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E3 Ligases in T Helper 2-mediated Pathogenesis

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Abstract

Development of immunological tolerance has emerged as a critical mechanism for prevention of inflammatory and autoimmune diseases. Of the T helper (Th) subsets, the Th2 cells are the major players in allergic airway inflammation. Work in the past decade has been focused on active regulators of the Th2 differentiation program; however, the mechanisms that control Th2-mediated inflammation are poorly understood. E3 ubiquitin ligases (Cbl-b, Itch and GRAIL) which are implicated in T cell tolerance induction have been acknowledged as crucial factors in controlling the development and function of pro-allergic Th2 cells. Understanding of the molecular mechanisms underlying the function of these ligases in Th2 cells will lead to development of pharmacological approaches to promote the tolerance state in Th2 cells in order to treat allergic inflammation.

Keywords: Th2; Allergy; Asthma; E3 ligases

Introduction

Upon encountering antigen, naive $CD4^+$ T cells differentiate into various T helper (Th) subsets including Th1, Th2, Th9, Th17, follicular helper (Tfh) and regulatory (Treg) T cells which play critical functions in regulating adaptive immune responses [1-3]. The Th2 cells regulate immune responses against extracellular parasites and are involved in asthma and other allergic diseases [4,5]. Together with T cell receptor (TCR) ligation, IL-4/IL-4R-mediated signaling promotes Th2 differentiation and production of signature Th2 cytokines including IL-4, IL-5, and IL-13 [6,7] through activation of Th2 master regulators such as STAT6 (signal transduction and activator of transcription 6) and GATA3 [8]. Th2-derived IL-4 acts in an autocrine feedback loop to promote further Th2 differentiation [9].

Although a robust immune response is critical for successful elimination of pathogens, the immune system is also equipped with a mechanism to prevent excessive damage to normal cells and tissues [10,11]. Failed self-tolerance may lead to abnormal T helper cell activation and consequently to autoimmunity and inflammation [12]. Multiple mechanisms have been implicated in induction of peripheral immune tolerance, including T-cell intrinsic extracellular (positive and negative costimulatory molecules on APCs) and intracellular mechanisms (E3 ubiquitin ligases, transcriptional and epigenetic repressors) as well as extrinsic mechanisms (Tregs and tolerogenic dendritic cells) [10].

It is well known that decision of T-cell tolerance or effector function is determined by combinatorial costimulatory signals. In the absence of positive costimulatory molecules, such as CD28 and ICOS, negative costimulatory molecules, such as PD-1, B7-S1 and B7-H3, are required for tolerance induction in T cells [10]. In addition to costimulatory molecules, post-translational modification processes including ubiquitination of proteins within receptor signaling complexes is essential for eliciting adequate but confined immune responses [13]. Ubiquitination is a process in which 76 amino acid peptide ubiquitin is covalently attached to one or more lysine residues of a target protein. This process is a sequence of three events that is regulated by three classes of enzymes termed E1, E2 and E3 [13]. The first step includes the formation of linkage between ubiquitin and the E1. The next step catalyzes the transfer of ubiquitin from E1 to E2. The third and final step of ubiquitination is accomplished by an E3 ubiquitin ligase resulting in the formation of bond between the C-terminal glycine of ubiquitin and a lysine residue of a target protein. E3 ligases could be divided into subgroups according to their structure and mode of action: the homology to E6-assotiated protein carboxyl terminus (HECT) domain containing and the really interesting new gene (RING) domain family of E3 ligases [13]. Ubiquitin E3 ligases of both RING (Cbl-b, GRAIL) and HECT (Itch) types have emerged as major players in controlling undesired T cell activation [14-18]. Defects in E3 ligase activity have been implicated in the pathogenesis of a variety of different diseases, including autoimmunity and inflammation, underlining the importance of tight regulation of the expression and activity of these ligases.

Abnormal Th2 cell activation can result in asthmatic or allergic symptoms; thus it is crucial to understand mechanisms that control the Th2 fate. The E3 ligases, Cbl-b, Itch and GRAIL are known to influence the Th2 cell differentiation pathway and Th2 mediated pathogenesis [19-21]. This review attempts to summarize the recent advances in understanding the role of these ligases in controlling the Th2 cell development and pathogenesis.

Th2-mediated pathogenesis

By secreting a variety of cytokines including IL-4, IL-5, IL-13, IL-9, IL-25 and IL-33, Th2 cells play an important role in the pathophysiology of allergic diseases, including asthma [22]. The cytokine IL-4 is important for allergic sensitization, IgE production, expression of proinflammatory mediators like IL-6, GM-CSF, VCAM-I, histamine and serotonin [1,23] and its deficiency leads to abrogated airway inflammation [24]. IL-5 is crucial for eosinophil recruitment and along with IL-13 induces mucus secretion, airway hyperresponsivity and tissue damage in chronic asthma [25]. Administration of recombinant IL-13 is known to induce airway hyperreactivity, whereas neutralization of IL-13 attenuates asthma development [26,27]. IL-9 which is produced by both the Th9 and Th2 cells, plays a significant role in the development of asthma by activating mast cells, eosinophils, B cells and

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epithelial cells [28]. IL-25, which belongs to the IL-17 family, induces IL-4, IL-5 and IL-13 cytokine production and consequently enhances mucus production, eosinophilia and IgE production [29]. IL-33 is also known to contribute to Th2 immune responses and the development of asthma. IL33-deficient mice develop milder airway inflammation following allergen challenge [30].

Factors controlling Th2 differentiation

TCR and co-stimulation: TCR signaling plays an important role in early IL-4 production by naïve $CD4^+$ T cells. Low strength TCR activation leads to weak and transient extracellular signal-regulated kinase (ERK) activation, GATA3 induction and responsiveness to IL-2, and consequently to early IL-4 transcription [31-35]. The endogenously produced IL-4 and continued STAT5 activation by IL-2 provide a necessary survival and enhancing signal for development of high-rate IL-4-producing Th2 cells [35]. Apart from TCR signal, the co-stimulatory receptor CD28 favors Th2 differentiation by enhancing activation of NFATc1 and inducing NF-κB dependent GATA3 expression resulting in IL-2 and IL-4 expression [5,36-38]. Another member of the CD28 superfamily, ICOS by enhancing NFATc1 expression and initial IL-4 production during early T cell differentiation, regulates c-Maf expression in the effector phase and hence effector IL-4 production [4,39-41]. In addition, the tumor necrosis factor receptor family members OX40, CD30 and GITR play crucial roles in Th2 survival and expansion [42-46].

Cytokines: Although the TCR and costimulatory molecules dramatically influence the Th cell fate, cytokines are the major determinants of Th cell differentiation. Cytokines including IL-4, IL-2, IL-6, IL-25, IL-33 and TSLP are involved in Th2 differentiation [4,5]. IL-4 activates STAT6 that leads to the induction of transcriptional factor GATA3 and to transcription of Th2-cpecific cytokines including *IL-4, IL-5* and *IL-13* [9,47-50]. IL-2-mediated STAT5 activation is also crucial for IL-4 expression and Th2 differentiation [51,52]. The cytokine IL-6 is known to induce early IL-4 production through NFAT activation [53,54]. TSLP is sufficient to facilitate Th2 priming and expansion independent of IL-4 by activating STAT5 [3]. IL-25 via promoting TSLP and OX40L and up-regulation of GATA3 also contributes to Th2 development [29]. IL-33, similar to IL-25, can promote TSLP and in collaboration with TSLP and other STAT5 activators can induce Th2 cell activation independently of TCR ligation [30].

Transcription factors: STAT6 is a major signal transducer in IL-4 mediated Th2 differentiation [6,7]. While STAT6 activation is necessary and sufficient for inducing high expression levels of the Th2 master regulator GATA3 and Th2 cytokines [49,55], minimal Th2 cytokine production could be triggered by GATA3 via IL-4/STAT6 independent pathways [56-59]. Although GATA3 alone directly binds to the *IL-5* and *IL-13* promoters and transactivates these genes [60,61], coordinated activity of STAT5 and GATA3 is required to promote *IL-4* gene expression [3,51,52,62]. GATA3 also cooperates with STAT6 and binds to a large number of genes in Th2 cells [63]. In addition, GATA3 maintains Th2 cell identity by inhibiting the expression or function of factors defining other T helper subsets [58]. Recently, STAT3 was shown to be required for STAT6 interaction with relevant gene loci in the developing Th2 cells [64]. Another transcription factor IFN regulatory factor-4 (IRF4) also is involved in Th2 differentiation by upregulating GATA3 and by cooperating with NFATc2 to activate IL-4 [65,66]. c-Maf [67] acts in synergy with NFAT and activating protein (AP)-1 protein JunB to induce IL-4 expression [68,69]. Various other factors like Dec2, T cell factor-1 (TCF1) and Growth factor independent-1 (gfi-1) have been also identified to influence Th2 effector gene expression [58,70-73].

Protein ubiquitination and T cell tolerance

T cell activation and tolerance are tightly regulated to ensure effective elimination of foreign antigens while maintaining immune tolerance to self-antigens. Immunological tolerance in T cells is maintained by various mechanisms to prevent inflammatory and autoimmune diseases. This is initially mediated in thymus where self-reactive T cells are deleted by negative selection [10,11,74]. Although most self-reactive T cells are eliminated by this mechanism, it is incomplete and additional peripheral tolerance mechanisms are required [10,11,74].

Multiple molecular pathways including intrinsic extracellular (positive and negative costimulatory molecules on APCs) and intracellular (E3 ubiquitin ligases, and transcriptional and epigenetic repressors) as well as extrinsic (Tregs and tolerogenic dendritic cells) mechanisms are required for the implementation and maintenance of T-cell tolerance at the periphery [10,74]. In tolerant T cells, upon TCR engagement in the absence of positive CD28 and ICOS costimulation, over-activated NFAT without its transcriptional partner AP-1 imposes a genetic program of T-cell tolerance including expression of tolerance-associated genes such as E3 ubiquitin ligases (Cbl-b, Itch and GRAIL) [10,18,74-76]. Protein ubiquitination by E3 ligases is an essential dynamic mechanism by which T cell signaling pathways can be modified to regulate T cell responses. These ligases can specifically target TCR signaling components, such as PLC-γ, PKC-θ, CD3ζ or p85 [10,74,75] for degradation, resulting in the inability to sustain a stable immunological synapse between T cells and APCs and reduce the antigen responses in anergic T cells. Recent evidence has suggested E3 ubiquitin ligases including Cbl-b, Itch and GRAIL as crucial intrinsic regulators of T cell tolerance that maintain the balance between tolerance, activation and autoimmunity [10,74].

Cbl-b is a member of highly conserved family of Cbl proteins that function as E3 ubiquitin ligases via their RING finger domain. Cbl-b expression is up-regulated in T cells upon tolerance induction and depends on the zinc finger transcriptional factors Egr-2 and Egr-3 [75,77-79]. Genetic ablation of Cbl-b results in T cell hyper-responsiveness to TCR stimulation in the absence of CD28 costimulation and impaired induction of T-cell tolerance both *in vitro* and *in vivo* [80-83]. As a result, Cbl-b deficient mice display both spontaneous and antigen-induced autoimmunity. Mechanistically, it has been reported that Cbl-b-induced ubiquitination of p85, the regulatory subunit of PI3K, prevents its interaction to CD28 and TCRζ [84], thus attenuating the Akt-dependent and PKCζ-dependent NF-kB activation as well as Vav1-mediated TCR-clustering that are necessary for T cell activation [83, 85]. However, a recent study has revealed that Cbl-b does not regulate PI3K activity but rather suppresses CD3/CD28-induced inactivation of Pten, a negative regulator of PI3K/Akt pathway [86]. Cbl-b may exert its effect on Pten by inhibiting the association of Pten with Nedd4, which targets Pten for K63-linked polyubiquitination [86]. Thus Cbl-b regulates Pten via ubiquitin ligase-independent mechanism. In addition, Cbl-b plays a negative role in Crk-L-C3G-mediated LFA-1 activation which leads to destabilization of the immune synapse [87]. Cbl-b also by targeting PLC-γ and PKC-θ prevents their activation and calcium mobilization [75].

Besides Cbl-b, various other E3 ubiquitin ligases have been identified as critical regulators of T cell activation and tolerance; namely, the HECT type E3 ligase Itch and the RING-type E3 ligase GRAIL (gene related to anergy in lymphocytes) [10,74]. As in the case of Cbl-b, Itch and GRAIL expression is upregulated in T cells under anergizing stimuli and Itch and GRAIL knockout mice develop spontaneous autoimmune responses [10,74]. Additionally, Itch and GRAIL deficient T cells are

hyperresponsive to TCR stimulation and ablation of either of these E3 ligases renders T cells resistant to anergy induction, both *in vitro* and *in vivo* [10,74]. Itch regulates T cell anergy by targeting PLCγ and PKCθ, two key TCR signaling molecules induced by Ca^{2+}/calcineurin signaling [75]. Down-regulation of these signaling proteins associates with inability to sustain stable immunological synapse and with T cell unresponsiveness after TCR engagement. Hyper-activation of GRAIL deficient T cells is selectively associated with their inefficiency in TCR down-modulation [10,88]. Downregulation of TCR-CD3 complex by GRAIL is dependent on its E3 ligase activity and proteasome-mediated degradation [10, 88]. Thus, E3 ligases cooperate in the control of T cell responses and autoimmunity.

E3 ubiquitin ligases and allergic inflammation

Work in the past decade has revealed complex regulation of the Th2 cell differentiation program. While most of the previous studies have focused on active regulators of the Th2 differentiation program, including transcription factors like STAT6, GATA3, JunB, IRF4, c-Maf, and NFAT, the factors and mechanisms that control abnormal Th2 development and protect from Th2-mediated autoimmunity are poorly understood. Development of immunological tolerance is a crucial mechanism for prevention from autoimmune diseases. E3 ubiquitin ligases (Cbl-b, Itch and GRAIL) which are implicated in T cell tolerance induction have been acknowledged as crucial factors in regulating the development and function of pro-allergic Th2 cells by targeting the major Th2 transcriptional factors such as JunB, STAT6 and GATA3 for degradation, and thus preventing excessive Th2-mediated inflammatory responses [19-21] (Figure 1). These E3 ligases could potentially cooperate with each other to synergistically promote Th2 cell tolerance.

Cbl-b: Recent findings have elucidated the role of Cbl-b in the generation of proallergic Th2 and Th9 cells [20]. Interestingly, the expression of Cbl-b is lower in Th2 and Th9 cells compared to the Th1 and Th17 cell subsets, suggesting the negative role of Cbl-b in Th2 and Th9 development. In fact, T cells deficient in Cbl-b produced much more Th2 and Th9 cytokines such as IL-4, IL-5, IL-13 and IL-9, indicating that Cbl-b could inhibit the Th2 and Th9 programming [20]. Importantly, this observation was validated *in vivo* by utilizing the well-established model of allergic asthma which has been shown to be mediated by both Th2 and Th9 cells. Mice deficient in Cbl-b in T cells developed more severe airway inflammation accompanied by high

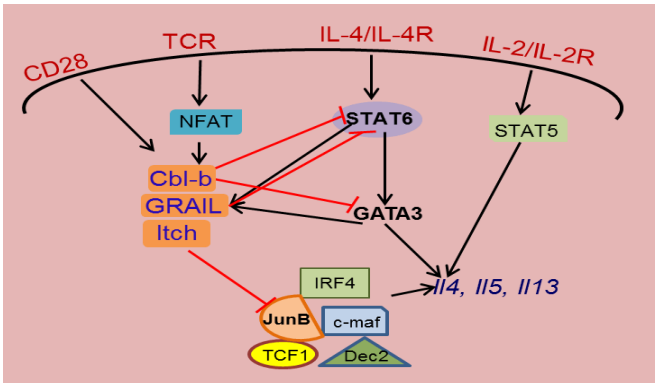

Figure 1: A schematic representation of E3 ligases mediated control of Th2 cells. T cell receptor (TCR), IL-4 receptor (IL-4R), IL-2 receptor (IL-2R) and CD28 signaling induced transcription factors are crucial for Th2 differentiation. TCR signaling induced NFAT induces the expression of E3 ligases such as Cbl-b, Itch and GRAIL in Th2 cells which modulate Th2 effector function by controlling Th2 master regulators STAT6, Gata3 and JunB through proteasome mediated degradation.

production of IL-4, IL-5, IL-13 and IL-9 cytokines in BAL fluid and increased IgE in the sera, strongly supporting that Cbl-b expression in T cells is sufficient to suppress Th2 and Th9 development and allergic inflammation [20].

At the molecular level, Cbl-b specifically associates with STAT6, a transcriptional factor which is critical for both Th2 and Th9 development, via either its TKB domain or phosphotyrosine residues in the cytosol and targets it for ubiquitination (at K108 and K398) and degradation in proteasome compartment [20]. STAT6 phosphorylation at Y641 is required for its ubiquitination. If the heightened Th2 and Th9 responses in Cbl-b deficient mice are mediated by STAT6, loss of STAT6 should abrogate these hyperresponses in Cbl-b KO mice. Loss of STAT6 in Cbl-b deficient mice completely abrogates only Th2 responses while moderately affects Th9 cell differentiation. These data suggest that Cbl-b controls Th2 responses in STAT6-dependent manner, while Th9 differentiation is regulated by Cbl-b via both STAT6-dependent and –independent mechanisms. In addition, in support of a critical role of Cbl-b in controlling of allergic inflammation, a Cbl-b mutation has been identified as an asthma susceptibility gene variant in children [89].

Impaired generation of iTregs in the absence of Cbl-b could also contribute to profound Th2 responses in Cbl-b deficient mice [90,91]. First, Wohlfert et al. suggested that Cbl-b deficiency impaired TGF-β-mediated phosphorylation of Smad2/3, which is critical for Foxp3 induction [90]. Later Yun-Cai Liu group determined that Cbl-b favors Foxp3 expression by controlling Akt-dependent inactivation of Foxo1 and Foxo3 [91]. In addition, recent studies have indicated that threshold of T cell activation, regulated by Cbl-b, determines the fate of iTreg development via Akt-2-dependent pathway [92]. Moreover, Zhang group revealed role for Cbl-b in controlling the development of thymus derived Treg cells and maintaining Foxp3 expression at a steady state through Cbl-b and Stub-1 dependent ubiquitination of Foxp3 [93].

Itch: Itch is primarily involved in Th2 tolerance induction, since severe airway inflammation and dermatitis in Itch-deficient mice are associated with excessive production of Th2 cytokines IL-4 and IL-5 as well as higher level of Th2 dependent immunoglobulins IgG1 and IgE in the sera [94]. At the molecular level, Itch WW domain binds to and induces ubiquitination of JunB, a transcriptional factor involved in Th2 differentiation [95]. Association between adaptor protein Nedd4 family-interacting protein 1 (Ndfip1) and Itch is required for Itch function towards facilitating degradation of JunB; thus preventing JunB-mediated Th2 cytokine production [96].

It has been acknowledged that Itch has a unique function in both natural (nTreg) and inducible (iTreg) regulatory T cells thus contributing to excessive Th2 response in Itch-deficient mice [97]. Itch plays a critical role in the regulation of TGF-β signaling in $CD4^+$ T cells, thus Itch deficient $CD4^+$ T cells are unable to differentiate into iTregs and are resistant to Treg-dependent immunosuppression [98]. TGFβ-dependent expression of Ndfip1 in iTregs promoted Itch-mediated degradation of JunB, hence preventing IL-4 production. Thus, Ndfip1 and Itch by silencing IL-4 production provide opportunity for iTreg cell differentiation [99]. Itch is also involved in the iTreg cell development by enhancing the function of transcription factor TIEG1 which transactivates expression of the key Treg transcription factor Foxp3 [98]. In addition to iTregs, it has been clearly revealed that nTregs require Itch to specifically restrain Th2 cell responses [19]. Compared to control mice, Treg-specific Itch-deficient mice exhibit more severe lung inflammation in an experimental model of asthma that is associated with dramatic increase in Th2 cytokines including IL-4, IL-5 and IL-

13 in BAL fluid and Ova-specific IgE in the sera [19]. Thus, lack of Itch in nTregs leads to systemic T cell activation, which skews towards Th2 cytokine production. Interestingly, Itch does not control nTreg generation, Foxp3 expression and their suppressive function or stability. However, Itch deficient nTregs and ex-nTregs produce greater amount of Th2 cytokines, suggesting the unique function of Itch in controlling Th2-like phenotype in nTreg cells [19]. In nTregs, Itch largely functions through suppression of STAT6 activation and Gata3 expression [19]. In addition, Itch-ablated Treg cell secreting Th2 cytokines could instruct non-Th2 cells to acquire Th2 inflammatory responses [19]. Thus, the function of Itch in nTregs is unique compared to other factors and is required to control Th2 inflammatory allergic responses rather than its suppressive activities.

GRAIL: In addition to Cbl-b and Itch, recent report by our group revealed the essential role of GRAIL in regulation of Th2 differentiation and Th2-mediated pathogenic responses [21]. Interestingly, high GRAIL expression in T helper cells is specifically associated with Th2 cells, suggesting the role of Th2 cytokines in controlling GRAIL expression [21]. In fact, our data indicates that IL-4 signaling components such as STAT6 and GATA3 determine GRAIL expression in Th2 cells [21]. Observed correlation between GRAIL and Th2 cytokine expression could project GRAIL as a marker of pro-allergic cells where IL-4 signaling determines GRAIL expression.

GRAIL expression in Th2 cells is essential for controlling pathogenic Th2 responses, since GRAIL deficiency in CD4+ T cells leads to augmented Th2 cell development and function *in vitro* and *in vivo* and greater susceptibility to allergic asthma [21]. Mechanistically, the enhanced function of GRAIL-deficient Th2 cells depends on the elevated level of transcription factor STAT6 and IL-4Rα-chain expression [21]. Both naïve T cells and Th2-polarized cells from GRAIL deficient mice exhibit increased STAT6 transcription factor expression. GRAIL interacts with STAT6 and targets STAT6 via ubiquitination and proteasome-mediated degradation. In addition, IL-4R expression was also enhanced in the GRAIL deficient T cells upon IL-4 treatment. Analysis of naïve $CD4^+$ T cells from Grail deficient mice revealed that the IL-4R promoter region is enriched in both permissive H3K4me3 and the restrictive H3K27me3 modifications**.** This bivalent chromatin could allow minimal IL-4R expression in naïve cells but nonetheless make it poised for rapid transcriptional activation in response to subsequent IL-4 signaling favoring Th2 differentiation. Thus, these results altogether suggest an additional mechanism for controlling peripheral Th2 tolerance, which will have implications in the prevention and treatment of allergic diseases.

Conclusion

The ubiquitination of key proteins including TCR signalling complexes and transcription factors is crucial for controlling the immune responses and prevent autoimmunity and inflammation. As more components in the ubiquitin pathway are linked to the immune system, identifying the targets and understanding the underlying biochemical mechanisms of their stringent regulation and precise specificity are becoming increasingly important. Therapeutically, understanding the molecular basis of ubiquitination in the immune system could lead to development of novel approaches for enhancing immunity and prevent breakdown of immune homeostasis. In recent years, we have come to appreciate the roles of E3 ligases like Cbl-b, Itch and GRAIL in regulating T cell responses, including Th2 cell development and effector functions (Figure 1). Dysregulation of these E3 ligases leads to pathogenesis of allergic diseases like asthma underlining the importance of their tight regulation and control. Further elucidation of the precise role of these ligases, their targets and the signaling events necessary to activate these proteins and their relative contributions *in vivo* are necessary to reveal therapeutic options for the treatment of allergic and infectious diseases.

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