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THEMIS Is Required for Pathogenesis of Cerebral Malaria and Protection against Pulmonary Tuberculosis

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We identify an *N*-ethyl-*N*-nitrosourea (ENU)-induced I23N mutation in the THEMIS protein that causes protection against experimental cerebral malaria (ECM) caused by infection with *Plasmodium berghei* ANKA. *Themis*^{I23N} homozygous mice show reduced CD4$^+$ and CD8$^+$ T lymphocyte numbers. ECM resistance in *P. berghei* ANKA-infected *Themis*^{I23N} mice is associated with decreased cerebral cellular infiltration, retention of blood-brain barrier integrity, and reduced proinflammatory cytokine production. THEMIS^{I23N} protein expression is absent from mutant mice, concurrent with the decreased THEMIS^{I23N} stability observed *in vitro*. Biochemical studies *in vitro* and functional complementation *in vivo* in *Themis*$^{I23N/+}$:*Lck*$^{-/+}$ doubly heterozygous mice demonstrate that functional coupling of THEMIS to LCK tyrosine kinase is required for ECM pathogenesis. Damping of proinflammatory responses in *Themis*^{I23N} mice causes susceptibility to pulmonary tuberculosis. Thus, THEMIS is required for the development and ultimately the function of proinflammatory T cells. *Themis*^{I23N} mice can be used to study the newly discovered association of *THEMIS* (6p22.33) with inflammatory bowel disease and multiple sclerosis.

The inflammatory response to microbial stimuli is a multistep process that involves sensing of a danger signal, recruitment of myeloid (neutrophils, basophils, monocytes, macrophages) and lymphoid (CD4$^+$ and CD8$^+$ T lymphocytes, NK cells) cells, production of proinflammatory cytokines (tumor necrosis factor alpha [TNF-α], interferon gamma [IFN-γ], and interleukin-1 [IL-1]) and chemokines (IL-8, monocyte chemoattractant protein 1, and KC), elimination of the microbial threat, and tissue destruction and repair (1, 2). In the presence of persistent tissue injury or of an unusual infectious or environmental insult, overexpression of proinflammatory mediators or insufficient production of anti-inflammatory signals (prostaglandin E2, IL-10, TGF-β, and IL-1Ra) causes acute or chronic states of pathological inflammation. Population studies of chronic inflammatory diseases such as inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, and others have identified a complex genetic architecture of disease susceptibility, with additional effects of microbial triggers that initiate and sustain pathological inflammation (3–5). Many of the mapped disease loci and genes are common to two or more such diseases, suggesting that some critical features of pathogenesis are shared by these conditions.

Cerebral malaria (CM) is an acute, life-threatening encephalitis that is a complication of *Plasmodium falciparum* infection in children and pregnant women (6). CM-associated neuroinflammation has been studied in a mouse model of experimental CM (ECM) induced by infection with *Plasmodium berghei* ANKA (7). In this model, brain endothelial cells activated by trapped parasitized red blood cells (pRBCs) produce cytokines and chemotactic factors that recruit neutrophils and activated CD8$^+$ and CD4$^+$ T cells. Release of cytotoxic molecules by inflammatory leukocytes leads to loss of integrity of the blood-brain barrier (BBB), microthrombosis, and hypoxia of the brain parenchyma, leading to neurological symptoms, including seizures and coma, and ultimately death (8, 9). Recent findings show that elevated levels of inflammatory molecules (TNF-α, IFN-γ, IL-1β, macrophage inflammatory protein 1α [MIP-1α], MIP-1β, CXCL10, and complement component 5a [C5a]) are associated with an increased risk of CM, supporting a neuroinflammatory component of human CM (10–12). Antibody-mediated cell ablation experiments have demonstrated a strong pathological role for CD8$^+$ and CD4$^+$ T cells, NK cells, and neutrophils in ECM (7). Conversely, we and others have demonstrated an ECM-protective effect of mutations in major proinflammatory genes such as those for IFN-γ (*Ifng*) and its receptor (*Ifngr1*), lymphotoxin (*Lta*/*Ltb*), complement component 5a (*Hc*) (reviewed in reference 13), and certain transcription factors that regulate the expression of these genes in myeloid and lymphoid cells, including IFN regulatory factor 1 (IRF1) (14), IRF8, and STAT1 (15). Whole-brain transcript profiling along with chromatin immunoprecipitation and sequencing data comparing ECM-susceptible and -resistant (*Irf8*^{myls} BXH2 strain) mice identified a core transcriptome activated during ECM (15). This transcriptome contains several genes, including those for IRF1, IRF8, and STAT1, that have been identified as risk factors for acute and chronic human inflammatory conditions. Thus,

Received 5 September 2014 Returned for modification 7 October 2014
Accepted 24 November 2014
Accepted manuscript posted online 1 December 2014
Citation Torre S, Faucher SP, Fodil N, Bongfen SE, Berghout J, Schwartzentruber JA, Majewski J, Lathrop M, Cooper AM, Vidal SM, Gros P. 2015. THEMIS is required for pathogenesis of cerebral malaria and protection against pulmonary tuberculosis. Infect Immun 83:759–768. doi:10.1128/IAI.02586-14.
Editor: J. H. Adams
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doi:10.1128/IAI.02586-14

studies using the mouse model of ECM may identify critical regulatory genes and pathways that underlie the shared etiology and pathogenesis of acute and chronic human inflammatory diseases.

To uncover novel host factors that, when inactivated, protect against the development of ECM, we employed *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screening of mice. We report a recessive mutation in the gene *Themis* (thymus-expressed molecule involved in selection; Mouse Genome Informatics accession no. 2443552) that protects mice from lethal neuroinflammation upon infection with *P. berghei* ANKA. The effect of this mutation on immune cell function has been characterized at the cellular and molecular levels.

MATERIALS AND METHODS

Ethics statement. This study was performed in accordance and compliance with the strict guidelines of the Canadian Council on Animal Care. Protocols were approved by the ethics committee of McGill University (protocol 5287) and the Trudeau Institute Institutional Animal Care and Use Committee of (protocol IACUC 02-191 [Cooper]). Mice were euthanized by carbon dioxide inhalation, and every effort was made to minimize animal suffering.

Mice. Inbred C57BL/6J (B6) and C57BL/10J (B10) mice were purchased from The Jackson laboratory (Bar Harbor, ME). *Lck* mutant mice (Lck^{tm1Mak}, referred to as $Lck^{-/-}$) were provided by André Veillette (IRCM, Montreal, QC, Canada). Eight week-old B6 males were administered 3 weekly doses of ENU (90 mg/kg) by intraperitoneal injection. Mutagenized G0 males were bred with wild-type (WT) B10 females to generate G1 offspring, which were backcrossed again with B10 females (G2 offspring). Two G2 females per pedigree were backcrossed with their G1 father to generate G3 mice for phenotyping. Homozygous $Themis^{I23N}$ G3 mice were intercrossed to produce a stable mouse line.

Parasites and infections. *P. berghei* ANKA parasites from the Malaria Reference and Research Reagent Resource Center were maintained as frozen stocks at −80°C. Blood parasitemia was determined on thin blood smears stained with Diff-Quik reagents. Seven-week-old G3 mice were intravenously (i.v.) infected with 10^6 pRBCs. Mice were monitored three times daily for the appearance of neurological symptoms. In other experiments, mice were infected (i.v.) with 5×10^5 *Plasmodium chabaudi* AS pRBCs and blood parasitemia was monitored over time. *Plasmodium* strains were provided by Mary M. Stevenson (McGill University Health Center Research Institute, Montreal, QC, Canada).

Genomic analyses. *Melvin* G3 mice were genotyped by using a panel of 193 single nucleotide polymorphism markers informative for parental B6 versus B10 strains (16). Linkage analysis was performed with the R/qtl software package by using the binary model, where survival past day 13 (ECM resistance versus susceptibility) was used as a phenotype to detect linkage. Whole-exome sequencing of two ECM-resistant *Melvin* G3 mice was carried out. Exome capture was performed with a SureSelect Mouse All Exon kit (Agilent Technologies) and parallel sequencing on an Illumina HiSeq 2000 (100-bp paired-end reads). Reads were aligned with genome assembly mouse/July 2007 (NCBI37/mm9) by using the Burrows-Wheeler alignment tool (17), and coverage was assessed with BEDTools (18). Variants were called by using Samtools pileup and varFilter (17) and annotated with ANNOVAR (19). The *Themis* mutation was genotyped by PCR (primers 5′-CCACCCCCATGTGTTTCTAC-3′ and 5′-CACTTTGTTTGCTGGGTGTG-3′), followed by sequencing of the PCR product.

RT-qPCR. Reverse transcriptase quantitative PCR (RT-qPCR) was performed with primers 5′-TGAAATCCAAGGTGTGCTGA-3′ and 5′-CGTCCGTAGACAGCAACTGA-3′. *Themis* mRNA was expressed relative to the hypoxanthine phosphoribosyltransferase (HPRT) reference control.

Protein expression in transfected cells. A full-length WT *Themis* cDNA was PCR amplified from a B6 thymus mRNA template with primers 5′-ACTGGAATTCCCACCATGGCTTTATCTCTGGAAG-3′ and 5′-CAGTCTCGAGTCACAGTGGTGCTTGCGG-3′. Restriction sites for EcoRI (GAATTC) and XhoI (CTCGAG) were introduced into the primers to facilitate cloning into expression plasmid pcDNA3. A full-length $Themis^{I23N}$ mutant was generated by site-directed mutagenesis with primers 5′-CCTGACTGGTTTTCTAGGA-3′ and 5′-TCCTAGAAAACCAGTCAGG-3′. HEK293 (ATCC CRL-1573) cells were transfected with Lipofectamine 2000 reagent (Life Technologies), followed by selection in Geneticin (G418, 500 μg/ml; Invitrogen). Protein expression was monitored by immunoblotting with an anti-THEMIS antibody (3D4; R. H. Schwartz, NIH). For protein stability studies, stably transfected cells were incubated with cycloheximide (CHX; 20 μg/ml). Cells were lysed in 50 mM Tris (pH 7.5)–150 mM NaCl–1% Triton X-100–0.1% sodium dodecyl sulfate (SDS) supplemented with protease/phosphatase inhibitors.

THEMIS tyrosine phosphorylation. HEK293T (ATCC CRL-3216) cells (3×10^6) were cotransfected with WT or $Themis^{I23N}$ constructs and either the WT or a hyperactive F505 *Lck* variant (André Veillette, IRCM, Montreal, QC, Canada), and 24 h later, cells were lysed in 50 mM Tris (pH 7.5)–150 mM NaCl–1% Triton X-100–0.1% SDS. THEMIS was immunoprecipitated (IP) with anti-THEMIS antibody (16 h, 4°C) and then captured with protein G agarose beads. IP products were separated on gel and then immunoblotted with a mouse antiphosphotyrosine antibody (P-Tyr-100; Cell Signaling Technology) and a horseradish peroxidase-conjugated anti-mouse TrueBlot ULTRA IgG secondary antibody (eBioscience). Blots were reprobed with anti-THEMIS antibody, and blots of whole-cell lysate were probed with anti-LCK antibody (André Veillette) to validate the efficiency of immunoprecipitation and transfection, respectively.

Evans blue dye extravasation. *P. berghei* ANKA-infected mice (day 6 postinfection) were injected (i.v.) with 0.2 ml of 1% Evans blue dye (Sigma-Aldrich, Oakville, Canada). One hour later, mice were exsanguinated and perfused with phosphate-buffered saline. Brains were excised and incubated with 1 ml of dimethyl formamide for 48 h to extract the Evans blue dye from the tissues. Optical density at 610 nm was measured, and measurements were converted into micrograms of dye extravasated per gram of tissue.

Immunophenotyping. Thymus and spleen cells (1×10^8 to 2×10^8) were stained with anti-CD4–phycoerythrin (PE)/Cy7 and anti-CD8–PE, and doubly negative (DN; $CD4^-$ $CD8^-$), doubly positive (DP; $CD4^+$ $CD8^+$), CD4 singly positive ($CD4^+$; $CD4^+$ $CD8^-$), and CD8 singly positive ($CD8^+$; $CD4^-$ $CD8^+$) T cells were isolated with a fluorescence-activated cell sorter (FACS). Leukocytes infiltrating the brains of infected animals were isolated as previously described (20). Thymus and spleen cells from control and *P. berghei* ANKA-infected mice were analyzed by FACS by using markers of lymphoid cells (anti-CD45–allophycocyanin [APC]-efluor780, anti-CD8–Bv421, anti-CD4–PE, anti-TCRβ–fluorescein isothiocyanate [FITC]) and myeloid cells (anti-CD45–APC-efluor780, anti-CD11b–APC, anti-Ly6G–FITC). All of the antibodies used were purchased from BioLegend. Viable leukocytes were gated as $CD45^+$ cells in the spleen and thymus and as $CD45^{hi}$ cells in the brain. Spleen cells were stimulated with either CD3/CD28 (eBioscience) or IL-12p70/IL-18 (BioLegend) for 48 h; this was followed by measurement of TNF-α and IFN-γ by enzyme-linked immunosorbent assay (ELISA; BioLegend). Cytokines were also measured in sera of naive and *P. berghei* ANKA-infected mice.

Infection with *M. tuberculosis*. Eight-week-old mice were infected via the aerosol route with ~200 CFU of *Mycobacterium tuberculosis* H37Rv. Bacterial replication was determined by colony counts on organ homogenates. Histological analysis of the lung caudal lobe was performed with formalin-fixed sections stained with hematoxylin and eosin (H&E) or stained for acid-fast bacilli with Ziehl-Neelsen stain.

RESULTS

We used ENU mutagenesis in mice to identify genes that, when inactivated, protect against lethal ECM induced by *P. berghei* ANKA infection (21, 22). In this screening, G3 offspring were

FIG 1 Resistance to *P. berghei* ANKA-induced CM in the *Melvin* pedigree. (A) Breeding scheme for the production of ENU-induced mutant mice (see Materials and Methods for details). (B) G2 females, Danelle and Nina, were backcrossed with their G1 *Melvin* father, and their offspring infected with 10^6 *P. berghei* ANKA pRBCs and monitored for neurological symptoms and survival. Mice that survived beyond day 13 were considered ECM resistant. G3 [G1 × G2 Danelle], $n = 17$; G3 [G1 × G2 Nina], $n = 25$; B6, $n = 18$. (C) Genome-wide linkage analysis of 22 G3 DNA samples from pedigree *Melvin* (8 resistant [R], 14 susceptible [S]) was performed with a panel of 193 informative markers that distinguish B6 versus B10 mice. The LOD score trace detects significant linkage to proximal Chr10 (LOD, ~8.63; position, 27.6 Mb; $P = 0.05$). (D) Compiled haplotype data from pedigree *Melvin* animals show enrichment for homozygous B6 (A) alleles between positions 25.1 and 33.4 Mb on Chr10 in resistant mice, indicating that an ENU-induced mutation generated in the B6 background of these mice is conferring ECM resistance. Susceptible mice have either a heterozygous (H) or a homozygous B10 (B) genotype.

generated (Fig. 1A) and infected with *P. berghei* ANKA. ECM-susceptible B6 and B10 controls develop fatal neurological symptoms (paralysis, tremors, and seizures) between days 5 and 8 postinfection. Conversely, *P. berghei* ANKA-infected G3 mice that do not develop cerebral symptoms and survive beyond day 13 are considered ECM resistant. Mating of G1 male *Melvin* mice with two G2 females produced G3 mice, 19% of which were ECM resistant (8/34 pups; Fig. 1B). Linkage analysis revealed a linkage peak on chromosome 10 (Chr10; logarithm of odds [LOD], ~8.63; position, 27.6 Mb) (Fig. 1C). Haplotype analysis of ECM-resistant mice showed enrichment for homozygosity of B6-derived alleles (A; from ENU-treated G0) on proximal Chr10, while ECM-susceptible mice displayed enrichment for homozygosity of WT B10 alleles (B) (Fig. 1D). This suggests that ECM resistance in the *Melvin* pedigree is caused by homozygosity for an ENU mutation on proximal Chr10.

Whole-exome sequencing of two ECM-resistant G3 mice identified an ENU-associated homozygous mutation under the linkage peak on Chr10. The T-to-A transversion in exon 1 of *Themis* causes a change from isoleucine (I) to asparagine (N) at position 23 (I23N) of the cysteine-containing, all-β in Themis-1 (CABIT-1) domain of THEMIS (Fig. 2A). I23 is invariant in the THEMIS family, and the I23N substitution is nonconservative, suggesting that it is likely to be pathological (Fig. 2A). Genotype-phenotype correlations in additional *Melvin*-derived mice validated that homozygosity for I23N (*Themis*^{I23N}) is fully protective against ECM, while heterozygosity is mildly protective, and all WT mice are ECM susceptible (Fig. 2B). The ECM resistance phenotype of *Themis*^{I23N} mice is independent of *P. berghei* ANKA blood parasitemia between days 1 and 6 (Fig. 2C). In addition, challenging mice with *P. chabaudi* AS, a malarial parasite whose pathogenesis is limited to blood stage replication without cerebral disease, indicates that *Themis*^{I23N} has no effect on the ability of mice to control replication (days 4 to 9) and eradicate (days 9 to 12 and 14 to 18) blood stage parasites (Fig. 2D).

THEMIS is a 73-kDa protein expressed in the thymus, spleen, and lymph nodes (23–25). We found that expression of THEMIS protein was very low in the thymi of homozygous *Themis*^{I23N} mutant mice, while heterozygous mutants showed expression levels similar to those of B6 or 129S1/SvImJ WT mice (Fig. 3A). Expression of the THEMIS^{I23N} variant was extremely low in sorted CD4$^+$ CD8$^+$ DP thymic lymphocytes, and was undetectable in all other sorted T cell populations from the thymus or spleen (Fig. 3B). We found that the mutation had no effect on mRNA expression in

FIG 2 An ENU-induced mutation in the CABIT-1 domain of the THEMIS protein protects against ECM. (A) Whole-exome sequencing identified a T-to-A transversion in exon 1 of the *Themis* gene causing an isoleucine (I)-to-asparagine (N) amino acid substitution at position 23 in the CABIT-1 domain. Isoleucine at position 23 of the protein is highly conserved across species. Hom., homozygous; PRS, proline-rich sequence. (B) Survival plots of *P. berghei* ANKA-infected *Themis* homozygous mutants (*Themis*^{I23N}; $n = 24$), heterozygous mice (*Themis*$^{I23N/+}$; $n = 12$), and *Themis* WT or B6 ($n = 29$) mice. Statistically significant difference between homozygous mutant and WT mice was determined with the log rank test (****, $P < 0.0001$). Data are compiled from 13 independent infections. (C) The courses of *P. berghei* ANKA blood parasitemia in WT B6 controls and *Themis*^{I23N} homozygous mice are similar ($n = 5$/group). (D) *Themis*^{I23N} homozygous mutants ($n = 3$) challenged with *P. chabaudi* parasites are resistant to blood-stage malaria and display blood parasitemia similar to that of B6 controls ($n = 5$). Data are compiled from two independent experiments. (C, D) Data are expressed as means ± standard deviations for each group. Statistical analysis was performed with the two-tailed unpaired Student *t* test (n.s., nonsignificant).

sorted T cells (Fig. 3C). To investigate a possible effect of the mutation on protein stability, HEK293 cells stably expressing the WT or *Themis*^{I23N} variants were treated with CHX and analyzed by immunoblotting (Fig. 3D and E). The THEMIS^{I23N} variant shows a half-life drastically shorter (estimated at ~6 h) than that of the WT (>12 h), strongly suggesting that the I23N mutation impairs protein stability, resulting in low levels of THEMIS^{I23N} expression *in vivo*.

We investigated the effect of the *Themis*^{I23N} mutation on the number and function of T cells, both at steady state and during infection. FACS analysis indicated a significant depletion of singly positive CD4$^+$ (**, $P = 0.0026$) and CD8$^+$ (*, $P = 0.0335$) T cell compartments in the thymi of *Themis*^{I23N} mice compared to those of WT mice (Fig. 4A and C). Depletion of singly positive T cells was also evident in splenic CD4$^+$ (**, $P = 0.0069$) and CD8$^+$ (*, $P = 0.0152$) cells (Fig. 4B and D). Such depletion of CD4$^+$ and CD8$^+$ T cells has been reported for null mutant alleles at *Themis* (23–27), suggesting that I23N is a complete loss-of-function allele. The effect of the *Themis*^{I23N} mutation on the number and function of T cells was investigated at day 5 after *P. berghei* ANKA infection, a time at which peripheral T cells become activated (7). *P. berghei* ANKA infection caused a significant increase in the number of CD4$^+$ splenic T cells in both mutant and WT mice; however, the total number and relative frequency of CD4$^+$ T cells were much greater in the control than in the mutant (**, $P = 0.0079$) (Fig. 5A and B). We observed reduced IFN-γ production *in vitro* by *Themis*^{I23N} mutant splenocytes from *P. berghei* ANKA-infected mice upon T cell receptor activation via anti-CD3/CD28 stimulation (**, $P = 0.0012$) or following incubation with IL-12/IL-18 (**, $P = 0.0022$) (Fig. 5C). Decreased IFN-γ production by mutant splenocytes *in vitro* was paralleled *in vivo* by lower levels of serum IFN-γ in infected mutant mice (*, $P = 0.0147$) (Fig. 5E). Similarly, splenocytes from *Themis*^{I23N} mutants produced significantly less TNF-α in response to stimulation with anti-CD3/CD28 (*, $P = 0.0154$). Neuroinflammation in ECM is associated with breakdown of the BBB (28, 29). An Evans blue dye extravasation assay was used to assess the BBB integrity of *P. berghei* ANKA-infected mice. As opposed to the brains of *P. berghei* ANKA-infected B6 controls that cannot exclude the blue dye, the brains of *Themis*^{I23N} mutants remained unstained, indicating an intact BBB (Fig. 5G). By flow cytometry analysis, we observed less cellular infiltration of CD45^{hi} leukocytes, CD45^{hi} Ly6G$^+$/CD11b$^+$

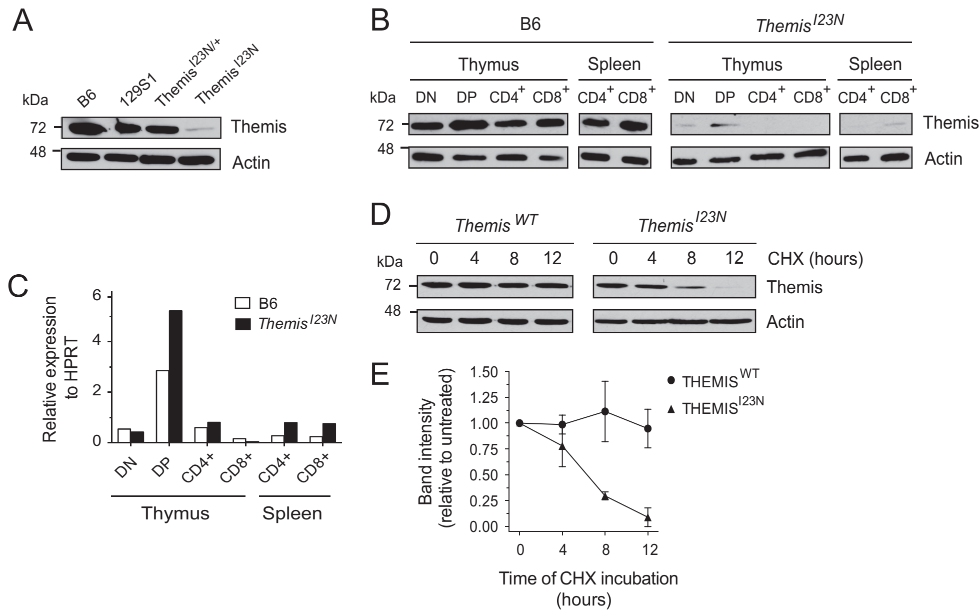

FIG 3 Reduced protein expression and reduced stability of the THEMIS^{I23N} variant *in vivo* and *in vitro*. (A) Cell extracts from the thymi of WT control B6 and 129S1/SvImJ (129S1) mice and *Themis* heterozygous (*Themis*$^{I23N/+}$) and homozygous (*Themis*^{I23N}) mice were analyzed for THEMIS protein expression by immunoblotting. (B) Extracts from sorted CD4$^-$ CD8$^-$ (DN), CD4$^+$ CD8$^+$ (DP), CD4$^+$ CD8$^-$ (CD4$^+$), and CD4$^-$ CD8$^+$ (CD8$^+$) spleen and thymic T cells were analyzed for expression of the WT and THEMIS^{I23N} variant proteins by immunoblotting. (C) RT-qPCR was performed with RNA isolated from sorted T cell populations from the thymus and spleen. *Themis* mRNA is expressed relative to the HPRT reference control. The data are from a representative experiment with RNA isolated from two mice per group. (D) Transfected HEK293 cells stably expressing the WT or THEMIS^{I23N} variant protein were treated with CHX (20 μg/ml) for 4, 8, and 12 h, and equal amounts of protein (50 μg) were analyzed by immunoblotting. Data represent three independent experiments assessing two different clones per construct. (E) Data from panel D were quantified by ImageJ. THEMIS band intensities were normalized to actin loading controls and expressed as a fraction of the protein expression present at time zero (untreated) for each construct. Data are expressed as means ± standard deviations.

neutrophils, and CD45^{hi} CD4$^+$ and CD8$^+$ T cells in the brains of *Themis*^{I23N}-infected mice than in those of controls (Fig. 5H). Hence, ECM resistance in *Themis*^{I23N} mice is concomitant to reduced numbers of CD4$^+$ and CD8$^+$ T cells, reduced proinflammatory cytokine production during infection, and preservation of BBB integrity.

THEMIS participates in T cell receptor signaling, and its activity is regulated by the CD4 and CD8 glycoprotein-associated

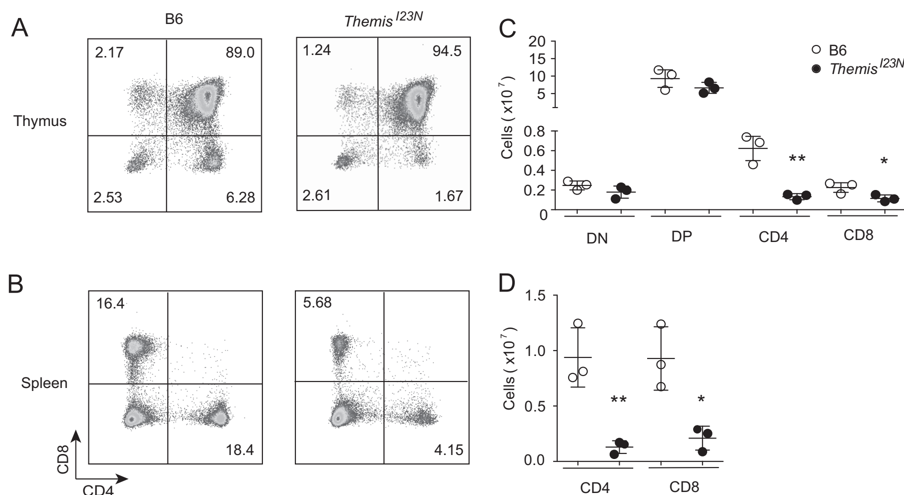

FIG 4 *Themis*^{I23N} mutants show a defect in thymocyte development. Thymocytes and splenocytes from WT and *Themis*^{I23N} mutant mice ($n = 3$/group) were stained with anti-CD45–APC efluor780, anti-CD4–PE, and anti-CD8–Bv421 and analyzed by flow cytometry. (A, B) Representative FACS plots showing CD4-versus-CD8 profiles expressed as a percentage of viable CD45$^+$ thymic (A) and splenic (B) cells. (C) Total numbers of T cell populations from the thymus. *Themis*^{I23N} mice have significantly lower numbers of CD4$^+$ T cells (**, $P = 0.0026$) and CD8$^+$ T cells (*, $P = 0.0335$) in the thymus than WT mice do. (D) Total numbers of T cells from the spleen. *Themis*^{I23N} mice have a significantly lower number of peripheral CD4$^+$ T cells (**, $P = 0.0069$) and CD8$^+$ T cells (*, $P = 0.0152$) than WT mice do. Data are expressed as means ± standard deviations for each group and represent data from two independent experiments. Statistical analysis was performed by two-tailed unpaired Student *t* test.

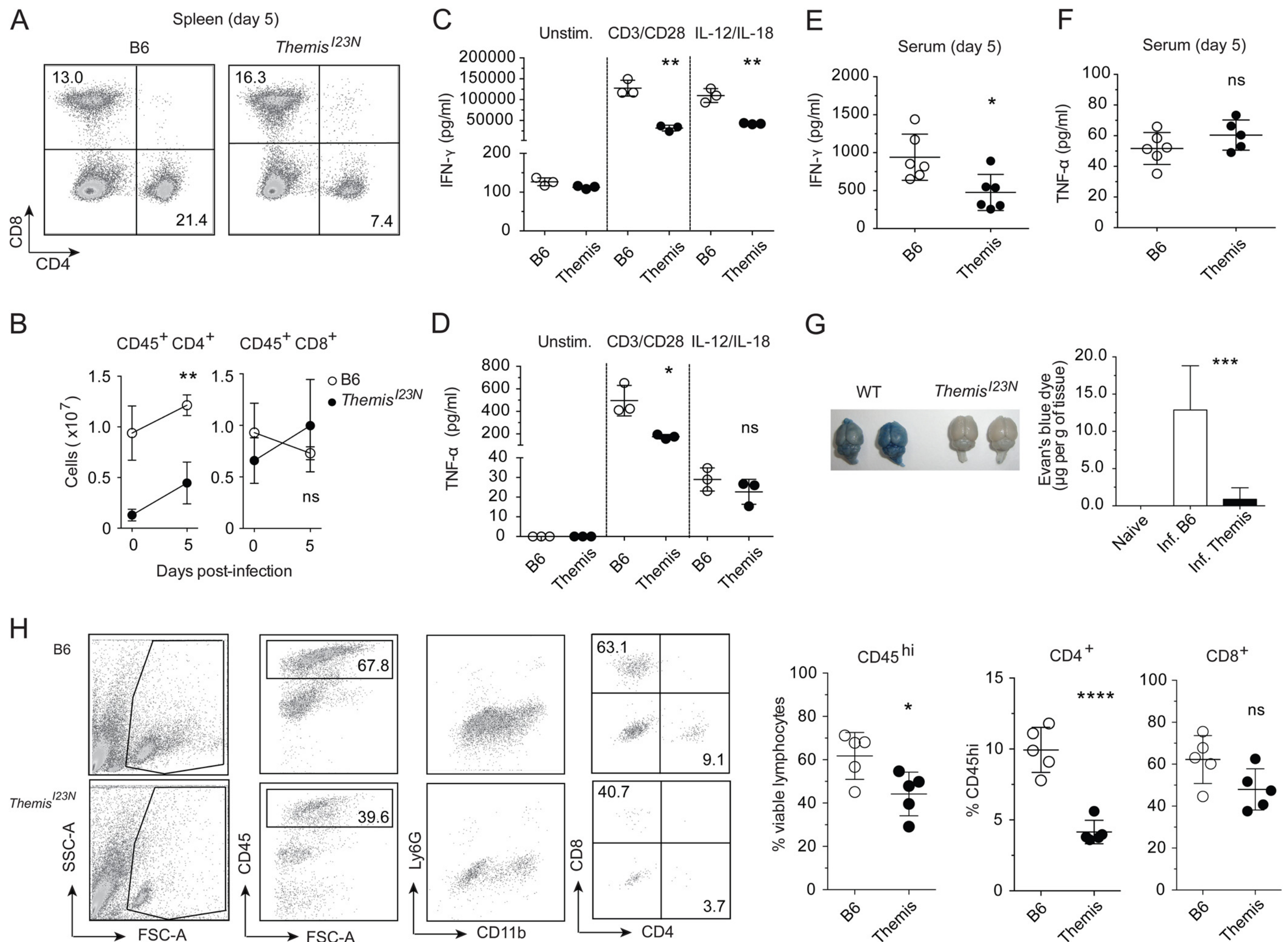

FIG 5 Immunophenotyping of *Themis*^{I23N} mutants following *P. berghei* ANKA infection. Control B6 and *Themis*^{I23N} mutant mice were infected with *P. berghei* ANKA, and 5 days later, the function of peripheral (spleen) T cells was examined. FACS analysis indicates a lower proportion (A) and absolute number (B) of CD45$^+$CD4$^+$ T cells in *P. berghei* ANKA-infected *Themis*^{I23N} mutants than in B6 WT controls (**, $P = 0.0079$; $n = 3$). (C) Splenocytes from *P. berghei* ANKA-infected *Themis*^{I23N} mice produce significantly less IFN-γ *ex vivo* in response to stimulation with anti-CD3/anti-CD8 or with IL-12/IL-18 (**, $P = 0.0012$ and $P = 0.0022$, respectively) than WT B6 controls. Unstim., unstimulated. (D) Same as panel C, but TNF-α was measured in culture supernatant. (E, F) Serum cytokines were measured by ELISA and show less circulating IFN-γ (*, $P = 0.0147$) in *Themis*^{I23N} mutants than in B6 controls. Data are expressed as means ± standard deviations for each group and represent data from two independent experiments. Statistical analysis was performed with the two-tailed unpaired Student *t* test. (G) Evans blue extravasation assay to assess integrity of the BBB of *P. berghei* ANKA-infected (Inf.) WT (B6 or 129S1/SvImJ) and *Themis*^{I23N} mutant mice. Six days following infection, mice were injected with Evans blue dye. The dye was extracted from the brain tissues, and the optical density at 610 nm was measured. Measurements were converted into micrograms of dye extravasated per gram of tissue. Brains from *Themis*^{I23N} mutants remained unstained by Evans blue (***, $P = 0.0003$). Naive WT mice, $n = 3$; *P. berghei* ANKA-infected B6 mice, $n = 3$; *P. berghei* ANKA-infected *Themis*^{I23N} mice, $n = 8$. Data are expressed as means ± standard deviations for each group and represent data from two independent experiments. Statistical analysis was performed with the two-tailed unpaired Student *t* test. (H) Five days following *P. berghei* ANKA infection, infiltrating leukocytes were isolated by Percoll gradient from brain homogenates and analyzed by FACS. Representative FACS plots of cellular infiltration in the brain indicate reduced infiltration of CD45^{hi} leukocytes, CD45$^+$ CD11b$^+$ Ly6G$^+$ neutrophils, CD45$^+$ CD4$^+$ T cells, and CD45$^+$ CD8$^+$ T cells in the brains of *Themis*^{I23N} mutants. Data are expressed as mean percentages ± the standard deviations and represent data from two independent experiments. Statistical analysis was performed by two-tailed unpaired Student *t* test. SSC, side scatter; FSC, forward scatter.

tyrosine kinase LCK (30–33). We examined the coupling of THEMIS and LCK in the pathogenesis of ECM. We first examined LCK-mediated tyrosine phosphorylation of WT and THEMIS^{I23N} variant mice *in vitro* (Fig. 6A). Hyperactive LCK^{F505Y} tyrosine phosphorylated WT and THEMIS^{I23N} mice with similar efficiencies. Furthermore, we examined the requirement of LCK for neuroinflammation in the ECM model. *Lck*$^{-/-}$ mice challenged with *P. berghei* ANKA were resistant to ECM (Fig. 6B), ultimately succumbing to hyperparasitemia later in infection (days 18 to 21), similarly to ECM-resistant *Themis*^{I23N} homozygous mice. We also conducted genetic complementation studies with mice that are doubly heterozygous mice for loss-of-function mutations at *Themis* and *Lck* (*Themis*$^{I23N/+}$:*Lck*$^{-/+}$). *P. berghei* ANKA-infected *Themis*$^{I23N/+}$:*Lck*$^{-/+}$ doubly heterozygous mice showed greater ECM resistance (43% survival) than mice that are heterozygous mice for *Themis*$^{I23N/+}$ alone (16% survival), although this difference did not reach statistical significance ($P = 0.088$, log rank statistical test) (Fig. 6B). However, *P. berghei* ANKA-infected *Themis*$^{I23N/+}$:*Lck*$^{-/+}$ doubly heterozygous mice are ECM resistant, unlike B6 WT controls (**, $P = 0.006$). Taken together, these

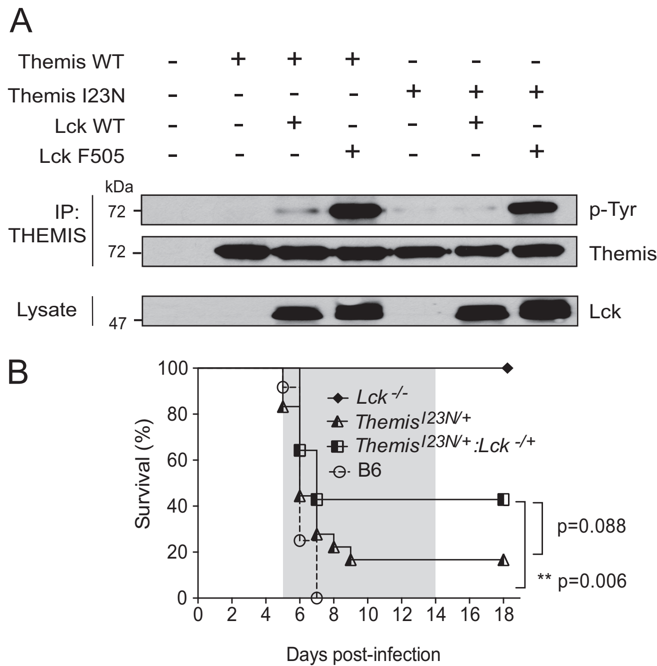

FIG 6 LCK protein tyrosine kinase mutants are resistant to ECM. (A) Plasmids encoding either the WT or the *Themis*^{I23N} variant were cotransfected with either an *Lck*^{WT} or a hyperactive *Lck*^{F505Y} plasmid into HEK293T cells. THEMIS protein was immunoprecipitated (IP) from total cell lysate and assessed for tyrosine phosphorylation by immunoblotting with an antiphosphotyrosine (p-Tyr) antibody. LCK (56 kDa) was assessed in total cell lysate to ensure successful cotransfection. In the presence of hyperactive Lck^{F505}, both WT and mutant THEMIS proteins are subjected to tyrosine phosphorylation. (B) Survival of *Lck* knockouts (*Lck*$^{-/-}$; $n = 12$), *Themis* heterozygous mice (*Themis*$^{I23N/+}$; $n = 18$), *Themis-Lck* doubly heterozygous mutants (*Themis*$^{I23N/+}$:*Lck*$^{-/+}$; $n = 14$), and B6 WT controls ($n = 12$) following infection with *P. berghei* ANKA. The log rank statistical test indicated significantly greater CM resistance of the *Themis-Lck* doubly heterozygous mice than of WT B6 (**, $P = 0.006$) and suggests resistance greater than that of *Themis*$^{I23N/+}$ mice, although this difference did not reach statistical significance ($P = 0.088$).

results show that LCK and THEMIS are individually required for pathological inflammation during ECM, with possible functional coupling between the two proteins.

Proinflammatory Th1 cytokines produced by T cells are required for protection against intracellular infections (15). We evaluated the response of *Themis*^{I23N} mice to infection with *M. tuberculosis*. Mice were infected with low-dose aerosol *M. tuberculosis*, and bacterial replication was assessed in the lungs, spleen, mesenteric lymph nodes (MLN), and liver at days 35, 60, and 90 postinfection (Fig. 7A). *Themis*^{I23N} mutants displayed 5- to 10-fold greater *M. tuberculosis* replication than controls. Histological analysis of *M. tuberculosis*-infected B6 lungs identified typical small mononuclear accumulations in the background of a normal lung alveolar network, while large segments of *Themis*^{I23N} mutant lungs were consolidated with extensive infiltration of leukocytes and with areas of necrosis (Fig. 7B). Increased microbial burden in the inflammatory lesions present in the *Themis*^{I23N} mutants was also evident upon staining for acid-fast bacilli (Fig. 7B). Together, these results indicate that *Themis* is required for protection against mycobacterial infections.

DISCUSSION

We have identified an I23N mutation in THEMIS that causes resistance to CM. In summary, the I23N mutation is phenotypically

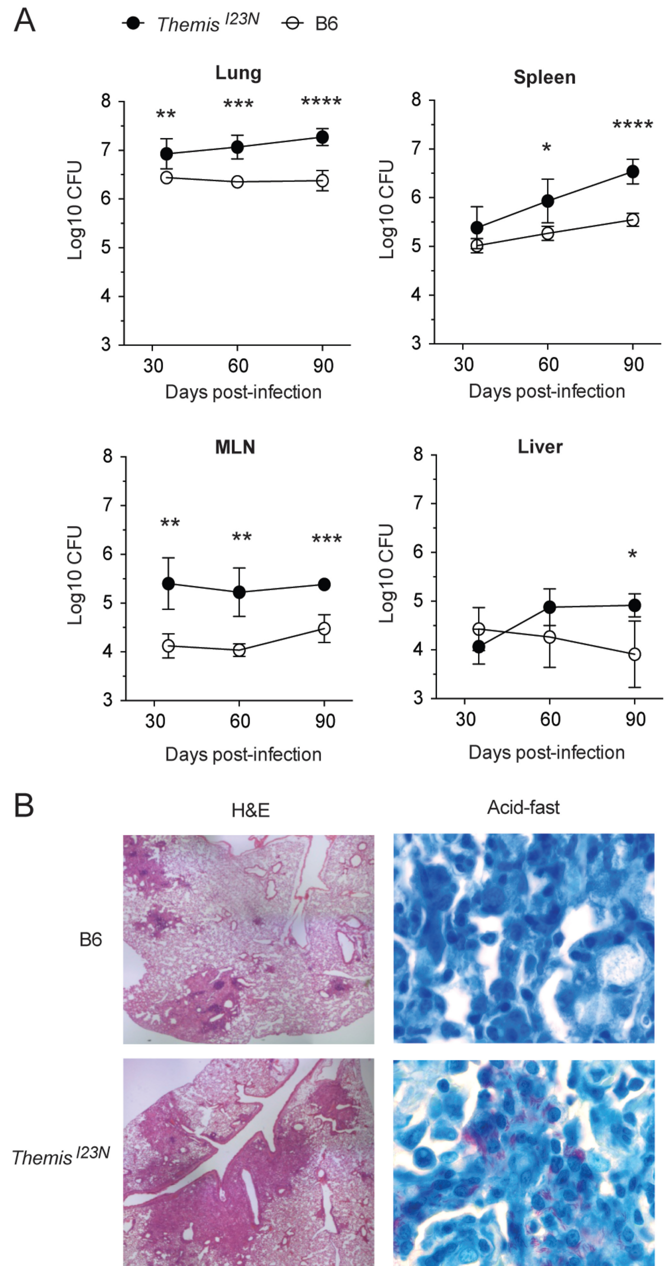

FIG 7 Loss of THEMIS function causes susceptibility to tuberculosis. (A) Control B6 and *Themis*^{I23N} mutants were infected with 200 CFU of *M. tuberculosis* H37Rv by the aerosol route, and 35, 60, and 90 days postinfection, organs were harvested and microbial replication was determined by CFU counting. Each group contained five mice, and statistical significance was estimated by the two-tailed unpaired Student *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). MLN, mesenteric lymph nodes. (B) Histological analysis of *M. tuberculosis*-infected lungs from B6 controls and *Themis*^{I23N} mutants at day 90 postinfection by H&E staining and visualization of acid-fast bacilli by Ziehl-Neelsen staining.

expressed as (i) decreased protein stability, (ii) reduced CD4$^+$ and CD8$^+$ T cell numbers in the thymus and spleen, (iii) decreased proinflammatory cytokine (IFN-γ, TNF-α) production by T cells at steady state and during infection, (iv) retention of BBB integrity, and (v) emergence of susceptibility to pulmonary tuberculosis. THEMIS acts as a regulator of positive selection of thymic

lymphocytes from $CD4^+$ $CD8^+$ DP cells to mature singly positive $CD4^+$ and $CD8^+$ cells (23–27). Mice lacking $CD8^+$ T cells or their secreted products (TCRαβ [34], TAP-1 [35], β2-microglobulin [36], and perforin [37]) or mice deficient in $CD4^+$ T cells (34, 35, 38) are ECM resistant. The effect of the *Themis*^{I23N} mutation on thymic T cell maturation and peripheral T cell activity is similar to those described for null mutations in *Themis* (23–27). Hence, studies in models of infectious diseases with our *Themis*^{I23N} mutant provide insight and are generally reflective of loss of THEMIS function. Additional work is necessary to confirm that THEMIS is specifically required for the proinflammatory function of peripheral T cells, independent of its role in T cell development.

The CM-protective effect of *Themis*^{I23N} may reflect the essential role of T cell receptor (TCR) signaling in the intrathymic development of functional T cell repertoires. Indeed, studies with mice lacking TCR structural components (34, 35) or downstream signaling molecules such as LCK (30), LAT (39), GRB2 (40), TESPA1 (41), and ITK (42) show severe defects in T cell development with significant immunodeficiency (reviewed in reference 43). LCK acts immediately downstream of the TCR, and upon its activation, it phosphorylates THEMIS (31–33). Phosphorylated THEMIS has been proposed to interact with additional members of the TCR signalosome to propagate TCR proximal signaling (44). In this study, we show that *Lck*$^{-/-}$ mutant mice are, like *Themis*^{I23N} mice, resistant to the development of ECM. In addition, genetic complementation studies with mice that are doubly heterozygous mice for loss-of-function mutations in *Themis* and *Lck* (*Themis*$^{I23N/+}$:*Lck*$^{-/+}$) also display increased resistance to ECM, unlike singly heterozygous control mice. These data establish that THEMIS and LCK are both required for neuroinflammation.

THEMIS has two amino-terminal globular CABIT domains (CABIT-1 and CABIT-2) that are predicted to form an extended β-sandwich-like fold or a dyad of six-stranded β-barrel units (23, 24, 44). Though the CABIT structural motifs have been identified by sequence conservation, their structure-function relationships have yet to be clearly defined. Recent studies have shown that deletion of a part of the CABIT-1 domain (Δ150-174) in mouse THEMIS causes a defect in T cell development (45). We show that the I23N mutation in the CABIT-1 domain causes protein instability in primary thymic cells and in transfected cells, suggesting that I23N may cause misfolding of the protein, which is consequently targeted for degradation. Hence, integrity of the CABIT-1 domain is required for THEMIS protein stability.

Finally, genome-wide association studies have recently identified *THEMIS* (6p22.33) as a candidate gene for the Chr6 locus associated with celiac disease in humans (Chr6; positions, 127.99 to 128.38 Mb, CRCh37/hg19) (46–48). Duodenal mucosa from active celiac patients showed higher *THEMIS* gene expression than that of patients undergoing effective treatment or that of healthy controls (2, 49). *THEMIS* was also recently identified as a risk factor for multiple sclerosis (50). The demonstration herein that *Themis* elimination protects against neuroinflammation in mice validates the proposed role of *THEMIS* in pathological inflammation in humans. Furthermore, the association of *THEMIS* with different inflammatory diseases that affect different anatomical sites and that follow different pathogeneses, places *THEMIS* as one of the core “inflammatory” genes that regulate common aspects of pathological inflammation *in vivo* and that participate in the etiology of these different inflammatory diseases.

ACKNOWLEDGMENTS

This work was supported by a Team Grant to P.G. and S.M.V. from the Canadian Institutes of Health Research (CIHR Team in ENU Mutagenesis and Infectious Diseases CTP-87520), and by a grant to P.G. from the U.S. National Institutes of Health (R01AI035237). P.G. is supported by a James McGill Professorship award. S.M.V. is supported by the Canada Chairs Research Program.

We are indebted to Patricia D’Arcy, Genevičve Perreault, Leigh Piercey-Brunet, Mifong Tam, and Susan Gauthier for expert technical assistance and to Sandra Salem and Vicki Leung for help with protein work and imaging, respectively.

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