Review Article

Regulatory T Cell Development from the Top Down: the Role of T Cell Receptor-Generated Second Messengers in Thymic Regulatory T Cell Development

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Abstract

Regulatory T cells (Treg)s are a subset of CD4^{+} T cells with suppressive properties. Their function and development relies on the expression of the lineage-determining transcription factor Foxp3. Mutations in Foxp3 result in the failure of Treg development, leading to widespread autoimmunity and death in mice and in humans. Although some Tregs acquire expression of Foxp3 in the periphery (inducible Tregs; iTregs), a larger proportion of Tregs called natural Tregs (nTregs) are generated in the thymus during T cell development. Thymic Treg development is dependent on intracellular signal transduction events transduced by the T cell receptor (TCR), co-stimulatory molecules, and cytokines. Here we review the proximal signals emanating from the TCR that are essential for proper thymic Treg development, with particular emphasis on the PLCγ pathway.

REGULATORY T CELLS

Regulatory T cells (Tregs) are a subset of CD4^{+} T cells that are defined by expression of the transcription factor Foxp3, as well as their ability to suppress T cell-mediated immune responses [1,2]. Genetic mutations resulting in dysfunctional Foxp3 production lead to failed Treg generation and subsequently to fatal T cell-mediated autoimmune inflammation in both mice and humans [3-7]. Tregs also prevent tissue damage resulting from excessive immune responses to foreign and commensal antigens [8-10]. Because of the importance of these cells in immune tolerance, the investigation of Treg development has been the focus of intense immunological research. A combination of T cell receptor (TCR), costimulatory receptor, and cytokine receptor ligation events are required for Foxp3 upregulation and entry of developing CD4^{+} T cells into the Treg repertoire [11]. Here, we review recent work elucidating the proximal signal transduction events downstream of the TCR that are required for formation of the Treg lineage.

High affinity TCR signals are required for natural Treg generation in the thymus

Like all T cells, naturally occurring (n)Tregs are generated through a tightly regulated development process occurring within the thymus, allowing for the production of a diverse population of Tregs with a wide array of TCR specificities [12]. Developing T cells perceive TCR-mediated survival signals generated from interactions with major histocompatibility complexes (MHC) bearing self peptides, allowing for positive selection, while overly strong signals lead instead to death by negative selection. Thus, the selection process acts both to impart T cells with the ability to respond to foreign peptides presented in the context of MHC, and to prevent overt T cell-mediated autoimmunity. While strong TCR-mediated signals can induce apoptosis in developing T cells, they can also instruct these cells toward Foxp3 upregulation and entry into the nTreg pool [13]. This phenomenon has been observed in multiple strains of TCR transgenic mice in which all T cells have been engineered to bear a TCR of a single specificity. In the absence of cognate peptide expression in the thymus, little to no Treg development is observed. However, when the cognate peptide is present during thymic development, an unusually high percentage of CD4^{+} T cells upregulate Foxp3 and obtain suppressive ability [14-18]. In addition, when the transgenically expressed self-antigen was altered such that the transgenic TCR bound to it with decreased affinity, Treg production was

diminished in these models [15,19]. Such findings favor the notion that strong TCR-mediated signals are required to promote Treg development.

In addition to the TCR transgenic mouse models, the role of strong TCR signals for the selection of Tregs has been examined in the polyclonal setting using TCR-driven immediate early gene Nur77 expression as a surrogate for TCR signal strength. Nur77-driven GFP reporter-based studies demonstrate that both thymic and peripheral Tregs perceive TCR signals of higher magnitude than other CD4+ T cells [20,21]. Additionally, when the Nur77-GFP reporter mouse was bred to mice lacking the pro-apoptotic molecule Bim, CD4+ T cells that were rescued from clonal deletion had levels of Nur77-driven GFP similar to the levels normally found in Tregs [21]. This suggests that negative-selecting TCR signals are of similar strength to those that induce Treg generation. Together, these data suggest that strong TCR signals play a critical role in the generation of Tregs.

TCR-driven PLCγ1 activation is required at multiple stages of nTreg development

TCR ligation induces a cascade of TCR-mediated signal transduction events, emanating from the formation of multimolecular protein complexes beneath the surface of the cell [22]. One key event downstream of these proximal signaling complexes is the activation of phospholipase C γ1 (PLC-γ1). PLC-γ1 cleaves phosphatidylinositol-4,5-bisphosphate (PIP$_2$) into a single molecule of inositol-1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG) [23]. TCR-mediated PLC-γ1 activation is an absolute requirement for Treg development, as mice harboring a mutation disrupting PLC-γ1 recruitment post-TCR engagement fail to generate Tregs even though T cell positive selection is maintained [24]. This finding specifically implicates signal transduction events downstream of PLC-γ1 as the crucial components of TCR-mediated signaling that promote Treg development. Indeed, both IP$_3$ and DAG generated by PLC-γ1 induce downstream activation of several transcription factors including NFAT, NF-κB, and AP-1; all of which have been shown to bind within promoter and/or enhancer regions of the Foxp3 genetic locus and augment Foxp3 expression in T cells [25-28].

Thymic Treg development is thought to occur in two sequential steps, one of which is TCR-dependent and another that is TCR-independent [29]. When developing CD4 single positive (SP) thymocytes encounter a strong TCR stimulus, they upregulate cell surface expression of the high-affinity interleukin-2 (IL-2) receptor subunit (CD25). This CD25+ CD4 SP population is highly enriched in Treg progenitors and is sensitive to IL-2 stimulation [29-31]. When the CD25+ CD4 SP Treg progenitors receive IL-2 signaling through CD25, Foxp3 expression is induced in a TCR-independent manner [29,30]. Thus, TCR-induced upregulation of CD25 in CD4 SP thymocytes represents an important step for nTreg generation, in addition to the induction of TCR-driven transcription factors necessary for Foxp3 expression. As is true for Foxp3 expression, multiple transcription factors induced downstream of IP$_3$ and DAG production promote CD25 expression in T cells [25,32-35]. Thus, regulation of these TCR-induced second messengers is very likely to influence Treg development on a molecular level both for CD25 upregulation and for subsequent Foxp3 upregulation itself.

Role of TCR-mediated IP$_3$ and Ca^{2+} production in nTreg development

IP$_3$ binds to receptor-gated Ca^{2+} channels (IP$_3$R) in the endoplasmic reticulum (ER) membrane, allowing Ca^{2+} ions to flow from the ER into the cytosol. The depletion of ER calcium stores causes activation of store-operated calcium channels (SOC) in the plasma membrane, allowing additional calcium to flow into the cell for maintenance of signaling and replenishment of ER stores. Calcium mobilization induces activation of the nuclear factor of activated T cells (NFAT) [36]. Multiple studies have shown that efficient calcium mobilization is required for Treg development, but how NFAT activation is ultimately involved in Foxp3 expression remains unclear [37]. While reporter-based assays have demonstrated that NFAT binding to the human Foxp3 promoter induces robust Foxp3 expression in human T cells, the relationship between NFAT and murine Treg development has been difficult to assess [27]. T cells express three different NFAT isoforms (NFAT1, NFAT2, and NFAT4), and several studies have shown that both single and double-isoform knockout mice exhibit largely normal Treg development, likely because of redundancy among the NFAT isoforms [38,39]. A recent study found Treg development to be diminished when NFAT2 was deleted in early T cell development in mice additionally lacking NFAT1 (*Nfat1$^{-/-}$Nfat2$^{fl/fl}$Lck-Cre*), suggesting NFAT2 may indeed drive Treg development at a specific stage of thymic maturation [40]. It is plausible that NFAT also promotes the formation of Treg progenitors, since NFAT binding is required at specific sites within the murine CD25 locus to achieve efficient CD25 expression [33,34]. Additionally, once Foxp3 expression occurs, NFAT/Foxp3 complexes can bind the IL-2 promoter to prevent IL-2 expression, as well as promote expression of the suppression-associated molecule *Cytotoxic T-Lymphocyte Antigen 4* (CTLA-4); both of which are distinct traits of the Treg transcriptional program driven by Foxp3 [41]. Indeed, mutations of Foxp3 that disrupt the Foxp3/NFAT interface site abrogate Foxp3-driven suppressive function in transduced T cells [41].

Role of TCR-mediated DAG production in nTreg development

DAG initiates multiple signal transduction events relevant to both CD25 and Foxp3 expression. DAG activates protein kinase C (PKC), leading to downstream induction of nuclear factor κB (NF-κB) transcription factors. Additionally, DAG activates RAS guanyl nucleotide-releasing protein (Ras-GRP), which leads to extracellular signal-related kinase (ERK) activation and subsequent induction of the activator protein 1 (AP-1) transcription factor family members (22, 23). DAG signaling is terminated by enzymes known as DAG kinases (DGK)s, which convert DAG into phosphatidic acid [42]. The ζ isoform of DGK plays a major role in controlling DAG signals downstream of the TCR [42,43]. Hence, T cells lacking DGKζ demonstrate a selective enhancement of DAG-mediated signals upon TCR stimulation [44-46]. We recently reported that DGKζ-deficient mice exhibit a cell-intrinsic increase in both nTreg and CD25+ nTreg precursor development, supporting the role of DAG signaling in promoting Treg development [47]. This augmentation was partially dependent on activation of the NF-κB subunit c-Rel, which was enhanced downstream of TCR stimulation in DGKζ-deficient T

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cells [47]. The contribution of c-Rel was not surprising, as several groups have found c-Rel to drive Treg development in recent years [26,28,48-52]. c-Rel has been shown to promote opening of the Foxp3 locus by binding a conserved non-coding DNA sequence element (CNS3), and additionally forms a multi-transcription factor enhanceosome that drives gene expression from the Foxp3 promoter [26,28]. In support of these findings, mice expressing a constitutively active form of an upstream NF-κB/c-Rel pathway member, inhibitor of IκB kinase β (IKKβ), exhibit increased Treg frequencies; while mice lacking c-Rel develop very few Tregs at all [50, 51].

In addition to c-Rel, ERK activation also plays an important role in the enhanced generation of Tregs in DGKζ-deficient mice [47]. When ERK phosphorylation is pharmacologically inhibited, no Foxp3 upregulation is observed by in DGK-deficient immature thymocytes. Additionally, there is a linear correlation between the degree of ERK phosphorylation and the frequency of Foxp3 upregulation observed *in vitro*. Consistent with these findings, a transgenic mouse with selective enhancement of ERK signaling exhibits increased Treg development *in vivo*. While it remains unclear exactly how ERK might act to drive thymocytes toward the Treg lineage, AP-1 transcription factors that are activated downstream of ERK have been implicated in both CD25 and Foxp3 expression, several AP-1 binding sites exist in the promoter and enhancer regions of both the CD25 and Foxp3 genetic loci [25,34]. Thus, the activation of AP-1 transcription factors are likely important for Treg precursor generation (upregulation of CD25) as well as for the expression of Foxp3. Indeed, reporter-based assays show that AP-1 binding sites within the Foxp3 promoter is required for Foxp3 expression peripheral human T cells [25]. In addition to AP-1 activation, ERK phosphorylates the transcription factor Runx1, allowing Runx1 to interact with binding partners to augment its transcriptional activity [53-56]. Interestingly, Runx1 has been reported to bind the CNS2 region of the Foxp3 locus along with its transcriptional coactivator Cbf-β, and this binding is required for stable expression of Foxp3 in dividing nTregs [28,53,54]. It thus seems plausible that ERK activation could be working in a variety of ways to promote nTreg development downstream of TCR-mediated DAG production. The proximal signaling pathways from the TCR that contribute to Treg generation are summarized and depicted in (Figure 1).

CONCLUDING REMARKS

Many recent studies have investigated the factors required to drive developing thymocytes toward Foxp3 upregulation and Treg lineage choice. The answer to this question may uncover strategies for Treg enhancement in autoimmune and bone marrow transplantation settings, which could prove highly beneficial for human health. It is now clear that efficient Treg development requires TCR-driven signals of a certain magnitude and/or specificity, which are likely produced by interactions with self-antigen during Treg development within the thymus. Determining the relative contribution of specific signaling events downstream of the immunological synapse is more difficult, however, as several signaling pathways seem to be simultaneously required for efficient Foxp3 upregulation. Due to the complex nature of this process, an effective strategy to promote Treg development therapeutically might be to target signaling pathways proximal to TCR engagement. Such treatment would work to broadly enhance the magnitude of
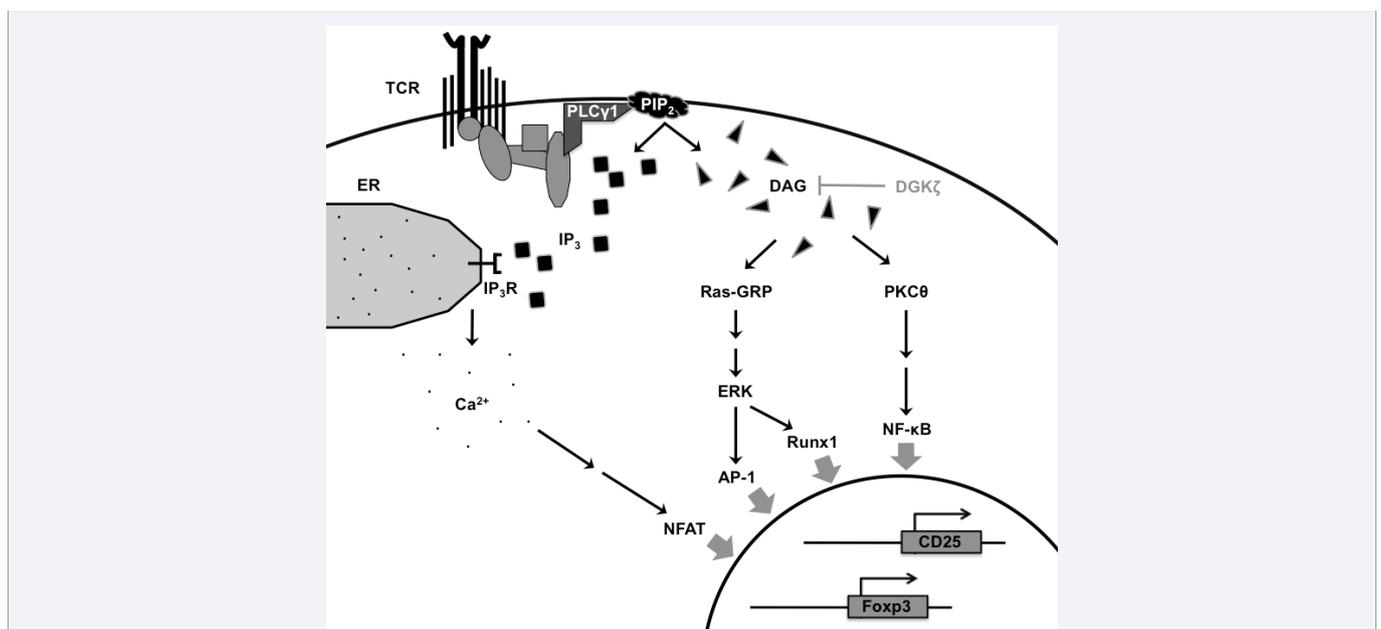

Figure 1 TCR-mediated second messenger production promotes Treg development through the initiation of multiple signal transduction pathways. TCR engagement leads to the formation of a multimolecular signaling complex, resulting in the activation of PLCγ1 and the production of the second messengers IP$_3$ and DAG. IP$_3$ production promotes calcium release from the ER, leading to downstream activation of NFAT, while DAG molecules activate the PKCθ and Ras-GRP pathways to ultimately promote transcriptional activation of NF-κB, AP-1, and Runx1. All of these transcription factors have been shown to bind regions of the CD25 and/or Foxp3 locus, where they are required for efficient upregulation of these genes and entry of developing CD4 SP T cells into the Treg lineage. Negative regulators of these second messengers, such as DGKζ, serve to inhibit these processes.

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a given TCR signal from the top down. Inhibition of negative regulators of signal transduction, including DGKζ, could be an attractive approach, although proper targeting of such inhibitors could be complicated to put into practice. Additionally, whether increasing Treg population size would actually improve disease outcome remains unclear for many autoimmune disorders. Future studies will likely provide insights into this important question and hopefully transition our extensive knowledge of Treg development toward effective clinical practices.

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