TGF-β and Regulatory T Cell in Immunity and Autoimmunity

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Abstract

Introduction The immune response is controlled by several inhibitory mechanisms. These mechanisms include regulatory T cells, which exist in multiple classes. Notable among these are Foxp3-expressing regulatory T cells (Treg), NKT cells, and Tr1 cells. Common to these mechanisms are inhibitory cytokines such as interleukin-10 and transforming growth factor-beta (TGF-β). TGF-β and Foxp3-expressing Treg cells are critical in maintaining self-tolerance and immune homeostasis. *Discussions* The immune suppressive functions of TGF-β and Treg cells are widely acknowledged and extensively studied. Nonetheless, recent studies revealed the positive roles for TGF-β and Treg cells in shaping the immune system and the inflammatory responses. In this paper, we will discuss the role of these mechanisms in the control of immunity and autoimmunity and the mechanisms that underlie how these molecules control these responses.

Keywords TGF-β · Treg · NKT · IL-10

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Introduction

Adaptive and innate immune strategies have evolved in mammals to defend against foreign pathogens to maintain health. With highly antigen-specific surface receptors, T cells are pivotal for adaptive immune responses. Most T cells activate immune responses. Through quasi-random recombinational mechanisms, thymic derived T cells potentially possess infinite numbers of specificities toward foreign as well as self-antigens. Dysregulated self-reactive T cells can lead to autoimmune diseases. Multiple processes therefore are in place to suppress the generation or the function of self-reactive T cells. These T cells can be deleted in the thymus and the periphery. Such elimination processes are however incomplete, resulting in small populations of mostly low-affinity self-reactive T cells in the periphery to potentially initiate an autoimmune response. Fortunately, active immune suppressive mechanisms exist to suppress the function of these autoreactive T cells. Dysregulated immune suppression often results in immune disorders. Autoimmunity and inflammatory diseases can be caused by decreased immune suppression, while cancers are often associated with increased immune suppression. Great progress has been made in understanding the cellular and molecular components of immune suppression. Active immune suppression is mediated mostly through either cytokines or through specialized cells. The pleiotropic cytokine, transforming growth factor-beta (TGF-β), and the immune-suppressive cell, previously called suppressor cells [1] and now usually termed regulatory T cells (Treg), play critical roles in suppressing the immune response. For years, we have been interested in the immune suppressive functions of TGF-β and Treg under normal and immune-pathological conditions. Recently, our and other studies suggest that, in a cell-type and environment-dependent fashion, TGF-β and Treg might also positively regulate immune responses. In this paper, we will

discuss our views on the functions of TGF-β and Treg in the immune regulation.

TGF-β and its Signaling

Consisting of a family of pleiotropic cytokines, TGF-β regulates multi-faceted cellular functions including proliferation, differentiation, migration, and survival [2].

Among the three known isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3), TGF-β1 is predominantly expressed in the immune system. TGF-β is synthesized in an inactive form, pre-pro-TGF-β precursor. Additional stimuli are required to liberate active TGF-β, enabling it to exert its function by binding to its receptor [3–7]. The active form of TGF-β can function in either a cell-surface-bound form or a soluble form [8, 9].

Activated TGF-β binds to its receptor consisting of two subunits, ALK5 and TGFβRII, and initiates signaling cascades [10]. Intracellular signal transduction of TGF-β is mediated to a great degree via Smad proteins [10, 11]. The eight vertebrate Smads identified thus far are grouped into three categories: five receptor-associated Smads (R-Smad1, 2, 3, 5, and 8), one common Smad (Co-Smad4) and two inhibitory Smads (I-Smad6 and 7). Upon TGF-β stimulation, activated ALK5 phosphorylates R-Smad-2 and R-Smad-3. Phosphorylated R-Smads associate with Co-Smad4 and translocate into the nucleus to bind to DNA containing a Smad-binding element [12–15]. Smad-independent TGF-β signaling pathways have also been reported [16, 17]. Through mechanisms yet to be determined, rapid activation of Ras-Erk, TAK-MKK4-JNK, TAK-MKK3/6-p38, Rho-Rac-cdc42 MAPK, and PI3K-Akt pathways occurs when cells are treated with TGF-β [18]. MAPKs also coordinate with Smads to modulate TGF-β responses [19–21]. Moreover, TGF-β receptors activate TRIP-1 and PP2A through direct protein binding to regulate translation initiation [22–24]. Therefore, TGF-β exerts its regulation of target cell function via many different signaling pathways. T-cell specific target genes of TGF-β are largely unknown; however, the expression of genes important for T-cell differentiation and function, such as GATA3, T-bet, STAT4, interferon-γ (IFN-γ), and granzyme-B, are suppressed by TGF-β [25–29].

Regulatory T cells

Immune suppression was proposed in the early 1970s by Gershon et al. [1, 30, 31]. It was not until the 1990s that Sakaguchi et al. [32] identified a subset of T cells with markedly increased expression of CD25 as suppressor T cells and later referred to as Treg cells. In recent years, substantial progress has been made in identifying different types of Treg cells and in understanding how these cells are generated and function. Based on cell surface markers or cytokine secretion profiles, Tregs can be generally grouped into two categories: naturally occurring Tregs (nTreg) and acquired Treg (aTreg).

Naturally Occurring Treg

A subset of CD4 T cells that develop in the thymus and constitutively express cell surface IL-2 receptor α chain (CD25) are termed as nTreg. $CD4^+$ $CD25^+$ nTregs comprise approximately 10% of peripheral CD4 T cells in mice and humans. nTregs are critical for maintaining self-tolerance, as disruption of thymic development or peripheral maintenance of these cells invariably results in the development of autoimmunity. Cell surface molecules, such as cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), and lymphocyte activation antigen-3 have also been used to differentiate nTreg from conventional T cells [33]. TGF-β is expressed in nTreg at high levels as a cell-surface-bound form [9, 34]. nTreg cells suppress immune responses in an antigen non-specific fashion in vitro and in vivo [33, 35–37]. Foxp3, an X-linked transcription factor belonging to fork-head family, is specifically and highly expressed in nTreg. Thus, Foxp3 is currently used as the most reliable molecular marker for nTreg.

Acquired Treg

Conventional T cells are able to gain immune-suppressive activities and become aTreg. Tr1 and Th3 cells are two reported types of aTreg. Tr1 cells are often found within the intestinal mucosa and suppress immune reactions toward a variety of cognate antigens [38]. There is no particular surface marker associated with Tr1 cells. However, these cells produce increased levels of interleukin-10 (IL-10) and TGF-β [39]. Tr1 cells do not express Foxp3 [40], suggesting that it is a subset of Treg distinct from nTreg. Th3 is another aTreg subset induced primarily from naive CD4 T cells after ingestion of a foreign antigen via the oral route, thereby eliciting oral tolerance [41, 42]. While no particular surface marker is associated with these cells, Foxp3 is expressed in Th3 cells [43]. In addition, TGF-β is produced at elevated levels by Th3 cells [42]. Whether Th3 cells form a distinct aTreg subset or are activated nTreg remains to be evaluated.

TGF-β Regulated Immune Responses

Critical Role for TGF-β in Immune Suppression

The inhibitory effect of TGF-β on the immune system was first described in 1986, where TGF-β was found to inhibit

the proliferation of human B and T cells [44, 45]. However, the definitive evidence to support the critical roles of TGF-β in immune suppression were not presented until the analysis of TGF-β1-deficient mice [46, 47]. Since then, using genetic-modified mouse models, our and other laboratories have carried out extensive studies to evaluate the immune functions of the components of TGF-β signaling networks, including their receptors and intracellular signaling molecules, and furthered the understanding of the suppressive role for TGF-β in the immune system. TGF-β regulates the adaptive immunity components, such as T cells, as well as the innate immunity components, such as natural killer (NK) cells [48–54]. TGF-β suppresses immune responses through at least two ways: inhibiting the function of inflammatory cells and promoting the function of Treg cells.

Multiple types of immune cells can be regulated by TGF-β [55]. In the early studies, TGF-β was found to suppress T-cell proliferation by inhibiting the production of IL-2, a lymphokine known to potently activate T cells, NK cells, and other types of cells of the immune system. Addition of exogenous IL-2 partially relieved TGF-β-mediated suppression [45]. TGF-β suppresses IL-2 production in T cells potentially through direct inhibition of IL-2 promoter activity; a *cis*-acting enhancer DNA element was identified to be critical in suppressing IL-2 production by TGF-β [56]. In addition, R-Smad3 is critical in mediating TGF-β inhibited IL-2 production, as TGF-β failed to suppress IL-2 production in murine T cells that lack this gene [57]. Moreover, the Smad-binding element has been located upstream of the human IL-2 promoter, which is important for Smad-mediated transcriptional suppression of IL-2 [58]. Because addition of exogenous IL-2 did not fully reconstitute T-cell proliferation [45], TGF-β inhibits T-cell proliferation also through yet-to-be-defined IL-2-independent mechanisms.

To perform immune function, naive CD4 T cells differentiate into three major types of effector T cells after activation [59]. Based on their cytokine production, CD4 effector T cells, also named T helper (Th), can be categorized as Th1, Th2, and Th17 cells [60, 61]. Th1 cells produce IFN-γ, Th2 cells secrete IL-4, IL-13, and IL-5, and Th17 cells express IL-17 and IL-22 [62]. While TGF-β partially suppresses T-cell proliferation, we have found that TGF-β potently inhibits effector T-cell functions and thus their differentiation into Th1 or Th2 effector cells under tissue culture conditions [63]. Further studies revealed that TGF-β regulates effector T-cell function through multiple mechanisms. Distinct sets of transcription factors are preferentially expressed in and are important for Th-cell differentiation. These include T-bet and Stat4 for Th1 differentiation and Gata-3 and Stat6 for Th2 differentiation [59]. While the detailed mechanism remains unknown, our and other studies showed that T-bet and Gata-3 expression

is inhibited by TGF-β [26, 27, 64, 65] possibly, in the latter case, through a mechanism via blocking Itk kinase activity and calcium influx [25]. Interestingly, effector cytokine production by fully differentiated Th2 cells is unaffected by TGF-β, while Th1 cells remain susceptible to TGF-β suppression [66]. Therefore, TGF-β exerts most of its inhibitory effects on the establishment of effector cell functions. Our recent study demonstrated that Th1-polarizing condition promotes CD122 (IL-2 receptor β chain) expression through T-bet [67]; thereby enhancing the clonal expansion and survival of Th1 cells [67]. Addition of TGF-β suppressed CD122 upregulation under Th1-skewing conditions. Therefore, TGF-β also limits Th1 effector cell numbers through inhibiting the upregulation of CD122. It was also noted that TGF-β inhibited T-cell differentiation independent of T-cell proliferation [68]. Thus, TGF-β potentially regulates T-cell proliferation and effector functions through discrete mechanisms with the greatest effects on suppressing their differentiation. While TGF-β inhibits the production of pro-inflammatory cytokines, it promotes T-cell production of IL-10, an anti-inflammatory cytokine, likely through direct activation of the IL-10 promoter via Co-Smad4 [69].

Besides regulating CD4 T cells, TGF-β controls CD8 T-cell proliferation and effector functions. The expression of effector molecules by CD8 T cells, such as IFN-γ and perforin, is inhibited by TGF-β [70–73]. Recent studies showed that TGF-β is important for Treg-induced inhibition of the exocytosis of granules and cytolytic function of CD8 T cells [74].

TGF-β is critical in immune suppression under physiological conditions because TGF-β1$^{-/-}$ mice develop a multifocal inflammatory disease associated with increased inflammatory cytokine production [46, 47, 75]. This phenotype is predominantly mediated through T cells, as depletion of CD4$^+$ T cells or crossing TGF-β1$^{-/-}$ mice onto an MHC class II null background prevented this inflammation [76]. However, from these studies, it was not clear whether T cells are direct targets of TGF-β since TGF-β1 acts on multiple cell types. Indeed, TGF-β plays an important role in suppressing innate immunity. By expressing a transgene encoding a dominant negative form of TGFRII under the control of CD11c promoter (CD11cTGFDNR), we blocked TGF-β signaling in NK cells and dendritic cells (DC) [51]. Blockade of TGF-β signaling in NK cells caused the accumulation of a large pool of NK cells secreting copious amounts of IFN-γ. Increased IFN-γ induced Th1 differentiation of CD4 T cells in these mice and resulted in their resistance to *Leishmania* infection. However, blockade of TGF-β signaling in DC from these mice did not affect DC homeostasis or interleukin 12 production, suggesting that TGF-β differentially affects NK and DC cells. In addition, TGFβRII deletion facilitated generation of a highly pathogenic T-cell

subset exhibiting hallmarks of NK cells. These TGFβRII-deficient NK-like T cells highly elevated IFN-γ expression [77]. Further studies are warranted to elucidate TGF-β function in the generation and function of innate components and the underlying mechanisms.

To further investigate the intrinsic function of TGF-β in T cells, several groups including ours have used transgenic approaches to block TGF-β signaling in T cells by expressing dominant negative TGF-β receptors [50, 52]. In this effort, we generated mice expressing a dominant-negative form of TGFβRII from the CD4 promoter (CD4-dnTβRII), whose CD4 and CD8 T cells are refractory to TGF-β signaling. These mice developed an autoimmune inflammatory phenotype associated with uncontrolled CD4$^+$ T-cell differentiation into Th1 effector cells [50]. Without TGF-β signaling, both CD4 and CD8 T cells from CD4-dnTβRII mice displayed increased effector functions, which led to drastically increased immune rejection of B16 melanoma and EL4 lymphoma in vivo [78]. Nonetheless, CD4-dnTβRII mice displayed much less immune pathology than TGF-β1$^{-/-}$ mice. This is possibly due to insufficient expression of the transgenes or incomplete inhibition of TGF-β signaling. Subsequent studies demonstrated that deletion of TGF-βRII in the bone marrow cells results in an immune pathology similar to that found in TGF-β1$^{-/-}$ mice [79]. However, the contribution of T cells to such a phenotype remained undetermined. Recently, more definitive studies from our laboratory have uncovered the essential role of TGF-β signaling in controlling the development, homeostasis, and tolerance of T cells through both Treg-dependent and Treg-independent mechanisms [67]. Mice with T-cell specific TGF-βRII deletion (4cre-RII/RII) developed a progressive wasting disease and succumbed to death by 5 weeks of age. In these mice, a great number of leukocytes infiltrated into multiple non-lymphoid organs, autoantibody levels were elevated, and peripheral T cells displayed activated phenotypes. In addition, deficiency of TGF-βRII caused mice to develop fatal autoimmune diseases similar to the TGF-β1$^{-/-}$ mice. This phenotype can be attributed to hyperactivation and exaggerated Th1 effector functions of immune cells, especially T cells [67, 79]. These findings are in accordance with the results from Rudensky's laboratory, where a different strain of T-cell specific TGF-βRII knockout mice were used [77].

T-bet encoded by the *Tbx21* gene is a transcription factor that is critical for IFN-γ production and Th1 differentiation of CD4$^+$ T cells [80]. We attempted to alleviate/rescue the Th1-type immune disorder observed in 4cre-RII/RII mice by creating 4cre-RII/RII mice deficient in the *Tbx21* gene. Much to our surprise, CD4$^+$ T cells from 4cre-RII/RII-Tbx21$^{-/-}$ mice remained activated but with much less IFN-γ production. Therefore, TGF-β suppresses T-cell activation through a T-bet-independent mechanism, while T-bet remains essential for IFN-γ expression. In addition, CD4$^+$ T-cell numbers were found to be decreased in these mice, likely due to decreased expression of CD122 (IL-2Rβ), a receptor that is important for both IL-2 and IL-15 signaling. Further analysis revealed that Th1-skewing conditions preferentially upregulated CD122 on CD4$^+$ T cells in vitro in a T-bet dependent manner [67]. More interestingly, addition of TGF-β inhibited the upregulation of CD122 on CD4$^+$ T cells, suggesting that physiologically TGF-β limits CD4$^+$ effector T-cell numbers through controlling IL-2- and IL-15-driven T-cell expansion. As TGF-β potently inhibits T-bet expression in Th1 cells [26], it remains to be addressed whether TGF-β inhibits CD122 expression through T-bet-dependent and/or T-bet-independent mechanisms.

Activation of T cells in 4cre-RII/RII mice might be due to decreased Treg numbers in the periphery [67]. However, using a transfer model, we and others found that spontaneous activation of T cells lacking TGF-βRII is refractory to Treg suppression [67, 81]. It thus suggests that wild-type Treg are not able to suppress T cells that cannot respond to TGF-β. This conclusion is consistent with previous reports [9, 82, 83]. It does not however establish a Treg-independent role of TGF-β in controlling T-cell activation, since Treg-mediated suppression might be through a TGF-β-dependent mechanism. Compelling evidence to support that TGF-β controls T-cell activation through Treg-independent fashion came from the analysis of 4cre-RII/RII mice with the OTII T cell receptor (TCR) transgene on a *Rag1*$^{-/-}$ background, where endogenous Foxp3$^+$ Treg cells failed to develop. Substantial portions of T cells from these mice displayed an activated phenotype, while only a small percentage of these cells produced effector cytokines, which could be due to lack of stimulation from cognate antigens. Treg-independent TGF-β-dependent regulation of immune functions is an area that is poorly understood, and future studies are needed to pursue the mechanisms involved.

Leukocytes and stromal cells are both able to generate TGF-β; which source of TGF-β is important and whether TGF-β regulates T-cell function as an autocrine, a paracrine, or an endocrine cytokine are interesting questions. We generated T-cell specific TGF-β1 knockout mice and addressed contribution of T-cell-made TGF-β1. These mice developed lethal immunopathology in multiple organs, associating with enhanced T-cell proliferation, activation, and differentiation. In a transfer model, TGF-β1 produced by Treg cells was shown to be required for Th1-cell differentiation and the onset of inflammatory bowel disease, while TGF-β1 generated by conventional T cells also contributed to the inhibitory effects [84]. These findings demonstrated that, to regulate T cells, TGF-β1 functions through an autocrine or a paracrine but may not be an endocrine mechanism. Whether such a mechanism could be

applied to other leukocytes or non-lymphoid cells and what mechanisms are involved in achieving localized effects of TGF-β are important questions to be addressed in the future.

Another mechanism by which TGF-β inhibits immune responses is through promoting the generation of Treg cells by inducing Foxp3 expression. Early studies demonstrated that TGF-β was necessary and sufficient for human CD8^{+} T cells to acquire suppressive activities [85]. In addition, regulatory activity was induced in human naive (CD45RA^{+} RO^{−}) CD4 T cells by TGF-β after stimulation [86]. TGF-β was subsequently demonstrated to induce the expression of Foxp3 in CD4^{+} CD25^{−} human T cells [87] and in activated murine CD4^{+} and CD8^{+} T cells as well. In the presence of TGF-β1, *Staphylococcus* endotoxin-B-activated CD8 T cells inhibited the proliferation and effector functions of CD4^{+} and CD8^{+} T cells. This was accompanied by elevated levels of IL-10 and TGF-β1 [88]. TGF-β was later demonstrated to convert mouse CD4^{+} CD25^{−} into CD4^{+} CD25^{+} T cells with elevated Foxp3 expression [87, 89].

Studies demonstrated that TGF-β is able to convert CD4^{+} CD25^{−} non-Treg into CD4^{+} CD25^{+} Treg cells, and this was accompanied with increased Foxp3 expression [87, 89]. However, a substantial portion of Foxp3^{+} Treg cells are negative for CD25 [90, 91]. In these studies, it was not distinguished whether such conversion is due to preferential expansion/survival of the existing Foxp3^{+} CD25^{−} population or due to de novo Foxp3 expression in the Foxp3^{−} CD25^{−} population. Evidence for TGF-β promoting the conversion of Foxp3^{−} cells into Foxp3^{+} cells came from our study using Foxp3-monomeric red fluorescent protein (mRFP) knockin mice, where Foxp3-expressing cells are marked by mRFP expression [91]. TGF-β induced de novo Foxp3 expression in Foxp3^{−} CD4 T cells. Furthermore, only Foxp3^{+} CD4^{+} cells but not Foxp3^{−} CD4^{+} counterparts possessed regulatory activities [91].

Although TGF-β promotes Treg generation in vitro, it has been controversial whether TGF-β is involved in the generation or maintenance of Foxp3-expressing Treg under physiological conditions. Transient expression of TGF-β by a transgene specifically expressed in islets promotes the generation of CD4^{+} CD25^{+} Treg in situ with high Foxp3 expression in diabetes-predisposed non-obese diabetic (NOD) mice [92]. This observation correlated with the suppression of diabetes. In addition, induced Treg cells suppressed the onset of diabetes after adoptive transfer of these cells into NOD mice [92]. These findings demonstrated that TGF-β is sufficient to promote the generation of Treg under physiological conditions. Conflicting results have been presented with regards to whether TGF-β is essential for the development and maintenance of nTreg. In one study, the CD4^{+} CD25^{+} Treg population was shown to be decreased in adult mice transgenic for a dominant negative form of TGF-β receptor II [93] under the control of the CD2 promoter

(hCD2-ΔkTβRII). After being transferred into mice subjected to dextran sodium sulfate-induced colitis, hCD2-ΔkTβRII transgenic CD4^{+} CD25^{+} cells proliferated poorly compared with wild-type CD4^{+} CD25^{+} nTreg cells, thus suggesting that TGF-β signaling was required for the maintenance and expansion of CD4^{+} CD25^{+} nTreg in vivo [94]. However, in another transgenic model where a similar form of TGFDNR (dnTβRII) is expressed under the control of the CD4 promoter [50], CD4^{+} CD25^{+} nTreg cells in CD4-dnTβRII transgenic mice developed normally. A slightly increased number of nTreg were found in the periphery of these mice compared to their wild-type counterparts ([83] and our unpublished observation). Peripheral but not thymic nTreg were found to be reduced in 8- to 10-day-old TGF-β1^{−/−} mice [81], suggesting an essential function for endogenous TGF-β1 in the maintenance of the peripheral population of Treg. These results contrast that of an earlier study where no defect of Treg development or maintenance was observed in TGF-β1^{−/−} mice [95]. A more recent definitive study from our laboratory demonstrated that Foxp3-expressing nTreg cells that lack TGFβRII developed normally in the thymus but were poorly maintained in the periphery [67]. Interestingly, in the same study, TGFβRII-deficient Treg proliferated faster than the wild-type counterparts in the periphery despite that lower numbers of TGFβRII-deficient Treg cells were found, suggesting that TGF-β signaling is required to promote the survival of peripheral Treg [67]. The reasons for these discrepancies are unknown but may be related to the different experimental systems and mouse genetic backgrounds used. In addition, because CD25 is also expressed by activated T cells, CD4^{+} CD25^{+} Treg identified in these studies may have been contaminated by activated T cells to varying degrees in the earlier studies. With the development of enhanced green fluorescent protein (EGFP)-Foxp3 and Foxp3-mRFP knockin mice and Foxp3 intracellular staining, these potential complications can be circumvented by identifying Treg based on Foxp3 expression [67, 90, 91]. It is coming to a consensus that TGF-β is required for the maintenance of Treg in the periphery. We investigated whether T-cell-made TGF-β is required for Treg maintenance by studying mRFP-expressing Treg cells from mice lacking TGF-β1. We found that T-cell-produced TGF-β1 is dispensable for the development and maintenance of Treg cells [84], despite the fact that TGF-β1-deficient Treg cells lost their suppressive activities in a transfer model. Thus, self-made TGF-β1 appears to be required for Treg function, while it remains an open question as what source of TGF-β is required for Treg maintenance.

TGF-β Promotes Certain Immune Responses

As discussed above, TGF-β is prevalently viewed as an immune suppressive cytokine. However, as a ploeitropic

cytokine, TGF-β has been found to exhibit immune-promoting properties, which has recently attracted much attention.

In early studies, TGF-β was found to enhance the proliferation of mouse CD8^{+} cells under certain conditions [96] and to increase TNF-α production by both CD4^{+} and CD8^{+} cells [97]. TGF-β accelerates T-cell death in some studies [98, 99], while an anti-apoptotic role for TGF-β has also been documented [100, 101].

Interestingly, TGF-β was recently identified to be important for the induction of IL-17-producing cells under inflammatory conditions [102–105]. Th-17 cell is a new class of effector T cells that is gaining increasing attention. Th-17 cells produce IL-17 and IL-22 and are critical for the induction of experimental autoimmune encephalomyelitis (EAE) in mice. Under culture conditions, TGF-β in combination with IL-6 promotes Th-17 differentiation. In addition, TGF-β is important for the generation of Th-17 cells and the induction of EAE in mice [103, 106]. By deleting the TGF-β1 gene specifically in T cells, we found that T-cell-made TGF-β1 is required for the induction of pathogenic Th-17 cells in the intestines and in the spinal cord. myelin oligodendrocyte glycoprotein-induced EAE is drastically ameliorated when T cells cannot generate TGF-β1 [84]. Considering that TGF-β1 can be generated by conventional and Treg cells, it would be important to discern whether either type of cells is required for the generation of Th-17 cells and the induction of EAE in mice.

The lack of TGF-β signaling is often associated with increased proliferation and effector function of immune cells. However, two recent independent studies demonstrated that TGF-β might promote proliferation and survival of immune cells under certain conditions. The numbers of canonical CD1d-dependent natural killer (NK1.1^{+}) T cells and CD8^{+} T cells were decreased upon TGF-βRII deletion, suggesting a positive role for TGF-β signaling in their development [67]. In the absence of TGF-β signaling, OT-II CD4 T cells, which only responded to OVA peptide from chicken ovalbumin, were prone to apoptosis in the periphery and failed to differentiate into effector cells. This phenotype correlates with reduced expression of CD122 on these cells. It suggests that TGF-β signaling is required for the survival of some cells. However, it remains to be addressed as to what is the relationship among TGF-β promoted T-cell survival, CD122 expression, and Th1 differentiation.

Collectively, these studies highlight the multi-faceted effects of TGF-β on various immune functions and emphasize the importance of cellular and environmental contexts in directing the discrete roles of TGF-β. What triggers and mediates TGF-β to perform such a broad spectrum of functions needs to be elucidated in the years to come.

Treg-Controlled Immune Responses

Early studies showing that thymectomy of neonatal rodents within 3 days after birth led to lethal autoimmunity suggested that a set of thymus-derived cells are important to maintain self-tolerance in the periphery [107, 108]. Upon identification of CD4^{+} CD25^{+} as markers for Treg cells, Sakaguchi's group used anti-CD25 antibody to deplete these cells, and such manipulation led to lethal autoimmunity due to the loss of peripheral tolerance in adult mice [33]. In 2003, an X-linked forkhead family transcription factor named Foxp3 was identified to be expressed specifically in nTreg among the lymphocyte populations [35, 109, 110]. Spontaneous monogenetic mutation in the *Foxp3* gene results in systemic autoimmunity in *Scurfy* mice and *IPEX* (X-linked neonatal diabetes mellitus, enteropathy, and endocrinopathy syndrome) patients [111–115]. The disease manifested in *Scurfy* mice was attributed to Treg deficiency. However, T-cell extrinsic elements were reported to contribute to the Scurfy phenotype [116], while counter-evidence were also presented [117]. In addition, targeted deletion of *Foxp3* gene in mice led to a phenotype reminiscent of *Scurfy* mice with undetectable CD4^{+} CD25^{+} T cells [118–120]. Moreover, ectopically overexpressed *Foxp3* gene endowed immune suppressive activities to conventional CD4 and CD8 T cells [112, 113, 120]. Therefore, Foxp3 controls the development and function of nTreg cells, a type of suppressor T cells that are essential in maintaining self-tolerance and immune homeostasis.

Mechanisms of Treg-Mediated Immune Suppression

The essential role of Treg in immune suppression is indisputable. Yet, the mechanisms by which Treg cells carry out their function remain ill-defined. Nevertheless, it is generally agreed that Treg suppress immune responses through multiple mechanisms including cell contact dependent and independent mechanisms. Several surface molecules preferentially expressed by Treg cells are proposed to be important for their function. For example, CD25, a high-affinity IL-2 binding receptor, is highly expressed by the nTreg cells [121]. In addition, the peripheral maintenance of nTreg is dependent on IL-2 signaling [122–124], which is also important for the proliferation and survival of activated effector T cells. It is therefore hypothesized that one mechanism of nTreg suppression of conventional T-cell activation is through competition for IL-2 consumption [125, 126]. Under culturing conditions, such competition may result in effector T cell death due to deprivation of growth factors, such as IL-2 [127]. However, studies showing that CD25-deficient nTreg cells possess intact suppressive activity question the validity of this hypothesis [123]. CTLA-4, another surface molecule preferentially

expressed by nTreg, is important for inhibiting immune activation by competing for costimulatory ligands on T cells [128] and inhibiting the function of antigen-presenting cells [129, 130]. Thus, it is suggested that CTLA-4 is important for nTreg-mediated immune suppression. Genetic evidence however argues against the critical roles for CTLA-4 in nTreg function, as the function of CTLA-4$^{-/-}$ nTreg functioned normally in vitro and in vivo [131, 132]. However, antibody-mediated blockage of CTLA-4 abrogated nTreg function [131, 132], while it remains unknown whether this is due to a non-specific effect or due to a simultaneous block of CTLA-4 and its potential homolog with redundant functions by the antibodies.

Recent studies have unraveled important roles of cytokines in nTreg function. TGF-β appears to be critical in mediating nTreg function, as T cells from CD4-dnTβRII mice that are unresponsive to TGF-β are refractory to nTreg-mediated suppression in vitro and in vivo [9, 82, 83]. Despite the fact that TGF-β mRNA is not elevated in nTreg cells, it is suggested that the membrane bound form of TGF-β is increased in nTreg cells and is important for their function [9, 34]. IL-10 is another immune suppressive cytokine preferentially expressed in Treg [133, 134] and is important in mediating the functions of these cells [134, 135]. Besides suppress effector T cell functions directly, recent study suggests that nTreg dampens immune responses through regulating innate component, such as DC. nTreg can potentially induce tolerogenic DC through CTLA-4 engagement induced tryptophan catabolism [136]. In addition, it appears that nTreg destabilize the interaction between antigenic DC with conventional T cell to prevent the activation of T cells [137]. It is clear now that multiple mechanisms are involved in Treg-mediated immune suppression. Cell-contact-dependent and cell-contact-independent mechanisms critical for controlling Treg function remain to be identified in the future.

Foxp3 Controls Treg Function

As a single molecule that controls the development and function of Treg, Foxp3 has been under close scrutiny since its discovery. We and others have developed knockin mouse models to track Foxp3-expressing cells with fluorescent proteins [90, 91]. Foxp3 can be expressed in thymic-derived Treg cells; its expression can also be induced in conventional T cells in vitro by TGF-β regardless of their proliferation status [91]. In addition, Foxp3 expression can be induced in vivo in conventional T cells under suboptimal stimulation [138]. More importantly, in these studies, Foxp3 expression has always been associated with the immune suppressive function. Thus, it is generally thought that Foxp3 serves as an on-and-off switch to positively regulate Treg function in a binary

fashion. However, a transient increase of Foxp3 expression in human CD4 T cells did not result in suppressive function. In addition, in many cases of human IPEX patients, functional Foxp3 protein is made but at much reduced levels [139]. Moreover, emerging evidence associates decreased Foxp3 expression in Treg cells with various human autoimmune disorders, such as graft-versus-host disease [140], autoimmune myasthenia gravis [141], and multiple sclerosis [142]. We have also found that intra-islet Treg cells expressed lower levels of Foxp3 than Treg cells from other peripheral lymphoid organs in diabetic NOD mice, while the frequencies of Foxp3-expressing Treg cells among the different compartments were comparable. Thus, Foxp3 may control Treg function in a dose-dependent, non-binary fashion, and decreased Foxp3 expression could lead to impaired Treg function and be causal for immune disorders.

Studying of a mouse model where attenuated Foxp3 expression is serendipitously achieved, we found that reduced Foxp3 expression led to the development of a lethal aggressive lymphoproliferative autoimmune syndrome reminiscent of Scurfy mice [143] and T-cell-specific Foxp3 knockout mice [120]. Compared with WT Treg cells, the surface expression of 'signature genes' for Treg cells, such as CD25, CTLA-4, and GITR [32, 144, 145], were decreased in these mice [146]. Thus, Foxp3 programs the gene expression of Treg in a tunable and dose-dependent fashion [146].

Further investigation revealed interesting phenotypic changes in the functions of Treg cells, which express decreased Foxp3. Foxp3 is required for the development and maintenance of Treg cells [120]. However, decreased Foxp3 expression did not lead to defective thymic development of Treg cells. This result agrees with the results from a separate study, where GFP complementary DNA was inserted into endogenous Foxp3-coding region to mark T cells with Foxp3 promoter activity but fail to produce functional Foxp3 protein [147]. Substantial numbers of GFP-expressing thymocytes were also detected in these mice [147]. By adoptive transfer assays, we found that attenuated Foxp3 expression did not result in intrinsic defects in the homeostatic expansion/maintenance of Treg cells in the periphery. Extra-thymic generation of Foxp3-expressing T cells can be promoted in vitro by TGF-β [89, 91, 106].TGF-β induced de novo Foxp3 expression normally in T cells with attenuated Foxp3 expression. Therefore, decreased Foxp3 expression did not affect the development, homeostatic expansion/maintenance, or TGF-β driven de novo generation of Foxp3-expressing T cells [146]. These findings present a possibility that defective and even ablated Foxp3 expression might not result in the total elimination of the "Treg lineage." Indeed, Foxp3-null mice generated "Treg lineage" cells in the thymus and can be
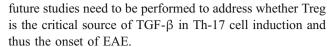
maintained in the periphery [147]. Thus, in *Scurfy* mice and *IPEX* human patients, *Foxp3*-expressing T cell subsets are likely to be generated in the thymus and maintained in the periphery. Further studies are warranted to identify and characterize these "Treg lineage" cells lacking functional Foxp3.

In vitro, hypoproliferative ('anergic') and immune-suppressive activities are two defining properties for Foxp3-expressing Treg cells that are thought to go hand-in-hand [33]. Intriguingly, upon TCR stimulation in vitro, while Foxp3 low-expressing T cells remained anergic; their immune-suppressive activities were greatly impaired. Thus, anergy and immune suppression are two separable properties of Treg cells that are affected differentially by the expression levels of Foxp3 [146]. In addition, in a T-cell transfer model, the immune-suppressive activities of Foxp3 low-expressing T cells were abolished in vivo, although these cells infiltrated efficiently into lymphoid and non-lymphoid organs. Thus, the suppressive function of Treg requires high level Foxp3 [146]. Interestingly, agreeing with these findings, Foxp3-deficient "Treg lineage" cells remain anergic but without immune suppressive function [147]. Therefore, unlike what has been recognized, Foxp3 may not be the "grand-master" of Treg development. It is emerging that Foxp3 appears to be required for stabilizing "regulatory function" of already differentiated "Treg lineage" cells in vivo or TGF-β induced Treg cells in vitro [147–149]. The molecular master-switch controlling the commitment of "Treg lineage" remains to be discovered. In addition, despite the fact that multiple molecules, such as CTLA-4 and CD25, have been suggested to contribute to the suppressive activities of Treg, it remains unsolved whether any of them may be sufficient to render Treg-suppressive function. It would be interesting to investigate whether any of these genes are able to reconstitute the Treg function in the aforementioned mouse models. Genomic-scaled DNA binding profiling studies have been performed to identify Foxp3-specific target genes that might be crucial for Treg function. Over 700 potential targets including promoters, intragenic regulatory elements, and small modulatory RNA/small interfering RNA have been identified [150]. Future efforts are need to sieve through these candidates to pinpoint the critical Foxp3 target genes in controlling the immune suppressive function of Treg cells.

Positive Roles for Treg in Inflammation

Treg contribute to inflammation by inducing Th-17 indirectly through TGF-β. TGF-β appears to be critical for the induction of Th-17 cells according to our and other studies [105]. High levels of TGF-β, probably existing as a membrane bound form, are found on Treg cells. Therefore, Treg cells have been identified to be an important inducer for Th-17 cells in vitro. Using TGF-β1 knockout mice,

future studies need to be performed to address whether Treg is the critical source of TGF-β in Th-17 cell induction and thus the onset of EAE.

Foxp3-expressing cells can convert into effector cells. Analysis of Foxp3 low-expressing cells revealed that they developed effector functions producing large amounts of type 2 effector cytokine, such as IL-4, while the changes of IL-2, IFN-γ, or IL-17 expression were modest [146]. In another study, "Treg lineage" cells without functional Foxp3 were able to express Th1, Th2, and Th17 effector cytokines [147]. These findings present an interesting possibility that Foxp3-expressing cells might not always suppress but rather, in some cases, can foster the immune response, e.g. under inflammatory conditions. In fact, TNF-α, a pro-inflammatory cytokine, has been shown to repress the expression of Foxp3 in human Treg cells [151]. It is reasonable to believe that in a highly inflammatory microenvironment, residential Treg decrease their Foxp3 expression, subsequently lose their suppressive function, and might even be converted into effector cells to contribute to the immune responses. Upon resolution of infection, inflammatory cytokines would subside and Foxp3 levels and suppressive activity would be restored.

Conclusions

The importance of active immune suppression is widely acknowledged. Studies on TGF-β and Treg have shed light on how immune suppression functions. Advances in these areas have been and are being translated into clinical benefits. Further investigations are warranted to elucidate the mechanism through which TGF-β and Treg control immune responses. In addition, we are starting to realize that under certain conditions, TGF-β and Treg can both serve as promoting factors to direct immune responses. To facilitate designing immune therapies against inflammatory diseases and cancers, more studies are needed to reveal alternative functions of TGF-β and Treg.

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