

The Development of Airway Hyperreactivity in T-bet-Deficient Mice Requires CD1d-Restricted NKT Cells¹

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T-bet^{-/-} mice have been shown to have a profound deficiency in the ability to generate invariant NKT (iNKT) cells in the periphery due to a halt in terminal maturation, but despite this deficiency, T-bet^{-/-} mice develop spontaneous airway hyperreactivity (AHR) and airway inflammation. Because in some situations the development of AHR requires the presence of iNKT cells, we sought to more clearly understand how AHR develops in T-bet^{-/-} mice by examining T-bet^{-/-} mice in several distinct mouse models of asthma, including spontaneous, OVA-induced and α -galactosylceramide (α -GalCer)-induced AHR. Surprisingly, we found that administration of α -GalCer, which very specifically activates iNKT cells, greatly increased the AHR response in the T-bet^{-/-} mice. Moreover, in T-bet^{-/-} mice, spontaneous AHR as well as AHR induced with OVA or α -GalCer were all eliminated by blocking CD1d, the restricting element of iNKT cells, using an anti-CD1d-blocking mAb. Although the number of the iNKT cells in T-bet^{-/-} mice was reduced compared with that in wild-type mice, the remaining iNKT cells produced primarily IL-4 and IL-13, and only minimal amounts of IFN- γ . We conclude therefore that the AHR that develops in T-bet^{-/-} mice is dependent on the presence of iNKT cells, and that whereas T-bet^{-/-} have reduced numbers of iNKT cells, these are sufficient for the development of AHR. *The Journal of Immunology*, 2009, 182: 3252–3261.

Asthma is an immunological disease that includes multiple inflammatory and clinical phenotypes, characterized by airway inflammation and airway hyperreactivity (AHR),³ and by symptoms of recurrent wheezing, coughing, and shortness of breath (1). Clinically, asthma is heterogeneous and includes several different forms (e.g., associated with allergy, infection, air pollution, or exercise), which depend on distinct cell types and pathways (e.g., eosinophils, lymphocytes, or neutrophils). In allergic asthma, the most common form of asthma, the airway inflammation is caused by Th2-driven inflammatory responses, which enhance eosinophilia, AHR, and Ag-specific IgE production (2, 3). However, a shared disease mechanism for all forms of asthma has not been established.

Invariant NKT (iNKT) cells are required for the development of AHR in several mouse models of asthma (4–6). iNKT cells comprise a unique subset of lymphocytes that express features of both classical T cells and NK cells. iNKT cells express a conserved or invariant TCR, which recognizes glycolipid Ags such as α -galac-

tosylceramide (α -GalCer) presented by CD1d. Upon stimulation, iNKT cells produce large amounts of IL-4, IL-13, and IFN- γ , which play critical roles in the regulation of immune responses (7–9). In animal models of asthma, AHR induced either by exposure to allergen, by exposure to ozone, or with virus, all failed to develop in NKT cell-deficient CD1d^{-/-} mice, which lack the restricting element of iNKT cells, and in α 18^{-/-} mice, which lack the α -chain of the invariant TCR (4–6). However, adoptive transfer of wild-type (WT) iNKT cells reconstituted the capacity of α 18^{-/-} mice to develop AHR (4, 5). Furthermore, iNKT cells can directly induce the development of AHR, because respiratory administration of glycolipids such as α -GalCer or glycolipids from *Sphingomonas* bacteria activated iNKT cells and caused AHR and airway inflammation, even in the complete absence of CD4⁺ T cells and adaptive immunity (10). Moreover, distinct subsets of iNKT cells are required in the different models of asthma. For example, CD4⁺ iNKT cells producing both IL-13 and IL-4 were involved in allergen-induced AHR (4); NK1.1⁺ iNKT cells producing IL-17 were required for ozone-induced AHR (5); and CD4⁺ iNKT cells producing IL-13 were involved in virus-induced AHR (6). Nevertheless, in all of these different models of asthma, iNKT cells appear to represent a common unifying element that is required for the development of AHR.

T-bet^{-/-} mice, which have a significant reduction in iNKT cells, develop AHR spontaneously (11), suggesting that AHR might develop in these mice independent of iNKT cells. T-bet is a member of the T-box family of transcription factors that is expressed in Th1, but not in Th2 cells (12). T-bet deficiency causes a defect in the development of the Th1 subset, with a significant reduction of IFN- γ , but a significant increase in IL-4, production (13). Recently, it has been reported that iNKT cells require the transcription factor T-bet for development, and that T-bet^{-/-} mice have a profound deficiency in the ability to generate iNKT cells due to a halt in the terminal maturation of iNKT cells (14). Although TNF- α , IL-4, and IL-13 levels were increased in the lungs of T-bet^{-/-} mice, the precise pathogenic mechanisms by which

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³ Abbreviations used in this paper: AHR, airway hyperreactivity; α -GalCer, α -galactosylceramide; BAL, bronchoalveolar lavage; iNKT, invariant NKT; WT, wild type.

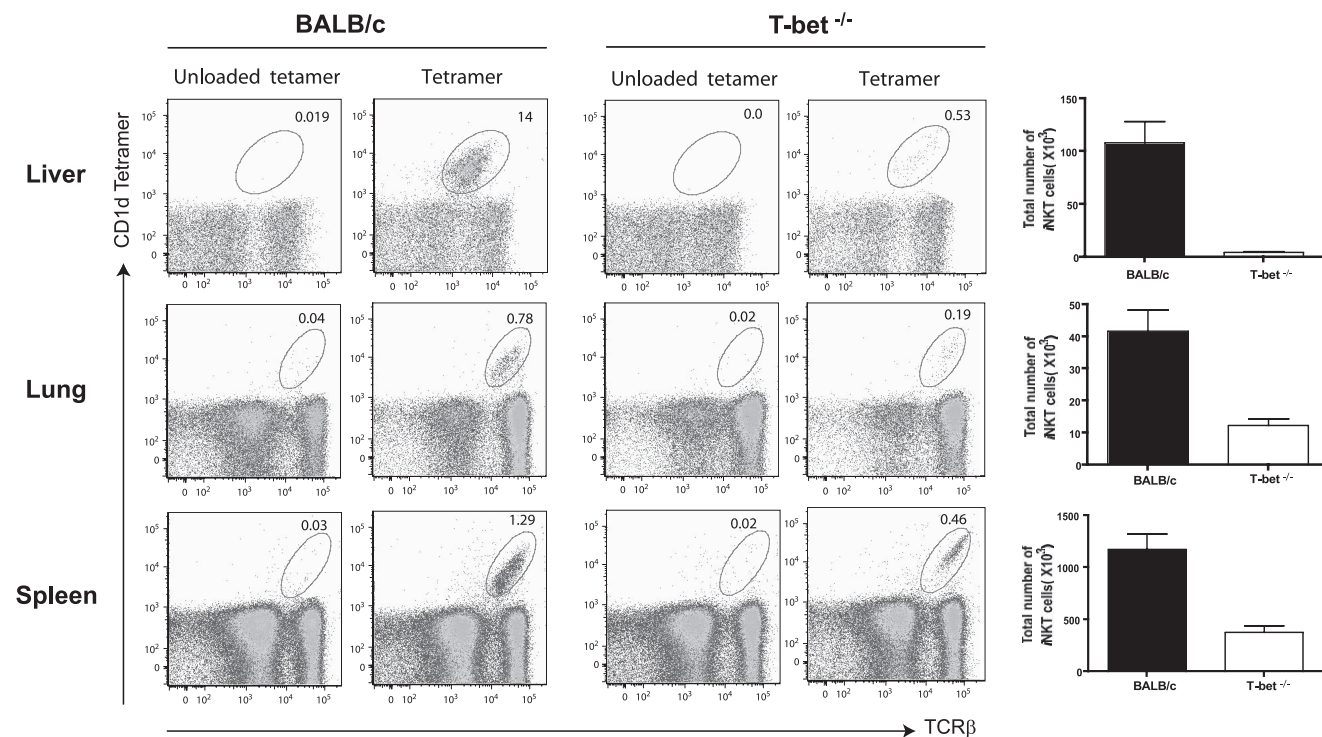


FIGURE 1. Peripheral population of iNKT cells in T-bet^{-/-} mice. T-bet^{-/-} mice have reduced numbers of iNKT cells. Cells were isolated from liver, spleen, and lung tissues of WT BALB/c and T-bet^{-/-} mice. iNKT cells were identified by first gating on anti-CD45 mAb-positive cells, then gating on lymphocytes. In the dot plots, the iNKT cells are indicated by the red circle (double-positive cells, stained with CD1d tetramers loaded with PBS57 and with TCRβ). The numbers of iNKT cells are shown as a percentage of total lymphocytes. The graphs are presented as a number of iNKT cells in each tissue in WT BALB/c and T-bet^{-/-} mice. These data are representative of at least three experiments.

AHR develops in T-bet^{-/-} mice, and whether iNKT cells are involved in this process, are not fully understood.

To better understand the role of iNKT cells in the development of AHR, particularly in T-bet^{-/-} mice, we examined T-bet^{-/-} mice in several distinct mouse models of asthma, including spontaneous AHR, OVA-induced AHR, and α-GalCer-induced AHR. Surprisingly, administration of α-GalCer, which specifically activates iNKT cells, greatly increased the AHR response in the T-bet^{-/-} mice. Moreover, T-bet^{-/-} mice sensitized and challenged with OVA developed severe AHR. Importantly, treatment of the T-bet^{-/-} mice with a blocking anti-CD1d mAb, which blocks the activation of iNKT cells, abolished spontaneous AHR, as well as the AHR responses induced by both OVA and α-GalCer. Although T-bet^{-/-} mice have reduced numbers of iNKT cells, the remaining iNKT cells produced significant amounts of Th2 cytokines (IL-4 and IL-13), but not IFN-γ. Thus, we conclude that T-bet^{-/-} mice develop AHR that requires the presence of iNKT cells, and that the reduced number of iNKT cells in T-bet^{-/-} mice is sufficient for the development of AHR.

Materials and Methods

Mice

Wild-type BALB/c ByJ mice and C.129S6-Tbx21^{tm1Glm}/J (T-bet^{-/-}) mice on the BALB/c background were purchased from The Jackson Laboratory. Jα18^{-/-} mice on the BALB/c background were generated by M. Taniguchi and T. Nakayama (Chiba University, Chiba, Japan) (15), and were bred in our animal facility. Age- and sex-matched animals were used between 7 and 8 wk of age. For some experiments (e.g., examination of spontaneous AHR in T-bet^{-/-} mice), 12- to 14-wk-old mice were used. The Animal Care and Use Committee, Children's Hospital Boston, approved all animal protocols.

Induction of AHR with α-GalCer

To induce AHR, mice were sensitized with 0.5 μg of α-GalCer intranasally. AHR was measured 24 h after α-GalCer challenge. α-GalCer was

synthesized by P. Savage (Brigham Young University, Provo, UT). For the CD1d-blocking experiments, 500 μg of anti-CD1d mAb (HB323; American Type Culture Collection) or control IgG1 mAb was injected i.p. 1 day before α-GalCer challenge. AHR was assessed by invasive measurement of airway resistance, in anesthetized, tracheostomized, and mechanically ventilated mice, using a previously described method (4).

Induction of AHR with OVA

To induce AHR, mice were sensitized i.p. with 100 μg of OVA (Sigma-Aldrich) in alum (2 mg). After 7 days, mice were exposed to intranasal Ag (50 μg of OVA per day) or PBS on 3 consecutive days, as described (4). For the CD1d-blocking experiments, 500 μg of anti-CD1d mAb (HB323) or control IgG1 Ab was injected 1 day before the start of sensitization and 1 day before the first intranasal OVA challenge.

Blocking spontaneous AHR

To block spontaneous AHR, 500 μg of anti-CD1d mAb (HB323) was injected i.p. once per week for 5 wk, starting at 8 wk of age. AHR was assessed by invasive measurement of airway resistance when the mice were 12 and 14 wk of age.

Adoptive transfer

iNKT cells were positively selected from spleens of WT BALB/c or T-bet^{-/-} mice using PE-conjugated PBS57-loaded CD1d tetramers (National Institutes of Health, National Institute of Allergy and Infectious Diseases MHC tetramer core facility) and anti-PE beads. iNKT cells were sorted with AutoMACS, according to the manufacturer's instructions. Purified iNKT cells (3×10^6) were adoptively transferred i.v. into Jα18^{-/-} mice 1 day before administration of α-GalCer.

Collection and measurement of cells in bronchoalveolar lavage (BAL) fluid

Mouse tracheae were cannulated, and the lungs were lavaged twice with 0.5 ml of 2% FCS in PBS and pooled, as described (4). For some experiments, BAL fluid cells were stained and analyzed by flow cytometry. The relative number of different types of leukocyte was determined by assessing cell morphology on slide preparations of BAL fluid cells stained with Diff-Quik solution (Dade Behring).

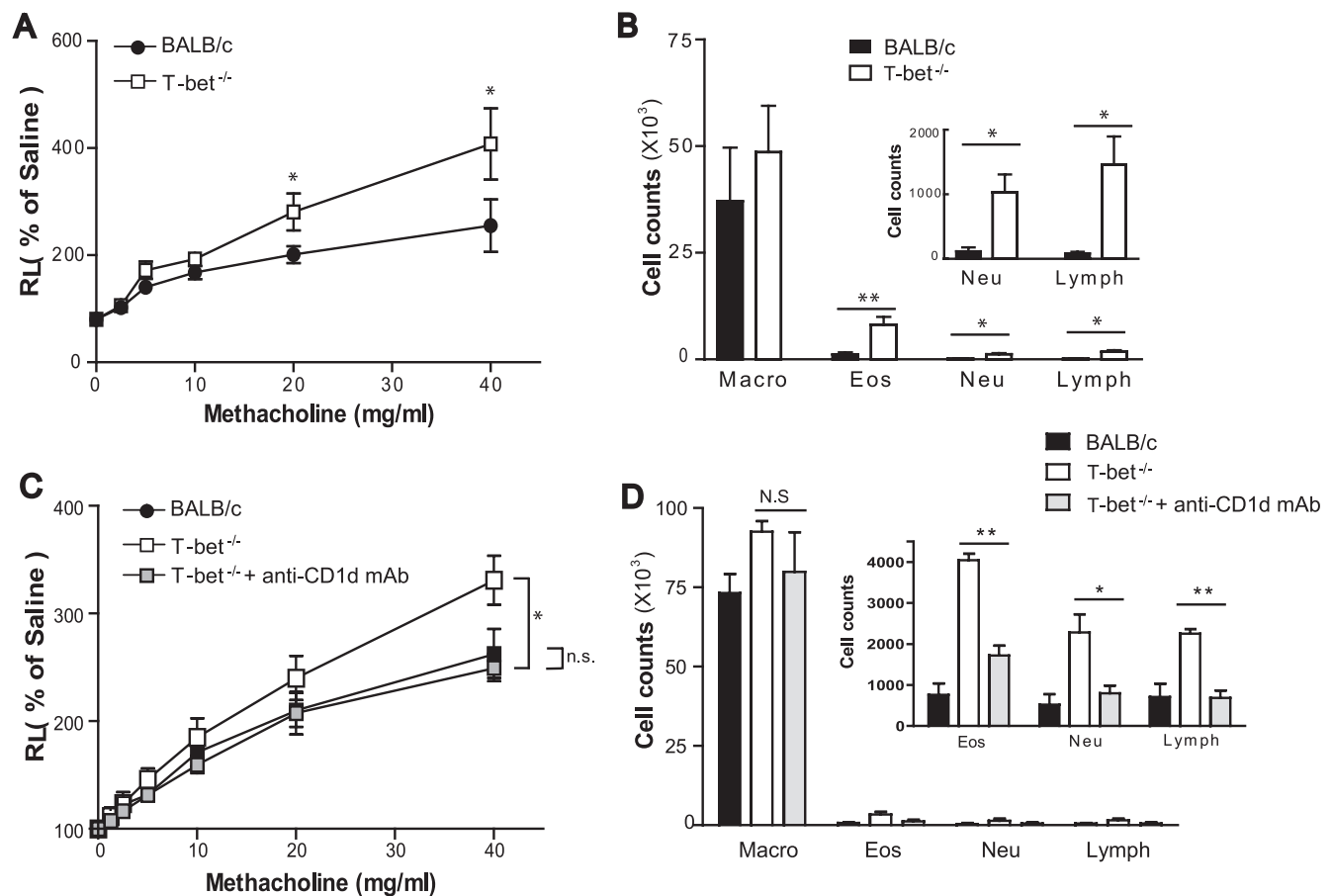


FIGURE 2. Development of spontaneous AHR in T-bet^{-/-} mice. *i*NKT cells in T-bet^{-/-} mice induce spontaneous AHR. Twelve- to 14-wk-old WT BALB/c mice and T-bet^{-/-} mice were used for this experiment. **A**, Changes in lung resistance (R_L) in response to methacholine were measured in anesthetized, tracheotomized, intubated, and mechanically ventilated mice. These data show the mean $\pm$ SEM percentage of saline value and are representative of three experiments ($n \geq 3$). $p \leq 0.05$ (*), T-bet^{-/-} mice were compared with WT BALB/c mice. **B**, BAL fluid from mice was collected immediately after AHR measurement. Data represent the number of cells/ml in BAL fluid. Macro, macrophages; Neu, neutrophils; Eos, eosinophils; Lymph, lymphocytes. The *inset* is a magnification of the data for neutrophils and lymphocytes. $p \leq 0.05$ (*) and $p \leq 0.01$ (**). **C**, Treatment with anti-CD1d-blocking mAb blocked the development of spontaneous AHR in T-bet^{-/-} mice. WT BALB/c and T-bet^{-/-} mice were treated with the HB323, anti-CD1d mAb, once per week for 5 wk, and AHR was measured as in **A**. **D**, Number of cells in BAL fluid is counted as **B**. Macro, macrophage; Neu, neutrophils; Eos, eosinophils; Lymph, lymphocytes. The *inset* is a magnification of the data for neutrophils and lymphocytes. $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

Lung tissue

Cell suspensions of lung tissue were prepared by first perfusing lungs with 2% FCS in PBS. The lung tissues were then diced and incubated in 10 ml of RPMI 1640 medium with 0.1% DNase I (fraction IX; Sigma-Aldrich) and 1.6 mg/ml collagenase (CSL4; Worthington Biochemical) at 37°C incubator for 60 min.

Flow cytometry

Hematopoietic cells were identified by gating on cells stained with anti-mouse PE-Texas Red-conjugated CD45 mAb. *i*NKT cells were identified using allophycocyanin-conjugated TCR β and PE-conjugated CD1d tetramers loaded with PBS57 (National Institutes of Health, National Institute of Allergy and Infectious Diseases MHC tetramer core facility). Unloaded CD1d tetramers were always used as a negative control. For intracellular staining, fixation and permeabilization were done on collected cells using Cytofix/Cytoperm kits (BD Pharmingen), according to the manufacturer's instructions. Cells were incubated with FITC-conjugated IL-4, Alexa Fluor 647-conjugated IL-13, and FITC-conjugated IFN- γ . The respective isotype control rat IgG1 κ Ab were used. All Abs were obtained from eBioscience. Flow cytometry was performed on a FACSCanto instrument (BD Biosciences) and analyzed with FlowJo 8.3.3 software (Tree Star).

ELISA

BAL fluid was collected immediately after AHR measurement. IL-4, IL-13, and mouse IFN- γ levels were measured by ELISA, as previously described (4).

Statistical analysis

Unpaired Student's *t* tests were used for comparisons between groups (Prism 4; GraphPad). Significance for all statistical tests was shown in figures for $p < 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***).

Results

T bet^{-/-} mice possess *i*NKT cells in liver, spleen, and lung

T-bet-deficient mice have a fault in the terminal maturation of *i*NKT cells (14). We therefore examined the number of *i*NKT cells in the liver, spleen, and lung of T-bet^{-/-} mice using CD1d-tetramer staining. The number of total lymphoid cells in each organ in T-bet^{-/-} mice was not different from that in WT BALB/c mice (data not shown). However, in the liver, which has the largest population of *i*NKT cells, the number of *i*NKT cells in T-bet^{-/-} mice was greatly reduced compared with that in WT BALB/c mice (41.5-fold reduced compared with WT BALB/c mice) (Fig. 1). However, significant numbers of *i*NKT cells were present in the spleen and lung tissues of T-bet^{-/-} mice, although in comparison with WT BALB/c mice, the number of *i*NKT cells in T-bet^{-/-} mice was still significantly reduced. *i*NKT cells in the spleen and lung of T-bet^{-/-} mice were ~ 3.2 - and ~ 3.3 -fold reduced, respectively (Fig. 1).

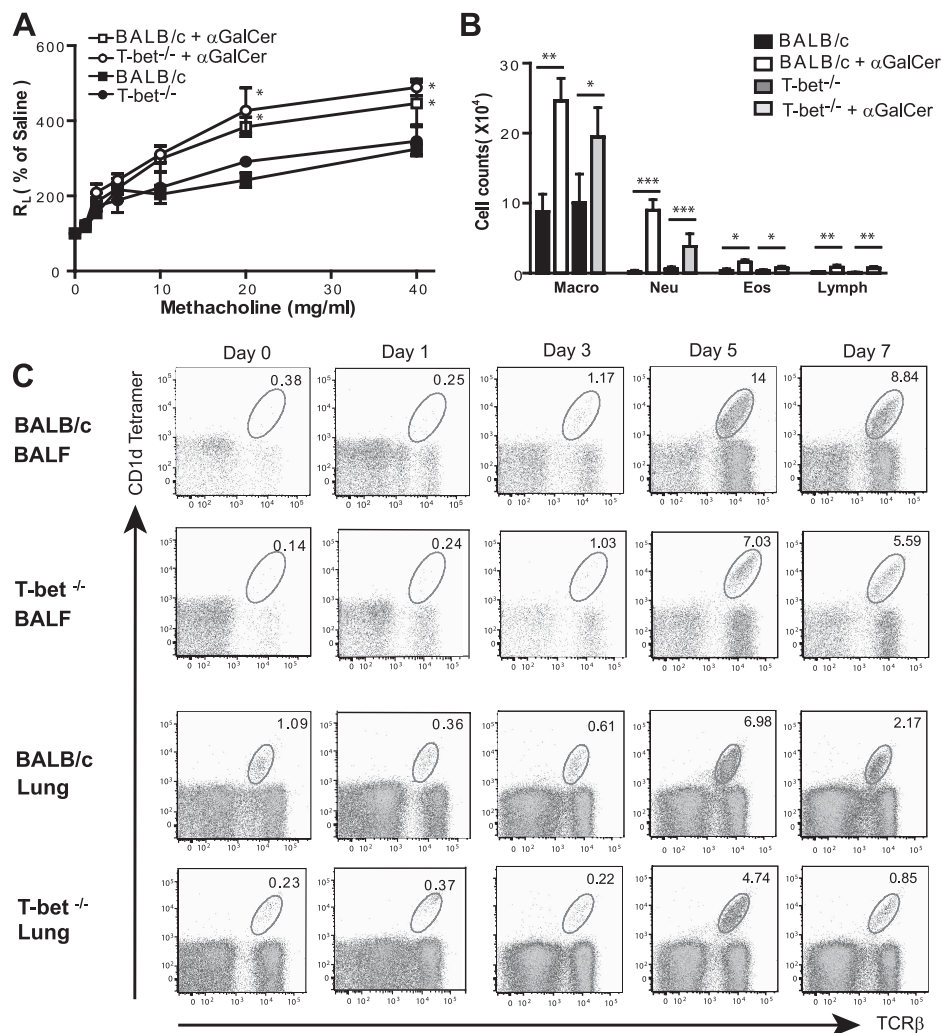


FIGURE 3. Development of α -GalCer-induced AHR in T-bet^{-/-} mice. In WT BALB/c and T-bet^{-/-} mice, AHR was induced by α -GalCer challenge. **A**, A total of 0.5 μ g of α -GalCer administered into WT BALB/c and T-bet^{-/-} mice 24 h before AHR measurement. Increasing concentrations of methacholine were used to measure AHR. Data represent the mean $\pm$ SEM percentage of saline value from three experiments ($n \geq 4$). Value of $p \leq 0.05$ (*) for the comparison between the α -GalCer group and control group. **B**, After AHR measurement, BAL fluids were collected immediately. Data represent the number of cells/ml in BAL fluid. Macro, macrophages; Neu, neutrophils; Eos, eosinophils; Lymph, lymphocytes. $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). **C**, BAL fluid and lung tissue were taken from WT BALB/c and T-bet^{-/-} mice challenged with α -GalCer. Collected cells were analyzed with PBS57-loaded CD1d tetramers and TCR β staining at days 3, 5, and 7 after α -GalCer challenge. The percentage of positive cells is indicated in each graph.

T-bet^{-/-} mice develop spontaneous AHR and inflammation

T-bet is a member of the T-box family of transcription factors and activates the IFN- γ gene (12). Therefore, T-bet deficiency results in enhanced Th2 cytokine production, associated with the development of spontaneous AHR and inflammation (11). We confirmed that T-bet deficiency results in spontaneous AHR, without exposure to allergen, particularly in older BALB/c T-bet^{-/-} mice, as demonstrated by an enhanced responsiveness to methacholine in 12- to 14-wk-old mice, assessed by direct measurement of airway resistance (R_L) ($p \leq 0.05$) (Fig. 2A). The development of spontaneous AHR was accompanied by airway inflammation consisting of macrophages, eosinophils, some neutrophils, and lymphocytes in BAL fluid (Fig. 2B). Although a previous study found that young BALB/c T-bet^{-/-} mice did not develop spontaneous airway inflammation as did young C57BL/6 T-bet^{-/-} mice (16), we found that T-bet deficiency in older BALB/c mice indeed resulted in both spontaneous airway inflammation as well as AHR that occurred without allergen exposure.

Because T-bet^{-/-} mice have significant numbers of iNKT cells in the periphery, we tested whether these iNKT cells in T-bet^{-/-} mice were involved in the development of spontaneous AHR. Treatment with an anti-CD1d-blocking mAb, which blocked the interaction between iNKT cells and CD1d, blocked the development of spontaneous AHR in the T-bet^{-/-} mice (Fig. 2C). Anti-CD1d mAb treatment also reduced the infiltration of cells (i.e., eosinophils, neutrophils, and lymphocytes) into BAL fluid (Fig. 2D). These results strongly suggest that CD1d-restricted iNKT cells in T-bet^{-/-} mice are required for the development of spontaneous AHR.

α -GalCer induces AHR and inflammation in T-bet^{-/-} mice

To directly examine the role of iNKT cells in the development of AHR in T-bet^{-/-} mice, we examined a model in which AHR was induced with α -GalCer, a marine sponge glycolipid that specifically activates iNKT cells (10). In these experiments, we used 6- to 7-wk-old BALB/c T-bet^{-/-} mice, which do not exhibit

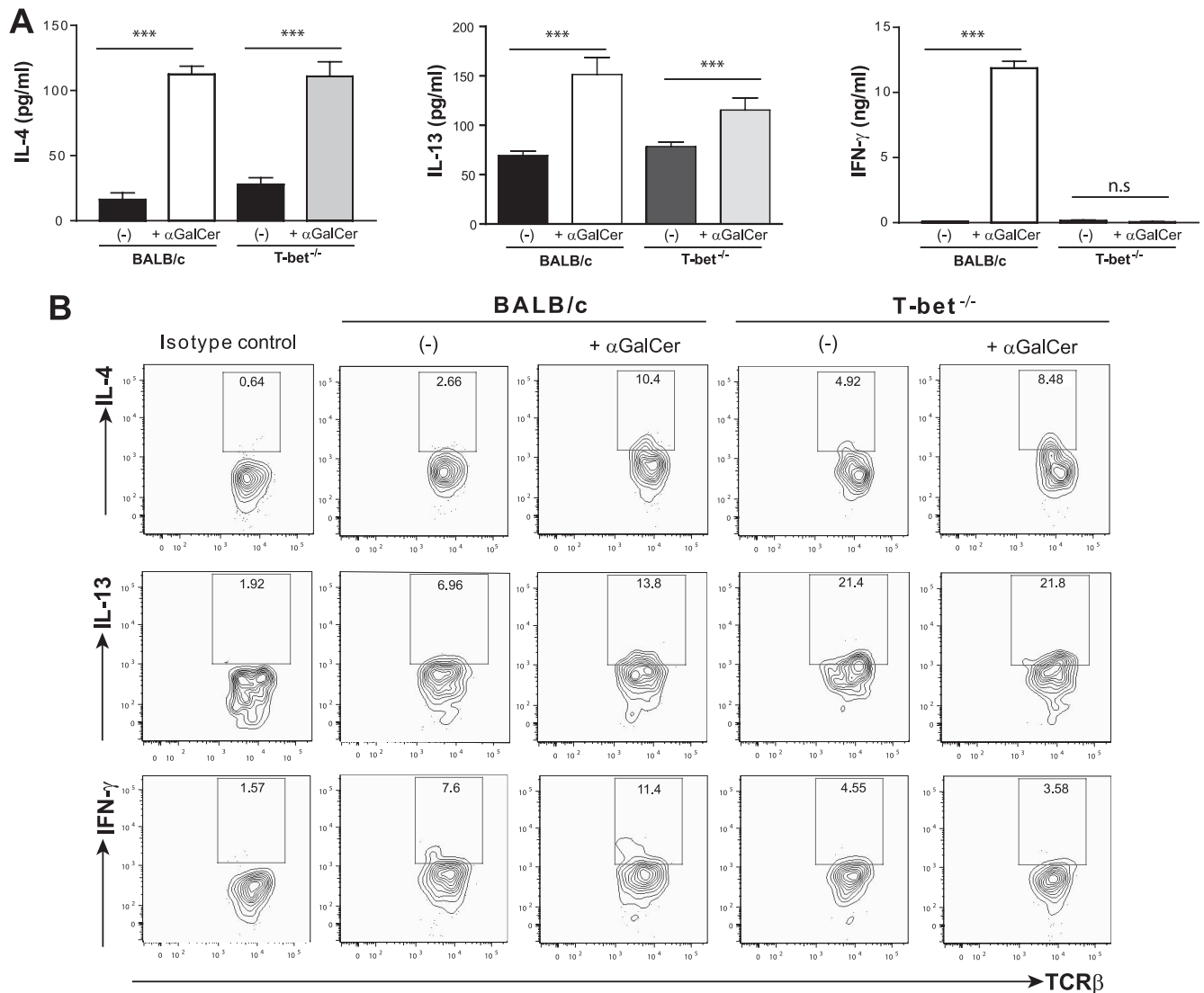


FIGURE 4. Characterization of *i*NKT cells in T-bet^{-/-} mice. *i*NKT cells in T-bet^{-/-} mice show a Th2-like phenotype. **A**, The amount of IL-4, IL-13, and IFN-γ in BAL fluid was measured using ELISA. BAL fluid from each group of mice was collected after AHR measurement (24 h after α-GalCer or saline challenge). Data represent the mean ± SEM, representative of three experiments. *p* ≤ 0.01 (**) and *p* ≤ 0.001 (***). **B**, Lung cells were obtained 24 h after α-GalCer or saline challenge in WT BALB/c and T-bet^{-/-} mice. Lymphocytes were stained with CD1d tetramer, anti-TCRβ mAb, and mAbs against IL-4, IL-13, or IFN-γ. The numbers shown in each graph represent the percentage of cytokine-producing T cells, after gating on CD1d-tetramer-positive cells.

spontaneous AHR. Fig. 3A shows that intranasal administration of α-GalCer induced a significant increase in AHR in T-bet^{-/-} mice and in WT BALB/c mice (*p* ≤ 0.05) and (*p* ≤ 0.05). The total number of cells, including neutrophils and eosinophils, in BAL fluid was also increased in T-bet^{-/-} mice after α-GalCer treatment (Fig. 3B). Importantly, the number of *i*NKT cells in the BAL fluid and in the lung tissue after challenge with α-GalCer increased and peaked on day 5 in both WT BALB/c and T-bet^{-/-} mice, although the percentage of BALB/c *i*NKT cells in T-bet^{-/-} mice was about half of that seen in WT BALB/c mice (Fig. 3C). These findings indicate that although the frequency of *i*NKT cells present in the lungs of T-bet^{-/-} mice is lower than in WT mice, the remaining *i*NKT cells are sufficient to respond on activation with α-GalCer, to infiltrate into the lungs and cause AHR.

We asked whether the efficiency of the *i*NKT cells from T-bet^{-/-} mice in causing AHR might be related to their cytokine profiles. Therefore, we examined BAL fluid cells from T-bet^{-/-} and BALB/c mice after challenged with α-GalCer, and observed

similar levels of IL-4 and IL-13 (Fig. 4A), which are cytokines important in the induction of AHR (17). However, IFN-γ was not found in the BAL fluid of T-bet^{-/-} mice. Cytokine production in *i*NKT cells was also examined by intracellular staining, and showed that a larger number of *i*NKT cells secreting IL-4 and IL-13 was present in the T-bet^{-/-} mice than in WT BALB/c mice (Fig. 4B). Taken together, *i*NKT cells from T-bet^{-/-} mice show Th2-like characters, and these features might be involved in causing the efficient development of AHR in T-bet^{-/-} mice. The overall effect, therefore, in T-bet^{-/-} mice, which contain fewer *i*NKT cells, but which secrete IL-4 and IL-13 at a higher frequency, is “normal” AHR (Fig. 3A) and “normal” levels of BAL fluid IL-4 and IL-13 (Fig. 4A).

Blockade of *i*NKT cells abolished the development of α-GalCer-induced AHR

To confirm the role of *i*NKT cells in the development of α-GalCer-induced AHR in T-bet^{-/-} mice, mice were treated with

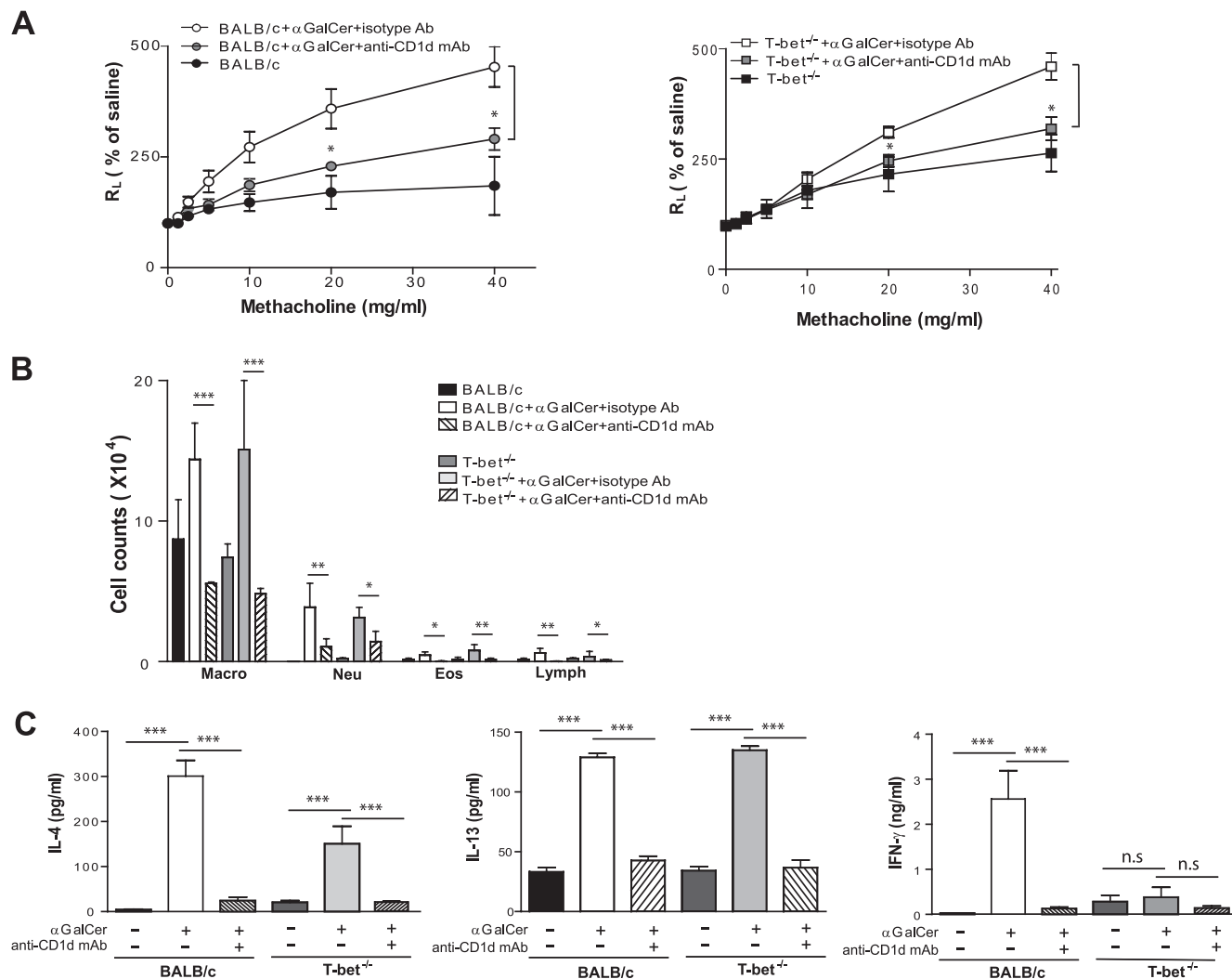


FIGURE 5. Blockade of *i*NKT cells in α -GalCer-induced AHR. Anti-CD1d mAb treatment reduced the development of α -GalCer-induced AHR in T-bet^{-/-} mice. **A**, Mice were treated with 500 μ g of anti-CD1d-blocking mAb (HB323), or isotype control mAb 24 h before α -GalCer challenge, and AHR was measured as in Fig. 2A. Data represent the mean $\pm$ SEM percentage of saline value from three experiments ($n \geq 3$). Value of $p \leq 0.05$ (*) for α -GalCer + anti-CD1d-blocking mAb vs α -GalCer + isotype control mAb in WT BALB/c mice, and $p \leq 0.05$ (*) for α -GalCer + anti-CD1d mAb vs α -GalCer + isotype control mAb in T-bet^{-/-} mice. **B**, BAL fluids were collected after AHR measurement. Data represent the number of cells/ml in BAL fluid. Macro, macrophage; Neu, neutrophils; Eos, eosinophils; Lymph, lymphocytes. $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). **C**, Supernatants from BAL fluid were collected and used for ELISA. The amounts of IL-4, IL-13, and IFN- γ in BAL fluid were measured. Data are the mean $\pm$ SEM, representative of three experiments. $p \leq 0.001$ (***).

anti-CD1d-blocking mAb or isotype control mAb. Blocking the interaction between *i*NKT cells and CD1d molecule reduced the development of AHR in WT BALB/c ($p \leq 0.05$) and in T-bet^{-/-} mice ($p \leq 0.05$) (Fig. 5A). Moreover, anti-CD1d-blocking mAb treatment reduced airway inflammation. The total number of cells and neutrophils in BAL fluids was significantly decreased after anti-CD1d-blocking mAb treatment (Fig. 5B). Furthermore, anti-CD1d mAb treatment significantly reduced IL-4 and IL-13 production in both WT BALB/c and T-bet^{-/-} mice (Fig. 5C). As noted earlier in Fig. 4, T-bet^{-/-} mice did not produce IFN- γ , and this did not change with anti-CD1d mAb treatment. These data confirmed that *i*NKT cells are critical cells for the development of α -GalCer-induced AHR in T-bet^{-/-} mice.

OVA induces CD1d-dependent AHR in T-bet^{-/-} mice

Next, we examined the role of *i*NKT cells in AHR using another AHR model induced by OVA. After OVA challenge, both WT

BALB/c mice and T-bet^{-/-} mice developed significant AHR ($p \leq 0.01$; $p \leq 0.05$, respectively) (Fig. 6A). The number of *i*NKT cells found in BAL fluid and in the lung increased after OVA challenge (data not shown). In both WT BALB/c and T-bet^{-/-} mice, OVA challenge significantly increased the number of cells in the BAL fluid compared with control mice, and infiltration of eosinophils into BAL fluid was increased (Fig. 6B). OVA treatment also induced IL-4 and IL-13, but not IFN- γ , production in the BAL fluid in T-bet^{-/-} mice (Fig. 6C). Analysis with intracellular cytokine staining indicated that a greater percentage of the *i*NKT cells in T-bet^{-/-} mice produced IL-4 than of the *i*NKT cells in WT BALB/c mice, although the percentage of IFN- γ -staining cells was reduced in T-bet^{-/-} compared with WT BALB/c mice (Fig. 6D). The role of *i*NKT cells in OVA-induced AHR was confirmed by blocking the development of AHR with anti-CD1d-blocking mAb, which significantly reduced AHR in both WT BALB/c ($p \leq 0.01$) and T-bet^{-/-} mice ($p \leq 0.01$) (Fig. 6E). Taken together, these data

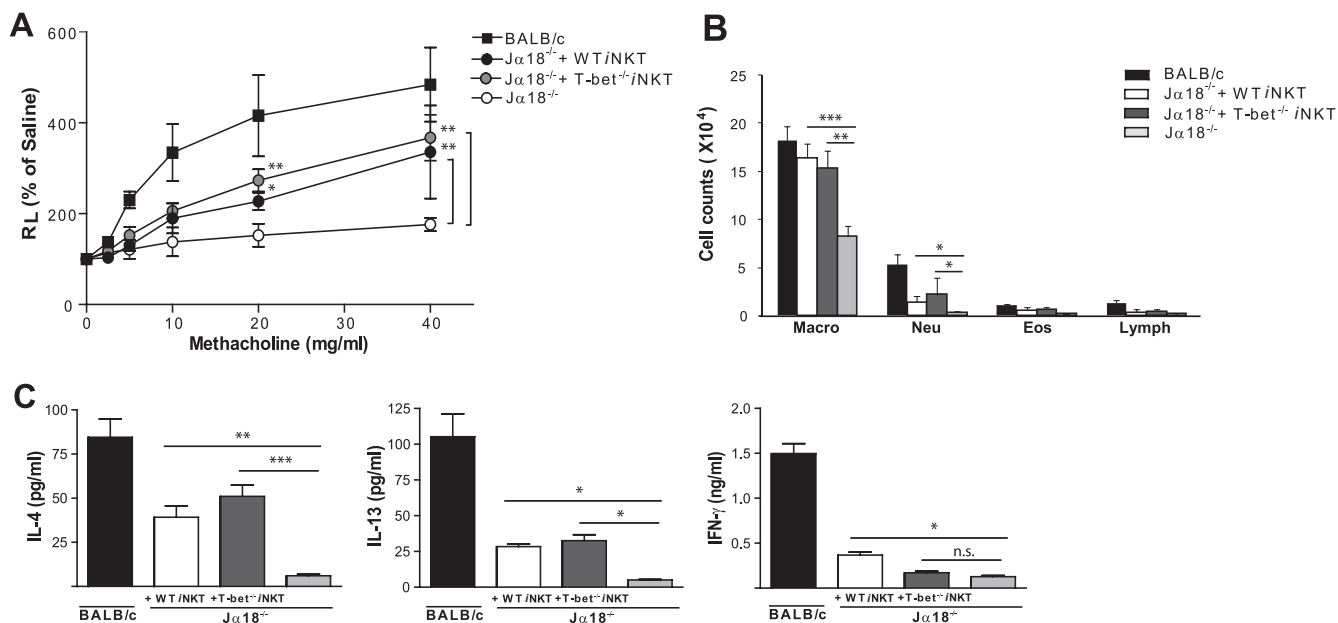


FIGURE 7. Restoration of AHR by adoptive transfer of iNKT cells. Adoptive transfer of iNKT cells from T-bet^{-/-} mice restores α-GalCer-induced AHR. **A**, A total of 3×10^6 of iNKT cells from WT BALB/c or T-bet^{-/-} mice was transferred into Jα18^{-/-} recipient i.v. A total of 0.5 μg of α-GalCer was administered 24 h after iNKT cell transfer. Increasing concentrations of methacholine were used to measure AHR. Data represent the mean ± SEM percentage of saline value from two experiments ($n \geq 4$). Value of $p \leq 0.01$ (**) for WT iNKT transferred into Jα18^{-/-} vs Jα18^{-/-} mice, and $p \leq 0.01$ (**) for T-bet^{-/-} iNKT transferred into Jα18^{-/-} vs Jα18^{-/-} mice. **B**, BAL fluid from mice in **A** was analyzed for cell counts. Data represent the number of cells/ml in BAL fluid and are the mean ± SEM, representative of three experiments. Macro, macrophage; Neu, neutrophils; Eos, eosinophils; Lymph, lymphocytes. $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). **C**, Cytokine levels in BAL fluid were analyzed by ELISA. Data are the mean ± SEM, representative of two experiments. $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

mice into Jα18^{-/-} mice also induced the infiltration of macrophages and neutrophils into the BAL fluid, whereas unconstituted Jα18^{-/-} mice failed to develop AHR and airway inflammation (Fig. 7, **A** and **B**). We also examined the cytokines in BAL fluid in each group, and found that adoptive transfer of iNKT cells from WT BALB/c or T-bet^{-/-} mice induced IL-4 and IL-13 production in BAL fluid. Transfer of iNKT cells from T-bet^{-/-} mice did not restore IFN-γ production in BAL fluid, consistent with the fact that iNKT cells from T-bet^{-/-} mice produce only low levels of IFN-γ (Fig. 7C). These results provide the direct evidence for the existence of an iNKT cell population in T-bet^{-/-} mice that can induce AHR.

Discussion

In this study, we showed that T-bet^{-/-} mice, which have a profound deficiency in the ability to generate iNKT cells (11), developed AHR that required CD1d-restricted iNKT cells. Thus, the AHR response in the T-bet^{-/-} mice, which was induced with either OVA, with α-GalCer, or which developed spontaneously, was blocked by treatment with anti-CD1d-blocking mAb, suggesting that the limited number of iNKT cells present in T-bet^{-/-} mice was responsible for the development of AHR. In addition, we found a significant number of iNKT cells was present in the lungs of T-bet^{-/-} mice, and that these iNKT cells produced a Th2 profile of cytokines (increased amounts of IL-4 and IL-13, and reduced levels of IFN-γ), which presumably enhanced their capacity to induce AHR. Moreover, we demonstrated that iNKT cells purified from T-bet^{-/-} mice were capable of restoring the development of AHR in iNKT cell-deficient Jα18^{-/-} mice. Therefore, the reduced number of iNKT cells in T-bet^{-/-} mice does not prevent iNKT cells from playing a critical role in the development of AHR, particularly because the iNKT cells produce IL-4 and IL-13, which critically regulate the development of AHR (1).

Previous studies have shown that T-bet^{-/-} mice produce enhanced levels of IL-13 and IL-4, which have been implicated in the development of AHR in these mice (18). The absence of IFN-γ may result in an overexuberant Th2 differentiation, a pattern observed in the lungs of patients with asthma (11), and in animal models of allergic asthma (17). This enhanced production of Th2 cytokines, without IFN-γ, may explain why the TBX21 (the human T-bet gene) functions as a human asthma susceptibility gene (19, 20). Although Th2 cells play a critical effector cell role in allergic asthma by producing Th2 cytokines in response to allergens, Th2 cells by themselves are not sufficient to support the development of AHR. This is very apparent in humans, in which only 30–40% of individuals sensitized to respiratory allergens (e.g., individuals with allergic rhinitis) develop asthma (21), indicating that factors in addition to Th2 cells are required for the development of asthma. This is also apparent in mice, in which AHR fails to develop in allergen-sensitized mice lacking iNKT cells (4). Based on our current studies, we suggest that the enhanced production of IL-13 and IL-4 that occurs in both Th2 cells and iNKT cells in T-bet^{-/-} mice predisposes T-bet^{-/-} mice to develop spontaneous AHR.

Although Th2 cells play a critical role in allergic asthma and are required for immune recognition of protein allergens, we previously showed that AHR that develops in response to α-GalCer and in response to ozone can occur independent of class II MHC-restricted conventional CD4⁺ T cells (5, 10). These observations indicate that iNKT cells play a critical role in the development of AHR independent of Th2 cells. In our current studies, we showed that the development of allergen-induced, α-GalCer-induced, as well as spontaneous AHR in T-bet^{-/-} mice is CD1d dependent. Furthermore, transfer of iNKT cells from T-bet^{-/-} mice restored AHR in iNKT cell-deficient mice. These results together suggest that the iNKT cells in T-bet^{-/-} mice not only mediate α-GalCer-induced

AHR, but also represent a critical component in spontaneous AHR and in allergen-induced AHR that develop in T-bet^{-/-} mice. Therefore, although T-bet^{-/-} mice have reduced numbers of *i*NKT cells, the remaining *i*NKT cells perform and contribute an essential function in the development of multiple forms of AHR.

These AHR studies in T-bet^{-/-} mice are particularly significant, because the requirement for *i*NKT cells in the development of allergen-induced AHR has met with recent skepticism, even though an important role for *i*NKT cells in the development of AHR has been demonstrated in many models of asthma (4–6, 10). For example, investigators have suggested that *i*NKT cells are dispensable for asthma, because studies have shown that eosinophilic airway inflammation can occur in the absence of *i*NKT cells (22, 23). However, although some airway inflammation can occur in *i*NKT cell-deficient mice, airway inflammation is not an adequate measure of asthma, because airway inflammation does not always correlate with AHR (24, 25), the sine qua non of asthma (26). Other investigators have suggested that *i*NKT cells are not required for asthma, because AHR can occur in β_2 -microglobulin^{-/-} mice, which lack class I molecules, including CD1d, and which were thought not to contain NKT cells. However, β_2 -microglobulin^{-/-} mice do in fact possess significant numbers of NK1.1⁺, TCR⁺ NKT cells (27, 28), which are restricted by a β_2 -microglobulin-independent form of CD1d (29–32). Blockade of CD1d in the β_2 -microglobulin^{-/-} mice prevented the development of AHR (33), consistent with the idea that NKT cells are indeed required for the development of AHR. Moreover, other investigators have suggested that *i*NKT cells are not important in human asthma, because only small numbers of *i*NKT cells (up to 2% of the lung T cells) were found in the lungs of patients with asthma (34). This argument is inaccurate, because the development of AHR requires very few pulmonary *i*NKT cells (10, 35), presumably because *i*NKT cells produce very large quantities of cytokines when activated. Furthermore, multiple studies have shown that the number of *i*NKT cells in the lungs of asthmatics is considerably greater than that of normal nonasthmatic individuals, although varying greatly in number in different patients (from 1 to >60%) (6, 36–41), presumably due to the great heterogeneity in the clinical manifestations of asthma. Finally, *i*NKT cells most likely play an important role in human asthma, because functional studies in nonhuman primates demonstrate that the activation of *i*NKT cells results in significant AHR (35).

Because T-bet^{-/-} mice have a deficiency in *i*NKT cell development, but develop spontaneous AHR and airway inflammation, one might conclude from these observations that *i*NKT cells are dispensable for asthma. However, we demonstrated that although the number of *i*NKT cells in the liver of T-bet^{-/-} mice is greatly reduced, a sizeable population of *i*NKT cells preferentially producing IL-4 and IL-13 is present in the spleen and in the lungs of T-bet^{-/-} mice, and that the presence of these CD1d-restricted *i*NKT cells is required for the development of several forms of AHR in these mice. Only small numbers of *i*NKT cells are needed to induce AHR, particularly if they produce IL-4 and IL-13 (10, 35), and it appears that the number of *i*NKT cells present in T-bet^{-/-} mice is sufficient for AHR to develop in response to allergen, α -GalCer, or which occurs spontaneously. Thus, the development of AHR in T-bet^{-/-} mice requires *i*NKT cells, extending previous studies demonstrating the importance of *i*NKT cells in the development of AHR.

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