Regulatory T Cells: Molecular and Cellular Basis for Immunoregulation

Yosuke Togashi and Hiroyoshi Nishikawa

Abstract $CD4^+$ regulatory T cells (Tregs) are a highly immune-suppressive subset of $CD4^+$ T cells, characterized by expression of the master regulatory transcription factor FOXP3. Tregs are proven to play central roles in the maintenance of self-tolerance in healthy individuals. Tregs are involved in maintaining immune homeostasis: they protect hosts from developing autoimmune diseases and allergy, whereas in malignancies, they promote tumor progression by suppressing anti-tumor immunity. Elucidating factors influencing Treg homeostasis and function have important implications for understanding disease pathogenesis and identifying therapeutic opportunities. Thus, the manipulating Tregs for up- or down-regulation of their suppressive function is a new therapeutic strategy for treating various diseases including autoimmune disorders and cancer. This review will focus on recent advances in how Tregs integrate extracellular and intracellular signals to control their survival and stability. Deeper mechanistic understanding of disease-specific Treg development, maintenance, and function could make disease-specific Treg-targeted therapy more effective, resulting in an increase of efficacy and decrease of side effects related to manipulating Tregs.

Contents

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1 Introduction

$CD4^+$ regulatory T cells (Tregs) are a highly immune-suppressive subset of $CD4^+$ T cells, characterized by expression of the master regulatory transcription factor FOXP3 (Sakaguchi et al. 1995; Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003). Tregs were originally identified as $CD4^+CD25^+$ T cells by Sakaguchi et al. (1995) and are proven to play central roles in the maintenance of self-tolerance in healthy individuals (Sakaguchi et al. 2010; Wing and Sakaguchi 2010). Mutations of the human FOXP3 result in impaired development or dysfunction of Tregs and, consequently, the occurrence of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome accompanying severe autoimmune diseases, inflammatory bowel disease, and allergy (IPEX syndrome) (Bennett et al. 2001). Likewise, mice that carry a mutation or genetic deletion of FOXP3, called Scurfy mice are deficient in Tregs and develop fatal systemic autoimmunity (Brunkow et al. 2001; Fontenot et al. 2003). In addition, forced expression of FOXP3 is able to confer Treg-like suppressive activity on naive conventional T cells (Tconvs) (Fontenot et al. 2003; Hori et al. 2003). FOXP3 has therefore been considered as a lineage-specifying transcription factor of Tregs or a master regulator of its functions.

Tregs are involved in maintaining immune homeostasis: they protect hosts from developing autoimmune diseases and allergy, whereas in malignancies, they promote tumor progression by suppressing anti-tumor immunity (Onizuka et al. 1999; Shimizu et al. 1999; Sakaguchi et al. 2010; Wing and Sakaguchi 2010). Elucidating factors influencing Treg homeostasis and function have important implications for understanding disease pathogenesis and identifying therapeutic opportunities. Thus, manipulating Tregs for up- or down-regulation of their suppressive function is a new therapeutic strategy for treating various diseases including autoimmune disorders and cancer. This review will focus on recent advances in how Tregs integrate extracellular and intracellular signals to control their survival and stability. We will discuss how these new insights can be utilized for the development of new approaches to promote and stabilize Tregs in many illnesses.

2 Development and Maintenance of Tregs

Tregs are separated into natural/thymic and peripheral/induced Tregs based on the sites where they are generated (Sakaguchi et al. 2010, Adeegbe and Nishikawa 2013). $FOXP3^+$ natural Tregs are generated in the thymus as an antigen-primed and functionally mature T cell subpopulation specialized for immune suppression (natural/thymic Tregs; nTregs). Some of $FOXP3^+$ Tregs also differentiate from Tconvs in the periphery under certain conditions (peripheral/induced Tregs; iTregs). The main task of $FOXP3^+$ nTregs is to migrate to inflammatory sites and suppress various effector lymphocytes, especially helper T (Th) cell subsets and $CD8^+$ cytotoxic T cells (Chaudhry et al. 2009; Koch et al. 2009; Chung et al. 2011; Linterman et al. 2011). nTregs reportedly express high levels of Helios (a member of the Ikaros transcription factor family) and Neuropilin-1 (a type-1 transmembrane protein). In contrast, iTregs that develop in the periphery often lack or have a low-level expression of these molecules. According to data from animal models, these iTregs are readily converted from Tconvs by in vitro stimulation with TGF-β or retinoic acid (Coombes et al. 2007). However, in humans, $FOXP3^+$ T cells induced from Tconvs by in vitro TCR stimulation with TGF-β fail to gain suppressive function and rather produce pro-inflammatory cytokines (Walker et al. 2005; Tran et al. 2007). At present, the function of iTregs such as TGF-β-induced ones in humans is obscure though there are some reports showing that several cytokines or a specific microbiota environment can induce Tregs with a suppressive function from $CD4^+CD25^-$ T cells (Ellis et al. 2012; Atarashi et al. 2013; Hsu et al. 2015). Yet it remains to be determined whether these peripherally induced $FOXP3^+$ Tregs are functionally stable in vivo.

2.1 TCR, CD28, and IL-2

nTreg development is initiated by TCR signal followed by a sequential activation of CD25 (IL-2 receptor α chain) expression, IL-2 signal, and FOXP3 expression (Lio and Hsieh 2008; Weissler and Caton 2014). Although not fully clarified in humans, nTregs stem from self-reactive thymocytes present in the thymus (Sakaguchi et al. 2010). A fraction of $CD4^+CD8^-$ thymocytes receive T cell receptor (TCR) stimulation by complexes of major histocompatibility complex (MHC) plus self-peptide and acquire expression of CD25, through which IL-2 signals are delivered via STAT5, resulting in expression FOXP3 and differentiation into Tregs (Jordan et al. 2001; Boyman and Sprent 2012; Malchow et al. 2013). nTreg development can be enhanced through the constitutive activation of STAT5 and directly binds cis elements in the FOXP3 promoter and enhancer to stabilize FOXP3 expression (Burchill et al. 2008). In addition to induction of CD25, TCR and CD28 signal also contribute to establishing and stabilizing the Treg lineage commitment in the thymus by inducing epigenetic and differentiation events in Tregs (Salomon et al.

2000; Tai et al. 2013; Zhang et al. 2013; Franckaert et al. 2015). Thus, antigen and IL-2 signal provided through TCR, CD28, and CD25 are essential for Treg lineage commitment in the thymus.

In the periphery, mature Treg survival for their homeostasis and function depends on TCR, CD28, and CD25, but their roles appear to be distinct from those in the thymus. Tregs proliferate more than Tconvs in steady state in a CD28 dependent fashion, suggesting that Tregs continuously recognize cognate antigens driving their cell cycle progression (Tang et al. 2003; Walker et al. 2003). Indeed, analysis of Treg subsets in the periphery shows that continuous stimulation through the TCR is required to maintain this population (Levine et al. 2014; Vahl et al. 2014). TCR-deficient Tregs proliferated less and expressed fewer effector molecules such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4), IL-10, and Ebi3.

Proliferating Tregs have a tendency to lose their FOXP3 expression and lineage stability in vitro and in vivo in lymphopenic hosts (Hoffmann et al. 2006; Zhou et al. 2009; Rubtsov et al. 2010). The conserved noncoding sequence 2 (CNS2) enhancer element, also known as Treg-specific demethylation region, is crucial for safeguarding lineage stability of proliferating Tregs (Feng et al. 2014; Li et al. 2014). However, stimulation via TCR with limited IL-2 leads to a loss of FOXP3 expression in Tregs with intact CNS2. CNS2 harbors binding sites for both the TCR-triggered transcription factor nuclear factor of activated T cells (NFAT) and IL-2-induced transcription factor STAT5, providing a transcriptional basis for Treg stability by coordinating TCR and IL-2 signal. Interestingly, forced expression of constitutively active STAT5 prevented the loss of FOXP3 in CNS2-deleted Tregs, demonstrating that STAT5 can stabilize FOXP3 expression independent of CNS2 (Feng et al. 2014). This may be explained by the NFAT-mediated looping between CNS2 and the FOXP3 promoter, also having NFAT and STAT5 binding sites (Li et al. 2014). Together, TCR-mediated signals are important for mature Treg function but pose a threat to their stability unless they are balanced by the IL-2 signal.

2.2 PI3K-Akt-mTOR

Phosphatidylinositide 3 kinase (PI3K), protein kinase B (Akt), and mammalian target of rapamycin (mTOR) form an intracellular signal hub common to the TCR, CD28, and IL-2 receptor. PI3K is directly activated when these receptors are engaged, leading to initial activation of Akt by the PH-domain containing protein PDK1 through phosphorylation of threonine 308. Akt is fully activated by additional phosphorylation on serine 473 by the mTOR complex 2 (mTORC2). Akt has many cellular targets; the Forkhead box O (Foxo) transcription factors and mTORC1 are most relevant to Treg biology. Foxo family transcription factors are crucial for Treg lineage commitment (Harada et al. 2010; Ouyang et al. 2012; Samstein et al. 2012) and are inhibited by Akt. mTORC1 coordinates anabolic

activities in cells and inactivates mTORC2, limiting further Akt activation. In the thymus, Treg development is enhanced by mutating the p110d catalytic subunit of PI3K (Patton et al. 2006) and is repressed by forced expression of a constitutively active Akt (Haxhinasto et al. 2008), demonstrating a negative role of the PI3K axis on nTreg development. However, deletion of mTOR (thus inactivating both mTORC1 and 2) or individual deletion of mTORC1 or 2 in T cells does not alter thymic development (Delgoffe et al. 2009), suggesting that the negative effect of PI3K and Akt on nTreg development is mTOR independent and mainly due to their role in Foxo1 inactivation.

Activation of PI3K is naturally antagonized by phosphatase and tensin homolog (PTEN). PTEN expression is progressively inhibited by stronger TCR stimulation, permitting efficient T cell activation and effector differentiation, an effect mediated by IL-2-inducible T cell kinase (Itk) (Gomez-Rodriguez et al. 2014). Thus, T cells with Itk deficiency fail to down regulate PTEN after activation and favor FOXP3 induction. In committed Tregs, the PI3K-Akt-mTOR signal axis continues to be repressed by high expression of PTEN. Treg-specific deletion of PTEN disrupted Treg homeostasis, function, and stability (Huynh et al. 2015; Shrestha et al. 2015). These PTEN-deficient Tregs lost both FOXP3 and CD25 expression but had a significant increase of mTORC2, but not mTORC1 activities. Additional deletion of mTORC2 in Tregs largely rescues the phenotype in mice with Treg-specific deletion of PTEN, demonstrating the normal function of PTEN in mature Tregs is to keep mTORC2 in check. In fact, the intact mTORC1 function is required for Treg function because mice with selective deletion of mTORC1 in Tregs die of multi-organ autoimmune diseases similar to FOXP3-deficient mice (Zeng et al. 2013). Mechanistically, mTOR is found to control Treg function in part by regulating metabolic programming. T cells rely on mitochondrial oxidative phosphorylation at steady state and switch to glycolysis after activation, a process essential for effector T cell differentiation (Wang and Green 2012). In contrast, Tregs preferentially use oxidative metabolism even after activation. An emerging concept is that metabolic input can also dictate T cell fate decision (Wang and Green 2012). PTEN-deficient Tregs show exaggerated glycolysis that is thought to contribute to Treg instability (Huynh et al. 2015; Shrestha et al. 2015). Additionally, functional defects in mTORC1-deficient Tregs are associated with disrupted lipid biosynthesis (Zeng et al. 2013). Thus, the impact of PI3K-Akt-mTOR axis on mature Treg function is controversial, while excessive activation of this pathway is clearly detrimental to Treg function as observed in PTEN-deficient Tregs; complete blockade of PI3K impairs Treg function as well (Patton et al. 2006; Patton et al. 2011).

2.3 Epigenetics

Epigenetic modifications, which include histone modifications, DNA methylation, microRNAs, nucleosome positioning, chromatin interaction, and chromosome

conformational changes, play indispensable roles in cell differentiation, especially for cell-lineage stabilization (Kim et al. 2009). In particular, DNA methylation and histone modifications critically contribute to cell-lineage determination and maintenance because they are heritable through cell divisions. Genomic DNA is mainly methylated by DNA methyltransferases (DNMT family members), whereas it can be demethylated by multiple steps, including methylcytosine hydroxylation mediated by TET family members (Bhutani et al. 2011; Pastor et al. 2011). Similarly, histones are modified for gene activation or repression by acetylation or deacetylation, methylation or demethylation, and phosphorylation or dephosphorylation (Teperino et al. 2010); therefore, epigenetic status is reversible. It is also known, however, that DNA methylation status modified in the early stages of development, such as genomic imprinting, is stably maintained throughout subsequent differentiation processes. Epigenetic changes of some specific loci are also stably sustained in specific cell lineages, including Tregs (Ansel et al. 2003; Schmidl et al. 2009; Ohkura et al. 2012). Recent genome-wide analyses have revealed several regions that show different patterns of DNA methylation or histone modification between Tconvs and Tregs in humans and mice (Floess et al. 2007, Schmidl et al. 2009; Wei et al. 2009; Ohkura et al. 2012). Such genes with Treg-specific DNA hypomethylation include those encoding Treg-function-associated or Treg-specific molecules, such as FOXP3, CTLA-4, and Eos (Ohkura et al. 2012). Furthermore, some Treg-specific changes in DNA methylation are highly stable in Tregs, whereas others are not. For example, *Foxp3* intron 1 (CNS2), *Ctla4* exon 2, and *Ikzf4* (encoding Eos) intron 1, are specifically demethylated in nTreg cells, and the hypomethylation status is stable after TCR stimulation, cell proliferation, or cytokine treatments (e.g., with IL-2 or TGF-β) (Ohkura et al. 2012). In contrast, the DNA methylation status of *Il2ra* intron 1, which is demethylated in nonactivated Tregs, is relatively unstable and demethylated in Tconvs by in vitro culture with or without TCR stimulation. In addition, enhanced H3 K4me3 histone modification of the Treg signature genes detected in nTregs is easily primed in Tconvs under a Th1-, Th2-, or Th17-cell-polarizing or iTreg-inducing condition (Wei et al. 2009). Along with these findings, a high-resolution DNaseI footprint analysis has shown that specific alterations in chromatin accessibility occur in Tregs in the course of their differentiation from their precursors (Samstein et al. 2012), although the DNaseI-hypersensitive regions do not differ mostly between $CD4^+FOXP3^-$ T cells and $CD4^+FOXP3^+$ T cells, indicating a small number of genes show increased hypersensitivity in Tregs, specific alterations in local nucleosome positioning and chromatin accessibility. The loci identified as newly accessible in Tregs are enriched in the genes known to be critical for Treg function, such as *Foxp3*, *Ctla4,* and *Ikzf2*. They are also classified as genes possessing Treg-specific DNA hypomethylated regions in Tregs, as discussed above (Schmidl et al. 2009; Ohkura et al. 2012). Together, Tregs acquire and sustain highly specific and stable epigenetic changes as exemplified by DNA hypomethylation at specific loci of a limited number of genes. This Treg-specific DNA hypomethylation is a reliable marker for assessing the epigenetic status of Tregs.

3 Functional Classification of Tregs

While Tregs are originally identified as $CD4^+$ T cells, expressing CD25, as CD25 is an activation marker and its expression is not confined to Tregs, additional markers are needed. Although $CD4^+CD25^+$ T cells with additional low-level expression of CD127 (IL-7 receptor α-chain) were reported to possess FOXP3 expression and suppressive function (Liu et al. 2006; Seddiki et al. 2006), CD127 is also down-regulated following recent activation of naive T cells that also express a low level of FOXP3 (Mazzucchelli and Durum 2007), suggesting a possible contamination of non-Tregs in the $CD127^{low}CD4^+CD25^+$ T cell fraction. FOXP3 is the master regulatory molecule in Tregs, and expression of FOXP3 represents the Treg population in mice. In contrast, to definitely identify Tregs in humans causes difficulty due to the upregulation of FOXP3 upon TCR stimulation of Tconvs (Tran et al. 2007). We have therefore proposed a classification of human Tregs based on the expression levels of a naive marker CD45RA and of FOXP3 (Fig. 1 and Table 1) (Miyara et al. 2009; Sakaguchi et al. 2010; Nishikawa and Sakaguchi 2014; Takeuchi and Nishikawa 2016). $FOXP3^+CD4^+$ T cells can thus be divided into three fractions: naive Tregs ($CD45RA^+FOXP3^{low}CD4^+$); effector Tregs (eTregs: $CD45RA^-FOXP3^{high}CD4^+$); and non-Tregs ($CD45RA^-FOXP3^{low}CD4^+$). The naïve Tregs have recently egressed from the thymus, have not yet been activated in the periphery and possess weak suppressive activity. Upon activation with TCR stimulation, naïve Tregs vigorously proliferate and differentiate into highly suppressive eTregs. In contrast, non-Tregs are not immune suppressive but are rather immune stimulatory T cells, producing inflammatory cytokines including IFN-γ and IL-17 (Miyara et al. 2009). This classification, based on Treg function, reflects the pathophysiology of autoimmune and inflammatory diseases. Both sarcoidosis patients lacking tuberculin reaction due to an immune-suppressive state and systemic lupus erythematosus (SLE) patients with systemic autoimmunity have increased $FOXP3^+CD4^+$ T cells in the peripheral blood (Miyara et al. 2009). In our classification with CD45RA and FOXP3 expression, highly suppressive eTregs ($CD45RA^-FOXP3^{high}CD4^+$) are the dominant component of $FOXP3^+CD4^+$ T cells in sarcoidosis, whereas $FOXP3^+$ non-Tregs ($CD45RA^-FOXP3^{low}CD4^+$) are increased in SLE (Miyara et al. 2009), clearly demonstrating the immune-suppressive state and a dysregulation of self-tolerance in sarcoidosis and SLE, respectively.

4 Suppressive Mechanism of Tregs

Tregs exhibit their suppressive activity by numerous cellular and humoral mechanisms (Fig. 2 and Table 2) such as suppression of antigen-presenting cells (APCs) via CTLA-4, secretion of inhibitory cytokines (IL-10, TGF-β and IL-35), expression of granzyme/perforin, consumption of IL-2, and degradation of ATP

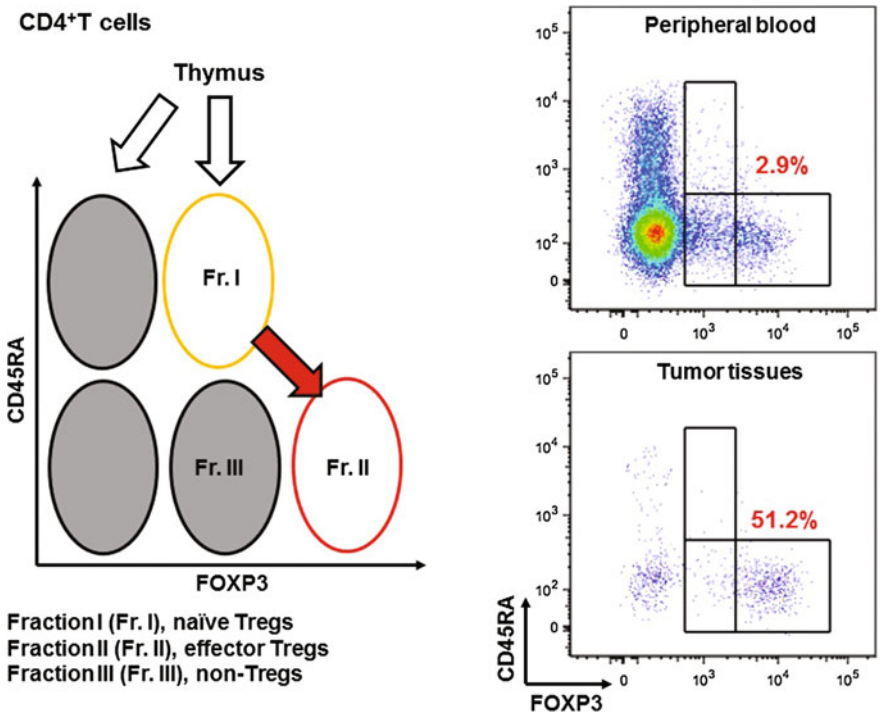

Fig. 1 Functional classification of human FOXP3$^+$CD4$^+$ T cell subpopulations. Human FOXP3$^+$ T cells in the peripheral blood and lymph nodes are composed of heterogeneous subpopulations containing suppressive Tregs (naïve and effector Tregs) and non-Tregs without suppression function. Human Tregs are classified into naive and effector Tregs by the expression levels of a naive marker CD45RA and of FOXP3. These subpopulations are designated as Fraction (Fr.) I, II, and III for naive Tregs, effector Tregs (eTregs), and non-Tregs, respectively. CD25 surface marker can be used in the place of FOXP3 because of their correlative expression in humans. In tumor tissues compared with peripheral blood, naive Tregs (Fr. I) numbers are reduced and highly suppressive eTregs (Fr. II) numbers are increased. The frequency of FOXP3$^+$ non-Tregs (Fr. III) is variable depending on cancer types

(Sakaguchi et al. 2010). Among these mechanisms, suppression via CTLA-4 (a co-inhibitory receptor constitutively expressed by Tregs) and IL-2 consumption via CD25 (IL-2 receptor α-chain, also constitutively expressed by Tregs) appear to play key roles for the following reasons: Treg-specific CTLA-4 deficiency impairs in vitro and in vivo Treg-mediated suppression (Wing et al. 2008); FOXP3 directly suppresses IL-2 gene transcription and upregulates *Ctla4* and *Il2ra* genes transcription (Hori et al. 2003); and high-dose IL-2 neutralizes in vitro Treg-mediated suppression (Takahashi et al. 1998; Thornton and Shevach 1998).

CTLA-4 engages with B7 molecules (i.e., B7-1 and B7-2; CD80 and CD86) on APCs with higher avidity compared with CD28 (Walker and Sansom 2011) and

Table 1 Classification of $FOXP3^{+}CD4^{+}$ T cells

Subset	Phenotype	Characteristics
Naïve Tregs (Fraction I, $CD45RA^{+}FOXP3^{low}CD4^{+}$)	CTLA-$4^{low}CD25^{high}CD127^{low/-}$ Ki-67^{-}	• Weak suppressive activity • Differentiate to effector Tregs upon TCR stimulation
Effector Tregs (Fraction II, $CD45RA^{-}FOXP3^{high}CD4^{+}$)	CTLA-$4^{high}CD25^{high}$ Ki-67^{+} PD-1^{+}, TIM-3^{+}, GITR^{+} Fas^{+}, IL-10^{+}, TGF-β^{+}	• Strong suppressive and proliferative activity • Prone to apoptosis • Tend to increase in peripheral blood with aging
Non-Tregs (Fraction III, $CD45RA^{-}FOXP3^{low}CD4^{+}$)	IL-2^{+}, IFN-γ^{+}, IL-17^{+}	• Heterogeneous population • No suppressive activity

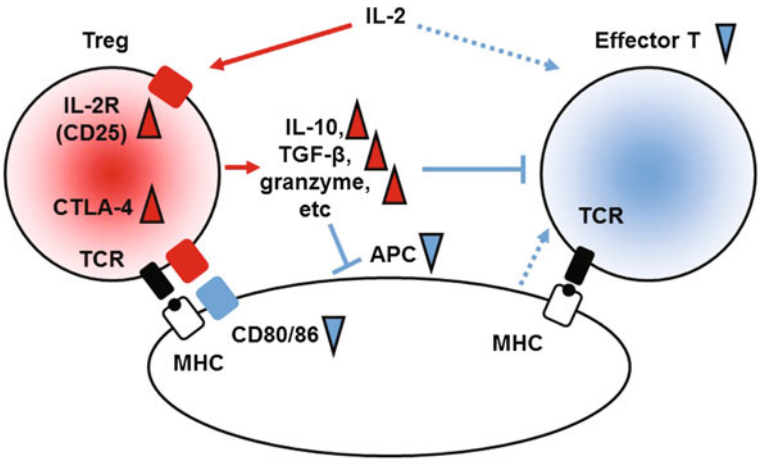

Fig. 2 Treg suppression mechanisms. Treg cells, which scarcely produce IL-2, deprive IL-2 from the surrounding via their high-affinity IL-2 receptor (IL-2R), making it unavailable for effector T cells. They also constitutively express CTLA-4, which down-modulates CD80/CD86 expression by antigen-presenting cells (APCs), thus depriving co-stimulatory signal to effector T cells. Tregs also produce immune-suppressive cytokines such as IL-10 and TGF-β, and secret granzymes, which also down-modulates APC and effector T cell functions. TCR, T cell receptor; MHC, major histocompatibility complex

provides inhibitory signaling to APCs. In mice, Treg-specific deletion of CTLA-4 elicits systemic hyper-proliferation of Tconvs, resulting in fatal autoimmune diseases affecting multiple organs, including severe myocarditis (Wing et al. 2008). Recently, heterozygous CTLA-4 mutations in humans were identified in patients with multiple autoimmune symptoms accompanied by impaired suppressive

Table 2 Key mechanisms of suppression by Tregs

Molecule	Mechanism of suppression
IL-2 receptor/IL-2	Constitutive expression of high-affinity IL-2 receptor α chain (CD25) and dependency on exogenous IL-2 by Treg cells together limit the amount of IL-2 available to Tconvs, thereby hindering the activation and proliferation of the latter
CTLA-4	Constitutively expressed CTLA-4 on Tregs preferentially binds to and downregulates CD80/CD86 co-stimulatory molecules on APCs, depriving Tconvs of the co-stimulatory signal
IL-10, TGF-β and other immune-suppressive cytokines and substances	Tregs produce immune-suppressive cytokines, such as IL-10 and TGF-β, form extracellular adenosine from ATP by CD39 and CD73, and can also mediate direct killing of Tconvs or APCs by secreting granzymes

function of Tregs (Kuehn et al. 2014; Schubert et al. 2014). In addition, B7 molecules are physically transferred to the surface or the cytoplasm of Tregs together with CTLA-4 (Walker and Sansom 2011). Then, maturation of APCs (via the co-stimulatory signal from B7 to CD28 on effector cells) is strongly inhibited. Treg expression of CTLA-4 is therefore essential for Treg-mediated immune suppression.

5 Tregs and Autoimmune Diseases

Reducing the number and function of Tregs compromises self-tolerance, leading to abnormal immune responses to self-antigens, thus resulting in autoimmune diseases (Wing and Sakaguchi 2010). It has been shown that Treg impairment is involved in the pathogenesis of many autoimmune diseases including rheumatoid arthritis (RA), SLE, and ANCA-associated vasculitis, etc. (Scheinecker et al. 2010; Free et al. 2013; Prakken et al. 2013). On the account of heterogeneity and complexity of autoimmune diseases, the function of Tregs needs to be evaluated deliberately. In some autoimmune settings, Treg numbers or frequencies reportedly are reduced compared to healthy controls, while the others not. This may suggest the existence of different Treg phenotypes in disease tissues (Bonelli et al. 2008; Monte et al. 2008). Even though the presence of dysfunctional Tregs in autoimmune diseases is commonly observed and manipulation of these Tregs are an essential issue (Ehrenstein et al. 2004; Venken et al. 2008; Rapetti et al. 2015). Cell therapy and/or reagents manipulating Tregs, therefore, are under intense scrutiny.

5.1 Treg Cell Therapy for Autoimmune Diseases

Mouse models reveal that Treg infusion can prevent/treat autoimmune diseases, and clinical application of Treg administration is now being tested in humans. The first reports of Treg infusion therapy for autoimmune diseases were in the context of type 1 diabetes (Marek-Trzonkowska et al. 2012; Bluestone et al. 2015). In these studies, polyclonal Tregs were administered with no safety concerns. Tracking Tregs with 6,6-$^{2}H_{2}$ glucose labeling showed that infused cells are present for at least a year, with no evidence for loss of the expected Treg phenotype (Bluestone et al. 2015).

It is, however, challenging to obtain therapeutic doses of Tregs due to the weak in vitro proliferative capacity. For example, by extrapolating data from mice, the therapeutic dose of polyclonal Tregs is estimated to be 3–5 $\times$ 10^{9} Tregs for a 70 kg patient (Tang and Lee 2012). To gain billions of Tregs is laborious and require to develop novel strategies to improve in vitro expansion of Tregs, such as high IL-2 and mTOR inhibition with rapamycin to stimulate Treg division and limit Tconv outgrowth, respectively. In addition to limited cell numbers, polyclonal Tregs carry the risk of non-specific suppression inducing side effects. Indeed a transient increase in viral reactivations was observed in hematopoietic stem cell transplantation patients treated with cord blood-derived Tregs (Brunstein et al. 2013). To overcome these limitations of polyclonal Tregs, methods to generate antigen-specific Tregs are being explored, including antigen-stimulated expansion (Lee et al. 2014), TCR transduction (Kim et al. 2015), and engineering with chimeric antigen receptors (CARs) (MacDonald et al. 2016; Yoon et al. 2017). All of these strategies should maximize disease control with lower numbers of Tregs as in mice, antigen-specific Tregs are 100 fold more potent than polyclonal cells (Hoeppli et al. 2016).

In addition, the suppressive function can be added into Tconvs by overexpressing FOXP3, or by culture with immunosuppressive cytokines such as TGF-β. The stability of cells arising from the latter, however, is unclear, with epigenetic analysis, suggesting that these "induced iTregs" may not be stable in humans (Rossetti et al. 2015). The first application of overexpressing FOXP3 will likely be as gene therapy for IPEX syndrome patients (Bacchetta et al. 2016). For wider application in autoimmunity, a better understanding of Treg function by simple FOXP3 overexpression is necessary to optimize the Treg cell therapy (Bhairavabhotla et al. 2016).

5.2 Non-Cell-Based Therapies

Because of the complexity and highly personalized nature of cell therapy, strategies to augment endogenous Treg numbers and function in vivo may be preferable.

Methods manipulating IL-2 availability are the deeply tested in clinical testing, with other methods promoting FOXP3 expression in the early stage of exploration. The unique requirement of Tregs for exogenous IL-2, constitutive expression of the high-affinity IL-2 receptor, and the association with poor IL-2 response in autoimmunity offers an ideal target for therapeutic manipulation. Whereas high-doses of IL-2 enhance Tconvs in vivo, low doses seem to specifically stimulate Treg survival/expansion. A trial of low dose IL-2 in type 1 diabetes found a dose-dependent increase in numbers of $CD4^{+}FOXP3^{+}$ Tregs and increased CD25, GITR, CTLA-4, and pSTAT5 (Rosenzwajg et al. 2015). Encouragingly, at the highest dose, Tconv responses against beta-cell antigens were suppressed in all patients, leading to the initiation of a larger phase-IIb trial (NCT02411253). This approach has also had success in the treatment of SLE (von Spee-Mayer et al. 2016), with additional trials of low-dose IL-2 planned in RA (NCT02467504), relapsing remitting multiple sclerosis (NCT02424396) and other several autoimmune/autoinflammatory disorders (TRANSREG study, NCT01988506). IL-2 has a short half-life, which can be prolonged through the administration of a cytokine-antibody complex. Careful selection of the anti-IL-2 antibody can allow tailored signal; the JES6-1 anti-mouse IL-2 antibody lowers the affinity of IL-2 for CD25, favoring signal to $CD25^{high}$ Tregs (Spangler et al. 2015). IL-2 itself can also be engineered, creating variants that have more or less affinity for the individual receptor chains, allowing preferential stimulation of Tconvs (Mitra et al. 2015) or, presumably, in the future, of Tregs. It is yet questionable whether these strategies will be feasible in humans due to the high CD25 expression on activated human Tconvs.

Rapamycin (sirolimus) preferentially favors Treg suppression by blocking Tconv proliferation and promoting FOXP3 mRNA expression and is now commonly used as a 'Treg sparing' immuno-suppressant in transplantation. Rapamycin is also being explored in autoimmunity, with a trial in multi-lineage autoimmune cytopenias showing rapid and long-lasting responses in a majority of children with the autoimmune lymphoproliferative syndrome, and encouraging results in those with SLE (Bride et al. 2016). Additionally, clinical trials are ongoing to test the effect of rapamycin in Crohn's disease patients with stenosis (NCT02675153) or in combination with islet transplantation in type 1 diabetes (NCT02505893; NCT00679042). With our knowledge for how peripheral Tregs develop, therapies that harness these processes are also being explored (Hardenberg et al. 2011). For example, Vitamin C can potentiate Tregs by regulating the activity of TET enzymes, which demethylate Treg-specific hypomethylated regions, including the FOXP3 locus (Yue et al. 2016). Similarly, all-trans retinoic acid, the metabolite of vitamin A, prevents human Tregs from becoming unstable by increasing histone acetylation in the FOXP3 promoter and demethylation of the Treg-specific demethylation region (Lu et al. 2014). Overall, there are many complementary strategies to enhance Tregs in vivo and it will be important to compare the effectiveness of these approaches with cell-based therapies.

6 Tregs and Malignant Tumors

The involvement of Tregs in tumor immunity was originally reported in 1999 (Onizuka et al. 1999; Shimizu et al. 1999). Mice treated with the anti-CD25 antibody (which depleted $CD4^+CD25^+$ Tregs) and nude (T cell deficient) mice transferred with splenocytes deprived for $CD25^+$ cells, exhibited tumor rejection and retardation of tumor growth. In the tumor microenvironment (TME) of melanoma, non-small cell lung, gastric and ovarian cancers, eTregs heavily infiltrate and account for 20–50% of $CD4^+$ T cells, as compared with 5–10%in the peripheral blood of healthy individuals (Sakaguchi et al. 2010; Nishikawa and Sakaguchi 2014; Saito et al. 2016; Takeuchi and Nishikawa 2016) (Fig. 1). High infiltration of Tregs in tumors is associated with a poor prognosis in various types of cancers including melanoma, non-small cell lung, gastric, hepatocellular, pancreatic, renal cell, breast and cervical cancers (Fridman et al. 2012; Nishikawa and Sakaguchi 2014). Yet in some cancers such as colorectal, head and neck, and bladder cancers, a higher infiltration of $FOXP3^+$ T cells is reportedly correlated with better prognosis (Fridman et al. 2012). In fact, in colorectal cancer, we have recently shown that $FOXP3^+$ non-Tregs heavily infiltrated a fraction of colorectal cancers containing high levels of inflammatory cytokines such as TGF-β and IL-12 and were associated with a better prognosis (Saito et al. 2016). The difficulty of distinguishing $FOXP3^+$ non-Tregs from $FOXP3^{high}$ eTregs in tumor tissues would have been a major confounding factor in previous studies evaluating the clinical significance of $FOXP3^+CD4^+$ T cells in colorectal cancers using immunohistochemistry.

6.1 Trafficking and Characteristics of Tregs in Cancer

Tregs are chemo-attracted to the TME although the combination of chemokines and their receptors differs in each cancer type (i.e., CCR4 with CCL22 in breast, colorectal, oral and ovarian cancer; CCR10 with CCL28 and CXCR4 with CXCL12 in ovarian cancer; and CCR5 with CCL5 in pancreatic cancer, etc.) (Curiel et al. 2004; Ishida et al. 2006; Wei et al. 2007; Gobert et al. 2009; Tan et al. 2009; Watanabe et al. 2010; Facciabene et al. 2011; Svensson et al. 2012). Blockade of chemotaxis by antibodies or small molecules may result in a reduction in Treg numbers in tumors (Tan et al. 2009; Spranger et al. 2013). These Treg-recruiting chemokines are generated in TMEs by macrophages and/or tumor cells. Additionally, $CD8^+$ T cells in tumors also produced Treg-recruiting chemokines with their exhaustion (Williams et al. 2017). In the TME, highly immune-suppressive eTregs with high-level expression of suppression-related molecules such as CTLA-4 and TIGIT are detected with reduced number of naïve Tregs, indicating a highly activated status of tumor-infiltrating Tregs (Sugiyama et al. 2013; Nishikawa and Sakaguchi 2014, Saito et al. 2016; Takeuchi and Nishikawa 2016). One possible mechanism of Treg activation in tumors is that proliferating and dying tumor cells provide a large amount of self-antigens, which Tregs might recognize, and be activated, as tumors

contain a subset of immature dendritic cells that promote the proliferation/stimulation of Tregs in a TGF-β-dependent manner (Ghiringhelli et al. 2005; Nishikawa et al. 2005). In accordance with this, the TCR repertoire of tumor-infiltrating Tregs is skewed and largely distinct from that of tumor-infiltrating Tconvs, suggesting that Tregs recognize certain skewed antigens and clonally expand in the TME (Hindley et al. 2011; Sainz-Perez et al. 2012). Yet whether these antigens are exclusively recognized by Tregs or recognition is shared by Th cells is unclear; however, Tregs usually harbor higher affinity TCRs compared with Tconvs and should be predominantly activated in tumors.

6.2 *Strategies for Treg-Targeted Therapy*

As discussed above, eTregs are present at a high frequency in tumors and need to be controlled for the generation/activation of anti-tumor immunity. Some clinical studies indicated the potential of depleting CD25-expressing lymphocytes to augment anti-tumor immune responses, yet other similar studies failed. As activated effector T cells also express CD25, CD25-based cell depletion may reduce activated effector T cells as well, canceling the effect of Treg depletion to augment anti-tumor immunity. Treg depletion by the CD25-depleting antibody daclizumab has been evaluated in clinical trials. When daclizumab was administered following dendritic cell vaccination in metastatic melanoma (n = 15), not only Tregs but also activated effector cells were depleted and neither anti-tumor immune responses nor antibody production was observed (Jacobs et al. 2010). In contrast, in breast cancer patients, administration of daclizumab followed by vaccination consisting of multiple tumor-associated peptides succeeded in Treg depletion and demonstrated favorable clinical responses (Rech et al. 2012). Additionally, one plausible concern is increased autoimmunity-related toxicities following Treg depletion. In order to secure the safety of Treg-targeted therapy, selective depletion of eTregs in tumors rather than the entire Treg population can be exploited to augment anti-tumor immunity without eliciting deleterious autoimmunity (Sugiyama et al. 2013). Targeting molecules and signals specific for eTregs is being tested in clinical trials as an effective strategy for eTreg depletion.

We showed that CCR4 was specifically expressed by a subset of suppressive eTregs abundant in melanoma, and treatment using anti-CCR4 antibody depleted the melanoma-infiltrating eTregs with CCR4 expression and efficiently induced/augmented cancer-testis antigen-specific both $CD4^+$ and $CD8^+$ T cells (Sugiyama et al. 2013). Mogamulizumab has been approved in Japan for the treatment of CCR4-expressing adult T cell leukemia/lymphoma (ATLL). Anti-CCR4 antibody markedly reduced eTregs as well as ATLL cells and augmented ATLL antigen (cancer-testis antigen)- specific $CD8^+$ T cell responses in an ATLL patient, possibly in association with the prolonged survival of this patient (Sugiyama et al. 2013). Based on these preclinical data, multiple early phase clinical trials with mogamulizumab as an eTreg depletion reagent are being conducted as monotherapy (trial

numbers NCT02281409 and NCT01929486 (Kurose et al. 2015) and in combination with anti-PD-1 antibody (NCT02476123 and NCT02705105), anti-PD-L1 (PD-1 ligand 1) antibody or anti-CTLA-4 antibody (NCT02301130) and anti-4-1BB agonistic antibody (NCT02444793) in advanced solid tumors, and in combination with docetaxel in non-small cell lung cancer (NCT02358473). A recent phase Ia study showed that mogamulizumab administration was safe and well tolerated and that 4 of 10 patients showed stable disease during treatment and were long survivors. The monitoring of eTregs in the peripheral blood mononuclear cells during treatment indicated efficient depletion of those cells, even at the lowest dose (Kurose et al. 2015).

OX-40 and GITR are members of the TNF receptor superfamily and are both co-stimulatory receptors expressed by activated T cells. On Tregs, OX-40 is induced after activation and GITR is constitutively expressed (Shimizu et al. 2002; Griseri et al. 2010). These signals reduce the suppressive activity of Tregs as well as enhancing activation of effector T cells. A phase I trial of an OX-40 agonist demonstrated anti-tumor activity in melanoma and renal cell cancer (Curti et al. 2013). Early phase clinical trials evaluating OX-40 agonists in head and neck, breast and prostate cancer and in B cell lymphoma are also being investigated (NCT01862900, NCT02274155, NCT02318394, and NCT02205333). Additionally, a combination of an OX-40 fusion protein (MEDI6383) and an anti-PD-L1 antibody, durvalumab, is also being investigated (NCT02221960). Similarly, phase-I clinical trials evaluating GITR agonists in solid tumors are under investigation (NCT 02583165 and NCT02628574).

Tregs are highly dependent on PI3K signals for their maintenance and function. Inactivation of PI3K signals in Tregs activates $CD8^+$ T cells and induces tumor regression (Ali et al. 2014). Therefore, not only molecules specifically expressed by Tregs but also signals on which Tregs specifically depend could become targets to control Tregs. CPA is an alkylating agent that reportedly depletes Tregs when used in low doses. In phase II clinical trial, patients with advanced renal cell cancer received a therapeutic vaccination of IMA901 consisting of multiple tumor-associated peptides and GM-CSF with or without preceding CPA administration (Walter et al. 2012). Patients treated with IMA901/GM-CSF/CPA showed Treg reduction with augmented anti-tumor immune responses. The OS tended to be extended in the IMA901/GM-CSF/CPA-treated group (n = 33) compared with the IMA901/GM-CSF-treated group (n = 35) (23.5 months versus 14.8 months). A phase III trial investigating the addition of IMA901/GM-CSF/CPA to the standard care of sunitinib was completed in 2015 and the results are awaited.

6.3 Involvement of Tregs in Immune Checkpoint Inhibitors

Immune checkpoint blockade—inhibiting the immunosuppressive signals from co-inhibitory molecules—allows a resurgence in the effector function of tumor-infiltrating T cells and provides clinical success in various types of cancers

including malignant melanomas and lung cancers (Hodi et al. 2010; Topalian et al. 2012; Borghaei et al. 2015; Brahmer et al. 2015). As immune checkpoint molecules such as CTLA-4 and PD-1 are expressed by both tumor-infiltrating effector T cells and Tregs, current immune checkpoint blocking agents could target Tregs as well. Analyses of anti-CTLA-4 antibodies in mouse models revealed that the anti-tumor efficacy was dependent on depletion of CTLA-4-expressing Tregs in tumors through the antibody-dependent cellular cytotoxic (ADCC) activity of the anti-CTLA-4 antibody; depletion of Fc function totally abrogated the anti-tumor effect of the anti-CTLA-4 antibody (Simpson et al. 2013; Matheu et al. 2015). Additionally, PD-1-expressing Tregs reportedly possess higher immune-suppressive function than Tregs without PD-1 expression in a mouse model (Park et al. 2015). Therefore, PD-1-blocking antibodies might act on Tregs to augment anti-tumor immunity as well as reversing the effector function of dys-functional effector cells. Yet, more than half of the treated patients did not respond to immune checkpoint blockade therapy, even if combinations. Immuno-monitoring of biomarkers to properly evaluate immune responses in cancer patients is critical for detecting responders.

There are two types of tumor antigens: tumor-specific antigens, which are either oncogenic viral proteins or abnormal proteins from somatic mutations (neoantigens); and tumor-associated antigens, which are highly or aberrantly expressed normal proteins. It is not yet determined how $CD8^+$ T cells specific for each antigen contribute to clinical tumor regression and whether activation of these $CD8^+$ T cells specific for self-antigens versus non-self-antigens are controlled differently. In vitro experiments comparing Treg-mediated suppression of self-antigen (Melan-A)-specific $CD8^+$ T cells versus non-self (cytomegalovirus)-specific $CD8^+$ T cells showed that cytomegalovirus-specific $CD8^+$ T cells were resistant to suppression by Tregs (Maeda et al. 2014), indicating that Treg-mediated suppression is more prominent on self-antigen-expressing tumor cells rather than those expressing neoantigens. It is therefore noteworthy that cancers in patients susceptible to immune checkpoint blockade monotherapy contain a large number of neoantigens (Snyder et al. 2014; Rizvi et al. 2015), and that $CD8^+$ T cells specific for the antigens can be resistant to Treg-mediated immune suppression. In contrast, cancers with a lower number of neoantigens did not respond to immune checkpoint blockade (Snyder et al. 2014; Rizvi et al. 2015), and $CD8^+$ T cells can be under the control of Treg-mediated immune suppression. Thus, integration of Treg-targeting therapies that reduce Treg function and/or number may expand the therapeutic spectrum of cancer immunotherapy.

7 Conclusion

Since the discovery of Tregs as a key mediator of immunological self-tolerance, a common immunological basis for Treg-mediated suppression of autoimmunity and tumor immunity has been extensively explored. The manipulating Tregs is under

active investigation as a new therapeutic approach for treating a wide variety of diseases including autoimmune diseases and cancer. Deeper mechanistic understanding of disease-specific Treg development, maintenance, and function could make disease-specific Treg-targeted therapy more effective, resulting in an increase of efficacy and decrease of side effects related to manipulating Tregs.

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