Understanding and Targeting Human Cancer Regulatory T Cells to Improve Therapy

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Abstract

Regulatory T cells (Tregs) are critical in maintaining immune homeostasis under various pathophysiological conditions. A growing body of evidence demonstrates that Tregs play an important role in cancer progression and that they do so by suppressing cancer-directed immune responses. Tregs have been targeted for destruction by exploiting antibodies against and small-molecule inhibitors of several molecules that are highly expressed in Tregs—including immune checkpoint molecules, chemokine receptors, and metabolites. To date, these strategies have had only limited antitumor efficacy, yet they have also created significant risk of autoimmunity because most of them do not differentiate Tregs in tumors from those in normal tissues. Currently, immune checkpoint inhibitor (ICI)-based cancer immunotherapies have revolutionized cancer treatment, but the resistance to ICI is common and the elevation of Tregs is one of the most important mechanisms. Therapeutic strategies that can selectively eliminate Tregs in the tumor (*i.e.* therapies that do not run the risk of causing autoimmunity by affecting normal tissue), are urgently needed for the development of cancer immunotherapies. This chapter discusses specific properties of human Tregs under the context of cancer and the various ways to target Treg for cancer immunotherapy.

Keywords

Regulatory T cells · Human cancer · Immunotherapy

12.1 Introduction

Regulatory T cells (Tregs) are a subset of immunosuppressive $CD4^+$ T cells that are critical for peripheral immunity, immune homeostasis, and self-tolerance. They play an important role in many conditions and diseases by preventing autoimmunity and overstimulation of the immune system in response to foreign pathogens, promoting resolution of inflammation, and suppressing anti-tumor immunity (Lin et al. 2018; Sakaguchi et al. 2010; Togashi et al. 2019). Indeed, research over the past 20 years has shown that tumors often have an increased density of Tregs, and they help promote the development of the immunosuppressive tumor microenvironment (TME), leading to the evasion of immune system by tumor cells and hence the consequent cancer

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S.-G. Zheng (ed.), *T Regulatory Cells in Human Health and Diseases*, Advances in Experimental Medicine and Biology 1278, https://doi.org/10.1007/978-981-15-6407-9_12

progression (Chaudhary and Elkord 2016; Togashi et al. 2019).

The existence of suppressive T cells has been dated back as far as to the 1970s; however, the study of these cells was limited by the lack of markers for the identity of these suppressive T cells (Gershon and Kondo 1970; Sakaguchi 2011). In the mid-1990s, a population of $CD4^+$ $CD25^+$ thymic T cells—later referred to as Tregs—was identified to play a role in suppressing autoimmunity (Asano et al. 1996; Sakaguchi et al. 1995). Forkhead Box P3 (FOXP3) was found to be the main transcription factor that drives Treg identity. In 2001, mutations in the mouse *Foxp3* gene were shown to be the cause of lethal autoimmunity and inflammation observed in the scurfy mice (Brunkow et al. 2001). In the same year, mutations in the human *FOXP3* gene were shown to be the cause of immune dysregulation, polyendocrinopathy, and enteropathy X-linked (IPEX) syndrome characterized by autoimmunity in several endocrine organs (Bennett et al. 2001; Wildin et al. 2001). These conditions share a similar phenotype that was observed in mice with depletion of $CD4^+$ $CD25^+$ T cells, which led to the discovery of *FOXP3* expression in $CD4^+CD25^+$ T cells, and that forced expression of FOXP3 in conventional $CD4^+CD25^-$ T cells converts them to functionally suppressive T cells with the phenotypic expression of several characteristic Treg genes such as CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and glucocorticoid-induced tumor necrosis factor receptor (GITR) (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003). Stable expression of FOXP3, which is achieved by demethylation of an evolutionarily conserved motif in the *FOXP3* gene (referred to as the Treg-specific demethylation region, TSDR), is required to thymic Treg stability (Floess et al. 2007; Ge et al. 2019; Huehn et al. 2009). In mice, FOXP3 is almost exclusively expressed in Tregs; however, in humans both Tregs and conventional $CD4^+$ T cells express FOXP3 following T-cell receptor (TCR) stimulation. As a result, the $CD4^+$ $FOXP3^+$ T-cell population may also contain some activated conventional T cells (Morgan et al. 2005; Roncador et al. 2005; Stockis et al. 2019). Demethylation of the *FOXP3* TSDR is the most distinguishing feature of human Tregs (Stockis et al. 2019). For the identification and isolation of functional human Tregs, neither demethylation of the TSDR nor FOXP3 expression is suitable; a combination of surface markers is required (Yang et al. 2019). In human, Tregs are generally identified by—though not perfect—the expression of CD4 and CD25 and low to no expression of the α-chain of the interleukin-7 receptor (IL-7R; CD127) (Liu et al. 2006; Romano et al. 2017).

12.2 Tregs in Cancer

The role of Tregs in suppressing anti-tumor immunity was first shown by Onizuka et al. and Shimizu et al. wherein they demonstrated that depletion of $CD25^+$ T cells in mice resulted in increased tumor rejection and reduced tumor growth. Similarly, adoptive transfer of Treg-depleted ($CD25^+$ depletion) splenocytes had the same effect (Onizuka et al. 1999; Shimizu et al. 1999). The role of Tregs in human cancers has been studied extensively and been reviewed numerous times (Chaudhary and Elkord 2016; Togashi et al. 2019). Tregs have been shown to be increased in the peripheral blood and lymph nodes of cancer patients and to accumulate in many solid tumors where they account for 10–50% of the tumor-infiltrating $CD4^+$ cells (Badoual et al. 2006; Hiraoka et al. 2006; Ichihara et al. 2003; Ling et al. 2007; Schaefer et al. 2005). The role of Tregs in suppressing anti-tumor immunity in humans is supported by several studies. Ladoire et al. showed that the pathologic complete response (PCR) to neoadjuvant chemotherapy in breast cancer patients was correlated with decreased Tregs and increased $CD8^+$ T cells. Depletion of Tregs using a previously FDA-approved CD25-blocking antibody improved the response to an experimental cancer vaccine in metastatic breast cancer patients (Rech et al. 2012). Transient depletion of Tregs via an IL-2-diphtheria toxin conjugate reduced metastatic lesions in melanoma patients (Rasku

et al. 2008). The elevated abundance of FOXP3$^+$ Tregs is generally associated with a poor prognosis in most non-mucosal-derived solid tumors (Chaudhary and Elkord 2016; Shang et al. 2015). This association between tumor-infiltrating Treg abundance and prognosis is particularly true when using the ratio between Tregs and conventional T cells, where a higher ratio is significantly correlated with a worse prognosis in breast cancer, lung cancer, melanoma, pancreatic cancer, and ovarian cancer (Curiel et al. 2004; Jiang et al. 2014; Leffers et al. 2009; Sayour et al. 2015; Tang et al. 2014; Tao et al. 2012; Yang et al. 2006). While Tregs tend to be higher in the peripheral blood in cancer patients, this is not always associated with the abundance of tumor-infiltrating Tregs (Adeegbe and Nishikawa 2013; Togashi et al. 2019). In contrast, a higher number of FOXP3$^+$ Tregs can be associated with good prognosis as well, such as in gastric and colorectal cancers (Haas et al. 2009; Salama et al. 2009). This may be due to the role of Tregs in suppressing tumor initiating and promoting inflammation in the colon associated with changes in the gut microbiome (Ladoire et al. 2011). Alternatively, recent studies have shown that colorectal tumors have high infiltration of FOXP3$^+$ non-Tregs that are inflammatory and associated with a good prognosis (Saito et al. 2016). Due to the difficulty of distinguishing Tregs in human with just FOXP3 expression (Morgan et al. 2005; Roncador et al. 2005), immunohistochemistry staining for FOXP3 may not be a viable method for determining the prognostic value of Treg infiltration in colorectal cancer. In support of this, infiltration of actual suppressive Tregs (defined by high expression of FOXP3 and negative for CD45RA) is associated with poor prognosis in colorectal cancer (Saito et al. 2016).

12.2.1 Cellular Source of Tumor-Infiltrating Tregs

Tregs can develop within the thymus by positive selection (thymic Tregs or tTregs) or arise from peripheral conventional CD4$^+$ FOXP3$^-$ T cells following prolonged T-cell receptor (TCR) stimulation in the presence of certain cytokines (pTregs, also referred to as induced Tregs (iTregs)) (Lee et al. 2011; Zheng et al. 2002, 2004). tTregs develop when the TCR of CD4 and CD8 double-positive cells in the thymus have a high-affinity interaction with self-antigens leading to the upregulation of CD25 as well as other Treg-associated receptors such as GITR (Burchill et al. 2008; Lio and Hsieh 2008). A second step for the development of stable CD25$^+$ FOXP3$^+$ Tregs involves IL-2 and STAT5 signaling, leading to stable FOXP3 expression (Burchill et al. 2007; Lio and Hsieh 2008). This development process results in a unique TCR repertoire relative to those from conventional CD4$^+$ T cells (Hsieh et al. 2006; Park et al. 2020; Wong et al. 2007). Zheng SG group first reported that iTregs arise from conventional CD4$^+$ FOXP3$^-$ T cells after prolonged TCR stimulation under certain cytokine conditions, such as in the presence of TGF-β and IL-2 (Davidson et al. 2007; Zheng et al. 2002, 2007). Thus, iTregs can share the TCR repertoire with peripheral conventional CD4$^+$ T cells. The stable expression of FOXP3 and thus the development of long-lived Tregs requires demethylation of the TSDR, which only happens in tTregs (Floess et al. 2007; Ge et al. 2019; Huehn et al. 2009).

Tumor-infiltrating Tregs can arise from several different sources, conversion of tumor-infiltrating CD4$^+$ FOXP3$^-$ T cells, recruitment of tTregs, and expansion of tissue-resident Tregs (Stockis et al. 2019). Studies have shown that several types of leukemias and lymphomas could induce the differentiation of conventional CD4$^+$CD25$^-$ T cells into Tregs (Deng 2018). For instance, malignant B cells from follicular lymphoma and non-Hodgkin's lymphoma could induce the expression of FOXP3 in CD4$^+$CD25$^-$ T cells (Ai et al. 2009; Mittal et al. 2008). Whether conversion of conventional CD4 T cells into Tregs occurs in human solid tumors is still debatable. In mice, adoptive transfer of conventional CD4$^+$CD25$^-$ T cells into tumor-bearing mice leads to the conversion of some of these cells to FOXP3$^+$ Tregs (Valzasina et al. 2006); however, whether this occurs in human and whether the

conversion of conventional $CD4^+CD25^-$ T cells to Tregs is a major source of tumor-infiltrating Tregs are unknown. Many tumor cells or other cells with TME can express TGF-β, so it is conceivable that the TME could induce the conversion of conventional $CD4^+CD25^-$ T cells to Tregs. $CD4^+CD25^-$ T cells and Tregs do not seem to share the same TCR repertoire in human and mouse tumors (Ahmadzadeh et al. 2019; Plitas et al. 2016), suggesting that tumor-infiltrating Tregs may not arise from $CD4^+CD25^-$ T cells. Ahmadzadeh et al. found that the clonality of tumor-infiltrating Tregs from melanoma, gastric, and ovarian cancers had little overlap with tumor-infiltrating or peripheral blood conventional $CD4^+CD25^-$ T cells, but tumor-infiltrating Tregs did share clones with their peripheral blood counterparts (Ahmadzadeh et al. 2019). Most importantly, the TCRs from tumor-infiltrating Tregs showed specificity to tumor antigens and could be expanded in an antigen-specific manner (Ahmadzadeh et al. 2019). This would suggest that tumor-infiltrating Tregs may arise from both the recruitment and clonal expansion of peripheral or tissue resident tTregs; or a second explanation is that tumor-infiltrating Tregs are able to extravasate from tumors and enter circulation. The expansion of tissue resident Tregs in tumors is supported by a study that revealed the tumor-infiltrating Tregs had a similar gene-expression pattern as normal tissue Tregs (Plitas et al. 2016). It should be noted that there is plasticity between specific subsets of $CD4^+$ T cells and Tregs, in particular between Th17 cells and Tregs (Wan et al. 2020). It was recently reported that Th17 cells could be converted into suppressive IL-17^+ $FOXP3^+$ and IL-17^- $FOXP3^+$ Tregs in the TME, indicating that the conversion of Th17 cells into Tregs could be an additional source of tumor-infiltrating Tregs (Downs-Canner et al. 2017).

12.2.2 Chemokine Receptors in Tumor-Infiltrating Tregs

The identification of chemokines and their receptors that potentially mediate the recruitment and retention of Tregs into the TME is an area of active research (Stockis et al. 2019). Tumor-infiltrating Tregs express a panel of chemokine receptors such as CC chemokine receptor 4 (CCR4) (ligands CCL22/CCL17), CCR5 (ligand CCL5), CCR6 (ligand CCL20), CCR8 (ligand CCL1), and CCR10 (ligand CCL28). Many studies have attempted to use these chemokine receptors to explain the recruitment of effector Tregs to the TME; however, these Treg chemokine receptors may have a more pronounced role of retaining Tregs within TME since all ligands are highly expressed within TME as well. CCR4—working through its ligands CCL22 or CCL17—is the best studied chemokine signaling in Treg recruitment into the TME. Several studies of ovarian, prostate, breast, gastric, and bladder cancers have shown that tumor-infiltrating Tregs and Tregs from malignant ascites express CCR4 and that the ligand CCL22, which is highly expressed in tumors by tumor cells or macrophages, can act as a chemoattractant for Tregs (Curiel et al. 2004; Gobert et al. 2009; Maeda et al. 2019; Miller et al. 2006; Mizukami et al. 2008). Recent studies have shown that secretion of CCL5 by tumors or cancer-associated fibroblasts can recruit the Tregs though its receptor CCR5 in mouse models of pancreatic adenocarcinoma, squamous cell carcinoma, colorectal cancer, and breast cancer (Tan et al. 2011; Wang et al. 2017; Ward et al. 2015) and that CCL5 could recruit Tregs to metastatic sites in the lung (Halvorsen et al. 2016). CCR6, a known chemokine receptor shared by memory Th1, Th2, Th17, and Tregs, was able to recruit Tregs into the TME via macrophage-produced CCL20 (Chen et al. 2013; Lee et al. 2017; Liu et al. 2011; Zhang et al. 2015). CCL28 can be induced via tumor-associated hypoxia within the TME and plays a role in the recruitment of Tregs though its receptor CCR10 (Facciabene et al. 2011). CCR8 was recently identified to be exclusively elevated in human tumor-infiltrating Tregs in breast cancer and several other cancer types (De Simone et al. 2016; Plitas et al. 2016). CCL1, expressed by Tregs, provides an autocrine signaling to upregulate its own receptor CCR8 on Tregs and STAT3-dependent upregulation of Foxp3,

CD39, IL-10, and granzyme B (Barsheshet et al. 2017) and is a major chemotaxis factor for Tregs in human breast cancer (Kuehnemuth et al. 2018). CCR8 can be targeted by monoclonal antibodies that have shown to reduce tumor-infiltrating Tregs (Villarreal et al. 2018).

12.2.3 Mechanisms of Action (Summarized in Fig. 12.1)

Tregs suppress effector T cells (Teff cells) via many different actions, either in a contact-dependent or -independent fashion. Many co-stimulatory (OX-40, GITR, 4-1BB, etc.) or co-inhibitory molecules (CTLA-4, PD-1, TIGIT, LAG3, TIM-3, etc.) are constitutively expressed on tumor-infiltrating Tregs. These co-inhibitory receptor–ligand pairs either promote the expansion of Tregs or suppress effector cells directly in a contact-dependent manner. Most studies support the contact-dependent mechanism for both human and mouse Tregs when using in vitro suppression assay (Dieckmann et al. 2001; Jonuleit et al. 2001; Piccirillo and Shevach 2001; Takahashi et al. 1998; Thornton and Shevach 1998). Tregs can also secrete peptides (TGF-β, IL-10, IL-35) or metabolize ATP to adenosine via CD39 and CD73, which provides an immunosuppressive microenvironment (Su et al. 2019). Many in vivo studies strongly support the role of cytokines or metabolites in Teff cell suppression (Asseman et al. 1999; Belkaid et al. 2002; Collison et al. 2007, 2009; Kingsley et al. 2002; Lan et al. 2012; Li et al. 2007; Powrie et al. 1996). We summarize the mechanisms of action for Tregs in Fig. 12.1. Most of these mechanisms were well-established in animal models with strong genetic evidence, whereas not all mechanisms are validated in human Tregs particularly relevant to human cancers. Here we briefly discuss the various mechanisms of action for Tregs and elaborate further in the next section for those related to human cancers.

The best-studied mechanism is via the co-inhibitory molecule CTLA-4. CTLA-4 is a high-affinity inhibitory receptor for the co-stimulatory molecules CD80 and CD86 expressed on antigen-presenting cells (APCs) that otherwise bind to CD28 on Teff cells to induce a co-stimulatory signal for T-cell activation, in conjunction with the primary activating signal from MHC-antigen complexes binding to the TCR on Teff cells (Ge et al. 2019; Togashi

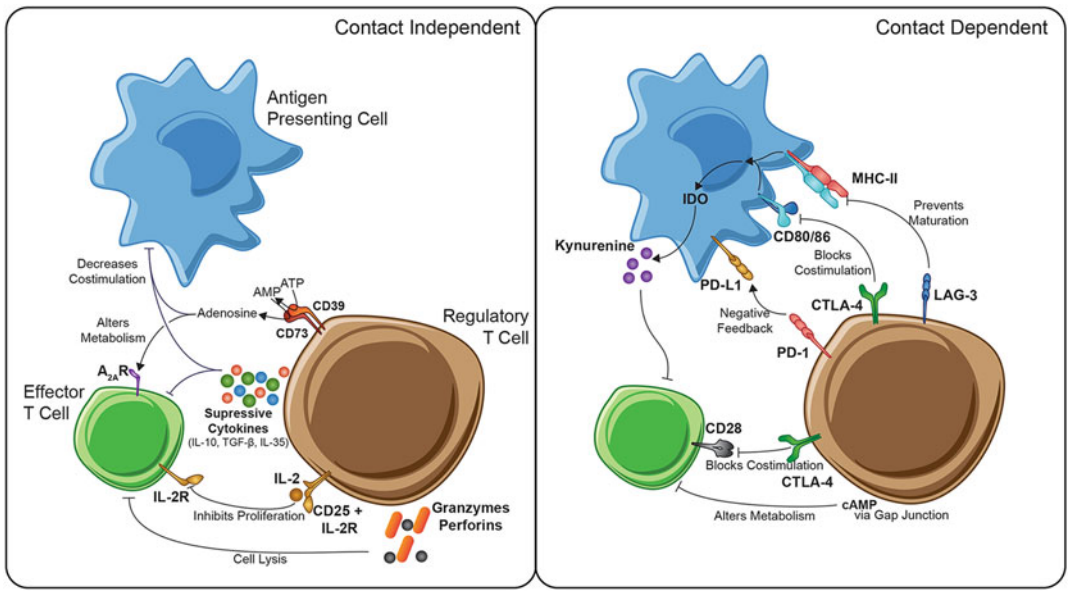

Fig. 12.1 The immunosuppressive mechanisms by Tregs

et al. 2019). Actually, CYLA-4-B7.1 signal also drives Treg development (Zheng et al. 2006). Tumor-infiltrating Tregs also express many other co-inhibitory molecules including T-cell immunoreceptor with Ig and ITIM domains (TIGIT) (Kurtulus et al. 2015), Tim-3 (HAVCR2) (Das et al. 2017; Gao et al. 2012; Sakuishi et al. 2013a), LAG-3 (CD223) (Camisaschi et al. 2010), and PD-1 (Kamada et al. 2019a; Lowther et al. 2016). TIGIT competes for binding of CD155 with CD226, preventing CD226-mediated co-stimulation of Teff cells and can also induce the expression of the suppressive cytokine IL-10 in dendritic cells (DCs) (Levin et al. 2011; Yu et al. 2009). LAG-3 binds to MHCII on APCs with a higher affinity than CD4, thus preventing antigen-specific stimulation of CD4 T cells (Huard et al. 1994; Sasidharan Nair and Elkord 2018). LAG-3 can also induce the secretion of indoleamine 2,3-dioxygenase (IDO) from DCs which can impair the function of Teff cells by producing kynuremine (Ge et al. 2019; Munn and Mellor 2013). Studies have also shown that Tregs express a large amount of cyclic adenosine monophosphate (cAMP) which they can directly transfer to Teff cells via gap junctions leading to downregulation of IL-2 and decreased proliferation (Klein and Bopp 2016). These mechanisms of suppression by Tregs require contact between the Tregs and Teff cells or APCs, and until recently, it was not known how Tregs come into the proximity of Teff cells to mediate suppression. Patterson et al. found that Tregs secrete the chemokines CCL3 and CCL4 which can actively promote the migration of Teff cells to close proximity with the Tregs to mediate suppression (Patterson et al. 2016). It is unknown whether tumor-infiltrating Tregs use the same mechanism or not, but our unpublished results indicate a common mechanism because tumor-infiltrating Tregs express several Teff chemokines—including CCL3, CCL4, and CXCL10—at much higher levels than those expressed by splenic Tregs (unpublished data).

Several contact-independent mechanisms of suppression have also been identified. Tregs highly and constitutively express CD25, which is a high-affinity receptor for IL-2. IL-2 is primarily produced by conventional T cells and is a critical cytokine for the proliferation of T and B cells. The high expression of CD25 on Tregs acts to sequester IL-2 from conventional T cells preventing their proliferation (Ge et al. 2019; Yau et al. 2012). The role of sequestration of IL-2 by CD25 in Treg suppression is supported by in vitro studies, showing that an excess of IL-2 can overcome Treg-mediated suppression of conventional T-cell proliferation (Takahashi et al. 1998; Yamaguchi et al. 2012). Tregs can also secrete several immunosuppressive cytokines including IL-10, TGF-β, and IL-35 (Chaudhary and Elkord 2016; Ge et al. 2019). Tumor-infiltrating Tregs from several human cancers including colorectal cancer, hepatocellular carcinoma, and pancreatic cancer can suppress the activity of autologous T cells by secreting TGF-β and IL-10 (Amedei et al. 2013; Kakita et al. 2012; Scurr et al. 2014; Yi et al. 2013). While IL-10 can inhibit DC activation, it can activate Teff cells under certain conditions and may not be a major mechanism of Treg-mediated suppression (Ge et al. 2019; Ouyang and O'Garra 2019). Tregs can also express the ectonucleotidases CD39 and CD73 which combine to convert extracellular adenosine triphosphate (ATP) into adenosine (Allard et al. 2020). Adenosine can bind to the adenosine receptors A_{2A} and A_{2B}, leading to increased intracellular cAMP which downregulates IL-2 in effector T cells (Blay et al. 1997; Klein and Bopp 2016; Ohta et al. 2006). Co-expression of CD39 and CD73 on human Tregs is rare, though studies have shown that CD39 is highly expressed on tumor-infiltrating Tregs in several human cancers and that $CD39^+$ cells can interact with $CD73^+$ cells or exosomes in the TME to produce adenosine (Jie et al. 2013; Schuler et al. 2014; Sundström et al. 2016).

12.3 Targeting Tregs for Cancer Immunotherapy

12.3.1 Immune Checkpoints as Therapeutic Targets for Tregs

12.3.1.1 Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4)

Discovery of CTLA-4 and its mechanisms of action. The Golstein group initially cloned CTLA-4 from mouse-activated $CD8^+$ T cells and called cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Brunet et al. 1987) that was later confirmed to be present within human genome (Dariavach et al. 1988). CTLA-4 was later defined as a negative regulator of T-cell activation (Krummel and Allison 1995)—later named as immune checkpoint—that completes with CD28 to bind to CD80/CD86, with 20-fold higher binding affinity than CD28 (Linsley et al. 1991). CTLA-4 knockout mice exhibited an alteration in the T-cell development in the thymus and resulted in highly proliferative and active T cells in periphery (Tivol et al. 1995). James Allison's group established the suppressive role of CTLA-4 in cancer immunosurveillance in 1996 and found that anti-CTLA-4 antibody induced a strong antitumor immunity (Leach et al. 1996), the primary reason for which James Allison won the Nobel Prize in Physiology or Medicine in 2018. The connection between CTLA-4 and Tregs was established by the Sakaguchi group in 2008, where Treg-specific deletion of *CTLA4* gene—driven by a constitutively expressed *FoxP3-IRES-Cre*—led to a similar phenotype as germline deletion of CTLA-4. These data established that the immunosuppressive function of CTLA-4 is mainly through its expression within tTregs (Wing et al. 2008). In contrast, peripheral Treg-specific deletion of *CTLA4* gene in adult mice—driven by a tamoxifen-inducible *Foxp3-eGFP/Cre-ERT2*—resulted in the expansion of both conventional $CD4^+$ T cells and peripheral Tregs. Transcriptomic analysis further confirmed that *CTLA4* deletion led to a compensatory overexpression of immunosuppressive molecules including LAG3, PD-1, IL-10, etc., which are essential to maintain the suppressive phenotype of $CTLA\text{-}4^-$ peripheral Tregs (Paterson et al. 2015).

At the molecular level, CTLA-4 is believed to be important for counteracting the co-stimulatory signal of CD28 to CD80/CD86 on antigen-presenting cells, either by direct suppression of antigen-presenting cells via CD80/CD86-mediated signaling transduction (Onishi et al. 2008; Wing et al. 2008) or by removing surface CD80/CD86 via trans-endocytosis (Qureshi et al. 2011). At the cellular level, CTLA-4 expression on the Teff cells, either conventional $CD4^+$ T cells or $CD8^+$ T cells, is important to limit the priming stage for T-cell activation and proliferation within secondary lymphoid tissues. A similar mechanism of CTLA-4 on Tregs is conceived to be within the secondary lymphoid tissues where CTLA-4 on Tregs inhibits APC function via CD80/C86 binding (Onishi et al. 2008; Wing et al. 2008). Nevertheless, CTLA-4 is not the only—sometimes not even the major—mechanism for the suppressive function of Tregs since polyclonal T-cell activation using anti-CD3 and anti-CD28 co-activation can be potently suppressed by Tregs, but such in vitro system is not involved in APC and the CD80/CD86 proteins.

CTLA-4 in cancer immunotherapy. The James Allison group established the potential of antagonizing CTLA-4 to activate antitumor immunity in 1996 (Leach et al. 1996). The FDA approved ipilimumab, a fully human anti-CTLA-4 antibody, to treat metastatic melanoma in 2011. This is a milestone of immune checkpoint inhibitors. As another immune checkpoint, i.e., the PD-1/PD-L1, has gained more success than targeting CTLA-4, lessons can still be learned from the mechanism of action for ipilimumab. We have pointed out above that CTLA-4 plays an immunosuppressive role in tTregs which have

been proven the major Treg populations in various cancer types; hence, it is not surprising to find that the efficacy of ipilimumab is positively correlated with the reduced Treg abundance in tumor microenvironment. Animal studies further support that anti-CTLA-4 works on both Teff activation and Treg depletion for its maximal efficacy (Bulliard et al. 2013; Selby et al. 2013; Simpson et al. 2013). One caveat of ipilimumab therapy is the high rate of treatment-related adverse events, and many patients receiving ipilimumab experienced level 3 or 4 immune-related adverse events. In the EORTC 18071 trial, five patients died of ipilimumab treatment-related colitis, myocarditis, or multiorgan failure associated with Guillain–Barre syndrome. Ipilimumab is a human IgG1 antibody with predicted deleting activity, it is likely that these severe immune-related adverse events are the cause of ipilimumab-mediated Treg depletion via FC-gamma receptors (Arce Vargas et al. 2018). Tremelimumab, a human IgG2 isotype without deleting activity, can also bind to FC-gamma receptors and deplete Tregs (Arce Vargas et al. 2018). Even though a recent study argued against the role of ipilimumab on human cancers, the sampling time (many weeks after the last ipilimumab treatment) may miss the point of Treg depletion from these clinical samples (Sharma et al. 2019). The Sakaguchi group re-engineered the Fc-portion of ipilimumab to enhance its binding affinity to human FC-gamma receptor IIIa, which leads to antibody-mediated cytotoxicity (ADCC)-mediated killing of Tregs as well as exhausted $CD8^+$ T cells (Ha et al. 2019). The distinct difference of CTLA-4 expression on Tregs versus Teff cells is that Tregs constitutively express CTLA-4 on the surface, whereas Teff cells only express CTLA-4 on the surface upon activation and to a much lower level than that on Tregs. This feature gives a window for Treg depletion first, followed by CD8 T-cell activation by other means such as vaccination or anti-PD-1 therapy that will be mentioned later (Ha et al. 2019).

12.3.1.2 Lymphocyte-Activation Gene 3 (LAG-3, CD223)

LAG-3 is a surface protein that is expressed by activated $CD4^+$ and $CD8^+$ T cells, as well as by Tregs. LAG-3 could be the third most promising immune checkpoint in cancer immunotherapy after CTLA-4 and PD-1/PD-L1. As an immune checkpoint, LAG-3 binds to MHCII on antigen-presenting cells (Liang et al. 2008) to block the TCR and CD4-co-receptor-mediated signals for T-cell activation at the priming phase of the tumor-immune cycle. In addition, cancer cells can produce another ligand, namely fibrinogen-like protein 1 (FGL1), as the major immune-inhibitory ligand to bind with LAG-3 independent of MHCII (Wang et al. 2019) and inhibit T-cell activation at the effector phase (Topalian et al. 2016). Elevated expression of LAG-3 in tumor-infiltrating lymphocytes is significantly associated with disease progression of many human cancers (Chen and Chen 2014; Gandhi et al. 2006; Hemon et al. 2011; Matsuzaki et al. 2010; Shapiro et al. 2017). Many inhibitory molecules/antibodies against LAG-3 have been developed and showed some clinical benefit either alone or in combination with other immune checkpoint inhibitors (Table 12.1). In combination with anti-PD-1 therapy, anti-LAG-3 facilitates the eradication of established tumors that are resistant to either single antibody treatment by inducing an active anti-cancer immune response (Matsuzaki et al. 2010; Woo et al. 2012). The expression of LAG-3 on Tregs is induced upon the activation of Teff cells. Genetic deletion of *LAG3* or the treatment with anti-LAG-3 antibody inhibits the proliferative and suppressive capacities of Tregs, supporting that LAG-3 is important for Treg-mediated immune suppression under physiological conditions (Huang et al. 2004). The role of LAG-3 in Tregs, however, can be reversed when Tregs are placed under chronic inflammation such as in autoimmune diabetes (Zhang et al. 2017). Treg-specific deletion of LAG-3 in non-obese diabetic mice (NOD, autoimmune type 1 diabetic model) resulted in Treg expansion in the islets but not peripheral

Table 12.1 Summary of clinical trials related to tumor-infiltrating Tregs

NCT	Cancer	Compound	Target	Additional agents	Phase	Status
NCT02946671	Solid tumors	Mogamulizumab	CCR4	Nivolumab (PD-1)	I	Completed
NCT01626664	Adult T-cell leukemia-lymphoma	Mogamulizumab	CCR4	None	II	Completed
NCT00888927	Peripheral T-cell lymphoma	Mogamulizumab	CCR4	None	I/II	Completed
NCT00355472	Relapsed adult T-cell leukemia-lymphoma and peripheral T-cell lymphoma	Mogamulizumab	CCR4	None	I	Completed
NCT01728805	Cutaneous T-cell lymphoma	Mogamulizumab	CCR4	None	III	Active, not recruiting
NCT04185220	Adult T-cell leukemia-lymphoma and cutaneous T-cell lymphoma	Mogamulizumab	CCR4	Recombinant IL-15	I	Recruiting
NCT04256018	Cutaneous T-cell lymphoma	Mogamulizumab	CCR4	Low-dose total skin electron beam	II	Not yet recruiting
NCT01611142	Peripheral T-cell lymphoma	Mogamulizumab	CCR4	None	II	Completed
NCT02476123	Advanced solid tumors	Mogamulizumab	CCR4	Nivolumab (PD-1)	I	Completed
NCT00920790	CCR4+ Adult T-cell leukemia-lymphoma	Mogamulizumab	CCR4	None	II	Completed
NCT02301130	Advanced solid tumors	Mogamulizumab	CCR4	Durvalumab (PD-1) and tremelimumab (CTLA-4)	I	Completed
NCT04128072	Cutaneous T-cell lymphoma	Mogamulizumab	CCR4	Low-dose total skin electron beam	II	Not yet recruiting
NCT02281409	Advanced and metastatic solid tumors	Mogamulizumab	CCR4	None	I/II	Completed
NCT03309878	Relapsed or refractory diffuse large B-cell lymphoma	Mogamulizumab	CCR4	Pembrolizumab (PD-1)	I/II	Recruiting
NCT02444793	Advanced solid tumors	Mogamulizumab	CCR4	Utomilumab (4-1BB)	I	Terminated
NCT02358473	Non–small cell lung cancer	Mogamulizumab	CCR4	Docetaxel	I	Completed
NCT02867007	Locally advanced or metastatic solid tumors	Mogamulizumab	CCR4	KHK2455 (IDO)	I	Active, not recruiting
NCT03767582	Pancreatic adenocarcinoma	BMS-813160	CCR2/ CCR5	GVAX	I/II	Recruiting
NCT03274804	Microsatellite stable metastatic colorectal cancer	Maraviroc	CCR5	Pembrolizumab (PD-1)	I	Active, not recruiting
NCT03631407	Microsatellite stable metastatic colorectal cancer	Vicriviroc	CCR5	Pembrolizumab (PD-1)	II	Active, not recruiting
NCT01736813	Metastatic colorectal cancer	Maraviroc	CCR5	None	I	Completed
NCT03838367	Metastatic triple-negative breast cancer	Leronlimab	CCR5	Carboplatin	I/II	Recruiting
NCT00128622	CEA-expressing malignancies	Denileukin diftitox	CD25	Tumor vaccine	I	Completed
NCT00847106	Advanced melanoma	Daclizumab	CD25	DC-based anti-tumor vaccine	I/II	Completed
NCT00082914	Metastatic melanoma and kidney cancer	Denileukin diftitox	CD25	None	II	Completed
NCT00278369	Metastatic renal cancer	Denileukin diftitox	CD25	None	I	Completed
NCT00425672	Breast cancer	Denileukin diftitox	CD25	None	I/II	Completed

(continued)

Table 12.1 (continued)

NCT	Cancer	Compound	Target	Additional agents	Phase	Status
NCT00726037	Metastatic pancreatic cancer	Denileukin diftitox	CD25	None	II	Withdrawn
NCT03621982	Select advanced solid tumors	ADCT-301	CD25-ADC	None	I	Recruiting
NCT03884556	Lymphomas and solid tumors	TTX-030	CD39	Pembrolizumab (PD-1), docetaxel, gemcitabine, paclitaxel	I	Recruiting
NCT02503774	Advanced solid tumors	Oleclumab	CD73	Durvalumab (PD-1)	I	Active, not recruiting
NCT04262388	Pancreatic adenocarcinoma, small cell lung cancer, and head and neck cancer	Oleclumab	CD73	Durvalumab (PD-1)	II	Not yet recruiting
NCT04262375	Non–small cell lung cancer and renal clear cell carcinoma	Oleclumab	CD73	Durvalumab (PD-1)	II	Not yet recruiting
NCT04148937	Advanced solid tumors	LY3475070	CD73	Pembrolizumab (PD-1)	I	Recruiting
NCT03454451	Advanced malignancies	CPI-006	CD73	Ciforadenant (A2A receptor) and pembrolizumab (PD-1)	I	Recruiting
NCT03616886	Metastatic triple-negative breast cancer	Oleclumab	CD73	Paclitaxel, carboplatin, and durvalumab (PD-1)	I/II	Recruiting
NCT03875573	Luminal B breast cancer	Oleclumab	CD73	Radiotherapy and durvalumab (PD-1)	II	Recruiting
NCT03835949	Advanced or metastatic cancers	TJ004309	CD73	Atezolizumab (PD-L1)	I	Recruiting
NCT03267589	Relapsed ovarian cancer	MEDI9447	CD73	Durvalumab (PD-1)	II	Recruiting
NCT03549000	Non–small cell lung cancer, triple-negative breast cancer, pancreatic adenocarcinoma, ovarian cancer, renal clear cell carcinoma, metastatic castration-resistant prostate cancer, microsatellite stable colorectal cancer	NZV930	CD73	Spartalizumab (PD-1) and NIR178 (A2A receptor)	I	Recruiting
NCT04104672	Pancreatic adenocarcinoma	AB680	CD73	Zimberelimab (PD-1), nab-paclitaxel, and gemcitabine	I	Recruiting
NCT02754141	Advanced solid tumors	BMS-986179	CD73	Nivolumab (PD-1) and rHuPH20	I/II	Recruiting
NCT03954704	Advanced solid tumors	GS-1423	CD73-TGFB	None	I	Recruiting
NCT02740270	Advanced solid tumors and lymphomas	GWN323	GITR	Spartalizumab (PD-1)	I	Active, not recruiting
NCT02697591	Advanced or metastatic solid tumors	INCAGN01876	GITR	None	I/II	Active, not recruiting

(continued)

Table 12.1 (continued)

NCT	Cancer	Compound	Target	Additional agents	Phase	Status
NCT03277352	Advanced or metastatic malignancies	INCAGN01876	GITR	Epacadostat (IDO1) and pembrolizumab (PD-1)	I/II	Active, not recruiting
NCT03126110	Advanced or metastatic malignancies	INCAGN01876	GITR	Ipilimumab (CTLA-4) and nivolumab (PD-1)	I/II	Active, not recruiting
NCT01239134	Malignant melanoma	TRX518	GITR	None	I	Completed
NCT02583165	Advanced tumors	MEDI1873	GITR	None	I	Completed
NCT04335039	Glioblastoma	INCAGN01876	GITR	INCAGN01876 (PD-1), SRS	II	Not yet recruiting
NCT04021043	Advanced lung, chest, and liver cancers	BMS-986156	GITR	Ipilimumab (CTLA-4), nivolumab (PD-1), SRS	I/II	Recruiting
NCT03799003	Advanced solid tumors	ASP195	GITR	Pembrolizumab (PD-1)	I	Recruiting
NCT03295942	Locally advanced or metastatic tumors	OMP-336B11	GITR	None	I	Terminated
NCT01216436	Metastatic melanoma	GITR-L-transfected DC	GITR	Anti-CTLA-4-transfected DC	I	Terminated
NCT02553499	Advanced solid tumors	MK-1248	GITR	Pembrolizumab (PD-1)	I	Terminated
NCT03489369	Advanced solid tumors and lymphomas	Sym022	Lag-3	None	I	Active, not recruiting
NCT02460224	Advanced malignancies	LAG525	Lag-3	Spartalizumab (PD-1)	I/II	Active, not recruiting
NCT02614833	Metastatic breast cancer	IMP321	Lag-3	Paclitaxel	II	Active, not recruiting
NCT02060188	Colorectal cancer	BMS-986016	Lag-3	Nivolumab (PD-1)	II	Active, not recruiting
NCT00351949	Metastatic renal cancer	IMP321	Lag-3	None	I	Completed
NCT00349934	Metastatic breast cancer	IMP321	Lag-3	None	I	Completed
NCT03252938	Advanced solid tumors	IMP321	Lag-3	Avelumab (PD-1)	I	Recruiting
NCT03250832	Advanced solid tumors	TSR-033	Lag-3	Anti-PD-1	I	Recruiting
NCT03005782	Advanced malignancies	REGN3767	Lag-3	REGN2810 (PD-1)	I	Recruiting
NCT01968109	Non–small cell lung cancer, gastric cancer, hepatocellular carcinoma, renal cell carcinoma	Relatlimab	Lag-3	Nivolumab (PD-1)	I/II	Recruiting
NCT02817633	Advanced solid tumors	SR-033	Lag-3	TSR-022 (Tim-3)	I	Recruiting
NCT03311412	Advanced solid tumors and lymphomas	Sym022	Lag-3	Sym021 (PD-1)	I	Recruiting
NCT02658981	Recurrent GBM	Urelumab	Lag-3	Nivolumab (PD-1)	I	Recruiting
NCT03607890	Advanced mismatch repair deficient cancers	Relatlimab	Lag-3	Nivolumab (PD-1)	II	Recruiting
NCT03538028	Advanced malignancies	INCAGN02385	Lag-3	None	I	Recruiting
NCT00732082	Pancreatic adenocarcinoma	IMP321	Lag-3	Gemcitabine	I	Terminated
NCT03849469	Select solid tumors	XmAb22841	Lag-3-CTLA-4	Pembrolizumab (PD-1)	I	Recruiting
NCT04082364	HER2+ gastric/GEJ cancer	MGD013	Lag-3-PD-1	Margetuximab (HER2)	II/III	Recruiting

(continued)

Table 12.1 (continued)

NCT	Cancer	Compound	Target	Additional agents	Phase	Status
NCT02274155	Advanced head and neck cancers	MEDI6469	OX40	None	I	Active, not recruiting
NCT02559024	Metastatic colorectal cancer	MEDI6469	OX40	None	I	Active, not recruiting
NCT02315066	Locally advanced or metastatic tumors	PF-04518600	OX40	PF-05082566 (4-1BB)	I	Active, not recruiting
NCT02528357	Advanced solid tumors	GSK3174998	OX40	Pembrolizumab (PD-1)	I	Active, not recruiting
NCT01862900	Metastatic breast cancer	MEDI6469	OX40	SBRT	I	Completed
NCT01303705	Metastatic prostate cancer	Anti-OX40	OX40	Radiation and cyclophosphamide	I	Completed
NCT01644968	Advanced cancers	Anti-OX40	OX40	None	I	Completed
NCT02410512	Locally advanced or metastatic solid tumors	MOXR0916	OX40	Atezolizumab (PD-L1)	I	Completed
NCT02221960	Select advanced solid tumors	MEDI6383	OX40	Durvalumab (PD-L1)	I	Completed
NCT02318394	Selected advanced solid tumors	MEDI0562	OX40	None	I	Completed
NCT02705482	Advanced solid tumors	MEDI0562	OX40	Tremelimumab (CTLA-4) and durvalumab (PD-1)	I	Completed
NCT03241173	Advanced or metastatic malignancies	INCAGN01949	OX40	Nivolumab (PD-1) and Ipilimumab (CTLA-4)	I/II	Completed
NCT04215978	Advanced solid tumors	BGB-A445	OX40	Tislelizumab (PD-1)	I	Not yet recruiting
NCT03092856	Metastatic kidney cancer	PF-04518600	OX40	Axitinib	II	Recruiting
NCT03831295	Advanced or metastatic solid tumors	BMS 986178	OX40	SD-101 (TLR9)	I	Recruiting
NCT03971409	Triple-negative breast cancer	PF-04518600	OX40	Avelumab (PD-1), binimetinib (MEK), and utomilumab (4-1BB)	II	Recruiting
NCT03410901	B-cell non-Hodgkin lymphoma	BMS 986178	OX40	SD-101 (TLR9) and radiation therapy	I	Recruiting
NCT04198766	Locally advanced or metastatic solid tumors	INBRX-106	OX40	Pembrolizumab (PD-1)	I	Recruiting
NCT03336606	Head and neck squamous cell carcinoma	MEDI6469	OX40	None	I	Recruiting
NCT03267589	Relapsed ovarian cancer	MEDI0562	OX40	Durvalumab (PD-1) and tremelimumab (CTLA-4)	II	Recruiting
NCT02554812	Locally advanced or metastatic solid tumors	PF-04518600	OX40	Avelumab (PD-1)	II	Recruiting
NCT03636503	Follicular lymphoma	PF-04518600	OX40	Rituximab (CD20), utomilumab (4-1BB), and avelumab (PD-1)	I	Recruiting

(continued)

Table 12.1 (continued)

NCT	Cancer	Compound	Target	Additional agents	Phase	Status
NCT03447314	Advanced solid tumors	GSK3174998	OX40	GSK1795091 (TLR4)	I	Recruiting
NCT02923349	Advanced solid tumors	INCAGN01949	OX40	None	I/II	Recruiting
NCT03758001	Advanced solid tumors	IBI101	OX40	Sintilimab (PD-1)	I	Recruiting
NCT03217747	Advanced malignancies	PF-04518600	OX40	Utomilumab (4-1BB), avelumab (PD-1), and radiation	I/II	Recruiting
NCT03390296	Acute myeloid leukemia	PF-04518600	OX40	Avelumab (PD-1) and azacytidine	I/II	Recruiting
NCT02205333	Aggressive B-cell lymphoma	MEDI6469	OX40	Durvalumab (PD-L1), rituximab (CD20), and tremelimumab (CTLA-4)	I/II	Terminated
NCT01689870	Metastatic melanoma	Anti-OX40	OX40	Ipilimumab (CTLA-4)	I/II	Withdrawn
NCT01416844	Metastatic melanoma	Anti-OX40	OX40	None	II	Withdrawn
NCT03782467	Advanced solid tumors	ATOR-1015	OX40-CTLA-4	None	I	Recruiting
NCT04116710	Advanced solid tumors	HS-130	OX40L-Ag fusion	HS-110	I	Recruiting
NCT03323398	Advanced malignancies	mRNA-2416	OX40L mRNA	Durvalumab (PD-1)	I/II	Recruiting
NCT03739931	Advanced malignancies	mRNA-2416	OX40L mRNA	Durvalumab (PD-L1)	I	Recruiting
NCT03894618	Advanced solid tumors and lymphomas	SL-279252	PD1-Fc-OX40L	None	I	Recruiting
NCT04140500	Advanced solid tumors	RO7247669	PD1-LAG3	None	I	Recruiting
NCT03563716	Non–small cell lung cancer	MTIG7192A	TIGIT	Atezolizumab (PD-L1)	II	Active, not recruiting
NCT04294810	Non–small cell lung cancer	Tiragolumab	Tigit	Atezolizumab (PD-L1)	III	Not yet recruiting
NCT04047862	Advanced solid tumors	BGB-A1217	TIGIT	Tislelizumab (PD-1)	I	Recruiting
NCT04256421	Small cell lung cancer	Tiragolumab	TIGIT	Atezolizumab (PD-L1), etoposide, carboplatin	III	Recruiting
NCT04262856	Non–small cell lung cancer	Zimberelimab	Tigit	Zimberelimab (PD-1) and AB928 (A2b receptor)	II	Recruiting
NCT03628677	Advanced malignancies	AB154	TIGIT	Zimberelimab (PD-1)	I	Recruiting
NCT03119428	Advanced solid tumors	OMP-313M32	TIGIT	Nivolumab (PD-1)	I	Terminated
NCT00986518	Metastatic colorectal cancer	Treg-depleted autologous cell transplant		None	I/II	Completed

tissues, ultimately reducing the autoimmune diabetes (Zhang et al. 2017). Cancer Tregs are considered to have near maximal suppressive activity (Delgoffe et al. 2013) with a population expressing high levels of LAG-3. Since most clinical trials related to anti-LAG-3 antibodies are earlier in clinical trials, there is insufficient information as of how anti-LAG-3 antibodies influence cancer Tregs. LAG-3$^+$ Tregs are significantly enriched in blood from melanoma and colon cancer patients and exhibit an effector/memory phenotype, along with the production of immunosuppressive cytokines TGF-β and IL-10 (Camisaschi et al. 2010). In colorectal cancer, LAG-3 and TIM-3 are co-expressed in more than 50% of cancer Tregs, along with other immunosuppressive molecules such as TGF-β, IL-10, and CTLA-4 (Ma et al. 2018). In addition to classic Foxp3-positive Tregs, co-expression of CD49b and LAG-3 identifies human regulatory type 1 (Tr-1) T cells (Gagliani et al. 2013) that are highly suppressive. It is anticipated that LAG-3-targeting interventions may result in cancer-specific Teff activation as well as Treg inhibition.

12.3.1.3 T-Cell Immunoreceptor with Ig and ITIM Domains (TIGIT)

TIGIT was first identified as a coinhibitory molecule expressed on Teff cells that gained attention by suppressing autoimmune responses (Joller et al. 2011; Levin et al. 2011). TIGIT binds to co-stimulatory ligand CD155 on DCs, which leads to the reduced production of IL-12, but induces IL-10 production (Yu et al. 2009). TIGIT was later found to be expressed on human Tregs with superior immune suppression toward Th1 and Th17 helper cells but not Th2 cells (Joller et al. 2014). TIGIT marks highly dysfunctional CD8 T cells in tumors as well as a highly immune suppressive subpopulation of TI-Tregs, but genetic evidence supports that TIGIT expression on Tregs is dominant in suppressing antitumor immunity (Kurtulus et al. 2015). The fact that TIGIT knockout mice are normal in development and do not develop autoimmune diseases, in addition to the highly immunosuppressive nature of TIGIT$^+$ Tregs, makes TIGIT a great candidate for Treg-based cancer immunotherapy. Several anti-human TIGIT antibodies have been developed and entered early clinical trials, most of which have negligible effect; however, the interest remains from the pharmaceutical industry likely due to its synergistic effects with anti-PD-1/PD-L1 blockade (Table 12.1).

12.3.1.4 T-Cell Immunoglobulin and Mucin-Domain Containing-3 (TIM-3)

TIM-3 is an immunoglobulin and mucin domain family and is originally identified on CD4 and CD8 T cells (Monney et al. 2002) with immune modulatory function. TIM-3 is later found to be expressed by Tregs and innate immune cells including dendritic cells, natural killer cells, monocytes, macrophages, and mast cells (Wolf et al. 2020). Four ligands have been identified—including galectin-9 (Gal-9) (Jayaraman et al. 2010), high-mobility group protein B1 (HMGB1) (Chiba et al. 2012), Ceacam-1 (Huang et al. 2015), and phosphatidylserine (DeKruyff et al. 2010)—that mediate different immune-modulatory function of CD4$^+$ or CD8$^+$ T cells. TIM-3 is expressed on tumor-infiltrating Tregs of many cancer types, with studies showing that TIM-3$^+$ Tregs are more immunosuppressive than their TIM-3$^-$ counterparts and are co-expressing other immune checkpoints such as TIGIT, CTLA-4, and PD-1 (Gao et al. 2012; Kurtulus et al. 2015; Liu et al. 2018; Ma et al. 2018; Sakuishi et al. 2013b). The genetic evidence of TIM-3 in the role of Tregs is lacking, and there is no clinical evidence that TIM-3 inhibition has direct impact on Treg function.

12.3.1.5 Programmed Cell Death-1 (PD-1)

PD-1 is another immune checkpoint protein that was initially identified on active CD8$^+$ and CD4$^+$ T cells. Ligation of PD-L1, mainly expressed by cancer cells or myeloid cells, with PD-1 leads to T-cell exhaustion and dysfunction. There are many outstanding reviews related to the PD-1/PD-L1 axis in the field of cancer immunotherapy (Chamoto et al. 2020; Iwai et al. 2017; Sanmamed and Chen 2018; Zou et al. 2016). Briefly, anti-PD-1/PD-L1 antibodies mainly

disrupt the PD-L1 ligation, which reverses the exhaustion phenotype of Teff cells—a process referred to as rejuvenation. As rejuvenation becomes the primary explanation for the mechanism of T-cell activation under anti-PD-1/PD-L1 therapies, a very recent paper provides a secondary opinion showing that anti-PD-1 antibodies (pembrolizumab and cemiplimab) were able to deplete tumor-infiltrating $CD8^+$ T-cell clones and replace them with novel $CD8^+$ T-cell clones against tumor neoantigens (Yost et al. 2019). In relation to Tregs, PD-1 was initially identified as an intracellular protein in resting Tregs and, upon TCR stimulation, moved to the surface of active Tregs (Raimondi et al. 2006). The role of PD-1/PD-L1 axis in the induced Tregs has been reviewed recently (Gianchecchi and Fierabracci 2018) and will not be covered here due to the irrelevance in most solid cancers. Tumor-infiltrating Tregs consist of a significant $PD\text{-}1^+$ population. Interestingly, limited literature points to a role of PD-1 in Treg suppression, including (1) in malignant gliomas where PD-1 marks dysfunctional Tregs with IFN-γ expression (Lowther et al. 2016); (2) The Nishikawa group identified $PD\text{-}1^+$ Tregs in gastric cancer that were amplified by anti-PD-1 antibody treatment, leading to the hyperprogression of cancers upon anti-PD-1 therapy (Kamada et al. 2019b); (3) similar Treg accumulation also occurs in the hyperprogressive adult T-cell leukemia/lymphoma when treated with anti-PD-1 therapy (Rauch et al. 2019). These data are consistent with animal models where Treg-specific deletion of *PDCD1* (gene encoding PD-1) led to the expansion of Tregs that are more suppressive to Teff cells (Kamada et al. 2019b). The PD-1 and PD-L1 axis may not be a very good therapeutic target for Treg-based immunotherapy.

12.3.2 Co-stimulatory Receptors as Therapeutic Targets for Tregs

Another field of interest in cancer immunotherapy is the agonistic activation of co-stimulatory receptors such as GITR, OX-40, and 4-1BB. Interestingly, TI-Tregs from human cancers preferentially express these co-stimulatory receptors at much higher levels relative to Tregs from peripheral blood. Several studies have shown that agonistic activation of these receptors results in the expansion of $CD8^+$ T cells while at the same time eliminating/inhibiting TI-Tregs (Arce Vargas et al. 2017; Bulliard et al. 2013, 2014). As these co-stimulatory agonists mainly activate $CD8^+$ T cells (Table 12.1), and the effects on human TI-Tregs are largely missing, we will not cover these receptors in detail.

12.3.3 Chemokine Receptors as Potential Therapeutic Targets for Tregs

We have briefly discussed the roles of chemokine receptors in the recruitment/retention of tumor-infiltrating Tregs. Here we choose three candidates, CCR4, CCR5, and CCR8, for further discussion.

12.3.3.1 CCR4

CCR4 is identified as the major chemokine receptor for Th2 and Tregs (Yoshie and Matsushima 2015), two $CD4^+$ T-cell subtypes that have tumor-promoting functions. The Zou group first established the function of CCR4—working through CCL22 produced by the TME—in the recruitment of Tregs to ovarian cancers (Curiel et al. 2004), which is further confirmed within several other cancer types (Curiel et al. 2004; Gobert et al. 2009; Maeda et al. 2019; Miller et al. 2006; Mizukami et al. 2008). The major drive to develop anti-CCR4 agents is the elevated expression of CCR4 in mature T-cell neoplasms including adult T-cell leukemia/lymphoma (ATL), cutaneous T-cell lymphomas (CTCLs), and peripheral T-cell lymphomas (PTCLs) (Ogura et al. 2014; Ohshima et al. 2004; Shimauchi et al. 2005; Yoshie et al. 2002). Mogamulizumab is a humanized anti-CCR4 antibody that was approved by the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) to treat $CCR4^+$ ATL in 2012 (Ishida et al. 2012). Mogamulizumab is also effective in

treating other T-cell neoplasms with CCR4 expression including relapsed CTCLs and PTCLs (Ogura et al. 2014). FDA approved its usage for the treatment of relapsed or refractory mycosis fungoides and Sézary disease in 2018 (Kasamon et al. 2019). It should be noted that the benefit of mogamulizumab comes hand-in-hand with some severe skin-relevant adverse effects including some fatal cases, largely attributed to its on-target elimination of skin-resident Tregs (Honda et al. 2015; Ishida et al. 2013; Maemoto et al. 2019). The mogamulizumab-mediated Treg depletion, however, can be repurposed to treat cancers and the Sakaguchi group confirmed that targeting CCR4 by anti-CCR4 monoclonal antibody selectively depletes effector-type Tregs and evokes the immune response to cancer (Sugiyama et al. 2013). Many following studies confirmed the Treg-depleting effect is through antibody-mediated cytotoxicity (ADCC) (Chang et al. 2016; Kurose et al. 2015; Maeda et al. 2019; Ni et al. 2015; Ogura et al. 2014; Remer et al. 2014; Winsett et al. 2017). The first reported Phase I cancer trial using mogamulizumab showed promising Treg depletion and limited toxicity, with additional on-target depletion of Th2 and Th17 cells (Kurose et al. 2015). Another Phase I study was recently reported and showed that mogamulizumab, in combination of nivolumab, provides a relative safety profile—with manageable level 3 or 4 treatment-related adverse events in 29% patients, showing evidence of anti-tumor activity and on-target Treg depletion (Doi et al. 2019). There are several other on-going early trials assessing the toxicity and anti-tumor activity in solid cancers (Table 12.1). A recent report puts some doubt on the recovery of Tregs after mogamulizumab treatment in a patient with severe graft-versus-host disease (GVHD), where the elimination of residual mogamulizumab by plasma exchange did not result in prompt recovery of donor Tregs (Sugiura et al. 2019). This situation, if it also turns out to be true, may be the primary reason for mogamulizumab-treatment related adverse events in the skin (Honda et al. 2015; Ishida et al. 2013; Maemoto et al. 2019) or in the long run may lead to chronic autoimmune diseases as seen in cancer patients treated with ICIs (Michot et al. 2016).

12.3.3.2 CCR5

CCR5 is expressed within and mediates the functions of several immune cell types, including T cells, macrophages, eosinophils, myeloid-derived suppressor cells (MDSC), and dendritic cells (Jiao et al. 2019). Cancer cells can have elevated CCR5 expression that provides them the proliferative, migratory, and/or invasive properties (Jiao et al. 2018; Nishikawa et al. 2019; Singh et al. 2018; Tang et al. 2016; Yang et al. 2017; You et al. 2018; Zhang et al. 2018). The initial burst of developing CCR5 inhibitors—either small molecules or antibodies—was due to the definition of CCR5 as a receptor for human immunodeficiency virus (HIV) with mutations that can resist HIV infection (Dean et al. 1996; Samson et al. 1996). Many CCR5 inhibitors are re-purposed for clinical studies in cancer patients (Table 12.1), though all these trials are not initially designed to target Tregs. CCR5 is expressed by tumor-infiltrating Tregs in several cancer types (Schlecker et al. 2012; Tan et al. 2009). We have shown that CCL5—a ligand for CCR5—from the TME can recruit Tregs to tumors (Tan et al. 2011). Preclinical studies using CCR5 inhibitor TAK-779 disrupts CCR5-dependent recruitment of Tregs (Tan et al. 2009). These results—along with strong genetic evidence that CCR5 deletion reduces tumorigenesis—indicate a potential therapeutic effect of targeting CCR5 on certain cancer patients. The first reported Phase I trial using maraviroc in colon cancer liver metastasis showed some therapeutic effects such as decreased proliferative index and elevated immune response to metastatic tumors (Halama et al. 2016). The result did not include an analysis of Tregs. While other trials are on-going, it is expected that there will be more clinical data to explore the impact of targeting CCR5 on Treg depletion in human cancers. The expression pattern of CCR5, however, dictates a nonspecific nature, a potential caveat leading to complicated clinical outcomes.

12.3.3.3 CCR8

CCR8 was initially identified as a human monocyte and thymus chemokine receptors (Tiffany et al. 1997). Similar to CCR4, CCR8 is also selectively expressed in Th2 cells (Zingoni et al. 1998) and recently found to be elevated in tumor-infiltrating Tregs in many human cancer types (Plitas et al. 2016), tissue-resident memory T cells in human skin (McCully et al. 2018), dendritic cells during allergic immune response (Sokol et al. 2018), as well as granulocytes (Blanco-Perez et al. 2019). $CCR8^{+}$ Tregs were later identified as a major regulator of autoimmune onset in the experimental autoimmune encephalomyelitis (EAE), a mouse model used for the study of multiple sclerosis (Barsheshet et al. 2017). Among Tregs, CCR8 expression is very specific to tumor-infiltrating Tregs relative to peripheral blood and normal tissue counterparts (De Simone et al. 2016; Plitas et al. 2016). This distinct expression of CCR8 in tumor-infiltrating Tregs is very intriguing as targeting CCR8—via antibody-mediated ADCC—will result in specific deletion of tumor-infiltrating Tregs while sparing normal tissue Tregs as shown in colon cancer (Villarreal et al. 2018). CCR8-targeted therapy holds great promise in Treg-based cancer immunotherapy; however, the clinical benefit for targeting CCR8 is yet-to-be established.

12.3.4 Other Targets for Tumor-Infiltrating Tregs

We have listed several other potential targets for tumor-infiltrating Tregs (Fig. 12.2), including anti-CD25 antibodies that block IL-2 sequestration, anti-TGF-β antibody that prevents the downstream immunosuppressive effect, and potential apoptosis inducers that target the highly proliferative but vulnerable tumor-infiltrating T cells.

12.4 Perspectives

It has been known for decades that tumor-infiltrating Tregs are outstanding suppressors for the antitumor immune responses. It is perceivable that tTregs—undergoing positive selection after

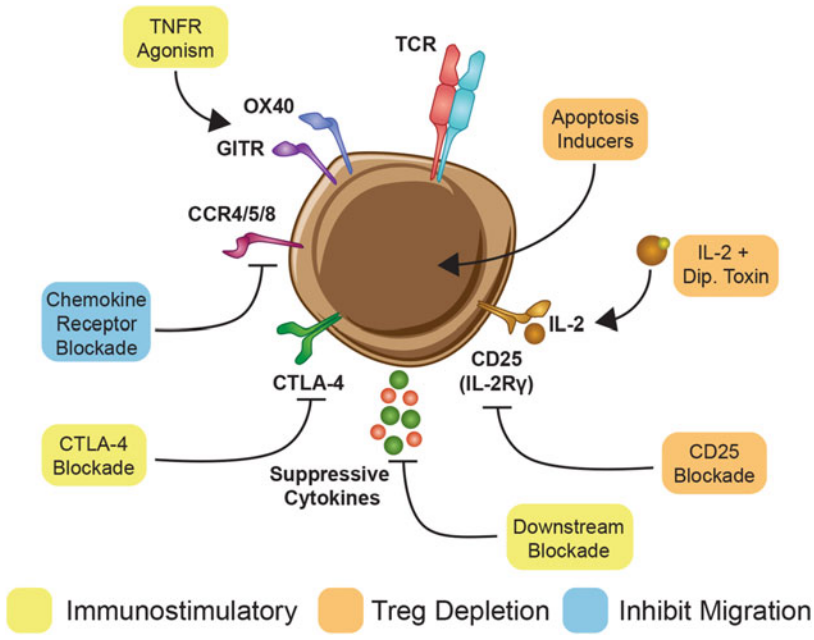

Fig. 12.2 Potential targets and therapeutics of tumor-infiltrating Tregs

encountering MHCII with self-antigens—are the major populations of tumor-infiltrating Tregs as most tumor antigens are self-antigens with a minor fraction of neoantigens. Now that we know the critical function of tumor-infiltrating Tregs in immune suppression, targeting or depleting tumor-infiltrating Tregs represents a viable approach to release anti-tumor immunity. The obstacles are (1) the efficacy of depletion/inhibition of tumor-infiltrating Tregs; (2) the specificity—including to normal Tregs and other immune cell populations; and (3) the identification of target cancer patient populations.

The first obstacle is relatively easy to conquer. For example, many outstanding publications have shown efficient Treg deletion using anti-CTLA-4 antibody, anti-CCR4 antibody, and others under the context of cancer immunotherapy in human clinical trials and mouse models of human cancer as discussed above.

The second obstacle has been a long-standing issue because all the current drug targets for Tregs are not specific to tumor-infiltrating or tumor-activated—those that are not located within tumors but are activated by tumor antigens—Tregs. This is the major reason that these Treg-targeting drugs often lead to treatment-related adverse events in cancer patients and that in many cases, these adverse events cause major problems to patients. The Sakaguchi group has tried to divide the $CD4^{+}FOXP3^{+}$ cells in the peripheral blood of healthy individuals into three distinct populations—based on the differential expression of CD45RA, FOXP3, or CD25—these including naïve/resting Tregs ($CD45RA^{+}FOXP3^{low}CD25^{low}$), effector Tregs which have high suppressive activity ($CD45RA^{-}FOXP3^{hi}CD25^{hi}$), and non-Tregs with no suppressive activity ($CD45RA^{-}FOXP3^{low}CD25^{low}$) (Miyara et al. 2009; Tanaka and Sakaguchi 2019). Numerous studies have shown that tumor-infiltrating Tregs tend to be effector Tregs which express immunosuppressive molecules, such as CTLA-4, LAG-3, and TIGIT, at higher levels than peripheral blood Tregs. As discussed previously, therapies targeting these molecules may have some selectivity in targeting tumor-infiltrating Tregs (Tanaka and Sakaguchi 2019). The problem still remains because effector Tregs exist at a significant amount within normal peripheral bloods and other tissues; hence, targeting these Tregs are inevitably leading to immune-related adverse responses. This leads to the ultimate demand for the identification of markers specific for tumor-infiltrating Tregs. Several other studies have looked at transcriptional differences between tumor infiltrating Tregs and peripheral blood Tregs or normal tissue Tregs (De Simone et al. 2016; Magnuson et al. 2018; Plitas et al. 2016). Zheng et al. used single-cell RNA sequencing of tumor-infiltrating, peripheral blood, and normal tissue lymphocytes from hepatocellular carcinoma patients to characterize tumor-infiltrating Tregs and found several differentially regulated genes; some that were identified by other studies including *CTLA4*, GITR (*TNFRSF18*), *TIGIT*, *LAYN*, 4-1BB (*TNFRSF9*), OX40 (*TNFRSF4*), and *CCR8*, as well as those that have not been identified before including *STAT3* and *RGS1* (Zheng et al. 2017). Apart from the expression of CCR8 in tumor-infiltrating Tregs and its potential prognostic value in breast cancer, Plitas et al. also found that a subset of tumor-infiltrating Tregs in breast, lung, and melanoma patients exclusively expressed *CD177*, a cell surface protein previously only studied in neutrophils (Plitas et al. 2016). Recently we identified an important function of epithelial-cell expression of CD177 in tumor suppression via attenuating β-catenin (Kluz et al. 2020) and that the expression of CD177 in tumor-infiltrating Tregs is critical in mediating the immunosuppressive function of Tregs in human cancers (Borcherding et al. 2018). These types of studies may be used to identify novel suppressive proteins and signaling pathways that are uniquely upregulated in tumor-infiltrating Tregs. Another potential avenue is to exploit the unconventional targets of tumor-infiltrating Tregs that are previously considered to be undruggable. For example, nuclear receptor 4A family (NR4A) has been shown to play a critical role in maintaining the abundance of tumor-infiltrating Tregs (Hibino et al. 2018). Our analysis indicates

that NR4A family genes are among the top of the differentially expressed genes in tumor-infiltrating Tregs in human cancers, along with a list of genes whose protein products are present intracellularly (Borcherding et al. 2018; Vishwakarma et al. 2019). These nuclear receptors and non-kinase intracellular proteins are traditionally considered as undruggable targets; however, the advance in bioengineering and medicinal chemistry makes them possible to be targeted such as using the *pro*teolysis-*ta*rgeting *c*himera (PROTAC) technology as we have recently published to better target BCL-X_L in cancer therapy (Khan et al. 2019).

The third obstacle is to choose cancer types and patient populations within certain cancer types. Like all currently available cancer immunotherapies, targeting Tregs will not benefit all cancer patients and sometimes may do more harm than good due to the broad immunosuppressive activity of Tregs including cancer-promoting immune cells. The primary consideration should be given for the abundance of immunosuppressive populations of Tregs and the immune landscaping within tumors. The threshold of Treg abundance should be the prerequisite for patients receiving anti-Treg therapy. The immune landscaping is able to dictate the potential influence of Treg depletion on shaping antitumor immunity. For example, some tumors exhibit dependence on other immunosuppressive cells such as MDSCs or on both Tregs and MDSCs, where depleting Tregs will be insufficient to invoke antitumor immunity.

The future Treg-based cancer immunotherapy should be able to compensate for ICI therapies since cancer types that benefit the most from ICI therapies show only an average of 25% response rate. As the primary ICIs targets are exhausted T cells, Treg-based therapy may benefit some patient populations as the frontline choice where ICIs are predicted to fail. Moreover, Tregs have been accused of the culprit for certain hyperprogressive cancers after nivolumab therapy (Kamada et al. 2019b), suggesting that Treg-based therapy could also benefit these hyperprogressive patients after ICI therapy.

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