Role of Th1/Th17 Balance Regulated by T-bet in a Mouse Model of *Mycobacterium avium* Complex Disease

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Role of Th1/Th17 Balance Regulated by T-bet in a Mouse Model of Mycobacterium avium Complex Disease

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Th1 immune responses are thought to be important in protection against intracellular pathogens. T-bet is a critical regulator for Th1 cell differentiation and Th1 cytokine production. The aim of this study was to determine the role of T-bet in host defense against Mycobacterium avium complex (MAC) infection. Wild-type mice, T-bet–deficient mice, and T-bet–overexpressing mice were infected with MAC via intratracheal inoculation. Macrophages and dendritic cells obtained from these mice were incubated with MAC. T-bet–deficient mice were highly susceptible to MAC, compared with wild-type mice and T-bet–overexpressing mice. Neutrophilic pulmonary inflammation was also enhanced in T-bet–deficient mice, but attenuated in T-bet–overexpressing mice, following MAC infection. Cytokine expression shifted toward Th1 in the lung and spleen of T-bet–overexpressing mice, but toward Th17 in T-bet–deficient mice. IFN-γ supplementation to T-bet–deficient mice reduced systemic MAC growth but did not reduce pulmonary inflammation. In contrast, neutralization of IL-17 in T-bet–deficient mice reduced pulmonary inflammation but did not affect mycobacterial growth in any organs tested. T-bet–deficient T cells tended to differentiate toward Th17 cells in vitro following exposure to MAC. Treatment with NO donor suppressed MAC-induced Th17 cell differentiation of T-bet–deficient T cells. This study identified that the fine balance between Th1 and Th17 responses is essential in defining the outcome of MAC disease. T-bet functions as a regulator for Th1/Th17 balance and is a critical determinant for host resistance to MAC infection by controlling cytokine and NO levels. The Journal of Immunology, 2014, 192: 1707–1717.

Non-tuberculous mycobacteria (NTM) are ubiquitous environmental organisms that cause a variety of infections in humans. NTM incidence is increasing in industrialized countries, where isolates of NTM outnumber those of Mycobacterium tuberculosis in mycobacterial pulmonary diseases (1). Mycobacterium avium complex (MAC) is the most common cause of NTM disease in humans. MAC causes disseminated infection in several immunodeficiency diseases, such as HIV/AIDS. Pulmonary MAC disease develops in individuals without clearly recognized immunodeficiency, especially in slender, postmenopausal women, suggesting that both environmental exposure and host susceptibility are required for the establishment of pulmonary MAC disease.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; iNOS, inducible NO synthase; MAC, Mycobacterium avium complex; MOI, multiplicity of infection; NTM, nontuberculous mycobacteria; qRT-PCR, quantitative RT-PCR; ROR, retinoic acid–related orphan receptor; SNAP, S-nitroso- N-acetylpenicillamine; T-bet−/− mice, T-bet knockout mice; T-bet+tg/− mice, T-bet-overexpressing transgenic mice.

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However, host factors that regulate MAC susceptibility have not been elucidated in human patients or animal models. Mycobacteria are intracellular pathogens that grow in macrophage phagolysosomes. Th1 immune responses are thought to be important in protection against intracellular pathogens. The Th1 cytokine IFN-γ activates NO production in macrophages, which subsequently enhances mycobacterial activities (2). IL-12 acts as a linker of innate and acquired immunities by inducing Th1 cell differentiation, releasing IFN-γ from Th1 cells, and activating macrophages (2, 3). Both cytokines are involved in protection against MAC infection because patients with defects in IFN-γ/IL-12 signaling are more susceptible to disseminated and extra-pulmonary MAC diseases (4, 5).

Naive CD4+ T cells differentiate into numerous Th cell subsets, such as Th1, Th2, Th17, and regulatory T cells, a process regulated by specific transcription factors (6, 7). Among them, T-bet is a critical regulator of the Th1 differentiation and Th1 cytokine production (8). T-bet also induces Th1 cytokine production and orchestrates Th1 cell migration by regulating the expression of chemokines and their receptors (9). In addition to promoting Th1 cell differentiation, T-bet blocks Th2 cell differentiation by sequestering the Th2-specific transcription factor GATA-3 from the IL-5 and IL-13 promoters (8, 10). Indeed, T-bet–deficient (T-bet−/−) mice exhibit greater susceptibility to several intracellular pathogens, including Leishmania major, M. tuberculosis, and Salmonella typhimurium (11–13). However, the role of T-bet in the pathogenesis of MAC diseases remains unclear.

In the present study, we established a mouse model of MAC disease by intratracheal inoculation of clinically isolated mycobacteria. We investigated the role of T-bet in the development of MAC infection using both T-bet−/− mice and transgenic (T-bet+tg/−) mice overexpressing T-bet. Unexpectedly, we observed that the Th1/Th17 balance, but not the Th1/Th2 balance, was regulated by
T-bet and was the key determinant for susceptibility and inflammatory responses to MAC in mice.

Materials and Methods

Mycobacteria

*M. avium* subsp. *hominissuis* was grown to mid-log phase in Middlebrook 7H9 liquid medium, aliquoted, and frozen at −80°C until use. Bacterial counts in each organ were determined by plating serial dilutions of organ homogenates of individual mice onto 7H10 agar plates and counting bacterial colonies 2 wk after infection. CFU are expressed as the mean CFU from eight individual mice.

Mice and infection

*T-bettg/tg* mice were generated as previously described (14) and backcrossed to BALB/c mice for eight generations. *T-bet−/−* BALB/c mice were obtained from the The Jackson Laboratory (Bar Harbor, ME). Wild-type BALB/c mice were purchased from Charles River Breeding Laboratories (Kanagawa, Japan). Female mice (8–12 wk old) were used in all experiments. Mice were anesthetized with isoflurane and intubated via the trachea with a 22-gauge i.v. catheter, followed by administration of 1 × 107 CFU *M. avium* in 50 μl sterile saline. Control mice were treated with 50 μl saline. All animal studies were approved by the Institutional Review Board.

Histology

Lung sections were stained with H&E. Zielhl–Neelsen stain was used to detect bacilli.

Bronchoalveolar lavage

Lungs were lavaged with six sequential 1-ml aliquots of saline. Cells were counted using a hemocytometer and differential cell counts were obtained after staining with Diff-Quick.

RT-PCR

Total RNA was extracted from lungs, CD4+ T cells, cultured macrophages, and cultured dendritic cells. Real-time quantitative RT-PCR (qRT-PCR) was performed using an ABI 7500 (Applied Biosystems). PCR primers used in this study are listed in Table I. The gene expression levels for each sample were amplified using the ΔΔCT method (15) and normalized against GAPDH mRNA.

Flow cytometry

Lungs were removed 2 mo after infection, digested with 75 U/ml collagenase (type 1; Sigma-Aldrich) at 37°C for 90 min, and isolated cells were filtered through a 20-μm nylon mesh. Cells were then stained with anti-CD4 and anti-TCRαβ Abs (BioLegend) to detect T cells, or with anti-F4/80 Ab (BioLegend) to detect macrophages, and analyzed by flow cytometry. T cell production of intracellular cytokines was determined by flow cytometric analysis using PE-conjugated anti-IFN-γ (BioLegend), anti-mouse IL-17 (BD Pharmingen), or anti-mouse TNF-α (BioLegend), as described previously (16). TNF-α production in macrophages was also determined by flow cytometry, as described previously (17).

Cytokine production in CD4+ T cells

Spleens were removed 2 mo after infection and digested with 75 U/ml collagenase and filtered through a 20-μm nylon mesh. CD4+ cells were purified using a MACS system with anti-CD4 mAb (Miltenyi Biotec). CD4+ T cells (2 × 106 cells/well) were activated for 24 h with anti-CD3 and CD28 mAb using a T cell activation kit (Miltenyi Biotec). TNF-α, IL-17, and IL-2 concentration were determined by ELISA (R&D Systems).

Supplementation with IFN-γ

*T-bet−/−* mice were treated with 10 μg recombinant murine IFN-γ (PeproTech) or PBS i.p. three times a week during 2 mo of MAC infection.

Anti–IL-17 Ab treatment

To neutralize IL-17, 900 μg rabbit anti–IL-17 polyclonal Ab (gift of Drs. Pam Lincoln and Steven L. Kunkel, University of Michigan) was injected i.p. into *T-bet−/−* mice three times a week during 1 mo of infection. Preimmune IgG was also used as a control.

Statistical analysis

Data are expressed as the means ± SEM. Data comparisons among the experimental groups were performed using one-way ANOVA followed by post hoc tests. Survival data were analyzed by a Kaplan–Meier and log-rank test. The p < 0.05 were considered to be statistically significant.

Results

Susceptibility to MAC is enhanced in mice lacking T-bet

To assess the influence of T-bet on the susceptibility to MAC, we evaluated the survival of wild-type, *T-bet−/−*, and *T-bettg/tg* mice following MAC infection. All *T-bet−/−* mice died within 2 mo following MAC infection, compared with 30% of wild-type mice or *T-bettg/tg* mice at 10 mo (Fig. 1A). The survival rate following MAC infection was significantly lower in *T-bet−/−* mice than in wild-type mice or *T-bettg/tg* mice (Fig. 1A). All saline-administered control mice survived during observation.

We then evaluated mycobacterial burden in wild-type, *T-bet−/−*, and *T-bettg/tg* lungs following MAC infection. Acid-fast bacilli were most prominently observed in macrophages and granulomatous lesions of *T-bet−/−* lungs, but they were scarcely observed in wild-type and *T-bettg/tg* lungs 2 mo after MAC infection (Fig. 1B). Measurement of organ CFU revealed elevated mycobacterial counts in lung, spleen, and liver of *T-bet−/−* mice relative to those in wild-type and *T-bettg/tg* mice (Fig. 1C). These results indicate that mice lacking T-bet have a high susceptibility to MAC infection.

MAC-induced pulmonary inflammation is regulated by T-bet

Inflammatory cell infiltration was observed in peribronchial and perivascular regions with granuloma formation in the lungs of wild-type mice following MAC infection (Fig. 2A). The inflammatory cell infiltration was more severe and extended to the alveolar region in *T-bet−/−* lungs, but it was less severe in *T-bettg/tg* lungs (Fig. 2A). No abnormal findings were observed in saline-administered controls (Fig. 2A).

The number of BAL-recovered inflammatory cells, particularly neutrophils, was increased in all genotypes following MAC infection. Among MAC-infected mice, the number of neutrophils and macrophages was significantly higher in *T-bet−/−* mice, but lower in *T-bettg/tg* mice, than in wild-type mice (Fig. 2B). The number of lymphocytes was higher in *T-bet−/−* mice than in other genotypes (Fig. 2B). These results indicate that pulmonary inflammation in response to MAC is regulated by T-bet.

Pulmonary Th1/Th17 balance is regulated by T-bet after MAC infection

We then assessed lung Th cytokine expression in wild-type, *T-bet−/−*, and *T-bettg/tg* mice following MAC infection using above-mentioned primers (Table I). Lung IFN-γ expression increased in
all MAC-infected mice, but it was significantly lower in T-bet−/− and higher in T-bet+/tg relative to wild-type mice (Fig. 3A). Similarly, lung inducible NO synthase (iNOS) expression was significantly lower in T-bet−/− and higher in T-bet+/tg relative to wild-type mice (Fig. 3A). Lung TNF-α expression was also significantly lower in T-bet−/− mice than in other genotypes (Fig. 3A). IL-4 expression was not induced in the lungs of any mice following MAC infection (Fig. 3A). Lung IL-17 and IL-6 expression increased in MAC-infected mice. Lung IL-17 expression was significantly higher in T-bet+/tg but lower in T-bet+/tg mice relative to wild-type mice, and IL-6 expression was elevated in T-bet−/− lungs relative to other genotypes (Fig. 3A). Pulmonary IL-10 expression was significantly higher in T-bet−/− mice than in other genotypes (Fig. 3A). Although pulmonary expression of IL-12p40 and IL-23p19 was induced in all genotypes following MAC infection, the expression levels were not different among all genotypes (Fig. 3A). These results indicate that Th1 cytokines are induced in wild-type lungs and more strongly in T-bet+/tg lungs following MAC infection. In contrast, Th17 cytokines but not Th2 cytokines are induced in the lungs of T-bet−/− mice after MAC infection. Thus, the pulmonary Th1/Th17 balance, but not Th1/Th2 balance, is regulated by T-bet during MAC infection.

Production of Th1 and Th17 cytokines in Th cells is regulated by T-bet

We assessed the production of IFN-γ and IL-17 in CD4+ T cells obtained from lungs of wild-type, T-bet−/−, and T-bet+/tg mice to assess the contribution of CD4+ T cells to Th1 and Th17 cytokine production. IFN-γ–producing CD4+ T cells increased in the lungs of all mice after MAC infection (Fig. 3B). Among MAC-infected mice, IFN-γ–producing CD4+ T cells were increased significantly in T-bet+/tg lungs but decreased in T-bet−/− lungs relative to wild-type mice (Fig. 3B). The proportion of IL-17–producing CD4+ T cells was elevated in wild-type and T-bet−/− lungs, but not in T-bet+/tg lungs, after MAC infection (Fig. 3B). Among MAC-infected mice, IL-17–producing CD4+ T cells were elevated in the lungs of T-bet−/− mice but reduced in T-bet+/tg mice relative to wild-type mice (Fig. 3B). These results indicate that production of Th1 and Th17 cytokines by CD4+ T cells is regulated by T-bet in MAC-infected lungs.

To clarify which cells are responsible for T-bet–regulated TNF-α production, we assessed the production of TNF-α in T cells and macrophages obtained from lungs of wild-type, T-bet−/−, and T-bet+/tg mice. TNF-α–producing CD4+ T cells were increased in the lungs of all mice after MAC infection (Fig. 3C). Among MAC-infected mice, the proportion of TNF-α–producing CD4+ T cells was significantly lower in T-bet+/tg lungs relative to wild-type and T-bet−/− lungs (Fig. 3C). Although TNF-α–producing macrophages were increased in the lungs of all mice after MAC infection, there was no significant difference in the number among mice genotypes (Fig. 3C). These results indicate that lower TNF-α expression in the lungs of T-bet−/− mice after MAC infection is due to lower production of TNF-α by T cells.
Th1/Th17 responses following MAC infection are systemically regulated by T-bet

To evaluate whether Th1/Th17 bias occurred systemically, we assessed the expression of Th-specific transcription factors and cytokine production in CD4+ splenocytes in wild-type, T-bet−/−, and T-bet+/− mice following MAC infection. T-bet expression was elevated in wild-type and T-bet+/− splenocytes, but not in T-bet−/− splenocytes following MAC infection (Fig. 4A). T-bet expression was significantly higher in T-bet+/− splenocytes than in wild-type splenocytes (Fig. 4A). In contrast, expression of retinoic acid-related orphan receptor (ROR)γt was markedly increased in CD4+ splenocytes from T-bet−/− mice relative to other genotypes (Fig. 4A). Expression of GATA-3 was not elevated in any mice (Fig. 4A). Expression of Foxp3 was elevated in T-bet+/− and other groups following MAC infection. Th1/Th17 cell differentiation and IL-17 secretion was increased from cells of all mouse genotypes, and lowest in T-bet+/− mice relative to other genotypes (Fig. 4B). There was a marked increase in IL-4 levels across all genotypes (Fig. 4B). These results indicate that Th1/Th17 cell differentiation and T-bet+/− cytokine production are systemically regulated by T-bet following MAC infection. These results also suggest that T-bet suppresses RORγt expression in CD4+ splenocytes.

IFN-γ supplementation increases resistance to MAC infection

Because IFN-γ production was reduced in T-bet−/− mice following MAC infection, we sought to assess whether IFN-γ supplementation affects the development of MAC disease in these mice. To assess the effect of IFN-γ on the susceptibility to MAC, organ CFU was evaluated in MAC-infected T-bet−/− mice treated with IFN-γ or vehicle. MAC CFU was significantly lower in the

Table I. Primers used for RT-PCR

<table>
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<th>Primer Target</th>
<th>Sequence</th>
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<tr>
<td>GAPDH</td>
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<tr>
<td></td>
<td>5′-CTTGATGTCAGCACAATCTCC-3′ (reverse)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5′-CCAGCAGACGTCCATGGAAGA-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-TCCTGTCATCCAGAGATTTTC-3′ (reverse)</td>
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<td>IL-17</td>
<td>5′-AAGACCTTCCGAGTTCGAAAC-3′ (forward)</td>
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<td></td>
<td>5′-TGGAACGGTTGAGGTAGTCTG-3′ (reverse)</td>
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<td>5′-CCGCACTTGCGAGAGCGTTC-3′ (reverse)</td>
</tr>
<tr>
<td>INOS</td>
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</tr>
<tr>
<td></td>
<td>5′-GTTTAAAGGTCGCGCCCT-3′ (reverse)</td>
</tr>
<tr>
<td>IL-23p19</td>
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<td>5′-TGGAACGGTTGAGGTAGTCTG-3′ (reverse)</td>
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<td></td>
<td>5′-GCTAGACGTGAGGCTCACAG-3′ (reverse)</td>
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Pulmonary cytokine expression is regulated by T-bet following MAC infection. (A) Expression of IFN-γ, iNOS, TNF-α, IL-4, IL-17, IL-6, IL-10, IL-12p40, and IL-23p19 in the lung of wild-type, T-bet−/−, and T-betigg/tg mice 2 mo after intratracheal inoculation of 1 × 10⁷ CFU MAC, analyzed by qRT-PCR (filled bars). Control mice were administered saline (open bars). The y-axis of each graph represents the relative expression of the respective genes calculated using the ΔΔCT method and normalized against GAPDH mRNA. (B) The proportion of IFN-γ–producing cells (left panel) or IL-17–producing cells (right panel) in CD4⁺ cells obtained from the lungs of wild-type, T-bet−/−, and T-betigg/tg mice 2 mo after intratracheal inoculation of 1 × 10⁷ CFU MAC or saline (Cont) was analyzed by flow cytometry. (C) The proportion of TNF-α–producing cells in CD4⁺ cells (left panel) or F4/80⁺ macrophages (right panel) obtained from the lung of wild-type, T-bet−/−, and T-betigg/tg mice 2 mo after intratracheal inoculation of 1 × 10⁷ CFU MAC or saline (Cont) was analyzed by flow cytometry. Experiments were performed in duplicate with four mice in each group. Data are expressed as means ± SEM. *p < 0.05 between T-bet−/− mice and other groups after MAC infection, #p < 0.05 between T-betigg/tg mice and other groups after MAC infection.
cell count and the numbers of macrophages, neutrophils, and lymphocytes in BAL fluids were similar regardless of IFN-γ treatment (Fig. 5B). These results indicate that IFN-γ supplementation reduces the growth and systemic expansion of MAC, but not pulmonary inflammation, in T-bet<sup>−/−</sup> mice.

Neutralization of IL-17 decreases MAC-induced pulmonary inflammation

We next sought to assess whether neutralization of IL-17 affected the development of MAC disease in T-bet<sup>−/−</sup> mice because IL-17 production was elevated following MAC infection. Treatment of T-bet<sup>−/−</sup> mice with anti–IL-17 Ab did not affect MAC CFU in the lung, spleen, or liver following MAC infection (Fig. 6A). However, there was a decrease in total cell count and, particularly, the number of neutrophils in BAL fluids when treated with anti–IL-17 Ab (Fig. 6B). The number of macrophages and lymphocytes was also decreased after treatment with anti–IL-17 Ab (Fig. 6B). These results indicated that neutralization of IL-17 decreased MAC-induced pulmonary inflammation, but did not affect mycobacteria growth.

T-bet does not affect intrinsic ability of macrophages and dendritic cells after MAC infection

Macrophages are known target cells for MAC infection and are the main effector cells for mycobacteria killing. To assess whether T-bet affects macrophage anti-MAC activity, the effects of IFN-γ on mycobacteria growth was evaluated in cultured macrophages obtained from wild-type, T-bet<sup>−/−</sup>, and T-bet<sup>tg/tg</sup> mice. Although MAC CFU were decreased in macrophages from all genotypes following IFN-γ treatment, the CFU were not different among the genotypes (Fig. 7A). Taken together, these results indicate that macrophage IFN-γ-stimulated antimicrobial activity is similar among all mice examined.

Dendritic cells are professional APCs that promote Th differentiation by producing several immunoregulatory cytokines. To assess whether T-bet affected cytokine production in dendritic cells, the expression of the Th1-directing cytokine IL-12 and the Th17-directing cytokines IL-6, IL-23, and TGF-β was examined in cultured dendritic cells obtained from wild-type, T-bet<sup>−/−</sup>, and T-bet<sup>tg/tg</sup> mice following exposure to MAC. Although the expression of IL-6 and IL-12p40 significantly increased in dendritic cells of all genotypes after exposure to MAC, the expression level was similar among all genotypes (Fig. 7A). The expression of IL-23p19 and TGF-β remained low after exposure to MAC in dendritic cells of all genotypes (Fig. 7B). The expression of these cytokines was also examined in cultured macrophages. Although the expression of IL-6, IL-12p40, IL-23p19, and TGF-β significantly increased in macrophages of all genotypes after exposure to MAC, the expression level was similar among all genotypes (Fig. 7A).
These results suggest that the T-bet did not directly affect anti-MAC activity or production of immunoregulatory cytokines in response to MAC in macrophages and dendritic cells.

**T-bet suppresses Th17 differentiation by controlling NO level after MAC exposure**

To assess Th cell differentiation in vitro, we cultured naive Th cells from wild-type, T-bet\(^{-/-}\), and T-bet\(^{tg/tg}\) mice with wild-type dendritic cells with or without exposure to MAC. The concentration of both IFN-\(\gamma\) and IL-17 was significantly higher in the coculture media of all genotypes 72 h after exposure to MAC than those without exposure to MAC, suggesting that naive T cells differentiated into both Th1 cells and Th17 cells in vitro in response to MAC (Fig. 8A). Among the genotypes, significantly lower levels of IFN-\(\gamma\) and higher levels of IL-17 were detected in the culture media of T-bet\(^{-/-}\) mice–derived Th cells. This indicated that naive Th cells from T-bet\(^{-/-}\) mice tend to differentiate toward Th17 but not toward Th1 by exposure to MAC (Fig. 8A). Similar to the lung tissue after MAC infection, the expression of iNOS was significantly lower in the culture cells derived from T-bet\(^{-/-}\) mice (Fig. 8B). A recent study demonstrated that NO plays a negative role in the regulation of Th17 cell differentiation (19). We therefore assessed the role of NO in Th17 cell differentiation using this coculture system. Treatment with SNAP, an NO donor, significantly suppressed the MAC-induced increase in IL-17 levels, and most prominently in the culture media of T-bet\(^{-/-}\) mice–derived Th cells (Fig. 8C). These results suggest that NO suppressed MAC-induced Th17 cell differentiation. Thus, Th cells in T-bet\(^{-/-}\) mice tended to differentiate toward a Th17 phenotype because of low induction of iNOS in response to MAC.

**Discussion**

In the present study, we demonstrated that susceptibility and lung inflammatory responses to MAC were regulated by T-bet. T-bet is a pivotal factor for Th1 development as well as the production of Th1 cytokines, such as IFN-\(\gamma\). It was demonstrated that T-bet\(^{-/-}\)
All experiments were performed in duplicate with four mice in each group. The expression of IL-6, IL-12p40, IL-23p19, and TGF-β in cultured dendritic cells (B) and cultured macrophages (C) obtained from wild-type, T-bet<sup>-/-</sup>, and T-bet<sup>tg/tg</sup> mice following exposure to MAC or saline and analyzed by qRT-PCR. The y-axis of each graph represents the relative expression of the respective genes calculated using the ΔΔCT method and normalized against GAPDH mRNA. All experiments were performed in duplicate with four mice in each group. *p < 0.05 between MAC- and vehicle-treated groups.

mice have greater susceptibility to several intracellular pathogens, including *M. tuberculosis*, *L. major*, and *S. typhimurium*. T-bet<sup>-/-</sup> mice exhibit Th2-biased responses after *L. major* infection with increased IL-4 and IL-5 levels (11). However, a fully polarized Th2 response was not present, although a selective elevation of IL-10 was observed in these mice after infection with *M. tuberculosis* and *S. typhimurium* (12, 13). In contrast, T-bet<sup>-/-</sup> mice were not susceptible to *Listeria monocytogenes* because compensatory IFN-γ was produced by NK cells and CD8<sup>+</sup> T cells through T-bet-independent pathways (20). These findings indicate that the T-bet-mediated host immune responses differ dependent on the infectious pathogen.

Cytokine analysis revealed IFN-γ was induced in the lungs of wild-type mice, and more strongly in those of T-bet<sup>tg/tg</sup> mice, whereas IFN-γ was decreased in T-bet<sup>-/-</sup> lungs following MAC infection. It is well known that IFN-γ plays a critical role in protection against *Mycobacterium* infections. A lack of or decrease in IFN-γ may increase susceptibility to systemic MAC infection, because patients with IFN-γ autoantibodies often develop disseminated and extrapulmonary MAC disease (5). Correspondingly, MAC growth was increased in multiple organs of T-bet<sup>-/-</sup> mice. Moreover, administration of exogenous IFN-γ inhibited their outgrowth. Cytokine analysis also revealed that TNF-α levels decreased in T-bet<sup>-/-</sup> lungs following MAC infection. TNF-α is essential for host resistance against *Mycobacterium* infection. Indeed, increased susceptibility to *Mycobacterium* infection is a recognized side effect of treatment with TNF-α antagonists (21). TNF-deficient mice are susceptible to *M. tuberculosis* with failure of granuloma formation in the infected organs (22). Consistent with this, we observed poor granuloma formation in T-bet<sup>-/-</sup> lungs after MAC infection. Macrophages and T cells are considered to be the main source of TNF-α. Production of TNF-α in macrophages is regulated by TLR via activation of NF-κB and by IFN-γ via activation of IFN regulatory factors during infection (23, 24). The production of TNF-α in Th1 cells is transcriptionally regulated by T-bet (25, 26). In the present study, we found that TNF-α-producing T cells were decreased in T-bet<sup>-/-</sup> lungs, whereas TNF-α-producing macrophages did not differ among all genotypes after MAC infection. These findings suggest that low levels of TNF-α in T-bet<sup>-/-</sup> lungs were due to the reduced expression of TNF-α in T cells. Thus, T-bet is a critical host factor for resistance to systemic MAC infection by controlling the induction of Th1 cytokines.

It was proposed that susceptibility to mycobacteria could be explained by immune dominance of either a Th1 or Th2 phenotype, because Th2 cytokines IL-4 and IL-13 inhibit Th1-mediated mycobactericidal activity (27). However, most studies failed to reveal the presence of mycobacteria-specific Th2 cells (28, 29). The lack of T-bet easily allows Th2-dominant conditions in other models (11). However, in the present study, significant Th2 development, as defined by IL-4 production and GATA-3 expression, revealed the presence of mycobacteria-specific Th2 cells (28, 29). It was proposed that susceptibility to mycobacteria could be explained by immune dominance of either a Th1 or Th2 phenotype, because Th2 cytokines IL-4 and IL-13 inhibit Th1-mediated mycobactericidal activity (27). However, most studies failed to reveal the presence of mycobacteria-specific Th2 cells (28, 29). The lack of T-bet easily allows Th2-dominant conditions in other models (11). However, in the present study, significant Th2 development, as defined by IL-4 production and GATA-3 expression, could not be detected following MAC infection in T-bet<sup>-/-</sup> mice. Similar results were described in T-bet<sup>-/-</sup> mice following infection with *M. tuberculosis* (12). Thus, classic Th2 activation might not be a dominant determinant of host susceptibility to MAC in type 1 immunity-diminished condition.

In contrast, IL-17 and IL-6 were significantly induced in lungs and CD4-positive T cells as well as the expression of ROtyt in T-bet<sup>-/-</sup> mice following MAC infection, suggesting Th17-biased responses occur in T-bet<sup>-/-</sup> mice in response to MAC infection. Although Th17 immunity plays a central role in protection against extracellular pathogens (30), its role in regulating intracellular pathogens is not fully understood. Initial studies suggested the IL-17 pathway was not critical for protection against mycobacteria, such as *M. tuberculosis* and *M. bovis* (31). However, recent studies demonstrated that IL-17/Th17 cells are involved in antimycobacterial immunity. IL-17 enhances the migration of neutrophils to the site
of infection at the early stage of tuberculosis through the induction of CXC chemokines (32, 33). Th17 cells accelerate the accumulation of Th1 cells and enhance Th1 antimycobacterial responses (33–35). IL-17 also plays a role in the formation and maintenance of granuloma in mycobacteria-infected lungs (36, 37). However, re-exposure of tuberculosis-infected mice to high levels of tuberculosis Ag promotes further Th17 responses that cause extensive lung damage associated with elevated neutrophil recruitment (38). In the present study, IL-17 neutralization did not exacerbate bacterial burden in T-bet−/− mice. Therefore, it is likely that IL-17 does not contain direct antimycobacterial activity in our MAC infection model.

Neutrophilic pulmonary inflammation was enhanced in T-bet−/− mice following MAC infection. IL-17 was considered as an important mediator for inflammatory cells that produce various mediators that provoke neutrophil influx into the inflamed sites (39). Correspondingly, neutralization of IL-17 clearly attenuated MAC-induced neutrophil recruitment in these mice. Therefore, enhanced neutrophilic inflammation is associated with an increase of IL-17 in T-bet−/− mice. Neutrophils were the predominant cell type in BAL fluids from patients with pulmonary MAC disease (40). In these patients, neutrophilic pulmonary inflammation with decreased CD4+ lymphocytes reflected disease progression (41). In our MAC infection model, neutrophils were not essential for Mycobacterium killing because elevated MAC CFU were observed in T-bet−/− mice. Thus, Th17-derived neutrophil recruitment might have pathological effects, rather than being protective, during MAC infection under Th1-diminished conditions.

It was generally accepted that lineage-specific transcription factors could inhibit the differentiation of other Th subsets. However, it was reported that Th1/Th17 subsets that express CD4+ IFN-γ+IL-17+ were observed in peripheral blood and pleural fluid from patients with tuberculosis. This suggests a complex process of transcription factor–regulated T cell differentiation during infection (42). Strong Th17 responses were observed in T-bet−/− mice during allograft rejection, autoimmune myocarditis, and parasite infection (43–45). Lazarevic et al. (46) demonstrated that T-bet suppressed the expression of genes encoding RORγt (Rorc) and Th17 cytokines by interacting with the transcription factor Runx1, which repressed the transactivation of the Rorc promoter and subsequent commitment to the Th17 lineage. However, a recent study demonstrated that NO derived from iNOS in activated T cells inhibited Th17 cell differentiation by nitration of tyrosine residues in RORγt (19). Correspondingly, we found reduced ex-
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Dendritic cells are professional APCs with an important role in the development of immune responses to Mycobacterium. Cross-talk between dendritic cells and Th cells is a critical process for Th cell differentiation, and it is mediated by various cytokines. Among them, IL-12 is essential for Th1 cell differentiation, whereas IL-6 and IL-23 play a key role in Th17 cell differentiation (3, 48). TGF-β is required for both Th17 and regulatory T cell differentiation (7). Because T-bet is expressed in APCs, such as dendritic cells and macrophages (8), it should be elucidated whether T-bet regulates cytokine production in these cells. The present study revealed that cytokine production was not different whether T-bet regulates cytokine production in these cells. The results were obtained in cultured macrophages. These observations suggest that APCs can produce both Th1- and Th17-directing cytokines as well as T-bet independently of stimulation by MAC. However, Th1 differentiation is selectively inhibited in T-bet-deficient naive T cells. Thus, T-bet may regulate susceptibility and inflammatory responses to MAC by modulating cell differentiation and cytokine production in Th cells but not by affecting the intrinsic ability of APCs. The putative mechanisms of T-bet-regulated host responses to MAC are summarized in Fig. 9.

In conclusion, we demonstrated that T-bet regulated susceptibility and inflammatory responses to MAC. T-bet-deficient mice displayed diminished Th1 responses and development of Th17 responses. Reduced Th1 responses increased susceptibility to systemic MAC infection, and Th17 development caused excessive neutrophilic pulmonary inflammation. Thus, the fine balance between Th1 and Th17 responses, maintained by T-bet, is critical in determining susceptibility and inflammatory responses to MAC.

FIGURE 9. Schematic presentation of the role of Th1/Th17 balance regulated by T-bet following MAC infection. APCs, such as macrophages and dendritic cells, can produce both Th1- and Th17-directing cytokines T-bet–independently by stimulation with MAC. (A) When T-bet is expressed in Th cells, it enhances mycobacterial activities by promoting Th1 cell differentiation and IFN-γ production, followed by NO production. T-bet also suppresses aberrant inflammatory cell recruitment by controlling Th17 cell differentiation directly or indirectly by NO production. (B) When T-bet is not expressed in Th cells, naive T cells cannot differentiate to Th1 cells but easily differentiate to Th17 cells. Reduced Th1 responses increase susceptibility to systemic MAC infection, and Th17 development causes excessive neutrophilic pulmonary inflammation. Thus, the fine balance between Th1 and Th17 responses, maintained by T-bet, is critical in determining susceptibility and inflammatory responses to MAC.

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Disclosures
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