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Foxp3 positive regulatory T cells: a functional regulation by the E3 ubiquitin ligase Itch

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Abstract

Regulatory T cells (Tregs) play a critical role in maintaining immune tolerance to self-antigens, whose development and activation is controlled by the master regulator and transcription factor Foxp3. Foxp3 acts as transcription repressor and exerts its suppressing function via directly associating with and inhibiting the function of other transcriptional regulators. The gene transcription of Foxp3 is regulated by diverse mechanisms at the cellular and molecular levels including the pleiotropic cytokine transforming growth factor-β (TGF-β). Itch is an E3 ubiquitin ligase whose deficiency is linked to excessive immune responses, abnormal T helper cell differentiation, and failed T cell anergy induction. Recent evidence indicates that Itch is involved in TGF-β-induced Foxp3 expression and Treg-regulated airway inflammation, thus identifying a ubiquitin-dependent pathway in modulating Tregs.

Keywords

Regulatory T cells; Foxp3; Immune tolerance; Ubiquitination; Itch; TIEG1; TGF-β

Introduction

One unique feature of the immune system is to distinguish self from non-self, which is achieved by the generation and selection of antigen-specific receptors on the surface of lymphocytes. Developing lymphocytes bearing receptors that recognize self-antigens will be deleted in the thymus (for T cells) or the bone marrow (for B cells) through a mechanism of central tolerance. Self-reactive mature lymphocytes that have escaped into the peripheral lymphoid tissues will be kept under control via the mechanism of peripheral tolerance including ignorance, lymphocyte unresponsiveness or anergy, activation-induced cell death, and functional immune suppression via regulatory T cells (Tregs). A proper balance of immunity versus tolerance ensures that the body can mobilize offensive attack to invading microorganisms or tumors, and at the same time, is protected from harming self-tissues or organs. Failure in self-tolerance can result in disastrous consequences such as the development of autoimmune diseases or airway inflammation.

The process of tagging the ubiquitin molecule to a protein substrate is carried out by a cascade of enzymatic reactions, with the E3 ubiquitin ligases being the critical components in targeting

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specific substrates for ubiquitin conjugation. Protein ubiquitination is involved in many biological processes including receptor downmodulation, cell cycle control, signaling transduction, or gene transcription. Several E3 ubiquitin ligases are involved in the regulation of immune responses including lymphocyte development, activation, differentiation, and tolerance induction [1]. This review will focus on the recent understanding of the cellular and molecular insights of Treg biology and regulation, with particular attention to a functional involvement of a protein ubiquitination pathway in regulating transforming growth factor-β (TGF-β) signaling and Treg-regulated allergic responses.

Regulatory T cells

Tregs are unique subpopulation of CD4+ T cells that play a pivotal role in maintaining immune tolerance to self-antigens and are characterized by the cell surface expression of CD25, the interleukin-2 (IL-2) receptor alpha chain [2]. Tregs can be divided into two types: the naturally occurring or the induced Tregs. Naturally occurring Tregs originate from thymus and comprise 5–10% of the CD4+ T cells in the peripheral lymphoid tissues. In addition to the CD25 marker, this subset of Tregs also expresses other cell surface molecules such as the co-inhibitory molecule cytotoxic T lymphocyte antigen 4 (CTLA-4) or the tumor necrosis factor receptor family member GITR. The development and function of naturally occurring Tregs is determined by the transcription factor Foxp3, since its mutation or deficiency is linked to excessive autoimmune diseases [3]. Inducible Tregs are converted from naïve CD4+CD25− peripheral mature T cells by in vitro TGF-β stimulation [4,5] or by in vivo chronic antigen administration [6]. Like the naturally occurring Tregs, inducible Tregs also suppress the proliferation of naïve CD4+ T cells in vitro and immune responses in vivo.

Multiple mechanisms have been proposed for Treg-mediated suppression. Such inhibitory effect occurs not only on naïve CD4+ T cells but also on CD8+ T cells, B cells, and dendritic cells (DCs) or natural killer T cells. Secretion of inhibitory cytokines such as IL-10, IL-35, or TGF-β and cell-to-cell contact is an important means for effective suppression. Other mechanisms are also involved, such as the modification of DCs, activation of the inhibitory adenosine receptors, or the apoptosis of responder cells.

Immune regulation by TGF-β signaling

The pleiotropic regulatory cytokine TGF-β exerts diverse biological functions such as cell fate decision, proliferation, apoptosis, and migration [7]. TGF-β binding to the type II receptor induces the complex formation with type I receptor, which results in the phosphorylation of the type I receptor serine/threonine kinase. The activation of the receptor complex in turn phosphorylates the intracellular transducers, Smad2/3, which then form complex with Smad4 and are translocated into the nucleus to regulate the transcription of target genes. One of the target gene products is Smad7, an inhibitory Smad, which negatively modulates TGF-β signaling via directly competing with Smad2/3 for receptor interaction. In addition to the Smad-dependent signaling pathways, TGF-β also activates Smad-independent signaling pathways including the activation of mitogen-activated protein kinases [8].

Previous studies have established that TGF-β signaling is important in regulating immune responses. Ablation of either TGF-β or the TGF-β receptor is linked to abnormal T cell responses and onset of autoimmunity [9–12]. TGF-β signaling regulates both Th1 and Th2 cell differentiation [13,14]. As described earlier, TGF-β also plays an important role in Treg generation and maintenance [4,5,10,15]. Although a functional role of TGF-β in the development of naturally occurring Tregs has been controversial, a recent study clearly demonstrated that TGF-β, together with IL-2, is critical for the thymic development of natural CD4+CD25+Foxp3+ Tregs [16]. In addition, recent studies have demonstrated a critical role

of TGF-β in the development of T helper 17 cells (Th17), a new subset of T helper cells, which are involved in autoimmune and inflammatory responses [17]. However, the detailed intracellular signaling pathways that TGF-β initiates in diverse processes of different types of T cells remain to be an open question.

Mechanisms of Foxp3 function

Foxp3 belongs to the forkhead box transcription factor family including Foxp1, Foxp2, Fopx3, and Foxp4, which are identified by a conserved forkhead/winged helix DNA-binding domain. This family of proteins is involved in development of many organs and tissues, whose dysregulation or mutation has been implicated in cancer and immunological diseases. For example, Foxp1 is linked to the development of several types of tumors [18] and to the motor neuron connectivity [19]; Foxp2 mutation results in speech and language disorders [20]. Mutations in human Foxp3 causes IPEX (immunodysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome), and in mouse, loss of Foxp3 gene leads to scurfy phenotypes manifested by massive lymphocyte infiltration in multiple organs and early death [21].

Foxp3 is a master regulator for Treg development and function. Retroviral expression of Foxp3 converts CD4+CD25− naïve T cells into CD4+CD25+ Tregs that are capable of suppressing other T cells in vitro and in vivo [22], and Foxp3-transgenic mice display higher numbers of CD4+CD25+ Tregs [23], whereas Foxp3-deficient mice lack CD4+CD25+ Tregs [24]. It was reported that Foxp3 acts as a transcriptional repressor by directly associating with IL-2 and IFN-γ promoters and modulates histone acetylation [25]. In addition, Foxp3, but not other Foxp family members like Foxp1 or Foxp2, inhibits NFAT and NF-κB-mediated transcription [26]. Foxp3 interacts with histone acetyltransferase, and such interaction is necessary for transcriptional repression [27]. It forms a complex with NFAT via its forkhead domain to repress IL-2 transcription, and such interaction is essential for Foxp3 function in inducing CD25 and CTLA-4 expression and for the inhibitory function of Tregs [28]. Foxp3 can also interact with AML1/Runx1, a transcription factor that is involved in both normal hematopoiesis and abnormal leukemogenesis [29]. A Runx1-binding site is identified in the upstream of IL-2 promoter, and Runx1 expression enhances IL-2 transcription. However, such enhancement is abrogated by Foxp3 expression, which requires a direct interaction between the two proteins.

Foxp3 expression in other tissues

In addition to Tregs, Foxp3 is expressed in other tissues or cell types. Female mice carrying Foxp3 mutation develop spontaneous and carcinogen-induced mammary carcinomas and show increased expression of Erb2, an oncogene linked to human breast cancer [30]. There are defects in Foxp3 expression in human breast cancer cell lines, which are correlated with the upregulated Erb2 abundance. It is thus concluded that Foxp3 is an X-linked tumor suppressor. In addition, Foxp3 acts as a transcriptional repressor for the breast cancer oncogene SKP2, the latter being a component of the SCF E3 ligase complex whose upregulation results in abnormal cell cycle and tumorigenesis [31]. Foxp3 is also detected in normal epithelial cells of other tissues including lung, prostate, and thymus as well as in human melanoma and other tumor cell lines [32,33]. The expression of Foxp3 in non-Tregs will help understand massive inflammation in scurfy mice.

Transcriptional regulation of Foxp3 gene

Analysis of the Foxp3 locus reveals several conserved non-coding sequences (CNSs) between human and mouse including the promoter region and the first intron [34,35]. Both histone acetylation and demethylation of DNA CpG islands have been identified in the CNSs of Foxp3 gene in Tregs (particularly, naturally occurring Tregs), suggesting that Foxp3 gene expression

is subjected to epigenetic regulation [36,37]. The Foxp3 promoter contains the binding sites for several transcription factors including Sp1, NFAT, and AP-1, which act downstream of the TCR signaling [34]. In addition, Foxp3 enhancers were also discovered in the CNSs of the first intron, which contain the binding sites for cyclin AMP response element binding protein (CREB)/activating transcription factor, which overlaps with a CpG island [35], and for the critical IL-2 signaling mediator, Stat5 [38], which is just upstream of the CREB site. In addition, a different Stat5-binding site is also found in the basic promoter region of the Foxp3 gene [39].

TGF-β stimulation induces Foxp3 expression in CD4+CD25− naïve T cells in vitro and converts them into inhibitory adaptive Tregs [4,5,10,15]. However, the signaling pathways leading to TGF-β receptor-mediated Foxp3 gene transcription were not clear. A recent study described a different enhancer region in the intron 1, which is upstream the Stat5- and CREB-binding regions and contains two very closely located binding sites for NFAT and Smad3, respectively [40]. More importantly, NFAT and Smad3 synergize to promote histone acetylation in the enhancer region and also Foxp3 induction.

Foxp3 gene expression is also subjected to negative regulation, particularly the IFN-γ, and IL-4 cytokines produced by Th1 and Th2 cells, respectively [41]. Retroviral transduction of either T-bet, a transcription factor for Th1 cells, and GATA-3 for Th2 cells resulted in the inhibition of Foxp3 expression [41]. The Th2-driving GATA-3 directly binds to a consensus motif in the Foxp3 basic promoter [42]. In addition, IL-4 can also inhibit Foxp3 expression via its downstream mediator, Stat6, whose DNA-binding site is located downstream of the NFAT/Smad3-binding region [43].

Other regulatory mechanisms of Foxp3 expression

One recently documented signaling pathway involved in Foxp3 expression is the PI3K-Akt-mTOR axis. Retroviral transduction of an active form of Akt impairs Foxp3 induction by TGF-β, which was reversed by the mTOR inhibitor Rapamycin [44]. A PI3K inhibitor LY294002 induces Foxp3 expression without the need of TGF-β stimulation, whereas loss of the lipid phosphatase Pten causes a reduction of Foxp3 expression in response to treatment with TGF-β or PI3K inhibitor [45]. It seems that such inhibition is independent of TGF-β signaling but affects the histone modification of both the Foxp3 basic promoter and enhancer regions in the first intron encompassing the Stat5/CREB-binding sites [45].

Notch signaling pathway plays a pivotal role in cell fate decisions of many tissues including the lineage decisions of lymphocytes like Th1 vs Th2 cells. Expression of Jagged1, a ligand for Notch1, in antigen-presenting cells, induces naïve CD4+ T cells into Tregs [46]. Similarly, Jagged-2-expressing hematopoietic progenitors promote Treg expansion via activating Notch3 [47]. In addition, expression of a stabilized form of β-catenin, a mediator of Wnt- or frizzled-signaling pathway, enhances the survival of Tregs [48], suggesting that this evolutionarily conserved pathway in cell differentiation and development also plays a role in Treg regulation.

One recent breakthrough in studying Foxp3 expression is the discovery of retinoic acid (RA), a vitamin A metabolite, induces Foxp3 expression in synergy with TGF-β [49–52]. RA is generated in functionally specialized mucosal DCs, which, when co-cultured with naïve CD4 + T cells, converts them into Foxp3+ T cells, whereas addition of either anti-TGF-β or RA antagonist abrogates the Foxp3 induction by this DC subset. More importantly, in vitro-generated Tregs by TGF-β and RA are more effective in preventing mice from colitis than the cells with only TGF-β treatment.

In addition to its effect on Tregs, TGF-β also induces the differentiation of Th17 cells in the presence of a pro-inflammatory cytokine, IL-6 [17]. In a sharp contrast to Tregs, which actively

suppress immune responses, Th17 cells are involved in promoting autoimmune and inflammatory responses. Intriguingly, RA inhibits Th17 differentiation [51] by reducing the expression of RORγt, a master transcription factor for Th17 cells [53]. Recent studies have documented that Foxp3 suppresses Th17 differentiation by antagonizing RORγt function [54,55].

Foxp3 expression in human T cells

Several studies suggested that in humans, as opposed to mice, Foxp3 expression might not always correlate with the suppressive capacity of T cells [21]. Transient expression of Foxp3 was observed in human conventional T cells upon TCR stimulation in vitro, probably due to low level of TGF-β in the culture medium in that neutralization of TGF-β function was reported to completely abrogate the activation-induced Foxp3 expression. In addition, in most cases, TGF-β-induced Foxp3+ T cells did not exhibit suppressive function [56–59]. Further, ectopic expression of Foxp3 in human naïve CD4+ T cells also failed to result in the acquisition of regulatory phenotype [60]. These findings indicate that induction of Foxp3 expression in human T cells is insufficient to convert conventional T cells into Treg-like cells or to define Treg cells.

Recent studies on epigenetic modification of Foxp3 promoter revealed a marked difference in the methylation status in natural Tregs and TGF-β-induced Foxp3+ Tregs. In natural Tregs, the CpG island within Foxp3 locus was reported to be demethylated, whereas in the latter cells as well as in Foxp3− T cells, it is methylated [37,61,62]. Consistent with these findings, pharmacological inhibition of DNA methyltransferase or knockdown of Dnmt1 gene did increase the stability of Foxp3 expression in peripheral non-Tregs [63]. It seems that the demethylation status is a prerequisite for stable Foxp3 expression and suppressive activity. As a consequence, methylation profile of the Foxp3 promoter would facilitate the distinction of truly committed Treg. Another difference in Foxp3 expression between humans and mice is the existence of a human-specific Foxp3 splice isoform, which misses exon 2 and consequently lacks the ability to bind and antagonize RORγt function [64]. The physiological role of this variant remains to be defined.

The E3 ligase Itch and its regulation

Itch was originally described by Neal G. Copeland and Nancy A. Jenkins' group from studies on mouse coat color alterations [65], whose mutation is linked to a skin scratching phenotype and immunological disorders, manifested by hyperplasia of lymphoid organs and inflammation in the lung and digestive tract. This mutation results from a chromosomal inversion, which disrupts the expression of a novel gene, called Itch [66]. Itch protein contains an N-terminal protein kinase C (PKC)-related C2 domain, four protein-interacting WW domains, followed by a carboxyl-terminal E3 ligase domain.

We found that Itch−/− T cells displayed enhanced cell proliferation and chronic activation [67]. Particularly, the mutant T cells produce more Th2 cytokines like IL-4 and IL-5, and sera from Itch−/− mice contain higher levels of IgG1 and IgE as compared with wild-type mice. At the molecular level, Itch WW domains bind to a PPXY motif in JunB, a member of Jun family proteins, and Itch promotes ubiquitin conjugation to JunB. Our results are consistent with previous publications in that JunB has been shown to be an important regulator in the differentiation of Th2 cells both in JunB transgenic mice and JunB gene-targeted mice [68, 69].

The E3 ligase activity and function of Itch is regulated by upstream kinases. It was demonstrated that a MEKK1-JNK-mediated signaling pathway controls the turnover of Jun proteins via the serine/threonine phosphorylation of Itch and its subsequent activation [70]. In

addition, we showed that Itch is also regulated by Fyn-mediated tyrosine phosphorylation. Unlike the serine/threonine phosphorylation, tyrosine phosphorylation of Itch does not affect its E3 ligase activity; rather, it negatively modulates its association with the substrate JunB [71].

Itch in T cell anergy

T cell anergy represents one of the peripheral tolerance mechanisms, in which T cells lose the ability to proliferate and produce IL-2 upon restimulation [72]. Early studies have documented that T cell anergy is due to defective TCR signal transduction starting from partial or reduced phosphorylation of upstream Src kinases, decreased Erk phosphorylation, or diminished activation of AP-1 transcription factors [73–75]. Recent studies have shown that E3 ubiquitin ligases such as GRAIL, Cbl-b, and Itch play a critical role in the process of T cell anergy induction [76–78]. Upregulation of these E3 ligases results in the downmodulation of critical signal molecules such as PLC-γ1 or PKCθ that blocks T cell activation even upon effective stimulation.

In a soluble antigen-induced tolerance induction mouse model, in which mice were injected systematically with high dose soluble antigen, followed by immunization with the same antigen plus either alum adjuvant to elicit Th2 response, or CFA adjuvant to induce Th1 response, Itch is primarily involved in the Th2 tolerance induction, since Itch−/− T cells continue to produce Th2 type cytokines, and Itch−/− mice develop severe airway inflammation [79]. In addition, mice deficient in either MEKK1 kinase domain or JNK1 displayed similar resistance to Th2 tolerance induction, indicating that MEKK1-JNK1 signaling converges with Itch-mediated ubiquitination to regulate Th2-mediated allergic responses.

Itch in Foxp3 expression

To understand how Itch is involved in the regulation of airway inflammation, we set up an intranasal tolerance protocol. Consistent with previous report [80], wild-type mice that inhaled the aerosolized antigen did not show airway inflammation. However, the same treatment failed to inhibit the lymphocyte infiltration in the lung of Itch−/− mice [81]. It looks that although Itch is not involved in the development of naturally occurring CD25+CD4+ Tregs, it affects the generation of TGF-β+ adaptive Tregs during tolerance induction.

Next we examined the responsiveness of Itch−/− T cells to Treg- or TGF-β-mediated suppression and found that Itch−/− CD4+CD25− T cells were resistant to the suppression by both Tregs and TGF-β. TGF-β treatment resulted in an upregulation of both Foxp3 gene transcription and protein expression in wild-type T cells, but to a much less degree in Itch−/− T cells. The in vitro converted Tregs from wild-type mice showed suppressive activity towards CD4+CD25− T cells, whereas TGF-β-treated Itch−/− T cells were much less inhibitory. The results collectively suggest that loss of Itch alters TGF-β signaling in T cells and affects TGF-β-induced Foxp3 expression.

Krüppel-like factors and TIEG1

The Krüppel-like factors (KLFs) are DNA-binding transcription factors that belong to the well-characterized Sp1 family transcription factors and are implicated in diverse biological processes including proliferation, development, differentiation, and apoptosis [82]. The family of about 17 members is characterized by the presence of highly conserved C-terminal triple zinc fingers that bind to GC-rich sequences or CACCC elements of the target gene promoters. The N-terminal transcription regulatory domains are highly variable and associate with either transcription co-activators such as p300/CREB-binding protein, C-terminal binding protein 2, or HDAC, the co-repressors that mediate transcriptional repression.

The TGF-β-induced early gene product 1 (TIEG1 or KLF10) is one of the family members whose expression is induced by the stimulation with TGF-β or EGF receptors [83]. A protein of 480 amino acids consists of the C-terminal conserved triple zinc fingers for DNA-binding and N-terminal transcriptional regulatory domains, with multiple proline-rich sequences spanning the entire protein. It has two very close homologues, TIEG2 (KLF11) and TIEG3, which are highly homologous in the Zinc finger domains, with some divergence in the regulatory region. Expression of TIEG1 mimics TGF-β-induced proliferative inhibition and enhances Smad-dependent transcriptional responses [84,85]. It also promotes myeloid-specific transactivation of the leukocyte integrin CD11d promoter [86]. TIEG1 null mice display reduced osteoblast differentiation in response to TGF-β-related bone morphogenetic protein 2 stimulation [87].

TIEG1 as a target for Itch

One critical question is how Itch regulates TGF-β signaling pathway and thereby modulates Foxp3 expression. Although we have observed that Itch modulates the phosphorylation status of Smad2 protein in mouse embryonic fibroblasts [88], such alterations were not obvious in Itch−/− T cells, which may reflect the difference in cell types. The next step is to screen further downstream signaling molecules that may act as the target protein(s) for Itch. One of the putative targets is TIEG1 protein, which contains multiple proline-rich sequences. A series of biochemical studies suggested that Itch associates with TIEG1 in vitro and in cells via Itch WW domains [81]. Importantly, Itch promotes ubiquitin conjugation to TIEG1 in both the mono-and polyubiquitinated forms. Functionally, coexpression of Itch and TIEG1 induces an augmented transactivation of Foxp3 promoter. In addition, TIEG1 directly binds to the Foxp3 promoter as revealed by the DNA-binding gel shift assay and chromatin immunoprecipitation assay. To examine a direct role of TIEG1 in Foxp3 expression, we expressed TIEG1 in mouse CD4+ T cells and found that TIEG1 expression resulted in Foxp3 expression in wild-type CD4 + T cells. However, the induction of Foxp3 was much less in Itch−/− T cells. The results pointed out that TIEG1 is a positive regulator of Foxp3 expression, whose activity is dependent on Itch-mediated ubiquitination.

The importance of TIEG1 in Foxp3 expression was further tested in the responsiveness of wild-type and TIEG1−/− T cells to TGF-β treatment. Like Itch−/− T cells, loss of TIEG1 in T cells resulted in a resistance to TGF-β-induced proliferative inhibition. Such defective Foxp3 expression could be reversed by retroviral TIEG1 reconstitution of TIEG1−/− T cells. To understand the biological relevance of TIEG1 in Treg function, we employed a murine model of airway inflammation. TGF-β-treated TIEG1−/− CD4+ T cells displayed much less inhibitory effect to the responder T cells in comparison with TGF-β-treated wild-type CD4+ T cells. The results provided solid genetic evidence that TIEG1 is involved in TGF-β-induced Foxp3 expression and the suppressive function of adaptive Tregs.

Concluding remarks

The abnormal immunological and inflammatory responses in itchy mice suggest that Itch is involved in the regulation of the key components in the immune system. The identification of a critical role of Itch in Th2 differentiation and T cell anergy induction and the recently documented role in induced Treg development provide with us the molecular insights into the mechanisms by which Itch participates in T cell regulation. More importantly, Itch acts as an E3 ubiquitin ligase to promote the ubiquitin conjugation to specific target proteins such as Jun proteins for proteasome-dependent degradation or TIEG1 for the proteolysis-independent functional modification. In the latter case, it remains to be addressed how Itch-mediated mono-ubiquitination of TIEG1 acts a positive regulator of Foxp3 expression.

Additional targets have been discovered for the E3 ligase Itch. One of those is the anti-apoptotic protein c-FLIP, an inhibitor of caspase-8, and a critical player in the pro-inflammatory cytokine TNFα-mediated cell survival and death pathway [89]. More recently, it was found that Itch forms complex with the ubiquitin-editing enzyme A20 and the adaptor protein RIP1 and affects RIP1 ubiquitination and its stability, thereby regulating TNFα-induced NF-κB activation [90]. These new findings will help explain the excessive inflammation manifested in the Itch mutant mice. Clearly, future studies will be aimed at delineating the multiple signaling pathways regulated by Itch and their relative importance in mediating the dysregulated immune and inflammatory responses in itchy mice. Such understanding will add in discovering novel therapeutic targets for clinical applications.

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