

Reduced airway hyperresponsiveness and tracheal responses during allergic asthma in mice lacking tyrosine kinase inducible T-cell kinase

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Background: Patients with allergic asthma have symptoms of a predominant T_H2 response, including airway eosinophilic inflammation and increased mucous production in the lungs. This accompanies increased airways responsiveness, which can be life threatening. Because T_H2 cells and cytokines have been implicated in contributing to these symptoms, pathways that control the development of these cells or that regulate their cytokine production represent good targets for controlling this disease.

Objective: We have previously shown that mice lacking the tyrosine kinase inducible T-cell kinase (ITK) have drastically reduced airway inflammation in a model of allergic asthma. However, it was not clear whether this translated into reduced airways hyperresponsiveness. We have analyzed tracheal responsiveness and airways hyperresponsiveness of wild-type (WT) and ITK null mice during induction of experimental allergic asthma.

Methods: Experimental allergic asthma was induced in WT and ITK knockout mice. Tracheal responses to carbachol, acetylcholine, and potassium chloride were analyzed. Airways hyperresponsiveness to methacholine challenge was also analyzed in allergen-challenged mice, along with lung and bronchoalveolar lavage fluid T_H2 cytokine message and protein.

Results: ITK null mice have reduced tracheal responses to cholinergic challenge *in vitro* before as well as after allergen challenge. These mice also have reduced airways hyperresponsiveness in response to allergen challenge, which could be rescued by transferring WT splenocytes or purified WT CD4⁺ T cells. This reduced airways response was

preferentially accompanied by reduced expression of T_H2 cytokines in the lungs.

Conclusion: Our results indicate that the tyrosine kinase ITK and its function in T cells represent an attractive target for antiasthmatic drugs.

Clinical implications: Modulating the expression or activity of ITK may be a novel strategy to block allergic airway inflammation. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Airways hyperresponsiveness, tracheal responses, T_H2 cells, cytokines, asthma, smooth muscle contractility

The prevalence of asthma has steadily increased, resulting in morbidity and in some cases mortality because of symptoms of this disease. Current views of this disease hold that the T-cell response is critical for its development—in particular, the development of allergen-specific T_H2 cells and their resultant cytokine production, IL-4, IL-5, and IL-13, which have effects on cells of the lung. These effects include smooth muscle proliferation, goblet cell hyperplasia, and mucous production, which usually accompany the development of airways hyperresponsiveness (AHR).^{1,2} Cytokines secreted by T_H2 cells seem to play a critical role in the development of AHR because transfer of allergen-specific T_H2 cells alone leads to AHR in mice.³ In addition, IL-4 or IL-13 alone can induce AHR in mice, and blocking these cytokines prevents the development of AHR in mice and human beings.^{4,7} Current treatments for asthma include bronchodilators, short-acting and long-acting β -agonists, anticholinergics, corticosteroids, antileukotrienes, antihistamines, and anti-IgE mAb.⁸

Targeting the activation and/or development of T_H2 cells has the potential to lead to more effective therapies for treating subjects with asthma. GATA binding protein 3 (GATA3) is a master regulatory transcription factor for T_H2 cytokines, whereas T_H1 specific T-box transcription factor (T-bet) is a master regulatory transcription factor for regulating T_H1 cytokines.⁹ Dominant-negative forms of GATA3 can inhibit airway inflammation observed in murine experimental allergic asthma, whereas mice lacking T-bet spontaneously develop immunological symptoms of asthma, and children with asthma carrying a particular allele of T-bet have improved responses to

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Abbreviations used

AHR: Airways hyperresponsiveness
 GATA3: GATA binding protein 3
 ITK: Inducible T-cell kinase
 Penh: Enhanced pause
 T-bet: T_H1 specific T-box transcription factor
 WT: Wild type

corticosteroids.¹⁰⁻¹⁴ Early signals that control T-cell activation are promising targets, and we have previously shown that the nonreceptor Tec family tyrosine kinase inducible T-cell kinase (ITK) is involved in regulating the development of airway inflammation in murine experimental allergic asthma.¹⁵ We have also shown that tracheal responses in nonmanipulated ITK null mice is reduced compared with wild-type (WT) mice.¹⁶ In this report, we show that ITK null mice exhibit reduced tracheal response both before and after airway allergen challenges. We also show that ITK null mice exhibit reduced AHR in response to airway allergen challenge, accompanied by reduced expression of T_H2 cytokines in the lung. The reduced AHR observed could be rescued by transfer of antigen-specific WT CD4⁺ T cells. Together, these data indicate that pharmaceutical targeting of ITK may be efficacious in treating asthma.

METHODS

Chemicals and other reagents

Unless otherwise noted, all reagents were from Sigma (St Louis, Mo). The CD4⁺ T-cell isolation kit was from Miltenyi Biotec GmbH (Auburn, Calif). Ovalbumin was from JT Baker (Phillipsburg, NJ) and Trizol from Invitrogen (Carlsbad, Calif).

Mice

C57BL/6 mice (WT and ITK null¹⁷) were used between 6 and 8 weeks old for these experiments, which were approved by the Office of Research Protection's Institutional Animal Care and Use Committee at Pennsylvania State University.

Induction of experimental allergic asthma

Mice were sensitized with ovalbumin as detailed in this article's [Methods](#) section in the Online Repository at www.jacionline.org.¹⁵ Twenty-four hours after the final intranasal exposure, mice were analyzed for AHR in response to methacholine as detailed below, killed at least 1 hour later, and trachea and lungs processed as detailed below, or for histology as previously described.¹⁵

Analysis of tracheal responses

Tracheae were isolated as detailed in this article's [Methods](#) section in the Online Repository at www.jacionline.org¹⁶ from mice immunized and challenged as described. Isolated tracheae were placed in modified K-H solution (see this article's [Methods](#) section in the Online Repository at www.jacionline.org) and used for experiments.¹⁶ Isometric force measurements were used to determine cumulative concentration-response curves in the tracheal smooth muscles to carbachol (10^{-9} to 10^{-3} mol/L), acetylcholine (10^{-9} to 10^{-3} mol/L), or KCl (10^{-3} to 10^{-1} mol/L). The results are expressed

as isometric tension developed per gram-wet weight of tissue (g/g; see this article's [Methods](#) section in the Online Repository at www.jacionline.org¹⁶).

Adoptive transfer of antigen-specific cell populations

Adoptive transfer of ovalbumin-specific cells was performed as detailed in this article's [Methods](#) section in the Online Repository at www.jacionline.org. In brief, WT or ITK null mice were immunized with ovalbumin/aluminum hydroxide and splenocytes isolated and cultured at 5×10^6 /mL with 2 mg/mL ovalbumin for 72 hours. Cells were then isolated and whole splenocytes, CD4⁺, or CD4⁺ cells (1×10^7 cells) transferred to naive WT or ITK null mice. Forty-eight hours after transfer, the mice were challenged intranasally 4× as described and AHR analyzed 24 hours after the last challenge. CD4⁺ cells were isolated by using a kit from Miltenyi Biotec.

Analysis of AHR

Airways hyperresponsiveness was analyzed by using a single chamber Buxco whole-body plethysmograph model PLY3211 coupled to a MAX1320 modular accessory unit and PLY1040 bias flow regulator, and data handled by using the BioSystem XA software for Windows (Wilmington, NC). Twenty-four hours after the final ovalbumin challenge, mice were analyzed for responses to PBS or escalating doses of aerosolized methacholine from 3.25 mg/mL to 25 mg/mL. Data were collected by using Drorbaugh and Fenn calculations for 5 minutes, logging a line of data every 25 good breaths, and enhanced pause (Penh) values plotted versus methacholine concentration.

Quantitative RT-PCR analysis

Lungs were isolated from WT and ITK null mice after analysis of AHR and homogenized in Trizol for isolation of RNA. Total RNA (5 µg) was used to generate cDNA with a kit from Amersham Biosciences (Piscataway, NJ). Quantitative RT-PCR was then performed by using an ABI 7300 Sequence Detection System in the Nucleic Acids Facility of the Huck Institutes for Life Sciences at Penn State University using specific primers for the following genes: IL-4, IL-13, IFN-γ, GATA3, T-bet, and GAPDH as a housekeeping gene. Data analysis is described in this article's [Methods](#) section in the Online Repository at www.jacionline.org.

ELISA

Analysis of IL-4, IL-13, and IFN-γ protein expression was performed by ELISA using kits from R&D Systems (IL-13; Minneapolis, Minn), or using a Multiplex Fluorescent Bead Immunoassay kit (IL-4 and IFN-γ; kind gift of Bender MedSystems, Vienna, Austria; see this article's [Methods](#) section in the Online Repository at www.jacionline.org).

Data analysis

Statistical evaluation was conducted by using the Student *t* test or ANOVA where appropriate, with a probability value of $P \leq .05$ considered statistically significant.

RESULTS

Inducible T-cell kinase null mice exhibit reduced AHR during allergic asthma induction. By using a murine model of allergic asthma, we have previously shown that mice lacking ITK exhibit reduced airway inflammation, airway

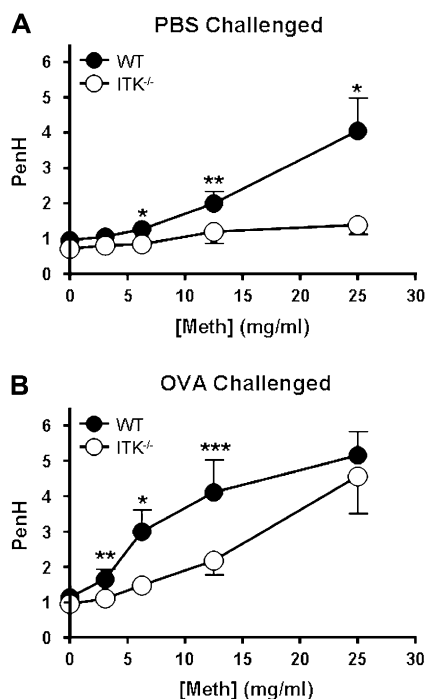


FIG 1. Reduced basal and ovalbumin (OVA) induced AHR in mice lacking ITK after induction of allergic asthma. AHR of WT and ITK^{-/-} mice immunized with OVA, then exposed intranasally to (A) PBS, * $P < .05$, Student t test, WT ($n = 8$), ITK^{-/-} ($n = 6$); or (B) OVA, * $P < .05$, ** $P < .07$, *** $P < .08$, Student t test, $n = 6$. Filled circles, WT mice; open circles, ITK^{-/-} mice; Meth, methacholine.

eosinophilia, and mucous production compared with WT mice.¹⁵ To determine whether this reduced immunological response correlated with reduced AHR, we performed experiments to analyze the response of WT and ITK null mice to methacholine challenge using a whole-body plethysmograph. Fig 1, A, demonstrates that control mice exhibit the expected changes in airway responses as a function of increased methacholine challenge. However, the mice lacking ITK exhibit significantly reduced airway responses compared with WT mice, in the absence of intranasal challenge with ovalbumin. By contrast, the response of the mice to PBS was not significantly different (see this article's Fig E1 in the Online Repository at www.jacionline.org). Similarly, when the mice were challenged with ovalbumin, WT mice exhibited increased responses, which were again significantly higher than those observed in ITK null mice (Fig 1, B). These data indicate that in addition to reduced immunological symptoms and airway inflammation in response to allergic asthma induction, mice lacking ITK also exhibited reduced AHR responses to allergen challenge.

Inducible T-cell kinase null mice exhibit reduced tracheal contractile responses to acetylcholine, carbachol, and KCl during allergic asthma induction. To determine if this reduced response observed in ITK null mice could also be observed in the isolated tracheae, we analyzed the contractile response of isolated tracheae from these mice to cholinergic stimulation using acetylcholine and

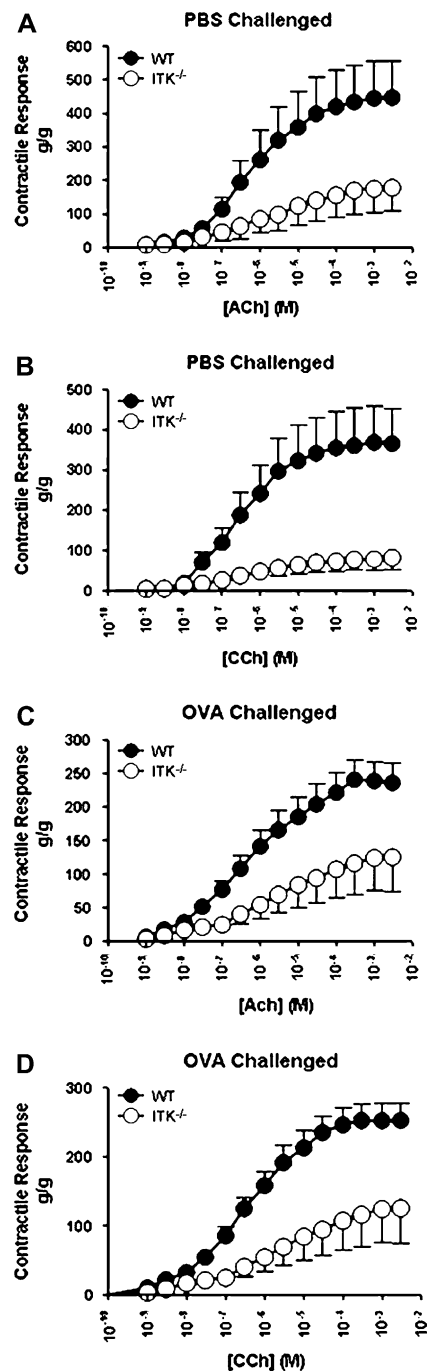


FIG 2. Reduced tracheal responses to cholinergic stimulation in mice lacking ITK. A, Tracheae from mice exposed to PBS intranasally stimulated with acetylcholine (ACh), WT ($n = 8$), ITK^{-/-} ($n = 5$). B, Carbachol (CCh), WT ($n = 8$), ITK^{-/-} ($n = 5$). C, Tracheae from mice exposed to ovalbumin intranasally stimulated with ACh ($n = 9$). D, CCh ($n = 9$). All graphs are $P < .05$ by 2-way ANOVA. Filled circles, WT mice; open circles, ITK^{-/-} mice.

carbarchol.¹⁶ Tracheae from WT and ITK null mice treated as described were isolated, and cumulative concentration-response curves to these agonists were generated from contractile measurements. Fig 2 demonstrates that similar to what was observed using the whole-body plethysmograph,

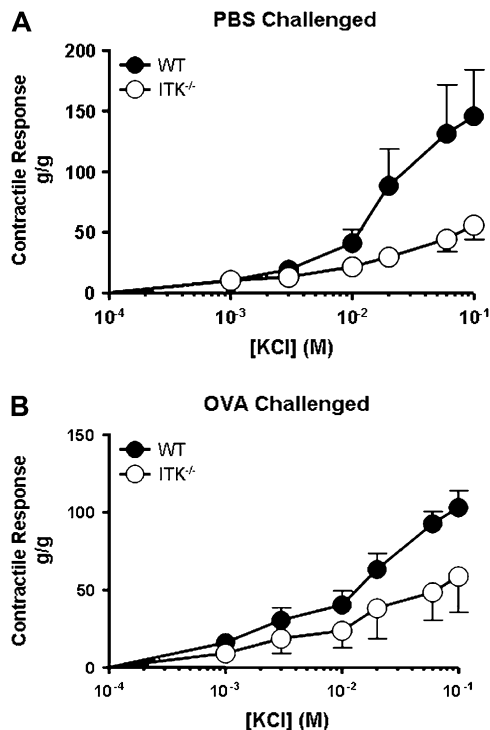


FIG 3. Reduced tracheal responses to KCl stimulation in mice lacking ITK. **A**, Tracheae from mice exposed to PBS intranasally, $n = 7$ for WT mice, $n = 6$ for ITK^{-/-} mice. **B**, Tracheae from mice exposed to ovalbumin (OVA) intranasally, $n = 10$ for WT and ITK^{-/-} mice. Both $P < .05$ by 2-way ANOVA. Filled circles, WT mice; open circles, ITK^{-/-} mice.

ITK null tracheae exhibited reduced contractile responses to stimulation with acetylcholine as well as carbachol compared with WT mice under conditions where they were challenged with PBS (Fig 2, *A and B*). However, although the effective concentration required for 50% increase (EC_{50}) values for the 2 mice and 2 agonists were similar (see this article's Table E1 in the Online Repository at www.jacionline.org), the contractile response at the effective concentration required for maximum increase (E_{max}) was significantly higher in WT tracheae than those from ITK null mice (Fig 2, *A and B*; see this article's Table E2 in the Online Repository at www.jacionline.org). The data indicate that under nonallergic asthma conditions—that is, control conditions—tracheae from mice lacking ITK exhibit significantly reduced responses to cholinergic stimulation compared with WT mice.

We next performed similar experiments to determine the tracheal responses to cholinergic stimulation from mice that had been induced to develop allergic asthma. Fig 2 demonstrates that similar to what was observed using the whole-body plethysmograph, tracheae from WT mice induced to develop allergic asthma exhibited significantly increased tracheal responses to acetylcholine and carbachol, with similar EC_{50} , but larger E_{max} than those from ITK null mice (see this article's Table E1 in the Online Repository at www.jacionline.org). However, the contractile response at the E_{max} was significantly

higher in WT tracheae than those from ITK null mice, excepting the carbachol stimulated trachea, which was higher in WT, but the difference did not reach statistical significance (Fig 2, *C and D*; see this article's Table E2 in the Online Repository at www.jacionline.org). To determine whether the responses we observed were specific to cholinergic stimulation, we used a depolarizing agent, KCl. Similar to what was observed in the whole-body plethysmograph in response to methacholine, as well as the *in vitro* response to acetylcholine and carbachol, WT tracheae stimulated with KCl had significantly greater contractile responses at E_{max} to KCl stimulation compared with ITK null mice (see this article's Table E1 and Table E2 in the Online Repository at www.jacionline.org). However, the EC_{50} values were similar, regardless of whether or not the mice had been induced to develop allergic asthma (Fig 3, *A and B*; see this article's Table E1 and Table E2 in the Online Repository at www.jacionline.org).

Thus, the absence of ITK results in reduced basal responses to methacholine as measured by whole-body plethysmography, as well as reduced responses to acetylcholine, carbachol, and KCl stimulation as measured using isolated tracheae from these mice. In addition, as previously observed for immunological symptoms and airway inflammation, in mice induced to develop allergic asthma, those lacking ITK exhibited reduced AHR responses to methacholine, as well as reduced tracheal responses to acetylcholine, carbachol, and KCl *in vitro*. We should note that ITK is not expressed in the trachea (data not shown), and thus, these responses may represent the effects of the immunological environment in the ITK null mice.

Reduced T_H2 cytokines in lungs of ITK null mice during allergic asthma induction

The observed reduced physiological responses as well as the previously observed reduced immunological symptoms and airway inflammation in ITK null mice compared with WT mice prompted us to examine the expression of cytokines that may be critical in controlling the physiological responses in these 2 mice. For these experiments, mRNA from the lungs of WT and ITK null mice that had been induced to develop allergic asthma were analyzed by quantitative RT-PCR for T_H1 and T_H2 cytokines shown to be important in regulating AHR, particularly IFN- γ , IL-4, and IL-13. In addition, because we had previously observed reduced T_H2 immunological responses in ITK null mice in this model, we also determined the expression of T-bet and GATA-3, 2 critical transcription factors for the induction of T_H1 and T_H2 responses, respectively. Our analyses indicate that lungs from ITK null mice had significantly reduced expression of the T_H2 cytokines IL-4 and IL-13, whereas IFN- γ expression was similar (Fig 4, *A*). We confirmed the differential expression of IL-4, IL-13, and IFN- γ from bronchoalveolar lavage fluid of the mice using ELISA or Fluorescent Bead Immunoassay (Fig 4, *B*). Interestingly, whereas expression of T-bet was not affected by the absence of ITK, GATA-3 was significantly reduced, suggesting that the difference in cytokine expression in the lungs of ITK

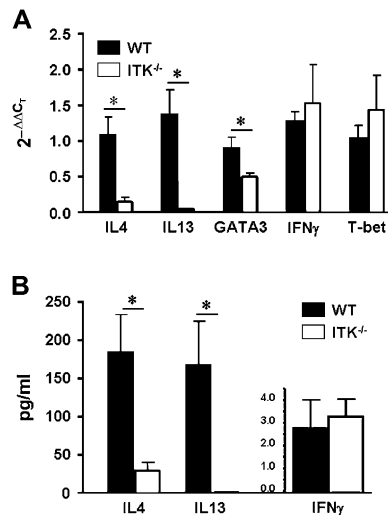


FIG 4. Reduced T_H2 cytokine message in lungs of mice lacking ITK during allergic asthma induction. **A**, Quantitative RT-PCR for the indicated genes from mRNA isolated from lungs of WT and ITK^{-/-} mice treated as in Fig 1, * $P < .05$ by Student t test, $n = 12$. **B**, ELISA for the indicated proteins. * $P < .05$ by Student t test, $n = 5$.

null mice may be a result of differential expression of these transcription factors (Fig 4, A).

Rescue of AHR responses in ITK null mice by transfer of antigen-specific WT CD4⁺ T cells

We have previously shown that ITK null T cells secrete less T_H2 cytokines during allergic asthma induction compared with WT T cells *in vitro*.¹⁵ To determine whether WT T cells could rescue AHR in ITK null mice, we transferred various populations of cells from ovalbumin-sensitized WT or ITK null mice into naive WT or ITK null mice, followed by intranasal challenges. In these experiments, WT or ITK null mice were sensitized with ovalbumin plus aluminum hydroxide, and the splenocytes were purified. These cells were then incubated *in vitro* with ovalbumin, washed, then directly transferred into naive ITK null mice. Fig 5, A, demonstrates that although transfer of splenocytes from sensitized ITK null mice into naive WT mice did not result in the development of AHR, transfer of splenocytes from sensitized WT mice into naive ITK null mice led to the development of AHR in the recipient mice. To determine whether WT CD4⁺ T cells could transfer AHR to naive ITK null mice, similar experiments were performed, except that we separated the splenocytes into CD4⁺ cells and CD4⁻ cells (including CD8⁺ T cells), followed by transfer into naive ITK null mice. Analysis of these recipient mice indicated that although transfer of the CD4⁻ population could not rescue the induction of AHR in naive ITK null mice, transfer of the CD4⁺ population was able to rescue AHR (Fig 5, B). As a control, transfer of splenocytes from sensitized ITK null mice could not transfer AHR to naive ITK null recipients (Fig 5, B). Analysis of histological sections indicated that these transferred T cells (and splenocytes; data not shown) also lead to increased airway inflammation in the naive ITK null

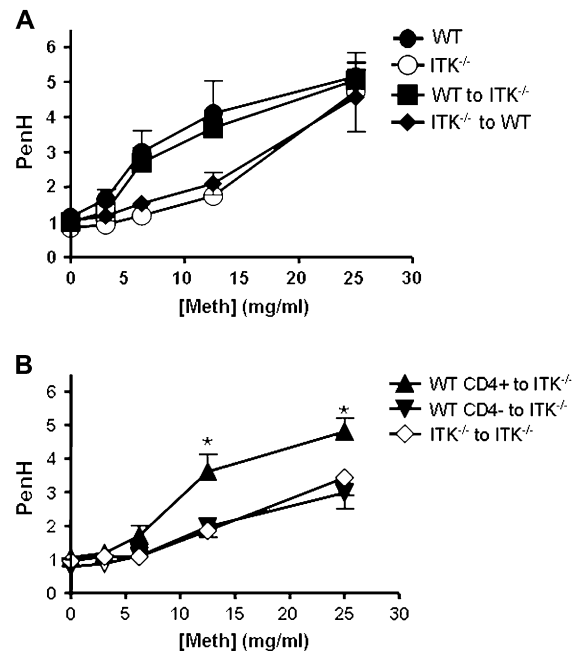


FIG 5. Rescue of AHR in mice lacking ITK by transfer of CD4⁺ T cells. **A**, (○-○), Naive ITK^{-/-} mice ($n = 9$); (◆-◆), naive WT recipients/ITK^{-/-} ovalbumin splenocytes ($n = 8$); (■-■), naive ITK^{-/-} recipients/WT ovalbumin splenocytes ($n = 12$). Data for ovalbumin-challenged WT strain from Fig 1 is included for ease of comparison (●-●). **B**, (◇-◇), Naive ITK^{-/-} recipients/ITK^{-/-} ovalbumin splenocytes ($n = 12$); (▼-▼), naive ITK^{-/-} recipients/WT ovalbumin CD4⁻ cells ($n = 13$); (▲-▲), naive ITK^{-/-} recipients/WT ovalbumin CD4⁺ T cells ($n = 8$). * $P < .05$, 2-way ANOVA. Meth, methacholine.

lungs after ovalbumin challenge (Fig 6). Altogether, these data suggest that ITK null mice have reduced airways response to allergic asthma induction, which could be rescued by transfer of WT CD4⁺ cells. They also indicate that ITK null tracheae have reduced responses to cholinergic stimulation in the absence or presence of conditions that lead to allergic asthma.

DISCUSSION

The Tec family kinase ITK is expressed in T cells and has been shown to regulate the production of several T-cell-derived cytokines, in particular IL-2 and the T_H2 cytokines IL-4, IL-5, and IL-13.^{15,18-20} Although the lack of ITK also affects T-cell production of IFN- γ , this effect does not seem as significant as that seen for IL-4.^{15,19,20} T_H2 cytokines have been demonstrated to be important for the development of allergic asthma.^{1,21-24} We demonstrate in this report that the lungs of ITK null mice have preferential reduced expression of T_H2 cytokines, along with reduced AHR during allergic asthma induction. We also demonstrate that the isolated tracheae from ITK null mice have reduced contractile response to both cholinergic agonists and depolarizing agents. This difference is observed both in control PBS-challenged mice as well as ovalbumin-challenged mice, suggesting

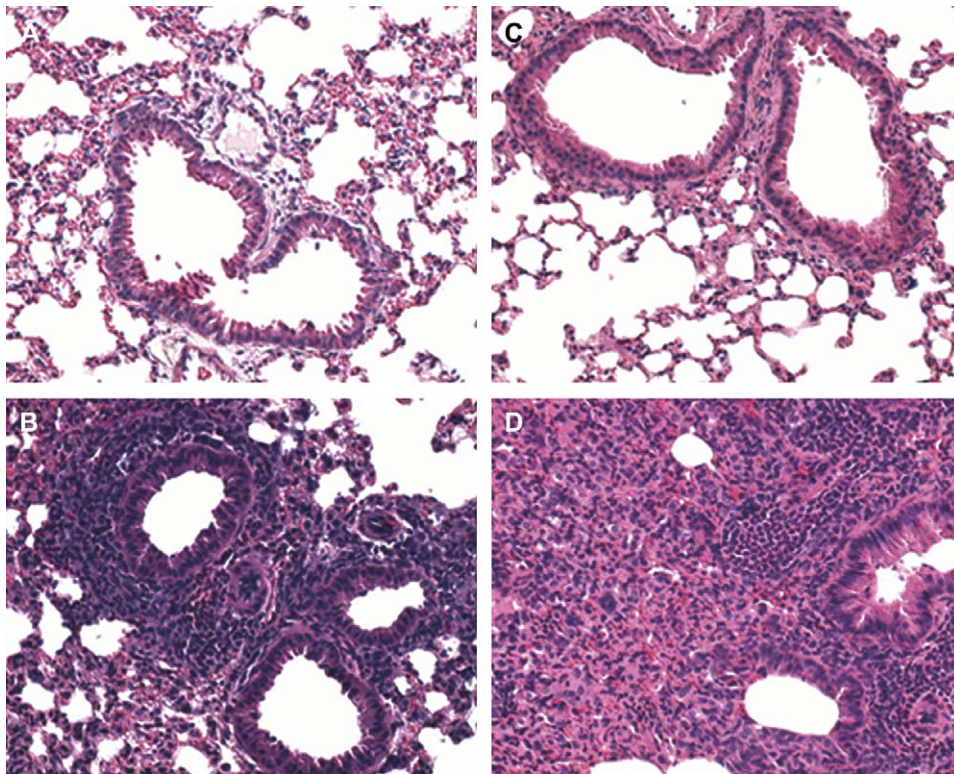


FIG 6. Rescue of airway inflammation in mice lacking ITK by transfer of CD4⁺ T cells. Lung sections from mice treated as in Fig 5 stained with hematoxylin and eosin for inflammation. **A**, Naive ITK^{-/-} mice. **B**, Naive ITK^{-/-} recipients/WT ovalbumin splenocytes. **C**, Naive ITK^{-/-} recipients/WT ovalbumin CD4⁻ cells. **D**, Naive ITK^{-/-} recipients/WT ovalbumin CD4⁺ T cells. All images were taken at 20 $\times$.

that ITK may indirectly regulate the tone of the tracheal responses in this mice, resulting in reduced AHR to allergic asthma induction.

Our finding of reduced tracheal responses to acetylcholine, carbachol, and KCl in the trachea from ITK null mice in the absence of challenge was surprising. This difference was not in the potency of the stimulating agents, but in their efficacy, suggesting that smooth muscle cells from ITK null mice exhibit altered responses to stimulation. Because ITK is not expressed in smooth muscle cells or other cells in the trachea (data not shown), our data would suggest that ITK may control the tone of the smooth muscle cells that line the trachea via mediators such as cytokines made by T cells and regulated by ITK. Indeed, IL-13 can affect the contractile response of smooth muscle cells, and we observed reduced IL-13 message and protein in lungs from ITK null mice.²⁴ We have also shown that ITK null T cells secrete reduced levels of IL-13 on rechallenge after immunization with ovalbumin.¹⁵ We speculate that reduced exposure to such cytokines may alter the response of ITK null trachea, and perhaps lungs, resulting in reduced responses on stimulation.²⁵ The same situation may apply under conditions of allergic asthma, in which WT mice are exposed to large amounts of these cytokines, whereas ITK null mice may not be, leading to altered AHR and tracheal responses. This view is supported by the fact that we can rescue the development of AHR in naive ITK

null mice by transfer of CD4⁺ T cells from sensitized WT mice and suggests that these cells and their cytokines are critical for the development of AHR in ITK null mice, similar to what has been reported by other investigators in WT mice.³ Thus, cytokines such as IL-5 and IL-13 alone can induce AHR if recombinant proteins are delivered to the lungs of WT mice, or if mice carry a transgene for these cytokines, and there is interplay among these cytokines in regulating AHR.^{4,22,26} These cytokines can then act on the smooth muscle cells of the lungs to induce AHR. Of interest is the fact that children with asthma were found to have better responses to inhaled corticosteroids if they carried a particular allele of T-bet.¹⁴ Transfer of WT CD4⁺ cells into ITK null mice may recover the production of these critical cytokines resulting in recovery of AHR in the ITK null mice.

The observed reduction in T_H2 cytokines in the lungs of ITK null mice may be the result of reduced T-cell differentiation to the T_H2 lineage, because although we observed little difference in the expression of T-bet, there was a significant reduction in expression of GATA3. Recently, data supporting a significant role for GATA3 in the development and maintenance of T_H2 cells were reported, and it is possible that mice lacking ITK have reduced GATA3 expression, and thus reduced T_H2 cytokine expression.²⁷⁻³⁰ Other investigators have also suggested that ITK may regulate either the expression

of T-bet or its tyrosine phosphorylation in T_H2 cells, therefore regulating T_H2 cytokine production.^{19,31} Our data would suggest that in the absence of ITK, the expression of T-bet is not affected in lung, which could be the result of other cells expressing this factors, or lack of an effect of ITK on the expression of this transcription factor.

Another role for ITK in regulating tracheal responses could be in regulating mast cell function via regulating signaling via the high-affinity Fc receptor for IgE on mast cells (FcεR). Mast cells can be found interacting with smooth muscle cells in the trachea and lungs, and ITK is expressed in mast cells.^{32,33} Indeed, ITK null mast cells have recently been reported to have some defects in degranulation.³⁴ Thus, ITK null mast cells may release reduced amounts of pharmacological agents that could affect smooth muscle function in the airways of these mice.³⁵

These results, taken together with our previously published work and that of others, suggest that inhibitors to the tyrosine kinase ITK being developed may represent a good target for developing drugs to treat allergic asthma, a view supported by recent efforts in the area in which inhibitors of ITK have been shown to be efficacious in a murine model of allergic inflammation.³⁶

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