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

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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⁺ CD4⁺ T cells and elevated levels of the eotaxin, MCP-1, and G-CSF cytokines was observed in the absence of T-bet. Our results suggest that in virulent malarias, immune modulation or therapy resulting in an early shift toward a Th2 response may help to ameliorate the most severe consequences of malaria immunopathogenesis and the prospect of host survival. *The Journal of Immunology*, 2013, 191: 4699–4708.

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 autoimmunity 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-

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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 ROR γ t, 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 *Ifng*; it is expressed in a variety of immune cells including dendritic cells, B cells, CD8 $^{+}$ T cells, and NK cells as well as Th1 cells; and it is essential for immunopathology and autoimmunity in numerous disease models (13). We demonstrate that T-bet regulates parasitemia and promotes pathogenesis during *Pb*-A infection and that ECM resistance in *Tbx21* $^{-/-}$ mice is associated with a diminished number of IFN- γ -expressing CD4 $^{+}$ T cells during the induction phase, a reduction of brain sequestered CD4 $^{+}$ and CD8 $^{+}$ T cells during the effector phase and amplification of a Th2 immune response characterized by an expansion of an activated GATA-3 $^{+}$ CD4 $^{+}$ T cell population and overproduction of the eotaxin, MCP-1, and G-CSF cytokines.

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 *Pb*-A parasites was used for all infections in this study. Infection was initiated in a donor mouse by injection of thawed *Pb*-A parasites. Once parasitemia reached ~5% in the donor mouse, blood was collected and diluted in PBS. Infection was then induced in experimental mice by i.p. injection of 10^6 *Pb*-A parasites and mice were monitored for clinical systems of ECM as described previously (27–29). Parasitemia (Parasitized RBCs / Total RBCs \times 100) was determined by examining Giesma-stained thin blood films.

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, CD8 $^{+}$ T cells, and T-bet $^{+}$, TNF- α $^{+}$ and IFN- γ $^{+}$ T cell subsets on days 0, 3, and 6 after infection and 2) CD69 $^{+}$ GATA-3 $^{+}$ CD4 $^{+}$ T cells on day 6 after infection in the spleen. Splenocytes were prepared as described previously (30), and brain-sequestered leukocytes (BSLs) were 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 $515 \times g$ for 30 min at 21°C on 33% Percoll (Sigma-Aldrich, St. Louis, MO).

Single-cell suspensions of splenocytes and BSLs were stained 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- $\alpha\beta$, PerCP-anti-CD4, Pacific blue-anti-CD4, APC/Cy7-anti-CD8, PE/Cy7-anti-CD69, PerCP-anti-CD69, 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-Tbet, PE-anti-GATA-3 in HBSS containing 1% BSA for 30 min at 4°C, washed three times in HBSS containing 0.1% BSA, fixed, and then 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 , Tbet, and GATA-3.

Real-time PCR

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 pulsed-homogenized to create a suspension. RNA was then purified by performing two chloroform extractions, an isopropanol precipitation, a wash with 70% ethanol, and a final resuspension in nuclease-free water. RNA was then treated with 8 U 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 magnesium chloride (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 Tbet, GATA-3, Foxp3, or ROR γ t gene fragments (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-1 α , IL-1 β , 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-1 α , MIP-1 β , 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

Tbet dependent immunity regulates parasite growth and induces ECM pathogenesis

To determine a role for Tbet in the pathogenesis of ECM, we measured the susceptibility of mice deficient for the *Tbx21* gene encoding the Tbet transcription factor to ECM. Simultaneously, we assessed the role of Tbet in the regulation of parasite burden by comparing the parasitemia of *Tbx21* $^{-/-}$ mice with WT controls. Following infection with *Pb*-A parasites, 10 of 10 (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 ($17.7 \pm 3.1\%$) had a 1.6-fold higher parasite burden than WT mice did ($10.9 \pm 1.5\%$). 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 (28.96 ± 3.27 ; $n = 3$) had significantly higher parasitemia (2.3-fold) than *Tbx21*^{-/-} mice without ECM (12.82 ± 2.53 ; $n = 7$; $p < 0.01$, Student *t* test; Fig. 1C). Parasitemia was also markedly higher in *Tbx21*^{-/-} mice with ECM compared with WT mice with ECM (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

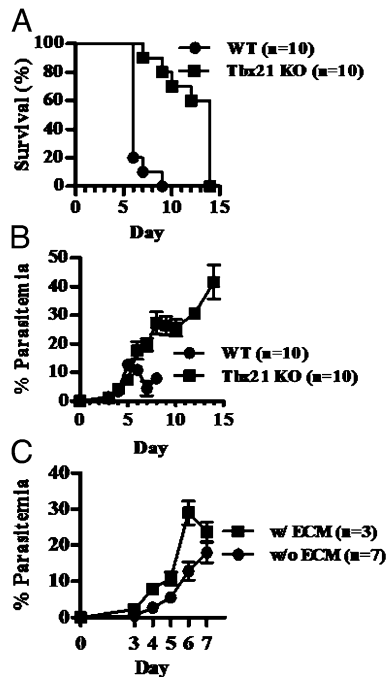


FIGURE 1. T-bet regulates parasite burden but promotes ECM during *Pb*-A infection of C57BL/6 mice. WT and *Tbx21*^{-/-} mice were infected with 10^6 *Pb*-A parasites, and susceptibility to ECM and parasitemia was measured over the course of infection. (A) *Tbx21*^{-/-} mice were significantly protected from ECM; 100% of WT mice versus 30% of *Tbx21*^{-/-} mice exhibited symptoms of ECM ($p < 0.01$, log-rank). (B) Parasite burden was greater in *Tbx21*^{-/-} mice during the cerebral phase of infection. (C) Among *Tbx21*^{-/-} mice, mice with ECM had significantly higher parasitemia than mice without ECM did ($p = 0.03$, two-way ANOVA), indicating that a threshold parasitemia was required to trigger ECM.

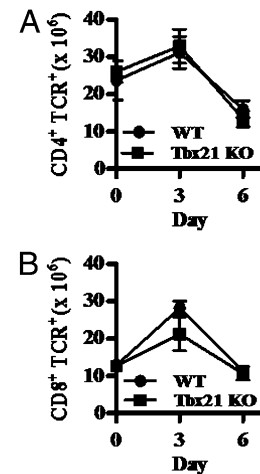


FIGURE 2. Effect of T-bet on the production of pathogenic CD4⁺ and CD8⁺ T cells during a *Pb*-A infection. WT and *Tbx21*^{-/-} mice were infected with 10^6 *Pb*-A parasites, and splenocytes were purified and stained with Abs specific to TCR- $\alpha\beta$, CD4⁺ and CD8⁺ T cells. Lymphocyte populations were then enumerated over the course of infection. There was no significant difference in the absolute number of (A) CD4⁺ and (B) CD8⁺ T cells over the course of *Pb*-A infection in WT versus *Tbx21*^{-/-} mice.

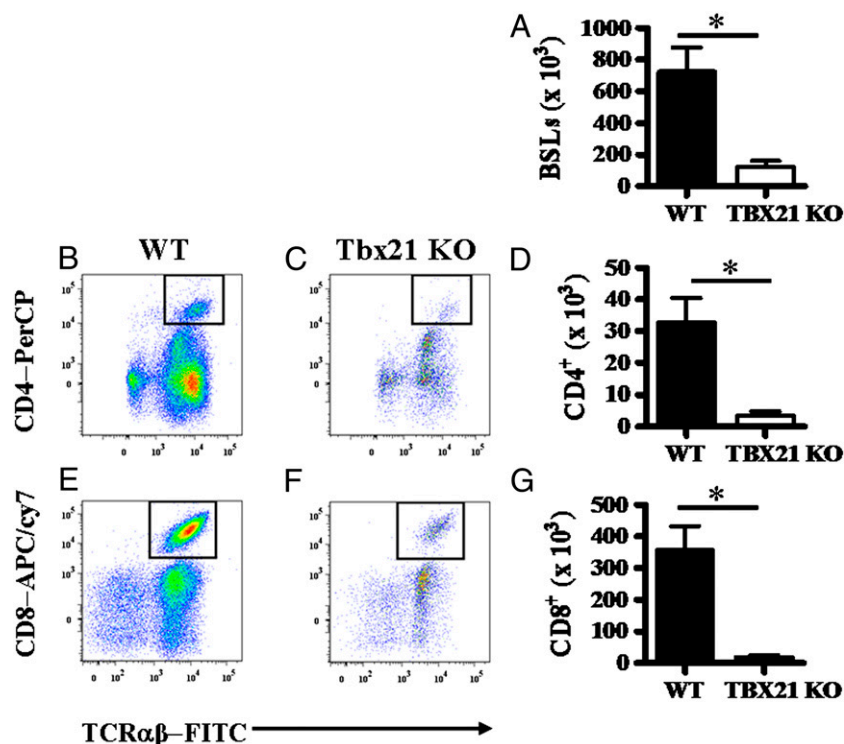
whether ECM resistance caused by the absence of T-bet is associated with a decrease in the induction or migration of pathogenic immune cell subsets to the brain. Remarkably, there was a 5.8-fold reduction in the number of BSLs in *Tbx21*^{-/-} ($125 \pm 35 \times 10^3$ BSLs) compared with WT ($725 \pm 152 \times 10^3$ 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. 3E, 3F) T cells in the absence of T-bet. *Tbx21*^{-/-} mice ($3.5 \pm 1.3 \times 10^3$) had a 9.5-fold reduction ($p < 0.04$, Mann–Whitney test) in brain-sequestered CD4⁺ T cells (Fig. 3D) compared with WT mice ($32.7 \pm 7.8 \times 10^3$). Furthermore, *Tbx21*^{-/-} mice ($19.8 \pm 6.0 \times 10^3$) had an 18-fold reduction ($p < 0.04$, Mann–Whitney test) in brain-sequestered CD8⁺ T cells (Fig. 3G) compared with WT mice ($356 \pm 77 \times 10^3$).

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 (α , β) receptor 1 (IFNAR1) (33). Among brain-sequestered CD8⁺ T cells in WT mice, $46.6 \pm 5.3\%$ ($158 \pm 20 \times 10^3$) and $50.9 \pm 4.6\%$ ($188 \pm 55 \times 10^3$) are central (CD44⁺CD62L⁺) and effector (CD44⁺CD62L⁻) memory cells, respectively (Fig. 4A), $31.9 \pm 3.4\%$ ($118 \pm 34 \times 10^3$) are activated (CD69⁺; Fig. 4C), $74.1 \pm 5.1\%$ ($258 \pm 41 \times 10^3$) express CXCR3 (Fig. 4E), and $8.5 \pm 0.9\%$ ($30 \pm 6 \times 10^3$) 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

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 10⁶ *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.



constitutively expressed in only $0.90 \pm 0.24\%$ of CD4⁺ T cells in noninfected mice. In comparison, T-bet expression was upregulated in $3.46 \pm 1.44\%$ of CD4⁺ T cells ($12.62 \pm 5.90 \times 10^5$ cells)

on day 3 after infection and peaked at $15.13 \pm 0.90\%$ ($24.14 \pm 3.73 \times 10^5$ cells) on day 6 after infection (Fig. 5C, 5D). T-bet has also been shown to be important for the generation of cytotoxic

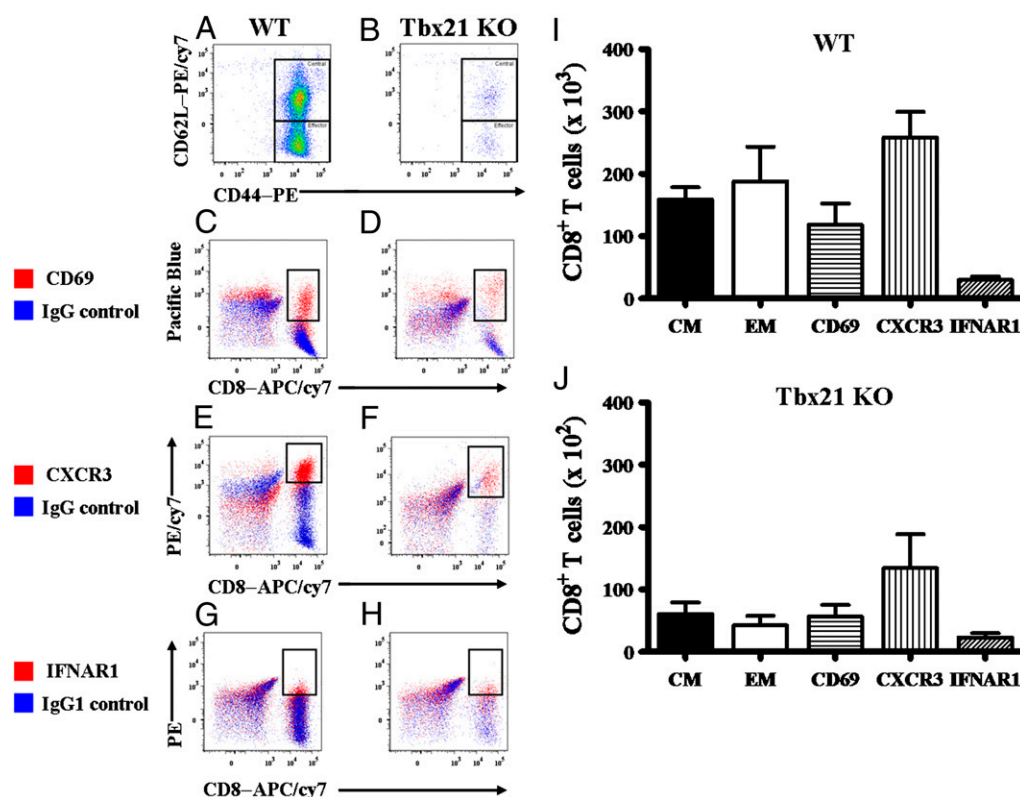
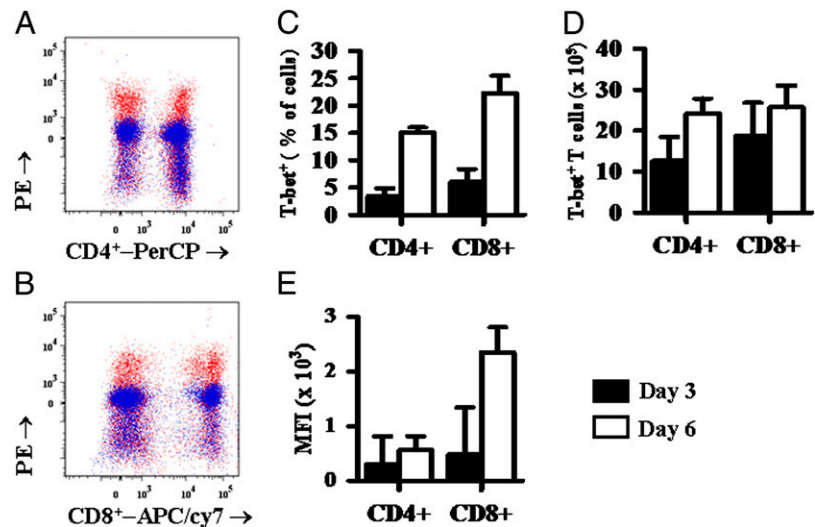


FIGURE 4. Phenotypic analysis of known biomarkers of brain-sequestered CD8⁺ T cells during ECM. Gated CD8⁺ T cells purified from WT and *Tbx21*^{-/-} mice on day 6 after infection with *Pb*-A parasites were analyzed for expression of (A, B) CD44 and CD62L, (C, D) CD69, (E, F) CXCR3, and (G, H) IFNAR1. (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 ($31.9 \pm 3.4\%$) express the CD69 activation marker. (J) Although *Tbx21*^{-/-} mice have significantly fewer brain-sequestered CD8⁺ T cells, these CD8⁺ T cells express known biomarkers.

FIGURE 5. T-bet expression in CD4⁺ and CD8⁺ T cells over the course of a *Pb-A* infection. WT mice were infected with 10⁶ *Pb-A* parasites, and T-bet expression was measured in CD4⁺TCRαβ⁺ and CD8⁺TCRαβ⁺ cells by flow cytometry. T-bet expression is shown in (A) CD4⁺ and (B) CD8⁺ T cells on gated TCR-αβ⁺ cells. T-bet was quantitated by subtracting the percentage of PE-anti-mouse IgG1⁺ cells (blue) from PE-anti-T-bet⁺ cells (red). The (C) percentage and (D) absolute number of T cells that express T-bet and the (E) MFI of T-bet are shown during the induction (day 3) and effector (day 6) phase of *Pb-A* infection. Expression of T-bet in CD4⁺ T cells is induced on day 3 and peaks on day 6. In contrast, expression of T-bet in CD8⁺ T cells at day 3 does not differ from baseline levels in naive mice (data not shown). However, on day 6 after infection, when CD8⁺ T cells are known to be pathogenic, T-bet is highly expressed in CD8⁺ T cells.



effector 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 $6.22 \pm 1.03\%$ of CD8⁺ T cells in noninfected mice. On day 3 after infection, the proportion ($6.04 \pm 2.36\%$) of CD8⁺ T cells expressing T-bet was similar to basal expression in naive mice. However, on day 6 after infection, $22.32 \pm 3.17\%$ of CD8⁺ T cells expressed T-bet ($25.74 \pm 5.27 \times 10^5$ 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 ($2.35 \pm 0.47 \times 10^3$) than in CD4⁺ T cells ($0.56 \pm 0.26 \times 10^3$) 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 ($13.58 \pm 2.04 \times 10^3$) to day 6 ($70.83 \pm 8.25 \times 10^3$) 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 ($22.38 \pm 1.56 \times 10^3$) versus *Tbx21*^{-/-} ($40.86 \pm 11.97 \times 10^3$) 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 ($2.95 \pm 1.25 \times 10^5$) had substantially more (5.2-fold) GATA-3⁺ CD4⁺ T cells than WT mice did ($0.57 \pm 0.19 \times 10^5$; $p < 0.01$, Mann-Whitney test). Furthermore, a larger portion 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) of disease. 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 *Pb-A* 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); $5.26 \pm 0.35\%$

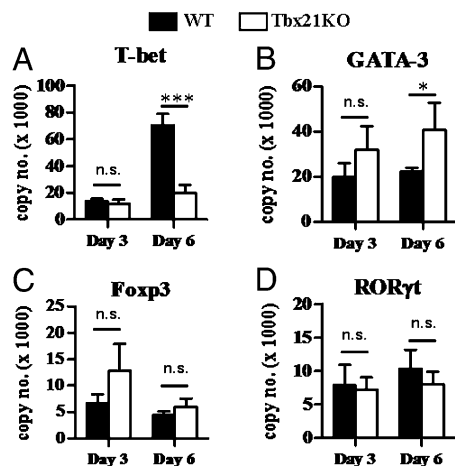


FIGURE 6. Relative copy number of transcription factors required for CD4⁺ T cell differentiation as detected by QRT-PCR. WT ($n = 4$) and $Tbx21^{-/-}$ ($n = 4$) mice were infected with 10^6 *Pb-A* parasites, and expression of (A) T-bet (B) GATA-3 (C) Foxp3 and (D) RORγt was determined by QRT-PCR of cDNA synthesized from RNA isolated from spleen tissue of infected mice on days 3 and 6 after infection. Similar to quantitation by flow cytometry, QRT-PCR results demonstrate that T-bet is maximally expressed ($70.83 \pm 8.25 \times 10^3$ transcripts) on day 6 after infection in spleen tissue from WT mice. Although there is no significant difference in expression of Foxp3 and RORγt between WT versus $Tbx21^{-/-}$ mice, $Tbx21^{-/-}$ mice express 1.8-fold more GATA-3 transcripts compared with WT mice. * $p \leq 0.05$, *** $p < 0.001$.

versus $2.93 \pm 0.25\%$ 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 ($2.42 \pm 0.54 \times 10^5$ cells) compared with WT mice ($4.41 \pm 0.70 \times 10^5$ cells) (Fig. 9F).

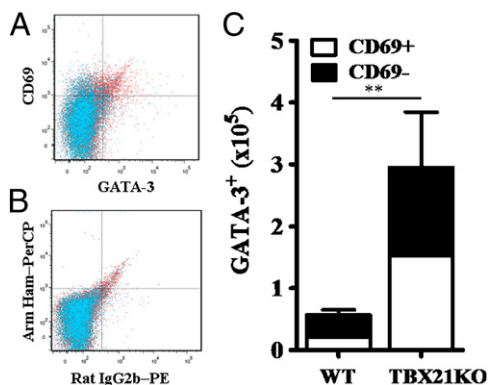


FIGURE 7. Expansion of activated Th2 (CD69⁺GATA-3⁺) CD4⁺ T cells in the absence of T-bet. WT ($n = 4$) and $Tbx21^{-/-}$ ($n = 4$) mice were infected with 10^6 *Pb-A* parasites, and flow cytometry was performed on splenocytes to determine GATA-3 expression and activation status (CD69) of CD4⁺ T cells. 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.

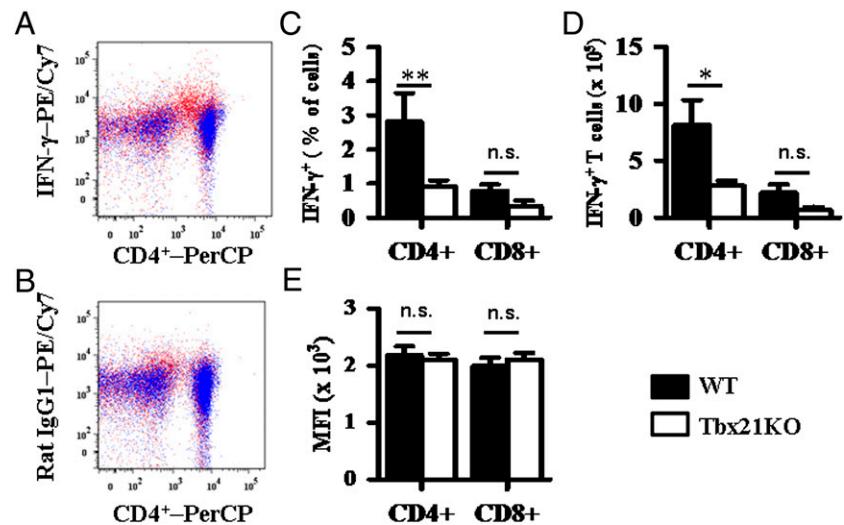
Discussion

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 $32.7 \pm 7.8 \times 10^3$ brain-sequestered CD4⁺ T cells and $356 \pm 77 \times 10^3$ brain

FIGURE 8. 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 *Tbx21* $^{-/-}$ ($n = 5$) mice were infected with 10^6 *Pb-A* parasites, and IFN- γ was quantitated in CD4 $^{+}$ TCR $\alpha\beta^{+}$ and CD8 $^{+}$ TCR $\alpha\beta^{+}$ 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 *Tbx21* $^{-/-}$ (blue) mice are shown. (C) There was a 3.11-fold reduction in the percentage and a (D) 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. * $p \leq 0.05$, ** $p < 0.01$



sequestered CD8 $^{+}$ T cells. In contrast, *Tbx21* $^{-/-}$ 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 *Tbx21* $^{-/-}$ 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 \pm 5.3% ($158 \pm 20 \times 10^3$) and 50.9 \pm 4.6% ($188 \pm 55 \times 10^3$) of brain-sequestered CD8 $^{+}$ T cells in WT mice were central (CD44 $^{+}$ CD62L $^{+}$) and effector (CD44 $^{+}$ CD62L $^{-}$) memory cells, respectively, 31.9 \pm 3.4% ($118 \pm 34 \times 10^3$) expressed the CD69 activation marker, 74.1 \pm 5.1% ($258 \pm 41 \times 10^3$) expressed CXCR3, and a small subset (8.5 \pm 0.9%) expressed IFNAR1 (Fig. 4A, 4C, 4E, 4G, and 4I). Although resistance to ECM in *Tbx21* $^{-/-}$ 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 *Tbx21* $^{-/-}$ 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

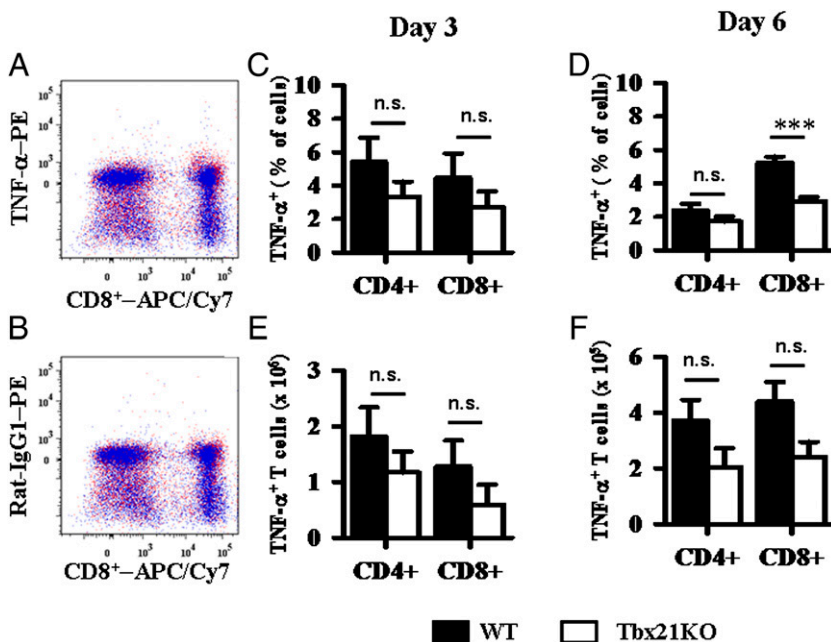


FIGURE 9. A reduction in TNF- α production by CD8 $^{+}$ T cells on day 6 after infection. WT ($n = 5$) and *Tbx21* $^{-/-}$ ($n = 5$) mice were infected with 10^6 *Pb-A* parasites, and TNF- α expression on CD4 $^{+}$ and CD8 $^{+}$ T cells on days 3 and 6 after infection was measured by flow cytometry. (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 *Tbx21* $^{-/-}$ (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 . *** $p < 0.001$.

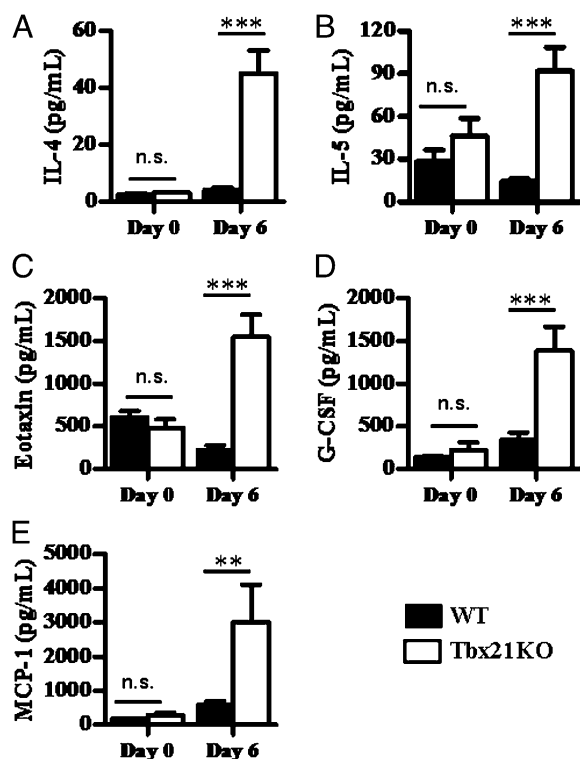


FIGURE 10. A panel of Th2-associated cytokines are elevated in the serum of *Tbx21*^{-/-} ($n = 3$, day 0 and $n = 8$, day 6) compared with WT ($n = 3$, day 0 and $n = 8$, day 6) mice. Serum levels of 23 cytokines were measured in naive (day 0) and *Pb*-A infected (day 6) mice using the Bio-Plex Pro Mouse Cytokine 23-plex assay. Only cytokines with a statistically significant differential expression in WT versus *Tbx21*^{-/-} mice are shown. *Tbx21*^{-/-} mice have significantly elevated levels of (A) IL-4, (B) IL-5, (C) eotaxin, (D) G-CSF, and (E) MCP-1 on day 6, during the cerebral phase of infection. ** $p < 0.01$, *** $p < 0.001$.

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 T cell subset depletion studies that demonstrate 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 ($0.48 \pm 0.86 \times 10^3$) to day 6 ($2.35 \pm 0.47 \times 10^3$) 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*^{-/-} C57BL/6 mouse spleen tissue over the course of a *Pb*-A infection by QRT-PCR. We found that *Tbx21*^{-/-} 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*^{-/-} 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).

Data from the murine experimental model of ECM and cytokine profiling of samples from field studies have long suggested that proinflammatory cytokines are the major contributors to CM pathogenesis (42). Studies depleting IFN- γ (10) or using IFN- γ -deficient mice (43) have demonstrated that IFN- γ is a critical mediator of ECM in susceptible strains of mice. In addition, IFN- γ R-deficient 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*^{-/-} mice (44). In accordance, we report a 2.9-fold reduction in the number of CD4⁺ T cells that express IFN- γ ⁺ in *Tbx21*^{-/-} 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 ⁺ CD8⁺ T cells was not reduced in *Tbx21*^{-/-} mice. It is likely that the reduced frequency of IFN- γ -expressing CD4⁺ T cells during the induction phase of disease in *Tbx21*^{-/-} mice contributes to the ECM-resistant phenotype observed in these mice.

We also found that there was a significant reduction in the percentage of CD8⁺ T cells that express TNF- α in *Tbx21*^{-/-} 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 lymphotoxin- 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*^{-/-} mice during the effector phase (day 6) of disease (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*^{-/-} mice also produced significantly higher serum levels of the cytokines eotaxin, G-CSF, and MCP-1 on day 6 after infection with *Pb*-A. *Tbx21*^{-/-} 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). Although eosinophil accumulation was not compared in WT versus *Tbx21*^{-/-} mice, it has previously been reported that Ghanian 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). Importantly, the eotaxin receptor is expressed by Th2 CD4⁺ T cells, and eotaxin is critical for the generation and maintenance of Th2 cells at allergic sites and promotes the production of IL-4 and IL-5 (49, 50). Therefore, it is likely that eotaxin participates in the amplification of the Th2 response observed in *Tbx21*^{-/-} mice.

Expression of serum G-CSF was increased by 4.07-fold in *Tbx21*^{-/-} (1384 ± 286.1) compared with WT mice (339.7 ± 92.8) on day 6 after infection (Fig. 10D). G-CSF was first identified as a growth factor for neutrophils (51). Indeed, administration of recombinant human G-CSF to attenuated *P. berghei* XAT-infected CBA mice resulted in a 5-fold elevation in the peripheral blood neutrophil count and a significantly lower peak parasitemia compared with control mice (52), and elevated G-CSF levels in pregnant

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 \pm 1102) by 5.16-fold on day 6 after infection compared with WT mice (580.4 \pm 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 murine 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 helminth 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 into different subsets has expanded tremendously (12). Nevertheless, how infections with different *Plasmodium* species modulate these differentiation programs in naive CD4⁺ T cells in HLA-disparate hosts and thus influence the outcome of pathogenesis of severe malaria has not been studied. Results from this study offer an initial insight on the contribution of CD4⁺ T cell differentiation programs on the pathogenesis of ECM in mice. Additional studies directed toward understanding the influence of malarial Ags and toxins on the differentiation program of naive CD4⁺ T cells in young children and partially immune adults could facilitate the design of superior antiparasitic and antidiarrheal vaccines against malaria.

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Disclosures

The authors have no financial conflicts of interest.

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