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The development of thymic Foxp3+ regulatory T cells: TGF-β matters

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Abstract

$CD4^+$ regulatory T cells expressing the transcription factor Foxp3 can be generated in the thymus (tTreg cells), but the cellular and molecular pathways driving their development remain incompletely understood. Transforming growth factor-beta (TGF-β) is essential for the generation of $Foxp3^+$ Treg cells converted from peripheral naive $CD4^+$ T cells (pTreg cells), yet a role for TGF-β in tTreg-cell development was initially refuted. Nevertheless, recent studies have unmasked a requirement for TGF-β in the generation of tTreg cells. Experimental evidence reveals that TGF-β in the context of TCR stimulation induces Foxp3 gene transcription in thymic Treg precursors, $CD4^+CD8^-CD25^-$ semi-mature and mature single-positive (SP) thymocytes. Intriguingly, thymic apoptosis was found to be intrinsically linked to the generation of tTreg cells, as apoptosis induced expression of TGF-β intra-thymically. In this short review, we will highlight key data, discuss the experimental evidence and propose a modified model of tTreg-cell development involving TGF-β. We will also outline the remaining unresolved questions concerning generation of thymic $Foxp3^+$ Treg cells and provide our personal perspectives on the mechanisms controlling tTreg-cell development.

Keywords

thymic Treg cells; TGF-β; apoptosis; IL-2; Foxp3

Introduction and background

During T-cell development some developing $CD4^+$ thymocytes differentiate into $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg cells), however a full description of this developmental pathway is yet to be achieved. It has become clear that thymic (t)Treg cells are largely, if not exclusively, generated in the medulla and/or at the medulla-cortex junctions in the thymus, and arise from the semi-mature and/or mature $CD4^+CD8^-$single-

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Conflict of interest

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positive (SP) thymocytes[1–3]. Although not unanimously agreed upon, it is generally considered that T-cell receptor (TCR) engagement is an essential, yet insufficient, factor for tTreg-cell development, and that additional co-stimulatory signals are required. In this regard, CD28, IL-2, and other signals via the γc receptor[4–9] have all been reported to promote acquisition of the Treg-cell fate. Several models have been proposed to explain the development of tTreg cells[10, 11], yet none manage to provide an explanation accounting for all aspects of tTreg-cell generation. Amongst these models, the suggestion that high-affinity T-cell receptor (TCR) signals to tTreg-cell precursors promotes tTreg-cell development remains the dominant and most popular paradigm[12]. This model suggests that tTreg-cell differentiation is initiated upon TCR recognition of high-affinity self-antigens in the thymus, and thus that TCR affinity is an intrinsic and key fate-determining factor promoting Treg-cell development in the thymus. Whilst many published reports support this model [13–16], recent studies have revealed that tTreg cells can be generated from $CD4^+$ thymocytes expressing TCRs with a wide spectrum of affinities for their cognate antigen[17, 18]. A second model proposes that tTreg-cell development can be divided into two steps, in which TCR stimulation drives the generation of $CD25^+Foxp3^-$ (GFP^-) thymocytes from $CD4^+CD8^-$ single positive precursors, which then become $Foxp3^+CD25^+$ tTreg cells in response to IL-2 [5, 19]. However, it now seems likely that IL-2 signals maintain tTreg-cell viability and protect Foxp3-expressing cells from undergoing apoptosis in the thymus [8, 20]. Interestingly, in mice tTreg cells cannot be detected in the thymus until three days after birth [20, 21]; this delayed emergence of tTreg cells cannot be explained in the context of either of these models of tTreg-cell development.

TGF-β signals have long been linked to acquisition of the Treg-cell fate, ever since our demonstration that TGF-β, in the context of TCR stimulation, could induce Foxp3 gene expression in mouse peripheral $CD4^+CD25^-$naive T cells, converting them into $Foxp3^+$ Treg cells (iTreg cells now called peripherally induced Treg cells, (pTreg cells)) capable of suppressing the proliferation of T cells [22]. This has been independently confirmed by many laboratories, and expanded to apply to human T cells, where it has been shown that IL-2 supports TGF-β-mediated pTreg-cell generation [23, 24]. In vivo, TGF-β is also required to induce naive T cells to become Treg cells in diverse experimental settings in mice [25, 26], including numerous experimental models of autoimmune disease. Although many experimental methods and approaches have been developed in an effort to generate pTreg cells, it is fair to conclude that perhaps most require TGF-β signaling. Thus, TGF-β is considered essential for the generation of Treg cells from peripheral naive $CD4^+$ T cells.

Deletion of TGF-β receptors causes a lack of initial tTreg-cell generation

In contrast to the consensus on the critical role for TGF-β in the generation of $Foxp3^+$ Treg cells from peripheral naive T cells, the function of TGF-β in the development of tTreg cells is a contentious area. It was initially thought that TGF-β played no role in tTreg-cell development, as mice in which T cells lacked expression of TGF-β receptor II were shown to have similar, or even higher, frequencies of $Foxp3^+$ tTreg cells in the adult murine thymus[27, 28]. These results would suggest that TGF-β signaling is differentially required during tTreg-cell and iTreg-cell/pTreg-cell generation. We revisited this area, examining tTreg-cell generation in neonatal T cells lacking TGF-β receptor I (from *Tgfbr1*$^{f/f}$LCK-cre$^+$

mice)[20], as it had been known that in normal mice, tTreg cells are not detected in the thymus until day 3 after birth[21, 29]. Surprisingly, we found that there was a profound deficiency in the number of tTreg cells at neonatal days 3–5 in *Tgfbr1*$^{f/f}$LCK-cre^{+} mice compared with that in littermate controls [20]. This finding indicated that TGF-β signaling in thymocytes played a critical role in the initial generation of tTreg cells. However, as was reported in the conditional TGFβRII knockout mice discussed above [27, 28], we also observed increased frequencies of tTreg cells in the thymus of older *Tgfbr1*$^{f/f}$LCK-cre^{+} mice, especially once the mice had developed systemic inflammation. Nevertheless, we subsequently showed that the enhanced frequencies of tTreg cells in the absence of TGF-β signaling were due to increased IL-2 levels in the thymi of knockout mice. The increased IL-2 drove tTreg-cell proliferation and hence increased the tTreg-cell frequencies in adult mice [20]. Consequently, it was shown that deletion of IL-2 in *Tgfb1*$^{f/f}$LCK-cre^{+} mice (*Tgfbr1*$^{f/f}$LCK-cre^{+} x IL-2$^{-/-}$ mice; called double knockout mice) abolished the tTreg-cell expansion in older mice. Indeed, both neonatal and adult double knockout mice showed a profound deficiency in the number of tTreg cells[20]. Based on these findings, a working model was proposed suggesting that TGF-β induces Foxp3 expression in thymic tTreg precursors, converting them into Foxp3^{+} tTreg cells[20]. Following tTreg-cell generation, IL-2 functioned to maintain this population, promoting the survival and expansion of Foxp3^{+} tTreg cells[8, 20]. Thus, although not fully elucidating the specific actions of TGF-β in this process, these data started to reveal a role for this cytokine in tTreg-cell generation.

Do TGF-β signals selectively protect tTreg cells from apoptosis?

The deficiency in tTreg-cell numbers in the neonatal thymi of mice in which TGF-βRII was selectively deleted on T cells, was subsequently confirmed by an independent study [30]. However, in this paper the authors suggested that the deficiency of tTreg cells in the neonatal thymus in the absence of TGF-β signaling was due to a failure of TGF-β to selectively protect tTreg cells from apoptosis [30], rather than TGF-β being required for the induction of Foxp3 expression. It is well established that TGF-β potently protects T cells from undergoing apoptosis [31], and it was demonstrated more than a decade ago that TGF-β could protect thymocytes from apoptotic cell death [32]. More recently, TGF-β was shown to protect the thymic precursors of a population of intraepithelial lymphocytes (IELs) from apoptosis [33]. These data raised questions about whether TGF-β specifically and/or selectively protected tTreg cells from death. To resolve this issue, we generated conditional knockout mice in which TGF-βRI expression, and therefore TGF-β signaling, was specifically deleted in only Foxp3-expressing cells[17]. In these mice, TGF-β signaling would be extinguished in Treg cells only following their generation (*Tgfbr1*$^{f/f}$Foxp3-cre^{+} mice). If it were the case that TGF-β signals specifically protected tTreg cells from apoptosis, one would expect to see a similar decrease in tTreg-cell frequency in the neonatal thymus of these knockout mice as that seen in the mice with conditional deletion of TGF-β receptor I on all T cells. However, neither neonatal nor adult thymi of the *Tgfbr1*$^{f/f}$Foxp3-cre^{+} mice showed any decrease in tTreg-cell frequencies[17]. These findings suggest that the role of TGF-β during tTreg-cell development is not to protect tTreg cells from apoptosis, but rather, collectively indicate that the role of TGF-β in tTreg-cell generation occurs prior to the induction and expression of Foxp3.

TGF-β signaling is required for Foxp3 gene transcription

Another step in answering the question of when and where TGF-β is involved in tTreg-cell generation was made when it was shown, in vitro and in vivo, that TGF-β is required for the induction of Foxp3 gene expression and subsequent tTreg generation[17]. In TCR transgenic systems, the intrathymic transfer of TCR-transgenic $CD4^+CD8^-$ single-positive thymocytes (containing no $Foxp3^+$ tTreg cells) into syngeneic hosts along with their cognate antigen, has been shown to result in tTreg-cell generation [34]. This system was employed to demonstrate that blockade of TGF-β by intrathymic co-administration of anti-TGF-β1,2,3 abrogates Foxp3 gene induction and subsequent tTreg-cell generation[17]. Moreover, when TCR-transgenic $CD4^+CD8^-$ thymocytes lacking TGF-β receptor I expression were intrathymically injected along with cognate peptide, these cells failed to differentiate into tTreg cells[17]. Collectively, these observations support a role for TGF-β in Foxp3 transcription and tTreg-cell generation.

A final, and perhaps more striking, piece of evidence supporting a role for TGF-β in tTreg-cell generation again came from in vivo experiments using intrathymic injection of polyclonal thymocytes [17]. Double negative (DN) thymocytes isolated from *Tgfbr1*$^{f/f}$CD4-cre$^+$ mice were intrathymically injected into syngeneic wild-type hosts, allowing the development of thymocytes unable to respond to TGF-β signals, to be assessed in a normal, un-inflamed, thymic environment [17]. DN thymocytes from *Tgfbr1*$^{f/f}$CD4-cre$^+$ mice failed to develop into tTreg cells. Taken altogether, these data establish TGF-β as an essential factor for Foxp3 induction and tTreg-cell generation in the thymus.

Linking thymic apoptosis to TGF-β production

Having established a critical role for TGF-β in the generation of tTreg cells, it was next vital to ascertain the factors controlling TGF-β production and expression in the thymus. To address this question, the expression of TGF-β in the neonatal thymus after birth was investigated.. Thymic tTreg-cell generation is temporally restricted after birth, with few tTreg cells seen until day 3 after birth [21]. Concordant with this observation, the total amounts of intrathymic TGF-β was observed to gradually increase over the neonatal period in C57BL/6 mice [17]. Interestingly, it was also noted that within the thymus, staining for active TGF-β1 was not universally distributed throughout the thymus; instead, the majority of active TGF-β1 staining was localized to the thymic medulla, a location in which tTreg-cell generation occurs [35, 36].

The positive correlation between thymic total, as well as active, TGF-β and the emergence of tTreg cells in the neonatal thymus, led to the question of what stimuli might be responsible for increased intrathymic TGF-β post birth. One largely overlooked process in tTreg-cell generation, aside from the report suggesting that TGF-β selectively protects tTreg cells from apoptosis by Ouyang et al. [25], is apoptosis; indeed thymocyte development occurs concomitant with considerable apoptosis, as thymocytes die as a result of both positive and negative selection. In an elegant study published in *Nature* in 1994, Suhr and Sprent showed that thymocyte apoptosis is accelerated after birth; at fetal day E18.5 few apoptotic cells were seen in the thymus, but by day 2 after birth populations of apoptotic

cells were easily identified within the thymus [37]. This timing in the induction of thymic apoptosis followed the same time course as the increase in TGF-β in the neonate thymus after birth [17]. Furthermore, it is well established that uptake of apoptotic cells by phagocytes induces TGF-β secretion from phagocytes [38, 39] and that apoptotic cells themselves, also release TGF-β [40]. Could thymocyte apoptosis, and consequent TGF-β production, be the initiation steps in tTreg-cell generation? We have observed that purified thymic macrophages produce significantly larger amounts of TGF-β upon exposure to apoptotic thymocytes in vitro (our unpublished data). Moreover, in vivo, by inducing thymocyte apoptosis with anti-CD3 treatment, γ-irradiation or administration of dexamethazone, it was shown that increased thymocyte apoptosis leads to increased levels of intrathymic TGF-β [17]. Importantly, following increases in apoptosis, enhanced TGF-β production by thymic macrophages and dendritic cells (DCs) was also seen. Collectively, these data indicated that thymic apoptosis stimulates production of TGF-β in the thymus.

Changes in levels of thymic apoptosis alter tTreg-cell generation

With the observations that thymic apoptosis could trigger TGF-β production in the thymus, and that TGF-β in turn drives Foxp3 expression in tTreg precursors, the next logical question was whether alterations in thymic apoptosis could influence tTreg-cell generation. In both adult and neonatal mice, increased levels of thymic apoptosis were shown to augment tTreg-cell frequencies [17]. Similarly, decreased frequencies of apoptotic thymocytes, were show to lead to a reduction in the numbers of tTreg cells. In particular, mixed bone marrow chimera mice with 75% DO11.10xRag$^{-/-}$ and 25% Balb/c bone marrow were generated, which were then treated with PBS or OVA [17]. Given that OVA administration to these chimeric mice would induce apoptosis in the DO11.10xRag$^{-/-}$ thymocytes, it would be possible to assess whether increased apoptosis of these thymocytes influences the generation of tTreg cells in the Balb/c polyclonal compartment [17]. Subsequently, it was shown that significantly more Balb/c tTreg cells were seen in the thymi of chimeric mice given OVA, compared with that in thymi of PBS-treated controls. In addition, when the levels of thymocyte apoptosis of day 1 neonatal mice were increased by exposing the mice to a low dose of γ-irradiation on the day they were born, the increased frequencies of apoptotic thymocytes were accompanied by enhanced levels of TGF-β in the thymi of irradiated neonates 24 hours later [17]. Importantly, 4–5 days after this, there was a significant increase in the tTreg-cell frequency in the thymi of neonates that had received γ-irradiation when compared with that in thymi of littermate controls. Finally, tTreg-cell generation in neonatal Bim$^{-/-}$mice has been examined [17]. As Bim$^{-/-}$mice exhibit reduced thymocyte apoptosis [41], it is possible to evaluate whether, at early time points after birth, these mice have a reduced tTreg-cell population. Indeed, the Foxp3^{+} tTreg-cell population in neonatal Bim$^{-/-}$mice was significantly reduced compared with that of littermate controls [17]. Collectively, these data show that in the neonatal thymus during the time period in which tTreg cells first start to develop, alterations in apoptosis influence the resultant size of the emerging tTreg-cell population. By altering apoptosis in the neonate thymus, and consequently intrathymic levels of TGF-β, the generation of tTreg cells can be augmented (more apoptosis) or reduced (less apoptosis). Altogether with the data showing TGF-β-

dependency of tTreg-cell generation, an intrathymic *apoptosis–TGF-β–Foxp3* axis mediating tTreg-cell development was proposed.

The role of TCR affinity in the development of tTreg cells

If an *apoptosis-TGF-β-Foxp3* axis controls the development of tTreg cells, how does this axis relate to the role of TCR affinity in tTreg-cell generation? Indeed, is TCR affinity an intrinsic, fate-determining factor mediating commitment to the tTreg-cell fate, or does TCR affinity play an indirect role by influencing thymic apoptosis, thereby subsequently influencing tTreg-cell development? To investigate this, an experimental system with TCR-transgenic T cells was employed to test the ability of peptides that have different affinities for the TCR to drive tTreg-cell generation [17]. When TCR-transgenic cells were injected into wild-type thymi along with peptides of increasing affinity, all of the TCR signals were capable of generating $Foxp3^+$ thymocytes[17], arguing that TCR affinity itself does not specifically determine tTreg-cell fate. This is in line with a previous report [18] and highlights that TCR signals of different affinities can drive tTreg-cell generation. Nevertheless, peptides of different affinities were shown to differ in the efficiency with which they induce the tTreg-cell fate as, similar to other TCR-mediated processes, tTreg-cell generation is influenced by the ability of peptide-MHC complexes to sufficiently activate the TCR [18]. However, high affinity TCR signals could also be better at inducing tTreg cells as high-affinity TCR signals drive negative selection, promoting thymocyte apoptosis [42]. In turn, increased thymic apoptosis creates a more favorable, TGF-β-rich, milieu to drive tTreg-cell generation from precursors. Importantly, irrespective of TCR affinity, blockade of TGF-β signaling results in decreased frequencies of tTreg cells being generated [17].

A modified model of tTreg-cell development

Based on the aforementioned data, and modified from our recent publication[17], we propose a model of tTreg-cell generation, outlining an *apoptosis–TGF-β-Foxp3* axis driving the development of Treg cells in the thymus (Fig. 1). Apoptosis of thymocytes, caused by high affinity TCR stimulation (negative selection) or other apoptotic stimuli, such as restricted access to growth factors, would promote thymic phagocytes, such as macrophages, to engulf and digest the apoptotic cells. In turn, phagocytes encountering apoptotic cells would be stimulated to produce TGF-β, creating TGF-β-rich microenvironments within the thymus. We suggest that this TGF-β-rich milieu is likely to be potent, highly localized to the producing cell and transient, with the highest concentrations of TGF-β located at the center. TGF-β produced could be activated by as-yet-unidentified mechanisms, including cleavage by enzymes (such as furin [43]), integrins [44] or by production of TGF-β by specific populations of antigen presenting cells within the medullary environment. Once a tTreg precursor enters this TGF-β-rich microenvironment, it will differentiate onto a tTreg cell. However this will only occur if two conditions are met. Firstly the precursor must engage its cognate antigen and therefore signal via its TCR. As such, to generate tTreg cells, TCR affinity can be high (but not high enough to initiate negative selection) medium or low (but not too low as to insufficiently activate the TCR). Secondly, the precursor must encounter sufficient levels of TGF-β to initiate the Foxp3 program. What this optimal TGF-β level is,

remains to be determined. Similarly, whether certain subpopulations of thymocytes are better able to receive or translate this TGF-β signal remains unaddressed. Under this model, the cognate antigens driving tTreg-cell generation could theoretically be presented by any type of thymic antigen presenting cells (APCs) including DCs, medullary thymic epithelial cells (mTECs) or other APCs[2]. However, it has been shown that certain thymic APCs might are better suited to this task [35,36]. This could be due to, for example, their location within the medulla, their ability to present antigen and/or their ability to activate TGF-β[35, 36]. If a developing $CD4^+$ SP thymocyte encounters its cognate antigen outside of the TGF-β-microenvironment, it will not differentiate into a tTreg cell (Fig. 1).

Following this model, it would be predicted that any precursor could differentiate into a tTreg cell, as long as it encountered both TCR stimuli and sufficient amounts of TGF-β. However, the frequency of thymic tTreg cells is about 3–5% of $CD4^+CD8^-$ SP thymocytes, and as such certain factors must limit tTreg-cell generation within the thymic environment. One such factor would be limited availability of TGF-β. Once activated, TGF-β rapidly binds its receptor and other surfaces; thus the TGF-β-rich milieu in our model is transient, such that not every precursor encountering cognate antigen would be stimulated by TGF-β. Competition between tTreg precursors has also been shown to limit the size of the tTreg-cell niche [45]. tTreg precursors would compete, not only for stimulation by their cognate antigens but also for TGF-β. Additionally, even once stimulated by both TGF-β and TCR, additional signals would support tTreg-cell generation including co-stimulatory signals, cytokines and other survival and/or novel factors. Only those precursors best able to compete for these signals will successfully complete their differentiation into the tTreg-cell fate. In this regard, a recent study has shown that Foxp3 itself is a death-promoting factor when expressed in tTreg cells [8]. To be rescued from death, Foxp3-expressing tTreg precursors must encounter IL-2 in the thymus [8]. Limited amounts of thymic IL-2 would further restrict the overall number of tTreg cells surviving (Fig.1).

A reconciled model of tTreg-cell generation?

Our proposed model of tTreg-cell development aligns with current models and, indeed, can provide some explanations for the mysteries and controversies surrounding the generation of Treg cells within the thymus:

High affinity agonists drive induction of tTreg cells

This model emphasizes that TCR affinity is a fate-determining factor instructing tTreg-cell differentiation. However, when considering our proposed *apoptosis-TGF-β-Foxp3* axis, it could be possible that high-affinity TCR signals themselves are not cell-intrinsic, qualitative factors controlling tTreg-cell generation. Rather, high-affinity TCR signals could be linked to tTreg-cell generation because they induce thymocyte apoptosis[42], and hence initiate the production of enhanced levels of intrathymic TGF-β. This TGF-β in turn induces Foxp3 expression in tTreg precursors.

Delayed appearance of tTreg cells in the neonatal thymus

The *apoptosis-TGF-β-Foxp3* axis also provides an explanation for the delayed appearance of tTreg cells in the neonatal thymus, which are not detectable until day 3 after birth, and is something that cannot be explained by current models. The sudden increase in thymic apoptosis at day 2 post birth[37] provides strong stimuli for the intrathymic production of TGF-β. As macrophages and immature DCs only need a few hours to produce and release TGF-β, the sudden increase in TGF-β could promote a rapid induction and accumulation of tTreg cells.

Intraclonal competition of tTreg cells or tTreg precursors

With transient and highly localized thymic microenvironments containing TGF-β, tTreg precursors will certainly compete for this cytokine alongside competing for TCR stimulation, survival and growth factors.

Remaining questions and challenges

Why is active TGF-β predominantly located in the medulla?

While this specific location provides a basis, and indeed perfect correlation, with the thymic regions best suited to tTreg-cell development[35], why TGF-β1 is specifically activated within the medulla remains unaddressed. What are the underlying mechanisms activating TGF-β within the thymus? As thymocytes in the cortex also become apoptotic, these apoptotic thymocytes will be engulfed and cleared by phagocytes localized in the cortex, but why the process in the cortex leads to less active TGF-β compared with that in the medulla is unknown. Among the possible mechanisms driving this, is the fact that there are different macrophage populations that sense and engulf apoptotic cells in the cortex ($F4/80^+$ macrophages) and medulla ($Mac3^+$ macrophages) [37]. Do these macrophages play some role in the differential activation of TGF-β in the medulla? Indeed, future work should define the mechanisms activating TGF-β within the thymus, and specifically within the medulla.

How are a few tTreg cells still generated in the thymus of TGF-β receptor knockout mice?

Although we have shown that enhanced levels of IL-2 in TGF-β-receptor knockout mice allows the expansion of tTreg cells [20], this observation means that there are still a small number of tTreg cells generated in the thymi of these knockout mice which are capable of responding to IL-2 and subsequently expand. If we hold that TGF-β signaling is required for the induction of Foxp3 in Treg precursors, and consequent tTreg-cell generation, how are these tTreg cells generated in the absence of TGF-β signaling? There is likely some inherent “leakiness” in the cre-lox systems used to assess tTreg-cell generation in TGF-β receptor knockout animals (both LCK-cre and CD4-cre systems have been employed [20]), and as such, the small population of tTreg cells in the TGF-β receptor-deficient animals may still express some level of the TGF-β receptor. Alternatively, whilst TGF-β is the dominant signal, it may not be the only TCR co-stimulatory signal capable of inducing Foxp3 expression. In the absence of TGF-β, other pathways may compensate for the Foxp3-inducing effects of TGF-β. A more clean and sophisticated knockout system will be needed to resolve this question.

What are the pathways downstream of TGF-β mediating Foxp3 expression?

Downstream of TGF-β, canonical Smad2/3 mediated pathways are important in driving *foxp3* expression [46], however, the detailed events linking Smads to Foxp3 gene transcription remain largely unknown. A Smad-binding site at a target gene is certainly important for Smad regulation of gene transcription. However, a Smad binding domain in a target gene is not a prerequisite for the involvement of TGF-β in gene transcription. The presence of Smad binding domains at target genes does not necessarily indicate that Smad-transcription factors must regulate target gene transcription, and, moreover lack of Smad binding sites also not eliminate a role for Smads in activation of gene transcription. Smad2/3 transcription factors do not effect gene transcription without first forming complexes with other co-transcription factors, co-activators and/or partners [47–49]. Thus, even if there is no obvious Smad binding site(s) in a gene promoter or regulatory element, it is still possible that Smad transcription factors can, through interaction with co-partners, indirectly bind to the target gene and regulate gene expression [47, 48]. Specifically relating to tTreg-cell generation, it has been shown that Smad3 binds at the *foxp3* promoter and regulates its activity, through a complex with other transcription factors [49]. Additionally, there are several Smad-independent, non-canonical pathways that are also downstream of TGF-β[50]; how these pathways function during tTreg-cell generation is not well understood, although some have been implicated in Treg development [51, 52]. A number of papers have suggested that TGF-β signaling plays no role in tTreg-cell generation as they show that Smad binding sites in the *foxp3* promoter are not critical for tTreg-cell development[53, 54]. However, bearing in mind the points above, these data do not eliminate a role for TGF-β signals in tTreg-cell generation. Thus, although the molecular mechanisms driving tTreg-cell generation remain to be fully elucidated, that TGF-β, does indeed play a vital role in tTreg-cell generation is starting to be recognized.

Conclusions and perspective

The current, and in fact dominate, view in the literature suggests that tTreg cells and pTreg cells are two different lineages, generated in response to disparate signals, with TGF-β-signaling vital for pTreg-cell generation but not tTreg-cell generation. However, more recent studies have started to reveal this might not be the case, and indeed, the generation and development of tTreg cells requires similar signaling pathways as pTreg cells, including TGF-β signaling. Therefore, the boundary that was previously proposed to distinguish these two populations of Treg cells, now becomes more ambiguous. Thus, it might be the time to reconsider the possibility that Treg cells are all generated via similar signaling pathways. The major difference between tTreg cells and pTreg cells would therefore be the location in which the Treg cell is generated and, as such, the antigens driving their development, rather than the requirement for TGF-β signaling in their generation.

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References

1. Klein L, Jovanovic K. Regulatory T cell lineage commitment in the thymus. Semin Immunol. 2011; 23(6):401–9. [PubMed: 21733719]
2. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat Rev Immunol. 2014; 14(6):377–91. [PubMed: 24830344]
3. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. Immunity. 2013; 38(3):414–23. [PubMed: 23521883]
4. Bayer AL, Yu A, Malek TR. Function of the IL-2R for thymic and peripheral CD4+CD25+ Foxp3+ T regulatory cells. J Immunol. 2007; 178(7):4062–71. [PubMed: 17371960]
5. Lio CW, Hsieh CS. A two-step process for thymic regulatory T cell development. Immunity. 2008; 28(1):100–11. [PubMed: 18199417]
6. Malek TR, Yu A, Vincek V, Scibelli P, Kong L. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. Immunity. 2002; 17(2):167–78. [PubMed: 12196288]
7. Tai X, Cowan M, Feigenbaum L, Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. Nat Immunol. 2005; 6(2):152–62. [PubMed: 15640801]
8. Tai X, Erman B, Alag A, Mu J, Kimura M, Katz G, Guinter T, et al. Foxp3 transcription factor is proapoptotic and lethal to developing regulatory T cells unless counterbalanced by cytokine survival signals. Immunity. 2013; 38(6):1116–28. [PubMed: 23746651]
9. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat Immunol. 2005; 6(11):1142–51. [PubMed: 16227984]
10. Hsieh CS, Lee HM, Lio CW. Selection of regulatory T cells in the thymus. Nat Rev Immunol. 2012; 12(3):157–67. [PubMed: 22322317]
11. Kitagawa Y, Ohkura N, Sakaguchi S. Epigenetic control of thymic Treg-cell development. Eur J Immunol. 2015; 45(1):11–6. [PubMed: 25348287]
12. Stritesky GL, Jameson SC, Hogquist KA. Selection of self-reactive T cells in the thymus. Annu Rev Immunol. 2012; 30:95–114. [PubMed: 22149933]
13. Caton AJ, Kropf E, Simons DM, Aitken M, Weissler KA, Jordan MS. Strength of TCR signal from self-peptide modulates autoreactive thymocyte deletion and Foxp3(+) Treg-cell formation. Eur J Immunol. 2014; 44(3):785–93. [PubMed: 24307208]
14. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, Naji A, Caton AJ. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol. 2001; 2(4):301–6. [PubMed: 11276200]
15. Apostolou I, Sarukhan A, Klein L, von Boehmer H. Origin of regulatory T cells with known specificity for antigen. Nat Immunol. 2002; 3(8):756–63. [PubMed: 12089509]
16. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, Hogquist KA. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. J Exp Med. 2011; 208(6):1279–89. [PubMed: 21606508]
17. Konkel JE, Jin W, Abbatiello B, Grainger JR, Chen W. Thymocyte apoptosis drives the intrathymic generation of regulatory T cells. Proc Natl Acad Sci U S A. 2014; 111(4):E465–73. [PubMed: 24474796]
18. Lee HM, Bautista JL, Scott-Browne J, Mohan JF, Hsieh CS. A broad range of self-reactivity drives thymic regulatory T cell selection to limit responses to self. Immunity. 2012; 37(3):475–86. [PubMed: 22921379]
19. Burchill MA, Yang J, Vang KB, Moon JJ, Chu HH, Lio CW, Vegoe AL, et al. Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. Immunity. 2008; 28(1):112–21. [PubMed: 18199418]
20. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. Nat Immunol. 2008; 9(6):632–40. [PubMed: 18438410]

21. Fontenot JD, Dooley JL, Farr AG, Rudensky AY. Developmental regulation of Foxp3 expression during ontogeny. J Exp Med. 2005; 202(7):901–6. [PubMed: 16203863]
22. Chen W, Jin N, Hardegen W, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med. 2003; 198(12):1875–86. [PubMed: 14676299]
23. Davidson TS, DiPaolo RJ, Andersson J, Shevach EM. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. J Immunol. 2007; 178(7):4022–6. [PubMed: 17371955]
24. Horwitz DA, Zheng SG, Wang J, Gray JD. Critical role of IL-2 and TGF-beta in generation, function and stabilization of Foxp3+CD4+ Treg. Eur J Immunol. 2008; 38(4):912–5. [PubMed: 18395858]
25. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, vom Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. Nat Immunol. 2005; 6(12):1219–27. [PubMed: 16244650]
26. Kasagi S, Zhang P, Che L, Abbatiello B, Maruyama T, Nakatsukasa H, Zanvit P, et al. In vivo-generated antigen-specific regulatory T cells treat autoimmunity without compromising antibacterial immune response. Sci Transl Med. 2014; 6(241):241ra78.
27. Li MO, Sanjabi S, Flavell RA. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. Immunity. 2006; 25(3):455–71. [PubMed: 16973386]
28. Marie JC, Liggitt D, Rudensky AY. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. Immunity. 2006; 25(3):441–54. [PubMed: 16973387]
29. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med. 1996; 184(2):387–96. [PubMed: 8760792]
30. Ouyang W, Beckett O, Ma Q, Li MO. Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development. Immunity. 2010; 32(5):642–53. [PubMed: 20471291]
31. Wahl SM, Orenstein JM, Chen W. TGF-beta influences the life and death decisions of T lymphocytes. Cytokine Growth Factor Rev. 2000; 11(1–2):71–9. [PubMed: 10708954]
32. Chen W, Jin W, Tian H, Sicurello P, Frank M, Orenstein JM, Wahl SM. Requirement for transforming growth factor beta1 in controlling T cell apoptosis. J Exp Med. 2001; 194(4):439–53. [PubMed: 11514601]
33. Konkel JE, Maruyama T, Carpenter AC, Xiong Y, Zamarron BF, Hall BE, Kulkarni AB, et al. Control of the development of CD8alphaalpha+ intestinal intraepithelial lymphocytes by TGF-beta. Nat Immunol. 2011; 12(4):312–9. [PubMed: 21297643]
34. Wirnsberger G, Mair F, Klein L. Regulatory T cell differentiation of thymocytes does not require a dedicated antigen-presenting cell but is under T cell-intrinsic developmental control. Proc Natl Acad Sci U S A. 2009; 106(25):10278–83. [PubMed: 19515822]
35. Cowan JE, Parnell SM, Nakamura K, Caamano JH, Lane PJ, Jenkinson EJ, Jenskinson WE, Anderson G. The thymic medulla is required for Foxp3+ regulatory but not conventional CD4+ thymocyte development. J Exp Med. 2013; 210(4):675–81. [PubMed: 23530124]
36. Aschenbrenner K, D'Cruz LM, Vollmann EH, Hinterberger M, Emmerich J, Swee LK, Rolink A, Klein L. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. Nat Immunol. 2007; 8(4):351–8. [PubMed: 17322887]
37. Surh CD, Sprent J. T-cell apoptosis detected in situ during positive and negative selection in the thymus. Nature. 1994; 372(6501):100–3. [PubMed: 7969401]
38. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest. 1998; 101(4):890–8. [PubMed: 9466984]

39. Xiao YQ, Freire-de-Lima CG, Schiemann WP, Bartton DL, Vandivier RW, Henson PM. Transcriptional and translational regulation of TGF-beta production in response to apoptotic cells. J Immunol. 2008; 181(5):3575–85. [PubMed: 18714031]
40. Chen W, Frank ME, Jin W, Wahl SM. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. Immunity. 2001; 14(6):715–25. [PubMed: 11420042]
41. Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, et al. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature. 2002; 415(6874):922–6. [PubMed: 11859372]
42. van, Santen HM.; Benoist, C.; Mathis, D. Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. J Exp Med. 2004; 200(10):1221–30. [PubMed: 15534371]
43. Pesu M, Watford WT, Wei L, Xu L, Fuss I, Strober W, Andersson J, et al. T-cell-expressed proprotein convertase furin is essential for maintenance of peripheral immune tolerance. Nature. 2008; 455(7210):246–50. [PubMed: 18701887]
44. Travis MA, Sheppard D. TGF-beta activation and function in immunity. Annu Rev Immunol. 2014; 32:51–82. [PubMed: 24313777]
45. Bautista JL, Lio CW, Lathrop SK, Fornush K, Liang Y, Luo J, Rudensky AY, Hsieh CS. Intraclonal competition limits the fate determination of regulatory T cells in the thymus. Nat Immunol. 2009; 10(6):610–7. [PubMed: 19430476]
46. Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI, Tone M. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. Nat Immunol. 2008; 9(2):194–202. [PubMed: 18157133]
47. Chen X, Weisberg E, Fridmacher V, Watanabe M, Naco G, Whitman M. Smad4 and FAST-1 in the assembly of activin-responsive factor. Nature. 1997; 389(6646):85–9. [PubMed: 9288972]
48. Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. Nature. 1998; 394(6696):909–13. [PubMed: 9732876]
49. Ruan Q, Kameswaran V, Tone Y, Li L, Liou HC, Greene MI, Tone M, Chen YH. Development of Foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome. Immunity. 2009; 31(6):932–40. [PubMed: 20064450]
50. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature. 2003; 425(6958):577–84. [PubMed: 14534577]
51. Maruyama T, Li J, Vaque JP, Konkel JE, Wang W, Zhang B, Zhang P, et al. Control of the differentiation of regulatory T cells and T(H)17 cells by the DNA-binding inhibitor Id3. Nat Immunol. 2011; 12(1):86–95. [PubMed: 21131965]
52. Wan YY, Chi H, Xie M, Schneider MD, Flavell RA. The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. Nat Immunol. 2006; 7(8):851–8. [PubMed: 16799562]
53. Schlenner SM, Weigmann B, Ruan Q, Chen Y, von Boehmer H. Smad3 binding to the foxp3 enhancer is dispensable for the development of regulatory T cells with the exception of the gut. J Exp Med. 2012; 209(9):1529–35. [PubMed: 22908322]
54. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature. 2010; 463(7282):808–12. [PubMed: 20072126]

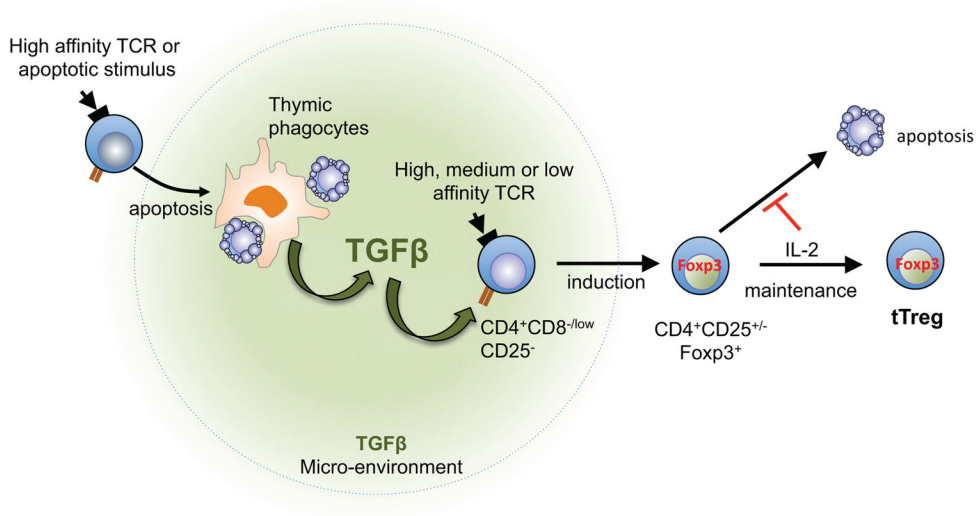

Figure 1. A new model for tTreg-cell development

An intrathymic *apoptosis-TGF-β-Foxp3* axis is responsible for development of Treg cells within the thymus. Thymocytes undergo apoptosis in the thymus (for example when undergoing negative selection) and are efficiently engulfed by thymic phagocytes. Uptake of apoptotic thymocytes by phagocytes stimulates the production of TGF-β, creating TGF-β-rich microenvironments within the thymus (indicated by the green shading), in particular in the thymic medulla. This TGF-β-rich milieu is likely to be potent, highly localized and transient, creating gradients of TGF-β within the thymus. If a tTreg precursor ($CD4^+CD8^-CD25^-$ thymocyte) engages its TCR and initiates TCR signaling within this TGF-β rich region of the thymus, *foxp3* expression will be induced and the $CD4^+$ SP thymocyte has the potential to differentiate into a mature tTreg cell. This will only occur if the *foxp3*-expressing thymocyte is able to efficiently compete for vital survival factors (for example IL-2) within the thymic environment.