The Transcription Factor T-bet Regulates Parasitemia and Promotes Pathogenesis during Plasmodium berghei ANKA Murine Malaria

Miranda S. Oakley, Bikash R. Sahu, Leda Lotspeich-Cole, Nehal R. Solanki, Victoria Majam, Phuong Thao Pham, Rajdeep Banerjee, Yukiko Kozakai, Steven C. Derrick, Sanjai Kumar and Sheldon L. Morris

*J Immunol* 2013; 191:4699-4708; Prepublished online 27 September 2013; doi: 10.4049/jimmunol.1300396

http://www.jimmunol.org/content/191/9/4699

---

References This article cites 62 articles, 33 of which you can access for free at: http://www.jimmunol.org/content/191/9/4699.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The Transcription Factor T-bet Regulates Parasitemia and Promotes Pathogenesis during *Plasmodium berghei* ANKA Murine Malaria

Miranda S. Oakley,* Bikash R. Sahu,† Leda Lottspeich-Cole,* Nehal R. Solanki,* Victoria Majam, †Phuong Thao Pham, † Rajdeep Banerjee, † Yukiko Kozakai, † Steven C. Derrick,* Sanjai Kumar, † and Sheldon L. Morris*

The pathogenesis of experimental cerebral malaria (ECM) is an immunologic process, mediated in part by Th1 CD4+ T cells. However, the role of the Th1 CD4+ T cell differentiation program on the ability to control parasitemia and susceptibility to ECM disease during blood stage malaria has never been assessed directly. Using the *Plasmodium berghei* ANKA murine model of ECM and mice deficient for the transcription factor T-bet (the master regulator of Th1 cells) on the susceptible C57BL/6 background, we demonstrate that although T-bet plays a role in the regulation of parasite burden, it also promotes the pathogenesis of ECM. T-bet–deficient (*Tbx21−/−*) mice had higher parasitemia than wild type controls did during the ECM phase of disease (17.7 ± 3.1% versus 10.9 ± 1.5%). In addition, although 100% (10/10) of wild type mice developed ECM by day 9 after infection, only 30% (3/10) of *Tbx21−/−* mice succumbed to disease during the cerebral phase of infection. Resistance to ECM in *Tbx21−/−* mice was associated with diminished numbers of IFN-γ–producing CD4+ T cells in the spleen and a lower accumulation of CD4+ and CD8+ T cells in the brain. An augmented Th2 immune response characterized by enhanced production of activated GATA-3 and mice deficient for the transcription factor T-bet (the master regulator of Th1 cells) on the susceptible C57BL/6 background, we demonstrate that although T-bet plays a role in the regulation of parasite burden, it also promotes the pathogenesis of ECM.

Cerebral malaria (CM) remains a major cause of death in African children younger than 5 years. Despite extensive research, the full picture of the molecular mechanisms that contribute to the pathogenesis of CM remain unclear. Because of limitations in studies that can be conducted in patients with CM, researchers have relied on the *Plasmodium berghei* ANKA (*Pb*−A) murine model of experimental cerebral malaria (ECM) to improve our knowledge of the genesis and downstream biological events that mediate the pathogenesis of CM.

During the course of a pathogen infection, the different CD4+ T cell subsets are an important component of adaptive immunity that contribute to the resolution of acute infection and help to establish sterilizing immunity or to suppress pathogen burden to subpatent chronic levels, allowing host survival. However, the proinflammatory cytokines that are generated to control rapid pathogen replication can also cause severe immunopathogenesis, sometimes with fatal consequences.

T cells have been shown to play an important role in the immunopathogenesis of ECM (1, 2). In the *Pb*−A C57BL/6 model, CD4+ T cells mediate the induction phase of immunopathogenesis of ECM, whereas CD8+ T cells mediate the effector phase of disease (3) by perforin and granzyme-dependent apoptosis of brain endothelial cells (4–6). Nonetheless, compared with the role of CD8+ T cells during ECM, the immune mechanism of CD4+ T cell–mediated pathogenesis of ECM is less understood.

A pathogenic role for CD4+ T cells during ECM was first documented by Grau et al. (7), who showed that depletion of CD4+ T cells daily for 7 d and then every other day in susceptible CBA mice during *Pb*−A infection results in resistance to ECM. Subsequent studies demonstrating that CD4-deficient mice are also resistant to ECM confirmed a role for CD4+ T cells in disease pathogenesis (3, 8). The proinflammatory cytokines IFN-γ and TNF-α have been shown to be required for the pathogenesis of ECM (9, 10). However, the immunologic pathway that results in the production of these cytokines during ECM has not been delineated. Interestingly, it was shown recently that mice deficient for IL-12Rβ2 but not IL-12p40 or IL-12p35 are resistant to ECM, suggesting that ECM induction through IL-12Rβ2 can occur via a novel proinflammatory pathway that acts independently of the IL-12 ligands (11).

CD4+ T cells can be classified into at least four distinct subsets that differentiate from naive CD4+ T cells (12). The Th1 subset requires IL-12 for differentiation, produces IFN-γ as its signature cytokine, is regulated by the transcription factor T-bet, and is important for immunity against intracellular pathogens but also promotes autoimmune and inflammation (13). The Th2 subset requires IL-4 for differentiation, is regulated by the transcription factor GATA-3, and mediates immune responses against extra-

---

*Division of Bacterial, Parasitic, and Allergic Products, U.S. Food and Drug Administration, Rockville, MD 20852; and †Division of Emerging and Transfusion Transmitted Diseases, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Rockville, MD 20852

Received for publication February 8, 2013. Accepted for publication June 17, 2013.

This work was supported by intramural grants from the U.S. Food and Drug Administration.

Address correspondence and reprint requests to Dr. Sanjai Kumar, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, 5516 Nicholson Lane, Kensington, MD 20895. E-mail address: sanjai.kumar@fda.hhs.gov

Abbreviations used in this article: BSL, brain-sequestered leukocyte; CM, cerebral malaria; ECM, experimental cerebral malaria; MFI, mean fluorescence intensity; *Pb*−A, *Plasmodium berghei* ANKA; QRT-PCR, quantitative real time PCR; WT, wild type.
cellular pathogens but also causes asthma and allergic diseases (14). The Th17 subset requires IL-21 for differentiation, produces IL-17 as its signature cytokine, is regulated by the transcription factor RORγt, and is important for immunity against extracellular bacteria and fungi, but is also responsible for organ-specific autoimmune diseases (15). The regulatory T cell subset requires TGF-β for differentiation, is regulated by the transcription factor Foxp3, and is critical for maintenance of self-tolerance and regulation of immunity (16).

Recently, efforts have been made to assess the contribution of each CD4+ T cell subset to the pathogenesis of ECM. Regulatory T cells can be beneficial or detrimental to the induction of ECM depending on the method and timing of regulatory T cell depletion and the genetic background of the host (17–24). Th17 cells, which induce tissue inflammation and are capable of disrupting the blood brain barrier (25), do not appear to contribute to the pathogenesis of ECM (26).

In the current study, we have directly assessed the role of Th1 cells in the immunopathogenesis of ECM using C57BL/6 mice deficient for the Tbx21 gene that encodes the T-bet transcription factor, the master regulator of Th1 cells. T-bet controls the Th1 genetic program in naive CD4+ T cells, directly activates Tbet, and is critical for maintenance of self-tolerance and regulation of immunity (16).

EFFECT OF T-BET ON ECM

Six- to eight-week-old female wild type (WT) and Tbx21−/− mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Importantly, Tbx21−/− mice were previously backcrossed on the C57BL/6 genetic background for eight generations. All mice were maintained at the U.S. Food and Drug Administration animal care facility and treated in accordance with the guidelines of the Animal Care and Use Committee. An uncloned parasite line of P. berghei that encodes the T-bet transcription factor to ECM. Simultaneously, we measured the susceptibility of mice deficient for the Tbx21 gene encoding the T-bet transcription factor to ECM. To determine a role for T-bet in the pathogenesis of ECM, we infected female wild type (WT) and Tbx21−/− mice on the C57BL/6 background with 106 parasites and sacrificed mice daily starting on day 5 to allow for observation of parasite sequestration by day 2. Parasitemia was assessed daily by examining a thin prep of infected blood on a Giemsa-stained blood film. Parasite density was analyzed using the Student test to determine if differences were significant. Differences in cell counts and serum cytokine levels were determined using the Student t test when parametric assumptions were met. Otherwise, the Mann-Whitney U test was applied. Lastly, differential expression of transcription factor RNA was analyzed using the mixed-effects model.

Materials and Methods

Mice and parasite infections

Six- to eight-week-old female wild type (WT) and Tbx21−/− mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Importantly, Tbx21−/− mice were previously backcrossed on the C57BL/6 genetic background for eight generations. All mice were maintained at the U.S. Food and Drug Administration animal care facility and treated in accordance with the guidelines of the Animal Care and Use Committee. An uncloned parasite line of P. berghei that encodes the T-bet transcription factor to ECM. Simultaneously, we measured the susceptibility of mice deficient for the Tbx21 gene encoding the T-bet transcription factor to ECM. To determine a role for T-bet in the pathogenesis of ECM, we infected female wild type (WT) and Tbx21−/− mice on the C57BL/6 background with 106 parasites and sacrificed mice daily starting on day 5 to allow for observation of parasite sequestration by day 2. Parasitemia was assessed daily by examining a thin prep of infected blood on a Giemsa-stained blood film. Parasite density was analyzed using the Student test to determine if differences were significant. Differences in cell counts and serum cytokine levels were determined using the Student t test when parametric assumptions were met. Otherwise, the Mann-Whitney U test was applied. Lastly, differential expression of transcription factor RNA was analyzed using the mixed-effects model.

Flow cytometry

We performed flow cytometry to determine the number of brain-sequestered CD4+ and CD8+ T cells as well as the phenotype of these CD8+ T cells in the brain on day 6 after infection and to enumerate the number of 1) CD4+ T cells, 2) CD8+ T cells, and 3) brain-sequestered leukocytes (BSLs) made from perfused tissue as follows: a single-cell suspension was first prepared by treatment with DNase (3 U/ml) and collagenase (0.5 mg/ml) under frequent agitation and trituration in a volume of 3 ml for 1 h at room temperature (Roche Applied Science, Indianapolis, IN). Leukocytes were then purified by centrifugation at 515 × g for 30 min at 21°C on 33% Percoll (Sigma-Aldrich, St. Louis, MO). Flow cytometric analysis of splenocytes and BSLs was performed with eFluor 506 viability dye (eBioscience, San Diego, CA), blocked with anti-CD16/CD32 (BD Biosciences, San Jose, CA), stained with the following Abs (purchased from BD Biosciences, BioLegend, or eBiosciences) specific for FITC–anti-TCR-α, fluorescein isothiocyanate (FITC)–anti-CD4, Pacific blue–anti-CD4, APC/Cy7–anti-CD8, PE–anti-CD90, Pacific blue–anti-CD69, PE–anti-CD44, PE/Cy7–anti-CD62L, PE/Cy7–anti-CXCR3, PE/Cy7–anti-IFNAR1, PE/Cy7–anti-IFN-γ, PE–anti-TNF-α, PE–anti-T-bet, PE–anti-GATA-3, and FITC–anti-IFNγ, and with the following PE– anti-T-bet, PE–anti-GATA-3, and FITC–anti-IFNγ. Stained cells were analyzed on an LSR II flow cytometer using FACS Diva (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) software. For intracellular staining, a 4-h incubation with brefeldin A (BD Biosciences) was included and cells were permeabilized prior to intracellular staining. Lastly, isotype controls were used for analysis of CD69, IFN-γ, TNF-α, T-bet, and GATA-3.

Real-time PCR

Flow fresh spleen tissue was collected simultaneously from WT and Tbx21−/− mice and stored at −80°C until use. For preparation of high-quality RNA, tissue was resuspended in Tri-Reagent (Molecular Research Center, Cincinnati, OH) and pulse-homogenized to create a suspension. RNA was then purified by two phenol-chloroform extractions, an isopropanol precipitation, a wash with 70% ethanol, and a final resuspension in nuclease-free water. RNA was then treated with 3 μg of Turbo DNA-free (Ambion, Austin, TX) for 30 min at 37°C, and cDNA was then synthesized from 1 μg of DNase-treated RNA in a 20-μl reaction volume containing iScript reverse transcriptase, random primers, deoxynucleoside triphosphates (dNTPs), and M-MLV reverse transcriptase (Bio-Rad Laboratories, Hercules, CA) at 42°C for 30 min (31). Quantitative real-time PCR (QRT-PCR) was performed in a 20-μl reaction volume containing 2 μl cDNA, 10 μl SsoFast EvaGreen Supermix (Bio-Rad), and 500 nM commercially available primers specific for mouse T-bet, GATA-3, Foxp3, or RORγt genes (Qiagen, Valencia, CA). Amplification and detection of specific product were performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with the following cycle profile: 1 cycle at 95°C for 30 s and 40 cycles with 1 cycle consisting of 5 s of denaturation at 95°C and 5 s of annealing and extension at 61°C. The relative concentrations of RNA were determined using a standard curve derived from the PCR products of 10-fold serial dilutions of plasmid containing a mouse β-actin gene fragment. Real-time PCR was performed on four mice per group in duplicate reactions.

Detection of serum cytokines

Serum cytokine profiles were assessed using the Bio-Plex Pro Mouse Cytokine 23-plex assay (Bio-Rad) specific for the IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α cytokines. Serum samples were incubated sequentially with beads coated with capture Ab, biotinylated detection Ab, and streptavidin-PE conjugate with three washes performed between each incubation step. Analytes were then assayed in a final volume of 125 μl, and data were acquired using the Bio-Plex 200 reader and analyzed using Bio-Plex Manager software version 6.0.

Statistical analysis

The log-rank test was used to determine differences in survival between WT and Tbx21−/− mice. Differences in parasitemia were analyzed by applying pairwise comparisons. Differences in cell counts and serum cytokine levels were determined using the Student t test when parametric assumptions were met. Otherwise, the Mann-Whitney U test was applied. Lastly, differential expression of transcription factor RNA was analyzed using the mixed-effects model.

Results

T-bet dependent immunity regulates parasite growth and induces ECM pathogenesis

To determine a role for T-bet in the pathogenesis of ECM, we measured the susceptibility of mice deficient for the Tbx21 gene encoding the T-bet transcription factor to ECM. Simultaneously, we assessed the role of T-bet in the regulation of parasite burden by comparing the parasitemia of Tbx21−/− mice with WT controls. Following infection with Pb−A parasites, 10% of 100% WT mice developed ECM by day 9 after infection. In contrast, only 3 of 10 (30%) Tbx21−/− mice succumbed to malaria during the cerebral phase of infection indicating that Tbx21−/− mice are significantly protected from ECM disease (p < 0.01, log-rank; Fig. 1A). Tbx21−/− mice that were resistant to ECM developed severe anemia and hyperparasitemia by day 14 after infection and were sacrificed.
The absence of T-bet also had an effect on parasite growth during a \( Pb \)–A infection. On day 6 after infection, when the majority of WT mice become moribund, \( Tbx21^{-/-} \) mice (17.7 ± 3.1%) had a 1.6-fold higher parasite burden than WT mice did (10.9 ± 1.5%). This difference was even more dramatic on day 7 after infection, when \( Tbx21^{-/-} \) mice (19.70 ± 2.0) had a 4.5-fold higher parasitemia (\( p = 0.03 \), Mann–Whitney test) than WT mice did (4.34 ± 2.44; Fig. 1B). These results indicate that T-bet also plays an important role in the regulation of \( Pb \)–A parasitemia.

Within the group of \( Pb \)–A–infected \( Tbx21^{-/-} \) mice, we noted a positive correlation between parasite burden and susceptibility to disease. On day 6 after infection, \( Tbx21^{-/-} \) mice with ECM had a 1.6-fold higher parasite burden than WT mice did (10.9 to 6). (A) Parasite burden was greater in \( Tbx21^{-/-} \) mice with ECM compared with WT mice (10.86 ± 1.49, \( n = 10 \); \( p < 0.01 \)). These results suggest that the parasitemia threshold for disease induction may be higher in \( Tbx21^{-/-} \) mice compared with WT mice.

**Measurement of pathogenic T cells during \( Pb \)–A infection**

To determine the relative contribution of pathogenic T cells in T-bet–mediated susceptibility to ECM, we quantitated CD4+ and CD8+ T cells in the spleens of naive mice (day 0) and during the induction (day 3) and symptomatic (day 6) phase of ECM by flow cytometric analysis. There was no significant difference in CD4+ or CD8+ T cell counts in WT versus \( Tbx21^{-/-} \)-infected mice over the course of infection (Fig. 2A, 2B).

We also compared brain-sequestered CD4+ and CD8+ T cells in WT versus \( Tbx21^{-/-} \) mice on day 6 after infection to determine whether ECM resistance caused by the absence of T-bet is associated with a decrease in the induction or migration of pathogenic immune cell subsets to the brain. Remarkably, there was a 5.8-fold reduction in the number of BSLs in \( Tbx21^{-/-} \) (125 ± 35 × 10^3 BSLs) compared with WT (725 ± 150 × 10^3 BSLs; Fig. 3A) mice, indicating that T-bet is important for the accumulation of BSLs in ECM susceptible mice. This reduction in BSLs correlated with a significant decrease in brain-sequestered CD4+ (Fig. 3B, 3C) and CD8+ (Fig. 3D) T cells in the absence of T-bet, \( Tbx21^{-/-} \) mice (3.5 ± 1.3 × 10^5) had a 9.5-fold reduction (\( p < 0.04 \), Mann–Whitney test) in brain-sequestered CD4+ T cells (Fig. 3D) compared with WT mice (32.7 ± 7.8 × 10^5). Furthermore, \( Tbx21^{-/-} \) mice (19.8 ± 6.0 × 10^5) had an 18-fold reduction (\( p < 0.04 \), Mann–Whitney test) in brain-sequestered CD8+ T cells (Fig. 3G) compared with WT mice (356 ± 87 × 10^5).

We next measured the expression of biomarkers of brain-sequestered CD8+ T cells that have previously been shown to correlate with ECM pathogenesis. Previous studies have demonstrated that brain-sequestered CD8+ T cells during the cerebral phase of ECM are differentiated and activated (4) and express the chemokine receptor CXCR3 (32) and the IFN (\( \alpha, \beta \)) receptor 1 (IFNAR1) (33). Among brain-sequestered CD8+ T cells in WT mice, 46.6 ± 5.3% (158 ± 20 × 10^5) and 50.9 ± 4.6% (188 ± 55 × 10^5) are central (CD44^-CD62L^-) and effector (CD44^CD62L+) memory cells, respectively (Fig. 4A), 31.9 ± 3.4% (118 ± 34 × 10^5) are activated (CD69^; Fig. 4C), 74.1 ± 5.1% (258 ± 41 × 10^5) express CXCR3 (Fig. 4E), and 8.5 ± 9% (30 ± 6 × 10^5) express IFNAR1 (Fig. 4G). Although \( Tbx21^{-/-} \) mice have an 18-fold reduction in brain-sequestered CD8+ T cells compared with WT mice, the phenotype of brain-infiltrating CD8+ T cells in WT (Fig. 4I) versus \( Tbx21^{-/-} \) (Fig. 4B, 4D, 4F, 4H, 4J) mice is similar. Thus, lower cell counts of brain sequestered CD8+ T cells rather than a difference in any identifiable CD8+ T cell phenotype can be attributed to resistance to ECM in \( Tbx21^{-/-} \) mice.

**Expression of T-bet in CD4+ and CD8+ T cells during \( Pb \)–A infection**

We measured the expression of T-bet in splenic CD4+ T cells over the course of \( Pb \)–A infection by flow cytometry (Fig. 5A). T-bet is not expressed in naive CD4+ T cells; we find that T-bet was.
constitutively expressed in only 0.90 ± 0.24% of CD4+ T cells in noninfected mice. In comparison, T-bet expression was upregulated in 3.46 ± 1.44% of CD4+ T cells (12.62 ± 5.90 × 10^5 cells) on day 3 after infection and peaked at 15.13 ± 0.90% (24.14 ± 3.73 × 10^5 cells) on day 6 after infection (Fig. 5C, 5D). T-bet has also been shown to be important for the generation of cytotoxic

![Figure 3](https://www.jimmunol.org/DownloadedFrom.jpg)

**FIGURE 3.** Tbx21−/− mice have a significant reduction in brain-sequestered CD4+ and CD8+ T cells during the symptomatic phase of Pb-A infection. WT and Tbx21−/− mice were infected with 10^6 Pb-A parasites, and BSLs were isolated from perfused brain tissue on day 6 after infection and then stained with fluorescent-labeled Abs specific for TCR, CD4, and CD8 for flow cytometry. (A) Significantly fewer BSLs were recruited to the brain in Tbx21−/− mice. Comparison of brain sequestered (B, C) CD4+ and (E, F) CD8+ T cells in WT and Tbx21−/− mice by flow cytometry demonstrated a (D) 9.5-fold reduction in CD4+ T cells and an (G) 18-fold reduction in CD8+ T cells in the absence of T-bet. *p ≤ 0.05.

![Figure 4](https://www.jimmunol.org/DownloadedFrom.jpg)

**FIGURE 4.** Phenotypic analysis of known biomarkers of brain-sequestered CD8+ T cells during ECM. Gated CD8+ T cells purified from WT and Tbx21−/− mice on day 6 after infection with Pb-A parasites were analyzed for expression of (A, B) CD44 and CD62L, (C, D) CD69, (E, F) CXCR3, and (G, H) IFNAR1. (I) In WT mice, the majority of brain-sequestered CD8+ T cells are central memory (CM) or effector (EM) memory cells and express the CXCR3 chemokine receptor, and a subset (31.9 ± 3.4%) express the CD69 activation marker. (J) Although Tbx21−/− mice have significantly fewer brain-sequestered CD8+ T cells, these CD8+ T cells express known biomarkers.
effect CD8+ T cells (34). Therefore, we also assessed the kinetics of T-bet expression by CD8+ T cells over the course of infection (Fig. 5B). T-bet was expressed in 6.22 ± 1.03% of CD8+ T cells in noninfected mice. On day 3 after infection, the proportion (6.04 ± 2.36%) of CD8+ T cells expressing T-bet was similar to basal expression in naive mice. However, on day 6 after infection, 22.32 ± 3.17% of CD8+ T cells expressed T-bet (25.74 ± 5.27 × 10^3 cells; Fig. 5C, 5D). Remarkably, although there was no significant difference between the percentage or absolute number of T-bet+ expressing CD4+ versus CD8+ T cells, the mean fluorescence intensity (MFI), a measure of the protein quantity per cell, of T-bet was 4.17-fold higher in CD8+ T cells (2.35 ± 0.47 × 10^3) than in CD4+ T cells (0.56 ± 0.26 × 10^3) on day 6 after infection (p < 0.02, Mann–Whitney test; Fig. 5E) indicating that T-bet is likely important for the generation of pathogenic CD8+ T cells as well as the differentiation of proinflammatory Th1 CD4+ T cells that produce the ECM-inducing cytokines in susceptible strains of mice.

**Tbx21−/− mice produce more GATA-3+ CD4+ T cells**

We next measured the splenic levels of the T-bet, GATA-3, RORγt, and Foxp3 transcription factors that regulate Th1, Th2, Th17, and Treg CD4+ T cell differentiation, respectively, by QRT-PCR (Fig. 6A, 6B, 6C, 6D). There was no significant difference in transcription factor levels on day 3 after infection. However, there was a substantial increase (5.2-fold) in the production of T-bet transcripts from day 3 (13.58 ± 2.04 × 10^3) to day 6 (70.83 ± 8.25 × 10^3) in WT mice (Fig. 6A). Furthermore, there was a significant difference (1.8-fold; p = 0.05, mixed model) in splenic mRNA levels of GATA-3 on day 6 after infection in WT (22.38 ± 1.56 × 10^3) versus Tbx21−/− (40.86 ± 11.97 × 10^3) mice, suggesting that ECM-resistant Tbx21−/− mice can produce more GATA-3+ CD4+ T cells during the symptomatic phase of infection (Fig. 6B).

Therefore, we next compared the number of GATA-3+ CD4+ T cells and their activation status by flow cytometry in WT versus Tbx21−/− mice on day 6 after infection (Fig. 7A, 7B). Similar to results determined by QRT-PCR, Tbx21−/− mice (2.95 ± 1.25 × 10^5) had substantially more (5.2-fold) GATA-3+ CD4+ T cells than WT mice did (0.57 ± 0.19 × 10^5; p < 0.01, Mann–Whitney test). Furthermore, a larger proportion of GATA-3+ CD4+ T cells were activated as determined by CD69 expression in Tbx21−/− (54.5%) compared with WT (37.3%) mice, resulting in a 7.3-fold difference in activated GATA-3+ CD4+ T cells between the two groups of mice (p < 0.01, Mann–Whitney test; Fig. 7C). These results indicate that the CD4+ T cell helper response is Th2 skewed in the absence of T-bet.

**The absence of T-bet is associated with a reduction in the number of IFN-γ-producing CD4+ T cells during the induction phase of ECM**

It is well established that T-bet is essential for the regulation of IFN-γ expression (35), and murine malaria studies demonstrating that IFN-γR−/− mice (36, 37) are resistant to ECM indicate that IFN-γ is required for the development of ECM. We therefore compared the expression of IFN-γ in CD4+ and CD8+ T cells in WT versus Tbx21−/− mice. Loss of T-bet did not alter the percentage or number of IFN-γ-producing CD8+ T cells over the course of infection. However, there was a significant difference in IFN-γ-producing CD4+ T cells during the induction phase (day 3; Fig. 8A, 8B), but not the effector phase (day 6; data not shown). There was a 3.11-fold decrease (p < 0.01, Mann–Whitney test) in the percentage of IFN-γ+ CD4+ T cells in the absence of T-bet (Fig. 8C) that translated into a 2.90-fold reduction (p = 0.05, Mann–Whitney test) in the number of CD4+ T cells that produce IFN-γ+ in Tbx21−/− mice (2.80 ± 10^5 cells) compared with WT mice (8.13 ± 10^5 cells; Fig. 8D). Despite this decrease in IFN-γ–producing CD4+ T cells in Tbx21−/− mice on day 3 after infection, there was no difference in the MFI of IFN-γ in CD4+ T cells between the two groups of mice (Fig. 8E). These results suggest that resistance to ECM in Tbx21−/− mice is caused by diminished production of IFN-γ+ CD4+ T cells during the early phase of disease pathogenesis. Consistent with this reduction in IFN-γ+ CD4+ T cells during the induction phase of disease, we observed a 1.8-fold reduction (p < 0.02, Mann–Whitney test) in serum IFN-γ on day 3 after infection in Tbx21−/− compared with WT mice (data not shown).

**Tbx21−/− mice produce diminished numbers of TNF-α+ CD8+ T cells during the effector phase of disease**

Because TNF-α is an important proinflammatory cytokine, we also compared the expression of TNF-α in CD4+ and CD8+ T cells over the course of P. falciparum infection in WT versus Tbx21−/− mice (Fig. 9A, 9B, 9C, 9D, 9E, 9F). Although there was no difference in the production of TNF-α+ CD4+ T cells, there was a significant reduction in the percentage of CD8+ T cells that express TNF-α during the effector phase (day 6) of disease (Fig. 9D). Loss of T-bet resulted in a 1.80-fold reduction in the percentage of TNF-α–producing CD8+ T cells (p < 0.01, Student t test); 5.26 ± 0.35%
versus 2.93 ± 0.25% of CD8+ T cells produced TNF-α in WT and Tbx21−/− mice, respectively. This difference translated into a 1.82-fold decrease in the number of TNF-α+ CD8+ T cells in Tbx21−/− mice (2.42 ± 0.54 × 10^5 cells) compared with WT mice (4.41 ± 0.70 × 10^5 cells) (Fig. 9F).

**FIGURE 7.** Expansion of activated Th2 (CD69+GATA-3+) CD4+ T cells in the absence of T-bet. WT (n = 4) and Tbx21−/− (n = 4) mice were infected with 10^6 Pb-A parasites, and flow cytometry was performed on splenocytes to determine GATA-3 expression and activation status (CD69) of CD4+ T cells. On day 6 after infection, splenocytes were harvested and stained with Abs specific for TCR, CD4, GATA-3, and CD69. (A) A significantly larger proportion of gated CD4+ T cells express both CD69 and GATA-3 in Tbx21−/− (red) compared with WT (blue) mice. (B) For gating purposes, CD4+ T cells were also stained with the PerCP-anti-Armenian Hamster IgG and PE-anti-rat IgG2b isotype control Abs for CD69 and GATA-3, respectively. (C) Tbx21−/− mice have 5.2-fold more GATA-3+ (Th2) and 7.3-fold more CD69+GATA-3+ (activated Th2) CD4+ T cells than WT mice on day 6 after infection, indicating that the absence of T-bet skews the CD4+ T cell repertoire during Pb-A infection. Values are expressed as absolute cell numbers rather than percentages to normalize for differences in splenic cell numbers between the two groups of mice. **p < 0.01.

Cytokine profile is Th2 skewed in Tbx21−/− mice

To delineate the mechanism of ECM resistance in Tbx21−/− mice, we compared the serum cytokine profiles of WT versus Tbx21−/− mice. Of the 23 cytokines examined, Tbx21−/− mice had significantly higher levels of cytokines associated with the Th2 response on day 6 after infection. In addition to elevated levels of IL-4 and IL-5 (Fig. 10A, 10B), Tbx21−/− mice had 6.9-fold more eotaxin (p < 0.01, Mann–Whitney test), 5.2-fold more MCP-1 (p < 0.01, Mann–Whitney test), and 4.1-fold more G-CSF (p < 0.01, Mann–Whitney test) than WT mice (Fig. 10C, 10D, 10E). Interestingly, there was no significant difference in serum levels of the Th1-associated cytokine IFN-γ, the anti-inflammatory cytokine IL-10, or the Th17-associated cytokine IL-17 between WT and Tbx21−/− mice on day 6 after infection.

Discussion

In mice, Pb-A parasites cause a highly virulent infection that is uniformly fatal. Depending on the genetic background of the host mouse strain, the cause of death is attributed to clinical symptoms of ECM (susceptible strain) or severe anemia (resistant strain). Earlier studies have demonstrated that in susceptible C57BL/6 mice, T cells play a key role in the pathogenesis of ECM. Although extensive studies have indicated that CD8+ T cells and IFN-γ are the important mediators of ECM, CD8+ T cells are not attributed as the primary source of IFN-γ in mice undergoing the pathogenesis of ECM (38). On the other hand, how CD4+ T cells contribute toward the clinical syndrome of ECM remain poorly understood. In this study, we examined the role of proinflammatory CD4+ Th1-type responses in the development of ECM by comparing the immunopathogenesis of WT C57BL/6 mice to Tbx21−/− mice that bear a genetic deletion in the Tbx21 gene. Depending on their cytokine milieu during TCR activation, naive CD4+ T cells can differentiate into several lineages of helper T cells that are defined based on their cytokine production and effector function (39). T-bet is a transcription factor that is expressed on a variety of immune cells. However, this molecule is best recognized for its critical requirement for the differentiation of naive Th0 cells into effector Th1 CD4+ T cells.

We find that Tbx21−/− C57BL/6 mice are highly resistant to ECM disease (100% WT versus only 30% Tbx21−/− mice developed ECM; Fig. 1A). Interestingly, this protection against ECM in Tbx21−/− mice is only partial, indicating that T-bet–independent mechanisms of stimulating a proinflammatory response (possibly mediated by CD8+ T cells) that can induce the pathogenesis of ECM exist. In addition to playing a role in the induction of ECM, we also found that presence of T-bet was detrimental for parasite growth. On day 7 after infection, Tbx21−/− mice had 4.5-fold higher parasitemia than WT C57BL/6 mice did (Fig. 1B). Lastly, our results indicate that the parasitemia threshold for disease induction could be higher in the absence of T-bet. On day 6 after infection, Tbx21−/− mice with ECM (28.96 ± 3.27, n = 3) had 2.3-fold higher parasitemia than Tbx21−/− mice without ECM did (12.82 ± 2.53, n = 7; Fig. 1C) and 2.67-fold higher parasitemia than WT mice with ECM did (10.86 ± 1.49, n = 10). Thus, although Th1-mediated proinflammatory cytokines might be necessary to control the acute phase of malaria infection, such responses can also promote the pathogenesis of disease in the nonimmune host.

CD4+ and CD8+ T cells are known to have a pathogenic role during ECM. In our studies, the numbers of CD4+ and CD8+ T cells were not distinguishable in the spleen between the two groups of mice (Fig. 2A, 2B). We enumerated brain-sequestered CD4+ and CD8+ T cells and found that WT mice had 32.7 ± 7.8 × 10^3 brain-sequestered CD4+ T cells and 356 ± 77 × 10^3 brain...
sequestered CD8+ T cells. In contrast, Thbx21−/− mice had 9.5-fold fewer brain-sequestered CD4+ T cells and 18-fold fewer brain-sequestered CD8+ T cells compared with susceptible WT mice. Thus, Th1 CD4+ T cells might also contribute to disease by promoting the recruitment and retention of pathogenic CD8+ T cells at the site of pathogenesis in brain tissue. These results are reminiscent of the murine diabetes model in which loss of T-bet expression in CD4+ T cells impaired cellular migration and subsequent infiltration into the pancreas, which is needed to promote diabetes (40).

Recent studies have identified several phenotypic characteristics of brain-sequestered CD8+ T cells during ECM. Because the majority of BSLs are CD8+ T cells during ECM and because CD8+ T cells are known to exert a pathogenic effect in the brain during the effector phase of disease, we compared the phenotype of brain-infiltrating CD8+ T cells in WT versus Thbx21−/− mice to determine whether the absence of T-bet is associated with the loss of a particular CD8+ T cell phenotype. The majority of brain-sequestered CD8+ T cells have been shown to be activated and differentiated memory T cells (4). In addition, 90% of brain-sequestered CD8+ T cells express the CXCR3 chemokine receptor (32), and this chemokine receptor has been shown to be essential for susceptibility to ECM (41). Furthermore, a recent study has demonstrated that expression of IFNAR1 by CD8+ T cells is required for ECM pathogenesis (33). Consistent with these studies, 46.6 ± 5.3% (158 ± 20 × 10^3) and 50.9 ± 4.6% (188 ± 55 × 10^3) of brain-sequestered CD8+ T cells in WT mice were central (CD44+CD62L+) and effector (CD44+CD62L−) memory cells, respectively, 31.9 ± 3.4% (118 ± 34 × 10^3) expressed the CD69 activation marker, 74.1 ± 5.1% (258 ± 41 × 10^3) expressed CXCR3, and a small subset (8.5 ± 0.9%) expressed IFNAR1 (Fig. 4A, 4C, 4E, 4G, and 4I). Although resistance to ECM in Thbx21−/− mice is associated with a dramatic reduction in the number of CD8+ T cells in the brain, the phenotype of brain-sequestered CD8+ T cells in Thbx21−/− mice appears to be similar to that of WT mice.

T cells contribute to ECM pathogenesis by secreting ECM-inducing cytokines. Th1 CD4+ T cells and CD8+ T cells can generate a panel of proinflammatory cytokines of overlapping characteristics and functions. Thus, the differential contributions of CD4+ and CD8+ T cells in the pathogenesis of ECM are difficult to discern. Although naive CD4+ T cells do not express T-bet, our data

![FIGURE 8](image-url) Production of IFN-γ by CD4+ T cells is diminished in the absence of T-bet on day 3 after infection. WT (n = 5) and Thbx21−/− (n = 5) mice were infected with 10^6 Pb-A parasites, and IFN-γ was quantitated in CD4+ TCRβ+ and CD8+ TCRβ+ cells in infected mice. Representative dot plots of expression of (A) IFN-γ and (B) rat IgG1 isotype control in CD4+ T cells on gated T cells in WT (red) and Thbx21−/− (blue) mice are shown. (C) There was a 3.11-fold reduction in the percentage and a 2.90-fold reduction in the absolute number of CD4+ T cells that express IFN-γ. (E) IFN-γ–specific MFIs did not differ between the two groups of mice. *p ≤ 0.05, **p < 0.01.

![FIGURE 9](image-url) A reduction in TNF-α production by CD8+ T cells on day 6 after infection. WT (n = 5) and Thbx21−/− (n = 5) mice were infected with 10^6 Pb-A parasites, and TNF-α expression on CD4+ and CD8+ T cells on days 3 and 6 after infection was measured by flow cytometry. (A) TNF-α and (B) rat IgG1 isotype control expression on CD8+ T cells is shown on gated T cells on day 6 after infection in WT (red) and Thbx21−/− (blue) mice. On day 3 after infection, there is no difference in the (C) percentage or (E) absolute number of CD4+ and CD8+ T cells that express TNF-α. However, on day 6 after infection, there is (D) a 1.80-fold reduction (p < 0.01, Mann–Whitney test) in the percentage and (F) a 1.82-fold reduction in the absolute number of CD8+ T cells that express TNF-α. ***p < 0.001.
Tbx21 was observed until day 6 after infection. These data support previous studies suggesting a shift toward a Th2 response that might confer protection.

We further assessed the effect of T-bet on the Th2 cytokines IL-4 and IL-5 (Fig. 10A, 10B). Tbx21 KO mice also produced significantly higher serum levels of the cytokines eotaxin, G-CSF, and MCP-1 than WT mice during the effector phase of disease (day 6) after infection (Fig. 10C, 10D). Eotaxin is well recognized as a potent chemoattractant for eosinophils (46, 47). Although eosinophil accumulation was not compared in WT versus Tbx21 KO mice, it has previously been reported that Ghanaian pediatric patients with CM had uniformly low eosinophil counts because of tissue sequestration and destruction rather than decreased production during acute illness followed by eosinophilia 30 d after cure (48).

We also found that there was a significant reduction in the percentage of CD8+ T cells that express TNF-α in Tbx21 KO mice compared with WT mice during the effector phase of ECM (Fig. 9D). TNF-α has long been considered a critical mediator of ECM pathogenesis (9). However, a subsequent study indicates that the related cytokine lymphoxygen-α rather than TNF-α is essential to the development of ECM (45). Despite this discrepancy in the role of TNF-α in the pathogenesis of ECM, TNF-α is also important for optimal immunoregulation of pathogen clearance by the host. It is plausible that diminished production of TNF-α by CD8+ T cells can contribute to the hindered parasite clearance observed in Tbx21 KO mice during the effector phase of disease (day 6) after infection (Fig. 1B). Thus, T-bet might exert multifactorial effects during a Pb-A infection, which lies beyond the CD4+ T cell differentiation program.

In addition to elevated serum levels of the classical Th2 cytokines IL-4 and IL-5 (Fig. 10A, 10B), Tbx21 KO mice also produced significantly higher serum levels of the cytokines eotaxin, G-CSF, and MCP-1 than WT mice after infection with Pb-A. Tbx21 KO mice (1547 ± 260.8) had 6.93-fold more serum eotaxin compared with WT mice (223.1 ± 59.5) on day 6 after infection (Fig. 10C). Eotaxin is well recognized as a potent chemoattractant for eosinophils (46, 47).

The factor regulating eosinophil accumulation is not known. However, a recent study demonstrated that IFN-γ is a critical mediator of ECM in susceptible strains of mice. In addition, IFN-γR−/− mice have been shown to be resistant to ECM, and this resistance is associated with reduced levels of CD8+ T cells in the brain (37). Importantly, a recent study indicates that the major source of IFN-γ that modulates ECM pathogenesis is CD4+ T cells (38). The frequency of IFN-γ-expressing CD4+ T cells has been found to be inherently lower in Tbx21 KO mice (44). In accordance, we report a 2.9-fold reduction in the number of CD4+ T cells that express IFN-γ in Tbx21 KO mice during the induction phase (day 3) of disease (Fig. 8D), when CD4+ T cells are known to exert a pathogenic effect (2). In contrast, the frequency of IFN-γ-expressing CD8+ T cells was not reduced in Tbx21 KO mice. It is likely that the reduced frequency of IFN-γ-expressing CD4+ T cells during the induction phase of disease in Tbx21 KO mice contributes to the ECM-resistant phenotype observed in these mice.

We showed an enhanced percentage of T-bet–expressing CD4+ T cells, but not CD8+ cells on day 3 after infection (Fig. 5C, 5D). In contrast, expansion of T-bet–expressing CD8+ T cells was not observed until day 6 after infection. These data support previous studies suggesting that the pathogenic role of CD4+ T cells during the induction (day 3 onward) phase versus CD8+ T cells during the effector (day 6) phase of ECM (3). T-bet expression in CD8+ T cells promotes the generation of cytotoxic effector T cells. Thus, in addition to inducing the differentiation of proinflammatory Th1 CD4+ T cells, T-bet might also play a crucial role in ECM pathogenesis by promoting the expansion and migration of pathogenic CD8+ T cells. A potential role for T-bet in the generation of cytotoxic CD8+ T cells that are pathogenic during ECM is supported by the 4.9-fold increase in the MFI of T-bet expression in CD8+ T cells from day 3 (0.48 ± 0.86 × 103) to day 6 (2.35 ± 0.47 × 103) after infection (Fig. 5E).

We further assessed the effect of T-bet on the CD4+ T cell differentiation program by comparing the expression of the transcription factors that regulate Th1, Th2, Th17, and Treg differentiation in WT versus Tbx21 KO C57BL/6 mouse spleen tissue over the course of a Pb-A infection by QRT-PCR. We found that Tbx21 KO mice expressed 1.8-fold more mRNA that encodes the GATA-3 transcription factor required for Th2 differentiation compared with WT mice on day 6 after infection (Fig. 6B). In accordance with these results, we also demonstrated a 7.3-fold increase in activated GATA-3+ CD4+ T cells in Tbx21 KO mice compared with WT mice by flow cytometry (Fig. 7A, 7B, 7C). These results suggest a shift toward a Th2 response that might confer protection from severe disease. These results are consistent with recent reports documenting higher GATA-3 expression by CD4+ T cells and a Th2-skewed response in the absence T-bet (39).
women with asymptomatic malaria may be involved in maintaining low parasitemia levels (53). However, G-CSF also plays an important role in adaptive immunity and has been shown to induce Th2 polarization in CD4+ T cells. CD4+ T cells treated with G-CSF display diminished IFN-γ and enhanced IL-4 production and upregulation of the GATA-3 transcription factor (54, 55). Furthermore, G-CSF has also been shown to impair the generation of cytolytic effector cells by posttranscriptional suppression of TNF-α (56).

Serum MCP-1 is elevated in *Tbx21−/−* mice (2995 ± 1102) by 5.16-fold on day 6 after infection compared with WT mice (580.4 ± 120.5; Fig. 10E). MCP-1 has been shown to play an important role in several neuroinflammatory diseases (57). However, in a study of 481 Thai patients with malaria, MCP-1 gene polymorphisms were not associated with CM (58) and in the Thai model of ECM, MCP-1 expression in the brain did not differ between susceptible versus resistant strains of mice (59). Although MCP-1 is well recognized for its ability to attract monocytes, it is also essential for Th2 polarization; MCP-1–deficient mice are unable to mount Th2 responses (60). In summary, although the eotaxin, G-CSF, and MCP-1 cytokines display diverse immune effector functions, each of these three cytokines have been shown to contribute significantly to Th2 immunity. Our studies indicate that resistance to ECM in *Tbx21−/−* mice can be attributed to a shift to a Th2 response. These findings are in general agreement with field studies and experimental studies showing that adults harboring helmint infections (61) and mice infected with *Schistosoma mansoni* have increased resistance to CM (62). Helminth parasites are generally known to trigger a Th2 response in their hosts. We think that the increased serum levels of these three cytokines observed in ECM-resistant *Tbx21−/−* mice may be a function of a generalized Th2 switch, and the precise association of these individual cytokines with resistance to ECM remains to be determined.

Recently, our knowledge of the complex interplay between networks of cytokines and transcription factors on differentiation of naive CD4+ T cells in young children and partially sequestered alpha beta CD8+ T cells in experimental cerebral malaria. *J. Immunol.* 169: 6369–6375.

References


