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TGF- β Regulation of T Cells

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Abstract

Transforming growth factor β (TGF- β) is a key cytokine regulating the development, activation, proliferation, differentiation, and death of T cells. In CD4⁺ T cells, TGF- β maintains the quiescence and controls the activation of naive T cells. While inhibiting the differentiation and function of Th1 and Th2 cells, TGF- β promotes the differentiation of Th17 and Th9 cells. TGF- β is required for the induction of Foxp3 in naive T cells and the development of regulatory T cells. TGF- β is crucial in the differentiation of tissue-resident memory CD8⁺ T cells and their retention in the tissue, whereas it suppresses effector T cell function. In addition, TGF- β also regulates the generation or function of natural killer T cells, $\gamma\delta$ T cells, innate lymphoid cells, and gut intraepithelial lymphocytes. Here I highlight the major findings and recent advances in our understanding of TGF- β regulation of T cells and provide a personal perspective of the field.

Keywords

CD4⁺ T helper cells; CD8⁺ T cells; regulatory T cells; innate lymphoid cells; intraepithelial lymphocytes; $\gamma\delta$ T cells; T cell quiescence and activation

If something in the cell is “off,” TGF- β may turn it “on,” while if something in the cell is “on,” TGF- β may turn it “off.”

—M.B. Sporn (1, p. 6)

1. INTRODUCTION

The delicate balance between effective immunity and proper tolerance is the prerequisite for an unerring immune system and for the well-being of the human body. Among the numerous immune cells, T cells are a key population in adaptive immunity. Developed in the thymus, T cell receptor (TCR) $\alpha\beta$ ⁺CD4⁺ and TCR $\alpha\beta$ ⁺CD8⁺ T cells and TCR $\gamma\delta$ ⁺ T cells migrate into the blood and peripheral lymphoid tissues and maintain their quiescence as naive T cells under intrinsic and extrinsic regulatory forces until they encounter their

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specific antigens processed and presented by antigen-presenting cells (APCs). Upon TCR stimulation, naive T cells can be activated with the assistance of positive costimulatory molecules such as CD28 and simultaneously by suppression and/or removal of negative factors. T cells can then produce IL-2, proliferate, and differentiate into effector cells, memory cells, or regulatory cells depending on the cytokines and molecules present in their microenvironments. Based on their function, cytokine production, and expression of lineage transcription factors, CD4⁺ T cells can be classified as T helper 1 (Th1), Th2, Th17, Th9, and T follicular helper (Tfh) effector cells; forkhead box P3 (Foxp3)⁺ regulatory T cells (Tregs); and T follicular regulatory T (Tfr) cells (2–9). On the other hand, CD8⁺ T cells can be divided into effector (Teff), exhausted (Tex), central memory (Tcm), effector memory (Tem), and resident memory (Trm) subpopulations, as well as CD8⁺ Tregs (10, 11). In addition, several nonconventional lymphocytes have been discovered and found to play important roles in immune responses. These include but are not limited to innate lymphoid cells (ILCs) (12), intraepithelial lymphocytes (IELs) in the gut (13), and natural killer T (NKT) cells (14). The development, activation, proliferation, differentiation, and death of T cells require extremely orchestrated signals, molecules, and cytokines no matter whether TCR engagement is needed or not. TGF- β is one of the most important cytokines in the regulation of T cells.

TGF- β was discovered in mouse tissues by Roberts, Sporn, and colleagues in 1981 (15). It is now known that the TGF- β superfamily is composed of at least 33 members including TGF- β , bone morphogenetic proteins (BMPs), activins, inhibins, and glial cell line–derived neurotrophic factors (GDNFs) (16). TGF- β has three mammalian isoforms: TGF- β 1, TGF- β 2, and TGF- β 3 (TGF- β 1, 2, 3). Although TGF- β 1, 2, 3 utilize the same receptors and reactive Smad2 and Smad3 (RSmad) signaling in vitro, the different isoforms may function differently in vivo. For example, TGF- β 2 is essential in controlling the development of multiple vital organs in the body, as null mutation of the *Tgfb2* gene results in a lethal embryo (17). TGF- β 3 also controls the development of some organs such as lungs and accelerates wound healing without an increase in scar formation (18, 19). TGF- β 1, however, is the dominant isoform in blood and immune cells and plays the most important roles in regulating immune responses, as null mutation of the *Tgfb1* gene (*Tgfb1*^{−/−}) causes systemic inflammation and untimely death in mice (20, 21). Importantly, specific deletion of TGF- β receptor I (TGF β RI) or TGF β RII or Smad2/3 in T cells leads to systemic T cell activation, uncontrolled inflammation, and early demise of all mice, similar to what is seen in *Tgfb1*^{−/−} mice (22–25). I use TGF- β to represent TGF- β 1 in this review unless otherwise specified.

TGF- β is produced by and responds to almost all immune cells and nonimmune cells and regulates both innate and adaptive immune responses. There have been numerous excellent reviews of TGF- β biology, including its functions in cell biology, cancer biology, and immune responses (16, 26–31). In this review, I focus on TGF- β regulation of T cells (Figure 1). I briefly summarize the general TGF- β signaling pathway and then discuss the major findings and recent advances in research on the cellular and molecular mechanisms of TGF- β regulation of T cells in physiological and pathological conditions including autoimmunity, cancer, and infectious diseases.

2. TGF- β SIGNALING

TGF- β is produced as a biologically inactive precursor protein and requires proteolytic processing. The latent form of TGF- β [LAP-TGF- β (LTGF- β)] contains a large N-terminal portion known as the latency-associated polypeptide (LAP) and a noncovalently associated C-terminal peptide, the active TGF- β . In some cases, LTGF- β can be chemically linked by disulfide bonds with two cysteine residues to form so-called latent TGF- β binding proteins (LTBPs) that can interact with and bind to proteins in the extracellular matrix (31). LTGF- β can be activated by removing the LAP portion from the precursors to release the bioactive TGF- β . Numerous proteinases and proteins in the matrix, such as matrix metalloproteinases (MMPs), and cell membrane-bound integrins, such as $\alpha_v\beta_8$ in Tregs and dendritic cells (DCs) and $\alpha_v\beta_6$ in epithelial cells, have been implicated in the process of TGF- β activation (31–33). T cell–conditional deletion of furin, which is involved in TGF- β activation, causes activation and proliferation of Teff cells, which produce less bioactive TGF- β 1 (34). It is conceivable that different factors and processes are required for TGF- β activation in different tissues and different immunological conditions, but the amounts of available bioactive TGF- β may determine the outcome for even the same T cells encountering the same antigen stimulation.

Once activated, TGF- β signals by binding to specific sets of heteromeric type I and type II receptor complexes (16, 26, 29). TGF- β first binds to T β RII and then recruits and activates T β RI (also called ALK5). In the canonical pathway, activated T β RI recruits and phosphorylates the receptor RSmad. Smad2/3 can be phosphorylated at the C terminus or in the linker region in response to TGF- β in the presence or absence of other signals. Phosphorylated Smad2/3 (pSmad2/3) normally forms a complex with the common Smad (Smad4) to be translocated into the nucleus to regulate their target genes. In general, the Smad2/3/4 complex is weak or even unable to regulate target gene transcription without interacting and/or collaborating with other cotranscription factors. Smad3 and Smad2 can compensate for each other in mediating TGF- β signaling in immune cells, as deletion of both Smad2 and Smad3, but not either one alone, in T cells results in complete block of TGF- β signaling (25). In addition to the canonical RSmad-mediated pathway, TGF- β can also signal through a TGF- β receptor–mediated but RSmad-independent noncanonical pathways (26). For example, the TNF receptor–associated factor 6 (TRAF6)-TGF- β -activated kinase 1 (TAK1) axis can serve as a critical route downstream of TGF- β receptors to mediate TGF- β signaling (35, 36) (Figure 1). Of special note, when considering TGF- β signaling in immune cells, especially in T cells, one must keep in mind that the presence of other stimuli and signals may determine and/or change the ultimate fate of the T cells.

3. TGF- β IN THYMOCYTES

Although compelling evidence indicates that TGF- β controls the development of thymic Foxp3⁺ Tregs (23, 37–40), invariant NKT (iNKT) cells (24), and precursors of TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ IELs (41) in the thymus, the function of TGF- β regulation of normal thymocyte development remains largely unclear (see Supplementary Text 1), with the exception of CD8 single-positive (SP) thymocytes. It has been reported that TGF- β is

required for the development of CD8 SP thymocytes. An elegant study by Singer's group has clearly demonstrated that TGF- β is indispensable in CD8 SP lineage decision (42). The authors revealed that four cytokines, namely IL-6, IFN- γ , TSLP (thymic stromal lymphopoietin), and TGF- β , together induce expression of the lineage-specifying transcription factor Runx3d (runt-related transcription factor 3d) and signal the generation of CD8⁺ T cells in the thymus. They conclude that it is a combination of signals (a) by IL-7 and IL-15 through common γ chain cytokine receptor; (b) by IL-6, IFN- γ , and TSLP through Jak-Stat; and (c) by TGF- β through Smad3 that completes the generation of CD8⁺ SP thymocytes (42). Consistent with this finding, it is reported that TGF- β upregulates IL-7 receptor expression in CD8⁺ SP thymocytes (43) (Supplementary Figure 1).

4. TGF- β IN CD4⁺ T CELLS

TGF- β regulates the quiescence, activation, proliferation, differentiation, and death of CD4⁺ T cells. The delicate balance of TGF- β regulation in all the aforementioned processes safeguards the normal function of CD4⁺ T cells.

4.1. TGF- β in CD4⁺ T Cell Quiescence and Activation

A fundamental question in immunology is how quiescence is enforced in naive T cells while activation by foreign antigens and self-antigens is allowed. After leaving the thymus, naive CD4⁺ T cells are present in the periphery in quiescence and survive in the steady state, which requires TCR tickling by self-MHC molecules (44). However, TCR tickling by self-antigens does not lead to autoimmunity in healthy individuals, as T cell quiescence is actively reinforced by extrinsic factors such as Tregs and potentially by intrinsic mechanisms. Naive CD4⁺ T cell activation requires two signals, the first being the signal of TCR engagement and the second being provided by costimulatory molecule CD28 (45). It is conceivable that in addition to these positive signals, the putative negative signal(s) from quiescence programs must be removed to achieve T cell activation. Indeed, Tu et al. (46) revealed that active TGF- β signaling occurs in both murine and human naive CD4⁺ T cells and strong, not weak, TCR engagement reduces TGF- β signaling by downregulating T β RI and consequent pSmad2/3 in CD4⁺ T cells. This T β RI downregulation occurs as early as 6–12 h after TCR stimulation and occurs through activation of CARD11 (caspase recruitment domain-containing protein 11) and NF- κ B. In examining which cytokines influence TCR-mediated T β RI downregulation, we found that of the panel of immune cytokines tested, only TGF- β prevents TCR-mediated T β RI downregulation. Interestingly, IL-6 is able to abolish TGF- β -mediated upregulation of T β RI, although IL-6 itself has no effects on TCR-mediated T β RI downregulation in CD4⁺ T cells (46). Functional analysis reveals that indeed, downregulation of T β RI and pSmad2/3 increases the sensitivity of T cell proliferation and production of cytokines such as IFN- γ in response to TCR stimulation, especially weaker TCR stimulation. Conversely, overexpression of T β RI in naive and activated T cells rendered T cells less responsive to TCR stimulation and suppressed autoimmunity (46). Significantly, naive CD4⁺ T cells isolated in newly diagnosed and untreated patients with systemic lupus erythematosus manifested reduced T β RI expression and increased TCR-driven proliferation compared to cells from healthy subjects (46). However, more studies are needed to validate these findings. Although these findings establish a key role

for TGF- β signaling in maintaining the quiescence and controlling the activation of naive CD4⁺ T cells, the molecular pathways downstream of TGF- β -Smad2/3 signaling remain unknown. Nonetheless, these findings indicate that TCR-mediated regulation of TGF β -T β RI signaling is a crucial criterion in determining T cell quiescence and activation (Figure 1), which should have important implications for our understanding of immune tolerance and immune responses, as well as the development and pathogenesis of autoimmunity, cancer, and infectious diseases (Supplementary Text 2).

4.2. TGF- β in CD4⁺ T Cell Differentiation and Function

The function of TGF- β in regulating CD4⁺ T cell differentiation is well recognized, although knowledge of the underlying mechanisms is incomplete. It is believed that TGF- β inhibits Th1 and Th2 differentiation but promotes the generation of Th17 cells, Th9 cells, and Tregs. It is one of the most potent immunoregulatory cytokines in Th1 cell differentiation and function (Supplementary Text 3), as discussed extensively by several excellent reviews (27, 28, 30, 47, 48). TGF- β suppresses the differentiation and function of Th2 cells in vitro and in vivo (2, 48; Supplementary Text 4). The regulation of TGF- β in the differentiation and function of murine Tfh and Tfr cells is incompletely understood, but it seems to play a role in human Tfh cell differentiation, at least in culture (Supplementary Text 5). Here, I discuss the major findings and the recent advances in the field, with a focus on Th17 cells, Th9 cells, and Tregs (Figure 2).

4.2.1. TGF- β in Th17 cells. The identification and characterization of Th17 cells is a significant advance in understanding CD4⁺ T cell differentiation and function beyond the classic Th1 and Th2 paradigm (49, 50). Th17 cells produce a panel of specific cytokines, including IL-17A, IL-17F, IL-22, and IL-21, and require primarily lineage-specific transcription factor ROR γ t and ROR α for differentiation (5, 6, 51). Although IL-23 was initially suggested to be important in the proliferation and function of Th17 cells (52), TGF- β plus IL-6 was later identified as a crucial factor for the initial differentiation of Th17 cells from naive CD4⁺ T cells (53–55). Based on the induction of Foxp3 by TGF- β in naive T cells (56), the addition of IL-6 suppresses the expression of Foxp3 and instead promotes the induction of IL-17 (53). Alternatively, coculture of Tregs with naive CD4⁺ T cells in the presence of IL-6 promotes IL-17 production, and the function of Tregs can be replaced by exogenous TGF- β (55). TGF- β is the primary factor inducing ROR γ t expression in CD4⁺ T cells, and this is optimized by the presence of IL-6 activating STAT3 (57, 58). Interestingly, STAT5, which is activated by IL-2, directly binds the same elements in the *Il17* gene as STAT3, displaces STAT3, and thus inhibits STAT3-mediated Th17 differentiation (59). Downstream of TGF- β signaling, activation of Smad2/3 is required for IL-17 expression (6, 51). One recent study shows that Smad4, in contrast to Smad2/3, inhibits TGF- β -induced Th17 cell differentiation by directly interacting with SKI, a transcriptional repressor that is degraded upon TGF- β stimulation (60). SKI controls histone acetylation and deacetylation of the *Rorc* locus and Th17 cell differentiation via Smad4: Ectopic SKI expression inhibits H3K9 acetylation of the *Rorc* locus, *Rorc* expression, and Th17 cell differentiation in a Smad4-dependent manner. In addition, several transcriptional factors have been identified as involved in Th17 cell differentiation. For example, basic helix-loop-helix protein E2A binds to and activates the *Rorc* gene and consequently Th17 differentiation in response to TGF- β

and IL-6 (61, 62). Trim33 (tripartite motif-containing 33), a modulator of TGF- β signaling that depends on Smad2, promotes the differentiation of proinflammatory Th17 cells and inhibits IL-10 (63).

Where TGF- β is produced and how it is activated for Th17 differentiation in vivo are exciting, unresolved questions. As TGF- β can be produced by almost all types of immune and nonimmune cells (16), it is likely produced in different tissues by different types of cells. For example, macrophages and immature DCs might produce and/or activate TGF- β upon phagocytosing apoptotic cells (39, 64–66). Indeed, this happens in facilitating regulatory Th17 cell differentiation in mice treated with anti-CD3 antibody, which depletes T cells during the viral infection (67). High doses of glucose can promote Th17 differentiation by activating LTGF- β through reactive oxygen species (ROS) production by T cells, thereby exacerbating the pathogenesis of Th17-mediated inflammation in mice (68). Tregs can also be a cellular source for TGF- β together with IL-6 for Th17 differentiation (55). T cell-derived TGF- β may also be a cellular source for autocrine Th17 differentiation (69).

Despite the required role of TGF- β and IL-6 in driving Th17 cell differentiation, it is evident that these Th17 cells are normally not pathogenic, which is partially due to their production of IL-10 (70). Inclusion of proinflammatory cytokines IL-23 and IL-1 β enhances pathogenic function by suppressing IL-10 (71, 72). Thus, Th17 cells are proposed to comprise two functionally different subsets: nonpathogenic Th17 cells induced by TGF- β and IL-6 and pathogenic Th17 cells induced by IL-1 β , IL-23, and IL-6. This notion is supported by some evidence that pathogenic Th17 cells do not require TGF- β 1 signaling (72), as CD4⁺ T cells with blockade of TGF- β signaling are still able to differentiate into pathogenic Th17 cells in response to IL-1 β , IL-23, and IL-6. However, it is suggested that TGF- β 3 may substitute for TGF- β 1 to induce pathogenic Th17 differentiation (73). In human Th17 cells, the role of TGF- β in Th17 cell differentiation has also been debated. Some studies have shown that TGF- β is dispensable and even suppressive in human Th17 cell differentiation (74–76). However, another report argued that naive/resting CD4⁺ T cells from human blood may not be truly naive and showed that TGF- β , IL-1 β and IL-6, IL-21, or IL-23 in serum-free conditions were necessary and sufficient to induce Th17 cells from naive human CD4⁺ T cells isolated from cord blood (77).

4.2.2. TGF- β in Th9 cells. Th9 cells produce IL-9 and play important roles in the pathogenesis of allergy and asthma and in antitumor immunity (7, 78). Although it was known about two decades ago that TGF- β regulated IL-9 production in CD4⁺ T cells (79), it was only in 2008 determined to be a critical factor in the differentiation of Th9 cells (80, 81). Two groups independently identified that TGF- β and IL-4 are required for *Il9* gene activation and Th9 cell differentiation. This can be accomplished either by coexposure of naive CD4⁺ T cells to TGF- β and IL-4 (80) or by TGF- β reprogramming Th2 cells to lose their characteristic profiles and switch to IL-9 secretion (81). Th9 cells do not produce IFN- γ , IL-4, or IL-17, but they may express IL-10. In addition to IL-4, IL-1 α , IL-1 β , IL-18, IL-33, IL-21, IL-6, and IL-10 together with TGF- β also enhance IL-9 production in CD4⁺ T cells in IL-4-dependent and -independent manners (82–84). IFN- γ and IL-27, however, are reported to inhibit Th9 differentiation (79, 84, 85). Interestingly, in polarizing conditions

based on TGF- β , stimulation of OX40 promotes differentiation of naive CD4⁺ T cells toward a Th9 phenotype (86, 87), and this costimulatory effect is mediated by the TRAF6 and NF- κ B pathways (87).

Human Th9 cells can also be differentiated from naive CD4⁺ T cells by TGF- β and IL-4 (82, 84, 86), although IL-6 and IL-1 β are also costimulatory for Th9 cells in the presence of TGF- β (82). Human CD4⁺ memory T cells require only TGF- β to produce IL-9, as neither IL-4 nor other proinflammatory cytokines are needed (82).

Several transcription factors have been reported to participate in the downstream signaling of TGF- β and IL-4 to activate *Il9* gene expression, including PU.1, IRF4, E2A, Stat5, Stat6, GATA3, BATF, Smad2/3, and NF- κ B (88). However, none of these transcription factors has been recognized as the lineage-specific transcription factor for Th9 cells, because they are also involved in the differentiation of other Th cells. It is reported that PU.1, IRF4, BATF, and GATA3 are all regulated by TGF- β signaling (88). It is possible that multiple transcription factors, rather than a specific single one, are required for Th9 differentiation, or that the specific transcription factor has not yet been identified. Immediately downstream of TGF- β signaling, Smad2, Smad3, and Smad4 are reported to be involved in Th9 cell differentiation (86, 89–91). It is suggested that Smad2/4 may promote *Il9* gene expression through EZH2 displacement (91), and that Smad2/3 and IRF4 cooperate in Th9 induction (90), but how these Smads function as direct or indirect mediators in *Il9* gene transcription is still largely unknown. In addition, TGF- β may also signal to activate the *Il9* gene through a Smad3-independent pathway. For example, it is reported that TGF- β and IL-4 activate TAK1, a critical component of the non-Smad-dependent pathways (35, 36), which downregulates Id3 expression and consequently promotes E2A and GATA3 binding to the *Il9* gene to activate its transcription (86).

It should be pointed out that although it is well established that Th9 cells can be differentiated in cell culture, Th9 cells are hardly detectable in the tissues in situ (92). This raises the question as to whether Th9 cells are only transiently present in vivo and transduce signaling to other immune cells, or whether they switch into a different population of T cells. This question is also related to the functional mechanisms by which Th9 cells carry out their antitumor activities and immunopathogenic effects with respect to allergy and asthma.

4.2.3. TGF- β in Tregs. CD4⁺CD25⁺Foxp3⁺ Tregs are essential to induction and maintenance of immune homeostasis and tolerance (8). Tregs regulate the immune responses in autoimmunity, inflammation, transplant rejection, allergy/asthma, infections, and cancer. They can develop in the thymus as natural, or thymic, Tregs (tTregs) and can also be generated in the periphery by conversion from CD4⁺Foxp3[−] naive T cells, in which case they are called induced Tregs (iTregs) or peripheral Tregs (pTregs). Foxp3 is the lineage-specific transcription factor for Tregs and controls their development (93–95). TGF- β plays a key role in the induction of Foxp3 and the development of and function of both iTregs and tTregs (32, 37, 56, 96), and this role is the focus of discussion in this section (Figure 2).

4.2.3.1. TGF- β in induced/peripheral Tregs. The identification and characterization of CD4⁺CD25⁺ Tregs by Sakaguchi et al. (97) has revolutionized our understanding of immunoregulation. Because Tregs freshly isolated from spleens and lymph nodes in normal mice not only are anergic to TCR stimulation but also exhibit potent suppressive activity against CD4⁺CD25⁻ responder T cells in an in vitro suppression assay (98), Tregs were once called CD4⁺CD25⁺CTLA-4⁺ anergic/suppressor T cells. It was once thought that Tregs only developed in the thymus, and not converted from naive CD4⁺ T cells in the periphery. Based on our longstanding interest in TGF- β regulation of T cells, in 2001 we discovered that TGF- β induces CD4⁺CD25⁺CTLA-4⁺ anergic/suppressor T cells from murine peripheral naive CD4⁺CD25⁻ T cells in the context of TCR stimulation in vitro (99). However, another group reported that in vitro stimulation of human blood CD4⁺ T cells with irradiated allogeneic peripheral blood mononuclear cells in the presence of TGF- β led to expansion of existing CD25⁺ cells rather than converted CD25⁺ cells from CD4⁺CD25⁻ T cells (100). Immediately after the discovery that Foxp3 is the key and lineage-specific transcription factor for the development and function of CD4⁺CD25⁺ Tregs (93–95), we discovered that TGF- β , in the context of TCR stimulation, induces *foxp3* gene expression from peripheral naive CD4⁺CD25⁻ T cells and converts them into CD4⁺CD25⁺Foxp3⁺ Tregs (56), a finding subsequently reported by numerous independent groups (101–105). Tregs were soon reported to have been converted from naive CD4⁺CD25⁻ T cells in vivo by using a TCR transgenic CD4⁺ T cell adoptive transfer system, or in non-transgenic settings in a TGF- β -dependent manner (65, 96, 106–109). For example, weak TCR stimulation with minute antigen doses and suboptimal DC activation favors Treg generation in a TGF- β -dependent manner (96), but this only occurs in naive mice. In gut-associated lymphoid tissues (GALTs), which are enriched with TGF- β , retinoic acid that can be produced by CD103⁺ DCs enhances TGF- β -induced Treg conversion (107, 109, 110). The successful conversion of Tregs from naive CD4⁺ T cells in vitro and in vivo by TGF- β has not only proven that *foxp3* can be induced from naive CD4⁺ T cells; it has also opened up a way to induce antigen-specific Tregs for potential immunotherapy for autoimmune diseases, allergy/asthma, and transplantation.

Indeed, efforts have been made to develop immunotherapy to experimental autoimmunity and allergy by manipulating TGF- β and Tregs. Adoptive transfer of in vitro induced antigen-specific Tregs by TGF- β could potentially be used to treat autoimmunity. However, it was reported that iTregs are less stable based on their DNA-demethylation status (111) and may be susceptible to losing their Foxp3 expression in vivo. Thus, several approaches have been developed to enhance the stability of iTregs. For example, vitamin C potentiates ten-eleven translocation (TET) activity and acts through Tet2/Tet3 to increase the stability of Foxp3 expression in TGF- β -induced Tregs (112). Whole-genome analyses show that the addition of vitamin C during TGF- β -induced iTreg differentiation in vitro potentiates the expression of Treg signature genes and alters the epigenetic landscape to better resemble that of Tregs generated in vivo (113).

On the other hand, progress has been made in inducing antigen-specific Tregs in vivo by manipulating TGF- β , especially in mice with established autoimmunity. Despite the success of iTreg conversion in naive mice (96), inducing antigen-specific Tregs in mice with

established autoimmunity was unsuccessful until only recently. Based on the findings that phagocytes produce and secrete TGF- β upon engulfment and digestion of apoptotic cells in vitro and in vivo (64, 66, 114), and that TGF- β is essential for the conversion of naive T cells into Tregs in the presence of TCR stimulation (56), we have successfully developed an experimental approach to induce autoantigen-specific Tregs in mice with autoimmune diseases. This protocol includes three functional steps, namely induction of a sufficient number of apoptotic immune cells such as T and B cells; contact and digestion of apoptotic cells by phagocytes to produce TGF- β ; and subsequent administration of low doses of autoantigenic peptides (TCR stimulation) in mice with disease. These in vivo induced autoantigen-specific Tregs potently suppress autoimmunity and inflammation in a variety of experimental autoimmune disease models, including models of experimental autoimmune encephalitis, experimental autoimmune uveitis, type 1 diabetes, and Sjögren syndrome (65, 115–117). Importantly, this treatment does not compromise immunity to bacterial antigens and tumors in mice (65, 116). In line with this, oral administration of hyperphysiological doses of d-mannose, an epimer of glucose, can suppress type 1 diabetes and asthmatic lung inflammation by inducing Tregs from naive CD4⁺ T cells in mice (118). Mechanistically, D-mannose can activate LTGF- β by upregulating integrin $\alpha_v\beta_8$ and increasing ROS production in T cells.

Generation of human CD4⁺CD25⁺Foxp3⁺ iTregs also requires TGF- β and IL-2 in addition to TCR stimulation (102, 104), although their in vitro immunosuppressive activity may vary (119). It was suggested that human Foxp3⁺ T cells can be induced in CD4⁺Foxp3⁻ T cells by TCR stimulation in the absence of exogenous TGF- β , but more careful analyses revealed that these TCR-driven Foxp3⁺ T cells from CD4⁺CD25⁻Foxp3⁻ T cells still require production and/or activation of TGF- β in serum-containing culture medium or in T cells (120, 121).

4.2.3.2. TGF- β in thymic Tregs. TGF- β signaling is also required for the development of tTregs in the thymus (37). Early studies suggested that TGF- β signaling might be dispensable for the development of tTregs, as adult transgenic mice with specific deletion of TGF β RII in T cells (T β RII^{ko}) did not show a significant reduction (22, 24), and actually showed an increase, in the frequency of tTregs (22). However, subsequent studies in mice with T cell-specific deletion of TGF β RI (T β RI^{ko}) revealed that a deficiency of TGF- β signaling in the thymocytes results in a profound defect of tTregs in the thymus during the neonatal stage (days 3–5) without significant changes in other populations of thymocytes (23). This was confirmed by another independent study with T β RII^{ko} mice (40). Consistent with an early report (22), T β RI^{ko} mice also gradually accumulated tTregs in the thymus with age, and by 2–3 weeks the frequency of tTregs in the knockout mice was actually higher than that in the age-matched wild-type mice (23). This paradox was resolved by the findings that deletion of IL-2 in T β RI^{ko} mice (IL-2^{-/-}T β RI^{ko}) completely abolished the increase in tTregs observed in the single-knockout T β RI^{ko} mice and that the double-knockout mice showed a profound decrease in tTregs, from neonatal through adult ages (23). Thus, the increased frequency of tTregs in the adult T β RI^{ko} mice is due to the accelerated expansion of a few tTregs driven by increased IL-2 in the T β RI^{ko} thymus. Thus, the crucial role of TGF- β signaling in the development of tTregs is now recognized (37, 38).

An important question, then, is the underlying mechanisms by which TGF- β controls tTreg development. Based on ample evidence that TGF- β is essential for *foxp3* gene transcription in naive CD4⁺ T cells and also in CD4⁺ SP thymocytes (39, 56), it is conceivable that the defect in tTregs in TGF β R knockout mice is due to the lack of TGF- β induction of the *foxp3* gene in thymic CD4⁺ SP precursors. However, a study suggested that the defect of tTregs in the T β RII^{ko} thymus was due to increased death of tTregs specifically in the absence of TGF- β signaling in T cells (40). While it is well known that TGF- β signaling is indeed important to protect thymocytes from unwanted death (122), this protection is unlikely unique to tTregs. Indeed, when T β RI was deleted only after Foxp3⁺ was expressed in tTregs (*Tgfbri*^{fllox/flox}*Foxp3-Cre*⁺), there was no difference in either the frequency or the absolute number of tTregs at neonatal age or in adult mice (39), eliminating the specific TGF- β protection of tTregs as the major mechanism for the deficiency of tTregs in the knockout mice. Through multiple experimental approaches involving in vitro FOTC (fetal organ thymus culture) cultures and in vivo intrathymic injections, in both normal and TCR transgenic mice, Konkel et al. (39) provided indisputable evidence that TGF- β signaling is indeed required for *foxp3* gene induction in CD4⁺ SP thymocytes. In understanding where and how TGF- β is produced and/or activated, we discovered that the apoptosis of thymocytes could drive thymic macrophages, DCs, and thymic epithelial cells to produce TGF- β that can be activated by as yet identified mechanisms (39). These findings prompt us to propose a modified model to explain the development of tTregs by linking thymic apoptosis (e.g., negative selection) to the development of tTregs in a TGF- β -dependent manner (Figure 2). This model could reconcile well with the current models of tTreg development (Supplementary Text 6).

The molecular mechanisms underlying TGF- β induction of *foxp3* in the tTreg SP precursors are still incompletely understood. The *foxp3* gene contains the conserved promoter sequence, located upstream of the transcriptional start site, and conserved noncoding sequence 0 (CNS0), CNS1, CNS2, and CNS3 (123–129). Downstream of TGF- β receptors, pSmad2 and pSmad3 are crucial for *foxp3* gene activation (25, 128). Smad3 may directly bind to CNS1 together with TCR signaling–induced NFAT to activate *foxp3* transcription (128), which was suggested to be responsible for iTreg/pTreg generation (129). An earlier argument against a role of TGF- β in tTreg development was based on the findings that there are no obvious Smad3-binding sequences in the *foxp3* promoter or in CNS3, which were proposed to be involved in the induction of tTregs (128, 129). However, recent studies have revealed that CNS0 is even more important than CNS3 in initiating *foxp3* transcription in tTregs (123, 126, 130). Binding of pStat5 to CNS0 is suggested to be the key factor in initiating *foxp3* transcription, and it is believed that pStat5 is activated by IL-2. However, it remains to be known whether the expression and activation of Stat5 in CD4⁺Foxp3⁺ SP thymocytes require TGF- β signaling.

Furthermore, even if there is no obvious Smad-binding site(s) in the *foxp3* promoter or in CNS0 or CNS3, it is still possible that Smad can, through interaction with copartners, indirectly bind to and regulate *foxp3* expression. Indeed, it has been shown that Smad3 binds at the *foxp3* promoter and regulates its activity, through an “enhancersome,” with other transcription factors like cRel that can directly bind the promoter and CNS3 (127). Thus, it might be time to reconsider the possibility that Tregs are all generated via similar signaling

pathways. The major difference between tTregs and iTregs/pTregs would therefore be the location in which the Treg is generated and, as such, the antigens driving their development, rather than the requirement for TGF- β signaling (37).

4.2.3.3. TGF- β in Treg function. The function of TGF- β in Treg-mediated immunosuppression is still incompletely understood. TGF- β -mediated Treg suppression can be classified into three types, namely autocrine TGF- β production and activation by Tregs, paracrine TGF- β production and/or activation between Tregs and other cells, and the effects of TGF- β signaling in Tregs on their suppression. Tregs can secrete a soluble form and express a cell membrane-bound form of TGF- β (32, 33, 131–133). However, the exact function of autocrine TGF- β in Treg-mediated suppression still remains controversial.

To better understand the role of autocrine TGF- β production by Tregs in mediating their immunoregulation, several groups have generated T cell-specific TGF- β 1 knockout mice (69, 134–137). However, these transgenic mice have generated more conflicting results. In a recent excellent review, Moreau et al. (32) carefully analyzed the contradictory phenotypes of the mice and explained that they are due to technical differences and some potential off-target effects during the generation of the T cell-specific *Tgfb1*^{-/-} mice. They believe that the evidence favors the notion that Treg-mediated immunosuppression is largely impervious to the loss of endogenous TGF- β under baseline conditions, and they conclude that Treg-derived TGF- β is dispensable for Treg-mediated immune suppression and that self-tolerance can be maintained in the absence of Treg-produced TGF- β (32). However, the potential roles of Treg-derived TGF- β in conferring Treg-mediated suppression in pathogenic conditions such as autoimmunity, chronic inflammation, cancer, and infection still cannot be excluded.

The identification of GARP (glycoprotein A repetitions predominant) as an anchor that complexes with LTGF- β on the surface of murine and human Tregs provided a structural basis for the early findings of cell membrane-bound LAP-TGF- β on Tregs (131, 132), which is required for the activation of LTGF- β to mediate Treg function in an autocrine or paracrine manner (32, 33, 133). This can be accomplished by integrin $\alpha_v\beta_8$ or $\alpha_v\beta_6$ binding to the tripeptide Arg-Gly-Asp (RGD) motif present in the LAP moiety of LTGF- β (138). Both murine and human Tregs express $\alpha_v\beta_8$ that activates TGF- β to increase in pSmad2 in Tregs or to induce Foxp3 from cocultured naive CD4⁺ T cells in vitro (133, 139). The GARP-LTGF- β complex can also be activated by interactions with the $\alpha_v\beta_8$ in DCs to induce iTregs from naive CD4⁺ T cells or to suppress other immune cells (140). In addition, GARP can also be proteolytically cleaved by thrombin and platelets to release active TGF- β 1 for cancer immune evasion (141). Moreover, the GARP-LTGF- β complex on Tregs may also be activated by $\alpha_v\beta_6$ on epithelial cells to regulate immune and nonimmune cells, which may be involved in tissue repair and wound healing or even fibrosis (32, 33) (Figure 2).

Finally, TGF- β signaling in Tregs also plays a role in Treg-mediated immunoregulation. It is generally believed that Tregs regulate immune responses through at least two functional mechanisms: general suppression of the activation and proliferation of T cells and other immune cells and specific regulation of individual Th subsets. For example, Treg expression of T-bet is required for suppression of Th1 responses (142), and Stat3 for suppression of

Th17 responses (143). By generating transgenic mice that lack T β RI specifically in Foxp3⁺ Tregs, we revealed that deletion of TGF- β signaling in Tregs does not compromise their general suppressive activity with respect to T cell proliferation (144). However, these T β RI knockout Tregs show increased suppressive function against Th1 cells by expressing higher levels of T-bet and CRCX3, but they exhibit defective suppressive activity toward Th17 cells by an as yet unidentified mechanism (144). These findings indicate that TGF- β signaling in Tregs is dispensable for the general suppressive activity against T cell proliferation but is required for the specific suppression of Th17 cells, yet inhibitory for Treg suppression of Th1 cells. Unexpectedly, these T β RI^{ko} Treg mice exhibit a specific defect in the recruitment and retention of Tregs in the gastrointestinal tract past 6 months, resulting in an inability to regulate inflammation in the gut. This is ascribed to the lack of CD103 expression in the T β RI^{ko} Tregs (144).

5. TGF- β IN CD8⁺ T CELLS

Based on their phenotypic, functional, transcriptional, and epigenetic state, CD8⁺ T cells can be classified into naive, effector (Teff), exhausted (Tex), and memory cells. Memory T cells can be further classified into circulating effector memory T (Tem) and central memory T (Tcm) and non-circulating and tissue-resident memory (Trm) cells (10, 11, 145). TGF- β regulates the activation, proliferation, differentiation, and function of CD8⁺ T cells. Also, TGF- β appears to play a role in their generation of a unique population, CD8⁺Foxp3⁺CD122⁺Ly49⁺ Tregs (146, 147). In this section, I highlight the recent findings and advances in TGF- β regulation of CD8⁺ T cells with emphasis on Teff, Trm, Tex, and CD8⁺ Tregs (Supplementary Figure 1).

5.1. TGF- β Suppression of Effector CD8⁺ T Cells

TGF- β suppresses CD8⁺ T cell activation and function through direct and indirect pathways. CD8⁺ T cells are major cytotoxic T lymphocytes that produce IFN- γ and express granzyme A and B and perforin to kill their target cells, especially tumor cells and virus-infected cells (48). In vitro, TGF- β suppresses the activation and differentiation of murine and human naive CD8⁺ T cells isolated from cord blood to effector CD8⁺ T cells (148, 149). The regulatory role of TGF- β in the CD8⁺ effector function is best evidenced by a variety of in vivo models of tumor, infection, and inflammation in mice in which TGF- β signaling is altered by either genetic manipulation or inhibition with antibodies or inhibitors. dnT β RII (dominant-negative TGF- β receptor II) mice exhibit a large expansion of tumor-reactive CD8⁺ T cells that produce high amounts of IFN- γ and granzyme B and eradicate tumors (48). The suppressive activity of TGF- β in antitumor CD8⁺ Teff cells can be performed either by specifically inhibiting the expression of perforin, granzymes A and B, Fas ligand, and IFN- γ in a Smad-dependent manner (150) or by indirect suppression through Treg-derived cell membrane-bound TGF- β (151) and via exclusion of CD8⁺ T cell infiltration to the tumor (152, 153). In addition, TGF- β enhances antigen-induced PD-1 expression through Smad3-dependent transcriptional activation in T cells in vitro and in tumor-infiltrating lymphocytes in vivo, which impedes antitumor activity (154). Systemic blockade of TGF- β with anti-TGF- β antibodies in combination with DNA vaccination or IL-2 treatment enhances tumor-infiltrating and tumor-reactive CD8⁺ T cells (155, 156).

Moreover, TGF- β might also control effector cell number by lowering BCL-2 amounts and promoting apoptosis of short-lived effector cells in mice, which interferes with anti-infection immunity in *Listeria* infection (157) and promotes medulloblastoma progression in SmoA1 (smoothened A1) transgenic medulloblastoma mice (158). In nonobese diabetic (NOD) mice, transgenic expression of TGF- β in the inflamed islets significantly delays diabetes development, in that TGF- β disables the transition of primed autoreactive CD8⁺ T cells to cytotoxic effectors within the pancreas, significantly impairing their diabetogenic capacity (159). Transgenic mice with T cell-specific deletion of TGF- β receptors or Smad2/3 results in massive CD8⁺ Teff cell activation and large amounts of IFN- γ secretion (22–25). In contrast to the CD4-Cre-driven system, adult mice with deleted T β RII, through the use of Cre driven by a promoter (dLck-Cre) that is active much later in T cell development, exhibit no obvious sign of autoimmunity or systemic inflammation and have milder activation of CD8⁺ T cells (160). However, adoptive transfer of these knockout T cells into lymphopenic hosts results in inflammation. Zhang & Bevan (160) propose that TGF- β may mainly regulate T cell proliferation and activation in response to exogenous stimuli, such as lymphopenia. However, an alternative explanation is that the late deletion of T β RII in T cells may spare TGF- β control of the development of tTregs in the neonatal window and that Tregs in turn function normally in maintaining immune homeostasis and tolerance in these knockout mice.

5.2. TGF- β in CD8⁺ Resident Memory T Cells

CD8⁺ Trm cells reside permanently in nonlymphoid tissues and appear phenotypically, functionally, transcriptionally, and metabolically different from Tcm and Tem cells. Trm cells lack CCR7 and CD62L and thus are unable to recirculate through the blood and lymphoid organs; instead, they express unique clusters of molecules that safeguard their residence in nonlymphoid tissues. This includes CD69, which inhibits sphingosine-1 phosphate receptor (S1PR1) to prevent Trm cells from egressing; CD103 (α E β 7), an integrin that binds to E-cadherin in epithelial cells; and CD49a, which tethers Trm cells to be retained in the tissues of residence (161, 162). Functionally, Trm cells are major CD8⁺ T cells in the tissues for the defense against infections and malignant cells; they produce IFN- γ and TNF- α and express cytotoxic molecules such as granzyme B to kill their target cells, although their expression may vary in different tissues (10).

TGF- β plays a central role in the differentiation and maintenance of Trm cells. Trm cells can be differentiated from circulating naive and memory precursors and/or from effector or memory cells in nonlymphoid tissues. Upon antigen activation, CD8⁺ T cells can be differentiated into either short-lived Teff cells (CD127[−]KLRG-1⁺) or memory precursor Teff cells (CD127⁺KLRG-1[−]) (157, 163). CD8⁺ T cells downregulate TGF- β receptors upon TCR stimulation (46, 164), but extracellular ATP senses the purinergic receptor P2RX7 on CD8⁺ T cells to help them regain TGF- β receptors and restore their sensitivity to TGF- β , which promotes differentiation of memory precursor Teff cells to CD103⁺CD8⁺ Trm cells (164).

TGF- β induces CD103 in CD8⁺ Trm cells through Smad3 binding to the *Itgae* gene. In addition, TGF- β also indirectly upregulates CD103 expression by suppressing T-bet and

TCF-1 expression, as both may bind to *Itgae* and interfere with Smad3-mediated CD103 gene transcription (165, 166). However, CD103⁺ Trm cells appear to be tissue specific, as liver CD8⁺ Trm cells do not express CD103 (145). Moreover, CD103⁻CD8⁺ Trm cells in the gut lamina propria do not require TGF- β , as TGF- β receptor knockout mice show no effects on these CD103⁻ Trm cells (167). Interestingly, TGF- β is also required for the formation of CD69⁺CD103⁺ Trm cells in the corneal epithelium, and these Trm cells patrol the cornea (168). In addition to CD103, TGF- β also enhances expression of CD49a in CD8⁺ T cells during antigen stimulation, such as in infections; however, CD49a is lost in most circulating memory T cells but is maintained in many Trm cells (169).

TGF- β is also critical in the suppression of Trm cell egress from the tissues. S1PR1 is a central molecule that promotes the egress of CD8⁺ Trm cells from the tissues (170). TGF- β suppresses S1PR1 expression in Trm cells through different mechanisms. TGF- β alone or in combination with IL-33 suppresses KLF2 through the PI3K/AKT pathway, which consequently inhibits S1PR1 expression, as KLF2 binds to the *Sipr1* gene promoter to promote *Sipr1* transcription. In addition, TGF- β also downregulates S1PR1 by upregulating CD69 in CD8 Trm cells (167).

The accumulated evidence indicates that integrin-mediated activation of LTGF- β is probably a major source of bioactive TGF- β that regulates Trm cells in the tissues. For example, in lymph nodes, not in the spleen, naive CD8⁺ T cells are conditioned through MHC-I-dependent interactions with peripheral tissue-derived migratory DCs that express $\alpha_v\beta_8$ to activate TGF- β 1, and this induces epithelial CD8⁺ Trm cells (171). In the tumor microenvironment, it is reported that type 1 Tregs express high amounts of $\alpha_v\beta_8$ integrin to activate TGF- β and promote Trm cell differentiation (172). Even tumor cells can also express $\alpha_v\beta_6$ integrin to activate TGF- β and enhance CD103⁺CD69⁺CD8⁺ Trm cells, and these Trm cells may interfere with anti-PD-1/anti-PD-L1-mediated immunotherapeutic effects (173).

5.3. TGF- β in CD8⁺ Exhausted T Cells

During chronic viral infections and cancer, CD8⁺ T cells can become Tex cells. CD8⁺ Tex cells exhibit defective production of IFN- γ and TNF- α cytokines; are marked with some group of surface molecules, such as PD-1, Tim3, LAG3, CD73, and 2B4 (174); and become dysfunctional and fail to form immunity against infections and cancer. Recent studies have provided evidence that TGF- β might also be involved in CD8⁺ T cell exhaustion during chronic infection and cancer. A breast cancer study showed that breast cancer cells secrete extracellular vesicles in the form of exosomes that carry PD-L1 and are highly immunosuppressive. TGF- β promotes breast cancer exosomal PD-L1 secretion, and this process facilitates CD8⁺ T cell dysfunction and exhaustion (175). Comparison of the transcriptome of “exhausted” CD8⁺ T cells infiltrating autochthonous melanomas to those of naive and acutely stimulated CD8⁺ T cells reveals that the transcriptional regulators Nr4A2 (nuclear receptor subfamily 4 group A member 2) and Maf are overexpressed in tumor Tex cells and are significantly upregulated in CD8⁺ T cells from human melanoma metastases. TGF- β and IL-6 are the main inducers of Maf expression in CD8⁺ T cells (176). In a model of chronic persistent murine cytomegalovirus infection, CD8⁺ T cells appear

exhausted, expressing PD-1, CD73, and CD39, and intriguingly, CD73 is activated on CD8⁺ T cells by TGF- β signaling (177). However, still other studies show that TGF- β -mediated dysregulation of CD8⁺ T cells may not necessarily be due to induction of exhaustion. For example, in a tumor model, TGF- β induces Foxp1 expression through Smad2/3-mediated c-myc repression, which prevents CD8⁺ T cell activation by tumor antigens, and this process is not associated with T cell exhaustion (178). Similarly, in a chronic virus infection model, it was shown that CD8⁺ T cell–intrinsic TGF- β signaling was responsible for virus-specific CD8⁺ T cell apoptosis and decreased cell numbers but was not necessary for their functional exhaustion (179). Nevertheless, a recent study showed that TGF- β repressed mTOR signaling in CD8⁺ Tex cells and was a critical determinant of the metabolism and function of precursors of Tex cells. These Tex cell precursors sustained Tex cells and self-renewed while continuously generating exhausted effector T cells (180). This underlines the metabolic involvement of TGF- β regulation of CD8⁺ T exhaustion.

5.4. TGF- β in CD8⁺ Tregs

Naive CD8⁺ T cells can also differentiate into Foxp3⁺ T cells in response to TCR stimulation in the presence of TGF- β (181). These Tregs manifest equal, if not stronger, suppressive activity with respect to CD4⁺Foxp3⁺ Tregs to inhibit immune cell activation and proliferation in coculture assays in vitro. However, it is mysterious that CD8⁺Foxp3⁺ Tregs are hardly detectable in normal mice in the steady state. This raises the question as to whether CD8⁺Foxp3⁺ Tregs even exist in mice and humans. About ten years ago, Kim et al. (182) reported on a population of CD8⁺ Tregs that expressed a unique set of molecules, namely CD122 and Ly49, but remained Foxp3⁻. Importantly, a more recent study indicated that the human counterparts of murine CD8⁺ Tregs have been discovered and are more numerous in those with autoimmune diseases such as systemic lupus erythematosus, Crohn disease, and multiple sclerosis as well as in patients infected with influenza virus or SARS-CoV-2 (183). These human CD8⁺ Tregs express a unique killer immunoglobulin receptor as well as Helios, which is also found in murine CD8⁺ Tregs. Of note, recent studies suggest that TGF- β is critical in the differentiation of CD8⁺ Tregs in mice (146, 147). In one study investigators generated T β RII and Eomes double-knockout mice (*Tgfb β 2*^{flox/flox}/*Eomes*^{flox/flox} *dlck-Cre*) and demonstrated that these mice specifically lack CD8⁺Foxp3⁻CD122⁺Ly49⁺ Tregs, whereas CD4⁺Foxp3⁺ Tregs and Tfr cells are not affected. Mechanistically, TGF- β induces and upregulates Helios in CD8⁺ T cells but not CD4⁺ T cells, although this has not been explained. In addition, TGF- β plus Eomes can preserve CD122 expression in these CD8⁺ Tregs (146).

6. TGF- β IN $\gamma\delta$ T CELLS

Recent studies have reported that TGF- β plays a role in the differentiation of IL-17-producing $\gamma\delta$ T cells and IL-9-producing V δ 2 $\gamma\delta$ T cells. In normal mice, IL-17⁺ $\gamma\delta$ T cells are an important cellular source of IL-17 and are involved in defense against bacterial infection and in autoimmune inflammation. TGF- β controls in the development of IL-17⁺ $\gamma\delta$ T cells in the thymus (184). Interestingly, $\gamma\delta$ T cells, by secreting IL-17F, drive adipocytes to express TGF- β via IL-17 receptor C (IL-17RC), and TGF- β promotes local sympathetic innervation to enhance thermogenesis (185). Human V δ 2 T cells are the

dominant $\gamma\delta$ T cell subset found in peripheral blood and recognize pyrophosphate molecules derived from microbes or tumor cells. TGF- β , in the presence of IL-15, induces Foxp3⁺ V δ 2 T cells that show some suppressive activity toward CD4⁺ T cells. However, these V δ 2 T cells express high amounts of the IL-9 gene (186).

7. TGF- β IN NATURAL KILLER T CELLS

NKT cells recognize self-glycolipid antigens and foreign glycolipid antigens presented by the nonclassical MHC-I-like molecule CD1d (187). NKT cells comprise two subsets, namely iNKT cells and type II NKT cells. iNKT cells utilize a semi-invariant TCR involving V α 14J α 18 in mice and V α 24J α 18 in humans (30, 47) to respond to α -galactosylceramide bound to CD1d. iNKT cells develop in the thymus, and ablation of TGF- β receptors in T cells block development of canonical CD1d-restricted NKT cells (24). However, the underlying mechanisms by which TGF- β controls the development of iNKT cells remains incompletely understood. CD1d^{-/-}dnT β RII mice, which lack CD1d-restricted NKT cells, exhibit significantly decreased hepatic lymphoid cell infiltrates and milder cholangitis compared with CD1d^{+/-}dnT β RII mice (188). In contrast to its positive role in the development of iNKT cells, TGF- β actually suppresses iNKT cell functions, such as IFN- γ production (30, 47). Interestingly, IL-9 production by iNKT cells is not imprinted during thymic development but is rather induced by TGF- β and IL-4 stimulation from mature peripheral iNKT cells (189) (Figure 3).

8. TGF- β IN INTRAEPITHELIAL LYMPHOCYTES

Intraepithelial lymphocytes (IELs) comprise heterogeneous populations of lymphocytes residing between intraepithelial cells (13, 190). Unconventional IELs (also called natural IELs) comprise TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ and TCR $\gamma\delta$ ⁺CD8 $\alpha\alpha$ ⁺ populations that develop in the thymus from CD4^{low}CD8^{low}Pd1^{hi} through TCR $\alpha\beta$ ⁺CD8⁻CD4⁻ α 4 β 7⁺ thymocytes and from CD4⁻CD8⁻TCRV γ 7⁺ thymocytes, respectively (190). Conventional IELs (also called induced IELs) include CD4⁺CD8⁻ and CD4⁻CD8 $\alpha\beta$ ⁺TCR $\alpha\beta$ ⁺ IELs and CD4⁺CD8 $\alpha\alpha$ ⁺TCR $\alpha\beta$ ⁺ cells (DP IELs). TGF- β plays a critical role in the development, differentiation, and maintenance of TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ and TCR $\alpha\beta$ ⁺CD4⁺CD8 $\alpha\alpha$ ⁺ DP IELs (Figure 3).

8.1. TGF- β in TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ IELs

TGF- β is a key factor to control the development and maintenance of TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ IELs, because transgenic mice with either *Tgfb1*^{-/-} or T cell-specific TGF β RI deficiency (T β RI^{ko}) lack TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ IELs (41). In addition, lack of Smad3 also results in a significant reduction of TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ IELs in mice. Conversely, mice with overexpression of active TGF- β 1 specifically in T cells (*Tgfb1glo*⁺*CD4cre*⁺) have more TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ IELs (41). Intriguingly, conventional TCR $\alpha\beta$ ⁺CD8 $\alpha\beta$ ⁺ IELs are not affected, and actually their frequency is increased in mice with TGF- β signaling deficiency. TGF- β protects DN TCR $\alpha\beta$ ⁺CD5⁺ thymocytes, the immediate precursors of TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ IELs (13), from apoptosis (41). Once leaving the thymus, these IEL precursors express α 4 β 7 integrin and CCR9, leading them to the gut epithelium (190), where on exposure to TGF- β they acquire expression of CD8 α and CD103 (α E β 7) (41, 191), but

downregulate the expression of $\alpha_4\beta_7$ (192). TGF- β induction of CD8 α in TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$ IEL precursors is mediated by downregulation of ThPOK leading to a reduced ratio of ThPOK to RUNX3 (41). In addition, TGF- β also maintains CD8 α expression on TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$ IELs. A recent report provided some evidence that humans also have CD8 $\alpha\alpha^+$ TCR $\alpha\beta^+$ intestinal IELs (193), although the role of TGF- β in their development remains to be shown.

8.2. TGF- β in CD4 $^+$ CD8 α^+ Double-Positive IELs

TCR $\alpha\beta^+$ CD4 $^+$ CD8 $\alpha\alpha^+$ DP IELs are also called induced IELs, as they are derived from peripheral CD4 $^+$ CD8 $^-$ T cells in the lymphoid tissues (194, 195). The first evidence supporting the notion that DP IELs are converted from CD4 $^+$ CD8 $^-$ T cells is that adoptive transfer of peripheral CD4 $^+$ CD8 $^-$ T cells from normal mice into Rag1 $^{-/-}$ mice leads to differentiation of DP IELs (194). Further studies reveal that in the context of TCR stimulation, TGF- β is the most important factor for the differentiation of DP IELs from peripheral CD4 $^+$ CD8 $^-$ T cells (41, 196). The antigens should be mainly derived from the microbiome in the gut, as germ-free mice lack these DP IELs (195). Interestingly, TGF- β upregulates only CD8 α mRNA, but not CD8 β expression, in CD4 $^+$ T cells in culture (41). In vivo, after adoptive transfer TGF β RI-deficient CD4 $^+$ CD8 $^-$ T cells failed to differentiate into DP IELs in Rag1 $^{-/-}$ mice (41). Although TGF- β induces both CD4 $^+$ CD8 $\alpha\alpha^+$ and CD4 $^+$ CD8 $\alpha\beta^+$ subsets in vitro (41), almost all DP IELs are CD4 $^+$ CD8 $\alpha\alpha^+$ (195). This paradox was resolved by the findings that retinoic acid in the gut together with TGF- β preferentially induces CD4 $^+$ CD8 $\alpha\alpha^+$ IELs (196). Mechanistically, TGF- β induces CD8 α by suppressing ThPOK and upregulating RUNX3 expression in CD4 $^+$ T cells (41, 196), and it was suggested that the upregulation of RUNX3 occurs before ThPOK downregulation in CD4 $^+$ T cells (196). It has been observed that CD8 α induction is in parallel with Foxp3 expression by TGF- β (41, 196), but only DP IELs, and not Foxp3 $^+$ Tregs, downregulate ThPOK expression (196). Interestingly, a recent study demonstrated that peripheral Foxp3 $^+$ Tregs, upon migration to the intestinal epithelium, lose their ThPOK and convert to DP IELs in a microbiota-dependent manner (197). However, the role of TGF- β in this conversion remains unknown. Functionally, DP IELs are considered to be cytotoxic T cells, as they express high levels of IFN- γ and granzyme B (196), although they also express IL-10 (194) and thus exhibit an immunoregulatory function in the gut. While TGF- β is required for differentiation of DP IELs, the role of TGF- β in their function remains largely unknown.

9. TGF- β IN INNATE LYMPHOID CELLS

ILCs do not express antigen-specific receptors, but they are important in protective immunity and regulation of homeostasis and inflammation (12). ILCs comprise three subsets. Group 1 ILCs (ILC1s) comprise the prototypical ILC1s and NK cells. ILC1s express NKp46, NK1.1, and transcription factor T-bet and produce IFN- γ . ILC2s express GATA-3 and produce IL-5 and IL-13. ILC3s produce IL-17A and IL-22 and depend on ROR γ t for differentiation. TGF- β has been reported to play roles in the differentiation of salivary gland ILC1s and the development of ILC2s (Figure 3).

9.1. TGF- β in ILC1s

TGF- β controls the differentiation of ILC1s in the salivary glands (198) and converts NK cells into ILC1-like cells in tumors and during viral infections (199, 200). Salivary gland ILC1s express a unique array of cell surface molecules, cytokines, and developmental transcription factors, namely surface CD103, CD49a, CD39, TRAIL, and CD69 (198). They also produce low amounts of IFN- γ and require T-bet and Eomes, but not Nfil3, for development. Conditional knockout mice with TGF β RII deletion specifically in ILC1s and NKp46⁺ cells (*Tgfb β 2^{flox/flox}Ncr1Cre⁺*) have fewer salivary gland ILCs, which lack their signature markers (198). Further studies reveal that TGF- β promotes salivary gland ILC1 differentiation by suppressing Eomes through Junk-dependent but Smad4-independent pathways. In addition, a recent report showed that TGF- β is also required for the maintenance of a granzyme C-expressing ILC1 subset in the salivary glands (201).

TGF- β may also convert NK cells into ILC1-like cells devoid of cytotoxic function (199, 200) in the tumor microenvironment. TGF- β induces conversion of NK cells (CD49a⁺CD49 β ⁺Eomes⁺) into intermediate ILC1 (CD49a⁺CD49b⁺Eomes⁺) populations and ILC1 (CD49a⁺CD49b⁻Eomes^{int}) populations in the tumor microenvironment, which is mediated by Smad-independent pathways (199, 200). Actually, Smad4 inhibits TGF- β -mediated conversion of ILC1-like cells from NK cells: Smad4 deficiency in NK cells does not affect ILC1 differentiation, but Smad4^{-/-} NK cells acquire an ILC1-like gene signature and are unable to control tumor metastasis or viral infection (199). Importantly, TGF- β -mediated conversion of NK cells into ILC1-like cells also occurs in human NK cells. NK cells from a SMAD4-deficient patient affected by polyposis were also hyperresponsive to TGF- β (199). IL-15 may synergize with TGF- β in this cellular conversion in human NK cells (202). Analysis of downstream TGF- β signaling suggests that TAK1-mediated activation of p38 MAPK as the critical pathway driving the conversion and that IL-15 enhanced TGF- β -mediated conversion through Ras:RAC1 signaling as well as via the activation of MEK/ERK. However, human circulating NK cells treated with TGF- β show heterogeneity in their potential to adopt an ILC1-like phenotype, as indicated by the upregulation of CD9 and CD103 in only a subset of cells in culture. On the other hand, murine and human ILC1s secrete TGF- β , driving expansion of CD44v6⁺ epithelial crypts in the intestine (203).

9.2. TGF- β in ILC2s

In the bone marrow, TGF- β signaling programs the development of ILC2s, but surprisingly not ILC1s or ILC3s, from their progenitors (204). This is accomplished by TGF- β -mediated generation and maintenance of ILC2 progenitors. Mechanistically, TGF- β upregulates expression of the IL-33 receptor gene *Il1rl1* (encoding IL-1 receptor-like 1, also known as ST2) in ILC2 progenitors and common helper-like innate lymphoid progenitors (CHILPs), at least partially through a MEK-dependent and Smad3-independent pathway (204). In addition, TGF- β also maintains ST2 expression in mature ILC2s and protects their survival. Adoptive transfer of T β RII^{ko} ILC2s into Rag1^{-/-}IL2 γ ^{-/-} mice followed by induction of airway inflammation with house dust mites results in far fewer ILC2 effector cells and consequently substantially decreased lung inflammation compared to mice receiving wild-type ILC2s (204). Consistent with this, it was shown recently that TGF- β induces

neuropilin-1 (Nrp1) in lung ILC2s and TGF- β -Nrp1 signaling enhances ILC2 function by upregulating ST2 expression (205). Moreover, TGF- β , whose activity is increased in systemic sclerosis, favored the expansion of KLRG1⁺ ILC2s while simultaneously decreasing their production of IL-10 in the skin of systemic sclerosis patients and increasing myofibroblast differentiation (206). Interestingly, TGF- β plays a role in promoting the conversion of c-Kit⁺ ILC2s into ROR γ t-expressing cells by inducing upregulation of IL23R, CCR6, and KIT mRNA in these cells (207).

9.3. TGF- β in ILC3s

Considering that ILC3s share some features of Th17 cells in that they produce IL-17 and IL-22, and that TGF- β is required for Th17 differentiation, it is somewhat surprising that no evidence thus far has been reported that TGF- β plays a role in the differentiation of ILC3s. ILC3s can be divided into two subsets based on the expression of the natural cytotoxicity receptor (NCR) NKp46 (encoded by *Ncr1*), namely NCR⁺ and NCR⁺ ILC3s (208, 209). Notch signaling is required for maintenance of NCR⁺ ILC3s and induces transition of NCR⁺ to NCR⁺ ILC3s (208). TGF- β , however, impairs the development of NCR⁺ ILC3s (209). A report suggests that TGF- β might initiate conversion of ILC3s toward regulatory ILCs (210) in the tumor microenvironment, but the significance of this conversion remains to be determined.

10. CONCLUSIONS AND PERSPECTIVE

Discovered as a transforming growth factor in nonimmune systems more than four decades ago (15), TGF- β has now been recognized as one of the most important cytokines regulating immune responses, especially T cell responses. Over the last 20 years, we have witnessed a conceptual revolution in how this ubiquitous and “nonspecific” cytokine acts, transforming from a simple suppressive performer that inhibits T cell activation and proliferation to a multifaceted conductor that orchestrates the development, homeostasis, differentiation, and death of almost all populations of T cells. Of particular note, the discoveries that TGF- β is critical for the differentiation of (a) CD4⁺ Tregs, Th17 cells, and Th9 cells; (b) CD8⁺ Trm cells, NKT cells, and TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ and CD4⁺CD8 $\alpha\alpha$ ⁺ IELs; and (c) salivary gland ILC1s and ILC2s have paved the way to a better understanding of T cell-mediated adoptive immunity and tolerance in immune homeostasis, as well as in the pathogenesis of autoimmunity, chronic inflammation, cancer, and infectious diseases. It is clear that the roles of TGF- β in the regulation of T cells not only are dependent on its signaling, but more importantly are also heavily influenced by signals from other molecules and cytokines in the microenvironment in which the T cells are located. Although we have learned a great deal about TGF- β function in T cells, many questions are yet to be resolved. For example, what is the missing link for the molecular and/or epigenetic mechanisms by which TGF- β signaling results in completely different Treg, Th17, and Th9 subpopulations in the presence of individual cytokines? How does TGF- β suppress CD8⁺ Teff cells but promote differentiation of Trm cells in the context of physiological and pathological conditions? What are the molecular mechanisms by which TGF- β is produced, processed, and activated in immune cells, particularly in T cells? What is the role of TGF- β during the cross talk between T cells and B cells or innate immune cells such as macrophages, neutrophils, and

NK cells? Does TGF- β play a part in T cells and other immune cells during their interaction with microbiota? What is the function of TGF- β during the cross talk between T cells and tumor cells, stromal cells, or blood endothelial cells in the tumor microenvironment? How does TGF- β function in T cell-mediated antitumor immunotherapy in the context of checkpoint blockade and CAR (chimeric antigen receptor) T cells? How can we optimize and better manipulate TGF- β signaling to develop antigen-specific Tregs for the treatment of autoimmunity, and conversely how can we suppress tumor-specific Tregs for the treatment of cancer? What is the function of TGF- β in T cells for the development and pathogenesis of viral infections, such as HIV and SARS-CoV-2 infections, and can we manipulate TGF- β function to treat diseases caused by these infections? It will be important to understand the exact mechanisms by which TGF- β regulates different populations of T cells so as to understand the pathogenesis of related human diseases and develop more effective immunotherapies. We are hopeful that research into TGF- β regulation of immune responses including T cells over the next two decades will help revolutionize our understanding of and therapy for relevant human diseases.

Supplementary Material

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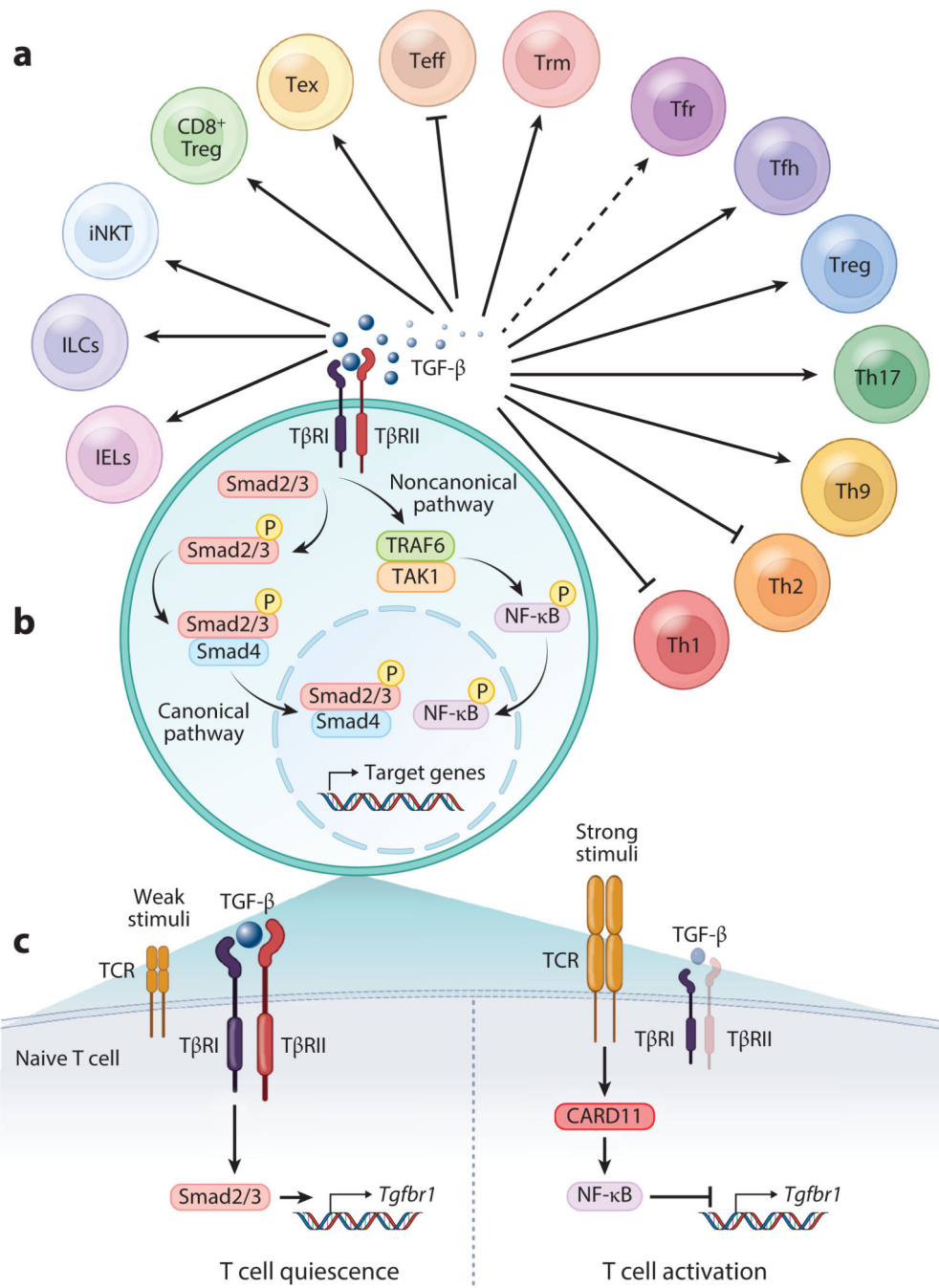


Figure 1.

TGF- β regulation of T cells. (a) In CD4⁺ T cells: Th1, Th2, Th9, and Th17 cells; CD25⁺Foxp3⁺ Tregs, including thymic Tregs and induced/peripheral Tregs; Tfh cells; and Tfr cells. In CD8⁺ T cells: Trm cells, Teff cells, Tex cells, and CD122⁺Ly49⁺Foxp3⁻ Tregs. In addition, iNKT cells, ILCs, and gut IELs. Dashed arrow indicates no experimental evidence available. (b) General TGF- β signaling pathways. TGF- β engages TβRII and then recruits and phosphorylates TβRI. The receptor complex then initiates the canonical Smad2- and Smad3-dependent pathway and/or noncanonical pathways such as the TRAF6-

TAK1-dependent pathway to regulate target gene expression. (c) TCR-mediated regulation of TGF- β -T β RI signaling acts as a third signal in determining T cell quiescence and activation. Naive CD4⁺ T cells in mice and humans have active TGF- β signaling, evidenced by T β RI, T β RII, and activated Smad2/3 (pSmad2/3). TGF- β signaling represents a key intrinsic negative signal in retaining quiescence and preventing unwanted activation in naive CD4⁺ T cells. After TCR engagement, naive CD4⁺ T cells show no reduction of, and very likely higher, T β RI expression in response to weak or low-dose TCR stimuli (*left*), but they exhibit rapid and profound reduction of T β RI when activated with strong or high-dose TCR stimuli in a CARD11-NF- κ B-dependent manner (*right*). Data not represented here indicate that TGF- β preserves and even upregulates the TCR-mediated downregulation of T β RI and that IL-6 abolishes TGF- β -mediated upregulation of T β RI in the context of strong TCR stimulation. Abbreviations: CARD11, caspase recruitment domain-containing protein 11; IEL, intraepithelial lymphocyte; ILC, innate lymphoid cell; iNKT, invariant natural killer T; pSmad2/3, phosphorylated Smad2/3; TAK1, TGF- β -activated kinase 1; T β RI, TGF- β receptor I; TCR, T cell receptor; Teff, effector T; Tex, exhausted T; Tfh, follicular helper T; Tfr, follicular regulatory T; Th1, T helper type 1; TRAF6, TNF receptor-associated factor 6; Treg, regulatory T cell; Trm, T resident memory. Figure adapted from images created with [BioRender.com](https://www.biorender.com).

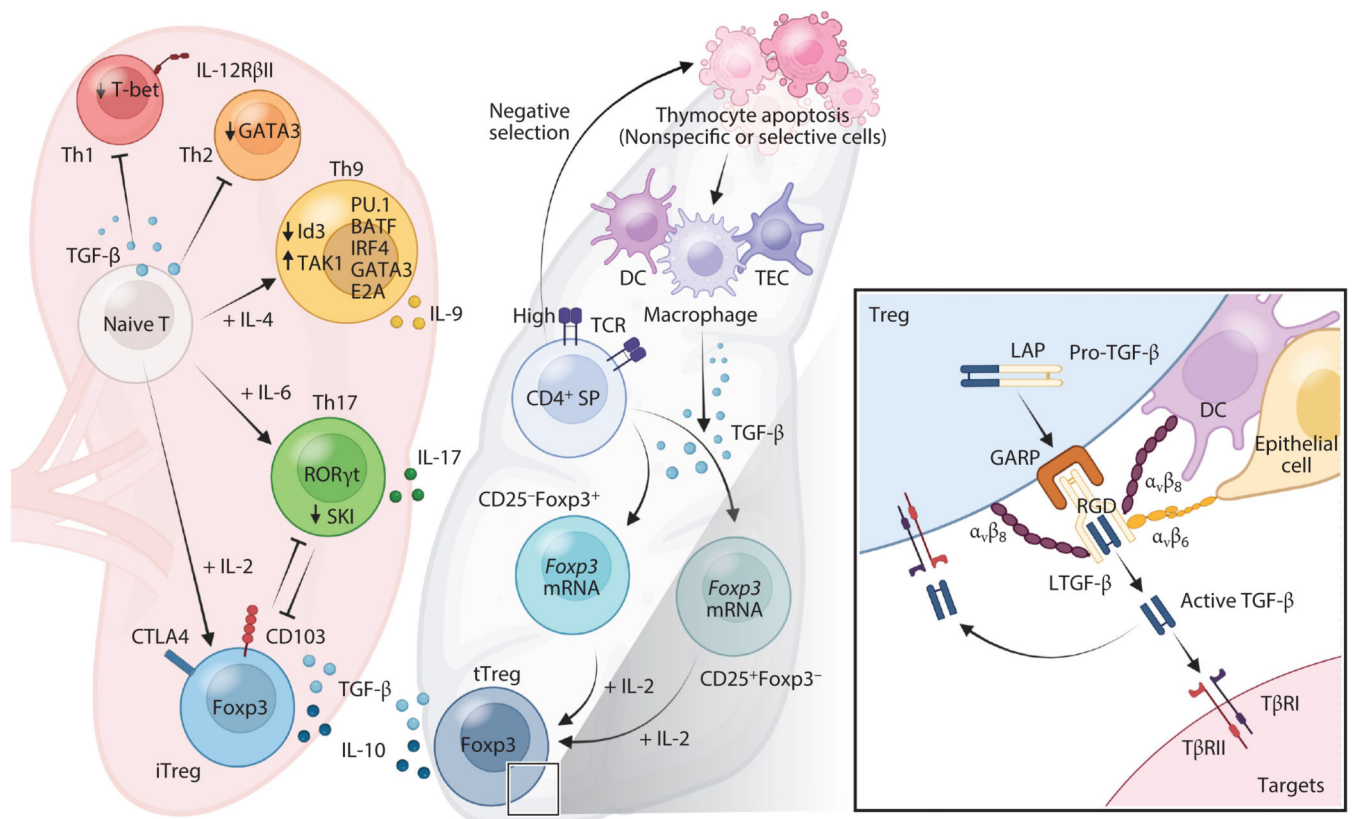
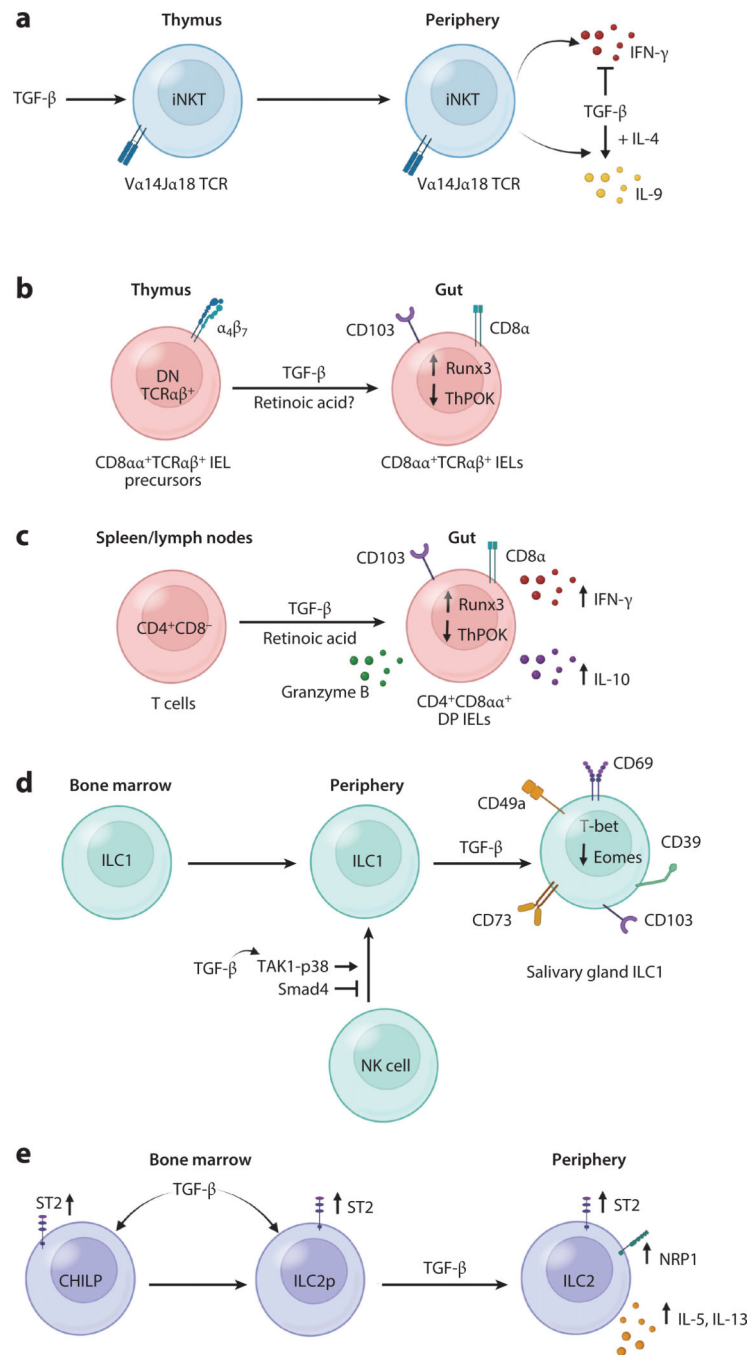


Figure 2.

(*Left*) TGF- β regulation of CD4⁺ T cell differentiation and function. TGF- β suppresses T-bet and IL-12R β II in Th1 cells, and GATA3 in Th2 cells. Multiple transcription factors, including PU.1, BATF, IRF4, GATA3, TAK1, ID3, and E2A, have been reported to be involved in regulation of *Il9* gene activation during Th9 differentiation in response to TGF- β and IL-4. For example, TGF- β suppresses Id3 expression through a TAK1-dependent pathway, and this process enhances the activity of E2A and GATA3 in *Il9* gene transcription. During Th17 differentiation, TGF- β induces ROR γ t expression, which can be enhanced by IL-6. Smad2/3 are required for Th17 differentiation, although it is still controversial whether Smad2/3 are required for TGF- β induction of ROR γ . In contrast, Smad4 inhibits Th17 differentiation by directly interacting with SKI, and TGF- β disrupts SKI and reverses Smad4-SKI complex-mediated inhibition of ROR γ t. TGF- β is essential for induction of Foxp3 in naive CD4⁺ T cells and converts them into Foxp3⁺ Tregs (iTregs/pTregs). (*Middle*) TGF- β is required for the development of tTregs. A proposed model of thymocyte apoptosis linked to the generation of tTregs in a TGF- β -dependent manner. Thymocytes undergoing apoptosis (e.g., negative selection) can be taken up and digested by macrophages, DCs, and thymic epithelial cells in the thymus, and this results in TGF- β production by these phagocytes and/or activation by yet unidentified mechanisms. TGF- β in turn induces the *foxp3* gene from Foxp3⁻CD4⁺CD8⁻ single-positive cells that simultaneously are engaged by their specific antigenic peptides (e.g., self-antigens). This results in two lineage-committed thymic Treg precursors (or immature thymic Tregs): CD25⁺Foxp3⁻ (*foxp3* mRNA⁺) and CD25⁻Foxp3⁺ precursors. These two precursors can then mature into CD4⁺CD25⁺Foxp3⁺

thymic Tregs in response to IL-2 in the thymus. This model could reconcile with the current models of thymic Treg development (Supplementary Text 6). (*Right*) TGF- β in Treg function. Tregs can secrete soluble TGF- β and also express cell membrane-bound LAP-TGF- β (LTGF- β). Surface LTGF- β is anchored by the protein GARP. The GARP-LTGF- β complex can be activated by integrin $\alpha_v\beta_8$ or $\alpha_v\beta_6$ binding to the Arg-Gly-Asp (RGD) motif present in the LAP moiety of LTGF- β , which releases bioactive TGF- β . Murine and human Tregs express $\alpha_v\beta_8$ that can activate GARP-LTGF- β to regulate Treg function (autocrine regulation). DCs also express $\alpha_v\beta_8$ that can activate GARP-LTGF- β on Tregs to regulate Treg suppression (paracrine regulation). In addition, epithelial cells and cancer cells can express $\alpha_v\beta_6$ to activate TGF- β from the GARP-LTGF- β on Tregs (paracrine regulation). Moreover, TGF- β signaling in Tregs also regulates Treg function (not depicted here), which is required for the specific suppression of Th17 cells by Tregs, yet is inhibitory for Treg suppression of Th1 cells. Abbreviations: DC, dendritic cell; GARP, glycoprotein A repetitions predominant; iTreg, induced Treg; LAP, latency-associated polypeptide; LTGF- β , latent TGF- β ; SP, single positive; TAK1, TGF- β -activated kinase 1; T β R1, TGF- β receptor I; TCR, T cell receptor; TEC, thymic epithelial cell; Th1, T helper type 1; Treg, regulatory T cell; tTreg, thymic Treg. Figure adapted from images created with [BioRender.com](https://www.biorender.com).

**Figure 3.**

TGF- β regulation of unconventional lymphocytes. (a) In the thymus, TGF- β regulates the development of iNKT cells. In the periphery, TGF- β represses their ability to produce IFN- γ , while, in combination with IL-4, promoting their ability to produce IL-9. (b) TGF- β is a key factor to control the development and maintenance of TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ IELs. It protects DN (CD4⁻CD8⁻) TCR $\alpha\beta$ ⁺CD5⁺ thymocytes (the precursors of TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ IELs) from apoptosis. TGF- β also induces the expression of CD8 α via downregulation of ThPOK and upregulation of Runx3 in the precursors. (c) TGF- β induces, together with

retinoic acid, the generation of CD4⁺CD8αα⁺ DP IELs by inducing CD8α expression in peripheral CD4⁺CD8⁻ T cells, and this is accomplished by suppressing ThPOK and upregulating RUNX3 expression. TGF-β also induces the expression of CD103. CD4⁺CD8αα⁺ DP IELs produce IL-10 and IFN-γ, and thus are considered cytotoxic T cells with regulatory function. (d) TGF-β promotes the differentiation of ILC1s in the salivary glands by suppressing Eomes, through Junk-dependent but Smad4-independent pathways. TGF-β also promotes the conversion of NK cells into ILC1s through the TAK1-p38 pathway, and Smad4 is inhibitory for this conversion. (e) In the bone marrow, TGF-β programs the development of ILC2s by upregulating the expression of ST2 in the CHILPs and the ILC2 precursors. TGF-β also maintains ST2 expression in mature ILC2s. Moreover, TGF-β induces Nrp1 expression in lung ILC2s further enhancing ILC2s functions by increasing ST2 expression. Abbreviations: CHILP, common helper-like innate lymphoid progenitors; DN, double negative; DP, double positive; IEL, intraepithelial lymphocyte; ILC1, group 1 innate lymphoid cell; ILC2p, ILC2 precursor; iNKT, invariant NK T; NK, natural killer; TAK1, TGF-β-activated kinase 1; TCR, T cell receptor. Figure adapted from images created with [BioRender.com](https://www.biorender.com).