Cytokine Signaling in the Development and Homeostasis of Regulatory T cells

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Cytokine signaling is indispensable for regulatory T-cell (Treg) development in the thymus, and also influences the homeostasis, phenotypic diversity, and function of Tregs in the periphery. Because Tregs are required for establishment and maintenance of immunological self-tolerance, investigating the role of cytokines in Treg biology carries therapeutic potential in the context of autoimmune disease. This review discusses the potent and diverse influences of interleukin (IL)-2 signaling on the Treg compartment, an area of knowledge that has led to the use of low-dose IL-2 as a therapy to reregulate autoaggressive immune responses. Evidence suggesting Treg-specific impacts of the cytokines transforming growth factor β (TGF-β), IL-7, thymic stromal lymphopoietin (TSLP), IL-15, and IL-33 is also presented. Finally, we consider the technical challenges and knowledge limitations that must be overcome to bring other cytokine-based, Treg-targeted therapies into clinical use.

C$D4^+Foxp3^+$ regulatory T cells (Tregs) are indispensable for the maintenance of immunological self-tolerance because of their ability to suppress autoreactive T cells that escape negative selection in the thymus. Humans and mice lacking the signature Treg transcription factor Foxp3 succumb to fatal lymphoproliferative disease early in life. In keeping with this role, clinical and experimental data have implicated Treg dysfunction in the pathogenesis of many autoimmune diseases (Grant et al. 2015), which collectively account for an enormous burden of morbidity and mortality. Meanwhile, the nonspecific therapies used to control these conditions (e.g., corticosteroids) often have limited efficacy and severe adverse effects.

As with other T-cell lineages, many aspects of Treg biology are regulated by a vast and intricate network of cytokine signals. A detailed understanding of how these cytokines impact Treg differentiation, homeostasis, and suppressive function will facilitate new therapies that disrupt autoimmunity through precise targeting of its underlying molecular pathways. The recent use of low-dose interleukin (IL)-2 immunotherapy has already shown the promise of this cytokine-based approach. Low-dose IL-2, which selectively expands and activates the Treg compartment in vivo (Aoyama et al. 2012; Kosmaczewska 2014; Yu et al. 2015), has proven beneficial to patients with systemic lupus erythematosus (He et al. 2016), hepatitis C virus–induced vasculitis (Saadoun et al. 2011), alopecia areata

(Castela et al. 2014), and graft-versus-host disease (Koreth et al. 2011; Matsuoka et al. 2013). Early phase clinical trials have also been conducted in the setting of type 1 diabetes (Hartemann et al. 2013; Todd et al. 2016).

Numerous cytokines besides IL-2 are now known to affect Tregs, and behind each one lies a potential new tool for the immunotherapy arsenal. Nevertheless, preliminary efforts to manipulate these pathways have often been hobbled by poor efficacy or severe off-target effects, a challenge resulting from the highly pleiotropic nature of cytokine signals. Exploiting the full range of opportunity provided by cytokine-based therapies will require more advanced insight into the complexities of cytokine signaling as it relates to the Treg compartment. Accordingly, the purpose of this review is to describe how several extensively studied cytokines, IL-2, IL-7, thymic stromal lymphopoietin (TSLP), IL-15, transforming growth factor β (TGF-β), and IL-33, are known to influence Tregs.

INTERLEUKIN-2

Originally identified as a growth factor for T cells in vitro (Morgan et al. 1976), IL-2 is produced primarily by activated $CD4^+$ and $CD8^+$ T conventional (Tconv) cells (Malek 2008). The high-affinity IL-2 receptor (IL-2R) comprises three subunits, IL-2Rα (CD25), IL-2Rβ (CD122), and the common γ chain (γ_c or CD132). Tregs and antigen-activated Tconv cells are the predominant populations that express all three simultaneously, positioning them to respond efficiently to IL-2 in vivo. Although Tregs do not produce IL-2, they express CD25 at uniquely high and consistent levels, whereas elevated CD25 expression in Tconv cells is generally a transient event during activation (Malek 2008). During signaling, IL-2 first binds to CD25, forming a dimer that recruits CD122 and γ_c (Stauber et al. 2006). Following receptor engagement, the Janus kinase (JAK)1 and JAK3 associate with the cytoplasmic tails of IL-2Rβ and γ_c, leading to phosphorylation of the JAKs along with three key tyrosine residues in the tail of IL-2Rβ. These phosphotyrosines activate three major intracellular signaling pathways:

mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), both mediated by the Shc adaptor protein, as well as the signal transducer and activator of transcription 5 (STAT5—composed of two similar proteins, STAT5A and STAT5B). These downstream signaling pathways govern survival, proliferation, and memory formation among antigen-activated Tconv lineages (Cheng et al. 2011), but are differentially regulated in the Treg compartment to support its suppressive function. Tregs are uniquely reliant on IL-2-dependent STAT5 activation, due in part to high levels of the lipid phosphatase and tensin homolog (PTEN), which acts to suppress IL-2-dependent PI3K and mechanistic target of rapamycin (mTOR) activity (Walsh et al. 2006; Huynh et al. 2015). IL-2 directly up-regulates Foxp3 and CD25 through STAT5 in a positive feedback mechanism to establish and maintain Treg transcriptional identity (Fontenot et al. 2005b; Burchill et al. 2007b; Feng et al. 2014). In conjunction with elevated CD25 expression, molecular adaptations in these pathways allow Treg development and homeostasis to be supported by a uniquely low threshold of IL-2 signaling, explaining the effectiveness of low-dose therapy (Yu et al. 2009). Tregs can also outcompete activated Tconv cells for available IL-2, and this cytokine deprivation has been highlighted as one mechanism of Treg-mediated immunosuppression (Pandiyan et al. 2007).

IL-2 is the predominant cytokine involved in regulating Treg development, stability, function, and peripheral homeostasis. Mouse models with abrogated expression of IL-2, CD25, or CD122 experience aggressive lymphoproliferation and fatal multiorgan autoimmunity (Sadlack et al. 1993, 1995; Suzuki et al. 1995; Willerford et al. 1995), and rare loss-of-function mutations in human CD25 produce a similar clinical syndrome in patients (Roifman 2000). This occurs because IL-2 signaling deficiency leads to a failure of Treg maturation and a consequent breakdown of self-tolerance (Fig. 1). Although $CD4^+Foxp3^+$ T cells are still present in IL-2/IL-2R-deficient mice, they show a ∼50% numerical reduction, and Foxp3 expression on a per-cell basis is reduced twofold (Jo-
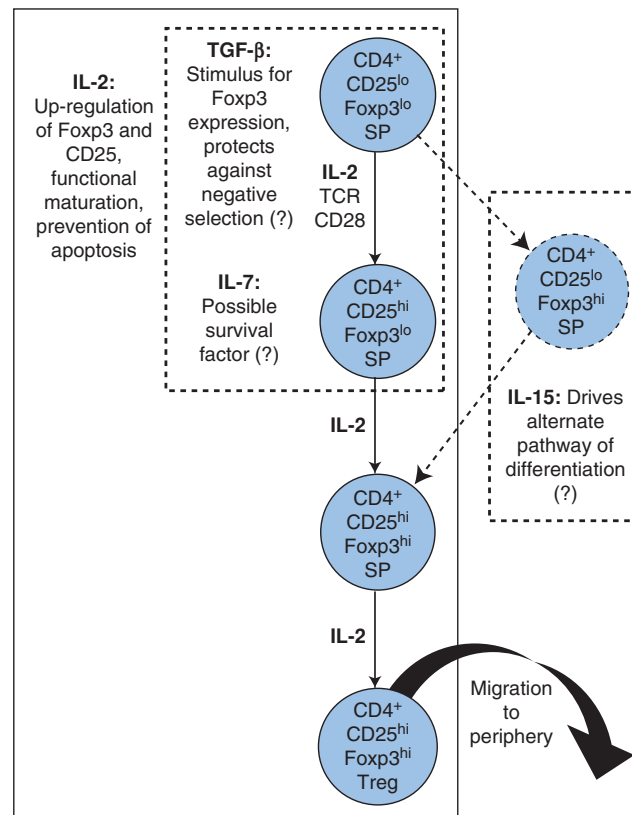
Figure 1. Cytokine contributions to regulatory T-cell (Treg) maturation in the thymus. Tregs develop from CD4 single-positive (SP) thymocyte precursors. Signaling through a high-affinity, self-reactive T-cell receptor (TCR) combined with CD28 costimulation is thought to trigger up-regulation of CD25. This potentiates the ability of Treg precursors to respond to interleukin (IL)-2, which prevents apoptosis, induces Foxp3 expression, and establishes Treg transcriptional identity and suppressive capacity. Boxes with dotted lines denote developmental stages where other cytokines may act. Dotted arrows denote a possible parallel pathway of Treg development. TGF-β, Transforming growth factor β.

sefowicz et al. 2012a). These Foxp3^{lo} cells have diminished expression of the suppressive mediators CTLA4, CD39, and CD73, suggesting that thymic Tregs generated in the setting of IL-2 deprivation lack adequate functional programming (Cheng et al. 2013). This idea is supported by adoptive transfer studies, as introducing a low number ($1-3 \times 10^5$) of CD4$^+$CD25$^+$ Tregs into neonatal *Il2rb*$^{-/-}$ mice provides full protection from autoimmunity, with treated animals retaining a donor-derived Treg compartment throughout life (Malek et al. 2002). Moreover, transgenic thymic rescue of IL-2Rβ signaling in these mice is sufficient to restore a normal Treg compartment and prevent autoim-

munity, further demonstrating a nonredundant requirement for the IL-2R in production of functional Tregs (Malek et al. 2000; Malek and Bayer 2004; Bayer et al. 2007).

Experiments using CD4 single-positive thymocytes confirm that IL-2 is an essential driver of Treg lineage commitment. Transgenic expression of a constitutively active *Stat5* transgene is sufficient to divert thymocyte precursors of naïve Tconv cells into the Treg lineage (Burchill et al. 2008). Mice deficient in the mediator TRAF3, which opposes IL-2R signaling, exhibit a two- to threefold increase in thymic Treg frequency (Yi et al. 2014). Active from the earliest stages of Treg differentiation, IL-2/IL-2R inter-

action is a critical inducer of Foxp3 expression in nascent Tregs (Bayer et al. 2007; Burchill et al. 2007a; Huehn et al. 2009), and also acts as a survival factor, up-regulating Bcl-2 to prevent their apoptosis (Tai et al. 2013). In vitro and in vivo experiments support a model whereby signals from a high-affinity, self-reactive T-cell receptor (TCR) and CD28 induce CD25 up-regulation in Treg precursors, leading to IL-2-driven induction of Foxp3 and resultant commitment to the Treg cell fate (Fig. 1) (Burchill et al. 2008; Lio and Hsieh 2008).

Although a majority of mature Tregs found in the periphery are of thymic origin, naïve Tconv cells can be instructed to differentiate into Tregs through a combination of molecular signals that includes IL-2. Treating cultured $CD4^+CD25^-$ T cells from humans or mice with the cytokine TGF-β (described below), in conjunction with TCR stimulation, can elicit Foxp3 expression and generate $CD4^+CD25^+$ "induced Tregs" (iTregs) with immunosuppressive function (Chen et al. 2003; Fantini et al. 2004). Although IL-2 on its own cannot convert naïve Tconv cells to a Treg cell fate (Xu et al. 2010), experiments using cytokine deprivation by antibody blockade and germline knockout (KO) show that IL-2 is essential for TGF-β-mediated induction of Foxp3, as well as subsequent iTreg clonal expansion and in vitro suppressor activity (Zheng et al. 2007). Adoptive transfer studies support the idea that iTreg persistence, transcriptional identity, and phenotypic stability are also IL-2-dependent in vivo (Shevach and Thornton 2014). iTregs are transcriptionally distinct from their thymic Treg counterparts on generation in vitro (Haribhai et al. 2009; Feuerer et al. 2010), and functional comparisons in some experimental models suggest that iTregs have lower phenotypic stability and suppressive capacity (Yadav et al. 2013; Shevach and Thornton 2014). These shortcomings have hindered the application of iTregs as a therapeutic tool, although cell-culture protocols for optimizing their potency and durability are a subject of continuing investigation (Kanamori et al. 2016; Schmidt et al. 2016).

Tconv precursors are also capable of differentiating into Tregs in vivo within peripheral tissue sites. Generation of these immunosuppressive cells, termed "peripheral Tregs" (pTregs) to distinguish them from cell culture–derived iTregs, is favored by subimmunogenic doses of peptide antigen in conjunction with TGF-β and IL-2 (Povoleri et al. 2013). Mice that undergo thymectomy at 3 days of age to abolish thymic Treg maturation develop autoimmune disease, demonstrating that pTregs alone are insufficient to maintain self-tolerance (Sakaguchi et al. 1995; Asano et al. 1996). Nevertheless, pTregs possess functional specializations that make them uniquely suited to suppressing inflammation in certain peripheral sites, particularly mucosal surfaces (Bilate and Lafaille 2012; Josefowicz et al. 2012b; Yadav et al. 2013). pTregs are key contributors to immune homeostasis in the intestine, a tissue microenvironment that appears to be especially favorable for their differentiation (Tanoue et al. 2016). pTregs present at this interface between host and external environment are integral for maintaining tolerance to an enormous array of antigens derived from self, ingested material, and microbiota (Littman and Rudensky 2010; Harrison and Powrie 2013). Crucially, gut-localized $CD4^+$ T cells in humans and mice exhibit an unusual degree of phenotypic plasticity between the pTreg and proinflammatory Th17 lineages, with IL-2 serving as an important regulator of this balance (Fig. 2). In vivo, TGF-β may be involved in differentiation toward either the Treg or Th17 cell fate (Konkel and Chen 2011), and additional cytokine signals are required to tip the scales toward the transcriptional program of either Foxp3 or the Th17-defining transcription factor RORγt (Hoechst et al. 2011; Kleinewietfeld and Hafler 2013; Ueno et al. 2015). Exposing Tconv cells to TGF-β in an environment rich in proinflammatory signals (particularly IL-6) favors Th17 differentiation (Kimura and Kishimoto 2010; Fujimoto et al. 2011). Conversely, IL-2-dependent STAT5 signaling promotes TGF-β-mediated differentiation of pTregs in the intestine (Povoleri et al. 2013; Yadav et al. 2013), while impeding the generation of Th17 cells from naïve precursors (Laurence et al. 2007; Zheng et al. 2008). Since the shift of this dynamic equilibrium away from pTregs and
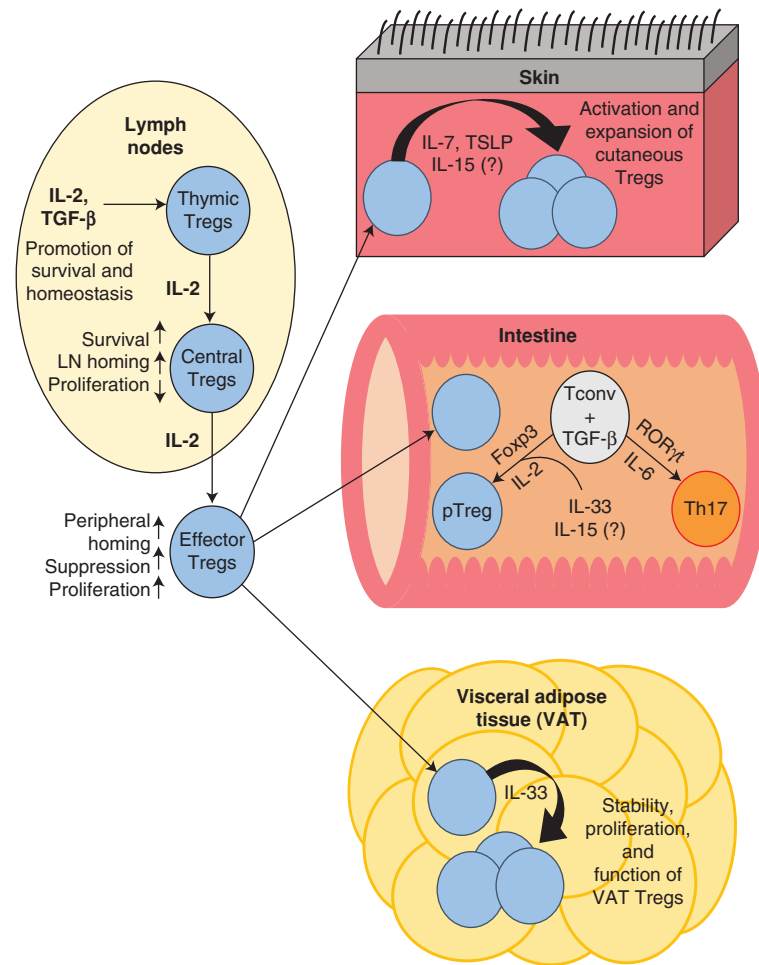
Figure 2. Effects of cytokine signaling on regulatory T cells (Tregs) in peripheral tissue sites. IL, Interleukin; TGF-β, transforming growth factor β; TSLP, thymic stromal lymphopoietin; LN, lymph node; Tconv, T conventional cell.

toward Th17 is essential to the pathogenesis of inflammatory bowel disease (Ueno et al. 2015), the role of IL-2 in this context has clear therapeutic implications.

Treg homeostasis does not require ongoing output from the thymus but is instead based on continuous self-renewal of thymic Tregs, which seed peripheral lymphoid tissues and undergo IL-2 driven expansion during early life (Bayer et al. 2005, 2007). There is substantial evidence that IL-2R signaling contributes to homeostatic maintenance of the peripheral Treg pool (Fig. 2), although the precise mechanisms have not been fully clarified. Many studies have positively

correlated CD25 with Foxp3 expression and phenotypic stability among Tregs in the periphery (Fontenot et al. 2005a; Barbi et al. 2014). IL-2 has also been found to participate in the epigenetic regulation that maintains Treg identity. Sustained heritability of Foxp3 expression depends on the *Foxp3* promoter as well as a *cis*-regulatory element within the *Foxp3* locus known as conserved noncoding sequence 2 (CNS2) or the Treg-specific demethylated region (TSDR). Both the *Foxp3* promoter and CNS2 contain STAT5-binding motifs, and CNS2 appears to function as an IL-2 sensor when this cytokine is present in limiting

amounts. IL-2-dependent STAT5 signaling in Tregs enables CNS2 to interact with the *Foxp3* promoter, which prevents loss of Foxp3 expression during cell-cycle progression or exposure to proinflammatory cytokines (Zheng et al. 2010; Feng et al. 2014; Li et al. 2014). Cellular adoptive transfer and bone marrow chimera studies, using IL-2/IL-2R-deficient mouse models, show an IL-2-mediated contribution to the survival, engraftment, and proliferation of Tregs in lymphoid tissues (Bayer et al. 2005, 2007; Cheng et al. 2013). Notably, administering exogenous IL-2 to $Il2^{-/-}$ mice not only rescues the numerical deficit in Tregs, but also corrects many of their transcriptional abnormalities, including low expression of suppression mediators such as CTLA4, CD39, and CD73 (Fontenot et al. 2005b; Barron et al. 2010). One report identified the antiapoptotic protein Mcl-1, which is known to be regulated by IL-2 signaling, as an essential survival factor for Tregs in the periphery (Pierson et al. 2013). However, it should be noted that interpretation of findings from IL-2/IL-2R-deficient models is problematic because it is often unclear whether Treg defects are attributable to impaired survival in the periphery, defective maturation in the thymus, or a combination of both. One study attempted to isolate peripheral signaling effects using antibody blockade of IL-2 in thymectomized adult mice. This treatment led to a selective reduction in Treg numbers and induction of autoimmunity, establishing an unequivocal role for IL-2 in peripheral homeostasis (Setoguchi et al. 2005). Emerging genetic tools, which permit fate mapping of Foxp3$^+$ cells and inducible deletion of signaling components in mice, have provided new opportunities to clarify the nature and importance of the IL-2R for Tregs in the periphery, although a clear consensus has yet to emerge (Rubtsov et al. 2010; Chinen et al. 2016).

These ambiguities surrounding the contribution of peripheral IL-2 signaling may be partially explained by heterogeneity within the Treg compartment itself. Current evidence indicates that mature Tregs can be divided into phenotypically and functionally distinct subpopulations. "Central" Tregs (cTregs) express high levels of the lymphoid homing marker CD62L, and exhibit a phenotype geared toward long-term survival and diminished functional activation. CD62L^{lo} "effector" Tregs (eTregs), which originate from cTregs, show comparatively reduced survival, elevated expression of activation markers, and efficient trafficking to nonlymphoid tissues (Fig. 2) (Yuan et al. 2014). Preliminary evidence indicates that Treg subsets vary in their degree of dependence on IL-2R-mediated signals, although the exact nature of this variation is still uncertain. In a mouse model with attenuated IL-2Rβ signaling, which possessed a grossly normal Treg compartment, a highly activated subset of tissue-resident eTregs bearing the surface marker Klrg1 failed to develop (Cheng et al. 2012). However, the functionally immature Tregs from $Il2^{-/-}$ or $Il2ra^{-/-}$ mice are skewed toward highly activated phenotypes, a fact that may suggest that cTreg survival is more reliant on IL-2 than that of eTregs (Campbell and Koch 2011). Resolving these multiple sites of action will require an understanding of Treg heterogeneity on a scale more refined than the cTreg/eTreg dichotomy. Accordingly, the search for cell-surface molecules capable of delineating functionally specialized cTreg and eTreg subsets is ongoing (Toomer et al. 2016).

TRANSFORMING GROWTH FACTOR β

The TGF-β signaling pathway provides a versatile mechanism to coordinate essential cellular functions, including proliferation, differentiation, and morphogenesis (Massague 2012), and is key to shaping immune responses and carcinogenesis. The three mammalian isoforms (TGF-β1, TGF-β2, and TGF-β3) are constitutively expressed across many tissues, with cells of the immune system predominantly expressing TGF-β1 (Prud'homme 2007). Among the major downstream targets of TGF-β signal transduction are the Smad family of transcription factors, which interact with each other and additional cofactors to form a diverse array of DNA-binding complexes (Massague et al. 2005).

Consistent with this intrinsic complexity, TGF-β can exert a vast range of proinflammatory and anti-inflammatory effects on many

types of immune cells (Prud'homme 2007; Li and Flavell 2008; Travis and Sheppard 2014). Yet remarkably, the *Tgfb1*$^{-/-}$ mouse was found to experience aggressive, multiorgan autoimmunity leading to premature death at 3–4 weeks of age (Kulkarni and Karlsson 1993), a phenotype strikingly similar to that of the *Foxp3* KO mouse. This observation suggested that TGF-β might exert its influence, in part, through the Treg compartment. In keeping with this idea, CD4$^+$CD25$^+$ T cells were found to express high levels of surface-bound TGF-β, and secreted this cytokine when activated (Nakamura et al. 2001; Green et al. 2003). Application of TGF-β-blocking antibodies or chemicals led to impairment of Treg-suppressive function (Aoki et al. 2005). Subsequently, a mouse model harboring a T-cell-specific deletion of TGF-β receptor II reproduced the lethal autoimmune phenotype, confirming that the central pathologic insult in this model is uncontrolled activation of self-reactive Tconv cells (Marie et al. 2006). This autoimmunity may be partially explained by the fact that TGF-β secreted from Tregs acts as a suppressive mediator in some models of inflammation (Schmidt et al. 2012). However, one study indicated that Tregs in the *Tgfb1*$^{-/-}$ mouse were diminished in number and had reduced expression of Foxp3. Following adoptive transfer into lymphopenic mice, both wild-type and *Tgfb1*$^{-/-}$ Tregs showed lower Foxp3 expression in hosts treated with TGF-β blocking antibody. Given that no phenotypic deficits were observed in CD4$^+$CD25$^+$ thymocytes, these findings suggest that TGF-β also makes a direct contribution to Treg maintenance and lineage commitment in the periphery (Fig. 2) (Marie et al. 2005). Notably, other studies do support a role for TGF-β in Treg thymic development. A T-cell-specific deletion, targeting the murine TGF-β receptor I, was found to temporarily block the appearance of CD4$^+$CD25$^+$Foxp3$^+$ thymocytes during the first week of postnatal life (Liu et al. 2008). Another study found evidence that TGF-β supports Treg development indirectly by curtailing negative selection of thymocyte precursors (Fig. 1) (Ouyang et al. 2010). The existence of new, more precise models of targeted genetic

deletion may help clarify the nature and extent of these TGF-β-mediated developmental effects (Chen and Konkel 2015).

As outlined previously, TGF-β is integral to the generation of Tregs from naïve Tconv precursors in vitro (iTregs) and in vivo (pTregs), an activity that has major physiological relevance within the intestinal microenvironment. The dual ability of this cytokine to provoke Th17 differentiation may be partly concentration-dependent, with larger amounts of TGF-β favoring Foxp3 induction (Omenetti and Pizarro 2015). Even at high levels, however, TGF-β alone cannot program the complete Treg transcriptional and functional profile. In addition to other cytokines (e.g., IL-2), the signals influencing Treg differentiation include retinoic acid, TCR affinity, dose and administration route of peptide antigen, and interactions with antigen-presenting cells (Povoleri et al. 2013; Yadav et al. 2013). As compared to thymic Tregs, iTregs boast a distinct pattern of epigenetic modifications at the *Foxp3* locus. In particular, the regulatory region known as conserved noncoding sequence 1 (CNS1) contains binding sites for the TGF-β downstream mediator Smad3, which acts cooperatively with other transcriptional regulators to induce Foxp3 expression (Tone et al. 2008). Deletion of CNS1 severely and selectively impairs iTreg differentiation, although it is dispensable for Foxp3 induction and Treg maturation in the thymus (Zheng et al. 2010; Josefowicz et al. 2012b).

INTERLEUKIN-7/THYMIC STROMAL LYMPHOPOIETIN

A critical factor for survival and maturation of T and B lymphocytes, IL-7 signals through a dimeric receptor consisting of the IL-7Rα chain (CD127) and the γ$_c$ (Fry and Mackall 2005; El Kassar and Gress 2010). IL-7Rα also forms one component of the dimeric receptor for the cytokine TSLP, a distant paralog of IL-7, which also exerts wide-ranging effects on the immune system (Ziegler et al. 2013). Unlike most cytokines, which are secreted by immune cells, IL-7 is produced mainly by nonlymphoid cells within lymphoid organs such as bone marrow

stroma and thymic epithelium (Jiang et al. 2005). $Il7^{-/-}$ (von Freeden-Jeffry et al. 1995) and $Il7r^{-/-}$ (Peschon et al. 1994) mice exhibit lymphoid hypoplasia with deficiencies in T- and B-cell compartments, γδ T-cell and natural killer T (NKT)-cell lineages (Maki et al. 1996; Boesteanu et al. 1997), and mutational loss of either IL-7Rα or the $γ_c$ causes severe combined immunodeficiency (SCID) in man (Puel et al. 1998). IL-7R regulates survival and expansion of double-negative thymocytes, and is down-regulated during the double-positive stage (Alpdogan and van den Brink 2005; Fry and Mackall 2005; Xiong et al. 2013). Re-expressed in the single-positive stage, it is retained on most mature Tconv cells and serves as an important signal governing peripheral homeostasis (Alpdogan and van den Brink 2005; Bradley et al. 2005; Fry and Mackall 2005; Mackall et al. 2011).

Despite its general importance for T-cell maturation in the thymus, evidence suggests that IL-7 does not play a fundamental role in the differentiation of Tregs. In vitro, IL-7 triggers pSTAT5 phosphorylation (albeit to a lesser extent than IL-2) and supports maturation of $CD4^+CD25^+Foxp3^-$ thymic Treg precursors (Vang et al. 2008), but this contribution is less clear in vivo. Although a block in thymic Treg development has been reported in $Il7r^{-/-}$ mice, interpretation of this finding is complicated by generalized defects in T-cell production (Bayer et al. 2008; Mazzucchelli et al. 2008). $CD4^+Foxp3^+$ cells are virtually absent in mice doubly deficient in IL-2Rβ and IL-7Rα, indicating a developmental impairment more severe than that produced by IL-2/IL-2R deficiency alone (Bayer et al. 2008). Despite these findings, selective thymic reconstitution in $Il2rb/Il7r$ double KOs showed that restoration of IL-2Rβ signaling alone can rescue Treg production, completely compensating for a lack of IL-7Rα (Bayer et al. 2008). Thus, IL-7Rα is dispensable for the generation of a functionally mature Treg compartment. It is possible that IL-7 provides a contributory signal to support survival of thymic Tregs during the CD4 single-positive precursor stage, but discerning where and how this effect might operate will require further investigation (Fig. 1).

A number of contradictory results have been obtained regarding the effects of IL-7 on Tregs in the periphery. Mature Tregs have traditionally been considered to express uniformly low levels of CD127, which should render them largely insensitive to IL-7-mediated effects (Liu et al. 2006; Seddiki et al. 2006). In keeping with this idea, mouse studies have found IL-7 signaling to be dispensable for survival, proliferation, phenotypic stability, and suppressive capacity in the peripheral Treg compartment (Peffault de Latour et al. 2006; Mazzucchelli et al. 2008). In vivo experiments using IL-7-transgenic mice and exogenous cytokine administration show that Tregs in the periphery can proliferate in response to IL-7 when it is present at elevated levels (Simonetta et al. 2012). However, this pathway does not appear to play a major role in overall Treg homeostasis under normal physiological conditions. One study examining lymphopenia-driven proliferation of $CD4^+$ T cells found that Tconv expansion was closely tied to IL-7 levels, whereas expansion of Tregs was instead governed by IL-2 (Le Campion et al. 2012). There is some evidence to suggest an IL-7-mediated contribution to Treg suppression. One study evaluating a specialized population of immunosuppressive dendritic cells in the context of diabetes onset showed that dendritic cell (DC)-produced IL-7 acted as a survival factor for $CD4^+CD25^+$ Tregs in vitro, as well as in vivo following adoptive transfer into nonobese diabetic mice (Harnaha et al. 2006). A possible mechanism behind this effect is suggested by another study, which reported that IL-7 enabled Tregs in culture to maintain CD25 expression, potentiating their ability to respond efficiently to IL-2. Adoptive transfers into IL-7-deficient or IL-7-overexpressing mice showed that this effect was maintained in vivo as well (Simonetta et al. 2014).

It appears that IL-7 may exert a more pronounced regulatory effect on specialized subpopulations of Tregs than on the cell lineage as a whole. For example, this cytokine has been reported as a positive regulator of Treg survival and function in the setting of cutaneous immunosuppression (Fig. 2). Using a mouse model of inducible self-antigen expression in skin, one

study reported that IL-7, rather than IL-2, was indispensable for the maintenance of highly activated cutaneous Foxp3$^+$ Tregs after self-antigen expression was turned off (Gratz et al. 2013). Another study examining Tregs in murine lymph nodes revealed that expression of the activation markers ICOS and CD103 defined a population of Tregs that express high levels of IL-7Rα (Simonetta et al. 2010). Tregs were found to up-regulate IL-7Rα during activation both in vitro and in vivo, suggesting an augmented responsiveness to IL-7-mediated signals (Simonetta et al. 2010). Both studies noted a CD127^{hi} phenotype in skin-resident Tregs characterized ex vivo, strengthening the idea that low expression of this receptor is not an inherent aspect of the Treg functional program (Simonetta et al. 2010; Gratz et al. 2013).

Emerging evidence suggests that IL-7Rα signaling through epithelium-derived TSLP is another important contributor to cutaneous Treg maintenance and function (Fig. 2). In one mouse model, cutaneous inflammation was initiated by a tamoxifen-inducible, tissue-specific deletion of the chromatin remodeler Mi-2β in the basal epidermis. This disruption of epigenetic regulation caused production of numerous inflammatory mediators and progression to a systemic autoimmune response, although the animals recovered completely (Kashiwagi et al. 2017). In this proinflammatory context, TSLP drove the proliferation of cutaneous Tregs as well as their differentiation into eTreg phenotypes, with corresponding up-regulation of transcripts related to activation, epithelial localization, and immunosuppression. Bone marrow chimera experiments showed that adoptively transferred Tregs with a deletion of *Crlf2* (which encodes the second "TSLPR" component of the heterodimeric TSLP receptor) were impaired in their ability to expand in skin following Mi-2β loss. Notably, in mice lacking TSLP-responsive Tregs, the progression to systemic autoimmunity went unchecked and resulted in a lethal phenotype (Kashiwagi et al. 2017). In another experiment, topical administration of a vitamin D3 analog to mice caused cutaneous Tregs to proliferate in a TSLP-dependent manner. This treatment reduced the inci-

dence of autoimmune diabetes in nonobese diabetic mice, and ameliorated clinical symptoms in experimental autoimmune encephalomyelitis-induced mice (Leichner et al. 2017). These results suggest that IL-7Rα-mediated activation and expansion of cutaneous Tregs can influence systemic immune homeostasis, an intriguing possibility that may be exploited by future therapeutic approaches.

INTERLEUKIN-15

The IL-15 receptor (IL-15R) shares two of its three subunits in common with the IL-2R, comprising a high-affinity IL-15Rα chain, CD122, and the γ$_c$ (Olsen et al. 2007). However, this structural similarity belies a vast divergence of effects in vivo, as IL-15 is highly pleiotropic and influences a much broader array of immune cell types than IL-2. *Il15*$^{-/-}$ and *Il15ra*$^{-/-}$ mice exhibit numerical reductions in various innate and adaptive cell populations, including CD8$^+$ T cells, natural killer (NK) cells, NKT cells, and γδ intraepithelial lymphocytes. In addition to acting as a prominent lymphocyte survival factor (Lodolce et al. 2002; Alpdogan and van den Brink 2005), IL-15 provides a specific and potent stimulus for memory-phenotype (CD44^{hi}) CD8$^+$ T cells in vivo (Zhang et al. 1998), and appears to regulate homeostasis and acquisition of memory functions in naïve CD4$^+$ T cells (Chen et al. 2014). IL-15 signaling also promotes antigen presentation and production of type I cytokines (IL-12 and interferon γ [IFN-γ]) by dendritic cells and macrophages (Ohteki et al. 2001), and potentiates NK cell proliferation and cytotoxicity (Becknell and Caligiuri 2005).

As for IL-7, IL-15 signaling promotes STAT5 phosphorylation, Foxp3 expression, and maturation in CD4$^+$CD25^{hi} single-positive thymocytes cultured in vitro, albeit to a lesser extent than IL-2 (Lio and Hsieh 2008; Vang et al. 2008). Nevertheless, an IL-15-mediated contribution to thymic Treg development has been challenging to identify in vivo. The similarity in clinical phenotype between CD25-deficient and CD122-deficient mice shows that IL-15 cannot independently support development of

a functional Treg compartment (Sakaguchi et al. 2008). IL-15-deficient mice have nearly normal numbers of Tregs, suggesting that this pathway is dispensable for their generation (Burchill et al. 2007b). Nevertheless, mice deficient in both IL-2 and IL-15 signaling pathways have a more profound deficit in Treg numbers than those lacking IL-2 alone (Burchill et al. 2007b; Sakaguchi et al. 2008). This finding suggests that IL-15 can contribute to Treg survival through IL-2Rβ/γ_c signaling when IL-2 is absent, although it evidently cannot rescue Treg function because the clinical course of lethal autoimmunity is unchanged. New mouse models for mapping the fates of thymocyte lineages in vivo suggest that IL-15 may make an ancillary contribution to Treg development. One such model featured deletion of the TCR signaling mediator Zap70, which abrogated thymic production of T cells. Reconstitution of T-cell development using a tetracycline-inducible Zap70 transgene allowed de novo–generated Tregs to be selectively analyzed by eliminating any mature Foxp3$^+$ cells of extrathymic origin (Marshall et al. 2014). This approach corroborated the generally accepted model that Tregs originate from CD4$^+$CD25^{hi} single-positive thymocytes that up-regulate Foxp3 expression in response to IL-2. However, among CD4 single-positive cells, a second lineage of CD25$^-$Foxp3$^+$ precursors were found to be capable of developing efficiently into Tregs. Survival and maturation of these precursors was dependent on IL-15 produced by the thymic stroma, indicating that this cytokine may support an alternative pathway of Treg differentiation (Fig. 1) (Marshall et al. 2014).

Although the possible influence of IL-15 on mature Tregs in the periphery is only beginning to be explored, emerging evidence suggests that this cytokine may potentiate Treg-suppressive function in certain specialized tissue environments. Using an explant culture system, it was reported that resident Tregs in human skin can be selectively expanded by treatment with IL-15 (Fig. 2) (Clark and Kupper 2007). One recent experiment suggests that IL-15 may contribute to immune homeostasis in the gut by regulating the equilibrium between Treg and Th17 differ-

entiation. Purified Tregs that were adoptively transferred into an IL-15-deficient RAG^{KO} mouse model showed skewing toward a Th17 phenotype, with accelerated loss of Foxp3 expression and up-regulation of RORγt. This led to acquisition of Th17 effector functions by the transferred cells, fueling aggressive colitis in the recipient hosts (Fig. 2) (Tosiek et al. 2016). These findings highlight the possibility of targeting the IL-15 signaling axis therapeutically, to drive expansion or differentiation of tissue-resident Tregs.

INTERLEUKIN-33

IL-33 is constitutively expressed at high levels in epithelial cells, lymphoid cells, and stromal cells of both humans and mice, and is also up-regulated in response to inflammatory stimuli (Cayrol and Girard 2014). A member of the IL-1 family of cytokines, IL-33, signals by binding ST2, a member of the Toll-like/IL-1-receptor superfamily. The resulting dimer then forms a complex with the ubiquitously expressed receptor component IL-1R accessory protein, whose cytosolic domain activates MyD88 signaling to trigger production of inflammatory mediators (Miller 2011). Given its abundance in epithelium, IL-33 is believed to function as an "alarmin," rapidly activating the innate immune system when tissue is damaged by trauma or infectious insults (Chan et al. 2012). IL-33 expressed under homeostatic conditions is retained in the cell nucleus, which may serve to increase the propagation speed of this "danger" signal by allowing the cytokine to be released preformed from necrotic epithelial cells (Cayrol and Girard 2014). IL-33 is unique among IL-1 family cytokines in promoting Th2-polarized immune responses, suggesting a conserved evolutionary function in stimulating antiparasite immunity at mucosal surfaces (Miller 2011). Current evidence suggests that IL-33 is an important contributor to Th2-driven allergic diseases such as asthma (Borish and Steinke 2011; Cayrol and Girard 2014; Saluja et al. 2015) and atopic dermatitis (Cevikbas and Steinhoff 2012), as well as rheumatoid arthritis, psoriatic arthritis, and systemic lupus erythematosus

(Miller 2011; Duan et al. 2013; Macedo et al. 2016).

As discussed previously, Tregs are known to acquire functional adaptations and unique transcriptional profiles that support efficient suppressive activity in nonlymphoid tissue sites (Feuerer et al. 2010; Campbell and Koch 2011). IL-33 has recently been identified as a candidate cytokine that appears to support these tissue-specific adaptations. Despite its established role as a mediator of Th2-mediated inflammation at environmental interfaces, IL-33 seems to play a dichotomous role in inflammatory bowel disease. Although levels of IL-33 and ST2 are increased in the intestines and serum of inflammatory bowel disease (IBD) patients, experimental findings suggest that this signaling axis also supports epithelial repair and resolution of inflammatory injury (Pastorelli et al. 2011; Duan et al. 2012; Grobeta et al. 2012; Sedhom et al. 2013). The recently discovered capacity of IL-33 to promote Treg activity in the gut may clarify this apparent contradiction. ST2 is preferentially expressed on colonic Tregs from mice, and signaling through this receptor promotes survival, proliferation, and suppressive activity of these Tregs both in vitro and in vivo. In addition, IL-33 was found to enhance TGF-β-mediated conversion of naïve T cells into pTregs (Fig. 2) (Schiering et al. 2014).

A similar role for IL-33 has also been characterized in visceral adipose tissue (VAT). VAT-resident Tregs are a specialized population of considerable clinical importance, mediating the link between obesity-induced inflammation and its associated disease outcomes, particularly insulin resistance leading to type 2 diabetes (Feuerer et al. 2009). ST2 was found to be very highly expressed in murine Tregs from visceral adipose tissue compared to those from lymphoid organs. Moreover, this expression was specifically enriched among Tregs with a highly activated phenotype (Vasanthakumar et al. 2015). ST2-deficient mice exhibited clinical evidence of insulin resistance, drastically reduced Treg frequency in the VAT, and impaired maintenance of Treg functional and transcriptional identity. Notably, in vivo administration of IL-33 was sufficient to drive proliferation of VAT Tregs in normal mice, and this treatment also rescued the numerical deficit of VAT Tregs in obese mice with corresponding improvements in glucose tolerance (Fig. 2) (Vasanthakumar et al. 2015).

CONCLUDING REMARKS

As illustrated by the preceding examples, we have a reasonable understanding of how several cytokines shape the Treg compartment. IL-2 is a critical cytokine for Treg development while also importantly influencing Treg homeostasis, stability, and function. TGF-β provides critical signaling for the development of pTregs. Emerging data also support the notion that IL-7, TSLP, IL-15, and IL-33 help to shape the activity of Tregs in various nonlymphoid tissue sites. However, in some other instances, the search for causal links between a particular cytokine signal and certain aspects of Treg development and function has yielded contradictory results. In particular, the roles of IL-7, IL-15, and TGF-β in the development and peripheral lymphoid homeostasis of thymic Tregs are less clear. Factors that contribute to these inconsistencies include functional redundancy among cytokines and their receptor subunits, the capacity of many cytokines to exert pleiotropic effects, and experimental variables such as choice of genetic model, inflammatory milieu, or tissue site.

Therapeutic applications of these cytokines to boost Tregs and promote immune tolerance must take into consideration such redundancy and pleiotropy. The promising clinical results using low-dose IL-2 to enhance the Treg compartment are largely because of our current understanding that many critical aspects of Tregs are supported by low levels of IL-2R signaling, whereas T effector and memory cells require more extensive IL-2R signaling. As such, low-dose IL-2 decreases the pleotropic activity and minimizes nonspecific toxicity of IL-2. Nevertheless, many essential parameters such as dose–response relationships, optimal administration schedule, and duration of treatment remain undefined for low-dose IL-2 (Klatzmann and Abbas 2015).

Therapies that exploit the Treg-promoting activities of IL-7, TSLP, IL-15, and IL-33 must also be devised to minimize their pleiotropic effects and render them selective toward Tregs. Unlike IL-2, we do not know whether signal thresholds for these cytokines might be favorably directed toward Tregs. Thus, a critical investigative frontier for this group of cytokines may be finding methods to deliver them with precision to minimize off-target effects. The evidence presented here suggests that even highly pleiotropic cytokines can exert defined and predictable influences on a Treg subpopulation specialized for a particular tissue compartment. Thus, rather than taking aim at the Treg pool in its entirety, it may be far more tractable to work toward precision delivery of an immunomodulatory agent to a peripheral anatomical site to engage one or more specialized tissue-resident Treg subsets with well-characterized signaling requirements. Accordingly, it appears that future progress in cytokine-based therapy will occur on a much finer scale.

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