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Regulation of T Cell Differentiation and Allergic Responses by the E3 Ubiquitin Ligase Itch

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Abstract. Itch is an E3 ubiquitin ligase that is originally identified by genetic analysis of a mutant mouse with aberrant immunological phenotypes and constant itching in the skin. Itch$^{-/-}$ T cells are biased toward the differentiation of T helper type 2 cells with augmented interleukin-4 cytokine production and serum IgE level. One of the mechanisms for Itch E3 ligase to regulate T cell responses is the induction of T cell anergy in which T cells become unresponsive upon restimulation. However, the detailed mechanisms underlying Itch-mediated protein ubiquitination and allergic responses remain to be investigated. Here we provide evidence that Itch is involved in the regulation of transforming growth factor (TGF)-β signaling in naïve T cells and TGF-β-induced expression of the tran-

scription factor Foxp3, a master regulator in regulatory T cells. Itch promotes ubiquitin conjugation to TGF-β inducible early gene 1 product (TIEG1). Moreover, monoubiquitinated TIEG1 positively modulates the transcription of *Foxp3* gene. The results suggest a novel mechanism by which Itch regulates regulatory T cells and subsequent allergic responses.

1 The Ubiquitin Conjugation System

The ATP-dependent protein degradation via conjugation to a protein substrate with a 76 amino acid peptide, ubiquitin, was discovered in early 1980s (Hershko et al. 1980). It has now been well established that this conjugation process, or protein ubiquitination, is carried out by a cascade of enzymatic reactions (Pickart 2001; Weissman 2001). The C-terminal glycine residue of ubiquitin is first activated by the ubiquitin activating enzyme or E1 to form a high-energy thiol-ester bond between the C-terminal glycine residue of ubiquitin and the active cysteine of E1. The activated ubiquitin is then transferred to one of the ubiquitin conjugating enzymes, or E2s, via a similar thiol-ester bond formation. The ubiquitin ligases, or E3s, recruit a protein substrate and bind to the ubiquitin-E2 complex and thereby help transfer ubiquitin from E2 to the lysine residue of a substrate via a covalent isopeptide bond formation. Based on the structure of E2 binding, the E3 ubiquitin ligases can be generally divided into two families, the really interesting new gene (RING)-type E3s, and the homologous to E6-associated protein C-terminus (HECT)-type E3s. In addition to E2 binding, the E3 ligases also contain well-defined protein interaction domains, through which the E3s confer the specificity of the substrate targeting in the ubiquitin system.

A substrate can be tagged with a single ubiquitin molecule to one lysine residue of a substrate, called monoubiquitination, or to multiple lysine residues to form multiple monoubiquitination (Hicke 2001). A substrate can also be tagged with more ubiquitin molecules via successive conjugation of one ubiquitin to another by utilizing lysine residues of the ubiquitin to form polyubiquitination. Depending on the usage of different lysine residues on a ubiquitin, the polyubiquitin chain can be

formed via the lysine 48 (K48) linkage, or K63 linkage. Evidence has been accumulated that monoubiquitinated protein occurs more often on cell surface receptors, which results in lysosome-dependent downmodulation, whereas K48-linked polyubiquitination leads to proteasome-dependent degradation, and K63-linked polyubiquitination is related to protein complex formation (Pickart 2001; Weissman 2001). In addition to ubiquitin, ubiquitin-like molecules such as SUMO (small ubiquitin-like modifier), or ISG15, are also tagged to the substrate in a manner similar to the ubiquitin conjugation pathway (Liu et al. 2005).

2 Ubiquitination in Immune Regulation

The process of protein ubiquitination has been implicated in various aspects of immune regulation, including the development, differentiation, activation, and tolerance induction of lymphocytes, viral and bacterial infection, antigen presentation, and immune evasion (Liu 2004). The identification of Cbl family proteins as RING-type E3 ubiquitin ligases clearly indicates that the lymphocyte function is tightly controlled by protein ubiquitination. Particularly, loss of Cbl-b results in excessive IL-2 cytokine production and T cell proliferation even without the necessity of CD28-mediated costimulation (Bachmaier et al. 2000; Chiang et al. 2000). Cbl-b was identified as an E3 ligase for the p85 subunit of PI3-kinase, which affects its association with upstream signaling molecules without directly affecting its degradation (Fang and Liu 2001). One of the recent findings is the involvement of Cbl-b in the induction of T cell anergy, a process of unresponsiveness upon T cell restimulation (Heissmeyer et al. 2004; Jeon et al. 2004). Cbl-b$^{-/-}$ T cells are not susceptible to either ionomycin-induced or high-dose soluble antigen-induced tolerance induction. In addition, in a mouse model of collagen-induced arthritis, Cbl-b$^{-/-}$ mice display early onset and severe joint inflammation in response to collagen immunization (Jeon et al. 2004). In addition to Cbl-b, other E3 ubiquitin ligases such as the RING finger protein Grail or the HECT-type E3 ligase Itch are upregulated during T cell anergy induction (Heissmeyer et al. 2004). Indeed, Grail E3 ligase is a critical player in T cell tolerance, as revealed by both in vitro and in vivo experimental approaches (Anandasabapathy et al. 2003).

Protein ubiquitination is a reversible process that is catalyzed by deubiquitinating enzymes. Analysis using a genomic bioinformatics approach suggests that more than 100 deubiquitinating enzymes are present in the human genome (Nijman et al. 2005). Both genetic and biochemical studies have implicated some of the deubiquitinating enzymes such as A20 or CYLD in the regulation of both innate and adaptive immune responses (Boone et al. 2004; Reiley et al. 2006).

3 The E3 Ligase Itch

Itch was originally described by Neal G. Copeland and Nancy A. Jenkins's group from studies on mouse coat color alterations (Hustad et al. 1995). Mutations in the agouti locus on mouse chromosome 2 that either upregulate or downregulate the expression of agouti protein cause the color changes in the hair shaft. One of these mutations, α18H, results from the decreasing expression of agouti, which leads to darker than normal coats. Interestingly, unlike mutations in other alleles, this mutation also causes a skin (in the back and neck) scratching phenotype and immunological disorders, manifested by hyperplasia of lymphoid organs and inflammation in the lung and digestive tract. Due to the constant itching in the skin, the mutant mice were also called itchy mice. It was hypothesized that in addition to the agouti gene, there was another mutation in the α18H allele, which is responsible for the itchy and immunological abnormality.

Subsequent genetic studies by the same group confirmed this hypothesis and revealed that the α18H mutation results from a chromosomal inversion that deletes 18 and 20 base pairs from the proximal and distal inversion breaks, respectively (Perry et al. 1998). This inversion affects the expression of agouti and disrupts the expression of a novel gene, named Itch. Sequencing of the Itch cDNAs identified an open reading frame of 2,562 base pairs, which encodes 854 amino acids with a molecular mass of approximately 113 kDa. Homology alignments of the predicted amino acid sequences showed that the Itch protein contains a carboxyl-terminal E3 ligase domain, proceeded by four protein-interacting WW domains, with high homology with E3 ligases such as the yeast Rsp5 or the mammalian Nedd4 proteins. This genetic study

suggests for the first time that Itch may act as an E3 ubiquitin ligase in regulating immune responses.

4 Itch Regulates Th2 Development

Given the immunological disorder in itchy mice, we initiated the study of Itch in immune regulation by identifying its potential target proteins. A genetic study on Notch signaling in *Drosophila* revealed a novel gene product, suppressor of Deltex or Su(dx), a negative regulator of Notch signaling (Cornell et al. 1999). Su(dx) belongs to the family of HECT-type E3 ligases similar to Itch. We found that Itch indeed is an E3 ligase for Notch by promoting Notch ubiquitination both in test tube and in transfected cells (Qiu et al. 2000). Itch$^{-/-}$ T cells displayed enhanced cell proliferation and chronic activation (Fang et al. 2002). Particularly, the mutant T cells produce more Th2 cytokines like interleukin-4 (IL-4) and IL-5 and sera from Itch$^{-/-}$ mice contain higher levels of IgG1 and IgE 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 (Hartenstein et al. 2002; Li et al. 1999).

Although those studies suggest that Itch is important in modulating critical signaling pathways by targeting specific substrates for ubiquitination, the mechanisms by which Itch-induced protein ubiquitination is regulated remain largely unclear. A recent study from Michael Karin's group, in collaboration with us, may shed light on this aspect, in which a MEKK1-JNK-mediated signaling pathway controls the turnover of Jun proteins via the serine/threonine phosphorylation of Itch and its subsequent activation (Gao et al. 2004). More recently, 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 ligase activity, rather it negatively modulates its association with the substrate JunB (Yang et al. 2006). These studies indicate that Itch is regulated by both tyrosine and serine/threonine kinases, but with

opposing effects, suggesting that Itch-mediated JunB ubiquitination is tightly controlled by upstream kinases via counterbalancing tyrosine vs serine/threonine phosphorylation.

5 Self-tolerance in the Immune System

Mature T cells are capable of mounting robust immune responses against invading pathogens, but at the same time are tolerant of self-tissues. The induction of T cell tolerance involves many mechanisms at different stages of T cell development. At first, self-reactive T cells are eliminated during thymocyte maturation via negative selection, a process called central tolerance. In addition to thymus-derived antigens, many antigens from other tissues or organs are expressed in the thymus antigen-presenting cells, which causes clonal deletion of T cells specific for self-peptide–MHC complexes. Evidence supporting the central tolerance mechanism includes the finding of AIRE, a transcription factor as well as an E3 ubiquitin ligase, which promotes the expression of many peripheral tissue antigens in thymus medullary epithelial cells (Anderson et al. 2002).

However, the central tolerance mechanism is not sufficient, since autoreactive T cells can escape into the secondary lymphoid organs, where the peripheral tolerance mechanisms take effect to keep them under control. Several mechanisms have been proposed to account for peripheral T cell tolerance to self-antigens, which include ignorance, activation-induced cell death, T cell anergy, and suppression by regulatory T cells (Tregs) (Walker and Abbas 2002).

6 Itch in Th2 Tolerance Induction

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 (Schwartz 2003). 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 (Fields et al. 1996; Gajewski et al. 1994; Li et al. 1996). Recent stud-

ies 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 (Anandasabapathy et al. 2003; Heissmeyer et al. 2004; Jeon et al. 2004). 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.

We went on to investigate the in vivo biological function of Itch during soluble antigen-induced tolerance induction. In this tolerance model, 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 (Venuprasad et al. 2006). 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. In addition, mice deficient in either MEKK1 kinase domain or JNK1 displayed similar resistance to Th2 tolerance induction, supporting a notion that MEKK1-JNK1 signaling converges with Itch-mediated ubiquitination to regulate Th2-mediated allergic responses.

7 Regulatory T Cells

Regulatory T cells, or Tregs, are a unique subset of the T cell population, characterized by the cell surface expression of CD4 and the IL-2 cytokine receptor alpha chain CD25, play a pivotal role in maintaining self-tolerance via actively suppressing the effector function of other T cells (Sakaguchi 2004). The transcription factor Foxp3 is a master regulator of Treg development and function, since loss or mutation of Foxp3 is linked to abnormal T cell responses and the development of autoimmune diseases. Tregs are generated in the early stage of thymic development, which become the naturally occurring $Foxp3^{+}CD4^{+}CD25^{+}$ Tregs in the periphery. In addition, peripheral $CD4^{+}$ T cells can be converted into $Foxp3^{+}$ Tregs by either tolerogenic antigen stimulation in vivo or TGF-β stimulation in vitro (Apostolou and von Boehmer 2004; Chen et al. 2003; Fantini et al. 2004; Kretschmer et al. 2005; Li et al. 2006; Wan and Flavell 2005). However, the molecular mechanisms for such conversion remain largely unclear.

Historically, instead of a systematic antigen injection as described above, oral or nasal antigen administration has been used to induce Th2 tolerance (McMenamin et al. 1994). Unlike the Th2 tolerance induced by high-dose antigen injection, tolerance via the oral or nasal route has been shown to result from the generation of Tregs (Mucida et al. 2005; Ostroukhova et al. 2004). Repeated exposure to inhaled low-dose antigen results in the generation of $Foxp3^+CD4^+$ Tregs that also express membrane-bound TGF-β (Ostroukhova et al. 2004). These Tregs from the tolerized mice inhibit the proliferation of normal $CD4^+$ T cells and suppress Th2-mediated allergic responses when adoptively transferred into a naïve host. A more recent study suggests that antigen-specific Tregs could be generated via oral tolerance in the absence of naturally occurring Tregs (Mucida et al. 2005). Like the inhaled antigen-induced Tregs, the oral tolerance-generated Tregs suppress $CD4^+$ T cell proliferation in vitro and inhibit IgE production and lung inflammation in vivo. In both studies, TGF-β was shown to be involved in the proper function of Treg-mediated suppression of the Th2 response, since administration of anti-TGF-β antibody abrogated the tolerance induction and hence the restoration of allergic responses. It remains unknown whether Itch affects Treg-regulated allergic responses.

8 TGF-β Signaling in Immune Regulation

TGF-β signaling is involved in diverse cellular responses such as cell proliferation, differentiation, apoptosis, and migration (Attisano and Wrana 2002). 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 by directly competing with Smad2/3 for receptor interaction. In addition to the Smad-dependent signaling pathways, TGF-β also activates Smad-independent signaling pathways (Derynck and Zhang 2003).

The intracellular signal transduction induced by TGF-β is regulated by the ubiquitin system, as E3 ligases such as Smurfs directly associate with Smad proteins and affects the stability of Smads or their binding partners (Izzi and Attisano 2004). We found that Itch$^{-/-}$ fibroblasts are resistant to TGF-β-induced proliferative inhibition (Bai et al. 2004). Itch E3 ligase directly promotes the ubiquitination to Smad2/3 and affects their phosphorylation by the receptor. However, whether Itch is involved in the TGF-β signaling in T cells remains unclear.

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 (Kulkarni et al. 1993; Li et al. 2006; Marie et al. 2006; Shull et al. 1992). TGF-β signaling regulates both Th1 and Th2 cell differentiation (Gorelik et al. 2000, 2002). As described earlier, TGF-β also plays an important role in Treg generation and maintenance (Chen et al. 2003; Fantini et al. 2004; Li et al. 2006; Wan and Flavell 2005). In addition, recent studies have demonstrated a critical role of TGF-β in the development of Th17, a new subset of T helper cells, which are involved in autoimmune and inflammatory responses (Weaver et al. 2007). However, the detailed intracellular signaling pathways that TGF-β initiates in diverse processes of different types of T cells remain to be investigated.

9 Itch in the Development of Regulatory T Cells

To understand how Itch is involved in the regulation of airway inflammation, we set up an intranasal tolerance protocol. Consistent with a previous report (McMenamin et al. 1994), 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 (Venuprasad et al. 2008). It seems 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 toward 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.

10 Itch Promotes Ubiquitination of TIEG1

To understand the mechanisms underlying the hyporesponsiveness of Itch$^{-/-}$ T cells to TGF-β treatment, we looked for further downstream signaling molecules that may act as the target protein(s) for Itch. One of the putative targets is the TGF-β-induced early gene 1 (TIEG1) protein, which contains multiple proline-rich sequences and is rapidly induced upon TGF-β stimulation and functionally mimics TGF-β-mediated transcriptional events in transiently transfection systems (Hefferan et al. 2000). A series of biochemical studies suggested that Itch associates with TIEG1 in vitro and in cells via Itch WW domains (Venuprasad et al. 2008). 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 released 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.

To further understand the involvement of TIEG1 in Foxp3 expression, we compared 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

defect in Foxp3 expression could be reversed by TIEG1 reconstitution of TIEG1$^{-/-}$ T cells. In addition, 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.

11 Perspectives

Previous studies have identified several substrates for the Itch E3 ubiquitin ligase, such as JunB, or c-FLIP (Chang et al. 2006; Fang et al. 2002; Gao et al. 2004), which go through proteasome-dependent degradation. The recent identification of TIEG1 as another target for Itch provides a novel mechanism of regulation, in which ubiquitin-conjugation to TIEG1 leads to its transcriptional activation of Foxp3 promoter. In this case, Itch most likely induces monoubiquitination of TIEG1, which in turn directly binds to the promoter region of *Foxp3* gene. How exactly the monoubiquitination of TIEG1 exerts its biological function, either via formatting complex with other transcription factors, or affecting its binding affinity to a particular DNA binding motif, remains to be investigated. Another issue is to fully comprehend the functional overlap of the Itch E3 ubiquitin ligase in differentially regulates Th2 vs Treg development. It is quite possible that the two mechanisms are not mutually exclusive: the regulation of Th2 differentiation by Itch may have an impact on its effect on Foxp3 expression in the Tregs, or vice-versa. Interestingly, it was recently shown that Foxp3 expression is correlated with Th2 cytokine production: Foxp3 deficiency results in a conversion of conventional CD4^{+} T cells into Th2 type effector T cells (Wan and Flavell 2007). Further investigation of the correlation of the two different T cell differentiation pathways may shed light on the mechanistic insight into the regulation of Th2-mediated allergic responses, which will eventually lead to the discovery of new therapeutic approaches for allergic and other immunological abnormalities.

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