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Review

TGF-β: the sword, the wand, and the shield of FOXP3$^+$ regulatory T cells

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Since its rediscovery in the mid-1990s, FOXP3$^+$ regulatory T cells (Tregs) have climbed the rank to become commander-in-chief of the immune system. They possess diverse power and ability to orchestrate the immune system in time of inflammation and infection as well as in time of harmony and homeostasis. To be the commander-in-chief, they must be equipped with both offensive and defensive weaponry. This review will focus on the function of transforming growth factor-β (TGF-β) as the sword, the wand, and the shield of Tregs. Functioning as a sword, this review will begin with a discussion of the evidence that supports how Tregs utilize TGF-β to paralyze cell activation and differentiation to suppress immune response. It will next provide evidence on how TGF-β from Tregs acts as a wand to convert naïve T cells into iTregs and Th17 to aid in their combat against inflammation and infection. Lastly, the review will present evidence on the role of TGF-β produced by Tregs in providing a shield to protect and maintain Tregs against apoptosis and destabilization when surrounded by inflammation and constant stimulation. This triadic function of TGF-β empowers Tregs with the responsibility and burden to maintain homeostasis, promote immune tolerance, and regulate host defense against foreign pathogens.

Keywords: Foxp3, TGF-β, LAP, LRRC32, GARP, Tregs, autoimmunity, tolerance, transplantation

Introduction

Since the rebirth of suppressor cells in the mid-1990s, there has been a relentless search to identify the weapon of choice that Tregs trigger to maintain peace and prevent riots (Sakaguchi et al., 1995; Shevach, 2011). Tregs appear to be the perfect superhero, possessing all types of power such as secretion of immunosuppressive cytokines [interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and IL-35], delivery of cytotoxic molecules (granzymes and perforin), conversion of molecules to immunosuppressants (CD39, CTLA-4), and consumption of vital cytokines [IL-1, IL-2, and tumor necrosis factor-α (TNF-α)] (Tang and Bluestone, 2008; Sakaguchi et al., 2009; Shevach, 2009). While the focus on TGF-β as the dominant mechanism of Treg-mediated suppression remains controversial, it appears that Tregs are equipped with many different backup weapons and use them for different circumstances. The crusade to prove that TGF-β is the Holy Grail and the heart of Treg power continues because of the similarity in phenotype and disease manifestation between mice that have defects in TGF-β pathway and those that are deficient in Tregs. Three issues remain unresolved regarding the purpose of TGF-β produced by Tregs in: (i) Treg-mediated suppression, (ii) infectious tolerance, and (iii) maintenance of function and stability. This review will provide updated evidence and data to address these issues and the difficulty and complexity that perpetuate this unsolved mystery. However, this quest is not endless and there might be land over the horizon.

TGF-β, the jack of all trades and mastery of all

TGF-β exists in three isoforms (β1, β2, and β3) with TGF-β1 being most common in the immune system. It was first discovered for its ability to induce anchorage-independent growth (Roberts et al., 1981). Thirty years later there is still so much to learn and discover about this incredible cytokine. It is amazing how extensive and pleiotropic TGF-β impacts biological processes such as embryogenesis, morphogenesis, tissue repair, and cell differentiation to aberrant neoplastic development. With regard to the immune system, TGF-β plays a complex and intertwined role in inflammation, T cell lineage commitment, antibody generation, immune suppression, and tolerance. TGF-β is critical for the development and differentiation of FOXP3$^+$ regulatory T cells (Tregs) (Dardalhon et al., 2008a, b). An enhancer element in the *Foxp3* locus has been identified that interacts with Smad3 and NFAT for induction of Foxp3 expression (Tone et al., 2008). TGF-β also is essential for the generation of IL-17 producing T helper cells (Th17) (Kozawa et al., 1997; Reape et al., 2008; Volpe et al., 2008). Most recently, evidence indicates that TGF-β is involved in the generation of IL-9 producing T helper (Th9) cells (Dardalhon et al., 2008a, b; Veldhoen et al., 2008).

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TGF-β signaling begins with high-affinity binding to TGF-β receptor II (TGF-βRII), a type II Ser/Thr kinase receptor. This receptor then phosphorylates and activates a second Ser/Thr kinase receptor, TGF-β receptor I (TGF-βRI) (Heldin et al., 1997). This receptor complex phosphorylates and activates Smad proteins 2 and 3, resulting in phosphorylation-dependent conformational changes to allow heteromerization with SMAD4 (Huse et al., 2001). This SMAD complex then translocates into the nucleus to regulate transcription (Shi and Massague, 2003; Wahl, 2007). Given its potency and diverse action, TGF-β has to go through another level of activation to mediate its effect, unlike other cytokines that are produced in an active form. Prior to binding to TGF-βRII, TGF-β must be converted to its active form. TGF-β is translated as pre-pro-TGF-β consisting of the signal peptide, latency associated peptide (LAP), and mature TGF-β peptide (Figure 1). Post-translational modifications in the endoplasmic reticulum result in the cleavage of the mature TGF-β peptide from LAP by a furin convertase. The homodimeric LAP non-covalently wraps around homodimeric mature TGF-β to form latent TGF-β (LTGF-β). This latent form is unable to bind to its receptor until proteolytic cleavage or structural modification to cause a conformational change to LAP that exposes TGF-β. There are multiple mechanisms of activating TGF-β from its latency by pathways that include acidification, protease, plasmin, matrix metalloproteases, thrombospondin-1, and certain α_v integrins (Lawrence, 2001; Annes et al., 2003; Li and Flavell, 2008). However, it is unclear how LTGF-β is activated *in vivo*. Three forms of TGF-β have been identified: (i) small latent form (LTGF-β) which is TGF-β associated with LAP, (ii) large latent form which consists of small latent form covalently linked with latent TGF-β binding protein (LTBP), and (iii) membrane latent form (mLTGF-β) which contains small latent form associated with glycoprotein A repetitions predominant (GARP) also known as leucine rich repeat containing 32 (LRRC32) (Stockis et al., 2009; Tran et al., 2009a, b; Figure 1).

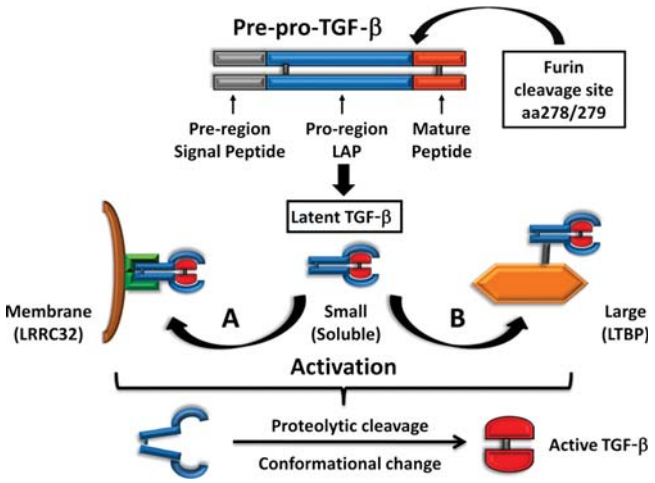


Figure 1 Schematic of the different forms of latent TGF-β. After translation, the pre-pro-TGF-β is modified in the endoplasmic reticulum and cleaved by a furin convertase to release mature TGF-β peptide from LAP. To protect TGF-β activity, LAP non-covalently binds active TGF-β to form a complex called latent TGF-β (small form). Latent TGF-β can be associated with (**A**) LRRC32 (membrane form) for transportation and anchorage to the surface membrane of activated Tregs or it can be linked covalently to (**B**) LTBP (large form) for secretion into the extracellular matrix. All three forms are latent and require activation by either proteolytic cleavage or conformational change to open LAP and liberate the active TGF-β.

Is TGF-β the sword of Tregs that severs cell activation and differentiation?

Since the resurrection of Tregs, there has been a persistent hunt to capture the secret sword that has been rumored to obliterate all forces with one single swing. This quest is vital to validate their legitimacy as suppressor cells. TGF-β became the primary focus because of its potent effect on inhibition of immune response, particularly T cell proliferation and differentiation (Kehrl et al., 1986; Wahl et al., 1988; Gorelik and Flavell, 2002; Li and Flavell, 2008). With that recognition sprung the first two studies that critically linked TGF-β with Treg suppressor function (Read et al., 2000; Nakamura et al., 2001). Read et al. (2000) first demonstrated, using an *in vivo* mouse model of colitis, that the immunosuppressive effect of Tregs could be abrogate with anti-TGF-β and anti-cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) antibodies (Abs), indicating the importance of TGF-β and its association with CTLA-4. Next, Nakamura et al. (2001) used *in vitro* experiments to show that Treg suppression was cell contact-dependent and mediated by mLTGF-β, which could further be enhanced by costimulation with CTLA-4. It was the very first study to demonstrate that TGF-β could be bound to the cell surface. A subsequent study came out that year by Piccirillo et al. (2002) challenging the notion that TGF-β was the dominant mechanism of Treg suppression. In that study, the investigators used purified Tregs from *TGF-β1*$^{-/-}$ mice to test their *in vitro* suppression and observed no difference when compared with wild-type (WT) Tregs. A major issue with *in vitro* suppression assay and the variability among different studies could be due to multiple real and *in vitro* effects such as IL-2 consumption and the absence of mechanisms for converting LTGF-β to active form. Therefore, those two studies ignited a heated debate and the search for the Holy Grail continued. However, given the similarities in phenotype consisting of lymphoproliferation, autoimmunity, and death within 3–5 weeks of age among *TGF-β1*$^{-/-}$, *TGF-βRI*$^{-/-}$, and *TGF-βRII*$^{-/-}$ mice when compared with scurfy and *Foxp3*$^{-/-}$ mice that are deficient in Tregs, the focus on TGF-β as the sword that mediates Treg suppression resumes (Shull et al., 1992; Gorelik and Flavell, 2000; Brunkow et al., 2001; Leveen et al., 2002; Fontenot et al., 2003; Li et al., 2006; Marie et al., 2006; Liu et al., 2008).

A major problem with those previous studies was the difficulty in identifying who was the knight and who was the pawn of TGF-β. Even if TGF-β is the weapon of choice, it remains unresolved whether Tregs mediate their suppression of T cell activation by stabbing dendritic cells (DCs) or directly at the T cells (Tran and Shevach, 2009; Tran et al., 2009a, b). Two studies attempted to address this issue by generating T cell-specific deletion of *TGF-βRII*, resulting in complete ablation of TGF-β signaling in T cells (Li et al., 2006; Marie et al., 2006). In both studies, the mice developed early onset fulminant multiorgan autoimmune

destruction and died by 3–5 weeks of age. Due to the massive activation of T cells and the dramatic decrease in Tregs in the periphery, both studies reconstituted these mice with WT TGF-βRII sufficient Tregs to assess whether they could control the autoimmunity. Because the presence of abundant WT Tregs was unsuccessful in controlling the disease, both studies concluded that the activation of these pathogenic T cells was not related to the lack of Tregs but instead was cell-intrinsic and autonomous due to absence of TGF-β regulation. However, both studies failed to demonstrate or rule out the possibility that due to the absence of TGF-βRII on T cells, the Tregs could not mediate their suppression and regulation via production of TGF-β. The mixed bone marrow chimera experiment by Marie et al. (2006) suggested that this mechanism was plausible since WT Tregs were capable of controlling TGF-βRII sufficient but not TGF-βRII deficient T cells in these chimeric mice. Li et al. (2007) subsequently generated mice with T cell-specific deletion of *TGF-β1*. Interestingly, instead of the aggressive autoimmunity and early lethality in germline *TGF-β1*$^{-/-}$ mice, these mice appeared healthy until 4 months of age when they started having progressive autoimmunity that led to death beginning at 6 months of age. In this study, the *TGF-β1*$^{-/-}$ Tregs were as suppressive as WT Tregs *in vitro*. However, similar to the results of Powrie et al. (1996) that used neutralizing TGF-β Abs, the co-transfer of *TGF-β1*$^{-/-}$ Tregs and WT naïve CD4$^+$ T cells into *Rag1*$^{-/-}$ mice resulted in severe colitis in contrast to co-transfer with WT Tregs. This experiment demonstrates convincingly that Treg-mediated suppression of colitis *in vivo* requires their production of TGF-β1. It also illustrates the discrepancy between *in vivo* and *in vitro* mechanisms of Treg suppression. The critical function of TGF-β in Treg-mediated suppression is also reiterated in mice with T cell-specific deletion of *furin* which produces a proprotein convertase that is critical for processing TGF-β to its latent form (Pesu et al., 2008). As early as 7–9 weeks old, these mice had increased numbers of activated, memory-like T cells in their periphery. By 6 months of age, these mice started developing a progressive wasting disease with systemic autoimmunity. Similar to the *TGF-β1*$^{-/-}$ Tregs, the *furin*$^{-/-}$ Tregs failed to suppress colitis when co-transferred with WT naïve CD4$^+$ T cells into *Rag1*$^{-/-}$ mice. To go a step further, Gutcher et al. (2011) published a successive study where *TGF-β1* gene was specifically deleted from Foxp3 expressing Tregs by crossing their floxed *TGF-β1* mice with *Foxp3-cre* transgenic mice. Interestingly, these mice appeared healthy and displayed no evidence of autoimmunity even at the age of 9 months. Instead, they observed an increase in the frequency and number of Tregs in the lymph nodes and concluded that the role of the TGF-β1 produced by Tregs was to regulate their proliferation. At a steady state, these mice did not spontaneously develop colitis or have an increase in the frequency of IFN-γ-producing Th1 cells. Unfortunately, they did not perform the same colitis experiment to assess whether these Tregs also were not protective against colitis. It would be informative to test these mice under disease conditions such as cancer or chronic infection model to assess the importance of TGF-β1 production only in Tregs.

Given the level of TGF-β produced by Tregs, particularly the unique membrane bound form, it is quite shocking that the lack of TGF-β production specifically in the Tregs resulted in no autoimmune or inflammatory phenotype. The results from Flavell's group would suggest that the sole purpose of TGF-β production by Tregs is to control their homeostasis, although there is a contradiction regarding its role in Treg-mediated suppression based on their previous results (Li et al., 2007) and the findings from other investigators (Oida et al., 2006; Gil-Guerrero et al., 2008; Wang et al., 2008a, b; Shen et al., 2011). The study by Ostroukhova et al. (2006) revealed the unique suppressive mechanism of mLTGF-β on Tregs and not soluble TGF-β by activating the Notch1–HES1 axis in target cells. Several studies have shown that Tregs can inhibit natural killer cell function in a cell contact-dependent, TGF-β-mediated process (Ghiringhelli et al., 2005; Wahl et al., 2006; Frimpong-Boateng et al., 2010). Other studies have unveiled the ability of Tregs to suppress CD8$^+$ cytotoxic T lymphocytes in a TGF-β-dependent manner (Chen et al., 2005; Mempel et al., 2006). Tregs have also been certified to suppress Th1 differentiation (DiPaolo et al., 2005; Shen et al., 2011). To address whether the TGF-β-mediated suppression of Th1 differentiation was secondary to induction of Foxp3, one study used scurfy mice deficient in Foxp3 and treated them with exogenous TGF-β to demonstrate that TGF-β could inhibit Th1 differentiation in the absence of Foxp3 expression (Takimoto et al., 2010). Thus far, it appears that TGF-β is Treg's Excalibur that mediates potent immune suppression and regulation of cell differentiation. The recent discoveries by us (Tran et al., 2009a, b) and Stockis et al. (2009) have elucidated the critical function of LRRC32 in transporting and anchoring LTGF-β to the cell membrane of activated Tregs and its importance in Treg-mediated suppressor function. It would be insightful to dissect whether this mLTGF-β plays a dominant role in suppression or maintenance of Tregs by generating mice with Foxp3-specific deletion of *Lrrc32*. Given the overwhelming evidence of the impact of TGF-β on Treg-mediated suppression of multiple cell types, it was quite surprising to discover that the generation of mice with *TGF-β1* specifically deleted from Foxp3 expressing Tregs appeared healthy. Because Flavell's group did not perform more extensive experiments with these mice and failed to fully explain their contradictory results, we are left unresolved. The Trojan horse remains standing.

Is TGF-β the wand of Tregs that converts the enemies into their fighters?

A powerful force is one that not only can halt the onslaught of destruction but can also convert it into peace and defense. While TGF-β could function directly to mediate Treg suppression of T cell activation, differentiation and proliferation, it could also function in a novel mechanism of infectious tolerance by converting naïve T cells into anergic and suppressive cells (Dieckmann et al., 2002; Jonuleit et al., 2002; Zheng et al., 2004; Qiao et al., 2007; Shevach et al., 2008). The term 'infectious tolerance' was originally introduced by Gershon and Kondo (1971) to explain the observation of suppression of naïve lymphocytes by cells with regulatory function. Subsequently this term was expanded to represent a phenomenon where suppressor cells, later shown to be Tregs, could educate naïve T cells to become suppressor cells (Qin et al., 1993; Cobbold et al., 2004). Applying sophisticated

transgenic and knockout mice, Andersson et al. (2008) convincingly demonstrated that Tregs could directly induce naïve $CD4^+$ T cells to become $Foxp3^+$-induced Tregs (iTregs) and that these iTregs possessed potent suppressive function both *in vitro* and *in vivo*. This conversion of iTregs by natural Tregs was mediated by TGF-β from Tregs, since *TGF-β1*$^{-/-}$ Tregs failed to generate iTregs. In support of this infectious tolerance mechanism, a recent study by Zheng et al. (2010) investigated whether conserved non-coding DNA sequence (CNS) elements in the *Foxp3* gene regulated the development of nTregs and iTregs. To examine the role of CNS1 in the generation of iTregs, they co-transferred WT or *CNS1*$^{-/-}$$CD4^+Foxp3^-Ly5.2^+$ T cells with WT $Ly5.1^+$ Tregs into *RAG1*$^{-/-}$ recipient mice. After 10 weeks, they assessed the induction of Foxp3 in the $Ly5.2^+$ naïve T cells and observed that CNS1 was essential for the generation of iTregs, particularly in the gut-associated lymphoid tissues. Since CNS1 contained a TGF-β-NFAT response element, the *in vitro* assay demonstrated a significant reduction in the induction of Foxp3 in naïve T cells deficient in CNS1 when stimulated with anti-CD3, TGF-β, and T-depleted splenocytes. All together, these experiments suggest the Tregs can convert naïve T cells into iTregs via TGF-β and its association with CNS1. This novel concept of infectious tolerance to explain how limited numbers of antigen-specific Tregs can efficiently exert their suppressive effect *in vivo* to control inflammation and induce tolerance is quite appealing. However, more definitive *in vivo* experiments are needed to prove this infectious tolerance mechanism by Tregs.

While Tregs are typically regarded as the peacekeeper to prevent riots, it appears that in certain situations they can send in special forces to fight a battle. To maintain peace and homeostasis, Treg function has been to arrest cell activation and differentiation. Additionally, it makes sense that Tregs possess the magic to convert T cells to become iTregs to help preserve harmony. However, it is quite surprising to learn that Tregs can empower instead of suppress naïve T cells to differentiate into effector Th17 (Lohr et al., 2006; Veldhoen et al., 2006; LeibundGut-Landmann et al., 2007; Xu et al., 2007; Vokaer et al., 2010; Pandiyan et al., 2011). The Th17 produced IL-17 cytokines that are important in host defense but have also been associated with inflammatory and autoimmune conditions (Korn et al., 2009). Given the requirement of TGF-β and the inhibitory effect of IL-2 in Th17 differentiation, it is conceivable that Tregs could promote Th17 differentiation because of their unique ability to express abundant level of TGF-β and the high-affinity IL-2 receptors (CD25) to sequester IL-2 (Scheffold et al., 2005; Pandiyan et al., 2007). The studies by Veldhoen et al. (2006) and Xu et al. (2007) used *in vitro* co-cultured conditions to illustrate that Tregs could induce naïve T cells to become Th17, and this process required the production of TGF-β by Tregs. Pandiyan et al. (2011) showed that the consumption of IL-2 by Tregs could contribute to the generation of Th17, but did not address the involvement of TGF-β from Tregs in their study. Contradictory to the contribution of TGF-β from Tregs in promoting Th17 development, Gutcher et al. (2011) used mice with *TGF-β1* specifically deleted from Foxp3 expressing Tregs and the *in vivo* experimental autoimmune encephalomyelitis (EAE) model to argue that the production of TGF-β from Tregs was dispensable for Th17 differentiation. In their studies, these mice were as susceptible to EAE and had no defect in generating Th17 when compared with littermate control. They went farther to show that the source of TGF-β1 was from the T cells that functioned in an autocrine manner. A recent study by Chen et al. (2011) also supports the findings of Gutcher et al. (2011) and Pandiyan et al. (2011) by demonstrating that Tregs promote Th17 differentiation but not maintenance *in vivo* through regulation of IL-2 and not TGF-β. Utilizing a similar strategy as Flavell et al., they generated mice with *TGF-β1* specifically deleted from Foxp3 expressing Tregs and used them as recipients for adoptive transfer of OT-II $CD4^+CD25^-$ T cells from transgenic mice expressing T cell receptor (TCR) specific for ovalbumin $OVA_{323-339}$ epitope. After immunization with $OVA_{323-339}$, there were similar frequencies of OT-II $CD4^+CD25^-$ T cells expressing IL-17 in these mice when compared with control, suggesting that Tregs were not required to produce TGF-β1 for Th17 development *in vivo*. A major potential problem in their study is that the Tregs in those mice are polyclonal while the responder cells contain TCR specific for $OVA_{323-339}$. Therefore, it is conceivable that during the immune response to OVA, the Tregs could be present in the vicinity to sequester IL-2 from OT-II T cells being stimulated by DCs in an antigen-specific manner. However, because of their lack of antigen specificity to OVA, the Tregs were not activated through their TCRs to express TGF-β. Consequently, this study could not conclusively rule out the role of TGF-β from Tregs in regulating Th17 differentiation, but it does reveal that OT-II differentiation into Th17 could receive their TGF-β signal from themselves or DCs. Shedding light on the complexity of life and the immune system, a provocative study from Ghoreschi et al. (2010) revealed that in certain conditions Th17 could develop in the absence of TGF-β but instead required IL-23. They showed that in the absence of TGF-βRI or the presence of dominant mutant TGF-βRII on $CD4^+$ T cells, Th17 could be generated *in vitro* and identified in the lamina propria of these mice due to signaling from IL-23. Most insightful was the revelation that unlike Th17 generated with TGF-β1, Th17 generated with IL-23 expressed T-bet, failed to produce IL-9 and IL-10, and were significantly more pathologic in causing EAE. Therefore, it seems that in the presence of Tregs, peace is maintained through the induction of iTregs and invasion of pathogens such as fungi and bacteria is controlled through the generation of host protective Th17; and in their absence, chaos ensue. The decision of whether to convert these responder cells into iTregs or Th17 could be secondary to the presence of certain cytokines or molecules in the environment, such as IL-2 or retinoic acid for iTregs and IL-6, IL-1, and/or IL-21 for Th17. Another potential mechanism permitting Treg's ability to control iTreg and Th17 differentiation could be dependent on the level or concentration of TGF-β expression. A study by Zhou et al. (2008) illustrates the importance of the concentration of TGF-β in selectively regulating Treg and Th17 development. Low concentrations of TGF-β favor Th17 differentiation by enhancing IL-23 receptor (IL-23R) expression, while high concentrations promote Treg differentiation by inhibit IL-23R up-regulation.

Once again, we are left with a huge bonfire and full of smoke. These mice with Foxp3-specific deletion of *TGF-β1*, which should be the ideal mice to address the function of TGF-β1 on Tregs, are providing opposite results from other studies. These mice reveal that TGF-β1 production from Tregs is not involved in the maintenance of tolerance and the induction of Th17. Instead, similar to T cell-specific deletion of *TGF-β1*, it appears that the TGF-β1 produced by Tregs functions primarily to limit their proliferation and TGF-β sources other than T cells provide their maintenance in the periphery. The controversies continue and we are unsettled. Further studies are needed to validate the results from these mice and to rectify these discrepancies.

Is TGF-β the shield of Tregs that protects them in the battlefield?

So far it appears that TGF-β plays an important role in the maintenance of Tregs, their suppressor function and the generation of iTregs and Th17. However, it has not been addressed whether mLTGF-β has a critical responsibility in Treg fitness by providing self-regenerating energy and a force field through autocrine feedback. How do Tregs protect themselves from friendly fires when homing to sites of inflammation? The studies by Marie et al. (2006) and Li et al. (2006) depict the requirement of TGF-β signaling into Tregs for maintenance in the periphery but not in the thymus during their development. Those studies were challenged by Liu et al. (2008) who examined the development of Tregs at a very early time point in mice with T cell deletion of *TGF-βRI*. They showed that at day 3 of life, there were virtually no detectable Tregs in the CD4$^+$CD8$^+$ (DN) and CD4$^+$CD8$^-$ (SP) thymocytes when compared with control. Even at days 4 and 5 of life, the SP Tregs were $<$1% compared with 2%–5% in control. The Treg numbers became similar to control by 1 week of age and died by 3–6 weeks of age from lethal inflammation, which supported comparable findings in the above studies with T cell-specific *TGF-βRII*$^{-/-}$ mice. This expansion of Tregs in the thymus after 1 week of age was dependent on IL-2. In this Cre-Lox recombination system used to generate T cell- and Foxp3-specific deletion of *TGF-β1*, *TGF-βRI*, and *TGF-βRII*, it might not be perfect so that a few cells could have expressed the targeted gene enough to allow for their selection to become Tregs prior to the gene deletion. The abundant amount of IL-2 in the thymus permitted their expansion but once departure into the periphery, their maintenance could not be sustained due to insufficient IL-2 and the lack of TGF-β signaling. It would be highly informative to analyze the TCR repertoire in these mice to assess whether it is restrictive from clonal expansion of these few Tregs. In support of the essential role of TGF-β signaling in Treg survival was the observation that mice with double knockout for *SMAD2* and *SMAD3*, critical downstream molecules of TGF-β signaling, also developed severe inflammation and died within 3–5 weeks of life (Takimoto et al., 2010). The investigators only examined the frequency of Tregs in 4-week-old mice and found similar percentages of Tregs in the thymus but dramatic reduction in the periphery when compared with littermate control. Clearly, all these studies demonstrate the importance of TGF-β signal into Tregs for their development in the thymus and maintenance in the periphery. It would be insightful to identify whether the few Tregs in the periphery of these mice are Helios$^+$ given the association of Helios with thymic-derived Tregs (Thornton et al., 2010). In the *TGF-β1*$^{-/-}$ germline mutant mice, the presence of Tregs could be secondary to compensatory expression of TGF-β2 and TGF-β3 in the thymus and periphery (Marie et al., 2005). Similarly this effect could occur in the mice with T cell- and Foxp3-specific deletion of *TGF-β1*, where the Tregs could alternatively expressed TGF-β2 and TGF-β3. We have shown that LRRC32 can also bind to TGF-β2 but not TGF-β3 to allow for its surface expression (Tran et al., 2009a, b). The compensatory membrane expression of TGF-β2 by LRRC32 in the Tregs of these mice might be adequate for their selection and maintenance but insufficient for their suppressive function.

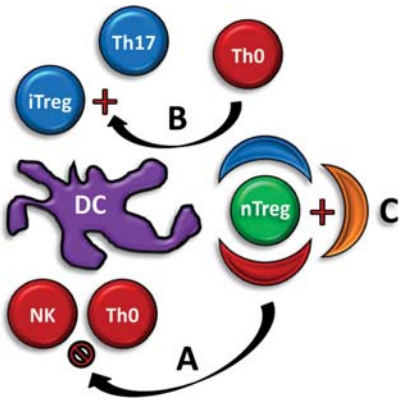
Thus far, the study by Liu et al. (2008) above is quite convincing in the argument that TGF-β signal is indispensible for Treg development in the thymus. However, it is unclear whether TGF-β is required for the induction of Foxp3 or for survival during the selection process. Even at the DP stage, there were no detectable Foxp3$^+$ cells at day 3 of life. Since the expression of TGF-β, particularly the membrane bound form, required strong anti-CD3 stimulation *in vitro*, it is possible that only those thymocytes destined to become Tregs are capable of expressing LRRC32 upon encounter with high-affinity self-antigens to deliver LTGF-β to their surface for activation and close contact autocrine signaling. We have shown that LRRC32 expression is independent of Foxp3 (Tran et al., 2009a, b). This concept makes sense since only a tiny portion of thymocytes are Foxp3$^+$. If TGF-β is so promiscuous and could be expressed by all cells or secreted in the thymus, a higher proportion of cells should be Foxp3$^+$ even if transient. In the thymus of human infants and mice, we do not detect significant membrane expression of LRRC32 and LAP in the DP and SP thymocytes (Supplementary Figures S1A and S2A). However, after 48 h stimulation of human infant thymocytes with anti-CD3/CD28 Abs, we can detect dramatic expression of membrane LRRC32 and LAP in the FOXP3$^+$ population of DP and SP cells (Supplementary Figure S1B). Since Tregs are believed to contain mostly self-reactive TCRs and therefore are constantly being stimulated and maintaining an activated phenotype, what protects them for activation-induced cell death and apoptosis (Pacholczyk et al., 2006; Rubtsov et al., 2010)? The study by Ouyang et al. (2010) investigated the function of TGF-β signal in preventing thymic negative selection to promote Treg development. Using T cell-specific *TGF-βRII*$^{-/-}$ mice, they demonstrated that TGF-β signal was important during the negative selection process to protect Tregs from apoptosis, which was mediated partly by the proapoptotic molecule Bim. Overall, it is conceivable that TGF-β signal into Tregs could play dual roles as an inducer of Foxp3 and an inhibitor of apoptosis.

Once Tregs have survived boot camp and are deployed to the battlefield, what will shield them from friendly fires and fuel them for combat? The studies with T cell-specific *TGF-βRI*$^{-/-}$ and *TGF-βRII*$^{-/-}$ mice have shed light into the vital energy source of TGF-β for their survival in the periphery. However, the outcome of those T cell- and Foxp3-specific *TGF-β1*$^{-/-}$ mice suggests that Tregs could survive without their own production of TGF-β1 and could absorb it from the environment. As mentioned before, the possibility still exists that a compensatory mechanism could have occurred that results in the expression of TGF-β2 and

TGF-β3 in the Tregs. Moreover, TGF-β can be found in the tissue and blood but it is usually in the latent form. Therefore, it is possible that *TGF-β1*$^{-/-}$ Tregs maintain their expression of LRRC32 upon activation to capture LTGF-β and compensate for their own deficiency. We have shown that LRRC32 transfected Jurkat cells expressed LRRC32 on their surface but lacked latent TGF-β and can bind exogenous LTGF-β1 and β2 but not β3 (Tran et al., 2009a, b). Other studies have suggested that LRRC32 could function in Tregs to maintain their Foxp3 expression, fitness, and suppressive activity, which would make sense since it facilitates LTGF-β expression (Wang et al., 2008a, b; Probst-Kepper et al., 2009). In the peripheral blood of humans, we are unable to identify significant expression of membrane LRRC32 and LAP on Tregs. The low level of LRRC32 and LAP in PBMCs is mostly coming from platelets attached to these cells based on the correlation with the platelet marker CD61 (Supplementary Figure S1C). Since the expression of mLTGF-β is dependent on TCR stimulation, it is conceivable that Tregs trafficking in the peripheral blood are no longer activated and those that were recently activated in the lymph nodes or tissues have already released this complex. In support of this mechanism, we can detect LRRC32^{+}LAP^{-} Tregs in the spleen and mesenteric lymph nodes of mice, suggesting that the Tregs were recently activated and have liberated the LTGF-β (Supplementary Figure S2B). However, after 48 h activation of human CD4^{+}CD25^{+} T cells, there is enormous expression of LRRC32 and LAP in the FOXP3^{high} and not the FOXP3^{low} cells (Supplementary Figure S1D). Similar observation is seen after 24 h stimulation of mouse CD4^{+} T cells (Supplementary Figure S2C). The studies by Marie et al. (2005) and Liu et al. (2008) indicated that in the absence of TGF-β signaling, Tregs have diminished Foxp3 level and suppressor function. Several studies have cautioned the vulnerability of Tregs to be brainwashed and switch side in the harsh inflammatory environment (Feuerer et al., 2009; Tsuji et al., 2009; Zhou et al., 2009a, b). Using sophisticated transgenic mice with inducible labeling and tracking of Tregs *in vivo*, Rubtsov et al. (2010) challenged the claim of Treg instability by demonstrating that they were in fact stable and anergic under physiologic, IL-2 depriving, Th1-type inflammatory, and autoimmune conditions. This discrepancy could be explained by the recognition that life is not tomato soup, so Tregs are composed of a heterogeneous population of mostly a stable lineage and a minor transitory or uncommitted subset (Bailey-Bucktrout and Bluestone, 2011; Hori, 2011). This uncommitted subset, which could be iTregs, represents versatile temporary workers hired to perform a specific task depending on the environment and therefore possess multiple skills. We are still left to ponder about the purpose of mLTGF-β on activated Tregs and whether it provides a force field to maintain their stability and prevent exhaustion from constant stimulation. With the available of Abs for surface detection of Lrrc32 on mouse and human Tregs, we can isolate the Lrrc32 positive and negative subsets to compare their potency and stability. The development of Treg therapy should be similar to those of drug manufacturing, which is to achieve the highest homogeneous purity and potency with minimal contaminations and side effects. The ability to detect, isolate and expand bona fide Tregs that possess stability and potency would be highly therapeutic with minimal potential complications for cell therapy to prevent or treat autoimmunity and transplant-related complications (Tran and Shevach, 2009). Hopefully the generation of mice with Foxp3-specific deletion of *Lrrc32* will offer us enlightenment into this complex world of Tregs and TGF-β.

Conclusions

If the notion is that the body does not waste energy without a reason and the effort spent in expressing mLTGF-β is not to serve a redundant function, then there must be an essential purpose for presenting this molecule every time Tregs get activated. In platelets, LRRC32 probably serves to retain LTGF-β at the site of injury for hemostasis and tissue healing similar to the function of LTBP for anchoring LTGF-β to the extracellular matrix (Rifkin, 2005; Macaulay et al., 2007; O'Connor et al., 2009). One confounding factor that might explain the controversies with Tregs and TGF-β is the mechanisms of LTGF-β activation. In the *in vitro* assays, depending on the condition and cell source, the mechanism of LTGF-β activation might be absence. Frequently in the *in vitro* suppression assay, the antigen presenting cells are irradiated and that process might disable the mechanism of LTGF-β activation. Moreover, the assay often uses either mouse T cell-depleted splenocytes which mostly contain B cells or human T cell-depleted PBMCs which have very few DCs. A recent study by Travis et al. (2007) has shed light on the critical role of integrin $\alpha_v\beta_8$ on DCs for activation of LTGF-β. They showed that in the absence of $\alpha_v\beta_8$ on DCs, the mice developed autoimmunity and colitis by 4–5 months of age. Using *in vitro* co-cultures, β8-deficient DCs could only induce $<$2% Foxp3^{+} cells in contrast to ∼4% from control, which overall was not that impressive. If all T cells have the potential to secrete TGF-β, should we not expect more induction of Foxp3 instead of having to add exogenous active TGF-β? Clearly *in vitro* cultures lack the mechanism of TGF-β activation, since acidification of the supernatant from activated cells is required to liberate the active form for detection. Moreover, it is possible that the TGF-β detected in the supernatant is only a tiny portion that has leaked out of the cells; and that in actuality the secretion of TGF-β is regulated by complex intracellular processes that include transporter molecules such as LTBP and LRRC32. In this study, they did not address the role of $\alpha_v\beta_8$ on DCs with regard to the activation of mLTGF-β on Tregs, particularly since TCR ligation is necessary for its expression. Their study also reveals that there are other mechanisms of TGF-β activation, since mice that have a mutation encoding an inactive RGD sequence in LAP of LTGF-β1 recapitulate the phenotype of *TGF-β1*$^{-/-}$ mice (Yang et al., 2007). This RGD region is vital for the interaction of integrins such as $\alpha_v\beta_6$ and $\alpha_v\beta_8$ to activate and liberate active TGF-β. A major breakthrough in our understanding of LTGF-β activation was the recent generation of a crystal structure from Springer's group (Shi et al., 2011). Simply binding of $\alpha_v\beta_6$ integrin to LTGF-β in solution was not sufficient to release active TGF-β. Instead the activation of LTGF-β requires anchorage of LTGF-β from one end, such as LTBP to the extracellular matrix, and integrin attachment to the cytoskeleton at the other end in order to generation a tensile force upon cellular contraction to unfold the LAP and liberate the active TGF-β. Based on their findings, it would appear that



A: Suppression	B: Conversion	C: Survival
❑ T cells ▪ Read et al., 2000 ▪ Nakamura et al., 2001 ▪ Chen et al., 2005 ▪ Mempel et al., 2006 ▪ Marie et al., 2006 ▪ Ostroukhova et al., 2006 ▪ Li et al., 2007 ▪ Pesu et al., 2008 ❑ NK cells ▪ Ghiringhelli et al., 2005 ▪ Wahl et al., 2006 ▪ Frimpong-Boateng et al., 2010	❑ iTregs ▪ Dieckmann et al., 2002 ▪ Jonuleit et al., 2002 ▪ Zheng et al., 2004 ▪ Qiao et al., 2007 ▪ Shevach et al., 2008 ▪ Zheng et al., 2010 ❑ Th17 ▪ Veldhoen et al., 2006 ▪ Xu et al., 2007	❑ Thymus ▪ Liu et al., 2008 ▪ Ouyang et al., 2010 ❑ Periphery ▪ Marie et al., 2005 ▪ Li et al., 2006 ▪ Marie et al., 2006 ▪ Takimoto et al., 2010

Figure 2 Schematic of proposed functions of LTGF-β from Tregs and references to key studies supporting these mechanisms. (**A**) Upon interaction and stimulation by the DC, the Treg expresses LTGF-β which is activated by the DC to liberate the active form of TGF-β for inhibition of DC or naïve T cell (Th0). (**B**) TGF-β could be directed at the Th0 in the presence of IL-2 for conversion into iTregs or in the presence of IL-6 or IL-1 for conversion into Th17. (**C**) LTGF-β could be activated by the DC to feedback in an autocrine manner for anti-apoptotic signal and enhancement of FOXP3 and its downstream function.

LRRC32 could serve the same function as LTBP to anchor the LTGF-β on the cell membrane for presentation and activation. However, we are left pondering about the mechanism of activation for soluble LTGF-β that is not bound to LTBP or LRRC32. A recent study has indicated that neuropilin-1 can bind and activate LTGF-β (Glinka and Prud'homme, 2008). Therefore, one possibility is that cells that are able to express unique molecules such as neuropilin-1 and LRRC32 can capture soluble LTGF-β to present for activation.

We are still in the fog with regard to the purpose of endowing Tregs with the ability to express mLTGF-β, although the fog is clearing up over time. Further knowledge is needed on the mechanisms of TGF-β activation, its processing and secretion and the phenotype of mice deficient in *Lrrc32*, both germline and Treg specific. Only then we will appreciate the impact of TGF-β produced by Tregs to regulate: (i) immune suppression, (ii) induction of iTregs and Th17, and (iii) autonomous stability (Figure 2). The generation of these *Lrrc32* knockout mice will offer potential development of therapeutic strategies to augment or abrogate Tregs by manipulating the processing, expression and activation of mLTGF-β on Tregs. These mice will also provide crucial insights into the function of LRRC32 on platelets as well as embryogenesis. So for all those enemies out there, do not underestimate the power of the almighty Treg, for in its grip is the Excalibur.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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