipid accumulation attributable to an increased rate of fatty acid synthesis, and Treg cells from human breast cancers engage a transcriptional program supporting lipid synthesis (Pacella et al. 2018). Furthermore, oxidative metabolism of lipids by Treg cells not only decreases their demand for glucose but also confers resistance to

fatty acid–induced cell toxicities (Howie et al. 2017). Metabolic competition between Treg cells and effector cells has also been proposed to lead to senescence in effector T cells as mediated by ATM-associated DNA damage, which can be reversed by TLR8 signaling (Liu et al. 2018). The decreased concentrations of glutamine in the TME may also contribute to the increased frequency of Treg cells, as glutamine deprivation has been suggested to promote FOXP3 expression. Specifically, the loss of glutamine-dependent α-ketoglutarate production and a decreased UDP-GlcNAc synthesis in T cells have been shown to favor the differentiation of pTreg cells (Araujo et al. 2017, Klysz et al. 2015). However, the degree to which pTreg cell differentiation contributes to the overall Treg cell pool in different types of cancers remains to be investigated.

Apart from adapting to the unique metabolic constraints of the TME, Treg cells can directly shape it. For example, the aforementioned Treg cell expression of CD39, acting along with C73 expressed by chronically stimulated intratumoral $CD8^+$ T cells and other cell types, promotes the conversion of ATP to adenosine (Maj et al. 2017). Since extracellular ATP is immunostimulatory and adenosine is immunosuppressive (Antonioli et al. 2013), therapeutic targeting of this pathway can promote antitumor immune responses and synergize with checkpoint inhibitors (Willingham et al. 2018). The increased local catabolism of tryptophan by IDO can also contribute to attenuated antitumor immunity in part through activation of Treg cells in the TME. Tumor cell expression of IDO correlates with an increased frequency of Treg cells in the TME (Godin-Ethier et al. 2011). IDO can also inhibit the reprogramming of Treg cells into Th17 cells by reducing IL-6 production by dendritic cells (Chai 2011). The PI3K-Akt-mTOR pathway is an important regulator of Treg cells' stability and function in the TME, leading to inhibition of suppressive function and reprogramming into effector-like cells in the setting of TCR activation (Munn & Mellor 2016; Sharma et al. 2009, 2015). IDO activity can help to preserve the stability of Treg cells in the TME by opposing the mTORC2-Akt pathway (Sharma et al. 2015). Kynurenine, a tryptophan metabolite, can increase the suppressive activity of Treg cells and promote their expansion by acting as an endogenous ligand of the aryl hydrocarbon receptor (Baban et al. 2009, Fallarino et al. 2006). It is becoming increasingly clear that the distinct metabolic programs of, and competition for metabolites between, tumors and different immune cell subsets in the TME have a significant impact on antitumor immunity and represent a promising space for therapeutic intervention.

REGULATORY T CELL–BASED THERAPEUTIC INTERVENTIONS

Studies in preclinical cancer models in mice strongly indicate that Treg cell depletion can induce strikingly effective antitumor immune responses (Bos et al. 2013, Green et al. 2017, Onda et al. 2019). The major issues with a depletion strategy have been that potential molecular targets are shared between Treg cells and effector cells, as well as the potential for devastating autoimmune toxicities that may result from an indiscriminate Treg cell depletion. Preclinical and clinical findings form the basis of current efforts to develop effective Treg cell targeting methods, including transient generalized depletion, local depletion, or inactivation of intratumoral Treg cells. Thus, advances in the understanding of basic principles of Treg cell biology are beginning to translate into clinical practice.

CD25

CD25 has been a recurring focus of strategies to therapeutically target Treg cells due to both its very high expression on Treg cells and IL-2 being a critical factor of Treg cell differentiation, maintenance, and function. The concern regarding immune-related toxicities has been of continued relevance in this line of research, as blocking IL-2 or CD25 in mice resulted in various manifestations of autoimmunity (Chinen et al. 2016, McHugh & Shevach 2002, Setoguchi

et al. 2005). Depending on the Fc receptor interaction of CD25-specific antibodies, CD25 can be blocked or used to deplete Treg cells, with very different systemic immune consequences (Huss et al. 2016). Initial attempts at Fc receptor–mediated depletion did demonstrate induction of antitumor immune responses; however, also evident were manifestations of autoimmunity (Shimizu et al. 1999).This strategy was translated to the clinic in trials of the anti-CD25 antibody dacluzimab, which were largely unsuccessful likely due to the propensity of dacluzimab to deplete Treg- and CD25-expressing effector cells (Jacobs et al. 2010, Rech et al. 2012). Recent work, however, suggests that conventional anti-CD25 antibodies did not effectively deplete Treg cells in the TME potentially due to the upregulation of the inhibitory Fc gamma receptor at the tumor site; engineering the antibodies to selectively bind activating Fc gamma receptors may be more efficacious (Arce Vargas et al. 2017). An alternative strategy of targeting IL-2R-expressing cells including Treg cells relies on the use of a recombinant IL-2 diphtheria toxin fusion protein, also known as ONTAK. Like Treg cells expressing high levels of CD25, ONTAK was efficacious for the treatment of cutaneous T cell lymphoma and its administration was also shown to significantly decrease Treg cells in peripheral blood (Mahnke et al. 2007, Olsen et al. 2001). However, further development was hampered by production issues and a major dose-limiting toxicity due to vascular leak syndrome, leading to the recent engineering of a second-generation agent (Cheung et al. 2019).

Cosignaling Molecules

Cosignaling molecules expressed by immune cells can be divided functionally into costimulatory (e.g., CD28, ICOS, TNFR2, OX40, and 4-1BB) and coinhibitory (e.g., CTLA-4, PD-1 TIM3, LAG3, and TIGIT) receptors. The differential expression and activity of these molecules directs immune responses in a context-dependent fashion by promoting activation and differentiation of T cells while providing negative feedback and affording fine-tuned immunoregulation. While conventional (effector) T cells generally require antigen exposure and subsequent activation to upregulate cosignaling molecules, many of them are constitutively expressed by Treg cells (CTLA-4, OX40, GITR, etc.)—likely owing to heightened TCR signaling required for their generation—and are important to their differentiation, survival, and suppressive activities. High cell surface expression of some such molecules, including PD-1, ICOS, TIGIT, LAG3, TIM3, TNFR2, and 4-1BB, can be viewed as rationale for antibody development for potential Treg-specific targeting (Anderson et al. 2016, Bour-Jordan & Bluestone 2009).

In addition to being highly expressed on and functionally important for Treg cells, CTLA-4 affords an essential cell-intrinsic negative feedback mechanism of effector T cell responses (Paterson et al. 2015). Ipilimumab, an IgG1 anti-CTLA-4 antibody, has been clinically validated as an effective immunotherapeutic agent (Robert et al. 2011). The mechanism of action of anti-CTLA-4 antibodies in preclinical tumor models is dependent on both Treg cell depletion and effector T cell activation (Peggs et al. 2009). Utilizing depleting rather than neutralizing CTLA-4 antibodies as a means of targeting Treg cells may have a greater impact, as Treg cells from CTLA-4-knockout mice are still capable of mediating immune suppression (Lutsiak et al. 2005). In humans, it is not clear if the predominant mechanism underlying therapeutic efficacy of CTLA-4 antibodies is Treg cell depletion or the expansion and activation of effector cells directly. While some analyses of clinical samples suggest that ipilimumab can deplete intratumoral Treg cells (Romano et al. 2015, Tarhini et al. 2014), other studies failed to observe it (Ferrara et al. 2018). In this regard, tremelimumab, an IgG2 isotype CTLA-4 antibody with minimal potential antibody-dependent cytotoxicity, appears to have similar clinical activity as ipilimumab, suggesting that Treg depletion is not a critical mechanism by which CTLA-4 antibodies work (Ferrara et al. 2018). The degree to which CTLA-4 antibodies deplete Treg cells in tumors and the contribution of this effect to

their overall therapeutic efficacy are likely influenced by host Fc receptor polymorphisms and the availability of effectors of antibody-dependent cellular cytotoxicity in the TME (Arce Vargas et al. 2018). There is current considerable interest in engineering CTLA-4 antibodies with enhanced Fc receptor activity to deplete Treg cells. However, there is the issue of concomitantly depleting activated effector cells, and it may be beneficial to incorporate this understanding in sequencing therapeutic modalities with vaccination strategies or other immunostimulatory strategies (Ha et al. 2019). Similar to CTLA-4, PD-1 is also highly expressed by tumor-resident Treg cells. Targeting the PD-1/PD-L1 pathway is the most effective of current immunotherapies, and anti-PD-1/PD-L1 antibodies are thought to mediate their effect by maintaining the phosphorylated state of Shp2 phosphatases downstream of CD28 costimulatory signals, leading to T cell activation (Dong et al. 2002, Hui et al. 2017, Iwai et al. 2002, Nishimura et al. 1999). Interestingly, loss of PD-1 expression on Treg cells also leads to enhanced suppressive function, which may appear counterintuitive considering the clinical activity of PD-1/PD-L1 blockade (Sage et al. 2013). However, this observation raises the possibility that the activity of Treg cells during PD-1-based therapies may limit effectiveness, and combination interventions targeting Treg cells along with PD-1 warrant investigation.

The TNFα (tumor necrosis factor alpha) superfamily is composed of more than 40 members and is associated with a similar number of membrane or soluble receptors (TNFR). Costimulation through the TNFR superfamily is critically important in Treg cell differentiation and functionality (Mahmud et al. 2014). GITR, a member of the TNFR superfamily constitutively expressed on Treg cells, can also be induced upon activation of effector $CD4^+$ and $CD8^+$ T cells (Schaer et al. 2012). GITR activation leads to effector T cell activation and inhibition of Treg cell activity in tumors (Aida et al. 2014, Ko et al. 2005). Agonistic GITR antibodies have shown antitumor activity in numerous preclinical models both alone and in combination with other immunotherapies (Kim et al. 2015, Leyland et al. 2017). The mechanisms underlying these responses are thought to be largely related to a reduction in the number of intratumoral Treg cells and a concomitant increase in effectors (Cohen et al. 2010). The mechanism by which agonistic GITR antibodies reduces the frequency of Treg cells in tumors is likely secondary to depletion of a relatively activated subset of Treg cells (Mahne et al. 2017). Several GITR agonists are currently in early-phase clinical trials, with two early reports that suggest that they have antitumor activity in combination with a PD-1 antibody but no such activity as monotherapy (Pacella et al. 2018, Siu et al. 2017). OX40 is expressed on several TIL subsets including Treg cells in multiple cancer types (Aspeslagh et al. 2016). Tumor-resident Treg cells preferentially express high levels of OX40, and intratumoral delivery of anti-OX40 antibodies in mice leads to Treg cell depletion, which can be synergistic with other local immunotherapeutic approaches (Marabelle et al. 2013, Piconese et al. 2008). However, these observations are in contrast to what has been observed in human clinical trials, where there was no significant depletion of Treg cells but there was an increase in the proliferation of effector cells and some clinical responses (Curti et al. 2013). Combination therapies are also being investigated clinically; however, the preclinical data are cautionary, as combining OX40 agonism with concurrent PD-1 blockade resulted in a less-effective therapy than OX40 agonism alone (Shrimali et al. 2017). RANKL is another member of the TNF-α superfamily (TNFSF11) and binds to a membrane receptor named RANK (receptor activator of nuclear factor-κB), a member of the TNFR superfamily (TNFRSF11A) (Ahern et al. 2018). RANK and RANKL are commonly expressed in the TME and can impact the antitumor immune response (Chu & Chung 2014). RANK-expressing tumor-associated macrophages potentiate Treg cell proliferation and expression of RANKL, which can potentiate breast cancer metastases (Gonzalez-Suarez et al. 2010, Li et al. 2010, Tan et al. 2011). This feedback loop can be therapeutically targeted by RANKL blockade in association with Treg cell depletion (Ahern et al. 2017). TNF binds to two distinct

receptors: TNFR1, which is ubiquitously expressed, and TNFR2, which is predominantly expressed by Treg cells, a subset of neural cells, and endothelial cells (Kalliolas & Ivashkiv 2016). TNFR2 signaling can expand Treg cells, which may be protective against graft-versus-host disease (Chopra et al. 2016). Similarly, antibodies antagonizing the function of TNFR2 inhibit the proliferation of Treg cells and may be an effective immunotherapy (Torrey et al. 2017).

Chemokine Receptors

Differential gene expression analyses have revealed that tumor-resident Treg cells may have a distinct expression pattern of chemokine receptors that may be exploited for therapeutic purposes (De Simone et al. 2016, Plitas et al. 2016). CCR4, which is highly expressed on Treg cells and other $CD4^+$ T cell subsets, is important for Treg cell migration, and accordingly, its ligands are expressed by various cells in the TME (Curiel et al. 2004, Faget et al. 2011). This interaction has been the focus of numerous therapeutic strategies. A small-molecule inhibitor of CCR4 designed in silico has been shown to prevent the interaction of CCL22 and CCL17 with CCR4 and consequently inhibit the recruitment of Treg cells in experimental models (Davies et al. 2009). Further work with this molecule showed that CCR4 antagonism could enhance dendritic cell–mediated $CD4^+$ T cell proliferation in vitro, as well as offer potent adjuvant activity in an in vivo viral infection model (Bayry et al. 2008). Similarly, in tumor models, CCR4 antagonism induced expansion of antigen-specific $CD8^+$ T cells and, in combination with vaccination, promoted tumor immunity (Pere et al. 2011). Treg cell depletion using mogamulizumab, a depleting (defucosylated to enhance cytotoxicity) CCR4 antibody, may augment antitumor immune responses, yet no significant clinical responses have been reported so far in current clinical trials. The latter can be due to depletion of several T cell subsets other than Treg cells, raising the concern that these populations may be important effector cells (Kurose et al. 2015). Further comparisons of Treg cells from malignant and normal tissues have revealed CCR8 to be a marker for tumor-resident Treg cells with greater specificity and displaying a considerably more restricted expression pattern than CCR4 (De Simone et al. 2016, Magnuson et al. 2018, Plitas et al. 2016). CCR8-deficient mice have a normal lifespan and do not develop spontaneous disease, suggesting that targeting CCR8 as a cancer immunotherapy may have a safer toxicity profile than molecules that are central to immune homeostasis such as CTLA-4 and IL-2 (Chensue et al. 2001). A murine model of colon cancer targeting CCR8 with an antibody led to reduced tumor growth, suggesting that targeting Treg cells via CCR8 may be efficacious (Villarreal et al. 2018).

Conventional Cytotoxic Agents

It has been repeatedly observed that conventional therapies that target malignant tumors are associated with enhanced antitumor immunity. One of the earliest examples is the association of low-dose cyclophosphamide, a chemotherapeutic agent, with enhanced immune responses (Rollinghoff et al. 1977). This effect was linked to the depletion of suppressor cells, which were eventually identified as Treg cells (Ghiringhelli et al. 2004). More detailed analyses revealed that low-dose cyclophosphamide can not only deplete Treg cells but also inhibit their functionality. The mechanism may in part be secondary to the increased proliferative state of the Treg cell population, rendering these cells susceptible to alkylating agents, and to the lack of the transporter necessary for extruding intracellular cyclophosphamide (Dimeloe et al. 2014). Interestingly, the impact of cyclophosphamide on Treg cells is not unique and has been observed with other chemotherapeutic agents (Galluzzi et al. 2012). Clinically, this effect has been utilized in many trials looking to increase the immunogenicity of therapeutic interventions by concomitantly depleting Treg cells with low-dose cyclophosphamide. Some encouraging associations have

been made between outcomes of patients with colorectal cancer treated with a vaccine-plus-cyclophosphamide combination and the degree of Treg cell depletion in the peripheral blood (Scurr et al. 2017). However, in larger trials the addition of cyclophosphamide did not improve outcomes (Rini et al. 2016). The observed discrepancies may be due to the depletion of effector cells or the induction of other suppressive immune cell subsets such as myeloid-derived suppressor cells (Sevko et al. 2013). Further research will hopefully elucidate if there will be a role for chemotherapy as an immunomodulatory agent in the treatment of cancer. Radiotherapy is also a commonly used cytotoxic modality through the induction of DNA damage and free radical formation. Radiation can lead to antitumor immune responses, which also can contribute to its therapeutic effectiveness (Demaria & Formenti 2013). Radiotherapy likely contributes to the activation of innate immune and tissue damage sensors and to antigen release from dying cancer cells, leading to enhanced antigen presentation by dendritic cells, while reducing the tumor mass and, therefore, the chances of tolerance induction in newly recruited effector T cells (Formenti & Demaria 2009). Thus, the adjuvant-type of immunological effects of radiotherapy may synergize with immunotherapy (Formenti et al. 2018). However, there are also known immunosuppressive effects of radiotherapy with an increase in the frequency of Treg cells featuring prominently in stereotactically irradiated tumors (Muroyama et al. 2017). This is likely secondary to the known relative radiation resistance of Treg cells (Komatsu & Hori 2007). Targeting Treg cells has been shown to markedly improve the therapeutic efficacy of radiotherapy and warrants further clinical investigation (Bos et al. 2013).

CONCLUDING REMARKS

Tumor growth requires the mutual adaptation of cancerous cells and surrounding accessory cells, encompassing cells of both the adaptive and innate immune systems. These adaptations include shaping of the cellular and molecular features of genetically unstable tumors by the host immune system. Central to these adaptive mechanisms is the recruitment of Treg cells, whose potent suppressive capabilities inhibit effector T cells, confer tumor-growth-promoting properties on myeloid cells, and may directly support the survival of tumor cells. Treg cell depletion has a profound therapeutic effect in animal cancer models, the clinical translation of which is hampered by the absolute necessity of Treg cells for maintaining immune homeostasis. Insights into the biology of Treg cells have revealed context-specific functions, which have led to several studies of the phenotype and function of tumor-resident Treg cells. These advances are paving the way for rational and theoretically safer targeting of tumor-specific Treg cells and are beginning to translate into clinical practice.

DISCLOSURE STATEMENT

The authors have submitted a patent application describing the use of CCR8 antibodies to deplete Treg cells. G.P. has consulting support from Merck and Tizona Therapeutics. A.Y.R. is an advisor to and holds stock options from FLX Bio, Surface Oncology, Vedanta Biosciences, and IFM Therapeutics; is an advisor to BioInvent International; and is a consultant to Omeros.

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