Cellular Immunology 340 (2019) 103916

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

Regulation of autoimmune disease by the E3 ubiquitin ligase Itch

Emily K. Moser[a,*], Paula M. Oliver[a,b,*]

[a] Children's Hospital of Philadelphia, Philadelphia, PA, United States
[b] University of Pennsylvania, Philadelphia, PA, United States

ABSTRACT

Itch is a HECT type E3 ubiquitin ligase that is required to prevent the development of autoimmune disease in both mice and humans. Itch is expressed in most mammalian cell types, and, based on published data, it regulates many cellular pathways ranging from T cell differentiation to liver tumorigenesis. Since 1998, when Itch was first discovered, hundreds of publications have described mechanisms through which Itch controls various biologic activities in both immune and non-immune cells. Other studies have provided insight into how Itch catalytic activity is regulated. However, while autoimmunity is the primary clinical feature that occurs in both mice and humans lacking Itch, and Itch control of immune cell function has been well-studied, it remains unclear how Itch prevents the emergence of autoimmune disease. In this review, we explore recent discoveries that advance our understanding of how Itch regulates immune cell biology, and the extent to which these clarify how Itch prevents autoimmune disease. Additionally, we discuss how molecular regulators of Itch impact its ability to control these processes, as this may provide clues on how to therapeutically target Itch to treat patients with autoimmune disease.

1. Introduction

1.1. Immune cell dysfunction in autoimmunity

Autoimmunity is a major health problem worldwide. The high impact can be attributed to its chronic nature, and dearth of effective and specific treatments for the many disease types with variable clinical manifestations. In all cases, the immune system becomes dysregulated, and the body's anti-pathogen arsenal targets its own organ systems. All autoimmune diseases feature self-reactive lymphocytes, and are often first detected by the presence of self-reactive antibodies in serum. Disease types can be identified by the nature of the self-antigens and organ system targeted, yet even when correctly diagnosed, most autoimmune diseases are clinically treated with the same few broadly immunosuppressive therapies. Understanding specific processes that underlie immune cell dysregulation would allow precise targeting and avoid the devastating side effects of current therapies.

Auto-reactive lymphocytes become activated inappropriately when there is a failure to initiate or maintain immune system tolerance. Immune tolerance describes the unresponsiveness of the immune system to self-antigens and other innocuous antigens (e.g. allergens). In other words, tolerance mechanisms exist to ensure that immune cells do not attack the host, but only become activated by threats, such as injury and pathogen exposure. Immune tolerance may be broadly categorized into central tolerance and peripheral tolerance. Central tolerance occurs during lymphocyte development. As T cell and B cell precursors undergo antigen receptor rearrangements, some autoreactive T cell receptors (TCRs) and B cell receptors (BCRs) are formed. However, these receptors are removed from the repertoire through receptor editing, cell deletion, or anergy induction [1,2]. Once mature lymphocytes leave the primary lymphoid tissues, additional peripheral tolerance mechanisms are required to prevent the development of autoimmune disease, although these mechanisms are still incompletely understood. The known mechanisms include immune cell suppression by regulatory T cells, escape from anergy, and gain of autoreactivity due to somatic mutations in antigen receptors [3–6]. Once tolerance is broken, feedforward amplification of the initial damage is precipitated by inflammation and tissue destruction [7]. The cause for the loss of tolerance is often linked with a variety of genetic susceptibility and environmental exposure factors, but there are some cases where mutation of a single gene can cause autoimmunity. The ubiquitin ligase Itch is one of these critical mediators of immune tolerance. Our understanding of how Itch regulates immune cell function comes from naturally occurring and targeted loss-of-function mutations in mice and humans.

1.2. Hallmarks of autoimmunity in Itch deficiency

Itch was first discovered to be an essential enforcer of immune tolerance in 1998. A mutation in the distal regulatory region of the mouse *agouti* locus was found to cause severe autoimmunity. These mice developed lung inflammation and alveolar proteinosis, immune cell and erythroid progenitor proliferation in the spleen, enlarged lymph nodes, inflammation in the stomach, and ulceration of the skin

* Corresponding authors.
E-mail addresses: mosere@email.chop.edu (E.K. Moser), paulao@pennmedicine.upenn.edu (P.M. Oliver).

https://doi.org/10.1016/j.cellimm.2019.04.004
Received 3 April 2019; Accepted 3 April 2019
Available online 05 April 2019
0008-8749/

due to scratching [8]. This mutation was mapped to an inversion that disrupted the promoter of the neighboring gene, encoding a previously unknown catalytic HECT-type ubiquitin ligase. This gene was named Itch, in reference to scratching behavior in mice lacking this protein [9]. Itch was found to be conserved in both flies [10] and humans [11], although the importance of Itch function in human autoimmunity remained unknown.

During the decade following its discovery, the characteristics of autoimmunity in Itch deficient mice were described in greater detail. These mice exhibited aberrant CD4 T cell activation that was skewed to the Th2 lineage, supporting that Itch maintained immune tolerance in part through a role in T cells [12,13]. Additionally, Itch deficient mice displayed aberrant humoral responses, with elevated levels of total IgM, IgG, IgE and anti-nuclear antibodies [12,14]. Itch was also determined to negatively regulate NFκB signaling in macrophages and T cells, and genetic deletion of Itch in models of systemic autoimmunity worsened disease, supporting that Itch likely contributed to disease amplification and feedforward inflammation during autoimmunity [15–19].

The ability of Itch to regulate autoimmunity gained in clinical relevance in 2010, when Itch function was found to regulate immune tolerance in humans; 10 Amish children with autoimmunity were discovered to harbor an identical homozygous recessive mutation in the *Itch* gene. The mutated gene was predicted to produce a severely truncated protein, with a deletion of the catalytic HECT domain, responsible for ubiquitin ligase function. Similar to the Itch deficient mice, Itch deficient patients displayed chronic lung disease, splenomegaly, and enteropathy, as well as autoantibodies to a variety of self-antigens. In contrast to Itch deficient mice, these patients showed no signs of Th2 disease, although it is possible that the agricultural lifestyle of the Amish families could have prevented Th2 skewing. Additionally, about half of the children displayed various tissue-specific autoimmune diseases, including hypothyroidism, hepatitis, and diabetes [20]. Therefore, although Itch deficiency causes immune-mediated diseases with many similarities between mice and humans, there are significant differences, and the described mechanisms of Itch activity (from murine studies) do not fully explain the multifaceted autoimmune disease that occurs in Itch-deficient patients.

In addition to immune system dysfunction, Itch deficient patients also exhibited non-immune features that were not described in mice. Patients had marked cranio-facial abnormalities, short stature, and developmental delay, supporting the idea that Itch also functions outside the immune system, but these functions are perhaps less evolutionarily conserved. Recently, another pediatric patient with an *Itch* mutation was reported with similar clinical manifestations to the Amish patients, including stunted growth, mental retardation, autoimmune hepatitis, type I diabetes, and thyroiditis. At age three, the child died of sepsis after receiving two failed liver transplants for acute liver failure [21]. These reports highlight the importance of single gene mutations in driving autoimmunity in humans, which is in contrast to the etiology of most classic autoimmune diseases, such as systemic lupus erythematosus, which is linked to a variety of poorly-understood genetic and environmental risk factors and is less common in children than adults [22]. The identification of an additional child with an Itch mutation (distinct from the Amish cohort) suggests that mutations in *Itch* may be more common than previously thought, especially among pediatric patients with severe multifaceted autoimmune disease.

The early studies in Itch deficient mice and characterization of clinical disease in Itch deficient humans have provided the best phenotypic description of the autoimmune disease that spontaneously develops in the absence of Itch. Subsequent studies have elucidated cellular and molecular mechanisms of Itch functions in immune cells using diverse approaches: *ex-vivo* immune function assays (e.g. *in vitro* T cell activation), *in vivo* models of induced inflammation (e.g. induced airway inflammation), and *in vitro* overexpression systems. Examination of both phenotypic and mechanistic data is useful in understanding how Itch controls immune cells to prevent autoimmunity.

2. Itch regulates immune cell function to prevent autoimmune disease

Most of the evidence surrounding the role of Itch in immune cells supports the idea that Itch promotes peripheral tolerance. Less is known about how Itch may regulate immune cell development and central tolerance. There is some evidence that Itch regulates hematopoiesis and B cell development [23,24] but it is not clear if this impacts central tolerance mechanisms and selection of the TCR and BCR repertoires. After lymphocyte development, it is evident that Itch is involved at multiple stages in autoimmune disease, preventing an initial break in tolerance, then shaping the subsequent inflammatory processes that occur.

Itch prevention of autoimmunity is dependent on its enzymatic function. Most of the molecular evidence for Itch ubiquitylation of substrates originates from overexpression systems in cell lines. Many substrates have been identified using this approach, but it remains unclear to what extent these mechanisms prevent the emergence and exacerbation of autoimmunity *in vivo*. In contrast, studies that used primary immune cells and that measure endogenous (i.e. not overexpressed) enzyme/substrate relationships provide stronger evidence for physiologically relevant Itch activities that might prevent autoimmunity. As we review the roles of Itch in immune cells, we also note the experimental systems that were used to define molecular substrates of Itch. Additionally, we discuss cellular pathways and substrates regulated by Itch in non-immune cells. Defining different Itch-dependent regulatory networks could allow for therapeutic targeting of specific aspects of Itch function that could be used to treat autoimmune disease.

2.1. Itch prevents a breach in immune tolerance

Itch is required to prevent the initial breach in immune tolerance, evidenced by the spontaneous emergence of autoimmune disease in Itch deficiency, including the development of autoantibodies [8,14]. The best mechanistic link for how Itch maintains peripheral tolerance is through its role in preventing conventional Th cell activation and expansion. Itch can do this through multiple mechanisms, by regulating TCR signaling to promote anergy, through promoting T_{reg} cell function, and by promoting apoptosis. Thus, loss of Itch in either T_{regs} or conventional T cells is sufficient to drive aberrant conventional T cell activation and autoimmunity. There is growing evidence that Itch plays an important role in T cell-B cell interactions, but how this contributes to autoimmunity remains unclear.

2.1.1. TCR signaling

T cell activation occurs when TCR and costimulatory molecules are stimulated to mount a strong intracellular signaling cascade. When T cells receive a weak stimulus, such as TCR stimulation without costimulation, T cells can become anergic, or unresponsive to future stimulation. T cell anergy is an important peripheral tolerance mechanism that prevents activation of autoreactive T cells that escape central tolerance [4,5]. Itch has been shown to limit proximal TCR and cytokine signaling. By doing so, Itch enforces a threshold for the activation of T cells. Itch-deficient T cells would lack these negative regulatory mechanisms thus explaining their aberrant activation. In a model of anergy induction, incomplete T cell activation prompted Itch to ubiquitylate the TCR signal transduction molecules PKCθ and PLCγ, targeting them for degradation. This rendered the cells unresponsive to subsequent full TCR stimulation [25]. Although not specifically linked to initiation of anergy, other studies found that Itch ubiquitylated additional TCR signaling molecules to limit the propagation of signals, thus preventing T cell activation in response to sub-optimal stimuli. For example, Itch ubiquitylated TCRζ, preventing its association with the signaling molecule ZAP70 [19]. Additionally, Itch was found to ubiquitylate MEKK1, targeting it for degradation and limiting its availability to transmit TCR

signals [26,27].

In addition to regulation of well-known TCR signaling intermediates, Itch can also regulate Notch, a transcription factor that can promote TCR signaling and, when dysregulated, drive autoimmunity [18,28,29]. In cell lines, Drosophila, and in mouse hematopoietic stem cells, Itch ubiquitylates and degrades the Notch receptor to limit Notch signaling [24,30–34]. It remains to be seen whether Itch limits Notch signaling within T cells to prevent inappropriate Th cell activation and autoimmunity.

Together, these studies show that Itch is an important negative regulator of TCR signaling. Through ubiquitylation of proximal signaling molecules downstream of TCR, Itch may prevent activation and expansion of autoreactive T cells that have escaped central tolerance, preventing a breach of tolerance.

2.1.2. T cell expansion

2.1.2.1. IL-2/IL-2R signaling. The magnitude of T cell expansion is determined by both cell proliferation and cell death. Initial T cell activation depends on the strength of TCR and costimulatory molecules, but expansion of T cells after activation is governed by additional factors, including interleukin 2 (IL-2)/IL-2 receptor (IL-2R) signaling [35,36]. Itch deficient T cells show increased proliferative expansion after activation [27], and Itch has recently been linked to IL-2R signaling in Th cells. T cells that could not activate Itch showed increased proliferation and IL-2 signaling due to a failure to ubiquitylate and degrade the IL-2R proximal signaling molecule Jak1 [37]. While ubiquitylation of Jak1 may not be unique to Itch, since the closely related family member Nedd4-2 may also fail to be activated in T cells from these mice, Itch likely helps to limit Jak1 levels and, by doing so, regulates cytokine signaling.

2.1.2.2. Cell death/survival. In addition to regulating proliferation, there is considerable evidence from cell lines that Itch regulates apoptotic pathways, which could contribute to T cell expansion in Itch deficiency. Death receptor (DR) mediated apoptosis, initiated by DR ligand/DR interactions (e.g. FasL/Fas, TNFα/TNFR1 and Trail/DR4, DR5), plays an important role in maintenance of immune tolerance [38,39]. cFLIP is a homolog of caspase 8 that is expressed in multiple isoforms and can prevent caspase-mediated apoptosis. T cell specific cFLIP (long isoform) transgenic mice exhibit a similar phenotype to Itch deficient mice, and they display increased T cell proliferation and a Th2 bias [40]. Additionally, mice and humans lacking Fas develop lymphoproliferative disease similar to SLE [41,42].

From studies in cell lines, there is ample evidence that Itch can ubiquitylate cFLIP, targeting it for degradation. In HEK293 cells, Itch interacted with the long isoform of cFLIP to promote its degradation, causing caspase 8-mediated apoptosis after TNFα exposure, and this process was enhanced by JNK phosphorylation of Itch or the cancer drug cisplatin [43–45]. The interaction of cFLIP and Itch was also described in HeLa cells [46], and ovarian cancer cells [47]. Additionally, both long and short isoforms of cFLIP could associate with Itch after TRAIL ligation to promote cell death in glioblastoma cells and melanoma cells [48,49]. Because cFLIP is an important regulator of immunity and autoimmunity, and because it can be ubiquitylated by Itch to limit its expression, investigation of the interaction between Itch and cFLIP in T cell expansion is warranted.

p63 and p73 are tumor suppressors that are related to p53 and are required for p53 function [50]. The role of p53 to respond to DNA damage and promote apoptosis has been extensively studied in cancer. Emerging evidence suggests that these pathways also contribute to regulation of immune cells, including activation induced cell death and DNA damage induced cell death in T cells [51,52]. In humans, p53 expression is dramatically altered in tissues and immune cells from autoimmune patients, with notably lower levels in lymphocytes from RA patients [53]. Additionally, p53 deficient mice develop worse experimental arthritis and EAE. Furthermore, T cell conditional p53 null mice develop spontaneous autoimmune disease, and p53 deficient neutrophils display enhanced phagocytosis [54–57].

In cell lines, Itch-mediated ubiquitylation enhanced degradation of both p63 and p73 to oppose DNA damage-induced cell death [58,59]. Further characterization of this regulatory network discerned that Itch interacted with the ubiquitin ligase MDM2 (responsible for p53 degradation) to promote degradation of p63 and p73 and prevent apoptosis [60,61]. In contrast, the adaptor N4BP1 and the Hippo pathway signaling intermediate Yap1 could inhibit Itch-mediated degradation of p63 and p73, thereby promoting apoptosis [62,63]. Because p63 and p73 are predicted to limit autoimmune disease, Itch mediated degradation of these molecules in immune cells is inconsistent with the role of Itch to prevent autoimmune disease. Given that Itch can also regulate cFLIP, which opposes apoptosis, it is possible that the dominant function of Itch will depend on the cell type involved and the pathways triggered. For example, because T cells express DRs after activation, Itch regulation of cFLIP may predominate over Itch control of p63/p73. These studies raise the possibility that Itch-dependent control of cell death (i.e. via ubiquitylation of cFLIP or p63/73) might contribute to the aberrant T cell expansion that occurs in Itch deficiency. Whether this is the case or whether Itch regulates cell proliferation via as yet unknown mechanisms remains to be revealed.

2.1.3. T_{reg} cell numbers and function

In addition to its ability to directly limit TCR signaling and expansion in conventional Th cells, Itch is also required for T_{reg} cell function. T_{reg} cells help to maintain peripheral tolerance by suppressing other immune cells, including Th cells, B cells, and innate immune cells [64]. In mice, most T_{reg} cells are defined by the expression of the transcription factor FoxP3. FoxP3 expressing cells may arise in the thymus during T cell development (termed natural, or nT_{regs}), or they may be induced at peripheral sites, or *in vitro*, by the cytokine TGFβ (termed inducible, or iT_{regs}) [65,66]. Itch has been reported to regulate both iT_{reg} cells and nT_{reg} cells through different mechanisms.

Itch function in T_{reg} cells was first demonstrated in a model of induced airway inflammation, when iT_{reg} cells lacking Itch were unable to suppress lung inflammation. Mechanistically, Itch ubiquitylated the transcription factor TIEG1, enabling it to enhance transcription of FoxP3 after TGFβ exposure [67]. Interestingly, subsequent studies using mice in which Itch was deleted or non-functional in T_{regs} did not identify a defect in iT_{reg} cell generation [68,69], even though TGFβ signaling on T cells is expected to dictate maintenance of T_{reg} cells *in vivo* [70]. Instead, Itch regulated iT_{reg} cell generation indirectly; TGFβ signaling enabled Itch to restrict IL-4 production, allowing iT_{reg} cell generation [71]. Thus, while there is strong evidence to support that Itch promotes iT_{reg} cell generation, multiple mechanisms contribute to this effect.

Additional mechanisms for Itch-dependent regulation of proximal TGFβ signaling intermediates have been demonstrated in non-T cells, but whether these mechanisms impact iT_{reg} cell generation is not known. TGFβ/TGFβ receptor signaling proceeds via phosphorylation of Smad 2/3 and can by inhibited by Smad 7 [72]. In non-immune cells, there is evidence that Itch directly enhances TGFβ signaling through ubiquitylation of Smad proteins. In MEFS and HEK293 cells, Itch ubiquitylated Smad2, promoting enhanced phosphorylation and signal transduction [73]. Additionally, in A549 and NUGC3 cells, Itch ubiquitylated Smad7 to target it for degradation, thus enhancing TGFβ signaling, and increasing Smad 2/3 nuclear localization and transcriptional activity [74,75]. Finally, in U2OS and HOP92 cancer cell lines, Itch could ubiquitylate and degrade RASSF1A, thus relieving inhibition of Smad2 transcriptional activity [76]. Taken together, the evidence suggests that Itch is an important regulator of TGFβ signaling networks, working both upstream and downstream of TGFβ.

In addition to its role in TGFβ and iT_{reg} cell generation, more recent studies show the importance of Itch in nT_{reg} cell function and maintenance. Using mice that either lacked Itch or could not activate Itch in

T_{reg} cells (i.e. Itch flox or Ndfip1 flox × FoxP3Cre mice), two independent investigators have found that Itch activity in T_{reg} cells is required to prevent spontaneous inflammatory disease. T_{reg} cells lacking Itch, or Ndfip1, gained effector T cell-like properties, such as production of inflammatory cytokines and upregulation of glycolysis [68,69]. These data show that Itch is required to promote appropriate T_{reg} cell function, and defective T_{reg} cells are likely to contribute to aberrant effector T cell activation and spontaneous autoimmunity caused by Itch deficiency.

2.1.4. T cell-B cell interactions

One of the insights gained from the discovery of patients lacking Itch was that Itch deficiency led to severe multifaceted autoimmune disease, accompanied by autoantibody production. Itch deficient mice also display increased serum antibody and autoantibody, but how Itch limits antibody production is still unclear. Antibody is produced by activated B cells and plasma cells, which may develop with or without T cell help. T cells provide help to B cells in a variety of ways that depend on soluble factors (cytokines) or cell-cell contact (costimulatory molecules and integrins) [77]. The majority of class-switched long-lived antibody responses are generated from germinal centers (GCs), where B cells and follicular helper Th cells (Tfh) collaborate to orchestrate B cell affinity maturation and plasma cell differentiation [78]. GCs are also an important source of autoantibody; autoreactive B cells are generated by somatic mutation of B cell receptors, but these cells generally die. Loss of immune regulators in GC B cells (e.g. Fas) can rescue these cells from death and drive autoimmunity [79]. The role of Itch in T cell-B cell interactions has only begun to be described (Fig. 1), but understanding how Itch directly and indirectly limits B cell responses to control autoantibody will be critical to helping Itch patients with severe autoimmunity.

The importance of Itch in *T*-dependent antibody responses was first explored with a series of experiments using mixed bone marrow (BM) chimeras. Mixtures of Itch-deficient or sufficient bone marrows were made to query the how the role of Itch in B cells, αβ T cells, and γδ T cells impacted production of antibody. From these studies, it was determined that the increased serum IgM and IgG in Itch deficient mice required αβ T cells. Additionally, loss of Itch in αβ T cells and increased IL-4 was responsible for increased IgM production by B1-B cells (Fig. 1A), and Itch activity in γδ T cells prevented the emergence of high levels of IgE. This evidence supports the idea that Itch regulates non-GC *T*-dependent antibody responses to limit IgM and IgE [14]. However, these studies did not determine how Itch limited IgG and autoantibody.

A more recent study identified a new role for Itch in regulating GC-derived antibody responses. Itch was shown to play an important role in Tfh cell differentiation. Using a model of viral infection, the authors showed that CD4 T cells lacking Itch were unable to differentiate into Tfh cells, and mice with Itch deficient T cells mounted defective virus-specific humoral responses. Mechanistically, Itch ubiquitylated Foxo1, promoting its degradation and allowing expression of the Tfh master regulator Bcl6 [80] (Fig. 1B). These results are at odds with the observation of increased serum antibody in Itch deficient mice and patients. It could be that Itch is particularly important in promoting Tfh responses in the context of viral infection; Th cells lacking Itch are skewed towards Th2 differentiation [12], so they may be unable to respond to the virus-induced cues (i.e. interferon gamma) to support virus-specific antibody production. Indeed, this study did not report the status of spontaneous Tfh that develop in $Itch^{fl/fl}$CD4-Cre mice.

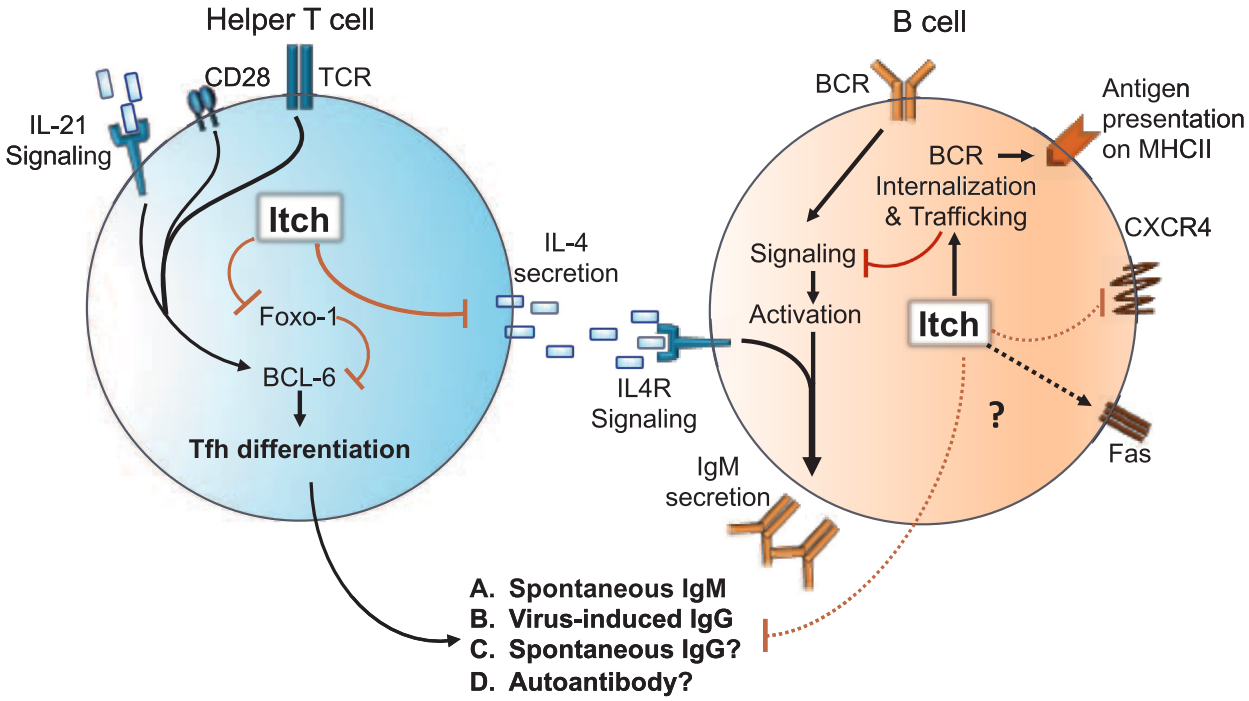

Fig. 1. The role of Itch to regulate T cell-B cell interactions and antibody **production**. Itch deficient patients with autoimmunity and mice lacking Itch develop abnormal serum antibodies. Itch limits levels of spontaneously produced IgM, IgG, and autoantibody, but Itch activity in T cells promotes virus-specific IgG production. A) Itch negatively regulates serum IgM levels through suppressing IL-4 production by T cells. The source of IgM is thought to be B1-B cells, suggesting that these *T*-B interactions are extrafollicular. B) Mice lacking Itch only in T cells mount defective antibody responses to viral infection. Itch promoted Tfh differentiation and germinal center responses by ubiquitylation and degradation of Foxo1, allowing protein expression of the Tfh master regulator, Bcl6. C) Itch deficient mice develop high spontaneous IgG, which is rescued in mice lacking T cells, supporting the idea that *T*-B interactions are required for high IgG. Whether Itch activity to prevent excess IgG occurs in germinal centers, extrafollicular interactions, within T cells or B cells is unknown. Speculations can be made by considering the roles of Itch that have been described in B cells and other cell types. Itch ubiquitylation of the signaling component of the BCR alters signaling, and this may dampen spontaneous IgG. Itch regulation of antigen presentation, CXCR4, or Fas would impact germinal center B cell proliferation, survival and IgG production. Itch might regulate spontaneous Tfh by a distinct mechanism from virus-induced Tfh. D) How Itch limits autoantibody is unknown. Determining how Itch limits autoantibody has high clinical relevance for autoimmune patients, including those with Itch deficiency.

Alternatively, it remains possible that B cells are negatively regulated by Itch. In Itch deficiency, GC B cells that are better able to become activated, proliferate, and survive with less reliance on Tfh cell help could be a major source of IgG and autoreactive antibody (Fig. 1C and D).

There is good evidence that Itch directly regulates B cell function, although how this role shapes antibody responses *in vivo* has not been determined. Itch was described to promote internalization and intracellular trafficking of the BCR. Mechanistically, Itch ubiquitylated Igβ, the signaling component of the BCR. Mutation of ubiquitylation sites on Igβ impaired its ability to traffic to MHCII containing lysosomes [81]. Reduced internalization and trafficking would be expected to both increase BCR signaling and decrease antigen presentation. Indeed, BCR signaling was altered in Igβ lysine mutant B cells, but there was no effect on antigen presentation to T cells [82]. These data support the idea that Itch regulates BCR signaling in a manner that does not restrict T cell-B cell interactions.

Itch has also been described to play a role in Epstein Barr Virus (EBV)-infected B cells. Itch was found to ubiquitylate the viral protein LMP2A, which is structurally and functionally similar to the BCR. LMP2A contains PY motifs that bind Itch [83], and it also recruits and initiates tyrosine kinase activity of Lyn and Syk. Thus, in EBV infected B cells, LMP2a acts as a scaffold to help Itch ubiquitylate and degrade proximal BCR signaling molecules, limiting BCR signaling [84]. Although the BCR itself does not contain PY motifs, it is possible that Itch could play a similar role in limiting endogenous BCR signaling, if an appropriate adaptor molecule was also expressed in B cells.

The ability of Itch to promote Fas-mediated apoptosis through degradation of cFLIP would be predicted to have a major impact on GC-derived antibody responses, if this mechanism occurs in B cells. GC B cells express high levels of Fas, but resist apoptosis due to upregulation of cFLIP after receiving survival signals from Th cells and follicular DCs [85]. In the absence of Itch-dependent degradation, increased cFLIP levels in GC B cells could permit survival of autoreactive B cells that are less reliant on Tfh help. However, this remains speculative. Future research investigating the link between Itch and cFLIP in B cells may shed new light on how Itch limits *T*-dependent antibody and autoantibody responses.

CXCR4 is a chemokine receptor that is expressed on a variety of cell types, including B cells. CXCR4 influences humoral responses by promoting plasma cell homing to bone marrow survival niches [86], and organizing the light and dark zones in GCs to promote affinity maturation [87,88]. Additionally, CXCR4 contributes to stability of the immune synapse that forms between T cells and antigen presenting cells [89]. Importantly, overexpression of CXCR4 is strongly linked to autoimmune disease in both mice and humans [90,91], and blockade of the CXCR4 ligand, CXCL12/SDF-1 can ameliorate a mouse model of SLE-like disease [92].

In cell lines, there is good evidence that Itch can directly ubiquitylate CXCR4 to regulate its expression levels, but whether Itch regulates CXCR4 in immune cells to prevent autoimmunity remains unexplored. Itch ubiquitylation of CXCR4 was required for its trafficking to the lysosome for degradation after ligand-mediated endocytosis [93–96]. Additionally, Itch can ubiquitylate members of the ESCRT endocytic trafficking machinery, namely Hrs and Stam1, to promote CXCR4 lysosomal targeting, as well as signaling from CXCR4 to activate ERK1/2 [93,97]. These studies raise the possibility that Itch could participate in targeting CXCR4, and other surface receptors, to the lysosome for degradation, with anticipated effects on antibody response and autoimmunity.

The evidence that Itch regulates both GC and non-GC T cell-B-cell interactions supports the idea that Itch activity in both B and T cells contributes to the regulation of serum antibody levels and autoantibody production. A better understanding of how Itch shapes T cell-B cell interactions, especially how Itch regulates B cells, is needed, as this might help to explain how Itch limits humoral responses and prevents autoimmune disease.

2.2. Itch shapes inflammatory processes after breach in tolerance

The most well-understood function of Itch is to modulate conventional Th differentiation, helping to define the inflammatory outcome of autoimmune disease. Itch has been described to be a negative regulator of both Th2, and more recently, Th17 differentiation. Itch defines Th differentiation by directly controlling the protein levels of lineage-specific transcription factors. In addition, by regulating TCR signal strength, Itch limits Th cell differentiation towards certain lineages.

2.2.1. Th2 cells

Soon after its discovery, Itch was found to negatively regulate Th2 cell differentiation in mice. TCR stimulation activated multiple transcription factors, including AP-1, composed of Jun and Fos. There are several isoforms of Jun and Fos, and one of these, JunB, preferentially pushes Th cells toward Th2 differentiation. Indeed, JunB overexpression in T cells is sufficient to cause Th2 differentiation [98,99]. Mechanistically, Itch ubiquitylated JunB, targeting it for degradation and preventing aberrant Th2 differentiation [12,13]. Overexpression experiments in cell lines demonstrated that Itch WW domains bound PY motifs on JunB (and the related c-Jun), and Y170 on JunB was required for interaction with Itch, ubiquitylation, and degradation [12]. These findings provided a mechanism by which Itch-deficient T cells gained a Th2 phenotype after activation, contributing to inflammation and the pathogenesis of autoimmunity in Itch-deficient mice. It is worth noting that in contrast to the evidence supporting that Itch binds JunB through its Y170 residue in T cells, in MEFs and in H1299 cells, a mutation in Y170 of JunB had no effect on its turnover rate [100]. These data support the idea that Itch interacts with distinct substrates in different cell types.

In addition to its role in regulating JunB protein levels, a collaboration between Itch and the E3 ubiquitin ligase WWP2 was recently shown to prevent Th2 differentiation through controlling TCR signal strength. Weak TCR signal strength has been implicated in driving Th2 differentiation. In this report, Itch prevented the TCR-inhibitory phosphatase SHP2 from dephosphorylating the TCRζ chain, thereby ensuring optimal TCR signaling and shunting activated Th cells away from Th2 differentiation [101]. This new finding is apparently at odds with previous findings that Itch inhibits strong TCR signaling through ubiquitylation of MEKK1, PLCγ, and PKCγ, and TCRζ. It is possible that Itch fine tunes the proportions of various signaling intermediates after TCR engagement, changing the quality of the signal in a way that discourages Th2 differentiation, which is then reinforced by Itch ubiquitylation of JunB.

2.2.2. Th17 cells

More recently, Itch has been found to inhibit Th17 differentiation through its ability to ubiquitylate RORγt, targeting it for degradation [102,103]. In addition, there is evidence that weak TCR signaling favors Th17 differentiation [104], raising the possibility that Itch could also oppose Th17 differentiation by its role in regulating the quality of TCR signaling, which would then be reinforced by RORγt degradation. Furthermore, Th17 differentiation is driven in part by TGFβ, in combination with other cytokines, including IL-6 [105]. Because Itch participates in TGFβ signaling networks by multiple mechanisms in Th cells and other cell types, Itch may also regulate Th17 differentiation in part through regulating TGFβ reponses.

In the context of autoimmunity, failure to limit Th2 and Th17 differentiation in Itch deficiency shapes the nature of disease that develops. Itch deficient mice develop severe inflammation at mucosal surfaces, important targets of Th2 and Th17 cells in pathologic immune responses. Because Itch deficient Th cells preferentially differentiate toward Th2 and Th17, therapeutics activating Itch-dependent immune processes could be well suited for autoimmune diseases that affect

mucosal sites and exhibit Th2 and Th17 activation, including allergic asthma, inflammatory bowel disease, and psoriasis.

2.2.3. Nfκb

In addition to regulating T and B cells, Itch also limits feed-forward inflammatory circuits in innate cells, largely through regulating NFκB activation. Unlike A20, another ubiquitin ligase that regulates NFκB, Itch regulation of innate immune function is secondary to its role in lymphocytes. This is supported by the observation that autoimmunity does not occur in Itch deficient mice that lack T and B cells. After activation with inflammatory stimli, Itch directly regulates several molecular components of NFκB signaling pathways to limit cytokine production by immune cells. Autoimmune tissue destruction releases cytokines (e.g. TNF, IL-6, IL-1b) and increases exposure to microbial ligands (e.g. LPS), that trigger NFκB activation in infiltrating immune cells. By opposing NFκB activation, Itch limits feed-forward inflammatory circuits that exacerbate and potentiate destruction of target organs in autoimmunity.

Most of the evidence supporting Itch function in limiting NFκB signaling comes from experiments using bone marrow derived macrophages (BMDMs). In BMDMs exposed to TNFα, Itch limited signaling by degrading Rip1, Tak1, and Tab1, thereby reducing TNFα-induced cytokine production [15,17,106]. Mechanistic studies in cell lines demonstrated that Itch directly ubiquitylated these proteins. Furthermore, in the cases of Rip1 and Tak1, Itch interacted with other ubiquitin editing enzymes (A20 and Cyld, respectively) to edit the type of ubiquitin chain on the substrate and ultimately target it for degradation [17,107]. In addition to TNFα-induced NFκB activation, Itch inhibited NFκB responses to pattern recognition receptor ligands. Itch-dependent degradation of Rip2 or Tak1 could limit NFκB activation after BMDM exposure to Haemophilus influenza and the microbial ligand muramyl dipeptide (MDP), respectively [16,108]. Although loss of Itch in innate cells is insufficient to initiate spontaneous autoimmunity, these studies suggest that Itch helps to dampen inflammatory responses that likely drive tissue destruction and exacerbate disease after the onset of autoimmunity.

2.2.4. EGFR signaling

Epidermal growth factor receptor (EGFR) signaling is not currently well-linked to immune cell function, although there is some emerging evidence that suggests EGFR likely contributes to immune cell function.

EGFR signaling has been attributed to promoting cell growth and differentiation in numerous tissues, including tumors. Interestingly, EGFR has also been found to exacerbate multiple experimental autoimmune diseases in mice, including arthritis, asthma, and SLE, and pemphigus in human patients, although the cell types and mechanisms involved remain unclear [109–113]. Multiple investigators have found that Itch interacts with EGFR to target it for degradation [114–118]. These observations raise the possibility that Itch-dependent regulation of EGFR signaling may impact immune cell function, and loss of EGFR regulation in Itch deficiency may shape the development of consequent autoimmune disease. Additionally, when considering targeting Itch regulatory networks for immune therapies, understanding how Itch therapeutics might impact these critical cellular pathways will be important for predicting drug specificity and toxicity.

3. Molecular regulation of Itch enzymatic activity

Itch is a catalytic E3 ubiquitin ligase. Itch exists in an inactive state, mediated by intramolecular interactions. The WW domains of Itch bind to its catalytic HECT domain, preventing Itch from ubiquitylating substrates [119]. Therefore, Itch must first be activated, through relief of intramolecular inhibition, in order to ubiquitylate its substrates. Itch enzymatic activity can be regulated by phosphorylation as well as protein-protein interactions. Most of the molecular regulators of Itch have been identified using ubiquitylation of the substrate JunB as the readout for Itch activity, but distinct activators have also been shown to regulate additional substrates in non-T cells, including CXCR4 and cFLIP. It is unknown why multiple mechanisms for Itch activation exist, but one possibility is that distinct activation mechanisms allow ubiquitylation of different substrates and in different cell types.

3.1. Phosphorylation-dependent regulation

The first evidence for Itch activation by phosphorylation came from the observation that MEKK deficient T cells and Itch deficient T cells shared a similar Th2 hyperactivation phenotype, raising the possibility that MEKK and Itch might function in the same pathway to regulate JunB levels and Th2 differentiation. Indeed, kinase activity of the MEKK substrate JNK could enhance ubiquitylation of JunB in an Itch-dependent manner [120]. Additionally, T222 of Itch was phosphorylated in primary T cells in a MEKK-dependent manner. When Itch mutants were overexpressed with JNK in cell lines, the phosphorylation of T222 or the T222D phosphomimetic could relieve intramolecular autoinhibition of Itch to make WW domains available for substrate binding [121].

In addition to activation of Itch by JNK-dependent phosphorylation of T222, Fyn-dependent tyrosine phosphorylation of Itch can inhibit Itch function, prolonging JunB half-life. Itch was found to be tyrosine phosphorylated in Jurkat cells after TCR stimulation, and primary T cells lacking the tyrosine kinase Fyn exhibited less tyrosine phosphorylated Itch and enhanced Jun degradation [122]. Additionally, Jun was also found to be less stable and more associated with Itch in T cells that lacked the tyrosine kinase c-Abl. The increased Jun degradation was Itch-dependent because mutation in the PY motif of Jun (Y170F, required for Itch binding) was stabilized in cAbl null cells [123]. Dephosphorylation of tyrosine on Itch also correlated with increased activity- CTLA4 ligation and SHP2 activation in T cells was associated with decreased Itch tyrosine phosphorylation and decreased IL4 secretion [124]. It is not clear which tyrosine(s) of Itch are required for this inhibition, or whether inhibition stabilizes the inhibitory intramolecular interactions or inhibits Itch in a different way.

In non-immune cells, phosphorylation was also shown to activate Itch. ATM kinase, a critical mediator of the DNA damage response, phosphorylated and activated Itch in hepatocyte cell lines through phosphorylation Itch S161 (distinct from JNK phosphorylation of T222), which enhanced ubiquitylation of c-Jun and cFLIP [125]. In HeLa cells, the kinase CISK phosphorylated Itch to inhibit its function and to prevent CXCR4 degradation [94]. The findings that Jun protein stability is regulated differently in different cells support the idea that Itch catalytic activity is likely regulated by different mechanisms and results in ubiquitylation of distinct proteins depending on the cell type and stimulus.

3.2. Protein-protein interactions

In addition to kinase-dependent activation, the small transmembrane protein Ndfip1 is an essential Itch activator in primary T cells. Ndfip1 deficient mice exhibit spontaneous autoimmune disease similar to Itch deficient mice, featuring aberrant T cell activation and Th2 inflammation at mucosal surfaces. In primary mouse T cells, Ndfip1 levels increased after TCR stimulation, and Ndfip1 was required for JunB ubiquitylation and degradation. Mechanistically, PY motifs within Ndfip1 interacted with Itch WW domains to relieve intramolecular autoinhibition, allowing the catalytic HECT domain to transfer ubiquitin to JunB [13,119]. Ndfip1 also was required for Itch-dependent degradation of RORγt to prevent Th17 differentiation [103] and Ndfip1 in macrophages was shown to activate Itch-dependent degradation of Tak1 to dampen NFκB signaling [108]. Recently, the Ndfip1 homolog, Ndfip2, has also been found to activate Itch. Loss of Ndfip2 was not sufficient to cause spontaneous autoimmune disease in mice, but lack of Ndfip2 exacerbated inflammation caused by Ndfip1 deficiency. Specific Itch substrates regulated by Ndfip2 have not yet been identified [37].

Of note, Ndfip1 deficient T cells still contain intact JNK signaling, yet they cannot degrade JunB. These results support the idea that JNK likely enhances Itch function, but may not be sufficient for relieving Itch autoinhibition in primary T cells. It is important to mention that the studies that identified JNK as capable to activate Itch were carried out in cell free assays, using overexpressed Itch that was pulled down from cell lysates [120]. It is possible that accessory molecules that help activate Itch also were present in the *in vitro* ubiquitylation assays, including the Itch activator Ndfip1.

In addition to Ndfip proteins, Tax1bp1 was shown to bind Itch through PY-WW domain interactions, allowing Itch to negatively regulate NFκB signaling in T cells [15]. Also, in HeLa cells and bone marrow mononuclear cells, intermolecular interactions between Missing-in-Metastasis (MIM), Itch, and CXCR4, enhanced Itch interactions with CXCR4 to promote its degradation [126]. Additionally, the proteins N4BP1 and Yap1 could inhibit Itch-mediated degradation of p63 and p73, thereby promoting apoptosis [62,63].

For the case of Itch activation by both phosphorylation and protein-protein interactions in T cells, TCR stimulation drives these molecular switches to turn on Itch activity. This supports the idea that Itch suppressive function is turned on as a TCR driven negative feedback mechanism that prevents a breach in immune tolerance, emergence of Th2 cells, and exacerbation of inflammation. In other cell types, it appears that additional mechanisms drive Itch activation and ubiquitylation of distinct substrates. Importantly, mechanisms of Itch activation in innate immune cells and B cells remain poorly understood.

4. Perspective on Itch regulatory networks and therapeutic targeting

Itch is a widely expressed ubiquitin ligase that functions to prevent autoimmunity. Itch can ubiquitylate a variety of substrates in multiple cell types, and cellular processes. Because of its dominant role in preventing autoimmunity and limiting inflammatory responses, therapeutic targeting of Itch may be a promising strategy for modulating the immune system- either to ameliorate autoimmune disease or to enhance protective immune responses. On the other hand, because Itch regulates cell functions extending beyond immune function and autoimmunity, including pathways prominent in many cancers (e.g. regulation of Hippo signaling, apoptotic pathways, Notch signaling, and EGFR signaling), very specific targeting of Itch function will be critical for safe and effective therapeutics. The very complexity of Itch regulatory pathways may actually aid feasibility of targeting specific immune functions. By understanding the relationships between molecular regulators (activators and inhibitors) and substrates of Itch in specific cell types, highly individual activator-substrate patterns may emerge. In other words, activators and inhibitors of Itch may provide a therapeutic handle to modify Itch function in specific cell types or cellular pathways. Using evidence of Itch activator-substrate mechanisms that have been demonstrated in primary cells, a picture of how Itch function might be differentially regulated in different cell types and inflammatory conditions emerges, although it is still incomplete.

The mechanisms governing Itch function in T cells are the most well-defined. Both Ndfip1 and JNK phosphorylation can activate Itch in T cells by relieving Itch autoinhibition through a conformational change, and tyrosine phosphorylation can inhibit Itch [122–124]. So far, both Ndfip1 and JNK can activate Itch to degrade JunB in T cells, but only Ndfip1 has been shown to promote ubiquitylation of RORγt, and Jak1 [13,37,69,103,119]. Tax1bp1 has also been shown to activate Itch in T cells, promoting its association with the ubiquitin editing enzyme A20 and degradation of a Rip1 to dampen NFκB signaling, which was not shown to be regulated by either Ndfip1 or JNK in T cells [15].

What would be the possible off-target effects of enhancing Ndfip1 to control T cell responses? Increasing Ndfip1 activity could limit cytokine production in macrophages, where it has been shown to function in mouse BMDMs to degrade Tak1 and downregulate NFκB signaling [108]. Whether Ndfip1 regulates Itch in any other cell types remains to be determined. Notably, enhancing Ndfip1 would not be expected to change NFκB signaling in T cells, because this signaling pathway is regulated by a separate Itch-activator-substrate combination; Tax1bp1-mediated activation of Itch promoted degradation of Rip1 to limit NFκB [15]. Targeting JNK to promote Itch activation in T cells is predicted to have liver toxicity because JNK-activated Itch promoted degradation of cFLIP and enhanced cell death in hepatocytes [43,125]. Together, the data from primary T cells support that targeted enhancement of Ndfip1 would be the best approach to activating Itch to regulate T cell lineage commitment to Th2 (JunB), Th17 (RORgt), T_{reg} cell cytokine production, and cytokine signaling (Jak1). These findings show the promise and challenge of identifying and targeting cell-type and substrate specific modulators of Itch to modify specific functional outcomes for immune therapies.

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