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IL-2–inducible T-cell kinase modulates T_H2-mediated allergic airway inflammation by suppressing IFN- γ in naive CD4⁺ T cells

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Abstract

Background—Asthma is a predominantly T_H2 cell–dominated inflammatory disease characterized by airway inflammation and a major public health concern affecting millions of persons. The Tec family tyrosine kinase IL-2–inducible T-cell kinase (Itk) is primarily expressed in T cells and critical for the function and differentiation of T_H cells. Itk^{−/−} mice have a defective T_H2 response and are not susceptible to allergic asthma.

Objective—We sought to better understand the role of Itk signaling in T_H differentiation programs and in the development and molecular pathology of allergic asthma.

Methods—Using a murine model of allergic airway inflammation, we dissected the role of Itk in regulating T_H cell differentiation through genetic ablation of critical genes, chromatin immunoprecipitation assays, and house dust mite–driven allergic airway inflammation.

Results—Peripheral naive Itk^{−/−} CD4⁺ T cells have substantially increased transcripts and expression of the prototypic T_H1 genes Eomesodermin, IFN- γ , T-box transcription factor (T-bet), and IL-12R β 1. Removal of IFN- γ on the Itk^{−/−} background rescues expression of T_H2-related genes in T_H cells and allergic airway inflammation in Itk^{−/−} mice. Furthermore, small hairpin RNA–mediated knockdown of Itk in human peripheral blood T cells results in increased expression of mRNA for IFN- γ and T-bet and reduction in expression of IL-4.

Conclusion—Our results indicate that Itk signals suppress the expression of IFN- γ in naive CD4⁺ T cells, which in a positive feed-forward loop regulates the expression of T_H1 factors, such as T-bet and Eomesodermin, and suppress development of T_H2 cells and allergic airway inflammation.

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Keywords

Tec kinase; T_H2; allergic asthma; T-bet; Eomesodermin; IFN- γ

Asthma is a major public health concern affecting millions of persons each year. Allergic asthma is a chronic inflammatory disease characterized by airway inflammation and airways hyper-responsiveness (AHR).¹ Patients with allergic asthma show increased infiltration of activated T_H cells, eosinophils, and mast cells into the lungs. Allergen exposure induces innate and adaptive immune responses that dictate the differentiation of naive CD4⁺ T cells into T_H2 cells and establish CD4⁺ T-cell memory.^{2,3} On re-exposure to the same allergen, T_H2 cells can rapidly secrete cytokines, providing help to B cells that secrete allergen-specific IgE but also affecting the response of the airway epithelia.²⁻⁴ T_H2 cells and their cytokines (IL-4, IL-5, and IL-13) can drive the development of allergic asthma, and blocking these cytokines has varying efficacy in alleviating symptoms of asthma both in experimental murine models and human subjects.⁵⁻⁸ Although we understand the basic details of the signaling events that regulate lineage choices of CD4⁺ T cells, there are still considerable gaps in the knowledge of pathways that regulate T-cell differentiation into T_H2 cells, gaps such as how early signals through the T-cell receptor (TCR) affect this differentiation process.⁹

IL-2-inducible T-cell kinase (Itk) is a member of the Tec family of nonreceptor protein tyrosine kinases that in T cells also includes Txk and Tec and is important for effective signaling through the TCR. In the TCR proximal signaling complex, Itk cooperates with other molecules, such as linker of activated T cells and SH2 domain containing leukocyte protein of 76 kDa, to phosphorylate and activate critical downstream effector molecules, such as phospholipase C γ , leading to increases in intra-cellular Ca²⁺ levels. This Ca²⁺ influx leads to the activation and nuclear translocation of nuclear factor of activated T cells through the calmodulin/calcineurin axis.^{10,11} In line with this, in the absence of Itk, there are severe defects in activation of key signaling components, including phospholipase C γ , which results in reduced influx of Ca²⁺ and defective activation of extracellular signal-regulated kinase/mitogen-activated protein kinase, with resultant reduction in the activation of the transcription factors nuclear factor of activated T cells, nuclear factor κ B, and activator protein 1. All of these signaling pathways have been shown to be critical for CD4⁺ helper subset differentiation. Thus Itk can both qualitatively and quantitatively affect TCR signaling.^{11,12}

Itk-deficient (Itk^{-/-}) mice are not susceptible to T_H2-mediated allergic asthma¹³ and exhibit an altered response to parasitic worm infections,^{14,15} which has been attributed to defects in the production of T_H2 cytokines by T_H cells in these mice.¹⁵⁻¹⁷ Itk has also been shown to negatively regulate T-box transcription factor (T-bet) expression in CD4⁺T cells.^{18,19} Here we show that Itk negatively regulates IFN- γ , indirectly negatively regulating T-bet expression. We found that naive Itk^{-/-} peripheral CD4⁺ T cells have substantially increased levels of prototypic T_H1 genes, including Eomesodermin (Eomes), IFN- γ , T-bet, and IL-12R β 1. Naive Itk^{-/-} CD4⁺ T cells preferentially upregulate T_H1-related genes, even when stimulated under T_H2-polarizing conditions. Analysis of naive CD4⁺ T cells from

mice lacking both Itk and T-bet revealed increased IFN- γ expression, even in the absence of T-bet. However, removal of IFN- γ from Itk^{-/-} CD4⁺ T cells led to reduced expression of T-bet, indicating that Itk negatively regulates IFN- γ , thus regulating T-bet. We further find that Itk does this at least in part by regulating the accessibility of the IFN- γ locus. More importantly, in the absence of IFN- γ expression, IL-4 expression is restored, along with susceptibility to T_H2-mediated allergic asthma in Itk^{-/-} mice. These signaling pathways seem to be conserved in human subjects because small hairpin RNA (shRNA)-mediated knockdown of Itk in human peripheral blood T cells results in increased expression of T-bet and IFN- γ and a reduction in IL-4 secretion. These findings provide critical understanding of the signaling pathways that regulate T_H1 and T_H2 differentiation and control the development of allergic asthma.

RESULTS

Itk suppresses T_H1 genes to regulate the induction of the T_H2 transcriptional program

We isolated naive CD4⁺CD44^{lo}CD62L^{hi} T cells from WT and Itk^{-/-} mice and determined expression of prototypic T_H1 genes. Naive Itk^{-/-} CD4⁺ T cells exhibited significantly higher basal levels of T-bet, IFN- γ , Eomes, and IL-12R β 2 mRNA compared with WT cells (Fig 1, A). Intracellular staining for protein confirmed the difference in T-bet and Eomes expression, which was further enhanced by a brief stimulation (5 hours) *ex vivo* with phorbol 12-myristate 13-acetate/ionomycin (P/I; Fig 1, B). Although there was no difference in basal IFN- γ protein levels, Itk^{-/-}, but not WT, T cells rapidly upregulated IFN- γ protein on stimulation with P/I (Fig 1, B). To assess the functional significance of this increase in mRNA (and protein) even before specific antigenic stimulus, we cultured isolated naive CD4⁺CD44^{lo}CD62L^{hi} T cells from these mice *in vitro* under T_H2-polarizing conditions and monitored the expression of T_H genes at various time points. Although WT T cells initiated a strong T_H2 transcriptional program as early as 24 hours after stimulation by upregulating GATA3 and IL-4, Itk^{-/-} cells exhibited less pronounced upregulation of GATA3 and IL-4 (mRNA: Fig 1, C; protein: Fig 1, D) during this polarization phase. Itk^{-/-} cells were also defective in inducing IL-13 mRNA expression (Fig 1, C). Instead, under the same T_H2-polarizing conditions, Itk^{-/-} T cells selectively upregulated T-bet and IFN- γ (mRNA: Fig 1, C; protein: Fig 1, D). This suggests that Itk signals downstream of the TCR during T-cell development, homeostasis, or both to suppress expression of T_H1 genes before encounter with specific antigen and that this is critical for induction of the T_H2 transcriptional program.

Itk negatively regulates IFN- γ in naive CD4⁺ T cells

Both T-bet and IFN- γ are upregulated in the absence of Itk, and there is a positive feed-forward loop between T-bet and IFN- γ .^{25,26} Therefore Itk potentially negatively regulates either T-bet or IFN- γ to affect the expression of the other gene. To ascertain which of the 2 effectors of this loop is regulated by Itk, we generated T-bet/Itk DKO and IFN- γ /Itk DKO mice and determined the expression of T-bet and IFN- γ in naive CD4⁺CD44^{lo}CD62L^{hi} T cells from these mice. We found that although the absence of T-bet had no effect on IFN- γ mRNA expression in naive Itk^{-/-} cells, the absence of IFN- γ led to a significant reduction in T-bet and Eomes transcripts (Fig 2, A). Examination of protein levels by means of

intracellular staining under nonstimulated or brief stimulation (by P/I) conditions confirmed these differences, with the exception that IFN- γ protein was not detected before stimulation (Fig 2, *B*). Thus Itk regulates the expression of T-bet and Eomes in naive CD4⁺ T cells at least in part by negatively regulating IFN- γ expression.

Itk signaling controls both T-bet and IFN- γ expression to regulate IL-4 secretion in human peripheral blood T cells

To determine whether Itk also suppresses the expression of T_H1 genes in human T cells, we transduced peripheral blood T cells with lentiviral particles expressing either an shRNA against Itk to knockdown the expression of Itk or a control shRNA and stimulated with anti-CD3/CD28 for 2 rounds of 5 days. Cells transduced with the shRNA against Itk showed a significant reduction in Itk expression at both the mRNA and protein levels (Fig 3, *A*). Cells were then restimulated with the same antibodies for 24 hours and then analyzed for T-bet and IFN- γ mRNA expression, and supernatants were analyzed for IL-4 expression. Strikingly, similar to murine T cells that were deficient in Itk, reduced expression of Itk in human peripheral blood T cells led to significant upregulation of T-bet and IFN- γ mRNA expression (Fig 3, *B*) and a reduction in the secretion of IL-4 (Fig 3, *D*). This difference in T-bet and IFN- γ expression was confirmed at the protein level by means of intracellular staining/flow cytometry, demonstrating increased percentages of T-bet and IFN- γ expression in these cells when Itk expression was reduced (Fig 3, *C*). Thus the Itk-mediated signaling pathways that control IFN- γ and T-bet expression are conserved in human T cells.

Removal of IFN- γ restores T_H2 cytokine gene expression in CD4⁺ T cells in the absence of Itk

We next determined whether removal of IFN- γ would restore the ability of Itk^{-/-} T cells to produce IL-4 by culturing naive Itk^{-/-} and Itk/IFN- γ DKO mice under T_H2-polarizing conditions. We found that the absence of IFN- γ in the Itk^{-/-} cells resulted in a substantial upregulation of IL-4 mRNA (and protein) levels compared with Itk^{-/-} T cells under the same conditions (Fig 4), indicating that increased IFN- γ levels act to suppress T_H2 differentiation of naive CD4⁺ T cells in the absence of Itk.

Itk signaling controls the accessibility of the IFN- γ locus

Given the expression of IFN- γ message in naive antigen-inexperienced Itk^{-/-} CD4⁺ T cells, we next determined whether signaling through Itk regulates epigenetic processes that control the accessibility of the IFN- γ locus in these cells. We evaluated the status of the IFN- γ locus before antigenic stimulus by performing ChIP assays on naive CD4⁺CD44^{lo}CD62L^{hi} T cells with antibodies against permissive (H3K4me2) and repressive (H3K27me3) histone marks. Immunoprecipitated DNA was amplified by using primers spanning either the promoter region or the proximal and distal regulatory and CNSs of the IFN- γ locus with primers described previously.^{21–23} We found that repressive H3K27me3 modifications at the IFN- γ promoter were significantly lower than WT cells in the absence of Itk and that Itk^{-/-} cells had significantly higher enrichment for permissive H3K4me2 marks in that region (Fig 5, *A*). In addition, naive Itk^{-/-} CD4⁺CD44^{lo}CD62L^{hi} T cells also exhibited significantly higher enrichment for permissive histones in different regions of the IFN- γ

promoter and a majority of the CNS regions we analyzed, including the proximal and distal CNS regions INF143+145 (–5.74kb), INF105+103 (–0.35kb), INF185+183 (19.36kb), CNS2A5+2A3 (30.27kb), and 137769 (48.88kb; Fig 5, B). In addition, in agreement with our previous finding that Itk regulates IFN- γ expression independent of T-bet (Fig 2), the absence of T-bet did not affect the enrichment of permissive histones in a number of IFN- γ CNS regions we analyzed (data not shown). These data indicate that Itk signaling is critical for maintaining the IFN- γ locus in a closed state, and in its absence, the region undergoes epigenetic modification to adopt an open chromatin conformation and contributes to the increased basal expression of IFN- γ in naive Itk^{–/–} CD4⁺CD44^{lo}CD62L^{hi} T cells.

Removal of IFN- γ rescues T_H2 cytokine gene expression and allergic airway inflammation in Itk^{–/–} mice

To determine whether this preupregulation of T_H1 genes in naive Itk^{–/–} CD4⁺ T cells influences T_H2 responses *in vivo*, we exposed mice to the allergen HDM to induce T_H2-mediated allergic airway inflammation and measured AHR. WT mice had a robust allergic airway inflammatory response, goblet cell hyperplasia, and mucus production in the lung, and as previously reported, Itk^{–/–} did not (Fig 6, A and B).¹³ Itk^{–/–} and Itk/T-bet DKO mice behaved similarly, with significantly lower goblet cell hyperplasia and bronchiolar lumen occlusion. By contrast, Itk/IFN- γ DKO mice responded more like IFN- γ ^{–/–} control mice, developing airway inflammation (Fig 6, A and B). Similarly, although we recovered significantly lower numbers of CD4⁺ T cells from the bronchoalveolar lavage fluid (BALF) of Itk^{–/–} mice compared with WT mice, as well as significantly lower numbers of CD4⁺ T cells and eosinophils from the BALF of Itk/T-bet DKO mice, the Itk/IFN- γ DKO mice had responses similar to those seen in IFN- γ ^{–/–} mice (Fig 6, C). Transcript levels for the T_H2 cytokines IL-4 and IL-13 were also consistently lower in Itk^{–/–} and Itk/T-bet DKO mice compared with those seen in WT and T-bet^{–/–} mice, respectively, whereas Itk/IFN- γ DKO mice behaved more like WT or IFN- γ ^{–/–} mice (Fig 6, D). These findings were supported by analysis of airways resistance by means of mechanical ventilation, which revealed that although WT mice exhibited an increased response to methacholine, Itk^{–/–} mice showed no such increase. IFN- γ ^{–/–} mice showed a slightly lower methacholine response when compared with WT control animals, but more importantly, the Itk/IFN- γ DKO mice exhibited an increase in AHR similar to that seen in IFN- γ ^{–/–} mice compared with Itk^{–/–} mice (Fig 6, E). Thus the absence of IFN- γ rescued airway inflammation in Itk^{–/–} mice with an increase in AHR (Fig 6, A and B). Thus the absence of IFN- γ on an Itk^{–/–} background results in the development of T_H2-driven allergic airway inflammation, suggesting that IFN- γ suppresses this response in Itk^{–/–} mice.

DISCUSSION

Although cytokine signals are critical in the differentiation process of T_H cells, it is being appreciated that early TCR signals are also key determinants of T_H lineage polarization.⁹ This has implications for our understanding of the development and maintenance of T_H-driven diseases, such as allergic asthma. Our results reported here show that signals regulated by the Tec kinase Itk, which acts downstream of the TCR, are critical in suppressing the expression of IFN- γ in peripheral naive CD4⁺ T cells. More importantly,

these pathways seem to be conserved in human subjects because the reduction in Itk signaling in human peripheral blood T cells results in enhanced expression of T-bet and IFN- γ and a significant reduction in the ability of these cells to make IL-4 on restimulation. Our data also suggest that suppression of IFN- γ by Itk regulated TCR signals prevent feed-forward induction of T-bet expression, which would commit T_H cells to the T_H1 lineage, thereby controlling the development of allergic asthma.

We therefore propose that in naive CD4⁺ T cells Itk regulates the strength, duration, or both, of TCR signals received during homeostatic expansion of naive CD4⁺ T cells after these cells leave the thymus. We suggest that strong signals during this period suppress the expression of IFN- γ , which, if expressed, might upregulate the expression of Eomes and T-bet. Weaker signals allow expression of IFN- γ , resulting in the expression of critical transcription factors. Indeed, when naive CD4⁺ T cells are transferred into lymphopenic hosts, they undergo homeostatic expansion driven by weak signals from the TCR and environmental cytokines, such as IL-7, IL-15, or both; develop the capacity to carry IFN- γ mRNA; and rapidly secrete this cytokine on subsequent stimulation.²⁷ Such cells have been referred to as HP or homeostatic proliferated cells; however, they generally have a memory phenotype (ie, CD44^{hi}CD62L^{lo}). In our analysis we have excluded such cells from our analysis by sorting naive (CD44^{lo}CD62L^{hi}) T cells to high purity. However, in the absence of Itk, these naive cells might have received signals during their expansion after leaving the thymus that result in upregulation of IFN- γ mRNA as part of this process. Such control of IFN- γ expression might be critical in maintaining the naive status of CD4⁺ T cells and allow these cells to have wide latitude in their response based on the antigenic stimulus and cytokine milieu.

The observed upregulation of IFN- γ mRNA in the absence of Itk in naive CD4⁺ T cells is likely the result of increased accessibility of the IFN- γ locus because the absence of Itk results in favorable epigenetic modifications along the promoter and a majority of the critical CNS regions of the IFN- γ locus. Therefore it is probable that normal CD4⁺ T cells carrying low-affinity TCRs might receive weak signals during the homeostatic expansion after leaving the thymus and have alterations in the IFN- γ locus that make it easier to upregulate IFN- γ mRNA. Alternatively, they can transcribe small amounts of IFN- γ mRNA, which can then influence subsequent cognate antigen activation events. The ability to probe the loci of single cells would allow the testing of this hypothesis. With regard to how this is regulated, we note that surprisingly, T-bet is not required for this upregulation of IFN- γ , suggesting non-T-bet-dependent regulation of IFN- γ by Itk in these cells. It is likely that Eomes or some other factor, such as signal transducer and activator of transcription 4, might be involved in this process, as recently suggested.²⁸

Our work supports and extends the findings of Miller et al,¹⁸ who showed that although Itk^{-/-} TCR-transgenic antigen-specific CD4⁺ T cells produce IL-4 under T_H2-skewing conditions, they noted that the Itk^{-/-} CD4⁺ T cells produce less IL-4 per cell. More importantly, they showed activation of Itk^{-/-} CD4⁺ T cells under T_H2-skewing conditions resulted in the production of significantly less IL-4 (and they carry less IL-4 mRNA) than seen in WT cells on restimulation. Importantly, this was peptide dose dependent. Furthermore, they showed that under non-skewing conditions, Itk^{-/-} CD4⁺ T cells are more

likely to produce IFN- γ under the same conditions in which WT CD4⁺ T cells produce IL-4 (ie, when stimulated with low-avidity peptide). At the time, they suggested that this was due to aberrant regulation of T-bet in the absence of Itk. Our work extends these findings *in vitro* and *in vivo* and shows that rather than regulating T-bet expression, Itk regulates IFN- γ expression. Because we used naive nontransgenic T cells, we cannot comment on the type of signaling the cells in our experiments receive (high vs low avidity), but our results suggest that naive nontransgenic Itk^{-/-} CD4⁺ T cells come prearmed with higher IFN- γ mRNA levels (perhaps because of their experience *in vivo*, as described previously), which predispose them to upregulate the T_H1 pathway. Thus our work adds significantly to the understanding of T_H differentiation by showing that Itk signals suppress the expression of IFN- γ in naive T cells *in vivo*, predisposing them to follow a more T_H1-type response when activated. We have previously reported that antigen restimulation of Itk^{-/-} lymph node T cells results in secretion of lower IFN- γ levels than seen in WT cells during the development of allergic airway inflammation.¹³ We have also reported that there is no difference in IFN- γ expression (as measured by means of ELISA) in BALF in the absence of Itk.²⁹ These data suggest that Itk^{-/-} T cells do not have reduced ability to secrete IFN- γ , and our previous data might reflect reduced recruitment of antigen-specific T cells to the lymph nodes, which reads out as reduced cytokine secretion on restimulation.

The data presented herein support a model in which Itk actively suppresses the expression of IFN- γ through an early TCR signal in naive CD4⁺ T cells. This signal, at least in part, regulates epigenetic processes that maintain the chromatin in the IFN- γ locus closed for transcription. In the absence of Itk, IFN- γ levels increase, leading to a positive feed-forward loop with Eomes and T-bet. This is then responsible for the inability of Itk^{-/-} CD4⁺ T cells to optimally induce the T_H2 transcriptional program and for the mice to have a T_H2 response on allergen challenge. In addition to providing mechanistic insight into the observed T_H2 defects in Itk^{-/-} mice, this work has implications for understanding Itk as a potential target for T_H2-mediated inflammation, T_H differentiation programs, and the pathology of asthma.

METHODS

Lentiviral transduction and activation of human peripheral blood T cells

Plasmids carrying lentivirus with shRNA targeting Itk or control shNull (Sigma) were transfected into HEK 293T cells along with the lentiviral packaging plasmids p 8.9 and pVSVg to generate lentiviral particles. The supernatant was collected 48 hours after transfection, passed through a 0.45- μ m filter (Whatman, GE Healthcare, Piscataway, NJ), and packaged virus was used to transduce 4×10^6 primary human peripheral blood T cells (Fig 3, A and B) or 2 to 4×10^6 human CD4⁺ T cells purified by using anti-human CD4 MicroBeads (Fig 3, C and D; Miltenyi Biotec) along with 8 μ g/mL Polybrene (Sigma). Cells were stimulated with 0.1 μ g/mL α -OKT3 CD3 (eBioscience) and 1 μ g/mL of α -human CD28 (eBioscience) at 2×10^6 /mL. Supernatants were analyzed for IL-4 using the Milliplex MAP human cytokine/chemokine magnetic bead kit (Millipore), with a limit of detection of 4.5 pg/mL for human IL-4 calculated by using StatLIA Immunoassay Analysis Software. All experiments were approved by the Office of Environmental Health and Safety at the Pennsylvania State University and Cornell University.

T-cell purification and polarizing conditions

All naive T cells ($CD4^{+}TCR\beta^{+}CD44^{low}CD62L^{high}$) were isolated from spleens and lymph nodes by using cell sorting with a Cytopeia Influx or a FAC-Saria Cell Sorter to a purity of greater than 95%. T cells were cultured alone or along with mitomycin C-treated APCs in RPMI 1640 with heat-inactivated 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 5 mmol/L β -mercaptoethanol. T_H2 -polarizing conditions included 1 μ g/mL α -murine CD3 and α -murine CD28 (BD Biosciences), 40 ng/mL IL-4 (PeproTech), and anti-IFN- γ (XMG1.2, BioLegend). Anti-IL-12 (10 μ g/mL; PeproTech) was also included when cells were cultured with APCs. Cells were analyzed for cytokine secretion, as detailed below. Antibody clone information is detailed in Table E1.

Analysis of cytokine secretion and expression of transcription factors

Cells were either analyzed directly (unstimulated) or stimulated briefly with P/I (50 ng/1 μ g/mL; Sigma) in the presence of brefeldin A (Sigma) for 5 hours. Some cells were differentiated under T_H2 -polarizing conditions for the indicated time and then restimulated with P/I in the presence of brefeldin A for 5 hours. All cells were fixed/permeabilized and then stained with α -CD4, α -TCR β , α -CD44, α -CD62L, α -human CD4, α -mouse IFN- γ , α -human IFN- γ , α -IL-4, α -mouse/human T-bet, α -GATA3, or α -Eomes (eBioscience). Data were acquired on an LSRII (BD Biosciences) and analyzed by using FlowJo software (Tree Star, Ashland, Ore).

Western blotting

Peripheral blood human T cells were transduced with either an shRNA targeting Itk or the control shNull and stimulated for the indicated length of time and lysed with RIPA buffer. Proteins were separated on an 8% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore), and immunoblotting was done with either α -Itk (Cell Signaling Technologies, Danvers, Mass) or α - β -actin (Sigma).

ChIP

ChIP assays were performed with the Magna ChIP G kit (Millipore), as recommended by the manufacturer. Fluorescence-activated cell sorting-sorted naive $CD4^{+}$ T cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature, the reaction was stopped with glycine, and the cells were lysed sequentially with cytoplasmic and nuclear lysis buffers. Chromatin was then sheared by means of sonication with a Bioruptor sonicator (Misonix, Farmingdale, NY). Lysate equivalent to 1 to 2 $\times 10^6$ cells was used per immunoprecipitation at 4°C overnight with α -H3K4me2, α -H3K27me3, or rabbit IgG antibodies (Abcam, Cambridge, United Kingdom). Enrichment of chromatin was analyzed with Fast SYBR Green qPCR (Roche) by using the 7500 Fast Real-Time PCR instrument (Applied Biosystems). Regions of the IFN- γ promoter/enhancer (along the indicated CNS regions detailed in Tables E2 and E3) were amplified by using qPCR with previously published primers.^{E1,E2} Data were normalized by using the percentage input method and then expressed as fold enrichment over respective IgG for respective ChIPs. Each experiment was performed on pooled cells from at least 5 mice per group.

Histopathologic analysis of lung sections

Histopathologic analysis of lung sections was performed by a veterinary pathologist (S.M.) based on a previously described scoring scheme.^{E3} In brief, sections stained with hematoxylin and eosin or periodic acid–Schiff were randomly assigned codes to blind the examiner to the specimen's identity. Lung sections were first evaluated for the overall distribution and severity of the lesions, followed by scoring at a magnification of $\times 200$, by examining at least 20 consecutive fields. Scoring was performed for various histopathologic features of lung inflammation and assigned a score of 1 (minimal), 2 (mild) 3 (moderate), and 4 (severe).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AHR	Airways hyperresponsiveness
APC	Antigen-presenting cell
BALF	Bronchoalveolar lavage fluid
ChIP	Chromatin immunoprecipitation
CNS	Conserved noncoding sequence
DKO	Double knockout
Eomes	Eomesodermin
HDM	House dust mite
Itk	IL-2–inducible T-cell kinase
P/I	Phorbol 12-myristate 13-acetate/ionomycin
qPCR	Quantitative PCR
shRNA	Small hairpin RNA
T-bet	T-box transcription factor
TCR	T-cell receptor
WT	Wild type

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Key messages

- Itk signals downstream of the TCR during T-cell development, homeostasis, or both, and this is critical for controlling the expression of T_H1 genes before cognate antigenic stimulus.
- In the absence of Itk, IFN- γ levels increase, leading to a positive feed-forward loop with T-bet, with the resultant inability of Itk^{-/-} CD4⁺ T cells to optimally induce the T_H2 transcriptional program and the mice to have allergic airway inflammation.
- A better understanding of these signaling events is critical for us to be able to manipulate effector responses of T cells without globally suppressing the immune system, offering attractive therapeutic opportunities for treatment of T cell-driven inflammatory diseases, such as allergic asthma.

FIG 1.

Itk negatively regulates TH1 genes, thus regulating TH2 differentiation. **A**, qRT-PCR on naive CD4⁺ T cells from WT and *Itk*^{-/-} mice and analyzed for the indicated genes. **B**, Fluorescence-activated cell sorting analysis of naive CD4⁺ T cells for the indicated proteins in unstimulated (US) or stimulated (S; P/I for 5 hours) conditions. Data are expressed as the mean fluorescence intensity (MFI) index, as follows: MFI $\times$ (% Stained – Background stain). **C**, Naive CD4⁺ T cells cultured under TH2-polarizing conditions and analyzed for the indicated genes at the indicated time points. **D**, Fluorescence-activated cell sorting analysis for the indicated proteins in TH2 culture conditions over the indicated time course. Data are expressed as the MFI index. qRT-PCR data were normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), followed by normalization to the respective unstimulated naive control cells. **P* < .05 by *t* test (mean $\pm$ SEM of 2 to 4

independent experiments; Fig 1, *A* and *B*) or by ANOVA (mean $\pm$ SEM of 2 independent experiments, with each experiment performed on pooled cells from at least 3 mice per group; Fig 1, *C* and *D*) comparing WT with Itk^{-/-} cells.

FIG 2.

Itk negatively regulates IFN- γ to regulate the expression of T-bet. **A**, qRT-PCR on naive CD4⁺ T cells from the indicated mice and analyzed for IFN- γ , T-bet, and Eomes. Values are presented as means $\pm$ SEMs of 2 to 3 independent experiments. * $P < .05$ by t test, Itk^{-/-} versus Itk/IFN- γ DKO cells. **B**, Fluorescence-activated cell sorting analysis for the indicated proteins in naive CD4⁺ T cells under unstimulated (US) or stimulated (S; P/I for 5 hours) conditions. Data are expressed as the MFI index (see the legend for Fig 1). Values are presented as means $\pm$ SEMs ($n > 3$). * $P < .05$ by t test, WT versus Itk^{-/-} and Itk^{-/-} vs Itk/T-bet DKO mice (IFN- γ); WT versus Itk^{-/-} and Itk^{-/-} versus Itk/IFN- γ DKO mice (T-bet); and WT versus Itk^{-/-} and Itk^{-/-} versus Itk/IFN- γ DKO mice (Eomes).

FIG 3.

Itk signaling controls the expression of both T-bet and IFN- γ to regulate IL-4 secretion in human peripheral blood T cells. **A**, Human peripheral blood T cells transduced with either control lentivirus (*shNull*) or carrying shRNA against Itk (*shItk*) were stimulated with α -CD3/CD28 then analyzed for Itk mRNA (*left*) or protein levels from total cell lysates probed with α -Itk (*top right*) or α - β -actin (*bottom right*). **B**, Cells treated as in Fig 3, A, were restimulated for 24 hours and analyzed for T-bet and IFN- γ expression by using qRT-PCR. Values in Fig 3, A and B, are means $\pm$ SEMs of at least 4 individual donors. * $P < .05$ by t

test. **C**, Purified CD4⁺ T cells treated as in Fig 3, A, were restimulated for 5 hours in the presence of brefeldin A and analyzed for the expression of T-bet and IFN- γ by using fluorescence-activated cell sorting (*top panels*) and quantified as the percentage of positive cells (*bottom panels*). Values are presented as means $\pm$ SEMs of 5 individual donors. * P < .05 by t test. **D**, Cells treated as above were restimulated for 24 hours, and the supernatant was analyzed for IL-4 by using ELISA (limit of detection, 4.5 pg/mL). Values are presented as means $\pm$ SEMs of 4 individual donors. * P < .05 by t test.

FIG 4.

Removal of IFN- γ restores IL-4 expression in $Itk^{-/-}$ T cells *in vitro*. **A**, IL-4 expression determined by using qRT-PCR from naive CD4⁺ T cells cultured under T_H2-polarizing conditions over the indicated time points (* P < .05). Data are normalized to naive unstimulated cells and means $\pm$ SEMs of 2 independent experiments, with each experiment performed on pooled cells from at least 3 mice per group. * P < .05 by ANOVA. **B**, Fluorescence-activated cell sorting analysis of IL-4 at day 3 of culture (data are expressed as the MFI index; see the legend for Fig 1). Values are means $\pm$ SEMs (n = 3). * P < .05 by t test.

FIG 5.

Itk signaling controls accessibility of the IFN- γ locus. **A**, ChIP assay on naive WT and *Itk*^{-/-} CD4⁺ T cells by using α -H3K4me2 or α -H3K27me3. The IFN- γ promoter region was amplified. **B**, ChIP assay on naive WT and *Itk*^{-/-} CD4⁺ T cells with α -H3K4me2. The promoter and indicated CNS regions along the IFN- γ locus were amplified by using qPCR. Data are expressed as fold enrichment over respective IgG controls after correcting for percentage input. Values are means $\pm$ SEMs of 2 to 3 independent experiments. **P* < .05 by *t* test.

FIG 6.

Removal of IFN- γ restores T_H2-driven allergic airway inflammation in Itk^{-/-} mice. **A**, Lung sections from the indicated mice exposed to HDM analyzed for mucus by staining for periodic acid–Schiff. **B**, Quantitative analysis of goblet cell hyperplasia and bronchiolar lumen occlusion from hematoxylin and eosin– and periodic acid–Schiff–stained lung sections. **P* < .05 by *t* test (*n* > 3). **C**, BALF from lungs of HDM-exposed mice were analyzed for the number of CD4⁺TCR β ⁺ T cells (*left*) or eosinophils (*right*). **P* < .05 by *t* test (*n* > 4). **D**, qRT-PCR analysis of total lung tissue RNA from the indicated mice exposed to HDM and analyzed for the indicated cytokines. **P* < .05 by *t* test (*n* > 8). **E**,

Analysis of the indicated mice for airway resistance after HDM exposure. $*P < .05$ by ANOVA ($n = 3-10$), WT versus $Itk^{-/-}$ and PBS control animals (WT/C; *left*; $Itk/IFN-\gamma$ DKO vs $Itk^{-/-}$). $Itk^{-/-}$ data in the right panel were taken from the left panel for ease of comparison (*right*).