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Transforming growth factor–β1 in regulatory T cell biology

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Abstract

Transforming growth factor–β1 (TGF-β1) is inextricably linked to regulatory T cell (T_{reg}) biology. However, precisely untangling the role for TGF-β1 in T_{reg} differentiation and function is complicated by the pleiotropic and context-dependent activity of this cytokine and the multifaceted biology of T_{regs}. Among $CD4^+$ T cells, T_{regs} are the major producers of latent TGF-β1 and are uniquely able to activate this cytokine via expression of cell surface docking receptor glycoprotein A repetitions predominant (GARP) and αv integrins. Although a preponderance of evidence indicates no essential roles for T_{reg}-derived TGF-β1 in T_{reg} immunosuppression, TGF-β1 signaling is crucial for T_{reg} development in the thymus and periphery. Furthermore, active TGF-β1 instructs the differentiation of other T cell subsets, including T_H17 cells. Here, we will review TGF-β1 signaling in T_{reg} development and function and discuss knowledge gaps, future research, and the TGF-β1/T_{reg} axis in the context of cancer immunotherapy and fibrosis.

INTRODUCTION

Transforming growth factor–β (TGF-β) was discovered biochemically in the 1970s in an attempt to isolate soluble factors that induce anchorage-independent growth or transform normal cells (1, 2). The high-affinity receptors of TGF-β1 (TGF-βRI/RII) were discovered shortly thereafter (3, 4). Human TGF-β cDNA was cloned in 1985 (5), followed immediately by cloning of mouse TGF-β cDNA (6). The successful cloning of TGF-β cDNA facilitated a boon to genetic studies and rapid unraveling of its roles in biology, including cancer, fibrosis, and immune tolerance. In the early 1990s, using loss-of-function genetic approaches, two groups independently reported that TGF-β1 deletion in mice resulted in extensive multiorgan inflammation and early death (7–9). The similarity of this phenotype to mice with major defects in regulatory T cells (T_{regs}) sparked an intense and

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ongoing interest in the intersection between T_{reg} and TGF-β1 biology. Figure 1 uses a timeline to outline key findings that helped establish the critical roles of TGF-β1 in immune tolerance and T_{reg} biology.

The biogenesis, activation, and signaling of TGF-β1 are complex [reviewed by (10)]. Nearly all cells can produce latent TGF-β1 (LTGF-β1), which can be secreted but requires activation before it can act in an autocrine or paracrine manner. Furthermore, because of the ubiquitous expression pattern of TGF-β receptors and their signaling modules, most cells can respond to active TGF-β1. Proper development of thymus-derived T_{regs} (tT_{regs}) (11) and induced T_{regs} (12) in the periphery requires active TGF-β1 signaling. In addition, TGF-β1 instructs the differentiation of other T cell subsets [e.g., T helper 17 (T_H17) cells] (13). Intriguingly, among $CD4^+$ T cells, T_{regs} are the major producers of LTGF-β1 and are uniquely able to activate LTGF-β1 via the expression of cell surface docking receptor glycoprotein A repetitions predominant (GARP) (14–16) and αv integrins (17). These observations suggest that TGF-β1 plays important roles in T_{reg} biology. Recent discoveries underscore the importance of TGF-β1 signaling in T_{reg} biology and emphasize that the function of this cytokine is highly context dependent. Here, we will review our current understanding of TGF-β1 biology as it specifically relates to T_{regs}. We will discuss key roles that extrinsic TGF-β1 signaling plays in regulating immune responses and T_{regs} and will also discuss how T_{reg}-derived TGF-β1 modulates the environment in contexts such as cancer and fibrosis. We will address current controversies in this field and outline future directions and potential therapeutic implications of targeting this pathway for the treatment of human diseases.

MECHANISM OF ACTIVATION AND SIGNALING OF TGF-β SUPERFAMILY

TGF-β1 biogenesis

The TGF-β family of ligands consists of three isoforms (TGF-β1, TGF-β2, and TGF-β3) that signal via TGF-β receptors (RI and RII) and co-receptors (such as betaglycan). These TGF-β isoforms are part of a larger superfamily, including bone morphogenetic proteins, activins, and growth differentiation factors (18). In this Review, we will focus our discussion on the TGF-β1 isoform. TGF-β1 is synthesized as a propeptide in the rough endoplasmic reticulum and is composed of the N-terminal latency-associated peptide (LAP) and the C-terminal TGF-β1 protein (Fig. 2). After initial biogenesis, two TGF-β1 propeptides combine to form a homodimer by intermolecular disulfide bonds and translocate to the Golgi, where furin performs a critical proteolytic cleavage step that separates TGF-β1 from LAP (19). Furin cleavage depends on a unique R-H-R-R sequence (20), but the two cleaved products remain noncovalently bound to each other. The resulting small latent complex (SLC) is the LTGF-β1 and is inactive because the mature TGF-β1 is held deep inside the structure to prevent its exposure and activation. TGF-β1 can be secreted extracellularly as the SLC or bound to LTGF-β–binding proteins (LTBPs) to form the large latent complex (LLC) (21). Downstream from biogenesis, the bioavailability and activity of TGF-β are controlled by multiple accessory molecules collectively called “TGF-β milieu molecules” (22), including LTBP (to anchor LTGF-β to the extracellular complex), GARP (16, 23, 24), and LRRC33

(to dock TGF-β onto the cell surface) (22) and αvβ6 and αvβ8 integrins (for activation of LTGF-β) (25).

Upon secretion, the LLC can bind to the extracellular matrix (ECM) via covalent interactions between LTBP and ECM proteins, such as fibronectin or fibrillin. Alternatively, the SLC can bind to GARP [e.g., on T_{regs}, platelets, and endothelial cells; (14–16, 26)] or LRRC33 [e.g., on macrophages and microglia; (22)]. GARP and its homolog LRRC33 are type I transmembrane cell surface docking receptors for LTGF-β, which modulate its bioavailability and downstream signaling. The binding of LTGF-β to LTBP, GARP, or LRRC33 requires disulfide bond formation with a key cysteine residue in the LAP (27, 28). The association of these complexes ultimately participates in the activation and release of TGF-β1 (29, 30). Unbound extracellular SLC may also exist, and it is sensitive to cleavage and subsequent activation by serine proteases such as matrix metalloproteinases, plasmin, and cathepsin D.

Release of mature and active TGF-β1 requires cleavage or conformational change of the SLC or LLC. Integrin-mediated activation of LTGF-β1 occurs via binding of αvβ6 or αvβ8 integrin heterodimers, expressed on specialized cells including T_{regs}, to tripeptide Arg-Gly-Asp motifs in the LAP. Such an interaction triggers a contractile force that unfastens the “straitjacket” conformation of LTGF-β1 liberating the active form (25, 31, 32). αvβ8 integrin was recently shown by cryo–electron microscopy to activate LTGF-β1 through a mechanism that does not require release of the mature TGF-β1 for subsequent signaling (33). One study also suggests that thrombin-mediated cleavage of GARP can activate LTGF-β1 from the cell surface GARP–LTGF-β1 complex (34).

TGF-β1 signaling cascade

Active TGF-β1 binds to TGF-β receptor II (TGF-βRII) on target cells, which recruits TGF-β receptor I (TGF-βRI) to create a heterotetrameric receptor complex. Binding and activation of this complex leads to phosphorylation of the cytoplasmic tails, which can lead to signaling via the canonical (Smad) or noncanonical pathways. Within the canonical signaling pathway, Smad2/3 phosphorylation results in the binding to Smad4 to create a signaling complex that translocates to the nucleus and induces downstream changes in gene expression. Upon nuclear translocation, the Smad complex binds to other transcription factors (e.g., NFAT and activating protein 1 in immune cells) or chromatin-remodeling proteins (e.g., histone acetylases) to regulate gene expression [e.g., Foxp3 in T_{regs}; (35)]. TGF-β1 can also signal via a plethora of noncanonical Smad-independent pathways [reviewed in (36)], including via the mitogen-activated protein (MAP) kinase pathway, which signals via extracellular signal–regulated kinase 1/2 or TGF-β–activated kinase 1 (TAK1).

TGF-β1 SIGNALING IN T_{REG} DEVELOPMENT AND FUNCTION

The majority of T_{regs} develop in the thymus (tT_{regs}) from CD4 single-positive T cell progenitors (37). However, some immunoregulatory T cells arise via conversion of mature $CD4^+$ conventional T cells in peripheral tissues [peripherally derived T_{regs} (pT_{regs})] (38, 39). While the importance of cell-intrinsic TGF-β1 signaling in tT_{reg} development and

homeostasis is not fully resolved, this cytokine is required for pT_{reg} induction (39). The underlying molecular mechanisms of this pathway have long been exploited in vitro to convert conventional T cells into a suppressive population [in vitro induced T_{regs} (iT_{regs})], which resemble in vivo T_{regs}. Manipulation of these cells presents a unique opportunity for developing immunoregulatory adoptive cell therapies (12, 40). Below, we discuss the relative contribution of TGF-β1 signaling to the biology of both tT_{regs} and peripheral T_{regs} (described in Fig. 3A).

TGF-β1 is a cryptic driver of thymic T_{reg} development

Foxp3 is a master T_{reg} transcriptional regulator whose expression first appears in CD4 single-positive thymocytes localized to the thymic medulla (41, 42). These T cell progenitors receive signals required for T_{reg} development, including T cell receptor (TCR)–mediated recognition of peptide/major histocompatibility complex II complexes, CD28 costimulation, and a variety of soluble cytokine cues, especially interleukin-2 (IL-2), IL-7, and IL-15. Active TGF-β1 is highly enriched in the medulla, and its expression temporally correlates with neonatal waves of thymocyte negative selection and T_{reg} differentiation. It is thought to be released in response to thymocyte apoptosis (43, 44). While accumulating evidence supports a role for TGF-β1 signaling in T_{reg} development, the molecular details remain poorly defined. Notably, TGF-β1 signaling negatively regulates medullary thymic epithelial cells (mTECs), which instruct T cell development (42, 45). Ablation of TGF-βRII in mTECs led to their expansion and enhanced function and ultimately increased production of tT_{regs} (45). This inhibitory role for TGF-β1 signaling in the thymic medulla illustrates the nuanced, complicated, and sometimes opposing pathways regulated by this highly pleiotropic cytokine.

Early in vivo experiments using animals with global TGF-β1 knockout (KO) or T cell–specific TGF-βRII deletion described the differential requirements for TGF-β1 by peripheral and thymic T_{regs} (46, 47). The loss of either TGF-β1 or TGF-βRII from T cells precipitated a substantial reduction of T_{regs} in the peripheral lymphoid organs of mice older than 8 days but induced no defect in tT_{regs}, with equivalent or higher numbers produced by KO mice (46–48). Supporting these experiments, T cell–specific deletion of TGF-β1 signaling proteins Smad2 and Smad3 results in mild expansion of tT_{regs}, whereas pT_{regs} are reduced (49, 50). These findings implied that TGF-β1 signaling is dispensable for tT_{reg} generation; however, subsequent analysis of neonate mice with T cells lacking TGF-βRI found a profound tT_{reg} deficiency. Thymic T_{reg} numbers recovered as the mice aged but correlated with increased levels of thymic IL-2 and proliferation of the remaining T_{regs}, suggesting that increased IL-2 production rescued the tT_{reg} phenotype. Co-deletion of IL-2 and TGF-βRI prevented tT_{reg} rebound (11). A later study confirmed the early loss of tT_{regs} in a TGF-βRII deletion model, which appeared to be due to a cell survival benefit that TGF-β1 sensing bestows on developing T_{regs}. TGF-βRII–deficient tT_{regs} underwent increased apoptosis, which correlated with reduced expression of the anti-apoptotic protein B-cell lymphoma 2. Double deletion of TGF-βRII and pro-apoptotic Bim rescued the defect in tT_{reg} generation (51). On the basis of these results, the authors proposed a model where TGF-β1 protects the tT_{reg} lineage from thymic negative selection and promotes survival and maturation (51,

52). However, TGF-β1 broadly protects thymocytes and other lymphocytes from apoptosis, indicating that this pathway is not specific to T_{reg} development (52–55).

Strikingly, tT_{regs} from *Foxp3*-Cre *Tgfbr1*$^{fl/fl}$ mice, which lose TGF-βRI expression only after Foxp3 induction, do not exhibit any defects at the neonatal or adult stage (43). Thus, TGF-β1 signaling appears to have a direct impact on commitment to the tT_{reg} lineage, which may be more important than its role in attenuating thymic negative selection. Intrathymic transfer of TCR-transgenic Foxp3^{-} CD4 single-positive uncommitted thymocytes into wild-type mice resulted in Foxp3 expression (43, 56). Blocking TGF-β1 signaling or using TGF-βRI–deficient cells prevented induction of Foxp3. Polyclonal double-negative thymocytes transferred into wild-type mice did not commit to tT_{reg} lineage if donor cells were TGF-βRI deficient (43). In vitro differentiation experiments also support a direct role for TGF-β1 in tT_{reg} development and Foxp3 expression because costimulation with TGF-β1 and common γ chain cytokines synergistically induced Foxp3^{+} cells (43).

Although these data suggest that TGF-β1 induces tT_{reg} development via Foxp3 signaling, the underlying molecular pathways remain undetermined. Foxp3 expression in CD4^{+} T cells is driven by conserved noncoding DNA sequence elements (CNS0 to CNS3) at the *Foxp3* locus (57). CNS1 contains binding sites for TGF-β1–responsive transcription factors that induce *Foxp3*, including NFAT and Smad3 (38, 58–60). However, although CNS1 is required for the generation of peripheral and induced T_{regs} (38, 58–60), in vivo competition experiments demonstrated equivalent numbers of CNS1-sufficient and CNS1-deficient Foxp3^{+} neonatal thymocytes (38). Therefore, TGF-β1–induced Foxp3 expression in tT_{regs} may occur through a noncanonical pathway, which does not require direct Smad binding to the *Foxp3* locus and may occur independently of Smad signaling altogether (36, 52). For example, TGF-β1 can activate MAP kinase signaling, including TAK1, which has been associated with thymic T cell development (36, 61, 62). In addition, TGF-β1 may repress negative regulators of T_{reg} fate, such as was recently hinted in deletion studies of the transcription factors MAZR and hematopoietically-expressed homeobox (63, 64).

TGF-β1 signaling is required for T_{reg} induction

Unlike its complicated role in tT_{reg} development, TGF-β1 is known to synergize with other signals to induce Foxp3 expression in mature CD4^{+} T cells. This signaling cascade induces CD4^{+} T cells to display immunoregulatory properties reminiscent, although not identical, to those of tT_{regs} (39, 65). The ability of TGF-β1 to endow naive T cells with suppressive function was first demonstrated in vitro using CD4^{+} T cells derived from human blood that were stimulated with irradiated allogeneic peripheral blood mononuclear cells in the presence or absence of TGF-β1. The suppressive cell fraction highly expressed CD25; however, the authors concluded that these suppressor cells were derived from existing CD25^{+} cells rather than converted from CD25^{-}CD4^{+} T cells (40). These findings were extended in a landmark paper that identified this phenomenon in vivo and determined that it was modulated via *Foxp3* expression (12). This aspect of TGF-β1 biology is of particular clinical interest because it opens the possibility of large-scale in vitro iT_{reg} generation for cell therapy applications (66, 67). In addition, pT_{regs} tend to be enriched and functionally

active in peripheral tissues, such as the gastrointestinal tract, where their augmentation could provide a strategy for antigen-specific targeting of inflammatory disease (38, 39, 66, 68, 69).

Induction of pT_{regs} is dependent on TGF-β1–mediated activation of Smad3. Binding of Smad3 at CNS1 in the *Foxp3* locus promotes *Foxp3* transcription, leading to acquisition of a T_{reg} phenotype (58–60). Intriguingly, CNS1 is only present in placental mammals; during pregnancy, pT_{regs} accumulate in the placenta, suggesting that this pathway evolved as a means to maintain maternal-fetal tolerance (59). TGF-β1 may also promote Foxp3 expression through CNS1-independent pathways. TGF-β1–activated STAT5 can demethylate CNS2, accessibility of which is critical for stable *Foxp3* gene expression (39, 70). Conversely, TGF-β1 signaling represses methylation by sequestering Uhrf1 from the nucleus (71). Recently, a posttranslational mechanism was also identified wherein TGF-β1 activation of TAK1 causes Nemo-like kinase to phosphorylate Foxp3 and protect it from proteasomal degradation (72). In addition, other factors such as moesin in $CD4^+$ T cells in the tumor microenvironment (TME) augment pT_{reg} induction through up-regulation of TGF-βRII (73).

Although TGF-β1 synergizes with other components of the in vivo milieu to generate pT_{regs}, these signaling factors do not guarantee that a T cell will commit to a regulatory lineage. By inducing *Foxp3*, TGF-β1 may allow integration of TCR signaling and effectively broaden the range of cognate ligand affinities favorable for conversion to a regulatory state but not necessarily enforce it (74). Interestingly, mechanisms do exist to prevent peripheral T cells from converting to pT_{regs}. Although naïve T cells readily express Foxp3 in response to TGF-β1, differentiated effector and memory populations resist conversion (75). T_H cytokines produced by these cells actively interfere with the potency of TGF-β1 (75). Poly(rC) binding protein 1, an RNA binding protein involved in posttranslational repression of TGF-β1 signaling, attenuates pT_{reg} induction (76). Nonetheless, secondary signals may be able to overcome such resistance, such as programmed cell death protein 1 (PD-1) ligation on T_H1 cells or combined rapamycin/retinoic acid stimulation of T_H2 cells (77, 78). As TGF-β1 mediates differentiation toward both the T_H17 and T_{reg} fate, experiments comparatively dissecting how this choice is made are instructive toward understanding how these elements coalesce in vivo. For example, metabolic requirements that drive differentiation and bias against glycolysis and mitochondrial respiration support T_{reg} differentiation (79). Similarly, bile acid metabolites enriched within the gastrointestinal tract promote T_{reg} differentiation. The metabolite isoallolithocholic acid directly increased T_{reg} induction in a TGF-β1–dependent mechanism involving CNS3, whereas another metabolite (3-oxo-5β-cholanoic acid) inhibited T_H17 differentiation (80).

The cellular and tissue context required for pT_{reg} generation and a precise molecular picture of how TGF-β1 signaling transactivates the Foxp3 promoter remain to be elucidated. A caveat in harnessing iT_{regs} for cell therapy is that TGF-β–induced Foxp3 expression in human T cells does not necessarily correlate with suppressive function (81). Although both in vitro and in vivo humanized mouse experiments demonstrate that Foxp3-expressing cells can be generated, confirming that suppressive activity will be essential for the development of clinically useful products (82, 83). A notable drawback to iT_{regs} is that they lack the functional stability of their in vivo counterparts (84). This is a critical issue

for the therapeutic potential of these cells as reversion to an effector phenotype after adoptive transfer has been reported (85). A specific difference between natural and in vitro induced subsets is the level of methylation at the CNS2 region (84). The demethylated state exhibited by tT_{regs} and pT_{regs} allows for efficient recruitment of transcription factors to the *Foxp3* locus and suggests activity of TGF-β1 synergizing factors (39, 86, 87). For example, signaling induced by IL-2, vitamin C, and rapamycin all lead to CNS2 demethylation and can augment iT_{reg} stability, although less potently in human T cells (88–91). Other molecules, such as retinoic acid, also enhance TGF-β1–induced T_{reg} stability but, apparently, through methylation-independent mechanisms (92). Nonetheless, given the substantial evidence linking pT_{reg} generation to peripheral organs (38, 39, 66, 68, 69), deciphering how tissue-specific signals interact with TGF-β1 signaling will be fundamental for developing strategies to modulate T_{regs} during inflammatory disease.

An unassuming role for TGF-β1 signaling in T_{reg} homeostasis and function

Although TGF-β1 signaling is required for T_{reg} induction, accumulated evidence does not suggest a central role for this cytokine in T_{reg} maintenance or function. Instead, TGF-β1 signaling likely fine-tunes T_{reg} function by modulating specific molecular pathways contextual to local tissue and inflammatory environments. One-year-old *Foxp3*-Cre *Tgfbr1*$^{fl/fl}$ mice do not exhibit overt inflammation or any defect in T_{reg} abundance in lymphoid organs, lung, and colon lamina propria (93). These observations are largely in agreement with earlier studies using a tamoxifen-inducible Cre-ERT2 system to delete *Tgfbr2* in peripheral $CD4^+$ T cells. After tamoxifen administration, increased T_{reg} proliferation and accumulation were noted in the spleen and lungs but not in the colon lamina propria or Peyer's patches, suggesting tissue-specific modulation of the T_{reg} compartment. Studies using mixed bone marrow chimeras indicated that T_{reg} expansion was a cell-intrinsic response and *Tgfbr2*$^{-/-}$ T_{regs} proliferated more robustly after anti-CD3 stimulation in vitro (94). Therefore, TGF-β does not appear to control T_{reg} maintenance under homeostatic conditions but may restrain T_{reg} proliferation in response to TCR signaling.

Although *Foxp3*-Cre *Tgfbr1*$^{fl/fl}$ mice do not exhibit overt inflammation over time, proinflammatory T_H17 cells accumulate within the colon lamina propria and skin. More T_H17 but fewer T_H1 cells infiltrated the central nervous system during experimental autoimmune encephalomyelitis, and T_{regs} from *Foxp3*-Cre *Tgfbr1*$^{fl/fl}$ were unable to control inflammation in a T cell transfer model of colitis (93). *Tgfbr1*-deficient T_{regs} demonstrated enhanced suppression of T_H1 cells in vitro but were less effective at restraining T_H17s (93). Mechanistically, the loss of TGF-β1 responsiveness led to increased expression of the T_H1-defining transcription factor T-bet, which facilitates T_{reg} suppression of type 1 inflammation (93, 95). In agreement with this imbalance in T_H1 and T_H17 control, TGF-β1 has been shown to induce expression of the transcription factor c-Maf (69). Generation of Rorγt$^+$ T_{regs} requires c-Maf, and these T_{regs} play a key role in maintaining homeostasis of the intestinal microbiota through T_H17 regulation (69, 96). In addition, *Foxp3*-Cre *Tgfb1*$^{fl/fl}$ animals with TGF-β1–deficient T_{regs} have a high frequency of interferon-γ–producing $Foxp3^+$ cells. In vitro culture assays indicated that autocrine TGF-β1 down-regulates IL-12 receptor genes to prevent IL-12–driven acquisition of a T_H1 phenotype (97). TGF-β may

also modify T_{reg} activity via metabolic regulation because signaling through this pathway has been associated with reduced expression of genes involved in glycolysis (98, 99).

TGF-β1 has important nonimmune functions in wound healing and barrier tissue homeostasis (100, 101). Given this tissue-centric biology, the gastrointestinal, skin, and central nervous system effects associated with loss of TGF-β signaling in T_{regs} hint at a broader role in tuning T_{reg} function within peripheral organs. Notably, T_{reg}-specific deletion of TGF-βRI altered expression of several molecules known to regulate immune cell trafficking and retention in gut tissues, including G protein–coupled receptor 15 and the integrin CD103 (93). Accordingly, *Tgfbr1*-deficient T_{regs} were unable to accumulate within the colon or control intestinal inflammation in a T cell transfer model of colitis (93). As CD103 also contributes to the persistence and function of $CD8^+$ tissue-resident memory T cells (Trm) in the skin, it is possible that TGF-β1 signaling broadly licenses T_{reg} activity at epithelial sites through modulation of integrin expression (102–104). However, when we deleted *Tgfbr2* in T_{regs}, these mice developed no immediate quantitative or qualitative defects in the skin (105). Long-term experiments will be necessary to determine whether TGF-β1 controls T_{reg} tissue homeostasis and function in the same manner as $CD8^+$ Trm (103, 106).

T_{REG}-DERIVED TGF-β1 IN T_{REG} FUNCTION

A lasting debate: Evidence for and against T_{reg}-derived TGF-β1 as an important mediator of immune tolerance

Our understanding of the immunological roles of TGF-β1 signaling started almost 30 years ago with the discovery that ubiquitous depletion of TGF-β1 from mice causes a severe inflammatory phenotype with fatal multiorgan insufficiency (7–9). Since then, there have been many studies to uncover the significance of TGF-β1 and its signaling cascade in conventional T cell and T_{reg} biology using different genetic mouse models (11, 46, 47, 49, 107–120) (Fig. 1). Unlike the well-defined roles of TGF-β1 and its signaling cascade in the establishment of pT_{regs}, the roles of endogenous TGF-β1 in the biology and function of committed T_{regs} remain controversial. TGF-β1 is produced in large quantities by T_{regs} (121), which has been speculated to be responsible for T_{reg}-suppressive function, a hypothesis reinforced by the discovery that T_{regs} can potently activate TGF-β1 via cell surface GARP and αv integrins. (Fig. 3B). However, despite the rationale of this premise, conflicting reports exist regarding the relative contribution of T_{reg}-produced TGF-β1 to their suppressive function. Here, we discuss the current controversy and provide clarity surrounding the roles of T_{reg}-derived TGF-β1 in immunosuppression. In essence, T_{reg}-intrinsic TGF-β1 is not required for the homeostatic suppressive function of T_{regs}.

The support for TGF-β1 to mediate T_{reg} immune suppression initially came from transfer colitis experiments. Shortly after T_{regs} were identified as the $CD4^+CD25^+$-expressing cell subpopulation with crucial immune suppressive properties, it was reported that their ability to prevent colitis induced by the transfer of naive $CD45RB^{high}$ $CD4^+$ T cells into immunodeficient mice was TGF-β1 mediated. This was based on the observation that the protective effect that they conferred over colitis was lost when TGF-β1 was neutralized with anti–TGF-β1 monoclonal antibodies (122, 123). In a follow-up study using mice whose

T cells are unresponsive to TGF-β1 signaling due to expressing a dominant-negative TGF-βRII (dnTβRII), T_{regs} fail to rescue the colitis phenotype (124). Incongruously, $CD4^+CD25^+$ T_{regs} purified from TGF-$\beta1^{-/-}$ mice showed no loss in their suppressive potential compared with T_{regs} from wild-type mice despite the lack of endogenous TGF-β1. However, the authors hypothesized that the TGF-$\beta1^{-/-}$ T_{regs} relied on exogenous TGF-β1 to induce their suppressive function. In line with the earlier reports, Nakamura *et al.* (125) showed that T_{regs} purified from TGF-$\beta1^{-/-}$ mice failed to rescue colitis in vivo, whereas they demonstrated normal capacity to block T cell proliferation in vitro. TGF-β1 has also been reported to be indispensable for T_{reg}-mediated immunosuppression in the context of cancer because T_{regs} suppressed wild-type but not dnTβRII tumor-specific $CD8^+$ T cells (126). When Li *et al.* (110) generated the first T cell–specific TGF-β1 KO mice, they showed that T_{regs} from these mice had decreased capacity to rescue T cell–induced colitis, although in vitro suppressive activity remained intact. To examine the role of T_{reg} produced TGF-β1 in their suppressive capacity, Pesu *et al.* (111) generated a mouse with T cell–specific deletion of furin, which is involved in the biogenesis of TGF-β1. T_{regs} from these mice were inferior at suppressing colitis compared with wild-type T_{regs}.

However, the literature on the roles of T_{reg}-derived TGF-β1 in their suppressive function has not always been supportive of its relevance. Various reports from experiments using similar settings to those described above, from anti–TGF-β1 neutralizing antibodies in vitro to various loss-of-function genetic mouse mutants, yield contradictory results. Early reports indicated that T_{regs} maintain their suppressive activity in vitro, even in the presence of anti–TGF-β1 neutralizing antibodies or genetic deletion (TGF-$\beta1^{-/-}$) (127–129). Similarly, piccirillo *et al.* (129) showed that neutralizing TGF-β1 in vitro was not sufficient to alter their suppressive activity, as was the case with T_{regs} isolated from *Tgfb1*$^{-/-}$ mice. They further reinforced this finding by using target T cells from either *Smad3*$^{-/-}$ or dnTβRII mice, both unable to respond to TGF-β1, which, however, were subject to suppression by T_{regs} as efficiently as the wild-type T cells. When the same *Smad3*$^{-/-}$ or dnTβRII T cells were used to induce colitis in vivo, the disease was still able to be rescued by T_{regs}, although the T cells were unable to respond to TGF-β1 (129, 130). Akin, TGF-β1–nonproducing T_{regs}, purified from *Tgfb1*$^{-/-}$ mice, were able to prevent T cell–induced colitis in vivo, indicating that they sustain their suppressive activity despite the lack of autocrine TGF-β1 (130, 131).

In most of the aforementioned studies, the role of T_{reg}-derived TGF-β1 in suppressive function was assessed either indirectly using target T cells that could not respond to TGF-β1 or directly using TGF-β1–nonproducing T_{regs} from *Tgfb1*$^{-/-}$ mice (110, 124, 125, 129–131). Gutcher *et al.* (112) generated the first T_{reg}-specific TGF-β1 KO mouse model, wherein T cells lose TGF-β1 production only after they commit to the T_{reg} fate. These mice remained healthy with no signs of autoimmune disease until late adulthood, providing the first concrete evidence that self-tolerance can be established and maintained in the absence of T_{reg}-produced TGF-β1. However, the technique by which they conditionally delete TGF-β1 from T_{regs} raises some concerns regarding the efficiency of TGF-β1 deletion from the progeny. Because of the importance of understanding the essence of the debate, some technical details regarding how the TGF-β1 KO mice were generated are provided here. Briefly, their mutant mice have both exon 1 of *Tgfb1* and exon 4 of the adjacent gene *B9d2* flanked with loxP (110). Although they rescued B9D2 expression by introducing

a *Tgfb1*-null allele with the intact *B9d2* locus, their T_{reg}-specific TGF-β1 KO mice are still B9d2 heterozygous because of the fact that the other KO allele contains a 5′ loxP site upstream of exon 4 of *B9d2*. When these *B9d2*^{exon4} *Tgfb1*^{exon1} floxed mice were later used to study the biological roles of B9D2, Town *et al.* (132) found that TGF-β1 was still produced upon Cre recombination. This expression resulted from an in-frame chimeric mRNA transcript that formed after excision of *B9d2* exon4 and *Tgfb1* exon1. A subsequent mouse model generated with a loxP-flanked *Tgfb1 exon6* allele was predicted to delete the active form of TGF-β1 only in Cre-expressing cells while sparing adjacent genes (133). However, the two loxP sites in these mice, deposited to the Jackson Laboratory, were later found to have reverse orientation, which was confirmed by our independent study (119). Although it is unclear whether this genetic anomaly occurred in the founder mice or represents genetic drift later during the propagation of this colony, such an anomaly makes these mice unreliable for the conditional deletion of *Tgfb1*. Therefore, until recently, as described below, it was unclear whether these models accurately represent TGF-β1 deletion from T_{regs}, and better genetic tools for the conditional deletion of TGF-β were needed.

Turner *et al.* (118) recently revisited the roles of T_{reg}-produced TGF-β1 in T_{reg} biology using the same *Tgfb1exon6* mice mentioned above. Mice with two loxP-flanked *Tgfb1exon6* alleles developed severe autoimmunity when crossed with mice that expressed Cre recombinase from the T_{reg} cell–specific *Foxp3* locus (*Foxp3*$^{YFP\text{-}cre}$), which led to their conclusion that T_{reg}-derived TGF-β1 is indispensable for self-tolerance (118). This conclusion was based on the presumption that *Tgfb1* is deleted from the Cre-expressing Foxp3$^+$ T_{regs} in the *Foxp3*$^{YFP\text{-}cre}$ *Tgfb1exon6*$^{fl/fl}$ progeny. However, a study from our group demonstrated that these mice develop autoimmunity because of a germline genetic lesion and not because their T_{regs} do not produce TGF-β1 (119). Briefly, we provided evidence that an inverted 3′-loxP site in the mice used by Turner *et al.* leads to chromosomal 7 abnormalities during Cre recombination, which ultimately render the T_{regs} highly apoptotic. Therefore, these mice develop severe autoimmunity because of T_{reg} depletion, similar to the phenotype seen in Scurfy mice (134–136). This was further supported by the fact that the phenotype of these mice was not rescued when crossed with *Tgfb1* knock-in mice, which have T_{regs} with restored TGF-β1 (119). We also showed that an alternative T_{reg}-specific TGF-β1 KO mouse strain generated by targeting exon2 of *Tgfb1* remained healthy with no signs of autoimmunity despite the complete absence of TGF-β1 from T_{regs} (119). Choi *et al.* (120) similarly provided evidence against the importance of T_{reg}-derived TGF-β1 in T_{reg}-mediated immunosuppression. By comparing T_{regs} from the *Tgfb1*^{exon1} floxed mice described earlier with wild-type T_{regs} (110), they performed in vitro and in vivo experiments to assess the functionality of T_{regs} in the absence of endogenous TGF-β1. They found no evidence for compromised T_{reg} function despite reliable deletion of TGF-β1 (120).

Together, this evidence favors the notion that T_{reg}-mediated immunosuppression appears largely impervious to the loss of endogenous TGF-β1 under baseline conditions. despite the initial premise and seemingly conflicting reports in the literature, we believe that the current debate has been settled that T_{reg}-derived TGF-β1 is dispensable for T_{reg}-mediated immune suppression and that self-tolerance can be maintained in the absence of T_{reg}-produced TGF-β1. This could simply be due to the unique ability of T_{regs} to activate LTGF-β1 in a paracrine fashion via GARP and αv integrin (discussed later), a hypothesis that we

are actively studying. However, the potential significance of T_{reg}-derived TGF-β1 under pathological conditions such as infection, autoimmunity, and cancer remains to be fully elucidated.

Role of LTGF-β1 bound to the surface of T_{regs}

Activated T_{regs} can store LTGF-β on their surface bound to GARP (14, 16, 23, 24, 121). The release of mature TGF-β1 from the LTGF-β/GARP complex on T_{reg} surface is primarily mediated by αv integrins, allowing the binding of active TGF-β1 to its receptor. In the gut, αv integrins expressed by a subset of $CD103^+$ dendritic cells have been reported to mediate TGF-β1 activation and iT_{reg} induction (137). Recently, it was reported that GARP-bound TGF-β1 can activate its receptor even without being dissociated from the complex (33). GARP can also be proteolytically cleaved by thrombin to release active TGF-β1 (34), a process for TGF-β1 activation that might be preferentially used by platelets compared with T_{regs} (26). Nonetheless, the role of the surface-bound LTGF-β in T_{reg} function has not been fully elucidated. Consistent with T_{reg}-specific deletion of TGF-β1, mice with conditional GARP ablation from T_{regs} successfully establish self-tolerance. However, these T_{regs} show reduced accumulation in the colon, which ultimately leads to enhanced T cell immunity and better control of azoxymethane/dextran sodium sulfate–induced colon cancer development (115).

T_{regs} themselves also express machinery for activating LTGF-β and may use this pathway to modulate immune function during active inflammation, tissue injury, and cancer. Both mouse and human T_{regs} have been reported to express the integrin αvβ8, which interacts with GARP to release mature TGF-β1 (17, 114). Deletion of αvβ8 on T_{regs} did not cause spontaneous autoimmunity but did interfere with the ability of these cells to ameliorate a T cell transfer model of colitis (114). Likewise, monoclonal antibodies that prevent TGF-β1 activation by targeting the GARP:TGF-β1 complex inhibited T_{reg}-mediated immunosuppression in a xenogeneic graft-versus-host disease model (138) and stimulated anticancer immunity (139, 140). We recently observed that specific deletion of αvβ8 on T_{regs} impaired skin responses to epithelial injury by repressing keratinocyte-driven innate inflammation. Disruption of this circuit exposed mice to uncontrolled bacterial infection through a compromised skin barrier (105). Therefore, although the ability of T_{regs} to produce LTGF-β per se is not a major contributor to self-tolerance during homeostasis, the regulatory mechanism of T_{regs} to active LTGF-β produced by neighboring cells in a paracrine fashion is likely important under specific disease and tissue conditions. Although speculative, it remains possible that it is the ability of T_{regs} to produce and activate LTGF-β that matters in immune tolerance. The proof of this hypothesis has to come from future creative strategies such as loss-of-function studies to examine the impact of simultaneous deletion of LTGF-β and its activating machinery from T_{regs}.

CLINICAL CONTEXT OF TGF-β1 SIGNALING IN T_{REG} BIOLOGY

Because of their ability to be induced by, produce, activate, and respond to TGF-β1, T_{regs} have long been implicated as key players in a wide spectrum of human diseases. Here, we will discuss the current knowledge on the role of T_{reg}/TGF-β1 axis in two major

pathological states, related to both immunological and nonimmunological roles of T_{regs}: fibrosis and cancer.

Fibrosis

Fibrosis is characterized by dysregulation of ECM deposition leading to gross disruption in tissue architecture and ultimately organ dysfunction. As an insidious component of a large number of diseases, fibrosis results in an enormous clinical burden. TGF-β1 is strongly implicated in the pathogenesis of fibrotic disorders because it can activate fibroblasts and drives their conversion into myofibroblasts. TGF-β1 also acts on many other cell types, including immune cells to establish a profibrogenic local environment (141). Therapeutic blockade of TGF-β1 in patients with systemic sclerosis decreased fibrotic biomarkers, highlighting the clinical relevance of this cytokine (142).

The role of T_{regs} in fibrosis has been controversial because they have been reported to both promote and control fibrosis across a variety of tissues and disease models. Experimental T_{reg} ablation worsened skin, lung, and liver fibrosis (143–145). Moreover, T_{reg} infiltration into the livers of chronically infected patients with hepatitis C correlated with reduced fibrotic pathology (146). In murine skin, T_{regs} are uniquely poised to control T_H2 immune responses (143, 147). Recently, it was shown that this function of skin T_{regs} results in an overall regulation of fibroblast activation and tissue fibrosis induced by T_H2 cytokines, both in the steady state and after treatment with bleomycin (143). It remains to be determined whether this is a unique feature of skin T_{regs} or whether this occurs in other tissues. Furthermore, it is currently unknown whether TGF-β1 biology plays any role in this process. In all the studies mentioned above, T_{regs} were found to restrain fibrotic disease by suppressing pathologic tissue inflammation, including through direct dampening of T_H1, T_H2, and T_H17 activity (143–145). Conversely, other studies observed improved fibrosis after T_{reg} depletion (148–150). This inconsistency may be due to the quality of T_{reg} activity in fibrotic tissues. T_{regs} exhibiting a proinflammatory and profibrotic phenotype reportedly accumulate in heart tissue after myocardial infarction. T_{reg} ablation and subsequent reconstitution generated cells with normal suppressive capability (150). Similarly, T_{reg}-specific deficiency of the costimulatory molecule CD226 exacerbated renal fibrosis secondary to their acquisition of a T_H2 phenotype (151). Therefore, local features within some fibrotic lesions may polarize T_{regs} toward a profibrotic phenotype.

It remains unclear how TGF-β1 contributes to the balance between pro- and antifibrotic T_{reg} function. However, pT_{regs} accumulate in and attenuate bleomycin-induced lung fibrosis after in vivo TGF-β1 stimulation (152). Therefore, secondary signals in the milieu of certain fibrotic lesions may co-opt pT_{reg} induction to generate unstable or pathogenic T_{regs}. Although TGF-β1 produced by T_{regs} could also contribute to fibrogenesis, the typically potent ability of these cells to suppress other profibrotic immune cells is likely dominant. In addition, T_{regs} in fibrotic tissues may act as TGF-β sinks and sequester this cytokine through either the TGF-β receptor or GARP (143). Together, it is likely that augmentation of bona fide T_{reg} activity will have therapeutic benefit in fibrotic disorders.

Cancer

The contribution of TGF-β1 to immune evasion during cancer is well known and has been recently reviewed elsewhere (153). It is also known that TGF-β1–rich stroma reduces the efficacy of immune checkpoint inhibitors (CPIs) due to restrained intratumoral T cell trafficking (154, 155). Importantly, rich infiltration of the TME by T_{regs} is the rule rather than the exception, which is a key roadblock for effective cancer immunotherapy (156). On the one hand, understanding how tumor-associated iT_{regs} are generated and maintained by TGF-β1 and how T_{regs} adopt to the TME and tolerize the tumor-specific immune responses holds promise for the design and development of next-generation immunotherapeutics. On the other hand, although T_{regs} are not the sole source of TGF-β1 within the TME, they are critical drivers of the TGF-β1 pathway. It was recently shown that αvβ8 integrin–expressing T_{regs} activate LTGF-β1 produced by tumor cells, promoting cancer immune evasion (157). Tumor cells and platelets positively regulate the local TGF-β1 levels and function via GARP (26, 158). Intratumoral TGF-β1 converts conventional T cells into pT_{regs}, which increases the T_{reg} pool in the TME and ultimately increases local TGF-β1 production through secretion and activation of LTGF-β via surface GARP (153, 158). As described in Fig. 4, despite some favorable roles of TGF-β1 in immunity (159), TGF-β1 signaling and T_{reg} activation for the most part negatively influence many aspects of adaptive and innate immunity, contributing to immune evasion, immunotherapy resistance, and tumor progression. For example, the TGF-β1/T_{reg} axis blunts the cytotoxic activity of $CD8^+$ cells (160), blocks T_H1 cells (161), and promotes tolerogenic function of dendritic cells (162). Moreover, TGF-β1 signaling potently suppresses the functions of natural killer cells by repressing the mammalian target of rapamycin pathway (163). It also polarizes macrophages toward a tumor-promoting M2 phenotype (164, 165). Last, the TGF-β1/T_{reg} axis has also been implicated in promoting oncogenesis and resistance to CPI therapy via enhancing the activity of cancer-associated fibroblasts (166).

However, targeting TGF-β1 therapeutically has proven to be challenging, partly because it is daunting to design a strategy to block TGF-β1 function in the cell type–specific fashion at the right place and the right time. The discovery of the GARP:TGF-β1 axis potentially provides a unique therapeutic approach to fine-tune TGF-β1 activation locally, avoiding the systemic side effects of global TGF-β1 deletion. Monoclonal antibodies that prevent active TGF-β1 release by blocking the GARP:TGF-β complex on T_{regs} have been shown to inhibit immunosuppression in a xenogeneic graft-versus-host disease model (138). The same anti-GARP targeting strategy seems to also stimulate anticancer immunity by eliminating the T_{reg}-mediated TGF-β1 activation in the TME, which contributes to immunotherapy resistance (139, 140), and is now being tested in a phase 1 clinical trial for the treatment of various cancers (NCT03821935). Similarly, other anti-GARP antibodies, which block the interaction of free T_{reg} GARP with LTGF-β1, could be also used to control local TGF-β1 levels by inhibiting the presentation and, ultimately, activation of LTGF-β1 by T_{regs} derived from other cellular sources (158). Furthermore, the expression of GARP on activated T_{regs} creates an opportunity to target GARP using chimeric antigen receptor–expressing T cells for not only GARP-expressing tumors but also the immunosuppressive T_{regs} in the TME. Future research is expected to define the exact place of GARP:TGF-β targeting strategies within the therapeutic arsenal against human diseases.

Other cancer therapeutic strategies targeting TGF-β1 pathway either alone or in combination with existing modalities include but are not limited to blocking integrins (139, 167), depleting LTGF-β pool systemically (168), $CD4^+$ T cell–specific TGF-β targeting using a bifunctional anti-CD4 antibody–TGF-β trap (4T-Trap) (169, 170) and others [reviewed in (171)]. Notably, bifunctional therapies combining anti–PD-L1 monoclonal antibodies and a TGF-β1 "trap" showed promising results in preclinical models and early-phase clinical trials (172, 173). However, this strategy suffered several setbacks including a failed phase 3 trial to demonstrate superiority over PD-1 blockade alone against non–small cell lung cancer. It is the high hope that continuing fundamental research coupled with innovative drug development strategies will eventually make the TGF-β pathway druggable for cancer.

CONCLUSIONS AND FUTURE PERSPECTIVES

The discovery of T_{regs} led to a paradigm shift in our understanding of how the immune system functions and maintains homeostasis. We now know that the immune system is always "on," reacting to both self and foreign antigens. It is effectively dampened by regulatory cells including T_{regs} that are also always on, preventing most individuals from developing chronic inflammatory and/or autoimmune diseases. Cancer diverts these regulatory mechanisms to prevent antitumor immunity, and pathologic fibrosing conditions may result from defects in regulatory cells that normally suppress profibrotic immune responses and subsequent fibroblast activation. T_{reg} biology and TGF-β1 biology are inextricably linked. iT_{regs} require TGF-β1 for their induction from naïve $CD4^+$ T cells, are capable of secreting large amounts of LTGF-β1, and highly express the molecular machinery to activate LTGF-β1 to act back on themselves or to signal to other neighboring cells. These tenets are widely accepted and supported by numerous experiments. Everything else regarding T_{reg} and TGF-β1 biology is not entirely clear. Given the multitude of cells that can express and/or activate this cytokine and the even larger number of cells that can respond to it, dissecting the functional consequences of any individual cell type activating or responding to TGF-β1 in a complex tissue environment has been difficult. T_{regs} have been no exception. Emerging data suggest that these cells use TGF-β1 to mediate their functions, but this is highly context dependent and is influenced by the tissue, the type of inflammation, and possibly the type of T_{regs}. In addition, it may be only in rare circumstances that T_{reg} utilization of TGF-β1 is not redundant with other cells using this cytokine in the local tissue environment. Teleologically, this redundancy makes sense, given the overall importance of the TGF-β1 pathway in preventing systemic inflammation and mortality. New transcriptome profiling platforms at the single-cell level have recently advanced our perception of T_{reg} diversity and functional heterogeneity, especially in the nonlymphoid sites. As cutting-edge omics technologies continue to evolve, it is very likely that distinct TGF-β1 signatures identified within specific T_{reg} subpopulations will help define the tissue and disease contexts where the T_{reg}–TGF-β1 axis plays a dominant role. The goal of future research will undoubtedly be to develop therapeutic strategies to selectively augment or inhibit this axis. Despite its complexity, this is an extremely exciting area of translational investigation, with the first hints of clinical efficacy just beginning to emerge.

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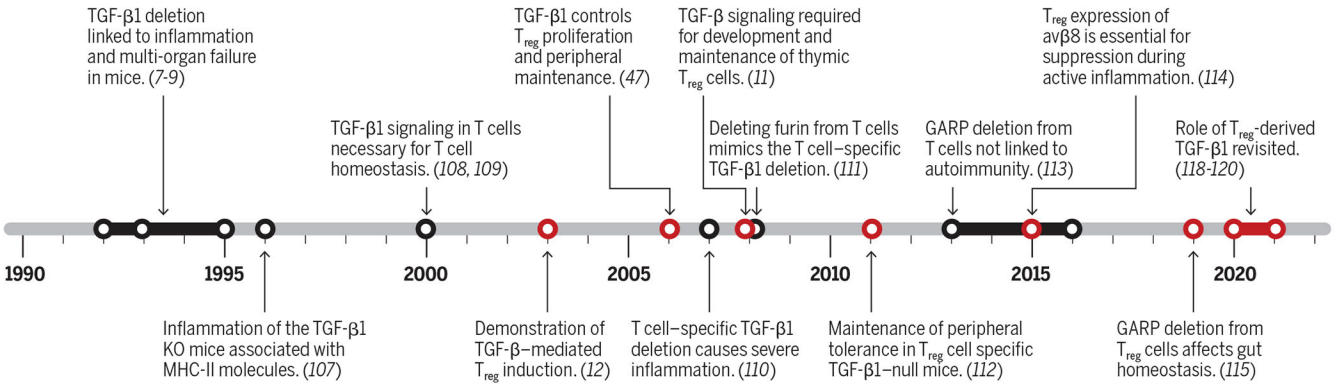

Fig. 1. Timeline of notable discoveries on the role of TGF-β1 and TGF-β1 signaling in the biology of T cells and T_{regs}.

Work on the roles of TGF-β in T_{reg} biology is highlighted in red.

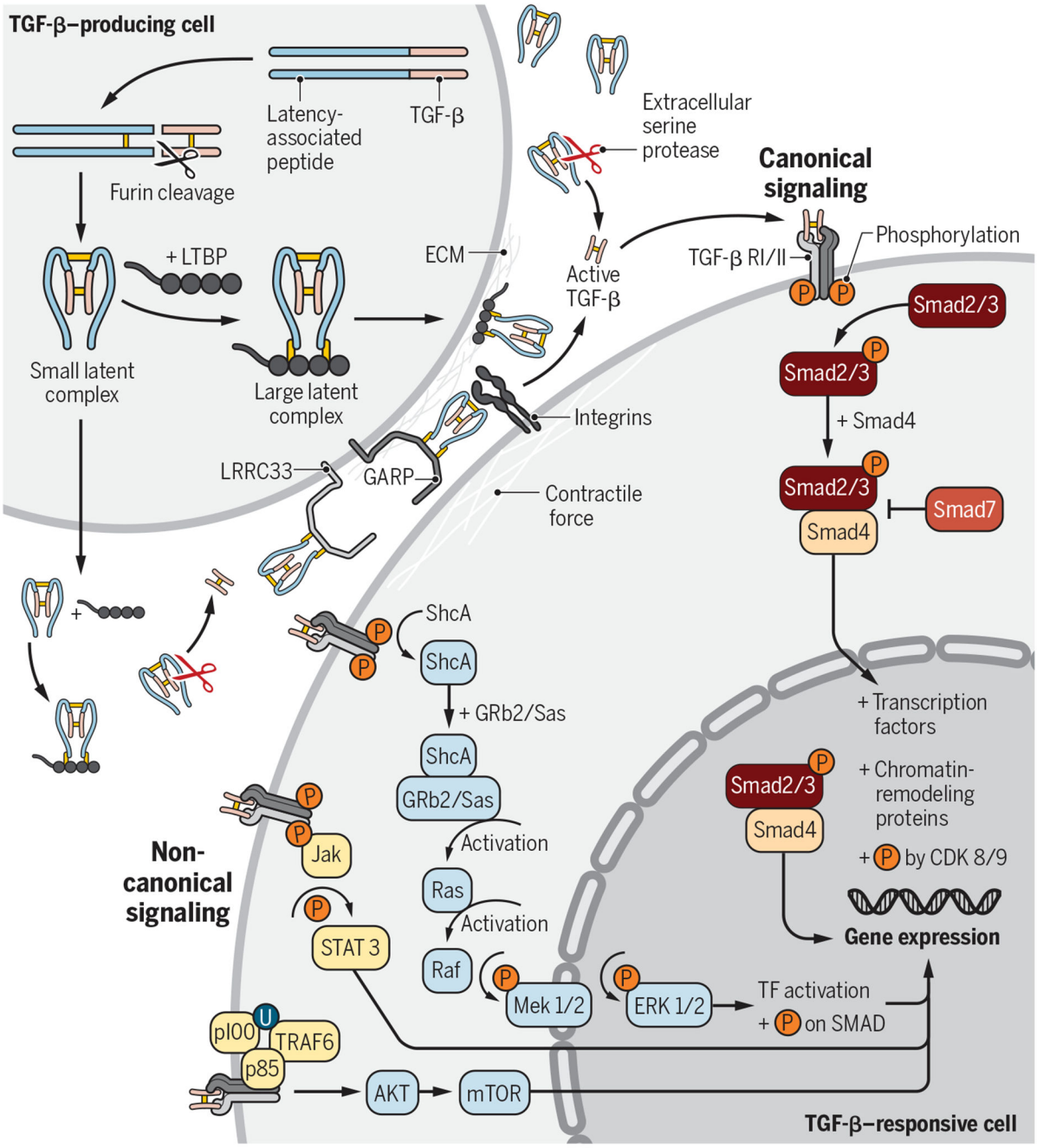

Fig. 2. TGF-β1 biogenesis, activation, and signaling.

TGF-β1 can be produced and secreted by a number of cell types, including T_{regs}. LTGF-β1 can be secreted in the SLC or LLC form. Upon secretion, the LLC can bind to fibronectin or fibrillin in the ECM. The SLC can bind to cell surface GARP (e.g., on T_{regs}, platelets, and endothelial cells) or LRRC33 (e.g., on macrophages and microglia). Activation of LTGF-β1 requires cleavage or conformational change of the SLC or LLC, which releases the mature TGF-β1 from the complex or exposes its active binding motif. Active TGF-β1 binds to TGF-βRII on target cells, which recruits and activates TGF-βRI intracellular domain.

Phosphorylation of the TGF-β RI cytoplasmic tails leads to activation and signaling via the canonical (Smad-dependent) or noncanonical pathways. All signaling pathways regulate downstream gene expression. TF, transcription factor.

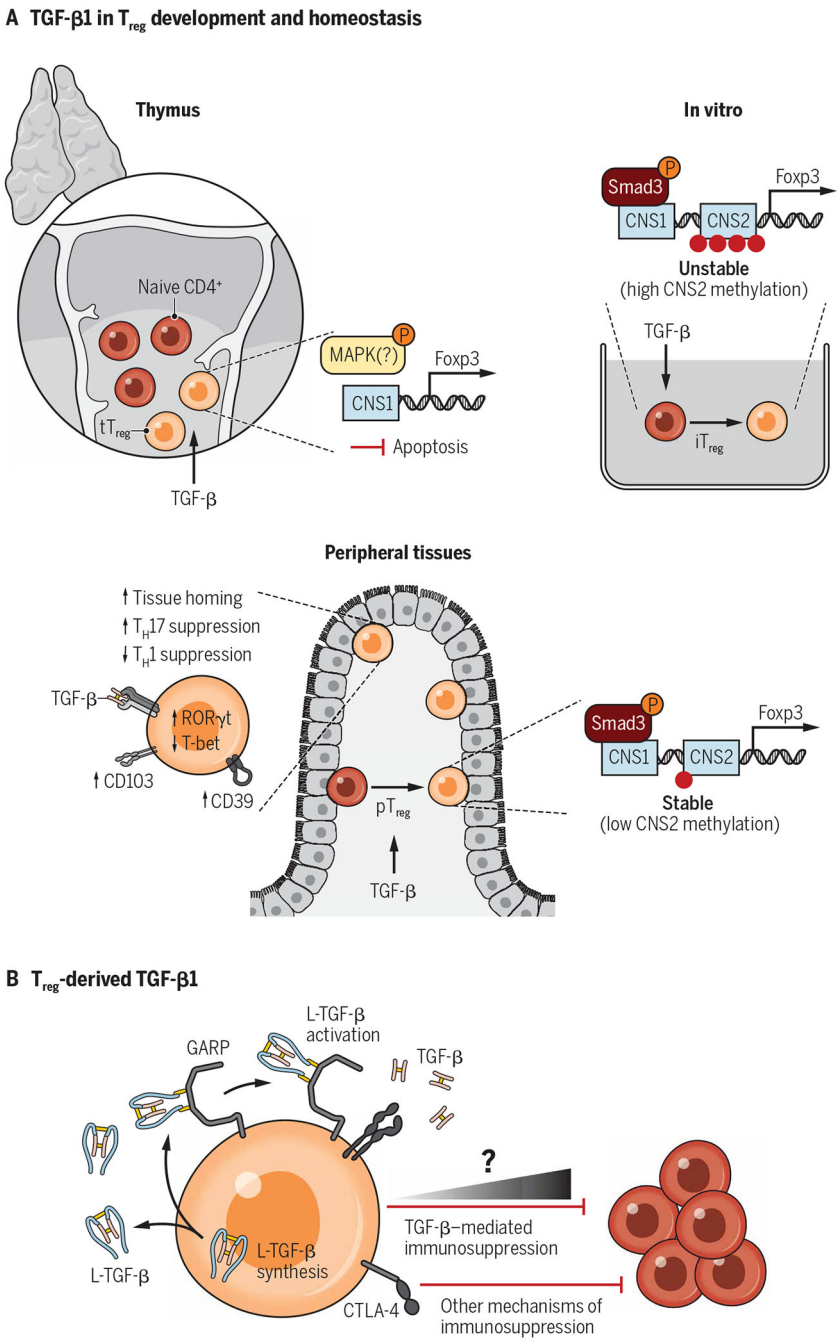

Fig. 3. An integrated view of TGF-β1 in T_{reg} biology.

TGF-β1 is uniquely poised to be a focal point in T_{reg} biology because T_{regs} highly express the TGF-β receptor, are major producers of latent TGF-β1, and have their activating machinery. (**A**) Signaling through receptor dimers of TGF-βRI and TGF-βRII is a requirement for both thymic T_{reg} development and induced T_{reg} differentiation. (**B**) T_{reg}-derived TGF-β1 has long been hypothesized to have a major role in T_{reg} function as these cells are a source of latent TGF-β1 and express both GARP and αvβ8 integrin, which work in tandem to activate TGF-β1. Accumulating evidence suggests that T_{reg}-produced

TGF-β1 contributes to immunoregulation but is one of several mechanisms in the T_{reg} arsenal. Although not critical to homeostatic function, T_{reg} generation of bioactive TGF-β1 is likely to have tissue- and context-specific immunomodulatory activity.

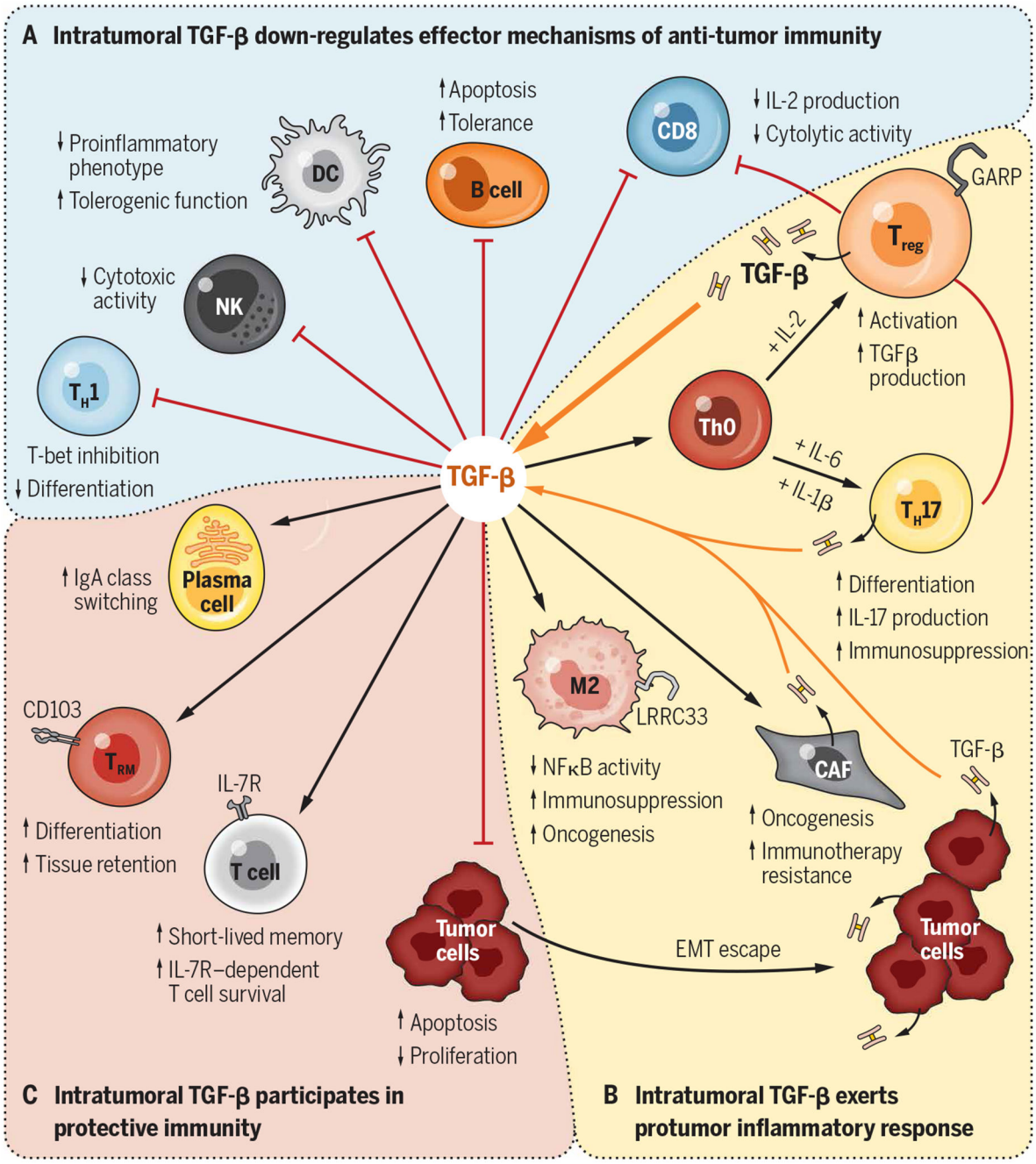

Fig. 4. Contributions of TGF-β1 to immune evasion and immunity in cancer.

The pleiotropic roles of TGF-β1 within the TME include (**A**) dampening the antitumor immune responses, (**B**) enhancing protumorigenic responses, and (**C**) promoting other elements of protective immunity through interactions with various cell types. Key functional aspects of these interactions are depicted. DC, dendritic cell; NK, natural killer; IgA, immunoglobulin A; NFκB, nuclear factor κB.