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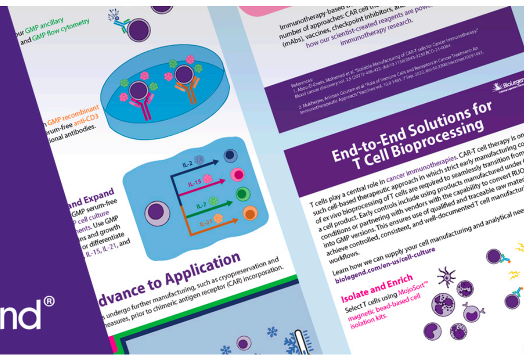

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Cynthia Mueller; ... et. al

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Attenuation of Immunological Symptoms of Allergic Asthma in Mice Lacking the Tyrosine Kinase ITK[1]

Cynthia Mueller and Avery August[2]

Allergic asthma patients manifest airway inflammation and some show increases in eosinophils, T_H2 cells, and cytokines, increased mucous production in the lung, and elevated serum IgE. This T_H2-type response suggests a prominent role for T_H2 cells and their cytokines in the pathology of this disease. The Tec family nonreceptor tyrosine kinase inducible T cell kinase (ITK) has been shown to play a role in the differentiation and/or function of T_H2-type cells, suggesting that ITK may represent a good target for the control of asthma. Using a murine model of allergic asthma, we show here that ITK is involved in the development of immunological symptoms seen in this model. We show that mice lacking ITK have drastically reduced lung inflammation, eosinophil infiltration, and mucous production following induction of allergic asthma. Notably, T cell influx into the lung was reduced in mice lacking ITK. T cells from ITK$^{-/-}$ mice also exhibited reduced proliferation and cytokine secretion, in particular IL-5 and IL-13, in response to challenge with the allergen OVA, despite elevated levels of total IgE and increased OVA-specific IgE responses. Our results suggest that the tyrosine kinase ITK preferentially regulates the secretion of the T_H2 cytokines IL-5 and IL-13 and may be an attractive target for antiasthmatic drugs. ***The Journal of Immunology,*** **2003, 170: 5056–5063.**

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The prevalence and severity of asthma has increased worldwide in the last 20 years. In the United States, morbidity and mortality are disproportionately high among inner city African Americans and Hispanics, especially children and young adults (up to three times the rate) (1). This results not only in morbidity caused by the disease, but also in lost opportunities in education and the workplace because of children and adults missing days. Patients with allergic asthma manifest airway inflammation and show increases in eosinophils, T_H2 cells, and cytokines, increased mucous production in the lung, and elevated serum IgE (2). These events combined impact the epithelial and smooth muscle cells of the lung leading to airway hyperresponsiveness.

The presence of so many hallmarks of a T_H2-type response has pointed to a prominent role for T_H2 cells and, in particular, T_H2-type cytokines IL-4, -5, and -13 in the pathology of this disease. Introduction of Ag-specific T_H2 cells alone or IL-4 and IL-13 alone can induce the majority of these events and lead to airway hyperresponsiveness in mice, and blocking these cytokines prevents the development of specific symptoms in mice (3–5). Furthermore, blocking IL-4 can relieve some of the symptoms of asthma in humans (6). The transcription factor GATA-3 regulates IL-5 production and dominant-negative forms of this protein can significantly inhibit experimental allergic asthma in mice (7). Finding further targets that are pharmaceutically tractable and that regulate the development and/or function of T_H2 cells would assist in treating this disease. The development of T_H2 cells is dependent on the cytokine milieu in the microenvironment where T cells encounter Ag such that in the presence of IFN-γ or IL-12, naive T cells differentiate into T_H1 cells and subsequently secrete IFN-γ. Similarly, in the presence of IL-4, naive T cells differentiate into T_H2 cells and subsequently secrete IL-4, -5, and -13 (for review, see Ref. 8). Although it has been proposed that specific types of dendritic cells can produce specific cytokines that lead to either T_H1 or T_H2 differentiation of T cells, the source of the cytokines responsible for T cell differentiation in vivo is not clear since T cells can also produce these cytokines (8). T cell cytokine production is dependent on early signaling events initiated by the TCR and costimulatory signals such as CD28. The combination of TCR and costimulatory signals result in activation of T cells and ultimately their differentiation into T_H1 and/or T_H2 cells and subsequent cytokine production (9). Costimulatory signals delivered by CD28 have been reported to be critical in the development of the two T cell subsets and their corresponding cytokine production (10, 11). Although there is a significant amount of data suggesting that CD28 may preferentially affect the differentiation to T_H2 cells in vitro (12, 13), as well as in vivo (11, 14), this remains an unclarified point because there are reports of CD28-independent T_H2 responses in vitro (15) and in vivo (16). Whether CD28 signals affect the ability of T cells to develop into T_H2 cells or their subsequent secretion of cytokine is not clear; however, CD28 signals have been demonstrated to be required for the development of allergic asthma in mice (17–21). The CD28 related costimulatory molecule inducible costimulator molecule has also been demonstrated to be involved in regulating the pathology of either a *Shistosoma mansoni* model of allergic airway disease (22) or an OVA model of asthma in mice (23). In addition, other targets proposed for regulating the development of allergic asthma include the adhesion molecule LFA-1 and the chemokine receptors CCR3, 4, and 8 (24–27). These molecules all lie downstream of T cell activation or are involved in T cell activation and trafficking of cells in the case of the chemokine receptors.

Triggering the TCR complex results in the activation of a number of attractive pharmaceutical targets, the tyrosine kinases of the Src, Syk, and Tec family of tyrosine kinases (for review, see Ref.

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[3] Abbreviations used in this paper: ITK, inducible T cell kinase; WT, wild type; IN, intranasally.

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28). These kinases are critical in the activation of immune cells and have limited expression patterns, with specific family members having lymphoid-specific expression (28). In T cells, members of the first two families of kinases, Lck and Zap-70, are critical for T cell activation as well as development (29). However, Txk/Rlk, a Tec family kinase, seems to be dispensable for T cell activation and development (30). By contrast, inducible T cell kinase (ITK),[3] the other Tec kinase expressed in T cells, seems to have a more prominent role in T cell activation and differentiation (30–37). Triggering the TCR also results in increases in intracellular calcium, which can activate the protein phosphatase calcineurin and mediate other downstream effects (38). The influx of calcium in T cells is controlled by ITK and mice lacking ITK have reduced calcium increases upon stimulation (37, 39). Indeed, the calcineurin inhibitors cyclosporin A and FK506 have been suggested as treatments for asthma patients (40). These mice also have reduced naive T cell function in IL-2 production and proliferation when stimulated via the TCR (32, 36, 39). In addition, T cells from mice lacking ITK either secrete no IL-4 or significantly less IL-4 than normal T cells, an event that seems to be dependent on the ITK-mediated calcium increase (35, 37).

These data suggest that by regulating the development and/or function of T_H2 cells, ITK may modify the development of allergic asthma. We have tested this hypothesis and now report that mice lacking ITK have drastically reduced lung inflammation and mucous production following induction of allergic asthma. This was probably due to a number of deficiencies in the ITK null mice: reduced Ag-specific recruitment of T cells to the lung; overall reduction in cytokine production, but preferential reduction in Ag-specific secretion of IL-5 and IL-13 by ITK null T cells; reduced T cell proliferative responses to challenge with the allergen OVA. However, these mice have high levels of serum and OVA-specific IgE. Our results suggest that the tyrosine kinase ITK may be an attractive target for antiasthmatic drugs.

Materials and Methods

Mice

Wild-type (WT) (The Jackson Laboratory, Bar Harbor, ME) and ITK null mice (kind gift from Dr. D. Littman, New York University School of Medicine, New York, NY; Ref. 32) on C57BL/6 backgrounds (6–8 wk old) were used for these experiments. The ITK null mice were backcrossed to the C57BL/6 at least 10 generations. All mice were kept in microisolater cages in the animal facilities at Pennsylvania State University and were provided with food and water ad libitum. All experiments were approved by the Office of Research Protection's Institutional Animal Care and Use Committee at Pennsylvania State.

Allergic asthma induction

Groups of mice (WT or $ITK^{-/-}$ mice) were primed with OVA (Sigma-Aldrich, St. Louis, MO) or carrier as follows: 50 μg/ml OVA complexed with aluminum hydroxide (10 μg OVA/1 mg alum; Pierce, Rockford, IL) were injected i.p. on days 0 and 5. Control mice were injected with aluminum hydroxide alone. Mice were then daily exposed intranasally (IN) with OVA (2 mg/ml, 40 μg total) on days 12 through 15 and sacrificed 24 h later for analysis.

Characterization of lung pathology

Following prime and challenge, mice were sacrificed and lungs were removed. Lungs were fixed in formaldehyde (3%) overnight before embedding and 5-μm sections were cut for staining. Sections were stained with H&E to examine infiltrating cells and periodic acid-Schiff (PAS) for analysis of mucous production. In some experiments, one lung was used for histology and the other was dissociated using collagenase (150 U/ml), and the resultant cell populations were analyzed using an Advia 1200 Hematology System (Bayer, Norwood, MA). To analyze the T cell population in the lung, mononuclear cells were isolated from collagenase-dissociated lungs on a 30%/60% Percoll gradient as described elsewhere (41). Cells were then reacted with directly conjugated Abs against CD3 (CyChrome; BD Pharmingen, San Diego, CA) and analyzed by flow cytometry. In some experiments, these stained cells were permeabilized and stained with anti-IL-4 (PE) and anti-IFN-γ (FITC; all from BD Pharmingen) and analyzed by flow cytometry, gating on the lymphocyte population as defined by their forward and side scatter characteristics.

Analysis of T cell response to OVA

Following prime and challenge, mice were sacrificed and splenocytes purified. Proliferation of T cells to OVA was analyzed by incubating the isolated splenocytes with OVA at the indicated concentrations. Cultures were then pulsed with [^{3}H]thymidine for 18 h 2 days after initiating the culture; the culture was harvested and incorporated radioactivity was determined by scintillation counting. Analysis of cytokine secretion was performed by stimulating 2×10^5 purified $CD4^+$ T cells with the indicated concentrations of OVA and T cell-depleted splenocytes for 96 h. Supernatants were then harvested and cytokine-specific (IFN-γ, IL-4, -5, -10, and -13) ELISAs were performed according to the manufacturer's instructions (BD Pharmingen for the IFN-γ, IL-4, -5, and -10, and R&D Systems (Minneapolis, MN) for IL-13).

Analysis of IgE levels

Before and following prime and challenge, mice were sacrificed and serum was obtained. Dilutions of sera were analyzed for total IgE and OVA-specific IgE by ELISA. In the former case, anti-murine IgE (2 μg/ml, 1/250) was used as capture Abs and HRP-conjugated anti-murine IgE (1/250) as detection reagents (Southern Biotechnology Associates, Birmingham, AL). To detect OVA-specific IgE, OVA was coated onto the ELISA wells (20 mg/ml) and dilutions of sera tested with HRP-conjugated anti-murine IgE were used as detection reagents.

Statistics

Values were compared using Student's *t* test.

Results

ITK null mice exhibit reduced lung inflammation following allergic asthma induction

The tyrosine kinase ITK has been reported to be involved in the activation of T cells leading to T_H2 cell differentiation in vitro and in T_H2 type responses to pathogens in vivo (35, 37). Since allergic asthma has many hallmarks of a T_H2-type response in humans and in animal models, we tested whether ITK may be involved in the allergic inflammatory response in the lung to the model allergen OVA in a murine model of allergic asthma. In this model, mice are primed with OVA, then challenged IN with OVA (42). This treatment results in increased inflammatory cell infiltration into the lung, thickening of the epithelial cells lining the bronchioles of the lung, mucous secretion, and increases in IgE in the serum of these mice. We primed mice (WT or $ITK^{-/-}$ on the same backgrounds) by i.p. injection of OVA/alum on days 0 and 5, then on days 12–15 they were challenged by exposure to OVA IN. On day 16, mice were sacrificed and analyzed for immunological symptoms of allergic asthma. Control mice were either not primed, but were challenged, or were primed and not challenged, with similar results (data not shown).

Isolated lungs were fixed and stained with H&E to determine leukocyte infiltration (Fig. 1). Our analysis demonstrated that although WT mice develop leukocyte infiltrates in the lung, mice lacking ITK had much reduced infiltration (compare Fig. 1, *a* and *b* and *c* and *d*). WT mice also exhibited thickening of the epithelial cell lining of the bronchioles, which was not observed in $ITK^{-/-}$ mice. Analysis of cells obtained following collagenase dissociation of lungs from similar experiments demonstrated a marked increase in eosinophil influx in lungs from WT mice primed and challenged with OVA, but not from unprimed mice or from mice lacking ITK under any of the tested conditions (Fig. 1*e*, $p < 0.068$). These data suggest that mice lacking ITK have defective inflammatory responses to priming and challenge with OVA in this model.

Mucous production in response to allergic asthma has been shown to be controlled by T_H2 cells and/or their cytokines (5). To

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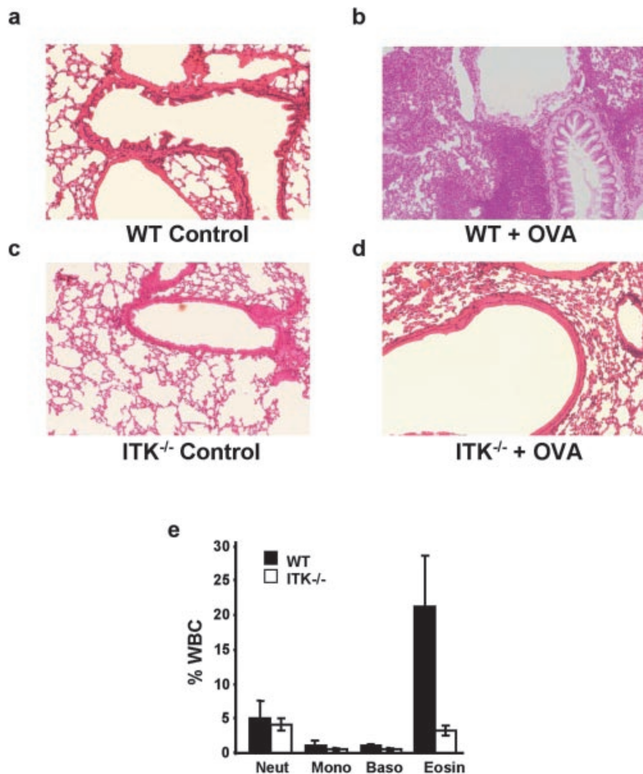

FIGURE 1. Reduced lung inflammation in mice lacking ITK following induction of allergic asthma. WT (*a* and *b*) or ITK$^{-/-}$ (*c* and *d*) mice were primed twice, 5 days apart with OVA/alum (*c* and *d*) or alum alone (*a* and *c*) then exposed IN to OVA (all mice) on days 12–15 as described in *Materials and Methods*. Twenty-four hours after the final OVA intranasal exposure, lungs from mice were fixed, paraffin-embedded, sectioned (5 μm), and stained with H&E. *a*, WT mouse primed with alum and exposed to OVA IN. *b*, WT mouse primed with OVA/alum and exposed to OVA IN. *c*, ITK$^{-/-}$ mouse primed with alum and exposed to OVA IN. *d*, ITK$^{-/-}$ mouse primed with OVA/alum and exposed to OVA IN. Note the thickening of the epithelial cell layer lining the bronchioles and inflammatory infiltration in the WT mouse primed and exposed (*b*) and the lack of such thickening and infiltration in the lung from the mouse lacking ITK (*d*). Original magnifications, ×10. *e*, Reduced eosinophil infiltration in the lungs of mice lacking ITK following induction of allergic asthma. Lungs from similarly treated mice were isolated and dissociated as described in *Materials and Methods*. The resultant cell populations were analyzed using an Advia 120 Hematology Analyzer for neutrophils (neut), monocytes (mono), basophils (baso), and eosinophils (eosin). Lymphocytes were also detected but no significant changes were detected in this population (data not shown). Profiles from the lungs of control mice profiles were similar to those seen in the ITK$^{-/-}$ mice and were omitted for clarity of presentation. Note the significant difference in the percentage of eosinophils in the lungs from the primed and challenged ITK$^{-/-}$ mice compared to WT mice ($p < 0.068$). Similar results were seen when control mice were primed with OVA/alum but were only exposed to PBS IN. Representative of more than seven separate experiments with at least three mice per group.

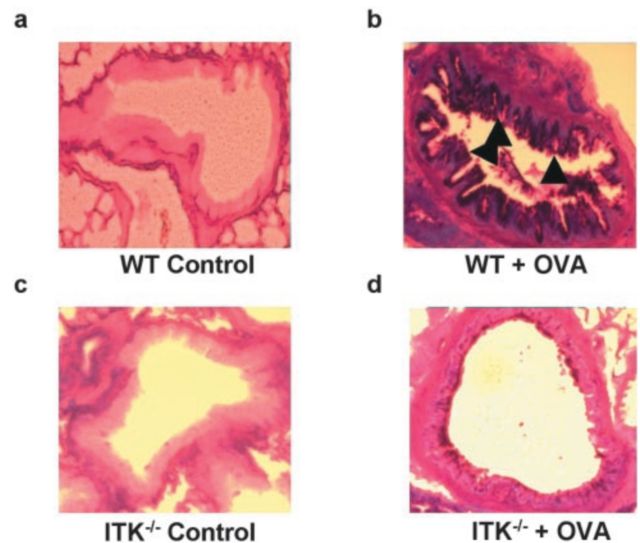

FIGURE 2. Reduced mucous production in mice lacking ITK following induction of allergic asthma. WT (*a* and *b*) or ITK$^{-/-}$ (*c* and *d*) mice were primed and challenged as described in Fig. 1 legend. Twenty-four hours after the final OVA IN exposure, lungs from mice were fixed, embedded in paraffin, sectioned (5 μm), and stained with PAS to detect mucous. *a*, WT mouse primed with alum and exposed to OVA IN. *b*, WT mouse primed with OVA/alum and exposed to OVA IN. *c*, ITK$^{-/-}$ mouse primed with alum and exposed to OVA IN. *d*, ITK$^{-/-}$ mouse primed with OVA/alum and exposed to OVA IN. Note that the dark areas lining the goblet cells in the lung of the WT mouse primed and exposed are stained with the PAS stain and mucous is being produced by these cells (*b*). Also note the very low production of mucous in the lung from ITK$^{-/-}$ mouse and the lack of hyperplasia of these cells (*d*). Original magnifications, ×20.

determine whether ITK$^{-/-}$ mice were also defective in mucous production in response to OVA challenge, we stained lung sections with PAS to detect mucous. Figure 2 confirms our findings in Fig. 1, indicating that although WT mice had increased mucous production by the goblet cells lining the bronchioles (Fig. 2, *a* and *b*), the bronchioles from ITK$^{-/-}$ had much reduced to almost absent mucous production (Fig. 2, *c* and *d*). These results suggest that ITK may regulate the T_H2-type response and/or the resultant cytokine production that leads to mucous production in this model of allergic asthma.

Reduced T cell infiltration in the lung of ITK null mice following allergic asthma induction

Examining lung-derived cells for the presence of T cells indicated that although there was an increase in the percentage of T cells (of the total lymphocyte population) in the lungs of WT mice upon OVA challenge, the lungs from the ITK$^{-/-}$ mice exhibited no increase in the percentage of T cells compared to the control ITK null mice (Fig. 3, *a* and *b*). Analysis of these lung-derived T cells for intracellular cytokine (IFN-γ and IL-4, classical T_H1 and T_H2 cytokines, respectively) indicated that while approximately 1.7% of the T cells from WT lungs had intracellular IL-4, none of the T cells obtained from ITK null lungs had intracellular IL-4. No IFN-γ was detected in the T cells from either the WT or ITK null lungs (Fig. 3*c*). These data suggest that reduced Ag-specific T cell recruitment to the lung may in part underlie the reduced responses we observe in the ITK null mice.

Increased total and Ag-specific IgE in ITK null mice

Serum IgE levels correlate with asthmatic symptoms (43). To determine whether the ITK$^{-/-}$ mice were generating an IgE response against the allergen, we tested their serum for total IgE and OVA-specific IgE. Under these immunization conditions, WT mice had increased serum IgE whereas WT control mice had low levels of IgE comparable to untreated naive WT mice (Fig. 4*a*). Surprisingly, mice lacking ITK had higher levels of serum IgE than control WT mice in the unimmunized state (Fig. 4*a*, $p < 0.004$). This increased serum IgE level was surprising given the proposal that ITK may be involved in regulating T_H2 responses. B cell class switch to IgE can be controlled by the levels of IL-4, IFN-γ, as well as IL-10 and perhaps ITK null mice have altered serum levels of these IFN-γ and IL-10, thus reducing the potential negative

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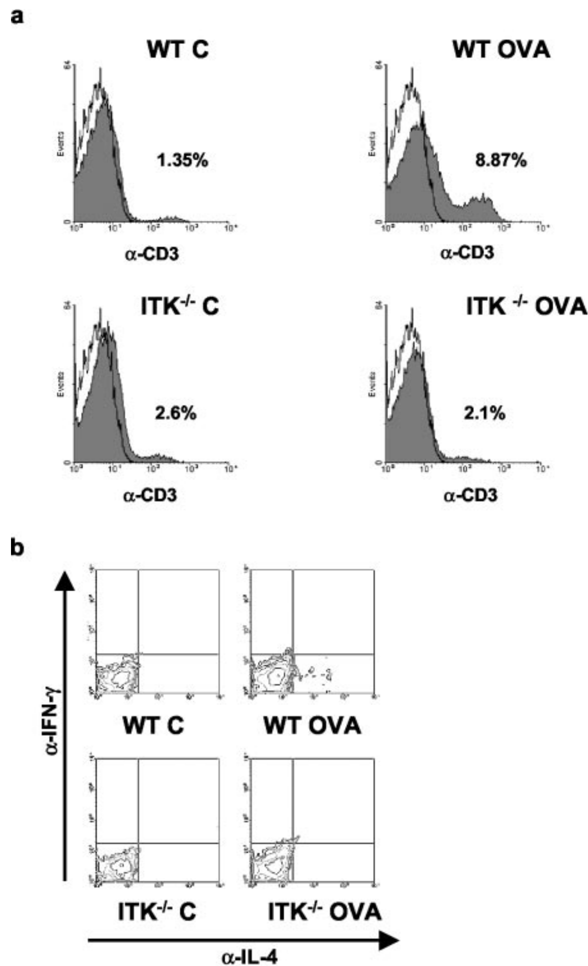

FIGURE 3. Reduced T cell infiltration in the lungs of mice lacking ITK following induction of allergic asthma. *a*, WT (*top panels*) or ITK$^{-/-}$ (*bottom panels*) mice were primed and challenged as described in Fig. 1 legend. Lungs were then isolated and dissociated as described in Fig. 1 legend and stained with Abs to CD3 and data were analyzed by gating on the lymphocyte population. Shaded areas indicate staining with anti-CD3 while the open lines are control Abs. *b*, Lung cells from WT (*top panels*) or ITK$^{-/-}$ (*bottom panels*) mice primed and challenged as described in Fig. 1 legend were stained with Abs to CD3, permeabilized, and then stained with Abs to IFN-γ and IL-4. Data shown represent the analysis of the CD3$^+$ population for intracellular IFN-γ (*y*-axis) and IL-4 (*x*-axis) expression.

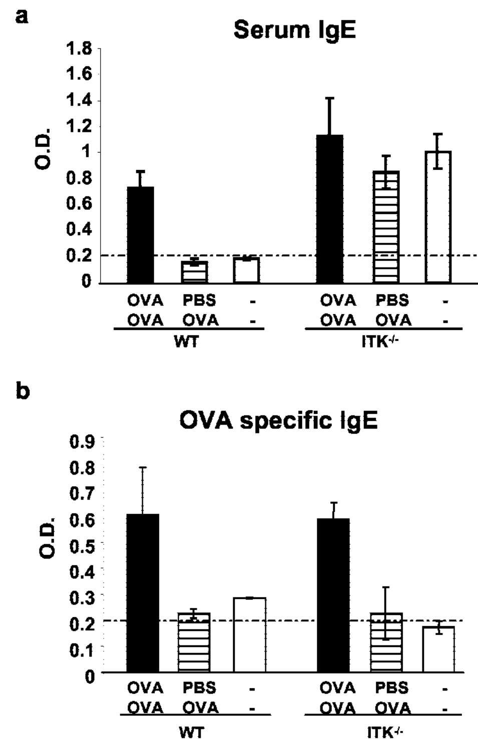

FIGURE 4. Increased IgE production following priming and challenge with OVA in mice lacking ITK. WT (*left*) or ITK$^{-/-}$ (*right*) mice were primed and challenged as described in Fig. 1 legend. Twenty-four hours after the final OVA intranasal exposure, serum from these mice as well as naive mice were analyzed for IgE levels by ELISA. Sera from the following mice were analyzed: primed with OVA/alum and exposed to OVA (■); primed with alum and exposed to OVA (striped bars); and naive mice (□). *a*, Total IgE levels (1/10 dilution). Note that naive and alum-only primed WT mice have low levels of total serum IgE levels (the horizontal line represents the limit of detection of the assay). By contrast, naive and unprimed ITK$^{-/-}$ mice have significantly elevated total serum IgE levels compared with similarly treated WT mice ($p < 0.004$). ITK$^{-/-}$ mice also exhibited significantly higher total serum IgE compared to WT mice following prime and challenge ($p < 0.002$). *b*, OVA-specific IgE levels (1/50 dilution). Note that naive and alum-only primed WT and ITK$^{-/-}$ mice have low levels of OVA-specific serum IgE levels (the horizontal line represents the limit of detection of the assay). By contrast, both primed WT and ITK$^{-/-}$ mice have significantly elevated OVA-specific serum IgE levels compared to unprimed mice ($p < 0.0007$ for WT mice and $p < 0.001$ for ITK$^{-/-}$). There was no significant difference in the levels of OVA-specific IgE seen in WT vs ITK$^{-/-}$ mice.

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influence of these cytokines on class switch to IgE (44, 45). To determine whether ITK$^{-/-}$ mice have altered serum levels of IL-4, IFN-γ, or IL-10, we tested serum from WT and ITK$^{-/-}$ mice for levels of IFN-γ, IL-10, and IL-4. Our analysis showed that ITK null and WT mice both had low levels of serum IL-4 and IL-10 and equivalent levels of IFN-γ (IFN-γ: WT, 681.6 ± 136.3 pg/ml; ITK$^{-/-}$, 611.5 ± 14 pg/ml; IL-4: WT, 8.5 ± 2.4 pg/ml; ITK$^{-/-}$, none detected; IL-10: WT and ITK$^{-/-}$, none detected). However, it is not clear whether these levels would lead to the increased circulating IgE levels in the ITK null mice.

Analyzing serum levels of IgE in ITK$^{-/-}$ mice primed and challenged with OVA revealed no change in total IgE, but similar to the WT mice, they did respond with increased OVA-specific IgE (Fig. 4*b*, $p < 0.001$ for ITK$^{-/-}$ mice and $p < 0.0007$ for WT mice). There was no significant difference in the levels of OVA-specific IgE in WT vs ITK$^{-/-}$ mice. Since OVA is a T-dependent Ag, these data indicate that although ITK$^{-/-}$ T cells may have altered T_H2 development and/or function, they may still be able to generate sufficient T_H2-type cytokines to allow for the development of an IgE-mediated response. This OVA-specific IgE response, however, did not accompany an increase in the inflammatory response in the lung, suggesting perhaps a difference in systemic vs mucosal immune responses in these mice or the lack of recruitment of eosinophils to the lungs that can respond, leading to the pathology seen in WT mice. However, it has been noted that increased IgE does not always accompany the lung pathology seen in allergic asthma (46).

Reduced T cell proliferation and altered cytokine production by ITK null T cells in response to allergen stimulation

Because it appeared that ITK$^{-/-}$ mice had a T_H2-type humoral immune response to OVA, which is a T cell-dependent Ag (47), we next determined whether ITK$^{-/-}$ T cells could respond to OVA following priming and challenge. Splenic cells from mice

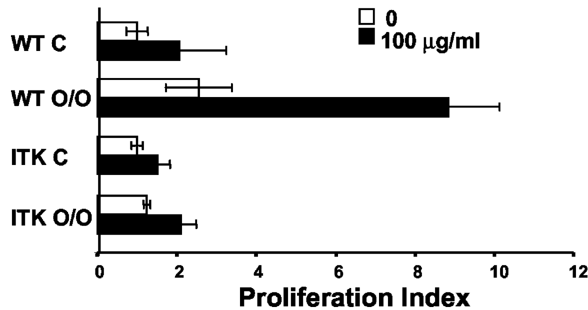

FIGURE 5. Reduced T cell responses following priming and challenge with OVA in mice lacking ITK. WT or ITK$^{-/-}$ mice were primed and challenged as described in Fig. 1 legend. Twenty-four hours after the final OVA intranasal exposure, splenocytes from these mice were incubated with the indicated concentration of OVA and proliferation was analyzed 48 h later by pulsing with [^{3}H]thymidine for 18 h. The cells were then harvested and incorporated ^{3}H was counted. The results are expressed as proliferation index, calculated as fold increase in [^{3}H]thymidine incorporation in OVA-stimulated cells over cells incubated without OVA. All cells proliferated similarly in the presence of Con A/PMA/ionomycin stimulation. Note that WT cells had significantly higher proliferation when stimulated with OVA compared to ITK$^{-/-}$ cells. Also note that although not statistically significant, OVA-stimulated ITK$^{-/-}$ cells also reproducibly proliferated higher than nonstimulated cells from control mice.

primed and challenged with OVA were incubated with OVA and T cell proliferation was determined after 3 days of incubation (Fig. 5). WT mice responded to OVA stimulation by proliferating (Fig. 5). Splenic cells from ITK$^{-/-}$ mice by contrast proliferated poorly, confirming that these mice have reduced T cell responses.

Analysis of cytokine production in response to OVA stimulation following the allergic asthma induction protocol indicated that ITK null T cells from draining lymph nodes produced fewer cytokines of both T_H1 and T_H2 type (Fig. 6*a*). Of note, however, is that while very little IL-4, -5 or, -13 was detected when these cells were stimulated in vitro, they were able to produce some IFN-γ and IL-10 (Fig. 6*a*). Qualitatively similar results were observed when splenic T cells were stimulated (Fig. 6*b*). Although T cells from ITK null mice were able to produce more cytokines of both T_H1 and T_H2 types, quantitatively they were more defective in the production of IL-4 and, to a greater extent, IL-5 and IL-13. Their production of IFN-γ and IL-10 was not affected as much (Fig. 6*b*). Thus, while mice lacking ITK have reduced T cell responses to antigenic stimulation, the production of T_H2 cytokines, in particular IL-5 and IL-13, is more affected by the lack of ITK. These data do suggest, however, that mice lacking ITK are able to secrete low levels of IL-4 that may be sufficient to generate an Ag-specific IgE response. However, the reduced levels of T cells in the lung, coupled with the reduced production of IL-5 and in particular IL-13, may be the reason for the significant lack of pathological response in the lung we observed during allergic asthma induction in these mice.

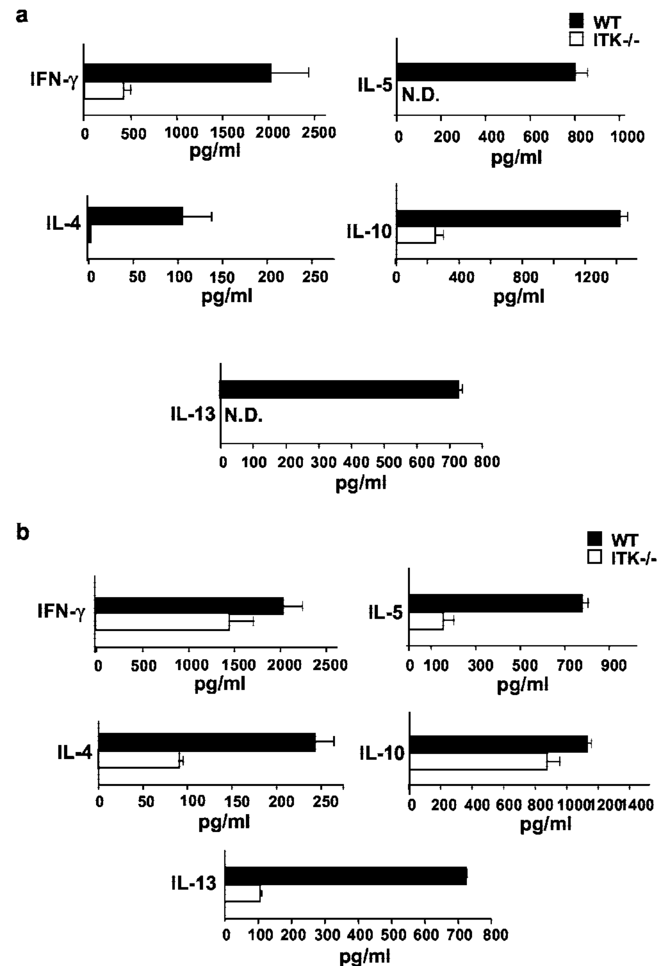

FIGURE 6. Reduced cytokine production by ITK null T cells following priming and challenge with OVA. WT or ITK$^{-/-}$ mice were primed and challenged as described in Fig. 1 legend. Twenty-four hours after the final OVA intranasal exposure, CD4$^+$ T cells were purified from the draining lymph nodes (*a*) or spleens (*b*) from these mice and were incubated with OVA (1 mg/ml) along with T cell-depleted APCs, and supernatants were harvested 96 h later for cytokine analysis. *a*, IFN-γ, IL-4, -5, -10, and -13 secretion in response to OVA stimulation by draining lymph node CD4$^+$ T cells. *b*, IFN-γ, IL-4, -5, -10, and -13 secretion in response to OVA stimulation by splenic CD4$^+$ T cells. ND, None detected.

Discussion

A large body of data suggests that T_H2 cells play a critical role in the development of allergic asthma, in both mouse models as well as in humans (2–5). Understanding the mechanisms regulating the development and/or function of T cell subsets will be critical for designing therapies that specifically control the development of and/or treatment of this disease. Although the manipulation of the development of these T cells by cytokine-specific targeting represents a promising avenue, these approaches generally tend to involve the use of large proteins that may be costly and difficult to administer. Further analysis of proteins that may control the development and/or function of these cells may result in the discovery of better pharmaceutical targets to which small molecule therapeutics can be developed. In this report, we demonstrate that in the absence of the Tec family tyrosine kinase ITK, mice are largely resistant to the immunopathological symptoms in a model of allergic asthma. Specifically, ITK null mice had drastically reduced levels of infiltration of eosinophils and, in particular, of T cells in the lung. The T cells that were in the lungs of ITK null mice were not producing detectable levels of IL-4. Similarly, lymph node T cells from ITK null mice produced no detectable IL-4, -5, and -13, while producing low levels of IFN-γ and IL-10. Similar results were observed using splenic T cells although these cells produced more IFN-γ and IL-10. Surprisingly, ITK null mice had elevated levels of total IgE and generated a normal anti-OVA IgE response, although this was not sufficient to have a significant effect on the pathological symptoms of the disease. Our data confirm and extend previous reports of ITK being involved in the development of a T_H2 response in vivo (35, 37).

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T cell activation is dependent on early signals delivered by the TCR and CD28. We and others have demonstrated that both the TCR and CD28 activate the Tec family kinase ITK (31–33, 36, 39). ITK activation leads to activation of phospholipase Cγ1 via an unknown mechanism, perhaps by tyrosine phosphorylation, leading to induction of calcium signaling (39). A number of transcription factors that regulate cytokine production are regulated by calcium levels, most notable NFAT (48). The lack of ITK (on the BALB/c background) has been reported to result in significantly reduced IL-4 production in the absence of a significant effect on IFN-γ secretion, which in vitro can be rescued by increased calcium levels by ionomycin (37). On the genetic background that we have used in our studies, C57BL/6, the lack of ITK results in a more generalized cytokine production defect, with decreases in both IFN-γ as well as IL-4 and IL-5 when T cells are polarized in vitro (35). However, in Ag-specific recall responses to *S. mansoni* egg Ag, reduced IL-4 and increased IFN-γ was observed in vitro (35). These published data suggest that although ITK may generally regulate T cell responses in vitro, in vivo, ITK may preferentially regulate T_H2-type responses.

Our finding of much reduced eosinophilic infiltration and mucous production in the lungs of ITK knockout mice may be the result of a number of factors that seem to be deficient in ITK null mice. The reduced T cell infiltration in the lung may result in a reduced overall T cell response to the allergen. However, this accompanied an apparently normal B cell/T cell-dependent IgE Ab response, suggesting that there is a systemic anti-OVA T cell response in these mice and either the level of IL-4 made in these mice is sufficient for efficient class switch or that another source of IL-4 exists that can regulate this response. The reduced T cell infiltration may be the result of reduced chemokine and/or chemokine receptor expression in these mice that drive T cell recruitment to the lung (49) or reduced adhesion of these cells in the lung. Indeed, ITK has been shown to be involved in TCR-induced adhesion via the β_1 integrins (34). Alternatively, the reduced production of IL-4 by ITK null T cells may underlie their lack of significant Ag-specific recruitment to the lung since IL-4 null T_H2 cells have been reported to be defective in this process (50). The production of IL-4 and chemokine and/or chemokine receptor expression for lung migration may be linked and future experiments will investigate these possibilities.

The reduced production of IL-5 is another factor that may underlie the reduced eosinophilic infiltration in the ITK null mice. IL-5 has been proposed as one of the major factors which regulate eosinophil infiltration in the lung during allergic asthma development (51). However, if the recruitment is hampered, then these cells are less likely to be able to contribute to the pathology of the disease. A similar situation exists for IL-13, which has been reported to be involved in the production of mucous by goblet cells lining the lung in allergic asthma (5). The ITK null T cells produced much less IL-13 than WT cells, and this coupled with the reduced T cell presence in the lung may underlie the almost complete lack of mucous production in these mice.

We found that IFN-γ and IL-10 production by ITK null T cells in the spleen, while also reduced, was less affected. In the lymph nodes, IFN-γ and IL-10 production was drastically reduced in response to Ag-specific stimulation. However, what may be important in these mice may not necessarily be the absolute amounts of cytokine being produced, as they can still mount a reasonable immune response (as witnessed by the production of OVA-specific IgE), but the ratio of these cytokines to each other. Indeed, although IFN-γ can be suppressive in the development of symptoms in this model, it can also cause increased lung inflammation although not mucous production (52). Viewed this way, ITK null T cells produce a higher ratio of IFN-γ:IL-4, IFN-γ:IL-5, and IFN-γ:IL-3, which may have a suppressive effect on the T_H2 cytokine-driven inflammation and accompanying eosinophil infiltration and mucous production. Similarly, the role of IL-10 in the development of allergic asthma has been controversial, with knockout studies suggesting that IL-10 can either enhance or suppress its development (53, 54). However, more recent studies have proposed a prominent role for IL-10 in suppressing the development of allergic asthma, although the source may be T regulatory cells (55, 56). One possibility is that the ratio of this cytokine in relation to the others being produced may be a critical factor in regulating the symptoms seen in this disease.

One seemingly paradoxical finding is the increased levels of serum IgE found in the ITK null mice. Although IL-4 is critical for B cells to class switch to IgE production (57) and the ITK null mice produce less IL-4 than WT mice, they produce almost normal levels of IL-10 (at least when assayed from splenic T cells). In human cells, IL-10 has been reported to inhibit IL-4-mediated B cell class switch to IgE; however, IL-10 can also increase IgE production by IL-4 and IL-4/CD40-stimulated B cells that have already class switched to IgE (44). Thus, ITK null mice may have elevated levels of IgE due to an increase IL-10:IL-4 ratio produced by ITK null T cells, leading perhaps to reduced class switch to IgE by B cells, but increased production of IgE by those cells that do undergo class switch. Alternatively, other cell types such as NK or NK-T cells, mast cells, dendritic cells, or eosinophils may be able to produce high levels of IL-4 in ITK null mice, leading to increased IgE production.

Fowell et al (37) have recently shown that T cells from BALB/c mice lacking ITK are defective in the production of IL-4 and in the typical T_H2-type immune response to *Leishmania*. They also demonstrated that even in the presence of exogenous IL-4, ITK null T cells could not secrete IL-4 in vitro, suggesting either that ITK regulates the ability of these T cells to respond to IL-4 and induce the necessary chromatin changes for IL-4 production or that ITK itself regulates those changes (37). Schaeffer et al. (35) however reported slightly different results, that T cells from C57BL/6 mice lacking ITK as well as those lacking both ITK and Rlk/Txk have an overall reduction in T_H1 or T_H2 cytokine production after T_H1 or T_H2 condition skewing in vitro. However, in a schistosome egg challenge model, mice lacking ITK continued to exhibit a T_H2-type defect along with reduced Ag-specific T_H2 cytokine production in vitro, whereas those lacking both ITK and Rlk/Txk had an apparently normal T_H2-type response in vivo and cytokine production in vitro (35). Thus, the in vitro and the in vivo responses differed in the latter animals; however, the in vivo results suggest perhaps that the absence of both ITK and Rlk/Txk results in a default T_H2 pathway in vivo for the production of these cytokines (35). The differences observed by these investigators may be the result of the different genetic backgrounds used for their studies. However, although the in vitro differentiation may have led to slightly different results, in vivo, both models point to a defect in either T_H2 cell differentiation or T_H2 cytokine production in mice lacking ITK.

A role for ITK in regulating other transcription factors has also been recently demonstrated by Miller and Berg (58), who recently showed that ITK null T cells are defective in Fas ligand expression due to defective up-regulation of transcription factors of the early growth response family (58). These transcription factors are regulated by NFAT (48). Thus, the previously published work along with our data suggest that T cells lacking ITK may not only be defective in T_H2 cell differentiation and/or cytokine production, but may also be defective in the expression of molecules regulating T cell migration to the lung. Taken together, these results suggest

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that the tyrosine kinase ITK may represent a good target for developing drugs to treat allergic asthma.

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