AN N-ETHYL-N-NITROSOUREA (ENU)-INDUCED MUTATION IN JAK3 PROTECTS AGAINST CEREBRAL MALARIA BUT CAUSES SUSCEPTIBILITY TO MYCOBACTERIA

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Address correspondence to: Philippe Gros, Professor, Complex Traits Groups, McGill University, 3649 Sir William Osler Promenade, room 366, Montreal, Canada, H3G-1Y6; philippe.gros@mcgill.ca**ABSTRACT**

Cerebral malaria (CM) is an acute, often lethal, neurological complication of malaria. The cell and molecular pathways involved in CM pathogenesis are poorly characterized and need to be better understood to identify novel therapeutic targets for intervention. CM can be modeled in mice by infection with *Plasmodium berghei ANKA*. To identify genes and proteins involved in the pathogenesis of CM, and whose~~ich~~ inhibition may be of clinical value, we set-up a forward genetic screen to identify recessive mutations that protect mice against otherwise lethal *P. berghei*-induced CM. We identified a pedigree (P48) segregating a resistance trait (in 31% of progeny) whose~~ich~~ protective effect was fully penetrant on C57BL/6J and 129S1 genetic backgrounds, and that was mapped to the proximal portion of chromosome 8. Whole genome sequencing of CM-resistant P48 animals identified homozygosity for a mis-sense mutation (W81R) in the Band 4.1/Ezrin/Radixin/Moesin (FERM) domain of the Jak3 protein (Janus-associated kinase 3). The causative effect of W81R was verified by complementation testing in *Jak3*$^{-/W81R}$ double heterozygotes. W81R shows co-dominance with increased survival of *Jak3*$^{+/W81R}$ over controls, suggesting a dominant-negative effect of the mutation. Immunological characterization of *Jak3*^{W81R} homozygotes shows defects in thymic development, with concomitant severe depletion of CD8^{+} T cells (spleen, thymus), B cells (spleen, bone marrow) and NK cells (spleen). There is also defective T-cell dependent production of IFNγ upon stimulation along different pathways. Adoptive transfer of infected splenocytes from *P. berghei* infected C57BL/10 mice abrogates CM-resistance in *Jak3*^{W81R} homozygotes, an effect largely attributed to the CD8^{+} T cell compartment. Paradoxically, *Jak3*^{W81R} homozygotes are found to be highly susceptible to infection with mycobacteria (BCG, *M. tuberculosis*) and *Citrobacter*, where a robust Th1 immune response is required for protection. Our findings highlight the critical role of Jak3 in CD8^{+} T cell and IFNγ driven CM pathogenesis, and identify this kinase as a possible novel target for pharmacological intervention in CM.

INTRODUCTION

Malaria, caused by infection with members of the *Plasmodium* family of parasites, still remains a global health problem, with close to 250 million clinical cases and almost a million deaths occurring each year, mostly in African children []. Cerebral malaria (CM) is the most severe complication of *P. falciparum* infection; Although CM develops in less than 1% of *Plasmodium* infected individuals, its sudden onset, rapid progression and limited treatment options (high dose quinine or artemisnin) contribute to an often-lethal outcome. CM is characterized by trapping of parasitized erythrocytes in the host microvasculature including the blood brain barrier (BBB) that triggers a strong inflammatory response *in situ*, vascular damage and hypoxia. Permeability of the BBB leads to seizures, paralysis, unrousable coma and death [1]. The cell and molecular pathways involved in CM pathogenesis are poorly characterized and need to be better understood to identify novel therapeutic targets for intervention. Clinical epidemiological studies in different geographical areas of malaria-endemicity have indicated that the onset, progression and outcome of CM involve a complex interplay between environmental factors, parasite-expressed virulence factors and host genetic factors influencing replication of the parasite or innate or acquired immunity [2-4]. Genetic studies in humans have pointed to a heritable component to susceptibility to CM [], while case control association studies have suggested a complex and heterogeneous genetic component in CM, including hemoglobin variants (hemoglobinopathies), polymorphisms in cytokine genes or gene promotors, and many others [reviewed by Kiatkowskie and Bongfen][1].

The complex genetic component of CM susceptibility has also been investigated in the mouse model for experimental CM caused by infection with *Plasmodium berghei* ANKA (*Pb*A). *P. berghei* infection in mice closely mimics *P. falciparum*-induced CM in humans [5], with susceptible mouse strains (e.g. C57BL/6J, CBA/J) developing an acute cerebral syndrome within 6-7 days characterized by ataxia, paraplegia, seizures and coma leading to uniform lethality by day 8-10 post-infection. Like in humans, studies in mouse mutants bearing inactivating mutations at specific genes have shown that host-driven inflammation plays a central role in CM pathogenesis [1, 6]. Indeed, local production of pro-inflammatory cytokines such as IFN-γ [7], TNF-α [8] and LT-α [9], upregulation of chemokine ligands [10] and cell adhesion molecules [11], as well as sequestration of immune cells ($CD4^+$, $CD8^+$, NK cells) [10, 12, 13] and parasites [13] in the brain microvasculature have all been shown to play a role in CM. On the other hand, inactivating mutations in genes coding for pro-inflammatory molecules [14, 15], and generation of anti-inflammatory cytokines like IL-10 [12] and IL-4 [16] have been associated with protection from CM.

Genome-wide linkage studies in informative crosses derived from mouse strains showing varying degrees of susceptibility to *P. berghei* induced CM have detected at least six quantitative trait loci (QTL) - designated *berghei* resistance (*Berr*) loci – as modulating response to infection including survival from acute infection: *Berr1* (chromosome 1*)*, *Berr2* (chromosome 11), *Berr3* (chromosome 9), *Berr4* (chromosome 4), *Berr5* (chromosome 19), and the Cmsc locus mapping to the H-2 region of chromosome 17 [17-20]. The *Berr5* locus co-localizes with three other immune loci, including *Trl-4* (tuberculosis resistance), *Tsiq2* (T-cell secretion of IL-4), and

Eae19 (experimental allergic encephalitis 19) suggesting the possibility of a common genetic effect underlying these phenotypes. Nevertheless, the modest effects of these individual loci, the relatively large size of the chromosomal regions mapping underneath the QTL peaks, and the large number of positional candidates have precluded the positional cloning of the genes involved.

ENU mutagenesis is a powerful experimental tool to introduce random mutations in the mouse germ-line. Such mutations can be propagated in informative pedigrees, where they can be bred to homozygosity, and where their effect on a given physiological system or host pathway can be investigated. In high throughput screening experiments, such mutations may manifest themselves as rare phenodeviant pedigrees displaying unique disease-associated phenotypes. The positional cloning of the mutant gene, facilitated by the *de novo* nature of the mutation (absent form the reference sequence), may in turn identify novel proteins that play a role in the specific phenotype and associated pathology. This strategy has been used successfully to identify genes, proteins and pathways in a broad range of disease states, including susceptibility to infections [], obesity [21], muscle development and function [22], cardiomyopathy [23] and thrombocytopenia [24]. In this study, we implemented a large scale ENU mutagenesis strategy to identify genes that play an important role in the pathogenesis of cerebral malaria. Intravenous infection of C57BL/6J and C57BL/10J mice with 10^6 *P. berghei* parasitized erythrocytes is uniformly lethal with all animal developing cerebral symptoms by day 5-6 and succumbing to infection by days 7-10. We searched for recessive mutations that would protect mice form *P. berghei* induced CM and associated lethality, and that would confer survival to this otherwise lethal infection. We aimed to identify novel protein and biochemical pathways that may constitute novel targets for small molecule inhibition and therapeutic intervention in this lethal infection.

In a first example of this screen, we report the identification of a pheno-deviant pedigree that displays segregation of a CM-resistance phenotype. We demonstrate that this resistance is phenotypically expressed as a severe depletion of several immune cells compartments including CD8+ T cell, B cells and NK cells, and caused by a mutation on chromosome 8 which we identify as a loss-of-function allele at the Jak3 kinase gene. Resistance to CM in this mutant is associated with impaired Th1 response which is concomitant with increased susceptibility to mycobacterial infections.

MATERIALS AND METHODS

Mice and parasites

Wild type C57BL/6J (B6), C57BL/10J (B10) and 129S1/SvImJ (129S1) mice were purchased from the Jackson laboratories (Bar Harbor, Maine, USA). ENU-mutagenized mice were bred at the animal facility of the Goodman Cancer Centre, McGill University. Mice were maintained under pathogen-free conditions and handled according to guidelines of the Canadian Council on Animal Care. *P. berghei* ANKA parasites were a kind gift from Dr. Mary M. Stevenson, Montreal General Hospital Research Institute, McGill University. Parasites were maintained as frozen stocks at -80°C, and passaged weekly in B10 mice. Infected B10 mice were monitored daily for parasitemia and when parasitemia reached 1-3%, mice were bled, and the blood was diluted for infection of ENU-mutagenized mice.

ENU Mutagenesis and breeding

Twenty 8-week-old wild type (WT) B6 male mice were mutagenized by intraperitoneal injection of a fractionated dose of 3 X 90mg/kg of ENU at 1-week intervals. After recovery of fertility (8-15 weeks post treatment), treated males were used in a breeding scheme designed to uncover recessive mutations as previously described [25]. Briefly, treated males (G0) were bred to WT B10 females to generate G1 animals, which are heterozygous for mutations across their genome. G1 males were crossed to B10 females to generate G2 animals, each of which has a 50% chance of inheriting any single mutation carried by their G1 father. Two G2 females were backcrossed to their G1 father to generate G3 animals, about a quarter of which were expected to be homozygous for mutations carried by the G1 male. In order to introduce a higher degree of polymorphism in the offspring to facilitate genetic mapping, G1 males from pedigrees with a confirmed heritable resistance trait (after phenotyping of G3 animals) were out-crossed to 129S1 female mice to generate G2 (aka F1) animals. F1 mice were intercrossed to generate F2 animals, 25% of which were expected to carry the mutation from the G1 male fixed to homozygosity (Figure 1).

Infection with *Plasmodium berghei* ANKA

G3 and F2 mice at >7 weeks of age were infected intravenously (i.v.) with 10^6 *P. berghei* ANKA-parasitized erythrocytes (obtained from parasite donor mice), and were monitored closely (twice daily) for the appearance of characteristic neurological symptoms, for weight loss and for survival. Mice that survived greater than 13 days post infection with no neurological symptoms were considered to be resistant to cerebral malaria. B10 and 129S1 mice were used as susceptible controls in all experiments, while IFN-γ knockout (KO) mice (on B6 background) were used as resistant controls.

DNA preparation and Genetic Mapping

Tail biopsies were obtained from all mice, and genomic DNA was isolated by a standard procedure using proteinase K digestion and phenol/chloroform extraction, as previously described [26], and DNA samples were diluted to 20ng/μl in distilled H_2O for genotyping. Genome-scanning was performed at the McGill University and Genome Quebec Innovation Centre (Montreal, Qc, Canada), using DNA samples from 15 resistant and 29 susceptible G3

mice from pedigree 48 (P48), and the massArray platform from Sequenom and a panel of 131 B6/B10 polymorphic markers (SNPs) distributed across the genome. Further linkage analysis was conducted in 211 additional (P48 X 129S1) F2 mice (see Figure 1) genotyped for microsatellite markers (Mouse Genome Informatics Database; www.informatics.jax.org) informative for B6 and 129S1 progenitors. Genotyping was carried out by a standard PCR-based method using [α-^{32}P] dATP labeling, and resolution on denaturing 6% polyacrylamide gels.

Immunophenotyping

Following isolation of cells from different tissues (spleen, thymus, bone marrow, lymph nodes and blood), the cells were surface stained with appropriate dilutions of antibodies (determined from titration experiments), for 20 minutes in the dark at 4°C, fixed in PBS containing 1% formaldehyde and stored at 4°C in the dark until FACS analysis (performed within 24 h). The following anti-mouse monoclonal antibodies were used: FITC anti-CD4 (RM4-5), PE anti-CD8a (53-6.7), PECy7 anti-CD19 (1D3), APC anti-CD11c (HL3), APCCy7 anti-GR1 (RB6-8C5), V450 anti-CD117 (2B8) (all from BD Pharmingen); PerCPCy5.5 anti-F4/80 (BM8), PerCPCy5.5 anti-CD3e (145-2C11) and eFluor 450 anti-CD11b (MJ7/18) (all from eBioscience). A minimum of 1 X 10^5 cells were collected by FACS for each tissue sample. Data analysis was performed using FACS DiVa version 6.0 software. Initial gating of each sample set used a forward scatter (FSC)-area versus a FSC-height plot to gate out cell aggregates. Immune cells were isolated, and the different cell populations stained with various antibodies (anti-CD3, -CD8, -CD4, -CD19, -CD11c, -CD117) and analyzed by flow cytometry.

Th1 differentiation and FACS staining

Single cell suspensions of splenocytes (≥95% viable by trypan blue exclusion testing), were labeled with carboxyfluorescein succinimidyl ester (1.25 µM, CFSE; In vitrogen) and transferred to 48-well plates (1x10^6 cells/well) in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 2 mM L-glutamine. Cells were stimulated for 3-4 days with plate-bound anti-CD3 (1µg/ml; clone 145-2C11) and soluble anti-CD28 (4µg/ml; clone 37-51) (all from BD) either in Th1 differentiation conditions [rIL-12 (5ng/ml; R&D, Minneapolis, MN); anti-IL-4 (10µg/ml; clone 11B.11, National Cancer Institute Biological Resources Branch preclinical Repository] or in control conditions (no exogenous cytokines or antibodies added). Activated splenocytes were re-stimulated with phorbol 12-myristate 13-acetate (PMA, 5 ng/ml) and ionomycin (375 ng/ml) in the presence of Brefeldin A (5 µg/ml; all from Sigma) for 4.5h in 96 V-bottom-well plates. Following incubation, cells were surface-stained with PerCPCy5.5 anti-CD3e (145-2C11), Pacific Blue anti-CD4 (RM4-5) and viability dye ViViD (Invitrogen) for 30 min in the dark at 4°C. After intracellular staining for IFN-γ and Tbet [27], cells were fixed in PBS containing 1% formaldehyde and stored at 4°C in the dark until FACS analysis (performed within 24 h). The following anti-mouse monoclonal intracellular antibodies were used: PECy7 anti-IFN-g (XMG1.2) and eFluor660 anti-Tbet (eBio4B10) (all from eBioscience). A minimum of 5 X 10^5 total cells were collected by FACS for each sample. Data analysis was performed using FACS DiVa version 6.0 software. Initial gating of each sample set used a forward scatter (FSC)-area versus a FSC-height plot, and a CD3 versus ViViD (Amcyan) to gate out cell aggregates and dead cells, respectively. Following this, $CD4^+$ T cells were selected by gating on small (FSC and side scatter properties), $CD3^+$ $CD4^+$ lymphocytes

to generate values for proliferation (CFSE) and IFN-γ- and Tbet-expression in this cell population.

Adoptive transfer experiments

Adoptive transfer was carried out as previously described [10]. Briefly, 8- to-10-week-old wild type (C57BL/10J; B10) or mutant mice (P48) were injected i.v. with 10^6 *P. berghei* ANKA-parasitized erythrocytes (RBC). Five days later, spleens were collected in RPMI-3% FBS, and single cell suspensions of viable cells were prepared. Cells were washed in RPMI-3% FBS by centrifugation, and RBC were lysed by re-suspending the final pellet in red blood cell lysis buffer (Sigma), and incubating for 1 minute at RT. Cells were washed again twice as before and counted. $CD8^+$ T cells, total T cells or NK cells were purified from infected B10 WT splenocytes by magnetic cell sorting (MACS; Miltenyi) according to the manufacturer's instructions. 5 million $CD8^+$ T cells, total T cells or NK cells from B10 WT infected spleens, or 20 million total WT or mutant splenocytes were transferred i.v. into P48/P48 mutant animals. Two hours later, control and reconstituted mice were infected with 10^6 *P. berghei* ANKA parasites and were monitored for appearance of cerebral symptoms and for overlall survival.

Infection with *Mycobacterium bovis* (BCG) and *Mycobacterium tuberculosis*

Single cell suspensions of *Mycobacterium bovis* BCG (strain Montreal) was prepared for *in vivo* infections as previously described [28]. Briefly, 5 x 10^4 colony-forming units (CFUs) were inoculated intravenously into 8-12 week-old mice. Six weeks after infection, mice were sacrificed, weighed and the spleen CFUs were determined by homogenization and plating on Dubos oleic agar base. The level of BCG infection was defined as the logarithm of the mean number of viable BCG recovered from spleens. The spleen index was defined as the square root of the spleen weight (x 100) divided by the body weight. For *Mycobacterium tuberculosis* (Mtb) infection, 8-12 week-old mice were infected with 50 CFUs/lung by the aerosol route, and survival was monitored.

Infection with *Citrobacter rodentium*

Mice were infected at four weeks of age with *Citrobacter rodentium* strain DBS100. *C. rodentium* was grown overnight in 3mL Luria-Bertani (LB) broth shaking at 37°C. Mice were infected by oral gavage of 0.1 mL of the overnight culture containing 3 X 10^8 CFU. Following infection with *C. rodentium*, the mice were monitored daily for 30 days post-infection. When any mouse became moribund or reached a clinical endpoint of infection (20% body weight loss, hunching and shaking, inactivity, ruffled fur, anal prolapse, overtly bloody stool, bleeding from the anus and body condition score <2), it was immediately euthanized.

Data Analysis

Mapping data were analyzed with the R/qtl software version 2.10.1. The binary model was used, and LOD scores calculated using survival as a phenotype. The cutoff for genome-wide significance ($p<0.05$) was 3.23.

RESULTS

Identification and characterization of a cerebral malaria resistant ENU mutant

To identify genes, proteins, and cellular pathways important for the pathogenesis of cerebral malaria (CM), we screened pedigrees derived from ENU-mutagenized mice, looking for the appearance of CM-resistant pheno-deviant pedigrees appearing on the otherwise CM-susceptible genetic background of C57Bl/6J (B6). Such pedigrees are believed to segregate protective mutations fixed for homozygosity, and affecting genes that are important for CM pathogenesis including host-driven detrimental effects. In our protocol, mutagenized B6 males were crossed to C57Bl/10J (B10, to facilitate susbsequent genetic mapping), and the resulting G1 males were backcrossed to B10 (Figure 1A); the resulting G2 females were backcrossed to their G1 father to produce G3 pedigrees where mutations are fixed to homozygosity in 25% of the animals. These G3 pedigrees were infected with *P. berghei* ANKA, and we monitored the presence of pheno-deviant progeny that fail to develop cerebral symptoms and survive this infection. When such positive pedigrees were detected, additional G3 animals from the same G2 females and G1 father were generated and phenotyped to validate the presence of a protective mutation. Screening a total of 3967 G3 mice from 153 pedigrees identified several such pheno-deviant pedigrees. One of these pedigrees, #48 (P48), displayed a fairly high percentage of resistant animals (~31% resistance), with both G2 females producing CM-resistant offspring (Figure 1B), and was chosen for further analysis.

A genome-wide scan was carried out in 44 G3 animals from P48 (15 CM-resistant and 29 CM-susceptible) using 130 informative polymorphic markers informative for B6 and B10. Linkage analysis (using R/qtl) identified a 17Mb region on the central portion of chromosome 8 (95% Bayesian credible interval: 60.4Mb-77.4Mb) as regulating differential CM-resistance in this pedigree, with a LOD score of 5.8 (Figure 2A). Haplotype analysis revealed that, as expected, resistance to CM at this locus was associated with homozygosity for B6-derived alleles (from mutated G0 male), while homozygosity for B10 alleles was associated with CM-susceptibility, and with B6/B10 heterozygotes being present in resistant and susceptible groups (Figure 2B). These findings suggested that the CM-protective effect of this locus is co-dominant in this cross. The P48 G1 male was also outcrossed to CM-susceptible 129S1 WT females, and the resulting offspring were intercrossed to generate a total of 211 F2 mice which were phenotyped by infection with *P. berghei* (Figure 2C). Approximately 25% of these F2 survived the cerebral phase, indicating that the resistance trait associated with P48 is fully penetrant on the distinct genetic background of 129S1. Genotyping of these animals verified that CM-resistance was controlled by the chromosome 8 locus, and also showed a co-dominant mode of inheritance of B6 protective alleles in this cross (Supplementary Figure 1).

Immunological phenotyping of P48 mutants

To gain insight into the cell population phenotypically expressing the CM-protective mutation, we examined the different lymphoid and myeloid organs, and monitored specific cell populations within them in mice fixed for homozygosity for B6-derived alleles at the chromosome 8 locus. Macroscopic examination readily identified severe thymic atrophy in homozygotes (Figure 3A), while heterozygotes were normal, suggesting a possible thymus development defect. FACS analysis further identified a severe depletion of the CD8+ T cell

compartment in thymus of these mice, while the CD4+ T cell compartment appeared unaffected. A similar severe depletion of the CD8+ T cells compartment was also detected upon analysis of spleen cells from these mice (Figure 3B), with an additional reduction in NK cells (Figure 3C). On the other hand, studies of bone marrow cells showed a complete absence of CD19+ B cells in mutant mice. In all tissues examined, there was no effect noted on the myeloid compartment (Gr1+ cells). All alterations in these cell populations were detected only in homozygotes, while heterozygotes showed normal cell numbers which were similar to C57Bl/6J controls. Furthermore, analysis of splenic $CD4^+$ T cells revealed a further defect in the function of these cells in homozygote mutants. Activation of these cells (via CD3 and CD28) under condition of Th1 polarization (rIL-12 and IL-4) showed impaired production of IFNg in response to PMA and ionomycin (Figure 4). These results indicated that although the mutation in P48 does not seem to affect the ontogeny of CD4+ T cells, it impairs the ability of these cells to produce IFNγ in response to Th1 polarizing stimuli.

Resistance to cerebral malaria is caused by a mutation in the Jak3 kinase

The 17Mb interval delineating the position of the CM-protective locus on chromosome 8 contains a number of positional candidates that have a) an established role in the immune system, b) are known to be modulated by IFN-γ, or c) show IFN-inducible STAT1 binding sites in their promoter. These include Interleukin 12 receptor beta 1 (IL12rb1; 73.3Mb), interferon gamma inducible protein 30 (Ifi30; 73.2Mb), Janus kinase 3 (Jak3; 74.2Mb), heme oxygenase (decycling) 1 (Hmox1; 77.6Mb), Interleukin 15 (IL15; 84.8Mb), interferon regulatory factor 2 (IRF2; 47.8Mb), Caspase 3 (47.7Mb), vascular endothelial growth factor C (Vegfc; 55.1Mb), unc-13 homolog A (Unc13a; 74.1Mb). Whole genome sequencing of genomic DNA from P48 mutant homozygotes was undertaken, and candidate variants in the above-mentioned genes that were absent form the B6 reference sequence were further validated by re-sequencing. This analysis revealed a unique T-to-A transversion in exon 2 of the *jak3* gene which causes a W81R substitution in the Band 4.1/Ezrin/Radixin/Moesin (FERM) homology domain at the N-terminus of the Jak3 protein. The FERM domain is involved in mediating interactions of Jak3 with different cytokine receptors in immune cells (Figure 5). The tryptophan at position 81 is absolutely conserved in Jak3 relatives from different species, suggesting a conserved structural/functional role of this residue in Jak3 activity. To confirm that the CM-resistance in P48 mutants was caused by a mutation in *jak3*, we carried out complementation testing using $Jak3^{-/-}$ null mice (B6.129S4-$Jak3^{tm1Ljb}$; MGI database, [29]). These animals have previously been shown to have atrophied thymuses, as well as low numbers of splenic $CD8^+$ cells, NK cells and a defect in B cell maturation [29]. Compound ($Jak3^{-/W81R}$) heterozygotes were generated by crossing $Jak3^{W81R}$ homozygotes to $Jak3^{-/-}$ null mice, and these together with $Jak3^{W81R/+}$ and $Jak3^{-/+}$ heterozygotes were phenotyped for susceptibility to *P. berghei* induced CM. Compound $Jak3^{W81R/-}$ heterozygotes $Jak3^{W81R/-}$ were found to be as CM-resistant as $Jak3^{W81R}$ homozygotes and as $Jak3^{-/-}$ null animals, confirming that the $Jak3^{W81R}$ mutation is indeed responsible for protection against CM in P48. On the other hand, $Jak3^{+/-}$ heterozygotes were as susceptible to *P. berghei* induced CM as the B10 WT controls. Finally, we noted that 50% of $Jak3^{W81R/+}$ heterozygotes were resistant to CM, in agreement with haplotype analyses of the original G3 animals. This observation together with the uniform susceptibility of $Jak3^{+/-}$ heterozygotes, confirmed the co-

dominant mode of inheritance of the *Jak3*^{W81R} mutation, and suggested that it may have a dominant negative effect (Figure 6).

Splenocytes from infected B10 WT mice restore CM susceptibility to P48/P48 mice

Sequestration of parasitized red blood cells at the brain microvasculature together with local inflammatory response *in situ*, have been shown to be necessary for development and progression of *P. berghei*-induced CM [13, 31]. We aimed to establish in cell transfer experiments, which of the immune cell population(s) missing from the *Jak3*^{W81R} mutants may be involved in CM pathogenesis, and which absence results in a CM-protective effect. First, total splenocytes from normal B10 or from *Jak3*^{W81R} mutants, harvested 5 days following infection with *P. berghei* ANKA, were transferred into *Jak3*^{W81R} mutant mice. Two hours later, recipient and control un-treated mice were infected with *P. berghei* and the effect of cell transfer on appearance of CM-associated phenotypes and lethality were monitored. The transfer of total spleen cells from wild type B10 controls into *Jak3*^{W81R} mutants eliminated CM-resistance in these animals and they all succumbed quickly in the cerebral phase of the disease, alike un-treated wild type controls (Figure 7A). On the other hand, the transfer of total spleen cells from *Jak3*^{W81R} mutant into *Jak3*^{W81R} mutant had no consequence on the CM-resistance trait of these mice (Figure 7A). To determine which of the absent spleen cell populations is associated with CM-resistance in *Jak3*^{W81R} mutants, we carried out further cell fractionation and transfer experiments (Figure 7B). While the transfer of NK cell from control B10 mice into *Jak3*^{W81R} mutants had no effect on CM-resistance, the transfer of either total T cells or of CD8+ T cells caused a similar and significant decrease in survival time following *P. berghei* infection of the transferred animals, with 50% of the transferred mice succumbing by day 9. Together, these results indicate that CD8+ T cells are critically important for the pathogenesis of *P. berghei* induced CM, and that their absence in the *Jak3*^{W81R} mutant is in part responsible for the CM-resistance phenotype of these mice.

***Jak3*^{W81R} mutant mice are highly susceptible to infections with *Mycobacterium* and with *Citrobacter*.**

Pro-inflammatory Th1 cytokines (IFN-γ, TNF-α and LT-α) play an important role in CM pathogenesis, and inactivating mutations in these molecules have a protective effect against *P. berghei*-induced CM. *Jak3*^{W81R} mutant mice lack CD8^{+} splenic T cells, and total spleen cells from *Jak3*^{W81R} mutants do not produce IFNγ in response to activation along the Th1 pathway (Figure 4). On the other hand, CD4^{+}, and CD8^{+} T cells, as well as Th1 cytokines produced by these and other cells are absolutely required for protection against intracellular pathogenic mycobacteria [32]. Therefore, we evaluated the response of *Jak3*^{W81R} mice to infection with mycobacteria (Figure 8). Mice were infected with low dose *M. bovis* BCG and bacterial replication was measured 6 weeks following infection (Figure 8A). *Jak3*^{W81R} mutants showed splenomegaly and increased spleen bacterial counts (by a factor fo 10 fold) compared to control B6 mice. Likewise, a large proportion (>75%) of *Jak3*^{W81R} mutants succumbed within 45 days following aerosol infection with virulent *M. tuberculosis* H37Rv, while all of the control B6 mice survived over the same period (Figure 8B). Together, these results strongly suggest that inactivation of *Jak3* kinase causes susceptibility to mycobacterial infection. Independently, it is know that effective protection against enteropathogenic bacteria such as *Citrobacter rodentium*, requires an intact T lymphocyte compartment [], and production of Th1 cytokines such as IFNg [] and TNF-a.

Therefore, we assessed the response of *Jak3*^{W81R} mutants to infection with *C. rodentium* (Figure 8C). In these experiments, C57BL/6J mice which are innately resistant to *C. rodentium* were used as genetic controls for the P48-associated *Jak3*^{W81R} mutation. Inactivation of *Jak3* in these mutants caused a dramatic increase in susceptibility to infection, leading to progressive mortality during the 30-day period following infection, compared to B6 controls. These results indicate that *Jak3* is required for effective Th1-driven host response to infections with extracellular and intracellular pathogens.

DISCUSSION

Cerebral malaria has a devastating impact on global health. The sudden appearance, rapid progression, and irreversible nature of the CM pathology together with the paucity of treatment (limited to high dose intravenous quinine and artemisinin)[], underlie the high rate of mortality and morbidity associated with CM. Therefore, there is a desperate need for novel therapeutic interventions. CM has is a complex pathology that involves multiple host tissues and physiological pathways, including the eyrthroid replicative niche of the parasite, the structure and secretion products of endothelial cells of the microvasculature, and different cell populations, cytokines and associated pathways of the innate and acquired immune system, the activity of which is triggered by variable parasite virulence determinants and further modulated by intrinsic genetic factors of the host []. A better characterization of the molecular pathways involved is required to identify novel targets for drug development and therapeutic intervention in CM. Studies in human clinical cases of CM and experiments in mouse models of *P. berghei*-induced CM have identified excessive TNFα and IFNγ-driven inflammatory response as a key determinant in CM pathogenesis []; novel strategies to blunt this response have shown promise in the prevention and treatment of CM [33-37]. We have implemented a genome-wide mutagenesis screen in mice to systematically identify genes and proteins that mediate pathological inflammatory response during *P. berghei* infection *in vivo*, and which pharmacological or genetic inactivation may protect from CM.

We report on the first mutant identified in this screen. Linkage analyses, genomic DNA sequencing, and complementation studies in double heterozygotes have established that CM protection in this mutant is caused by a mutation (W81R) in the amino-terminal FERM domain of Jak3 (Janus kinase 3; $Jak3^{W81R}$). Jak3 is cytosolic tyrosine kinase expressed primarily in the hematopoietic system that plays a critical role in a) the ontogeny of different myeloid and lymphoid cells, and b) the response of these cells to stimulation by different cytokines. Jak3 interacts with the common γc chain of type 1 and 2 cytokine receptors, which includes IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. This interaction causes recruitment and phosphorylation of STAT family members to trigger downstream transcriptional responses in cells expressing such receptors []. In humans, loss of either JAK3 or IL2RG (which codes for the common γc chain) causes autosomal and X-linked T^-B^+ SCID, respectively []. These patients lack T cells, and NK cells, have normal numbers of immature and poorly functional B-lymphocytes, a clinical picture in agreement with the established roles of IL7 (T cell development), IL-2 (peripheral T cell homeostasis and antigen-driven T-cell expansion), IL-15 (differentiation of NK cells), and IL-4 (B-cell maturation and isotype switching) and which associated signaling is impaired in JAK3 mutants []. *$Jak3^{W81R}$* homozygote mice showed a phenotype that overlaps T^-B^+ SCID in humans, and displayed an atrophied thymus, a low number of thymic and splenic $CD8^+$ T cells and NK cells, as well as a near absence of B cells in the bone marrow. $CD4^+$ T cells were present in normal numbers but appeared anergic and did not produce IFNγ in response to simulation with PHA or ionomycin (under conditions of Th1 polarization)(Fig 4b,c). This immune phenotype closely resembled that of previously described Jak3 knockout mice [29].

What is the mechanism underling protection against *P. berghei*-induced CM in *$Jak3^{W81R}$* homozygotes? IFNγ plays a critical role in initiating and amplifying pathological inflammatory response during CM, and mouse mutants lacking the IFNγ gene are protected against *P. berghei*-

induced CM []. Although the dominant cell type(s) responsible for early production of IFNγ *in vivo* during *P. berghei* infection has been debated, NK cells, $CD4^+$ and $CD8^+$ T cells have all been implicated [], and all three populations are affected in $Jak3^{W81R}$ homozygotes. In the case of NK cells, results either supporting or excluding a role for these cells in CM pathogenesis have been published. In one study, depletion of NK cells failed to alter the appearance of cerebral symptoms or the outcome of CM in *P. berghei* infected mice [], while another study found that IFNγ secretion by NK cells was important for recruitment of $CXCR3^+$ $CD4^+$ and $CD8^+$ T cells to the brain and development of cerebral disease [55][56]. On the other hand, cell depletion and cell transfer experiments *in vivo* have shown that IFNγ production by $CD4^+$ and $CD8^+$ T cells can both contribute to CM pathogenesis []. Results from our adoptive transfer studies in $Jak3^{W81R}$ homozygotes (Figure 7) provide additional insight into this question. We observed that a) total spleen cells from C57BL/10J mice were the only cell population that could fully restore CM-susceptibility in the mutants; b) total T cells and purified $CD8^+$ T cells had a similar effect and caused partial but significant reversion to CM-susceptibility of in $Jak3^{W81R}$ animals; c) transfer of purified wild type NK cells had no impact on the CM-resistance of the $Jak3^{W81R}$ mutants. These results strongly suggest that $CD8^+$ T cells are the major cell type contributing to CM pathogenesis, although other spleen cell populations or other cell:cell interaction, for example T cell dependent NK cell activation [57], appear to be required to observe the full effect. Nevertheless, our results clearly establish a role of the Jak3 kinase in the pathogenesis of cerebral malaria. This participation may reflect the function of Jak3 in the ontogeny of cell populations (NK cells, $CD8^+$ T cells) that produce of IFNγ and other soluble mediators of the pathological inflammatory response, and that are absent in the $Jak3^{W81R}$ mutant. The protective effect of $Jak3^{W81R}$ may additionally involve inhibition of γc chain containing cytokine receptor signaling in other cell types which ontogeny is not affected by the *Jak3* mutation. Nevertheless, our results suggest that pharmacological inhibition of Jak3 may be of therapeutic value in CM. Several small molecule Jak3 inhibitors have been developed and are undergoing clinical evaluation for inflammatory conditions such as rheumatoid arthritis, psoriasis and several autoimmune conditions including autoimmune encephalitis, and rejection of organ transplants [53, 54]. Our findings raise the interesting possibility that Jak3 inhibition by some of these molecules may represent a novel strategy for intervention in clinical cases CM, a proposition that can be tested experimentally.

An intriguing finding of our study is the intermediate CM-resistance phenotype characteristic of $Jak3^{W81R/+}$ heterozygotes, with a proportion of these animals either succumbing late in the cerebral phase or completely surviving the cerebral phase. This was first noticed in haplotype analyses of G3 mice of pedigree 48, with animals heterozygotes for the Chr. 8 markers being found in both the CM-resistant and CM-susceptible groups (Figure 2B), and subsequently verified during *P. berghei* infection of genotyped $Jak3^{W81R/+}$ hetrozygotes (Figure 6). The effect is not caused by the genetic background of the animals and is specific for $Jak3^{W81R/+}$ heterozygosity, as it seen when the mutation is introduced onto either B6/B10 or B6/B10-129S1 mixed genetic backgrounds (Figure 2B, S1). The cellular and molecular basis of co-dominance of the $Jak3^{W81R}$ mutation is intriguing. It could be explained either by a partial loss of Jak3 function in a dosage dependent pathway or by a specific dominant negative effect of the $Jak3^{W81R}$ allele. The observation that mice heterozygote for a null *Jak3* mutation ($Jak3^{+/-}$) are as susceptible to CM as wild type B6 controls clearly argues for the latter possibility. Interestingly, immunophenotyping

results of *Jak3*$^{W81R/+}$ heterozygotes show that CM protection in these animals is not associated with alterations in the numbers of NK, T and B lymphocytes, which are all present at normal levels when compared to controls (Figures 3B, 3C). Normal production of IFNγ in response to PHA and ionomycin stimulation under Th1 polarization assay conditions is also seen in *Jak3*$^{W81R/+}$ heterozygotes (Figure 4). This suggests the possibility of more subtle dominant negative effect of *Jak3*^{W81R} on the biochemical properties of Jak3 in cytokine signaling, and that would nevertheless be critical for establishing the inflammatory process during CM. Such a mechanism could take place in the context of sufficient Jak3 activity to would a) allow seemingly normal maturation of different immune cell lineages (NK, B, T cells), but b) not sufficient to mediate appropriate signaling during an acute inflammatory situation such as CM []. Interestingly, such a scenario has been previously proposed to account for incomplete penetrance and/or partial expressivity of the human SCID phenotype caused by homozygosity for loss of function *JAK3* mutations in certain familial cases [].

What would be the molecular basis of a dominant-negative effect of W81R on Jak3 function. Ligand-induced oligomerization of cytokine receptors and associated Jak3 kinases may position wild type and mutant Jak3 variants in close proximity in a signaling complex. In this context, inter-molecular dominant negative effects of gain-of-function Jak3 alleles such as W81R, may alter function of the wild type protein expressed in the same cell. W81 maps in the amino-terminal FERM domain, and several FERM domain mutations have been reported in SCID patients, including M1V, A58P, Del58A, 203DelG, Y100C, D169E and P151R [39, 40, 48]. The study of these and other site-directed FERM domain mutants indicate that this domain plays a key role in multiple aspects of Jak3 function [reviewed in XXX]. It is required for membrane targeting and for interaction with the γc chain of cytokine receptor []. It also acts as a positive regulator of Jak3 kinase activity: it physically interacts with the JH1-JH2 kinase domain to stimulate both ATP binding and tyrosine phosphorylation []. Such interactions may be critical in the early cross-phopsphorylation of Jak kinases that normally precedes phosphorylation of neighboring substrates []. A dominant negative effect of W81R could possibly act through inhibition of these early cross-phosphorylation events in heterodimers containing both wild type and mutant variants. Jak3 kinase activity is modulated by interaction with several proteins including JAB (Jak binding proteins), CIS, SOCS, SSI, STAM, PIAS and others []. A dominant negative effect of W81R may involve stabilization of an inhibited state following interaction of wild type and or mutant variants with these modulators. Additional biochemical studies will be required to elucidate the molecular mechanism of the W81R dominant negative effect.

Finally, although the full blown T^-/B^+ SCID disease is caused by complete loss of JAK3 function in humans (homozygosity or compound heterozygosity for mutant variants), our findings with the mouse *Jak3*^{W81R}allele suggest that heterozygosity for dominant negative human *JAK3* mutations may cause partially impaired immune response. Although immunodeficiency associated with heterozygosity for a *JAK3* mutation has rarely been reported [], our findings raise the possibility that spectrum of mild immunodeficiency associated with heterozygote *JAK3* mutations may be broader than previously suspected.

References

1. Hunt, N.H. and G.E. Grau, *Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria.* Trends Immunol, 2003. **24**(9): p. 491-9.
2. Bongfen, S.E., et al., *Genetic and genomic analyses of host-pathogen interactions in malaria.* Trends Parasitol, 2009. **25**(9): p. 417-22.
3. Kwiatkowski, D., *Genetic susceptibility to malaria getting complex.* Curr Opin Genet Dev, 2000. **10**(3): p. 320-4.
4. Weatherall, D.J., *Genetic variation and susceptibility to infection: the red cell and malaria.* Br J Haematol, 2008. **141**(3): p. 276-86.
5. de Souza, J.B., et al., *Cerebral malaria: why experimental murine models are required to understand the pathogenesis of disease.* Parasitology, 2010. **137**(5): p. 755-72.
6. Miu, J., et al., *Chemokine gene expression during fatal murine cerebral malaria and protection due to CXCR3 deficiency.* J Immunol, 2008. **180**(2): p. 1217-30.
7. Grau, G.E., et al., *Monoclonal antibody against interferon gamma can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor.* Proc Natl Acad Sci U S A, 1989. **86**(14): p. 5572-4.
8. Grau, G.E., et al., *Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria.* Science, 1987. **237**(4819): p. 1210-2.
9. Engwerda, C.R., et al., *Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria.* J Exp Med, 2002. **195**(10): p. 1371-7.
10. Campanella, G.S., et al., *Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria.* Proc Natl Acad Sci U S A, 2008. **105**(12): p. 4814-9.
11. Rudin, W., et al., *Resistance to cerebral malaria in tumor necrosis factor-alpha/beta-deficient mice is associated with a reduction of intercellular adhesion molecule-1 up-regulation and T helper type 1 response.* Am J Pathol, 1997. **150**(1): p. 257-66.
12. Amante, F.H., et al., *Immune-mediated mechanisms of parasite tissue sequestration during experimental cerebral malaria.* J Immunol, 2010. **185**(6): p. 3632-42.
13. McQuillan, J.A., et al., *Coincident parasite and CD8 T cell sequestration is required for development of experimental cerebral malaria.* Int J Parasitol, 2011. **41**(2): p. 155-63.
14. Senaldi, G., et al., *Protection against the mortality associated with disease models mediated by TNF and IFN-gamma in mice lacking IFN regulatory factor-1.* J Immunol, 1999. **163**(12): p. 6820-6.
15. Patel, S.N., et al., *C5 deficiency and C5a or C5aR blockade protects against cerebral malaria.* J Exp Med, 2008. **205**(5): p. 1133-43.
16. de Kossodo, S. and G.E. Grau, *Profiles of cytokine production in relation with susceptibility to cerebral malaria.* J Immunol, 1993. **151**(9): p. 4811-20.
17. Bagot, S., et al., *Identification of two cerebral malaria resistance loci using an inbred wild-derived mouse strain.* Proc Natl Acad Sci U S A, 2002. **99**(15): p. 9919-23.

18. Campino, S., et al., *Genetic control of parasite clearance leads to resistance to Plasmodium berghei ANKA infection and confers immunity.* Genes Immun, 2005. **6**(5): p. 416-21.
19. Berghout, J., et al., *Identification of a novel cerebral malaria susceptibility locus (Berr5) on mouse chromosome 19.* Genes Immun, 2010. **11**(4): p. 310-8.
20. Ohno, T. and M. Nishimura, *Detection of a new cerebral malaria susceptibility locus, using CBA mice.* Immunogenetics, 2004. **56**(9): p. 675-8.
21. Hong, C.J., et al., *ENU mutagenesis identifies mice with morbid obesity and severe hyperinsulinemia caused by a novel mutation in leptin.* PLoS One, 2010. **5**(12): p. e15333.
22. Miller, G., et al., *ENU mutagenesis reveals a novel phenotype of reduced limb strength in mice lacking fibrillin 2.* PLoS One, 2010. **5**(2): p. e9137.
23. Fernandez, L., et al., *An N-ethyl-N-nitrosourea mutagenesis recessive screen identifies two candidate regions for murine cardiomyopathy that map to chromosomes 1 and 15.* Mamm Genome, 2009. **20**(5): p. 296-304.
24. Chan, E.R., et al., *An ENU-induced recessive mutation in Mpl leads to thrombocytopenia with overdominance.* Exp Hematol, 2009. **37**(2): p. 276-84.
25. Balling, R., *ENU mutagenesis: analyzing gene function in mice.* Annu Rev Genomics Hum Genet, 2001. **2**: p. 463-92.
26. Fortin, A., et al., *Recombinant congenic strains derived from A/J and C57BL/6J: a tool for genetic dissection of complex traits.* Genomics, 2001. **74**(1): p. 21-35.
27. Sun, J. and E.J. Pearce, *Suppression of early IL-4 production underlies the failure of CD4 T cells activated by TLR-stimulated dendritic cells to differentiate into Th2 cells.* J Immunol, 2007. **178**(3): p. 1635-44.
28. Gros, P., E. Skamene, and A. Forget, *Genetic control of natural resistance to Mycobacterium bovis (BCG) in mice.* J Immunol, 1981. **127**(6): p. 2417-21.
29. Thomis, D.C., et al., *Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3.* Science, 1995. **270**(5237): p. 794-7.
30. Thomis, D.C. and L.J. Berg, *Peripheral expression of Jak3 is required to maintain T lymphocyte function.* J Exp Med, 1997. **185**(2): p. 197-206.
31. Baptista, F.G., et al., *Accumulation of Plasmodium berghei-infected red blood cells in the brain is crucial for the development of cerebral malaria in mice.* Infect Immun, 2010. **78**(9): p. 4033-9.
32. North, R.J. and Y.J. Jung, *Immunity to tuberculosis.* Annu Rev Immunol, 2004. **22**: p. 599-623.
33. Lackner, P., et al., *Glatiramer acetate reduces the risk for experimental cerebral malaria: a pilot study.* Malar J, 2009. **8**: p. 36.
34. Morrell, C.N., et al., *Beta interferon suppresses the development of experimental cerebral malaria.* Infect Immun, 2011. **79**(4): p. 1750-8.
35. Franklin, B.S., et al., *Therapeutical targeting of nucleic acid-sensing Toll-like receptors prevents experimental cerebral malaria.* Proc Natl Acad Sci U S A, 2011. **108**(9): p. 3689-94.
36. Cabrales, P., et al., *Nitric Oxide Protection Against Murine Cerebral Malaria Is Associated With Improved Cerebral Microcirculatory Physiology.* J Infect Dis, 2011.

37. Balachandar, S. and A. Katyal, *Peroxisome proliferator activating receptor (PPAR) in cerebral malaria (CM): a novel target for an additional therapy.* Eur J Clin Microbiol Infect Dis, 2011. **30**(4): p. 483-98.
38. Yamaoka, K., et al., *The Janus kinases (Jaks).* Genome Biol, 2004. **5**(12): p. 253.
39. Zhou, Y.J., et al., *Unexpected effects of FERM domain mutations on catalytic activity of Jak3: structural implication for Janus kinases.* Mol Cell, 2001. **8**(5): p. 959-69.
40. Roberts, J.L., et al., *Janus kinase 3 (JAK3) deficiency: clinical, immunologic, and molecular analyses of 10 patients and outcomes of stem cell transplantation.* Blood, 2004. **103**(6): p. 2009-18.
41. Ghoreschi, K., A. Laurence, and J.J. O'Shea, *Janus kinases in immune cell signaling.* Immunol Rev, 2009. **228**(1): p. 273-87.
42. Thomis, D.C. and L.J. Berg, *The role of Jak3 in lymphoid development, activation, and signaling.* Curr Opin Immunol, 1997. **9**(4): p. 541-7.
43. Russell, S.M., et al., *Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development.* Science, 1995. **270**(5237): p. 797-800.
44. Macchi, P., et al., *Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID).* Nature, 1995. **377**(6544): p. 65-8.
45. Park, S.Y., et al., *Developmental defects of lymphoid cells in Jak3 kinase-deficient mice.* Immunity, 1995. **3**(6): p. 771-82.
46. Nosaka, T., et al., *Defective lymphoid development in mice lacking Jak3.* Science, 1995. **270**(5237): p. 800-2.
47. Cornejo, M.G., T.J. Boggon, and T. Mercher, *JAK3: a two-faced player in hematological disorders.* Int J Biochem Cell Biol, 2009. **41**(12): p. 2376-9.
48. Notarangelo, L.D., et al., *Mutations in severe combined immune deficiency (SCID) due to JAK3 deficiency.* Hum Mutat, 2001. **18**(4): p. 255-63.
49. Chrencik, J.E., et al., *Structural and thermodynamic characterization of the TYK2 and JAK3 kinase domains in complex with CP-690550 and CMP-6.* J Mol Biol, 2010. **400**(3): p. 413-33.
50. Williams, N.K., et al., *Dissecting specificity in the Janus kinases: the structures of JAK-specific inhibitors complexed to the JAK1 and JAK2 protein tyrosine kinase domains.* J Mol Biol, 2009. **387**(1): p. 219-32.
51. Boggon, T.J., et al., *Crystal structure of the Jak3 kinase domain in complex with a staurosporine analog.* Blood, 2005. **106**(3): p. 996-1002.
52. Hamada, K., et al., *Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain.* EMBO J, 2000. **19**(17): p. 4449-62.
53. Kudlacz, E., et al., *The novel JAK-3 inhibitor CP-690550 is a potent immunosuppressive agent in various murine models.* Am J Transplant, 2004. **4**(1): p. 51-7.
54. Meyer, D.M., et al., *Anti-inflammatory activity and neutrophil reductions mediated by the JAK1/JAK3 inhibitor, CP-690,550, in rat adjuvant-induced arthritis.* J Inflamm (Lond), 2010. **7**: p. 41.
55. Hansen, D.S., et al., *NK cells stimulate recruitment of CXCR3+ T cells to the brain during Plasmodium berghei-mediated cerebral malaria.* J Immunol, 2007. **178**(9): p. 5779-88.

56. Yanez, D.M., et al., *Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria.* J Immunol, 1996. **157**(4): p. 1620-4.
57. McCall, M.B., et al., *Memory-like IFN-gamma response by NK cells following malaria infection reveals the crucial role of T cells in NK cell activation by P. falciparum.* Eur J Immunol, 2010. **40**(12): p. 3472-7.
58. Martin-Fontecha, A., et al., *Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming.* Nat Immunol, 2004. **5**(12): p. 1260-5.
59. Moretta, A., *Natural killer cells and dendritic cells: rendezvous in abused tissues.* Nat Rev Immunol, 2002. **2**(12): p. 957-64.
60. Belnoue, E., et al., *Control of pathogenic CD8+ T cell migration to the brain by IFN-gamma during experimental cerebral malaria.* Parasite Immunol, 2008. **30**(10): p. 544-53.
61. Waknine-Grinberg, J.H., et al., *Schistosoma mansoni infection reduces the incidence of murine cerebral malaria.* Malar J, 2010. **9**: p. 5.
62. Turcotte, K., et al., *A mutation in the Icsbp1 gene causes susceptibility to infection and a chronic myeloid leukemia-like syndrome in BXH-2 mice.* J Exp Med, 2005. **201**(6): p. 881-90.
63. Marquis, J.F., et al., *Disseminated and rapidly fatal tuberculosis in mice bearing a defective allele at IFN regulatory factor 8.* J Immunol, 2009. **182**(5): p. 3008-15.

FIGURE LEGENDS

Figure 1. *ENU-induced mutation that protects mice against P. berghei ANKA-induced cerebral malaria.* (**A)** Breeding scheme for the production and identification of ENU-induced recessive mutations that convey protection against cerebral malaria (CM). Details of the breeding strategy are described in "Materials and Methods". G3 and F2 pedigrees were phenotyped for the presence of animals resistant to *P. berghei*-induced CM. (**B)** Mice were infected with *P. berghei* ANKA (10^6 *P. berghei* ANKA-parasitized red blood cells, i.v.) and survival was monitored over time for individual pedigree P48 G3s (green and blue lines) derived from independent G2 females and a G1 male, and for susceptible C57BL/6J (B6; red line), and resistant mutant mouse strains bearing loss of function mutations in either the IFN-γ gene KO (IFN-γ KO; black line) or IRF8 (BXH2, brown line). Mice surviving past day 13 post infection were considered to be CM-resistant.

Figure 2. *Resistance to P. berghei ANKA-induced cerebral malaria in pedigree 48 maps to the central portion of chromosome 8.* Genome-wide linkage analysis of the CM-resistance trait (survival) was conducted in 44 G3 (15 resistant, 29 susceptible) mice from pedigree 48, and using polymorphic markers informative for the B6 and B10 progenitors. (**A)** LOD score traces identifying significant linkage to chromosome 8 and ($p = 0.05$, genome-wide significance shown as dotted line); the position of informative markers is shown, including rs33080067 and rs32729089 (LOD score ~5.8). (**B)** Haplotype analysis of the central portion of chromosome 8 in CM-resistant and CM-susceptible G3 animals from pedigree 48 (A, B6 homozygotes; H, B6/B10 heterozygotes; B, B10 homozygotes) showing exclusion of homozygote B6 haplotypes from the CM-susceptible group. (**C)** The G1 male from pedigree 48 was out-crossed to 129S1/SvlmJ to generate an F2 population that was phenotyped for response to *P. berghei* ANKA infection. Survival of F2 mice as well as parental 129S1/SvlmJ and C57BL/10J controls is shown.

Figure 3. *Phenotypic expression of resistance to cerebral malaria in mice from pedigree 48.* G3 and F2 mice homozygote (P48/P48) or heterozygote (P48/+) for the B6-derived mutant central chromosome 8 were identified by genotyping, and were subjected to several analyses, along with parental C57BL/6J, C57BL/10J and 129S1/SvlmJ controls. (**A)** Macroscopic examination of thymus from control and mutants showing severely atrophied thymus in homozygote mutants. (**B)** FACS density plots of different cell populations in thymus (top), spleen (middle) and bone marrow (bottom) stained for CD4, CD8, CD19, and CD117. The position of the different cell lineages in the scatter plots are identified at the extreme right panel and their numbers are expressed as a percentage (± SE ; n=5 mice per group) of total cells in this tissue. (**C)** Flow cytometric analysis of immune cell lineage composition expressed as the absolute number (mean ± SD; n=4-6 mice per group) of $CD4^+$ and $CD8^+$ single positive, $CD4^+CD8^+$ double positive (DP), B cells ($CD19^+$), granulocytes (GR; $Gr1^+$), hematopoietic stem cells (HSC; $lineage^-CD117^+$) and NK cells from 10^5 cells from spleen, thymus and bone marrow. Asterisks (one-way Anova test with Bonferroni post-test) identify significant differences between experimental animals and the C57BL/6J controls: $*p < 0.05$; $** p < 0.01$; $*** p < 0.001$.

Figure 4. *Th1 differentiation of CD4⁺ T cells in mice from pedigree 48.* CFSE-labeled splenocytes from wild-type (C57BL/6J; B6), homozygote (P48/P48) and heterozygote (P48/+) mutants mice were stimulated with anti-CD3 and anti-CD28 antibodies under non-Th1 or Th1-polarization conditions. Cells were then re-stimulated with PMA/ionomycin and IFN-γ production was measured by FACS. Dot-plots (left) show representative intracellular staining data for IFN-γ vs. CFSE fluorescence. Background-adjusted percentage of responding $CD3^+CD4^+Tbet^+$ viable T cells are shown in the upper right. Right: Absolute number (mean ± SEM) of IFN-γ expressing $Tbet^+$ $CD4^+$ splenocytes cultured in the presence of either non-Th1 or Th1-skewing conditions.

Figure 5. *Resistance to cerebral malaria in pedigree 48 is caused by a W81R in the conserved FERM domain of Jak3.* (**A)** Schematic representation of the Jak3 protein, showing the 7 JH structural domains, and associated functional annotation (below). The position of the W81R mis-sense mutation discovered in pedigree 48 is shown. (**B)** Multiple sequence alignment of the amino terminal portion of Jak3 surrounding W81 shows high conservation across Jak3 relatives (the corresponding species is identified).

Figure 6. *Resistance to cerebral malaria in $Jak3^{W81R/-}$ compound heterozygotes. $Jak3^{W81R}$* homozygotes were crossed to a mouse line bearing a null *Jak3* allele (*$Jak3^{-/-}$*) to create the *$Jak3^{W81R/+}$* compound heterozygotes. Single heterozygotes (*$Jak3^{W81R/+}$*; *$Jak3^{+/-}$*), homozygotes (*$Jak3^{W81R/W81R}$*; *$Jak3^{-/-}$*), compound heterozygotes (*$Jak3^{W81R/-}$*, gray line) and C57BL/10J controls were infected with 10^6 *P. berghei* ANKA-parasitized RBCs and monitored for survival. All surviving mice were sacrificed on day 15 post-infection (experimental end-point).

Figure 7. *Cell transfer experiments to suppress susceptibility to cerebral malaria in $Jak3^{W81R/W81R}$ mice*. Wild type C57BL/10J (B10) mice, *$Jak3^{W81R/W81R}$* homozygote mutants, or *$Jak3^{W81R/W81R}$* homozygote mutants having received the indicated cell populations (20 million total splenocytes or 5 million each of the indicated cell population; 2 hrs prior to infection) were infected i.v. with 10^6 *P. berghei* ANKA-parasitized RBCs, and survival from infection was monitored. Untreated wild type B10 and *$Jak3^{W81R/W81R}$* mutants were used as susceptible and resistant controls, respectively. All surviving mice were sacrificed on day 15 post-infection (experimental end-point).

Figure 8. *The $Jak3^{W81R}$ mutation confers susceptibility to infection with different bacterial pathogens.* (**A)** Control (C57BL/6J) and *$Jak2^{W81R}$* homozygote mutants were infected with $5x10^4$ colony-forming units (CFUs) of *Mycobacterium bovis* (BCG), and 6 weeks later, mice were sacrificed and the degree of infection was assessed by determination of spleen CFUs (left) and splenomegaly (spleen indez; right). (**B):** *$Jak3^{W81R}$* homozygotes and B6 controls were infected by aerosol inoculation of 50 CFUs/lung of *Mycobacterium tuberculosis* H37Rv, and monitored for survival. (**C):** *$Jak3^{W81R}$* homozygotes and B6 controls were infected by oral gavage with 3 X 10^8

CFUs of *Citrobacter rodentium* strain DBS100. Mice were monitored for survival 30 days post infection.

Figure S1. *Haplotype map of F2 mice from pedigree 48 for the central portion of chromosome 8 (51.9-84Mb).* F2 mice generated by crossing the G1 male (*Jak3*^{W81R}) to 129S1 progenitors were genotyped for microsatellite markers (Mouse Genome Informatics Database; www.informatics.jax.org) in the *51.9-84Mb* interval, and were phenotyped for resistance and susceptibility to *P. berghei* induced CM. Each row represents the haplotype (A, homozygote B6; H, heterozygote; B, homozygote 129S1) of an individual mouse for the indicated polymorphic markers.