

T-bet Deficiency Facilitates Airway Colonization by *Mycoplasma pulmonis* in a Murine Model of Asthma¹

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Epidemiological and clinical evidence suggest a correlation between asthma and infection with atypical bacterial respiratory pathogens. However, the cellular and molecular underpinnings of this correlation remain unclear. Using the T-bet-deficient (T-bet^{-/-}) murine model of asthma and the natural murine pathogen *Mycoplasma pulmonis*, we provide a mechanistic explanation for this correlation. In this study, we demonstrate the capacity of asthmatic airways to facilitate colonization by *M. pulmonis* and the capacity of *M. pulmonis* to exacerbate symptoms associated with acute and chronic asthma. This mutual synergism results from an inability of T-bet^{-/-} mice to mount an effective immune defense against respiratory infection through release of IFN- γ and the ability of *M. pulmonis* to trigger the production of Th2-type cytokines (e.g., IL-4 and IL-5), and Abs (e.g., IgG1, IgE, and IgA), eosinophilia, airway remodeling, and hyperresponsiveness; all pathophysiological hallmarks of asthma. The capacity of respiratory pathogens such as *Mycoplasma* spp. to dramatically augment the pathological changes associated with asthma likely explains their association with acute asthmatic episodes in juvenile patients and with adult chronic asthmatics, >50% of whom are found to be PCR positive for *M. pneumoniae*. In conclusion, our study demonstrates that in mice genetically predisposed to asthma, *M. pulmonis* infection elicits an inflammatory milieu in the lungs that skews the immune response toward the Th2-type, thus exacerbating the pathophysiological changes associated with asthma. For its part, airways exhibiting an asthmatic phenotype provide a fertile environment that promotes colonization by *Mycoplasma* spp. and one which is ill-equipped to kill and clear respiratory pathogens. *The Journal of Immunology*, 2006, 177: 1786–1795.

Infections of the lower respiratory tract potentially contribute to the initiation of asthma and recurrent exacerbations of asthma. Typical respiratory pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* do not initiate asthmatic exacerbations (1). However, respiratory syncytial virus and atypical bacterial pathogens such as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* are associated with acute exacerbations of asthma (2, 3). Additionally, *M. pneumoniae* has been detected in the lower airways of chronic, stable asthmatics with significantly greater frequency than in nonasthmatic control subjects (4, 5), suggesting that this bacterium has the capacity to influence both phases of the asthmatic phenotype. Infection of mice with *M. pulmonis*, a natural murine pathogen, recapitulates all the features of human respiratory mycoplasmosis (6). As such, this animal model serves as an invaluable tool to study the pathogenesis of community-acquired pneumonia and the interrelationship between mycoplasmosis and asthma.

An intriguing aspect of *Mycoplasma* infection that has drawn considerable attention over the past few years is the potential for this organism to be an initiator and/or exacerbator of asthma in children and adults (7, 8). Although numerous investigations have addressed the question of host defense in *Mycoplasma* infection of the respiratory tract (6, 9), the exact role of *Mycoplasma* in the

pathogenesis of asthma remains poorly understood. It is known that *M. pulmonis* triggers both Th1- and Th2-type immune responses in the lungs (10). IFN- γ is the key Th1 cytokine produced by dendritic, NK, NKT, and $\gamma\delta$ -T cells in addition to CD4⁺ and CD8⁺ cells in response to *M. pulmonis* infection (10, 11). IFN- γ activates alveolar macrophages (AMs),³ thus enhancing their antimicrobial activities through the up-regulation of NO and oxygen radicals (12). Owing to the induction of proinflammatory cytokines such as IFN- γ , Th1 responses also may contribute to the development of inflammatory lesions. For their part, Th2 cytokines down-modulate the antimicrobial activities of macrophages and thus may impair clearance of *M. pulmonis* and contribute to the establishment of a persistent infectious state. Th2 responses to *M. pulmonis* also may exacerbate asthma because they engender many of the pathophysiological changes associated with asthma (13, 14).

The present study employs a recently described murine model of asthma to explore further the immunological basis for the association between mycoplasmal pneumonia and the exacerbation of asthma. Mice deficient for T-bet, a member of the T-box family of transcription factors, spontaneously develop asthma-associated symptoms characterized by lung inflammation, airway remodeling, an increased number of bronchial myofibroblasts, and a dose-dependent increase in airway hyperresponsiveness (AHR) in response to methacholine challenge (15, 16). Importantly, it has been shown that T-bet expression is significantly lower in the lungs of allergic asthma patients than in nonasthmatic individuals (15). Development of an asthmatic phenotype in T-bet^{-/-} mice is thought to result from an inability of CD4⁺ Th cells, NK cells, and dendritic cells to produce IFN- γ (15, 17) and from overproduction of IL-13 by hyperactivated CD4⁺ memory cells (16). T-bet^{-/-} mice also exhibit an increased susceptibility to *Staphylococcus aureus*,

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³ Abbreviations used in this paper: AM, alveolar macrophage; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; PI, postinoculation; CBA, cytometric bead array; iNOS, inducible NO synthase.

Salmonella, *Mycobacteria*, and *Leishmania major* (17–20). Given the critical role played by IFN- γ in the development of innate and adaptive immunity, we hypothesized that the airways of T-bet $^{-/-}$ mice may represent a permissive environment for colonization by *M. pulmonis*, which, in turn, would result in a higher bacterial burden, severe lung pathology, airway remodeling, and increased obstruction of the airways.

Materials and Methods

Mice

T-bet $^{+/+}$ BALB/c mice were provided by Dr. L. H. Glimcher (Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA) and were bred in a specific pathogen-free environment in the Animal Resources Facility at Albany Medical College to obtain a population of homozygous wild-type (T-bet $^{+/+}$) and T-bet-deficient (T-bet $^{-/-}$) animals. Female mice were used at 6–8 wk of age, and all animal procedures conformed to the Institutional Animal Care and Use Committee guidelines.

Cultivation of *M. pulmonis* and inoculation of mice

The UAB-CT strain of *M. pulmonis* was provided by Dr. J. Simecka (Department of Molecular Biology and Immunology, University of North Texas Health and Science Center, Dallas, TX). *M. pulmonis* cultures were grown to late log phase in PPLO broth (Difco), and aliquots were stored in liquid nitrogen. Aliquots of *M. pulmonis* were spun at $10,000 \times g$ for 20 min, washed with sterile saline, and resuspended in saline to a final concentration of $\sim 10^6$ CFU/20 μ l for intranasal inoculation of anesthetized T-bet $^{-/-}$ and T-bet $^{+/+}$ mice. Sham control mice received an equivalent volume of uninoculated PPLO medium diluted in saline.

Serum and bronchoalveolar lavage fluid (BALF)

Whole blood was collected from anesthetized mice at 6 h, 1, 3, 7, 14, 21, and 28 days postinoculation (PI), and serum was used to determine cytokine levels and to characterize the humoral immune response to *M. pulmonis* as described previously (21). A BAL was performed by flushing lungs with 0.5 ml of saline twice, and the BALF was analyzed to determine differential cell counts and *M. pulmonis*-specific Ab titers.

Lung homogenates and quantification of mycoplasmal burden

Lungs were inflated with sterile PBS, collected aseptically in PBS containing a protease inhibitor mixture (Roche Diagnostics), and subjected to mechanical homogenization using a MiniBeadBeater-8 (BioSpec Products). The lung homogenates were spun briefly at 1,000 rpm for 10 s in a microcentrifuge to pellet the tissue debris. Supernatants were diluted 10-fold in sterile saline, and 10 μ l of each dilution (undiluted, 1/10, 1/100 and 1/1,000) was spotted onto PPLO agar plates in duplicate and incubated at 37°C for 7 days. The number of colonies on the plates were counted and expressed as log $_{10}$ CFU per gram of lung tissue. The remaining lung homogenate was spun at $14,000 \times g$ for 20 min, and the clarified supernatant was stored at -20°C .

Histological evaluation

The lungs were inflated with sterile PBS, and a portion of the left lobe of the lung was embedded in Tissue-Tek OCT compound for cryosectioning and phenol red staining, the remaining tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin-embedded sections (5 μ m) were stained with H&E, and disease severity was characterized based upon the cellular infiltrate within the peribronchiolar and perivascular regions and lung parenchyma. Collagen deposition was assessed by Masson's trichrome staining using the Accustain Trichrome stain kit (Sigma-Aldrich).

Cytokine measurement

Mouse Inflammation and Th1/Th2 cytometric bead array (CBA) kits (BD Biosciences-BD Pharmingen) were used for the simultaneous measurement of multiple cytokines in serum and lung homogenates. Data were acquired on a FACSArray Instrument (BD Immunocytometry Systems; BDIS) and analyzed using CBA software version 1.1 (BDIS).

Measurement of nitrite/nitrate levels

Quantification of NO levels was accomplished by measuring nitrite and nitrate concentrations in lung homogenates from uninfected and *M. pulmonis*-infected T-bet $^{-/-}$ and T-bet $^{+/+}$ mice. The nitrite and nitrate levels

were collectively measured as NOx by chemiluminescence (22). Briefly, NOx in the lung homogenate was reduced to NO by refluxing with a saturated solution of vanadium chloride in hydrochloric acid. The resultant NO was detected using a chemiluminescence NO analyzer (model 270B; Sievers Instruments). The samples were referenced to a standard curve generated from a range of nitrate concentrations (0.05–500 μ M) for the determination of nitrite/nitrate levels.

In situ detection and quantification of eosinophils

The extent of eosinophilic infiltration in the lungs of both uninfected and *M. pulmonis*-infected T-bet $^{-/-}$ and T-bet $^{+/+}$ mice was determined by cryosectioning OCT-embedded lung tissue followed by phenol red staining as described previously (23). For quantification of eosinophils at least five randomly chosen fields from each section were counted ($n = 4$ –6 mice/group), and the number of eosinophils was expressed as a mean $\pm$ SEM (21).

Serology

Serum and BALF were assayed for *M. pulmonis*-specific IgM, IgG1, IgG2a, IgG2b, IgA, and IgE Abs. *M. pulmonis* lysate was prepared and used as Ag in an ELISA as described previously (24). Due to the typical low levels of Ab raised against this pathogen, titers cannot be determined, and investigators routinely report Ab levels as OD (25, 26). A sample was considered positive if its OD reading was ≥ 2 SD above the mean OD of the control serum sample (27).

Assessment of AHR

Whole body, unrestrained plethysmography (Buxco) was used to monitor the respiratory dynamics of mice in a quantitative manner as described previously (23). Mice were allowed to acclimate unrestrained in the sampling chambers of the plethysmograph and were exposed for 1 min to nebulized methacholine or PBS. Recordings were taken for 3 min after each nebulization. Penh, a correlate of pulmonary airflow and resistance, is a dimensionless value that represents a function of the ratio of peak expiratory flow to peak inspiratory flow and the timing of expiration. The degree of airway obstruction was determined in *M. pulmonis*-infected or sham-treated T-bet $^{-/-}$ and T-bet $^{+/+}$ mice ($n = 10$) at 6 h, 1, 3, 7, 14, 21, and 28 days PI. The Penh values measured during each 3-min interval were averaged and expressed as mean $\pm$ SEM.

Statistical analysis

Data are presented as means $\pm$ SEM, and comparisons between groups were made by using the nonparametric Mann-Whitney *U* test and Student's unpaired *t* test. Pairwise comparisons for AHR measurements between the groups at different time points were made via repeated measures ANOVA. A *p* value of <0.05 was considered significant.

Results

T-bet $^{-/-}$ mice fail to control *M. pulmonis* growth

Perturbation of host defenses often is reflected in altered ability to control bacterial replication. To begin assessing the impact of T-bet deficiency on host immunity, T-bet $^{-/-}$ and syngeneic T-bet $^{+/+}$ control mice were challenged intranasally with a single dose of $\sim 10^6$ CFU of *M. pulmonis*, and bacterial burden in the lungs was determined over a 28-day period of time. During the acute phase of infection, a state of equilibrium was found to exist between the rate of replication and clearance of *M. pulmonis* in T-bet $^{+/+}$ mice (Fig. 1). However, by day 21, clearance mechanisms reduced the bacterial numbers, but did not eliminate the organisms completely. In contrast, T-bet $^{-/-}$ mice experienced an increase in bacterial burden such that at 3 and 7 days PI, mice harbored 100- to 1000-fold more bacteria than did the T-bet $^{+/+}$ mice. Although, as in T-bet $^{+/+}$ mice, clearance mechanisms predominated later in the disease process, bacterial burden in the lungs of T-bet $^{-/-}$ mice remained significantly higher than in T-bet $^{+/+}$ animals. Thus, T-bet deficiency appears to severely undermine the host's ability to control the growth of *M. pulmonis*.

T-bet $^{-/-}$ mice develop severe lung pathology in response to *M. pulmonis*

We investigated whether increased bacterial burden was associated with greater histopathology in the lungs of T-bet $^{-/-}$ vs T-bet $^{+/+}$

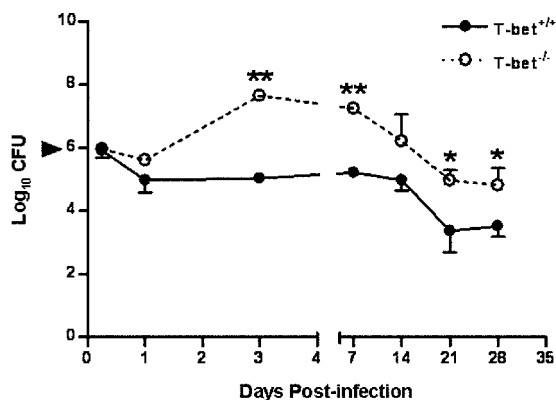


FIGURE 1. T-bet^{-/-} mice fail to limit *M. pulmonis* replication in the lungs. T-bet^{+/+} and T-bet^{-/-} mice were challenged intranasally with 10⁶ CFU of *M. pulmonis*. At the times indicated, mice were sacrificed, and homogenates of the lungs were plated for determination of bacterial burden. Results represent the mean $\pm$ SEM of CFU determined for each group ($n = 4$ –6/group) and are cumulative results of two independent experiments. Comparison between the groups was made using the nonparametric Mann-Whitney *U* test (*, $p < 0.05$; **, $p < 0.01$).

mice. The T-bet^{+/+} mice developed mild, rather diffuse pneumonia characterized by an accumulation of inflammatory cells in peribronchial and perivascular regions. In contrast, the lungs of T-bet^{-/-} mice presented with intense peribronchial and perivascular inflammatory infiltrates and pneumonia. Neutrophils were the principal cell type in pneumonic lesions, whereas the perivascular and peribronchial infiltrates consisted mostly of AMs and lymphocytes (Fig. 2). No obvious inflammatory response was observed in sham-inoculated mice (Fig. 2, A and B). At each 1-wk interval PI, the lungs of T-bet^{+/+} mice had fewer cellular infiltrates (Fig. 2, C, E, G, and I) than their T-bet^{-/-} counterparts (Fig. 2, D, F, H, and J). Unlike in T-bet^{+/+} mice, extensive infiltration of lung parenchyma, patchy interstitial pneumonia, thickening of the intra-alveolar septa, hyperplasia of smooth muscles around bronchioles, dysplasia of airway epithelium, and focal hemorrhagic patches was a frequent histological finding in the lungs of *M. pulmonis*-infected T-bet^{-/-} mice (Fig. 2, D and F). By day 21 and 28 PI, the inflammatory responses were mainly restricted to bronchioles and blood vessels with reduced cellular infiltrates in these areas (Fig. 2, H and J). Differential cell counting revealed a striking bias in the repertoire of cells infiltrating the lungs of T-bet^{-/-} and T-bet^{+/+} mice. Despite similarities at day 14, by days 21 and 28 PI, the BALF of T-bet^{+/+} mice contained a greater number of lymphocytes than macrophages, whereas this ratio was reversed in T-bet^{-/-} mice (Fig. 3). Collectively, these results demonstrate an association between bacterial burden and histopathological changes and show that infection of the airways of T-bet^{-/-} mice results in more severe lung pathology. Furthermore, whereas pulmonary inflammation in T-bet^{+/+} mice resolves by day 14 PI, a persistent inflammatory response exists in the lungs of T-bet^{-/-} mice.

At an early stage of infection T-bet^{-/-} mice produce significantly lower levels of nitrite/nitrate than do T-bet^{+/+} mice

An important element of host immunity to bacterial respiratory infections is the rapid clearance of organisms from the lungs. AMs are a major source of NO production during the acute inflammatory response (12). Given that levels of nitrate/nitrite, the metabolic products of NO, correlate with the efficiency of host defenses, we measured reactive nitrogen levels in lung homogenates of sham-inoculated and *M. pulmonis*-infected T-bet^{-/-} and

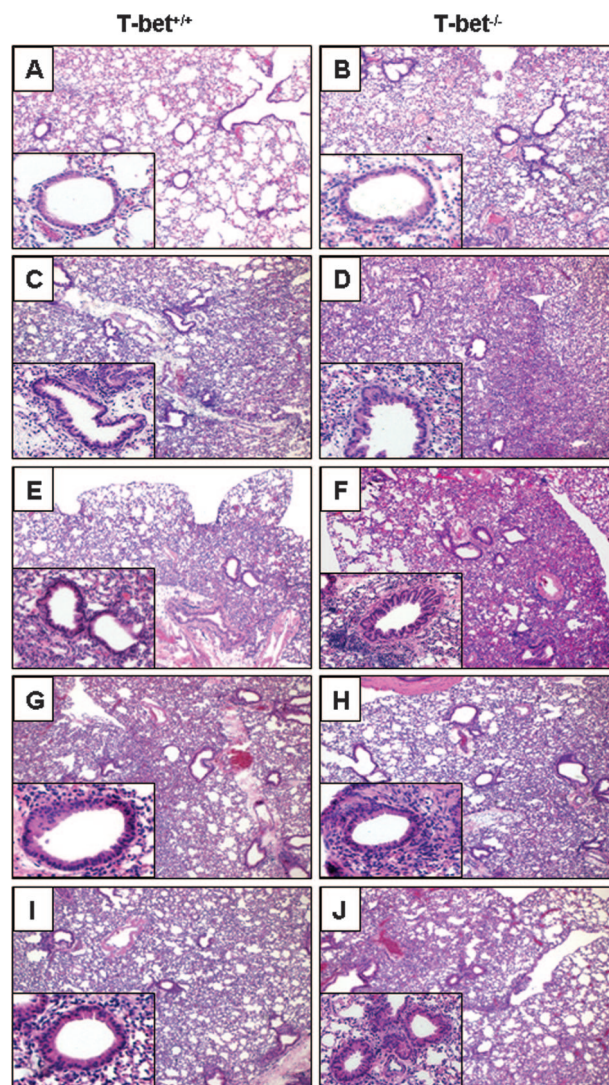


FIGURE 2. T-bet^{-/-} mice show more extensive histopathological changes than T-bet^{+/+} mice. Histopathological changes in the lungs of T-bet^{+/+} and T-bet^{-/-} mice were evaluated at day 7 (C and D), 14 (E and F), 21 (G and H), and 28 (I and J) PI with 10⁶ CFU of *M. pulmonis*. Lung sections of sham-inoculated T-bet^{+/+} and T-bet^{-/-} mice served as a control (A and B) (magnification, $\times 40$). Insets show the pathological changes observed in and around the bronchioles (magnification, $\times 200$).

T-bet^{+/+} mice. The T-bet^{+/+} mice were found to contain significantly higher levels of nitrite/nitrate compared with T-bet^{-/-} mice at 6 h PI (Fig. 4). These levels gradually returned to baseline by day 7 PI, whereas nitrite/nitrate levels in T-bet^{-/-} mice remained unaltered throughout the course of the study. These data demonstrate that inflammatory cells in the lungs of T-bet^{-/-} mice fail to produce significant amounts of NO, a scenario that may underlie the inability of these mice to restrict mycoplasmal replication early in the disease process.

M. pulmonis infection induces both Th1- and Th2-associated humoral immunity

Another key mediator of host defense against respiratory infection is the elaboration of pathogen-specific Abs. We measured *M. pulmonis*-specific IgM, IgG1, IgG2a, IgG2b, IgA, and IgE levels in the sera and BALF of T-bet^{-/-} and T-bet^{+/+} mice by ELISA. A clear pattern of IgM responses was not observed, and Abs were not

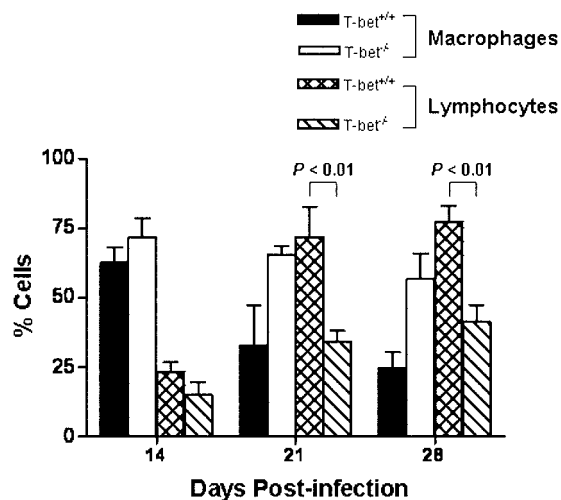


FIGURE 3. Differential cell count in the BALF of T-bet^{+/+} and T-bet^{-/-} mice infected intranasally with 10^6 CFU of *M. pulmonis* at 14, 21, and 28 days PI. Less than 5% of neutrophils and eosinophils were observed at the indicated time points and hence not shown. Results represent the mean $\pm$ SEM of cells determined for each group ($n = 4-6$ /group) and are the cumulative results from two independent experiments. Comparisons between the groups were made using nonparametric Mann-Whitney *U* test ($p < 0.01$).

detected in either mouse strain until day 14 PI, at which time only levels in T-bet^{-/-} mice were elevated above those observed in sham-inoculated mice (Fig. 5A). However, it is important to note that the murine IgM assay is not as specific as the IgG assay, and nonspecific color development is a common finding (25). In T-bet^{+/+} mice, release of IgG1, indicative of a Th2-type response, followed a bell-shaped distribution over a 3-wk period of time. In contrast, IgG1 levels in T-bet^{-/-} mice were elevated above those of sham-inoculated mice by day 14 and 21, and on day 28 PI they were significantly higher than in *M. pulmonis*-infected T-bet^{+/+} mice. IgG2a, an isotype associated with Th1-type responses, was observed in both T-bet^{-/-} and T-bet^{+/+} mice, but only reached levels significantly above their respective sham-inoculated controls at day 28 PI. The levels of IgG2b Abs, which facilitate bacterial clearance, were elevated in infected T-bet^{-/-} and T-bet^{+/+} mice above those observed in their sham-inoculated counterparts at all time points studied. However, the levels found in *M. pulmonis*-infected T-bet^{+/+} mice were significantly higher at days 14 and 28 PI than those observed in T-bet^{-/-} mice (Fig. 5A). Levels of serum IgA, a critical isotype in mucosal immunity and AHR, were significantly elevated above baseline values in T-bet^{+/+} mice throughout the experiment; however, levels in T-bet^{-/-} mice only reached statistical significance at day 28 PI (Fig. 5B). IgE Abs were undetectable in sera from T-bet^{-/-} or T-bet^{+/+} mice throughout the course of the study. Contrary to serum levels, amounts of IgA in the BALF from T-bet^{+/+} mice remained unaltered, whereas those in T-bet^{-/-} mice were elevated above those of sham-inoculated controls at days 14 and 21 and were significantly higher than those of T-bet^{+/+} mice at day 21 PI. IgE levels in the BALF from T-bet^{-/-} mice exhibited a similar trend with significant differences from the corresponding T-bet^{+/+} mice being observed at day 14 PI (Fig. 5B). Thus, *M. pulmonis* infection in T-bet^{-/-} and T-bet^{+/+} mice induces both Th1- and Th2-associated Ab isotypes. Importantly, Th2-type Abs (i.e., serum IgG1 and BALF IgE) predominate in infected T-bet^{-/-} mice.

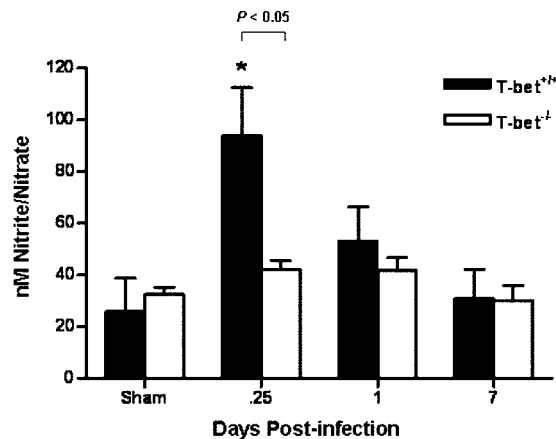


FIGURE 4. T-bet deficiency results in decreased NO production. Lung homogenates of T-bet^{+/+} and T-bet^{-/-} mice infected intranasally with 10^6 CFU of *M. pulmonis* from 6 h to day 7 PI were assayed for nitrite/nitrate levels as an indicator of NO production. Sham-inoculated mice served as controls. The results show representative data of two independent experiments ($n = 4-6$ mice/group). Comparison between the groups is made by one-way ANOVA. Asterisks represent significant differences from the corresponding sham values ($p < 0.05$).

T-bet^{-/-} mice display an impaired Th1- and enhanced Th2-type cytokine response to M. pulmonis infection

Multiplex Mouse Inflammation and Th1/Th2 CBA Kits were used to measure cytokine release. Levels of TNF- α , MCP-1, and IL-6 in the lung homogenates of *M. pulmonis*-infected T-bet^{-/-} and T-bet^{+/+} mice were elevated above their respective sham controls and often peaked as early as 6 h PI, returning to baseline levels by day 3. However, levels of IL-12p70 or IL-10, if produced, remained below detectable levels in both strains of mice for the duration of the experiment (data not shown). IFN- γ plays a key role in the activation of macrophages and the subsequent induction of NO, the principal mediator of bactericidal activity in macrophages. Thus, it was of interest to determine the IFN- γ concentrations in lung homogenates of *M. pulmonis*-infected T-bet^{-/-} and T-bet^{+/+} mice. In T-bet^{+/+} animals, IFN- γ levels were elevated as early as 6 h PI and differed significantly from those in T-bet^{-/-} mice by day 1 (Fig. 6A). IFN- γ production appeared to be bimodal with initial levels declining gradually to a low at day 7 PI and then rising again and remaining high for the duration of the experiment. The kinetics of IFN- γ production in T-bet^{-/-} mice differed in that levels remained unchanged from baseline until day 7, at which time the trend was similar to that seen in T-bet^{+/+} mice. These results suggest that the airways of T-bet^{-/-} mice, which have significantly lower levels of IFN- γ early in the infectious process, may provide a more permissive environment for bacterial replication within the lung.

Given the bias *M. pulmonis* demonstrates toward induction of Th2-type Ab responses, we predicted that the release of key Th2 cytokines (e.g., IL-4 and IL-5) might be augmented in the airways of T-bet^{-/-} mice. Following infection, we observed that the lung homogenates of T-bet^{-/-} mice contained significantly higher levels of Th2 cytokines than did the control samples. T-bet^{-/-} mice had elevated IL-4 levels at day 3 PI, which were significantly higher than levels in T-bet^{+/+} mice (Fig. 6B). Elevated IL-4:IFN- γ ratios that were observed in T-bet^{-/-} mice, but not in their T-bet^{+/+} counterparts, reflected the Th2-biased immune environment in the T-bet^{-/-} mice (Fig. 6B, inset). These ratios remained unaltered in T-bet^{+/+} mice throughout the course of the study.

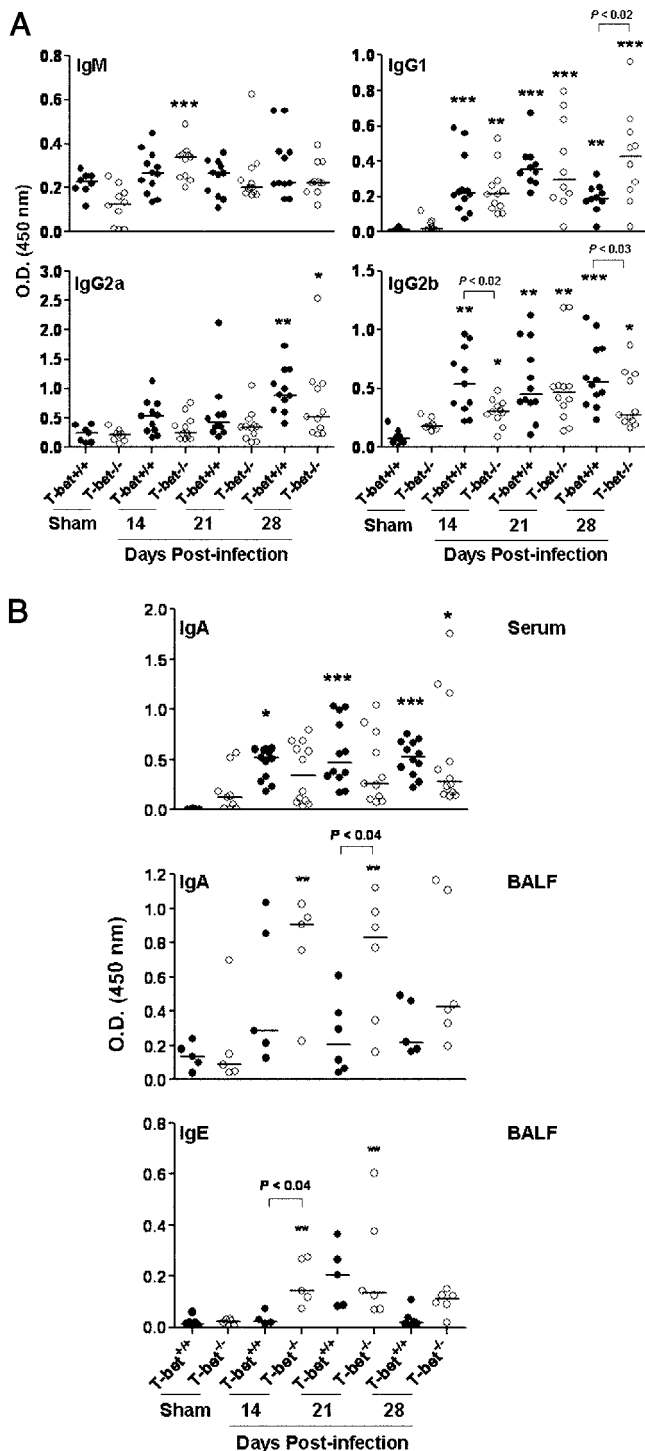


FIGURE 5. Production of *M. pulmonis*-specific Abs in T-bet^{+/+} and T-bet^{-/-} mice. Mice were challenged intranasally with 10⁶ CFU of *M. pulmonis*. Serum and BALF was collected at the indicated times. Serum IgM, IgG1, IgG2a, IgG2b (A), serum IgA and BALF IgA, and IgE (B) levels were determined by ELISA. Results represent the cumulative median values of the Ab levels observed in two independent experiments ($n = 5-6$ /group). Comparison between the groups is made by one-way ANOVA. Asterisk represents significant differences from the corresponding sham values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

IL-5 levels were elevated in T-bet^{-/-} mice above that of T-bet^{+/+} mice as early as 6 h and remained high at day 1 PI (Fig. 6C). After a slight decline, seen on days 3 and 7 PI, IL-5 levels rose again and remained elevated above the sham controls for both groups of mice

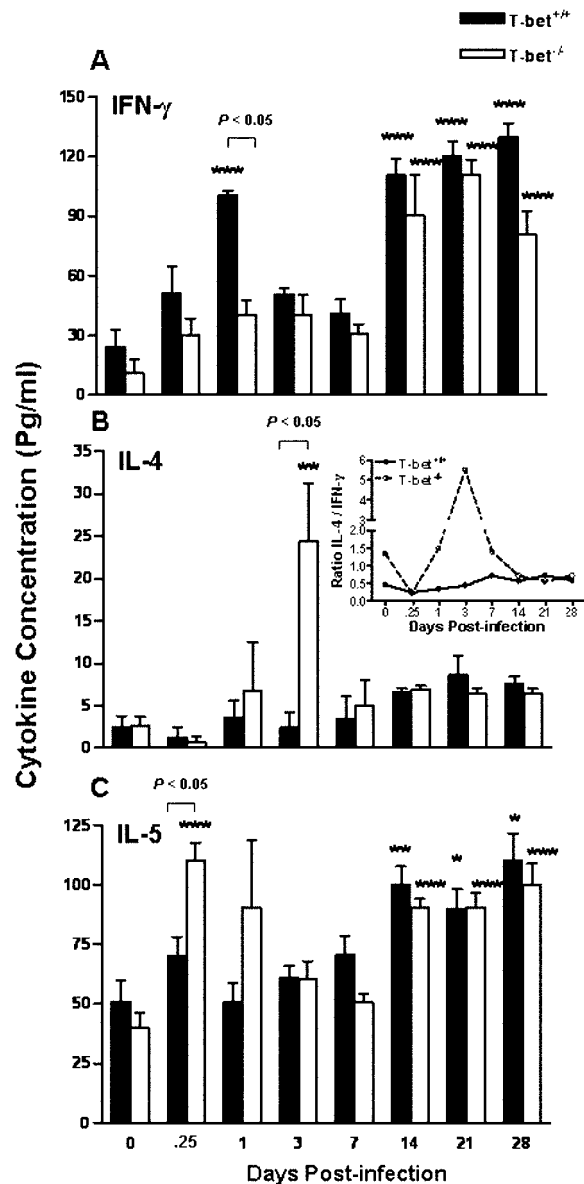


FIGURE 6. *M. pulmonis*-infected T-bet^{-/-} mice exhibit a Th2-biased cytokine response. Lung homogenates were prepared, and IFN- γ (A), IL-4 (B), IL-4:IFN- γ ratio (B, inset), and IL-5 (C) levels were measured. Sham-inoculated mice were used as a control. Results represent the mean $\pm$ SEM of the cytokine concentrations from three to four independent experiments ($n = 12-18$). Comparison between the groups is made by one-way ANOVA. Asterisk represents significant differences from the corresponding sham values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

until day 28 when the study was terminated. These results indicate that exposure to *M. pulmonis* augments the Th2-biased immune environment associated with asthma. Furthermore, an association appears to exist between IL-4:IFN- γ ratios and increased asthma-associated Ab isotypes in T-bet^{-/-} mice.

M. pulmonis infection induces markedly higher lung eosinophilia in T-bet^{-/-} than in T-bet^{+/+} mice

Because a Th2-biased cytokine milieu plays a critical role in the development of allergic lung pathology, we assessed the impact of this immune environment on the recruitment of eosinophils into the lungs of T-bet^{-/-} and T-bet^{+/+} mice. Lungs of the

sham-inoculated T-bet^{+/+} mice did not show eosinophilia, whereas those of the T-bet^{-/-} mice revealed a few scattered eosinophilic infiltrates surrounding bronchioles in the lung (Fig. 7A, *a* and *b*). Following infection with *M. pulmonis*, lung sections from T-bet^{+/+} mice showed increased eosinophilia at 6 h and day 1 PI. At days 3 and 7 PI, infiltrating eosinophils were localized mainly in alveolar walls and rarely in peribronchial regions (Fig. 7A, *c*, *e*, *g*, and *i*). In contrast, T-bet^{-/-} mice showed marked peribronchial eosinophilic infiltrates at 6 h and day 1 and exhibited extensive parenchymal eosinophilia at days 3 and 7 PI (Fig. 7A, *d*, *f*, *h*, and *j*). Enumeration of eosinophils indicated that the lungs of T-bet^{-/-} mice had significantly more eosinophils than that of T-bet^{+/+} mice (Fig. 7B). These observations suggest that *M. pulmonis* stimulates the recruitment of eosinophils into the lung and that this process is enhanced in T-bet^{-/-} mice, perhaps by virtue of increased IL-5 secretion in response to infection.

M. pulmonis infection of T-bet^{-/-} mice is associated with AHR

Measurement of AHR by whole body plethysmography was performed in uninfected T-bet^{-/-} and T-bet^{+/+} mice by subjecting them to increasing doses of methacholine. T-bet^{-/-} mice exposed to 12.5 and 25 mg of methacholine experienced 12- to 15-fold increases in Penh values over T-bet^{+/+} mice, respectively (Fig. 8, *inset*). To explore whether mycoplasmosis leads to obstruction of the air passages and increased airway resistance, we performed a time course experiment wherein T-bet^{-/-} and T-bet^{+/+} mice were infected with a single dose of ~10⁶ CFU of *M. pulmonis*. Neither sham-inoculated T-bet^{-/-} nor T-bet^{+/+} mice showed alterations in their baseline Penh values throughout the course of study. However, *M. pulmonis*-infected T-bet^{-/-} and T-bet^{+/+} mice had significantly increased Penh values at days 3, 7, and 14 PI, which in the T-bet^{+/+} mice returned to baseline by day 21 (Fig. 8). Importantly, *Mycoplasma*-triggered Penh, which correlates to increased obstruction of the air passages and airway resistance, was significantly greater in T-bet^{-/-} mice than in their T-bet^{+/+} counterparts and remained elevated above baseline for the duration of the experiment. These data demonstrate that there is a role for *M. pulmonis* infection as an inducer of airway obstruction and resistance in both the T-bet^{+/+} and T-bet^{-/-} mice and that these pathophysiological changes are worse in T-bet^{-/-} mice. Similar processes may be at play in juvenile and adult asthma patients suffering from mycoplasmal pneumonia.

T-bet^{-/-} mice show extensive airway remodeling in response to *M. pulmonis* infection

Increased subepithelial deposition of collagen is a prominent feature of airway remodeling in asthmatic individuals. To determine whether excessive fibrosis consequent to *M. pulmonis* infection underlies the obstruction of the air passages observed in *Mycoplasma*-infected T-bet^{-/-} mice, lung sections were stained for collagen. Sham-inoculated T-bet^{+/+} mice revealed only a thin and uniform layer of collagen, whereas T-bet^{-/-} mice had prominent deposits of collagen in subepithelial regions surrounding the bronchioles (Fig. 9, *A* and *B*). Fourteen, 21, and 28 days PI, the T-bet^{-/-} mice (Fig. 9, *D*, *F*, and *H*) exhibited greater collagen deposition in the subepithelial layers of bronchioles and perivascular regions of the lung than did T-bet^{+/+} mice (Fig. 9, *C*, *E*, and *G*). Dense fibrils of collagen in the submucosal and subepithelial areas with entrapped inflammatory cells also were seen more frequently in *M. pulmonis*-infected T-bet^{-/-} mice than in T-bet^{+/+} animals. Taken together, these results demonstrate that *M. pulmonis* has the capacity to induce fibrosis and that this process occurs

to a much greater extent in the lungs of infected T-bet^{-/-} than in T-bet^{+/+} mice. Similar events may contribute to the more severe airway remodeling associated with chronic asthma patients suffering from bacterial pneumonia.

Discussion

Detection of *Mycoplasma* spp. in the airways of asthmatic patients and the ability of these organisms to cause persistent AHR and induce airway remodeling have implicated this respiratory pathogen in asthmatic exacerbations (4, 28–30). Despite a strong clinical association and evidence to suggest that *Mycoplasma* spp. can impact both the acute and chronic phase of asthma, a mechanistic understanding of the immune processes that underlie the relationship between community-acquired pneumonia and asthma remains incomplete. Thus, the present study sought to elucidate the cellular and molecular bases for the “connection” between these two chronic respiratory etiologies.

Mice deficient for T-bet were used as they exhibit eosinophilia, AHR, and airway remodeling; a hallmark of human asthma that is not faithfully recapitulated in other mouse models of lung inflammation (15, 16). Another rationale for using T-bet^{-/-} mice is that animals spontaneously develop asthma-associated symptoms without the need for repeated allergen sensitization, an artificial means of inducing lung inflammation (15, 16). Mice lacking T-bet fail to develop Th1 cells, display a dramatic reduction in IFN- γ production by CD4⁺ T cells, NK cells (17), and dendritic cells (31), and overproduce IL-13, a potent Th2 cytokine. However, it is important to note that T-bet is not required for IFN- γ gene transcription in the CD8⁺ T cell lineage (17). Because CD4⁺ T cells, NK cells, and dendritic cells play a critical role in combating infection, decreased production of IFN- γ by these cell types may result in increased susceptibility to bacterial infections (18, 19).

IFN- γ is a pleiotropic cytokine essential for both innate and adaptive immune responses. Mice lacking IFN- γ or the IFN- γ receptor have profoundly impaired innate and adaptive immunity often leading to death of the host from infection (11). It has been reported that loss of IFN- γ results in more severe disease and higher mycoplasmal burden (32). Thus, not surprisingly, we observed that T-bet^{-/-} mice produced substantially less IFN- γ than the T-bet^{+/+} mice early during infection with *M. pulmonis* and that these mice had three log higher levels of *Mycoplasma* in their lungs than did T-bet^{+/+} mice. In contrast, by virtue of the ability of T-bet^{+/+} mice to produce IFN- γ during the acute phase of the infection, bacterial replication is effectively controlled. Later in the infectious process when T-bet^{-/-} mice also begin producing IFN- γ , likely released by CD8⁺ T cells, both mycoplasmal burden and inflammation in the lungs is diminished. This finding is consistent with the observation that during *M. pulmonis* infection, CD8⁺ T cells are a major source of IFN- γ and that their depletion results in the increased severity of mycoplasmosis (10). T-bet deficiency also may hinder the activation of macrophages due to lowered production of IFN- γ . Macrophages mediate killing of *Mycoplasma* through IFN- γ -stimulated generation of NO and its toxic metabolites (33). IFN- γ mediates the production of NO by regulating the expression of inducible NO synthase (iNOS) in murine AMs through JAK/STAT1 signaling (34–36). Previous studies have reported that inhibitors of NO synthesis abolish the bactericidal capacity of AMs (37, 38) and that iNOS-deficient mice are more susceptible to viral and bacterial respiratory pathogens including mycoplasmas (39–41). Despite a predominant AM-associated inflammation, the significantly lower NO levels observed in T-bet^{-/-} mice suggests that diminished levels of IFN- γ might be

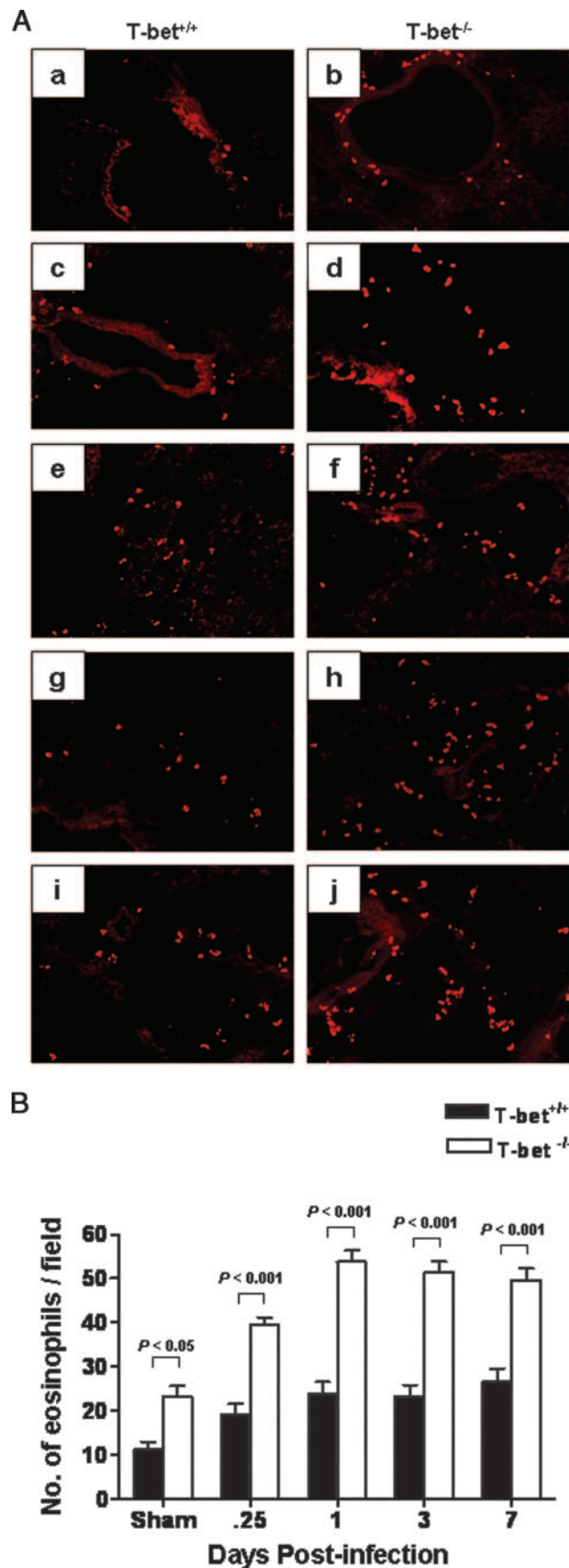


FIGURE 7. T-bet^{-/-} mice show greater infiltration of eosinophils in the lungs following *M. pulmonis* infection. Phenol red stained cryosections from *M. pulmonis*-infected T-bet^{+/+} and T-bet^{-/-} mice were evaluated at

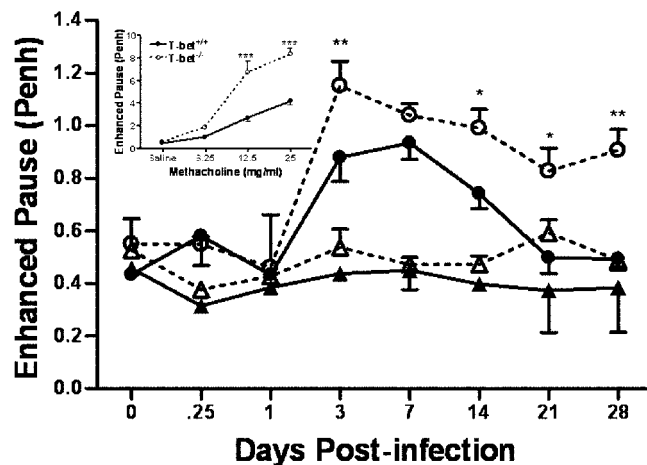


FIGURE 8. Airway obstruction and resistance induced by *M. pulmonis* in T-bet^{+/+} and T-bet^{-/-} mice. Data represent the Penh values for sham-inoculated T-bet^{+/+} (▲) and T-bet^{-/-} (△), and *M. pulmonis*-infected T-bet^{+/+} (●) and T-bet^{-/-} mice (○) measured at various times ranging from 6 h to 28 days PI. Data shown are representative of two independent experiments. Error bars represent the mean $\pm$ SEM ($n = 5$ /group), and the asterisks denote significant differences from the corresponding *M. pulmonis*-infected T-bet^{+/+} mice (*, $p < 0.05$; **, $p < 0.01$). Inset shows the AHR of uninfected T-bet^{+/+} and T-bet^{-/-} mice in response to increasing doses of nebulized methacholine.

responsible for insufficient expression of iNOS leading to decreased NO production. Lower IFN- γ levels often are associated with the establishment of a Th2 environment with respect to cytokine production. This bias toward a Th2-type immune response was reflected in the elevated IL-4:IFN- γ ratio found in *M. pulmonis*-infected T-bet^{-/-} mice, a similar elevation also is seen in patients with mycoplasmal pneumonia (42). Remarkably, an imbalance in the IL-4:IFN- γ ratio also has been observed in individuals with a family history of atopy (43), a heritable predisposition to developing asthma and other allergic etiologies. Atopic individuals, particularly children with moderately severe chronic asthma, produce significantly more IL-4 and less IFN- γ than do nonasthmatic individuals. Taken together, these results suggest a synergistic relationship exists between *Mycoplasma* infection and asthma wherein both respiratory syndromes are exacerbated by their coincidence and an imbalance between Th2 and Th1 cytokines that ensues. Despite the fact that IL-4 levels in T-bet^{-/-} mice were higher than in their T-bet^{+/+} counterparts at day 3 PI, overall release of this Th2 cytokine by either mouse strain was negligible, suggesting that its disease-modulatory abilities are limited. In keeping with this notion, IL-4 fails to play a critical role in the control of *Mycoplasma* infection in the lower respiratory tract (32), or in *Mycoplasma*-induced AHR (44), a pathologic feature also associated with atopy. Levels of IL-5, another important Th2 cytokine, also were elevated in T-bet^{-/-} mice early in the infectious process. However, unlike IL-4, IL-5 may be a contributory

6 h (c and d), 1 (e and f), 3 (g and h), and 7 (i and j) days PI (A). The eosinophils were stained bright red and were found in greater numbers in the lungs of T-bet^{-/-} mice and quantified by counting the number of eosinophils as described in *Materials and Methods* (B). The results are representative of three independent experiments ($n = 4$ –6/group) (magnification, $\times 200$). Comparison between the groups was made using one-way ANOVA.

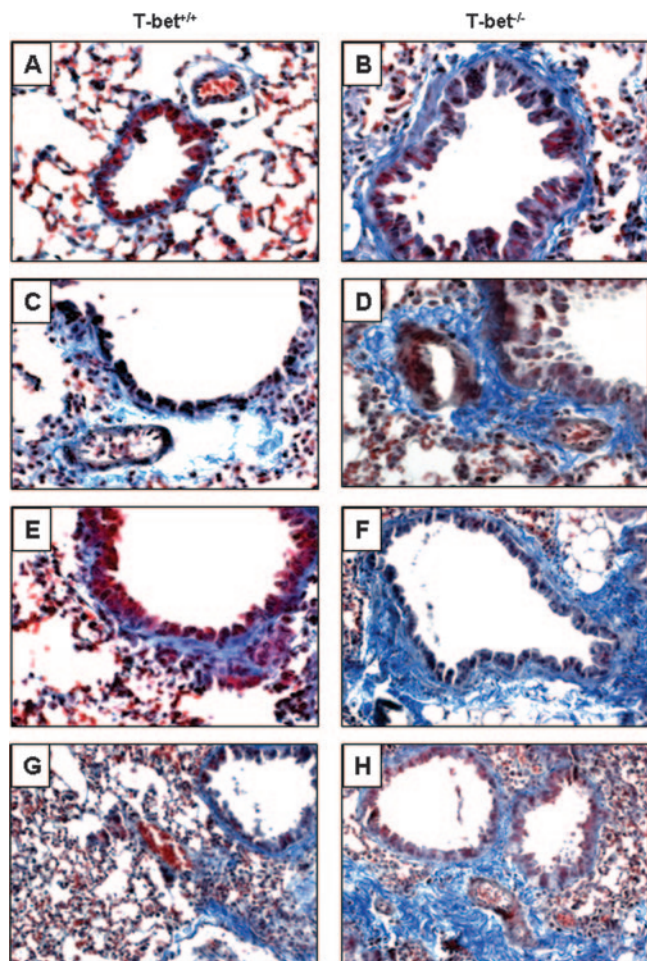


FIGURE 9. *M. pulmonis*-induced collagen deposition in the airways of T-bet^{+/+} and T-bet^{-/-} mice. Lung sections of T-bet^{+/+} and T-bet^{-/-} mice infected with *M. pulmonis* were stained with Masson Trichrome stain and evaluated for the deposition of collagen around the airways at day 14 (C and D), 21 (E and F), and 28 (G and H) PI. Sham-inoculated T-bet^{+/+} and T-bet^{-/-} mice were used as controls (A and B) (magnification, $\times 200$).

factor in the pathophysiological changes associated with both mycoplasmosis and asthma. Namely, increased production of IL-5 might support increased infiltration of eosinophils into the lung parenchyma, a feature of *M. pulmonis*-infected T-bet^{+/+} mice and one that was more prominent in asthmatic mice.

T-bet deficiency does not solely affect innate immune responses, but also impacts the development of adaptive immunity, which is essential for host defense against respiratory pathogens. We found that T-bet^{-/-} mice had an altered repertoire of cells infiltrating the lungs. The BALF of T-bet^{-/-} mice had significantly lower numbers of lymphocytes compared with their T-bet^{+/+} counterparts, a finding consistent with the observation that selective Th1 lymphocyte trafficking is impaired in T-bet^{-/-} mice (45). Inappropriate homing of lymphocytes to the lungs of T-bet^{-/-} mice may increase their susceptibility to *M. pulmonis* infection. T-bet also has been identified as an Ab isotype-specific regulator. T-bet transactivates the classical IFN- γ -related Th1 Ig isotypes IgG2a, IgG2b, and IgG3 and inhibits the Th2-related isotypes IgG1 and IgE (46). T-bet^{-/-} mice were found to produce excess amounts of *M. pulmonis*-specific IgG1 in the serum, IgA and IgE in BALF, and lower levels of serum IgG2b. Increasing evidence suggests that IgG1 is capable of mediating not only immediate hypersensitivity

independent of IgE, but also AHR (47, 48). In addition, IgG1 and IgE mediate activation of mast cells and the increased release of Th2 cytokines such as IL-4, IL-5, and IL-13, which enhance eosinophil recruitment to sites of inflammation (49). Elevated IgE and IgA levels in the BALF of T-bet^{-/-} mice likely reflect persistent stimulation of the respiratory mucosa by *M. pulmonis*. Elevated IgA levels also are associated with the asthmatic phenotype and AHR (50, 51). IgG2b Abs play a major role in the clearance of *M. pulmonis*, and failure to mount an IgG2b response is associated with increased disease susceptibility (52, 53). Thus, our results suggest that bacterially induced elevations in IgG1, IgE, and IgA levels exacerbate the asthmatic phenotype, whereas reduced production of IgG2b enhances the susceptibility of T-bet^{-/-} mice to infection by *M. pulmonis*.

Finally, we observed that *M. pulmonis* infection significantly increased airway obstruction (as demonstrated by elevated Penh) and the deposition of type III collagen in T-bet^{-/-} mice, pathophysiological changes also seen in asthmatic patients (15, 54). The higher Penh values seen in both the T-bet^{-/-} and T-bet^{+/+} mice coincides with suppressed IFN- γ levels. It is this lack of IFN- γ production early in the infectious process that may underlie the greater *M. pulmonis*-induced obstruction of airways in an asthmatic background. In addition to depressed IFN- γ production and elevated Th2-type Ab levels, the greater neutrophilic and eosinophilic infiltration seen at days 3 and 7 PI may underlie the sudden rise in Penh observed in T-bet^{-/-} and T-bet^{+/+} mice, though to a greater extent in T-bet^{-/-} mice. An association between eosinophils and AHR is well documented in human subjects, but in mice their relationship is still controversial (55–57). Recent evidence suggests that eosinophil-independent pathways contribute to AHR wherein murine IgG1 potentiates lung eosinophilic inflammation and AHR (49). The increased obstruction of airway observed in infected T-bet^{-/-} mice also is associated with more severe tissue inflammation and greater remodeling of the asthmatic airways. Compelling evidence now suggests these effects may be mediated by profibrotic cytokines such as IL-13 and TGF- β . Neutralization with anti-IL-13 Abs reduces airway inflammation, AHR, and subsequent airway remodeling in T-bet^{-/-} mice, and neutralization of TGF- β ameliorates collagen deposition as well (16). Furthermore, *M. pneumoniae* infection of allergen-challenged mice induces collagen deposition, which is associated with increased expression of TGF- β in the airway wall (58).

In conclusion, we have shown that in T-bet^{-/-} mice that spontaneously develop asthma-like symptoms, the airways provide a fertile environment that promotes colonization by *Mycoplasma* spp. and one which is ill-equipped to kill and clear respiratory pathogens. For its part, *M. pulmonis* infection triggers the early release of immunomodulators, which furthers the development of a Th2-type inflammatory milieu in the lungs and exacerbates the symptoms associated with asthma. Future studies directed at the identification of cytokines (e.g., IL-13 and TGF- β) and cell types (e.g., eosinophils and macrophages) that may sit at the immunomodulatory “nexus” of mycoplasmosis and asthma should provide rational targets for therapeutic intervention.

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Disclosures

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