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'Yin-Yang' functions of TGF-β and Tregs in immune regulation

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Summary

Transforming growth factor-β (TGF-β) and Forkhead box p3-expressing T-regulatory (Treg) cells are critical in maintaining self-tolerance and immune homeostasis. 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. This review discusses our and other's efforts in understanding the negative (Yin) as well as the positive (Yang) roles for TGF-β and Treg cells in immune regulation.

Keywords

TGF-β; Treg; Foxp3; Th2; IL-17

Introduction

Complex immune strategies have evolved in mammals to defend against foreign pathogens to maintain health. The two fundamental arms of the immune response, adaptive and innate immunity, together form a defensive front against these pathogens. Innate immunity, mediated by cells such as macrophages, is less specific and works by recognizing classes of micro-organisms. T and B cells are the major cells carrying out the adaptive immune responses through their highly antigen-specific surface receptors. By using quasi-random recombinational mechanisms, thymic derived T cells can potentially generate infinite numbers of specificities, being reactive to foreign as well as self-antigens. Self-reactive T cells can cause autoimmune diseases in the host if they are not tightly controlled. Multiple processes therefore are in place to suppress the generation or the function of self-reactive T cells. Self-reactive T cells can be deleted in the thymus as well as in the periphery. Such elimination processes are incomplete, however, resulting in small populations of mostly low-affinity self-reactive T cells in the periphery that can potentially initiate an autoimmune response. Fortunately, active immune suppressive mechanisms exist to suppress the function of these auto-reactive 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 cytokines or through specialized cells. The pleiotropic cytokine, transforming growth factor-β (TGF-β), and the immunosuppressive cell, previously called suppressor cells (1) and now usually termed T regulatory (Treg) cells, play critical roles in suppressing the immune response. For years, we have investigated the immune suppressive functions of TGF-β and Tregs under normal and

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immune-pathological conditions. Recently, our and other's studies suggest that, in a cell type and environment-dependent fashion, TGF-β and Tregs might also positively regulate immune responses. Thus, while TGF-β and Tregs are dominantly viewed as critical mediators for immune suppression, they in fact exert both 'Yin' (negative) and 'Yang' (positive) effects on the immune system. Here, we will review the evolution of our views on the functions of TGF-β and Tregs in the immune regulation.

TGF-β and its signaling

Consisting of a family of pleiotropic cytokines, TGF-β regulates multifaceted cellular functions including proliferation, differentiation, migration, and survival (2). Bone morphogenetic proteins (BMPs), activins, and growth differentiation factors (GDFs) also belong to the TGF-β family (3).

TGF-β1, -β2, and -β3 are the three isoforms that have been identified in mammals. While sharing similar functions, these isoforms are differentially expressed in a spatially and temporally dependent manner (4). In the immune system, TGF-β1 is the isoform predominantly expressed. 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 (6–10). Through proteolytic processing, active TGF-β is produced through its dissociation from latency associated protein (LAP) or latent-TGF-β-binding protein (LTBP), whose association keeps TGF-β inactive (5). The active form of TGF-β can function in either a cell surface-bound form or a soluble form (11,12).

Activated TGF-β binds to a heterodimeric receptor complex consisting of a type I and II trans-membrane serine/threonine kinase subunits. Although more than 35 TGF family members have been identified, only five type I [activin-like receptor kinase (ALK) family] and seven type II receptors have been reported (3). Binding of the TGF-β dimer with the tetrameric ALK5 and TGFβRII receptor complex initiates signaling cascades (13). Much of our knowledge of TGF-β downstream signaling is from the studies performed in non-lymphoid cells. TGF-β binding tethers the type II receptor with ALK5 and triggers the phosphorylation and activation of ALK5 by the type II receptor. Intracellular signal transduction of TGF-β is mediated to a great degree via Smad proteins (13,14). The eight vertebrate Smads identified thus far are grouped into three categories: five receptor-associated Smads (R-Smad1, 2, 3, 5, 8), one common Smad (Co-Smad4), and two inhibitory Smads (I-Smad6, 7). Upon TGF-β stimulation, activated ALK5 phosphorylates R-Smad-2 and -3. Phosphorylated R-Smads associate with Co-Smad4 and translocate into the nucleus to bind to DNA containing a Smad binding element (SBE) (15–18). As an inhibitory Smad protein, I-Smad7 suppresses TGF-β signaling (19). At least two mechanisms are used by I-Smad7 to inhibit TGF-β signaling: one is by competing with R-Smads for the binding to ALK5, and the other is by recruiting ubiquitin ligase complexes to degrade ALK5 via the proteasome (20,21). In addition, Smad-independent TGF-β signaling pathways have been reported (22,23). Through mechanisms yet to be determined, rapid activation of Ras-Erk (extracellular signal-regulated kinase), TAK-MKK4-JNK (TGF-β-activated kinase-mitogen-activated protein kinase kinase 4-c-Jun N-terminal kinase), TAK-MKK3/6-p38, Rho-Rac-cdc42 MAPK, and PI3K (phosphatidylinositol 3-kinase)-Akt pathways occurs when cells are treated with TGF-β (24). MAPKs also coordinate with Smads to modulate TGF-β responses (25–27). Moreover, TGF-β receptors activate TRIP-1 (TGF-β receptor-interacting protein-1) and PP2A (phosphatase 2A) through direct protein binding to regulate translation initiation (28–30). Therefore, TGF-β exerts its regulation of target cell function via many different mechanisms. 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 (signal transducer and activator of transcription 4), IFN-γ (interferon-γ), and granzyme-B, are suppressed by TGF-β (31–35).

Tregs

The concept of immune suppression was proposed in the early 1970s by Gershon *et al*. (1,36,37). Being unable to identify the cell types performing immune suppression impeded the validation of this concept and hampered development in this research area. It was not until a study performed by Sakaguchi *et al*. (38) in 1995 that a subset of T cells with markedly increased expression of CD25 was discovered as suppressor T cells and later referred to as Tregs (38). In recent years, substantial progress has been made in identifying different types of Treg cells as well as 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 (nTregs) and acquired Treg (aTregs).

nTregs

A subset of $CD4^+$ T cells that develops in the thymus and constitutively expresses cell surface IL-2 receptor α chain (CD25) is termed nTregs. $CD4^+CD25^+$ 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. A cluster of cell surface molecules besides CD25, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family related gene (GITR), and lymphocyte activation antigen-3 (LAG-3), have also been used to differentiate nTregs from conventional T cells (39). TGF-β is expressed in nTregs at high levels in a cell surface-bound form (12,40). nTreg cells suppress immune responses in an antigen-independent fashion *in vitro* and *in vivo* (39,41–43). Forkhead box protein 3 (Foxp3), an X-linked transcription factor belonging to forkhead family, is specifically and highly expressed in nTreg. Thus, Foxp3 is currently used as the most reliable molecular marker for nTregs.

aTregs

Conventional T cells are able to gain immune suppressive activities and become aTregs. Tr1 (T regulatory1) and Th3 (T-helper 3) cells are two reported types of aTregs. Tr1 cells are often found within the intestinal mucosa and suppress immune reactions towards a variety of cognate antigens (44). There is no particular surface marker associated with Tr1 cells. However, these cells produce increased levels of IL-10 and TGF-β (45). Tr1 cells do not express Foxp3 (46), suggesting that it is a subset of Tregs distinct from nTregs.

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 (47,48). While no particular surface marker is associated with these cells, Foxp3 is expressed in Th3 cells (49). In addition, TGF-β is produced at elevated levels by Th3 cells (48). Whether Th3 cells form a distinct aTreg subset or are activated nTregs remains unknown.

TGF-β inhibits immune responses

Identified as a growth factor for transformed tumor cells, TGF-β in fact inhibits the proliferation of non-transformed cells, such as epithelial cells and fibroblasts. 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 (50,51). However, the definitive genetic evidence to support the critical roles of TGF-β in suppressing immune responses *in vivo* were not presented until the analysis of TGF-β-deficient mice (52,53). Since then, using

genetic modified mouse models, our and others' 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 (54–60). TGF-β suppresses immune responses through two means: inhibiting the function of inflammatory cells and promoting the function of Treg cells (Fig. 1).

TGF-β inhibits the function of inflammatory cells

Multiple types of immune cells can be regulated by TGF-β. The role of TGF-β in controlling T-cell functions and immune responses has been studied extensively (61). The anti-proliferative function of TGF-β on T cells was first documented by studies performed *in vitro* using activated human T cells (51). TGF-β can suppress T -cell proliferation by inhibiting the production of interleukin-2 (IL-2), a lymphokine known to potently activate T cells, NK cells, and other of the immune system cells. Addition of exogenous IL-2 partially relieved TGF-β-mediated suppression (51). 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 (62). 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 (63). 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 (64). Because addition of exogenous IL-2 did not fully reconstitute T-cell proliferation (51), TGF-β inhibits T-cell proliferation also through IL-2-independent mechanisms that warrant further investigation.

TGF-β regulates cell proliferation through controlling the expression of cell cycle regulators. Upon TGF-β treatment, cyclin-dependent kinase inhibitors (CKIs), such as p15, p21, and p27, are upregulated, while cell cycle promoting factors, such as c-myc, cyclin D2, CDK2, and cyclin E, are downregulated (65–70). The functional significance of these expression changes in TGF-β-induced inhibition and how TGF-β modulates these genes in T cells remain to be elucidated. Although TGF-β inhibits naive T-cell proliferation, it has minimal effects on activated T cells, which may be due to reduced TGF-β receptor II expression on these cells (71). It nevertheless suggests that TGF-β functions in a cell type-dependent fashion.

To perform immune function, naive $CD4^+$ T cells differentiate into three major types of effector T cells following activation (72). Based on their cytokine production, $CD4^+$ effector T cells, also named Th cells, can be profiled as Th1, Th2, and Th17 cells (73,74). Th1 cells produce IFN-γ and lymphotoxin (LT), Th2 cells secrete IL-4, IL-13, and IL-5, and Th17 cells express IL-17 and IL-22 (75). While TGF-β partially suppresses T-cell proliferation, we have found that TGF-β potently inhibits effector functions of activated T cells and thus their differentiation into Th1 or Th2 effector cells under tissue culture conditions (76). 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 (72). While the detailed mechanism remains unknown, our and others' studies showed that T-bet and Gata-3 expression is inhibited by TGF-β (32,33,77,78), possibly, in the latter case, through a mechanism via blocking Itk kinase activity and calcium influx (31). Interestingly, effector cytokine production by fully differentiated Th2 cells is unaffected by TGF-β, while Th1 cells remain susceptible to TGF-βksuppression (79). Therefore, TGF-β exerts most of its inhibitory effects on the establishment of effector cell functions. Our recent study demonstrated that the Th1

polarizing condition promotes CD122 (IL-2 receptor β chain) expression through T-bet, thereby enhancing the clonal expansion and survival of Th1 cells (80). 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 (81). 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 (82).

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 (83–86). Recent studies showed that TGF-β is important for Treg-induced inhibition of the exocytosis of granules and cytolytic function of $CD8^+$ T cells (87).

The critical role for TGF-β in regulating immune suppression under physiological conditions has been demonstrated by studying mouse models where TGF-β signaling was altered. TGF-$\beta1^{-/-}$ mice develop a multifocal inflammatory disease associated with increased inflammatory cytokine production (52,53,88). This phenotype is predominantly mediated through T cells, as depletion of $CD4^+$ T cells or crossing TGF-$\beta1^{-/-}$ mice onto an major histocompatibility complex (MHC) class II null background prevented this inflammation (89). 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 important role in suppressing the function of innate immunity. By expressing a transgene encoding a dominant negative form of TGFβRII under the control of CD11c promoter (CD11cTGFDNR), we blocked TGF-β signaling in NK cells and dendritic cells (DCs) (57). Blockade of TGF-β signaling in NK cells caused the accumulation of a large pool of NK cells secreting copious amounts of IFN-γ. Such 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 DCs from these mice did not affect DC homeostasis or IL-12 production, suggesting TGF-β differentially affects NK cells and DCs. 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 (90). 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 (56,58). 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 (56). 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* (91). Nonetheless, CD4-dnTβRII mice displayed much less immune pathology than TGF-$\beta1^{-/-}$ 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-$\beta1^{-/-}$ mice (92). 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 independent mechanisms (80). Mice with T cell specific TGF-βRII deletion (4cre-RII/RII) developed a progressive wasting disease and succumbed to death by five weeks of age. In these mice, great numbers of leukocytes infiltrated into multiple non-lymphoid organs, auto-antibody levels were elevated, and peripheral T cells displayed activated phenotypes. In addition, deficiency of TGFβRII caused mice to develop fatal autoimmune diseases similar to the TGF-β1$^{-/-}$ mice. This phenotype can be attributed to hyper-activation and exaggerated Th1 effector functions of immune cells, especially T cells (80,92). These findings are in accordance with the results from Rudensky's laboratory (90), where a different strain of T cell specific TGFβRII knockout mice were used.

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 (93). 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, 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 (80). 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 (32), it remains to be addressed whether TGF-β inhibits CD122 expression through T-bet-dependent and/or -independent mechanisms.

The T-cell activation phenotype observed in 4cre-RII/RII mice might be attributed to decreased Treg numbers in the periphery (80). However, using a transfer model, we and others found that the existence of substantial numbers of wildtype Treg cells, which apparently cannot prevent the spontaneous activation of T cells lacking TGF-βRII (80,94). It thus suggests that wildtype Tregs are not able to suppress T cells that cannot respond to TGF-β. This conclusion is consistent with previous reports (12,95,96). 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 a Treg-independent mechanism came from the analysis of 4cre-RII/RII mice with the OTII 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-produced TGF-β1. These mice developed lethal imunopathology in multiple organs, associated 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 (97). These findings demonstrated that to regulate T cells, TGF-β1 functions through

an autocrine or a paracrine but not 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 future studies.

TGF-β promotes the generation and function of Treg cells

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 (98). In addition, regulatory activity was induced in human naïve ($CD45RA^+RO^-$) $CD4^+$ T cells by TGF-β following stimulation (99). TGF-β was subsequently demonstrated to induce the expression of Foxp3 in $CD4^+CD25^-$ human T cells (100), and in activated murine $CD4^+$ and $CD8^+$ T cells. In the presence of TGF-β1, Staphylococcus enterotoxin B (SEB)-activated $CD8^+$ T cells inhibited the proliferation and effector functions of $CD4^+$ and $CD8^+$ T cells. This inhibition was accompanied by elevated levels of IL-10 and TGF-β1 (101). TGF-β was later demonstrated to convert mouse $CD4^+CD25^-$ into $CD4^+CD25^+$ T cells with elevated Foxp3 expression (100,102).

Studies demonstrated that TGF-β is able to convert $CD4^+CD25^-$ non-Treg cells into $CD4^+CD25^+$ Treg cells, and this conversion was accompanied with increased Foxp3 expression (100,102). However, a substantial portion of $Foxp3^+$ Treg cells are negative for CD25 (103,104). 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. Unequivocal evidence for TGF-β conversion of $Foxp3^-$ cells into $Foxp3^+$ cells came from our study using Foxp3-mRFP knockin mice, where Foxp3-expressing cells are marked by mRFP expression (104). 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 (104).

Although TGF-β promotes Treg generation *in vitro*, it is controversial whether TGF-β is involved in the generation or maintenance of Foxp3-expressing Tregs under physiological conditions. Transient expression of TGF-β by a transgene specifically expressed in islets promotes the generation of $CD4^+CD25^+$ Tregs *in situ* with high Foxp3 expression in diabetes-predisposed NOD (nonobese diabetic) mice (105). This observation correlated with the suppression of diabetes. In addition, induced Treg cells suppressed the onset of diabetes following adoptive transfer of these cells into NOD mice (105). These findings demonstrated that TGF-β is sufficient to promote the generation of Tregs under physiological conditions. Conflicting results have been presented with regards to whether TGF-β is essential for the development and maintenance of nTregs. In one study, the $CD4^+CD25^+$ Treg population was shown to be decreased in adult mice transgenic for a dominant negative form of TGFβRII (106) under the control of the CD2 promoter (hCD2-ΔkTβRII). After being transferred into mice subjected to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate)-induced colitis, hCD2-ΔkTβRII transgenic $CD4^+CD25^+$ T cells proliferated poorly compared with wildtype $CD4^+CD25^+$ nTreg cells, thus suggesting that TGF-β signaling was required for the maintenance and expansion of $CD4^+CD25^+$ nTregs *in vivo* (107). However, in another transgenic model where a similar form of TGFDNR (dnTβRII) is expressed under the control of the CD4 promoter (56), $CD4^+CD25^+$ nTreg cells in CD4-dnTβRII transgenic mice developed normally. A slightly increased number of nTregs was found in the periphery of these mice compared to their wildtype counterparts (96, authors' unpublished observations). Peripheral but not thymic nTregs were found to be reduced in 8–10-day-old TGF-$\beta1^{-/-}$ mice (94), suggesting an essential function for endogenous TGF-β1 in the maintenance of the peripheral population of Tregs. These results

contrast those of an earlier study, where no defect of Treg development or maintenance was observed in *TGF-β1*$^{-/-}$ mice (108). A more recent definitive study demonstrated that Foxp3-expressing nTreg cells that lack TGFβRII developed normally in the thymus but were poorly maintained in the periphery (80). Interestingly, in the same study, TGFβRII-deficient Tregs were found to proliferate faster than the wildtype 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 Tregs (80). 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$^+$ Tregs 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 fluorescence 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 (80,103,104). A consensus is forming that TGF-β is required for the maintenance of Tregs in the periphery. We investigated whether T-cell-produced 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 (97), 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.

The downstream signaling through which TGF-β mediates the generation of Foxp3-expressing Tregs remains unclear. *R-Smad3*$^{-/-}$ mice developed by two different groups showed distinct phenotypes in the immune system. While no apparent abnormality was observed when exon1 of the *R-Smad3* gene was knocked out in mice (109), impaired mucosal immunity associated with activated T cells was found when the exon8 of the *R-Smad3* gene was targeted (60). However, there is no evidence to suggest that the phenotype found in the latter study is due to perturbed Treg compartments. A recent study proposed a cooperative role for TGF-β in upregulating CD25 expression following TCR stimulation, and this function of TGF-β appeared to be mediated through the R-Smad3/Co-Smad4 pathways (110). Thus R-Smad3/Co-Smad4 regulation of the generation and/or maintenance of Tregs may be through cooperation with IL-2 signaling. However, because the Treg population appeared to be normal when Co-Smad4 is deficient in T cells (60, 110, authors' unpublished observation), it is questionable that Co-Smad4 and R-Smad-dependent TGF-β signaling is critically involved in Treg generation under physiological conditions.

TGF-β also promotes immune responses

TGF-β is prevalently viewed as an immune suppressive cytokine. However, as a pleiotropic 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$^+$ T cells under certain conditions (111) and to increase TNF-α production by both CD4$^+$ and CD8$^+$ T cells (112). TGF-β accelerates T-cell death in some studies (113,114), while an anti-apoptotic role for TGF-β has also been documented (115,116).

TGF-β was recently identified to be important for the induction of IL-17-producing cells under inflammatory conditions (117–120). The Th17 cell is a new class of effector T cells that is gaining increasing attention. Th17 cells produce IL-17 and IL-22, and they are critical for the induction of experimental autoimmune encephalitis (EAE) in mice. Under culture conditions, TGF-β in combination with IL-6 promotes Th17 differentiation. In addition, TGF-β is important for the generation of Th17 cells and the induction of EAE in mice

(118,121). By deleting the TGF-β1 gene specifically in T cells, we found that T-cell-produced TGF-β1 is required for the induction of pathogenic Th17 cells in the intestines as well as in the spinal cord. Myelin oligodendrocyte glycoprotein (MOG)-induced EAE is drastically ameliorated when T cells cannot generate TGF-β1 (97). Considering that TGF-β1 can be generated by conventional T as well as Treg cells, it would be important to discern whether either type of cells is required for the generation of Th17 cells and the induction of EAE in mice.

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 NK ($NK1.1^+$) T cells and $CD8^+$ T cells were decreased upon TGF-βRII deletion, suggesting a critical role for TGF-β signaling in their development (80). In the absence of TGF-β signaling, OT-II $CD4^+$ T cells, which only responded to ovalbumin (OVA) peptide from chicken OVA, 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. This finding 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.

Tregs are critical in mediating immune suppression

Early studies showing that thymectomy of neonatal mice within three days after birth led to lethal autoimmunity suggested a set of thymus-derived cells are important to maintain self-tolerance in the periphery (122,123). Upon identification of CD4 and CD25 as markers for Treg cells, Sakaguchi's group (30) 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. In 2003, an X-linked forkhead family transcription factor named Foxp3 was identified to be expressed specifically in nTregs among the lymphocyte populations (41,124,125). 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 (126–130). The disease manifested in *Scurfy* mice was attributed to Treg deficiency. However, T-cell extrinsic elements were reported to contribute to the Scurfy phenotype (131), while counter-evidence was also presented (132). In addition, targeted deletion of *Foxp3* gene in mice led to a phenotype reminiscent of *Scurfy* mice, with undetectable $CD4^+CD25^+$ T cells (133–135). Moreover, ectopically overexpressed the *Foxp3* gene endowed immune suppressive activities to conventional $CD4^+$ and $CD8^+$ T cells (127,128,135). 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 (Fig. 2).

Tregs function through cell contact-dependent and -independent mechanisms

The essential role of Tregs 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 Tregs 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 (136). In addition, the peripheral maintenance of nTregs is dependent on IL-2 signaling (137–139), 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 (140,141). However, studies showing that CD25-deficient nTreg cells possess intact suppressive activity question the validity of this hypothesis (138). CTLA-4, another surface molecule preferentially expressed by nTregs, is important for inhibiting immune activation by competing for costimulatory ligands on T cells (142) as well as inhibiting the function of antigen-presenting cells (143,144). Thus, it is suggested that CTLA-4 is important for nTreg mediated immune suppression. Genetic evidence argues against the critical roles for CTLA-4 in nTreg function, as the function of nTregs deficient in CTLA-4 appeared to be normal *in vitro* and *in vivo* (145). However, antibody-mediated blockage of CTLA-4 abrogated nTreg function (145), but it remains unknown whether this effect 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 revreveled 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* (12,95,96). 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 (12,40). IL-10 is another immune suppressive cytokine preferentially expressed in Tregs (146,147), and IL-10 is important in mediating the functions of these cells (147,148). Besides suppressing effector T-cell function directly, a recent study suggests that nTregs dampen immune responses through regulating an innate component, such as DC. nTregs can potentially induce tolerogenic DCs through CTLA-4 engagement-induced tryptophan catabolism (149). In addition, it appears that nTregs destabilize the interaction between antigenic DCs with conventional T cells to prevent the activation of T cells (150). It is clear now that multiple mechanisms are involved in Treg-mediated immune suppression. Cell contact-dependent and -independent mechanisms critical for controlling Treg function remain to be identified.

Treg function is controlled by Foxp3

As a single molecule that controls the development and function of Tregs, 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 (103,104). 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 (104). In addition, Foxp3 expression can be induced *in vivo* in conventional T cells under sub-optimal stimulation (151). 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. Moreover, emerging evidence associates decreased Foxp3 expression in Treg cells with various human autoimmune disorders, such as graft-versus-host disease (152), autoimmune myasthenia gravis (153), and multiple sclerosis (154). 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, we hypothesized that Foxp3 controls 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.

Support for our hypothesis came from the generation and analysis of a knockin mouse model in our laboratory. In these mice, a gene cassette co-expressing luciferase and EGFP, whose translation is under the control of two tandem internal ribosomal entry sites (IRES), was inserted into the 3′-untranslated region (UTR) of the endogenous *Foxp3* locus of C57BL/6 mice to generate Foxp3-IRES-Luciferase-IRES-eGFP (*FILIG*) allele. Our initial intention was to co-mark Foxp3-expressing cells with GFP and luciferase, facilitating the isolation and *in vivo* tracking of these cells. Nevertheless, the resulting animal expressed decreased levels of Foxp3. The exact mechanism on how the attenuated Foxp3 expression is achieved is not entirely clear. It is likely that the four AU-rich elements (AREs) located in the luciferase cDNA that was inserted into 3′ UTR region of endogenous *Foxp3* gene destabilized Foxp3 mRNA resulting in the lower expression, because AREs localized in the 3′ UTR are known to destabilize mRNA. Such serendipitously generated mice allowed us to investigate the effects of decreased Foxp3 expression on the physiology of Foxp3-expressing T cells.

While heterozygous *FILIG* female mice (*FILIG/+*) were fertile and phenotypically normal, hemizygous *FILIG* male mice (*FILIG/Y*) were infertile and runted compared to age-matched wildtype mice. Over 50% of *FILIG/Y* mice developed scaly skin, and nearly all of them developed eyelid defects resembling blepharitis, a Th2 disorder, around four weeks of age. By three months of age, all the *FILIG/Y* mice succumbed to an aggressive lymphoproliferative autoimmune syndrome, manifested by extremely enlarged spleens and peripheral lymph nodes, infiltration of leukocytes into non-lymphoid organs, drastically increased levels of auto-antibodies in the serum, and activated peripheral $CD4^+$ and $CD8^+$ T cells. Overall, *FILIG/Y* mice displayed phenotypes reminiscent of *Scurfy* mice (155) and T-cell-specific *Foxp3* knockout mice (135). The transcription of the IRES-Luciferase-IRES-eGFP gene cassette was normal, as luciferase expression was detected in the lymphoid as well as the non-lymphoid organs of sick *FILIG/Y* mice, and GFP expression was only detected in $CD4^+$ T cells but not other cell types. By intracellular Foxp3 staining, Foxp3 was found to be expressed in GFP^+ *FILIG* T cells but at a 5–10 fold lower level. Compared with wildtype Treg cells, the surface expression of ‘signature genes’ for Treg cells, such as CD25, CTLA-4, and GITR (38,156,157), were decreased in GFP^+ $CD4^+$ T cells. mRNA levels of *Foxp3*, *Cd25*, *Ctla4*, and *Gitr* were also decreased, suggesting that the downregulation of these genes was at the mRNA level. These results demonstrated that decreased Foxp3 expression altered the properties of Treg cells and resulted in an aggressive lymphoproliferative autoimmune syndrome in hemizygous male mice. Thus, Foxp3 programs the gene expression of Tregs in a tunable and dose-dependent fashion (158).

Further investigation revealed interesting phenotypic changes in the functions of Treg cells that express decreased Foxp3. Foxp3 is required for the development and maintenance of Treg cells (135). However, decreased Foxp3 expression in *FILIG* T cells did not lead to defective thymic development of Treg cells. This result agrees with those from a separate study, where GFP cDNA was inserted into the endogenous *Foxp3* coding region to mark those T cells with Foxp3 promoter activity but that failed to produce functional Foxp3 protein (159). Substantial numbers of GFP-expressing thymocytes were also detected in these mice (159). 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. However, in the presence of Treg cells with normal levels of Foxp3 expression, Foxp3 low-expressing cells poorly repopulated the periphery. Decreased CD25

expression on GFP$^+$ *FILIG* CD4$^+$ T cells compared to wildtype Treg cells could account for this phenomenon, since Treg maintenance is dependent on IL-2 signaling (137–139). Extra-thymic generation of Foxp3-expressing T cells can be promoted *in vitro* by TGF-β (102,104,121). TGF-β induced *de novo* Foxp3 expression in GFP$^+$ *FILIG* CD4$^+$ T cells to a similar extent as in wildtype Treg cells. Therefore, these results demonstrated that decreased Foxp3 expression did not affect the development, homeostatic expansion/maintenance, or TGF-β-driven *de novo* generation of Foxp3-expressing T cells (158). 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 (159). Thus, in *Scurfy* mice and in 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 immunosuppressive activities are two defining properties for Foxp3-expressing Treg cells that are thought to go hand-in-hand (39). Intriguingly, upon TCR stimulation *in vitro*, while GFP$^+$ *FILIG* cells remained anergic, their immunosuppressive activities were greatly impaired. Thus, anergy and immunosuppression are two separable properties of Treg cells that are affected differentially by the expression levels of Foxp3 (158). In addition, using a transfer approach, we demonstrated that the intrinsic immunosuppressive activities of Foxp3$^+$ *FILIG* CD4+ T cells were also abolished *in vivo*, although adoptively transferred GFP$^+$ *FILIG* cells infiltrated efficiently into lymphoid as well as non-lymphoid organs. Thus, the suppressive function of Tregs requires high levels of Foxp3 (158). Interestingly, Foxp3-deficient Treg lineage cells remain anergic but without immunosuppressive function (159). Therefore, unlike what has been recognized, Foxp3 may not be the 'grand-master' of Treg development, as its expression is only required for stabilizing 'regulatory function' of already differentiated Treg lineage cells. 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 Tregs, it remains unsolved whether any of them may be sufficient to control 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. Genome-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 smRNA/siRNA have been identified (160). Future efforts are needed to sift through these candidates to pinpoint the critical Foxp3 target genes for controlling the immunosuppressive function of Treg cells.

Positive roles for Tregs in immune responses

Tregs contribute to inflammation by inducing Th17 cells indirectly through TGF-β. TGF-β appears to be critical for the induction of Th17 cells, according to our and others' studies (120). High levels of TGF-β, probably existing as a membrane-bound form, are found on Treg cells. Therefore, Treg cells have been identified as an important inducer for Th17 cells *in vitro*. Using TGF-β1 knockout mice, future studies need to be performed to address whether Tregs are the critical source of TGF-β in Th17 cell induction and thus the onset of EAE.

Foxp3-expressing cells can convert into effector cells. Analysis of Foxp3$^+$ *FILIG* cells revealed that these cells developed effector cell functions that likely contribute to these immune disorders. GFP$^+$ (Foxp3$^+$) cells from healthy *FILIG*/+ mice did not exhibit an effector cell phenotype, suggesting that they did not spontaneously activate and convert into

effector cells when substantial numbers of wildtype Treg cells are present (158). Surprisingly, however, a large portion of GFP^+ cells from diseased *FILIG/Y* mice produced IL-4, while the percentages of the cells expressing IL-2, IFN-γ, or IL-17 were only modestly increased compared to wildtype cells. The percentage of IL-4-producing cells also increased in $Foxp3^-CD4^+$ T cells from *FILIG/Y* mice, consistent with the Th2 disorder observed in these mice. Similarly, upon transfer, GFP^+ *FILIG* cells preferentially converted into Th2-type effectors, despite the fact that the coexisting $Foxp3^-$ cells produced large quantities of IFN-γ and therefore provided Th1-polarizing conditions (158). Thus, Foxp3-expressing cells with decreased Foxp3 expression preferentially converted into Th2-type effector cells *in vivo*, even in the presence of IFN-γ-producing Th1 cells. Upon conversion into Th2-type effectors, GFP^+ *FILIG* cells potently promoted the Th2 differentiation of conventional T cells *in vivo* and *in vitro* regardless of the Th1-skewing conditions imposed by the cytokine milieu (158). In another study, Treg lineage cells without functional Foxp3 were able to express Th1, Th2, as well as Th17 effector cytokines (159). These findings present an interesting possibility that Foxp3-expressing cells might not always suppress but rather in some cases might 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 (161). It is reasonable to believe that in a highly inflammatory micro-environment, resident Tregs 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, as well as suppressive activity, would be restored.

Conclusions

The importance of active immune suppression is widely acknowledged. Studies on TGF-β and Tregs have shed light on how immune suppression applications. 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 Tregs control immune responses. In addition, we are starting to realize that under certain conditions, TGF-β and Tregs can both serve as promoting factors to direct immune responses. To facilitate designing immunotherapies against inflammatory diseases and cancers, more studies are needed to reveal alternative functions of TGF-β and Tregs. Moreover, as TGF-β and possibly Foxp3 function in the non-lymphoid system, further studies on the roles for TGF-β and Foxp3 in the non-lymphoid systems and on the interaction between lymphoid and non-lymphoid systems are certainly needed to achieve a global comprehensive view of our immune system.

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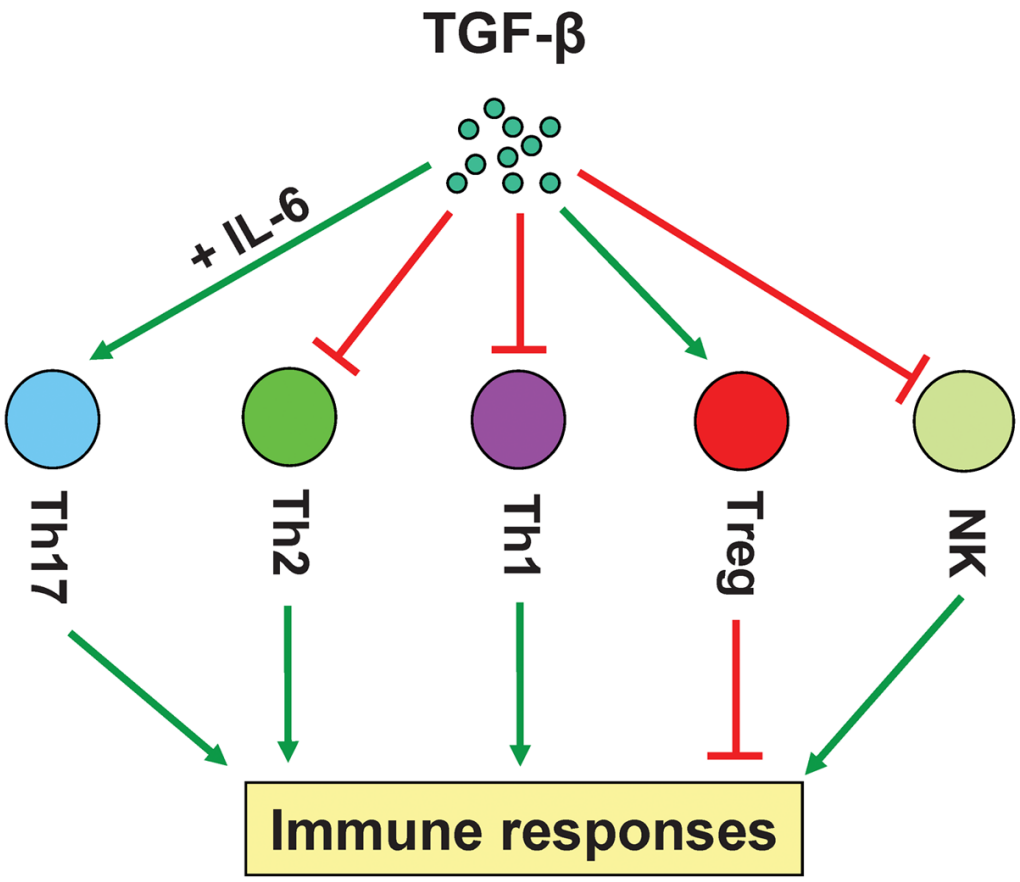

Fig. 1. TGF-β inhibits and promotes immune responses by modulating the functions of immune cells

To inhibit immune responses**,** TGF-β suppresses the functions of Th1 and Th2 $CD4^+$ effector cells and NK cells, and promotes the generation of Treg cells. To promote immune responses, TGF-β induces the generation of Th17 cells in combination with IL-6.

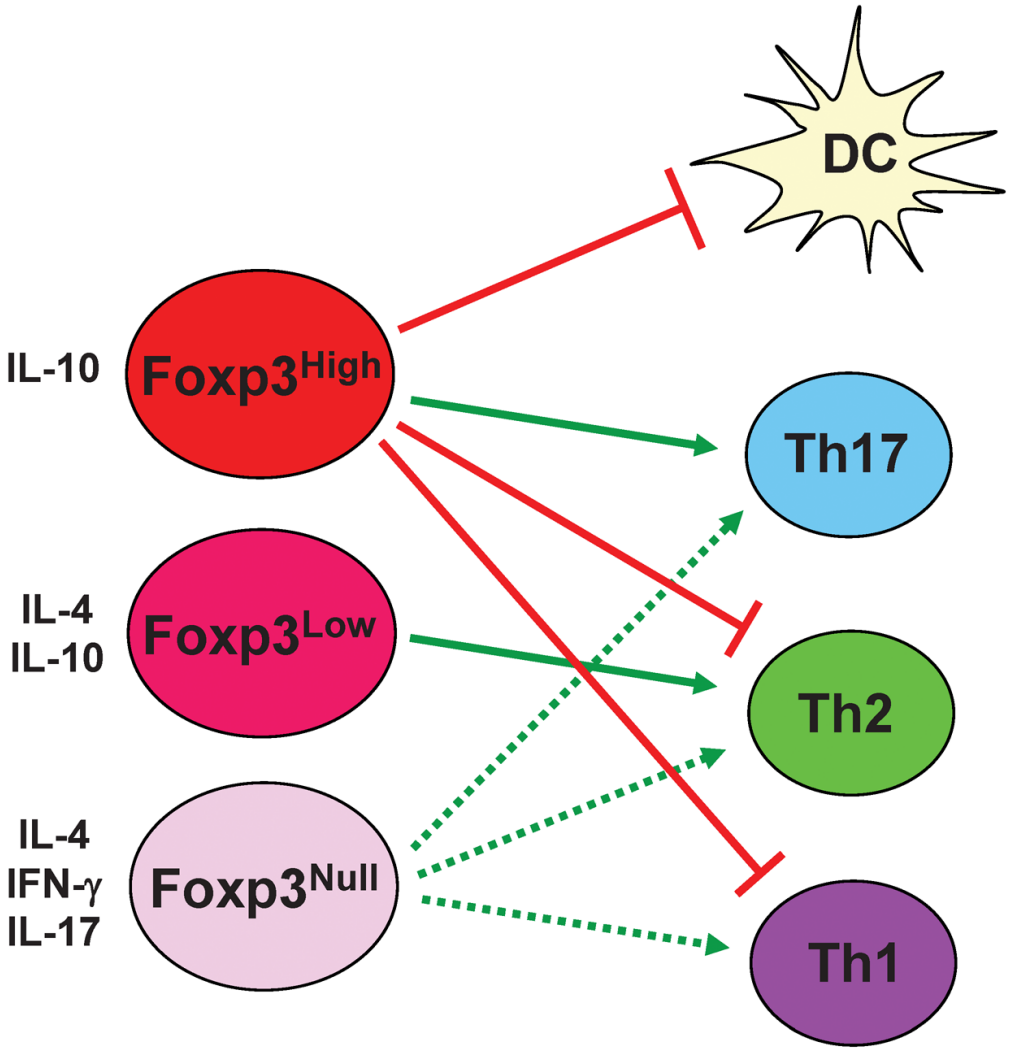

Fig. 2. Yin-Yang roles for Foxp3-expressing cells in immune regulation

High levels of Foxp3 expression endow T-cell immune suppressive activities to suppress the function of effector T cells and DCs through cell contact-dependent and -independent mechanisms. T cells with high levels of Foxp3 expression promote Th17 cell differentiation, potentially through TGF-β. With decreasing Foxp3 expression, T cells can be converted into different types of effector T cells and direct the differentiation of conventional T cells towards different lineages.