Diverse Roles for T-bet in the Effector Responses Required for Resistance to Infection

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Diverse Roles for T-bet in the Effector Responses Required for Resistance to Infection


The transcription factor T-bet has been most prominently linked to NK and T cell production of IFN-γ, a cytokine required for the control of a diverse array of intracellular pathogens. Indeed, in mice challenged with the parasite Toxoplasma gondii, NK and T cell responses are characterized by marked increases of T-bet expression. Unexpectedly, T-bet−/− mice infected with T. gondii develop a strong NK cell IFN-γ response that controls parasite replication at the challenge site, but display high parasite burdens at secondary sites colonized by T. gondii and succumb to infection. The loss of T-bet had a modest effect on T cell production of IFN-γ but did not impact on the generation of parasite-specific T cells. However, the absence of T-bet resulted in lower T cell expression of CD11a, Ly6C, KLRG-1, and CXCR3 and fewer parasite-specific T cells at secondary sites of infection, associated with a defect in parasite control at these sites. Together, these data highlight T-bet–independent pathways to IFN-γ production and reveal a novel role for this transcription factor in coordinating the T cell responses necessary to control this infection in peripheral tissues. The Journal of Immunology, 2015, 194: 000–000.

The T-box transcription factor T-bet is expressed in cells of the innate and adaptive immune system (1–4), but it is perhaps most prominently linked to the production of IFN-γ in T cells and NK cells (1, 2, 5). Although many studies have focused on the role of T-bet in CD4+ T cell production of IFN-γ, there are studies in which it also impacts on the ability of NK and CD8+ T cells to produce IFN-γ (6–9). In addition, T-bet has been shown to bind to the promoter region of other genes, suggesting a broader function in the immune response. For example, T-bet controls key checkpoints in NK cell maturation (10) and also inhibits T cell production of IL-2, IL-4, and IL-17, thus repressing other CD4+ T cell differentiation fates that include Th2 and Th17 cells (1, 2, 11). Furthermore, T-bet induces expression of the chemokine receptor CXCR3, and in vitro studies have identified a role for T-bet in the chemotaxis of CD4+ T cells (12). Consistent with a key role for T-bet in the development of Th1 cells, this transcription factor is required for IFN-γ-mediated resistance to Leishmania major, Salmonella, Mycobacterium tuberculosis, and HSV-2 (2, 13–15). Although production of IFN-γ is also required to limit replication of Listeria monocytogenes and LCMV, the IFN-γ produced in the T-bet−/− mice is sufficient for controlling these pathogens (9, 16). One explanation for this phenomenon is that these T-bet–independent pathways to IFN-γ production are mediated through a related T-box transcription factor, Eomesodermin (5, 17).

In current models, challenge of mice with the intracellular parasite T. gondii results in the production of IL-12 by dendritic cells and macrophages, which promotes the activation and expansion of NK cell and T cell populations that express high levels of T-bet and are associated with the secretion of IFN-γ (18–22). The production of IFN-γ, which engages numerous antimicrobial mechanisms (23, 24), is essential for the local control of T. gondii in multiple tissues. The studies presented in this article demonstrate that when mice deficient in T-bet are challenged with T. gondii, they control parasite replication at the site of initial challenge, associated with strong NK cell responses, and can generate parasite-specific T cells that produce IFN-γ. However, these mice do not survive acute infection, and secondary sites of parasite colonization that include the heart, thymus, lung, and brain have high parasite burdens. The increased mortality is associated with alterations in the phenotype of parasite-specific T cell populations that include reduced expression of markers of activation (CD11a), effector status (Ly6C and KLRG-1), and trafficking (CXCR3), as well as a marked reduction in their numbers at these peripheral sites. The use of a mixed bone marrow chimeric approach revealed a cell-intrinsic requirement for T-bet for the development of appropriate parasite-specific effector T cells. Together, these data highlight a novel role of T-bet in coordinating the CD4+ and CD8+ T cell responses that are essential for the control of infection in peripheral tissues.

Materials and Methods
Mice, infection, and Ab treatment
T-bet-deficient (T-bet−/−), CBA/CaJ, Thy1.1+C57BL/6, and Swiss Webster mice were purchased from The Jackson Laboratory. Wild-type C57BL/6 (WT) mice were purchased from Taconic. Wild-type C57BL/6 mice were purchased from the National Cancer Institute. All mice were housed in a specific pathogen-free environment at the University of Pennsylvania School of Veterinary Medicine in accordance with federal regulations.

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Abbreviations used in this article: ALT, alanine aminotransferase; MFI, mean fluorescence intensity; MIIC-II, MHC class II; PEC, peritoneal exudate cell; qPCR, quantitative PCR; STAg, soluble T. gondii Ag; WT, wild-type C57BL/6.

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guidelines and with approval of the Institutional Animal Care and Use Committee. The ME49 strain of T. gondii was maintained in Swiss Webster and CBA/Ca mice, and was used as a source of tissue cysts for i.p. (10–20 cysts) infections. Soluble Toxoplasma Ag was prepared from the RH strain of T. gondii as previously described (25). For IFN-γ neutralization experiments, mice were treated with 1 mg anti–IFN-γ or isotype control on days 0, 3, and 6 of infection. For depletion of NK cells, mice were treated with 50 μl anti-Asialo gm-1 or isotype control on days −1, 3, and 6 of infection. For infections of bone marrow, mice were irradiated (1000 rad) and i.v. injected with a 1:1 mixture of 6 × 10⁶ cells infected with WT (CD45.2⁺Thy1.1⁺) and T-bet⁻ (CD45.2⁺Thy1.1⁻) bone marrow. Mice were given water containing sulfamethoxazole for the first 2 wk after irradiation. Mice were allowed to reconstitute >8 wk after irradiation and were infected with T. gondii as described.

Isolation and analysis of immune populations

Single-cell suspensions from the spleens, lymph nodes, and peritoneal exudate cells (PECs) were prepared as previously described (26). Lungs were inflated with a solution of 1 mg/ml collagenase A (Roche) and 2% normal goat serum, the sections were incubated either with anti-Asialo gm-1 or isotype control on days 1, 3, and 6 of infection. Cells were stained for intracellular markers in Foxp3 Permeabilization buffer (eBio-science), fixed with Foxp3 Fixation buffer (eBioscience), and stained for intracellular markers in Foxp3 Permeabilization buffer (eBio-science). To measure intracellular cytokine production, we cultured isolated cells in cRPMI (1% penicillin/streptomycin, 2 mM l-glutamine, 10% FBS, 0.1% 2-ME, 1% nonessential amino acids, and 1 mM sodium pyruvate) in triplicate at 1 × 10⁶ cells/ml in a 96-well U-bottom plate, with PMA and ionomycin for 4 h with brefeldin A (Sigma-Aldrich) and monensin (eBioscience). Cells were stained for surface markers at 4˚C, and intracellular cytokines were detected by staining in FACS buffer containing 0.5% saponin (Sigma-Aldrich). Splenocytes were also restimulated at 1 × 10⁶ cells/ml in cRPMI alone (media) or with either anti-CD3 (1 μg/ml) or soluble T. gondii Ag (STAg; 12.5 μg/ml) and incubated at 37˚C for 72 h. Sera were assayed for alanine aminotransferase (ALT) and supranatants and sera were assayed for IL-12p40 and IFN-γ by ELISA.

Flow cytometry and imaging

The following Abs were purchased from BD: CD3 FITC, K67 FITC, CD49b (DX5) PE, K67 AF700, CD4 Pacific Blue, PSGL-1 BV421, CD8α PE-CF594, CD3 PE-CF594, CD122 biotin, and streptavidin PE-Texas Red. The following Abs were purchased from eBioscience: CD25 FITC, Foxp3 AF488, CD11a FITC, NK1.1 FITC, CD19 FITC, CD4 FITC, KLRC1-FITC, CD8 PE, PerCP-Cy5.5, CD69 FITC, CD3 PerCP-Cy5.5, NKP46 eFluor660, T-bet eFluor660, CD11c PE-Cy7, KLRC1-PE-Cy7, IFN-γ PE-Cy7, CD25 allophycocyanin-eFluor780, CD3 allophycocyanin-eFluor780, Ly6C (clone HK1.4) PerCpCy5.5, CD69 PE-Cy7, NKP46 PE-eFluor660, CD11c PE-Cy7, NK1.1 FITC, CD8 PE, CD45RO PE, CD49b PE-Cy7, CD49b AF700, MHC class II (MHC-II) Pacific Blue, CD11a PerCp-Cy5.5, CXCR3 PE-Cy7, TCRβ AF700, CD3 Pacific Blue, and NK1.1 Pacific Blue. Invitrogen live/dead Aqua stain was used to determine viability. Biotinylated Tgd-057 MHC-I monomers were kindly provided by E. John Wherry (University of Pennsylvania) and tetramerized by incubation with streptavidin-conjugated PE. PE-conjugated AS-15 MHC-II tetramers were kindly provided by the National Institutes of Health Tetramer Facility and Marion Pepper (University of Washington). All samples were run on an LSRFortessa (BD) and analyzed using FlowJo software (Tree Star). Analysis and presentation of distributions was performed using SPICE version 5.35 (downloaded from http://exon.niaid.nih.gov/space) (27). Images were obtained using the ImageStream (Amnis) and analyzed using IDEAS software (Amnis). To determine T-bet localization, we made nuclear and cytoplasmic masking functions using DAPI staining; these masks were then applied to T-bet expression.

Parasite detection

For IHC detection of T. gondii, tissue samples were fixed in 10% formalin solution and then paraffin embedded and sectioned. Sections were deparaffinized, rehydrated, Ag retrieved in 0.01 M sodium citrate buffer (pH 6.0), and endogenous peroxidase blocked by 0.3% H₂O₂ in PBS. After blocking with 2% normal goat serum, the sections were incubated either with anti-Toxoplasma Ab or isotype control. The sections were then incubated with biotinylated goat anti-rabbit IgG (Vector, Burlington, CA), and ABC reagent was applied (Vectastain ABC Kit; Vector Labs). Then DAB substrate (Vector Labs) was used to visualize specific staining according to manufacturer’s instructions, and slides were counterstained with hematoxylin.

Results

Expression of T-bet during the immune response to Toxoplasma gondii

As part of studies to understand the role of T-bet during the immune response against T. gondii, the populations involved in protective immunity were surveyed for infection-induced alterations in T-bet expression and localization. In naive WT mice, there is heterogeneous expression of the integrin CD49b (DX5) within the NK cell population, with high expression indicative of NK cell maturity. In infected mice, T-bet is not highly expressed in NK cells, but after challenge with T. gondii, a population of mature DX5⁺ NK cells that express high levels of T-bet emerged (Fig. 1A). Similarly, based on the use of Ly6C and KLRC-1 as markers of CD4⁺ and CD8⁺ T cell effector populations, respectively (28–30), naive mice
T-bet+ CD4+ T cells expressed at least one of these activation markers CD11a, an integrin that, together with CD18, comprises LFA-1, CXCR3, and Ly6C; the number of these T-bet+Ly6C hi CD4+ T cells that emerged postinfection was significantly increased over naive mice (Fig. 1B). Similarly, at this time point, there was an increase in the number of T-bet+CD11a hi CD8+ T cell population compared with naive mice (Fig. 1B). Among the CD8+ T cells, T-bet was highly expressed in the infection-induced polyclonal CD11a hi population (Fig. 2C), and T-bet expression was closely associated with expression of CXCR3 and KLRG-1 (Supplemental Fig. 1). Moreover, consistent with the polyclonal CD11a hi population, when parasite-specific CD8+ T cells were identified using an MHC-I tetramer for the parasite Ag Tgd057 (36), these cells highly expressed T-bet, KLRG-1, and CD11a (Fig. 2D). Thus, postinfection with T. gondii, T-bet is expressed in activated CD4+ and CD8+ T cells, and this correlates closely with the expression of CD11a, Ly6C, and KLRG-1 by parasite-specific effector populations.

Recently, human effector CD8+ T cells have been shown to have heterogeneous levels of T-bet protein that are associated with differential localization of T-bet in the nucleus and cytoplasm (37). Because T-bet expression closely correlated with expression of CXCR3 and KLRG-1, proteins that aid in defining effector populations in murine T cells, ImageStream analysis was combined with these surface molecules to visualize T-bet localization. Because there were relatively few numbers of cells that bound to the MHC tetramers, the polyclonal CD11a hi CD4+ and CD8+ T cell populations were analyzed. Among the Ly6C hi CD11a hi CD4+ T cell (memory precursor) population, T-bet was present primarily in the nucleus, as quantified by colocalization with DAPI (Fig. 2E). In contrast, within the Ly6C hi CD11a hi CD4+ T cell (effector) population, the expression of T-bet in ~75% of cells did not colocalize with DAPI, indicating that T-bet was cytoplasmic in these cells (Fig. 2E). Analysis of the CD8+ T cells revealed that within the KLRG-1 hi CD11a hi CD8+ T cell (memory precursor) population, a portion of cells expressed low levels of T-bet that was difficult to localize, but in those cells that highly expressed this protein, it was...
present primarily in the nucleus (Fig. 2F). However, the majority of KLRC-1<sup>+</sup>CD11<sup>+</sup>CD8<sup>+</sup> T cells (effectors) had T-bet present in the cytoplasm (Fig. 2F). Together, these data demonstrate that among the Ag-specific (CD11a<sup>+</sup>) T cells, T-bet is differentially localized among the effector and memory precursor T cell populations.

**T-bet knockout mice are susceptible to infection with *T. gondii***

To directly assess the significance of the infection-induced increases of T-bet in activated NK and T cells, we challenged WT and T-bet<sup>−/−</sup> mice with ME49, an avirulent strain of *T. gondii*. WT mice survived this challenge, yet mice lacking T-bet succumbed to infection between days 9 and 14 postinfection (Fig. 3A). In many instances, susceptibility to *T. gondii* is either a consequence of an inability to control parasite replication (21, 38, 39) or the development of T cell–mediated immune pathology (40–43). To determine the cause of the increased mortality seen in T-bet<sup>−/−</sup> mice, we assessed parasite burden and levels of immune-mediated damage. Analysis of the PECs revealed that there was a small but reproducible increase in parasite burden in the T-bet<sup>−/−</sup> mice at days 5 (Fig. 3B) and 9 postinfection (data not shown). A more comprehensive analysis of the tissues from infected WT and T-bet<sup>−/−</sup> mice by qPCR (Fig. 3C) and immunohistochemistry (Fig. 3D–G) revealed that in WT mice, few parasites were detected at peripheral sites of infection such as the thymus, heart, lung, and brain. However, in T-bet<sup>−/−</sup> mice, these tissues all contained areas of extensive parasite replication. Liver lesions (areas of coagulative necrosis in the liver that are typically associated with CD4<sup>+</sup> T cell–mediated immunopathology) were present in the tissues from WT mice but were absent or reduced in those from T-bet<sup>−/−</sup> mice (Fig. 3H). Although there was no difference in the frequency of NK cells in the livers from infected WT and T-bet<sup>−/−</sup> mice, there was a marked reduction in the number of T cells in the livers from the T-bet<sup>−/−</sup> mice (data not shown). Furthermore, WT mice had higher levels of infection-induced ALT, which is indicative of liver damage, in the sera than T-bet<sup>−/−</sup> mice at day 9 postinfection (Fig. 3I), consistent with the elevated pathology. Thus, the major cause of susceptibility of the T-bet<sup>−/−</sup> mice is a failure to control parasite replication at secondary sites of infection.

**Innate responses in the absence of T-bet***

To better understand the underlying cause of the increased susceptibility of the T-bet<sup>−/−</sup> mice, we characterized the innate response to *T. gondii*. These studies revealed no significant differences in serum levels of infection-induced IL-12 or IFN-γ in the WT and T-bet<sup>−/−</sup> mice at days 5 (data not shown) and 9 postinfection (Fig. 4A, 4B). Moreover, at day 5 postinfection, a period of enhanced NK cell activity (44, 45), there was equivalent expansion of DX5<sup>+</sup> NK cells from T-bet<sup>−/−</sup> and WT mice (Fig. 4C), and these cells expressed similar levels of the activation marker CD69 (Fig. 4D). Analysis of NK cells from the spleens of infected and uninfected mice showed that there were basal differences in IFN-γ production from WT and T-bet<sup>−/−</sup> NK cells (data not shown), but after challenge these cells produced similar levels of IFN-γ (Fig. 4E, 4F). In addition, when WT and T-bet<sup>−/−</sup> mice were treated with an IFN-γ blocking Ab, there was a marked increase in the percentage of infected PECs over the isotype control-treated mice (Fig. 4G, 4H). Furthermore, depletion of NK cells in T-bet<sup>−/−</sup> during infection with *T. gondii* also resulted in a similar increase in the percentage of infected PECs (Fig. 4I). Together, these data establish that T-bet is dispensable for NK cell activation, and the early IFN-γ produced in the absence of T-bet...
FIGURE 4. NK and cytokine responses are intact in infected T-bet−/− mice. (A–F) WT and T-bet−/− mice were infected i.p. with T. gondii. (A and B) Serum was harvested from day 9 infected and uninfected WT and T-bet−/− mice, and assayed by ELISA for (A) IL-12 and (B) IFN-γ. (C) Splenocytes from uninfected and day 5 infected WT and T-bet−/− mice were analyzed by flow cytometry for mature (DX5+) NK cells (NK1.1+CD3− live cells). (D) NK cells (NK1.1+CD3− live cells) from day 5 infected WT and T-bet−/− mice were analyzed by flow cytometry for expression of CD69 (WT CD69: MFI = 1246 ± 89.41, T-bet−/− CD69: MFI = 1086 ± 24.83). (E and F) Whole splenocytes from uninfected and day 5 infected WT and T-bet−/− mice were restimulated in vitro with PMA and ionomycin in the presence of brefeldin A and monensin for 4 h. Cells were then fixed and stained for cytokine production by NK and cytokine responses are intact in infected T-bet−/− mice. (G and H) T-bet−/− mice were infected i.p. with T. gondii. On days 0, 3, and 6, mice were given 0.5 mg anti–IFN-γ or isotype control (anti-IgG) i.p. On day 7 postinfection, peritoneal lavage was performed and PECs were collected for cytospin preparation and analysis of percentage of cells infected. Original magnification ×40. (I) T-bet−/− mice were treated with 50 μl anti-Asialo gm-1 or isotype control on days −1, 3, and 6 of T. gondii infection. At day 9 postinfection, peritoneal lavage was performed and PECs were collected for cytospin preparation and analysis of parasite burden. Data are representative of two to three experiments, n = 3 per experiment. *p < 0.05.

Role of T-bet in the T cell response to T. gondii

To assess the role of T-bet in the generation of the adaptive T cell response to T. gondii, we infected WT and T-bet−/− mice with T. gondii and analyzed responses 9–11 d later. Splenocytes from infected and uninfected mice were harvested and stimulated in vitro with either anti-CD3 or STAg and assessed for IFN-γ production (Fig. 5A, 5B). Intracellular staining revealed that among WT cells, the majority of CD4+ T cells producing IFN-γ were T-bet+ (Fig. 5C). Although infection induced an increase in IFN-γ production by both WT and T-bet−/− CD4+ T cells (13-fold increase in the WT and 10-fold increase in the T-bet−/−), the percentage of IFN-γ–producing CD4+ T cells was reduced in the T-bet−/− mice compared with the WT mice (Fig. 5C), but this was not associated with increases in IL-4 (data not shown). Similarly, the majority of WT CD8+ T cells producing IFN-γ also expressed T-bet; however, WT and T-bet−/− CD8+ T cells produced comparable levels of IFN-γ (Fig. 5D). Thus, a significant proportion of CD4+ and CD8+ T cells produce IFN-γ independently of T-bet.

To characterize the impact of T-bet on T cell activation, we used a panel of markers that included CD25, CD11a, Ly6C, KLRG-1, and CXCXR3 to compare the T cell populations in WT and T-bet−/− mice. This analysis revealed that after challenge, WT CD4+ T cells had high levels of CD11a, and this was decreased in the T-bet−/− CD4+ T cells (Fig. 6A). Interestingly, although there was no difference in CD25 expression between naive WT and T-bet−/− mice (data not shown), T-bet−/− CD4+ T cells from infected mice displayed higher levels of CD25 than CD4+ T cells from WT mice (Fig. 6A). However, there was no difference in the frequency or numbers of Foxp3-expressing cells between the WT and T-bet−/− CD4+ T cell populations (data not shown). In the CD8+ T cell population, there were CD11a+ and CD11a− populations of WT CD8+ T cells, and this heterogeneity was not apparent in the T-bet−/− CD8+ T cells (Fig. 6B). Moreover, similar to the CD4+ T cells in the T-bet−/− mice, CD8+ T cells expressed higher levels of CD25 than WT CD8+ T cells (Fig. 6B). Initially, these data on CD11a expression suggested that in infected T-bet−/− mice, there are fewer parasite-specific CD4+ T cells. However, the use of a T. gondii–specific AS15 MHC-II tetramer (35) revealed that in the spleens of WT and T-bet−/− mice, there was an equivalent expansion of these CD4+ T cells (Fig. 6C). In addition, there were no differences in PSGL-1, CD44, or CD62L expression between the WT and T-bet−/− mice, and assayed for cytokines (Fig. 6D). In addition, although these T cells did produce IFN-γ, the mean fluorescence intensity (MFI) was reduced compared with WT T cells (Fig. 6D).

In naive WT and T-bet−/− mice, the frequency of splenic Tgd057-specific CD8+ T cells is low, and infection with T. gondii results in an equivalent expansion of this population (Fig. 6E), which expressed similar levels of CD11a (Fig. 6F). In addition, there were no differences in CD44 or CD62L expression between the WT and...
T-bet−/− Tetramer+ CD8+ T cells (data not shown). However, although the majority of Tgd057-specific WT CD8+ T cells expressed KLRG-1 and CXCR3, the parasite-specific CD8+ T cells from T-bet−/− mice expressed significantly lower levels of these proteins (Fig. 6F), but the WT and T-bet−/− parasite-specific CD8+ T cells expressed comparable levels of IFN-γ (Fig. 6F). These data indicate that T-bet is not required for the activation and expansion of parasitestate CD4+ and CD8+ T cells but is required for these cells to acquire an effector phenotype associated with expression of CD11a, CXCR3, Ly6C, and KLRG-1.

Role of T-bet in T cell responses in peripheral sites

Given that the T-bet−/− mice had an increased parasite burden in peripheral tissues, the decreased expression of CXCR3 and CD11a by parasite-specific T cells from the spleens of these mice suggested a defect in their ability to either access distal sites of infection or function within these sites to control parasite replication. Therefore, WT and T-bet−/− mice were infected and the T cell responses were assessed at peripheral sites. In the lungs of WT mice, there was a large population of parasite-specific CD4+ T cells characterized by high levels of Ly6C. In the T-bet−/− mice, the numbers of these cells were markedly reduced and those that were present expressed reduced levels of Ly6C (Fig. 7A, 7B). Similarly, there were significantly more parasite-specific CD8+ T cells in the lungs of WT mice than in the T-bet−/− mice (Fig. 7C), and the WT cells expressed higher levels of KLRG-1 (Fig. 7D). Immunohistochemical analysis of brain sections taken at day 9 postinfection showed T cells associated with

FIGURE 5. T-bet–deficient T cells produce IFN-γ during infection with T. gondii. WT and T-bet−/− mice were infected i.p. with T. gondii. Splenocytes were restimulated in vitro with (A) anti-CD3 or (B) STAg, and supernatants were assayed for IFN-γ by ELISA. (C and D) Splenocytes from uninfected and infected WT and T-bet−/− mice were restimulated in vitro stained for IFN-γ production by (C) CD4+ T cells (CD3+CD4+CD8a− live cells) and (D) CD8+ T cells (CD3+CD4− CD8a+ live cells). Data are representative of three experiments, n = 3–4 per experiment. **p < 0.01.

FIGURE 6. Altered phenotype of parasite-specific T cells in infected T-bet−/− mice. WT and T-bet−/− mice were infected i.p. with T. gondii. (A and B) Splenocytes from infected mice were harvested and stained for analysis by flow cytometry for CD11a and CD25 expression levels on (A) CD4+ and (B) CD8+ T cells. (C and D) Splenocytes from infected or uninfected mice were stained for parasite-specific CD4+ T cells using an MHC-II tetramer and analyzed for (C) quantification, and (D) activation and effector status markers CD11a, Ly6C, CXCR3, and IFN-γ by flow cytometry. In the IFN-γ flow plots, the IFN-γ FMO is light gray. (E and F) Splenocytes from infected or uninfected mice were stained for parasite-specific CD8+ T cells using an MHC-I tetramer, and this population was (E) quantified and analyzed for (F) activation and effector status markers CD11a, KLRG-1, CXCR3, and IFN-γ by flow cytometry. In the IFN-γ flow plots, the IFN-γ FMO is light gray. Data are representative of three to five experiments, n = 3–4 per experiment.
and KLRG-1 expression was assessed on these cells. (E) T-bet steady-state in all four experiments performed, cells from the T. gondii. (experimental setting. In contrast, the CD8+ T cell compartment an increased frequency of WT CD8+ T cells in the lung (Fig. 8C). Changes in the CD8+ T cell response (46, 47), and the studies Ly6C expression was assessed on these cells. (C) also stained for parasite-specific CD8+ T cells using an MHC-I tetramer, and parasite colonization. WT and T-bet mice are comprised 65–75% of the total CD4+ T cells in the spleen, and CD11a (data not shown) than T-bet−/− parasite-specific CD4+ T cells postinfection. In addition, there was reduced IFN-γ from the T-bet−/− CD4+ T cell compartment compared with the WT CD4+ T cells (Fig. 8G). In contrast, there were significantly more WT parasite-specific CD8+ T cells in both the spleen and the lung after challenge (Fig. 8H), and these WT parasite-specific CD8+ T cells from the spleen and lung expressed higher levels of KLRG-1 (Fig. 8I) and CD11a (data not shown) than the T-bet−/− parasite-specific CD8+ T cells. Surprisingly, there was more IFN-γ production from the WT CD8+ T cells than from the T-bet−/− CD8+ T cells (Fig. 8J). These data indicate that after challenge with T. gondii, there is a cell-intrinsic requirement for T-bet for the activation and acquisition of effector status of the CD4+ and CD8+ T cell populations, and these effector populations are required for control of the parasite in peripheral tissues.

Discussion
The experiments presented in this article reveal that during experimental toxoplasmosis, T-bet is not required for the innate NK cell–dependent mechanism of resistance or for the development and expansion of parasite-specific CD4+ and CD8+ T cells. Indeed, the ability of NK and CD8+ T cells produce IFN-γ appears intact. However, T-bet is required for optimal IFN-γ production by CD4+ T cells. It has been reported that in response to restimulation with anti-CD3 and anti-CD28, a significant proportion of T-bet−/− CD4+ T cells from mice infected with T. gondii produced IFN-γ, and a smaller subset produced IL-4 (22). In the studies presented in this article, the parasite-specific T-bet−/− CD4+ T cells restimulated with STAg also produce IFN-γ, but not IL-4, and thus appear to be Th1 cells (48). Because the loss of CD4+ T cells alone does not lead to acute susceptibility to T. gondii (49, 50), this partial defect in CD4+ T cell IFN-γ production is not sufficient to explain the susceptibility of the T-bet−/− mice challenged with T. gondii. Rather, the increased parasite numbers at many secondary sites of parasite dissemination was associated with reduced numbers of parasite-specific effector T cells. However, in the spleen, despite the presence of parasite-specific effector T cells, there was still a marked increase in parasite burden. These latter results have to be interpreted with care because the parasite burden detected in the spleen also reflects the numbers of T. gondii in the blood, but suggest that the reduced expression of CD11a, CXCR3, Ly6C, and KLRG1 may also compromise the function of these parasite-specific T cells. Thus, these data highlight that additional functions of T-bet are required for the ability of effector T cells to access and operate within local sites of infection. To the best of our knowledge, this is the first report that, in the context of infectious disease, highlights this key role for T-bet in coordinating multiple facets of the effector response and localization to peripheral sites, and may help to explain the basis for the susceptibility of T-bet−/− mice in other infectious settings (2, 13–15, 51).

A common feature of the acute phase of many intracellular pathogens is the presence of an IFN-γ–dependent, NK cell–mediated mechanism of resistance (52–54). Because T-bet controls key checkpoints in NK maturation (10) and has been linked to the
ability of these cells to produce IFN-γ (2, 15), it seemed likely that the NK response during toxoplasmosis would be T-bet dependent. However, others have reported that T-bet is not required for the production of IFN-γ from these cells (8, 10), and after challenge of the T-bet−/− mice with T. gondii, NK cell IFN-γ production appeared normal. Similarly, when T-bet−/− mice are infected with L. monocytogenes, the early NK cell response is intact and is associated with acute resistance to the bacteria (16), but the long-term consequences of T-bet deficiency on the T cell responses to this bacterium remain unclear. Similarly, T-bet−/− mice survive for >2 wk after challenge with Salmonella (13), indicating that early IFN-γ production in this model is intact. These results from multiple experimental systems are consistent with a model in which high levels of inflammation associated with many parasitic and bacterial infections provide sufficient signals to overcome the requirement for T-bet in NK cell maturation.

Previous in vitro studies have identified a role for T-bet in T cell chemotaxis through its induction of CXCR3 (12), whereas more recent work has implicated T-bet in trafficking of regulatory T cells in vivo (55). The findings presented in this article that T-bet is required in vivo for the ability of parasite-specific T cells to express CXCR3 and the integrin CD11a (a component of LFA-1) reinforce this notion. CXCR3 and/or its ligands have a key role in the trafficking of effector T cells necessary to control a number of pathogens including T. gondii, respiratory syncytial virus, and influenza (56–59). Thus, the reduced number of effector T cells at
peripheral sites of infection in the T-bet−/− mice could be a result of defective T cell trafficking to these sites. However, this alteration in trafficking is not always detrimental; during toxoplasmosis, immune-mediated pathology in the liver is caused by CD4+ effector T cells (50, 60, 61), and in our studies, T-bet−/− mice have reduced levels of liver pathology. Likewise, the loss of T-bet or CXCR3 provides protection against experimental cerebral malaria, associated with a reduced number of T cells in the brains of infected mice (62, 63), and T-bet deficiency confers protection in a murine model of type 1 diabetes that is associated with reduced islet infiltration by T cells (7). These findings from diverse infectious and autoimmune models highlight that the ability to target T-bet (64) may also influence T cell trafficking and limit inflammation. Interestingly, T-bet was not restricted to the nucleus in highly activated cells. Although the function of T-bet as a cytoplasmic protein remains unknown, differential T-bet localization has been linked to effector status (37), activation (65), cell cycle (66), and protein stability (67). The biological significance of the cytoplasmic localization of T-bet, the mechanisms that underlie this partitioning, and whether localization influences T cell phenotype and/or migration remain unclear but may provide opportunities to target different functions of this transcription factor.

Although the studies presented in this article highlight the impact of T-bet on expression of CD11a and CXCR3 and their links to T cell trafficking, these molecules are involved in many facets of T cell activation, which may contribute to the defect in effector populations. For example, CD11a is upregulated on T cells after TCR signaling, and its expression has been used as a marker of activation for infection-induced polyclonal T cell populations (31–33). Consequently, the reduced levels of CD11a expression on T cells from infected T-bet−/− mice initially suggested that T-bet was required for their ability to generate parasite-specific populations. However, the use of tetramers demonstrated that the generation of parasite-specific CD4+ and CD8+ T cells was intact in the T-bet−/− mice and implied that upregulation of this integrin was, in part, dependent on T-bet. Thus, because CD11a is important during priming for the generation of Ag-specific effector CD8+ T cells (68, 69), upregulation of CD11a is a potential correlate of natural killer cell maturation. Immunology 20: 477–494.


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Disclosures

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