Prevention of Experimental Cerebral Malaria by Flt3 Ligand during Infection with *Plasmodium berghei* ANKA^{▽†}

Takahiko Tamura,^{1,2} Kazumi Kimura,¹ Masao Yuda,³ and Katsuyuki Yui^{1,2*}

Division of Immunology, Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences,¹ and Global COE Program,² Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, and Department of Medical Zoology, School of Medicine, Mie University, 2-174 Ed. bashi Tsu, Mie 514-8507,³ Japan

Received 19 December 2010/Returned for modification 24 January 2011/Accepted 20 July 2011

Dendritic cells are the most potent antigen-presenting cells, but their roles in blood-stage malaria infection are not fully understood. We examined the effects of Flt3 ligand, a cytokine that induces dendritic cell production, *in vivo* on the course of infection with *Plasmodium berghei* ANKA. Mice treated with Flt3 ligand showed preferential expansion of CD8⁺ dendritic cells and granulocytes, as well as lower levels of parasitemia, and were protected from the development of lethal experimental cerebral malaria (ECM). Rag2 knockout mice treated with Flt3 ligand also showed inhibition of parasitemia, suggesting that this protection was due, at least in part, to the stimulation of innate immunity. However, it was unlikely that the inhibition of ECM was due simply to the reduction in the level of parasitemia. In the peripheral T cell compartment, CD8⁺ T cell levels were markedly increased in Flt3 ligand-treated mice after infection. These CD8⁺ T cells expressed CD11c and upregulated CXCR3, while the expression of CD137, CD25, and granzyme B was reduced. In the brain, the number of sequestered CD8⁺ T cells was not significantly different for treated versus untreated mice, while the proportion of CD8⁺ T cells that produce gamma interferon (IFN- γ) and granzyme B was significantly reduced in treated mice. In addition, sequestration of parasitized red blood cells (RBCs) in the brain was reduced, suggesting that altered CD8⁺ T cell activation and reduced sequestration of parasitized RBCs culminated in inhibition of ECM development. These results suggest that the quantitative and qualitative changes in the dendritic cell compartment are important for the pathogenesis of ECM.

Malaria is one of the most serious infections in the world and is responsible for more than 1 million deaths each year. Infection with *Plasmodium falciparum* induces a wide range of severe pathologies, including cerebral malaria (CM), one of the major causes of mortality due to this important parasite (14, 29, 36). Infection with *Plasmodium berghei* ANKA, a rodent malaria parasite, induces neurological symptoms and death in C57BL/6 (B6) and CBA mice and is widely used as a mouse model of experimental cerebral malaria (ECM) (9). Previous studies using the *P. berghei* ANKA infection model indicate the importance of brain-sequestered CD8⁺ T cells in the pathogenesis of ECM. Depletion of CD8⁺ T cells in C57BL/6 mice prevented the development of ECM, while reconstitution of CD8⁺ T cells in recombination-activating gene (RAG)-deficient mice, which lack both T and B cells, resulted in the development of ECM after infection with *P. berghei* ANKA (3, 26). In addition, concomitant accumulation of parasitized red blood cells (pRBC) in the brain is critical for the development of ECM (1). The recruitment of CD8⁺ T cells to the brain and the pathogenesis of ECM are dependent on chemokine receptor CCR5 (2, 26) and on CXCR3 expressed on CD8⁺ T cells, as well as on the CXCR3 ligands CXCL9/

CXCL10 (6, 24, 37). In the effector phase, the production of proinflammatory cytokines, such as gamma interferon (IFN- γ), and the cytotoxic activity of CD8⁺ T cells play critical roles in the pathogenesis of ECM (26, 40).

CD11c⁺ dendritic cells (DCs) are professional antigen-presenting cells that can prime naïve T cells, leading to the development of effector T cells. DCs can phagocytose malaria-parasitized red blood cells during infection and can present malaria antigen in both the major histocompatibility complex (MHC) class I and class II pathways, activating malaria-specific CD8⁺ and CD4⁺ T cells, respectively, and thus playing critical roles in the induction of protective immunity against *Plasmodium* infection (15, 17, 19, 20, 25, 33). However, DCs also play an important role in the pathogenesis of ECM, a T cell-dependent disease. It has been shown previously that depletion of conventional DCs, but not plasmacytoid DCs, resulted in reduced activation of malaria-specific T cells and inhibition of ECM development (10). The regulatory function of DCs in the pathogenesis of CM, however, is not completely understood.

Flt3 ligand (Flt3L) is an important cytokine for the differentiation and homeostasis of DCs (32). DCs differentiate from Flt3⁺ progenitor cells at steady state (16). Administration of Flt3L induces a drastic increase in the number of DCs in the spleen and lymph nodes (21). In contrast, the lack of Flt3L leads to severe reductions in DC numbers in many tissues (22). It has been shown previously that the number and phenotype of DCs in the spleen fluctuated during infection with malaria parasites (33, 39). However, it was not clear what effect this fluctuation of DC numbers had on the priming of malaria-specific T cells and the pathogenesis of ECM. In this study, we

* Corresponding author. Mailing address: Division of Immunology, Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Phone: 81-95-819-7070. Fax: 81-95-819-7073. E-mail: katsuyui@nagasaki-u.ac.jp.

† Supplemental material for this article may be found at <http://iai.asm.org/>.

[▽] Published ahead of print on 1 August 2011.

stimulated the expansion of DCs *in vivo* by administration of Flt3L prior to infection with *P. berghei* ANKA and examined the effects of Flt3L on the activation of the immune system as well as on the development of ECM. The results showed that Flt3L-treated mice were protected from ECM and exhibited altered T cell activation phenotypes. These studies suggest an important regulatory function for DCs in the activation of T cells as well as in the pathogenesis of ECM.

MATERIALS AND METHODS

Mice and plasmid transduction. Rag-2^{-/-} mice of the C57BL/6 background (31) were provided by Y. Yoshikai (Kyushu University, Fukuoka, Japan), and were maintained in the Laboratory Animal Center for Animal Research at Nagasaki University. C57BL/6 mice were purchased from SLC (Hamamatsu, Japan). The animal experiments reported here were conducted according to the guidelines of the Laboratory Animal Center for Biomedical Research at Nagasaki University.

The coding sequence for the extracellular domain (amino acids 1 to 189) of mouse Flt3L was obtained by PCR from cDNA prepared from mouse spleen using primer pairs 5'-GATCCACCATGACAGTGCTGGCGCCAGC-3' and 5'-GATCTACTGCCTGGCCGAGGCTCTG-3'. After confirmation of the sequence, the PCR product was cloned into pCAGGS, resulting in pCAGGS-Flt3L. The plasmid was purified using the PureYield Plasmid Maxiprep system (Promega, Madison, WI), and its endotoxin level was 2.43 endotoxin units (EU) (equivalent to 0.55 ng endotoxin) per µg DNA, as determined by a *Limulus* test (Wako, Osaka, Japan). A solution containing the plasmid (5 µg in phosphate-buffered saline [PBS]) was injected into mice using the hydrodynamics method, as previously described (12).

GFP-expressing parasites and *P. berghei* ANKA infection. *P. berghei* ANKA was originally obtained from R. E. Sinden (Imperial College London, London, United Kingdom). Recombinant *P. berghei* strain ANKA parasites that constitutively express green fluorescent protein (PbKA-GFP) were engineered as previously described (25). The gene construct, based on pBluescript KS(+) (Stratagene), contains a *P. berghei* ANKA dihydrofolate reductase-thymidylate synthase (DHFR-ts) gene, the *P. berghei* ANKA hsp70 5' untranslated region, its N-terminal coding sequence, and the coding sequence of GFP. *P. berghei* ANKA merozoites were transfected with the DNA construct by electroporation and were selected in rats by using pyrimethamine. Surviving parasites were cloned by limiting dilution in mice.

P. berghei ANKA was maintained by passage through BALB/c mice. For infection, mice were inoculated intraperitoneally (i.p.) with parasitized red blood cells (pRBC) (1×10^6 , except where otherwise indicated). Parasitemia of infected mice was monitored by microscopic examination of Giemsa-stained tail blood smears. Typically, 5 to 6 days after infection with *P. berghei* ANKA, mice began to show neurological signs, such as hunching, paralysis, and ataxia, and they succumbed to death within 10 days of infection (6). A PBS solution containing 2% Evans blue dye was injected into mice on day 6 after *P. berghei* ANKA infection. After 1 h, mice were euthanized, and their brains were removed, fixed in a 3% paraformaldehyde solution, and photographed.

Flow cytometry. For the preparation of DCs, spleens were cut into small fragments, incubated with RPMI 1640 containing collagenase (100 U/ml), mechanically disrupted, and filtered to prepare single-cell suspensions. After the lysis of RBC, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (FITC-anti-CD4), allophycocyanin (APC)-anti-CD8, FITC-anti-MHC class II, FITC-anti-CD40, FITC-anti-CD80, FITC-anti-CD86, phycoerythrin (PE)-anti-CD11c, PE-Cy7-anti-CD3e, PE-Cy7-anti-CD19, PE-Cy7-anti-NK1.1, PE-Cy7-anti-Ter119, FITC-anti-T cell receptor beta (TCRβ), FITC-anti-F4/80, PE-anti-Gr1, APC-anti-Ly6G, APC-anti-CD45, APC-Cy7-anti-Ly6C, APC-Cy7-anti-CD45, biotin-anti-DX5, biotin-anti-CD11c, and biotin-anti-CD11b antibodies (Abs) and PE-Cy7- or APC-streptavidin. Antibodies were purchased from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), or BioLegend (San Diego, CA). For intracellular staining of IFN-γ and granzyme B, splenocytes or brain-sequestered leukocytes were cultured on plates coated with an anti-TCR monoclonal Ab (MAb) (H57; 2 µg/ml) for 5 h, with the addition of GolgiStop (BD Biosciences) during the final 4 h. The cells were collected and stained with an anti-IFN-γ or anti-granzyme B MAb according to the manufacturer's instructions. For intracellular staining of Foxp3, splenocytes were stained using an anti-Foxp3 staining set (eBioscience) according to the manufacturer's instructions. Cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed using

CellQuest software. The following cell populations were defined: DCs (CD11c^{high} CD3e⁻ CD19⁻ DX5⁻), NK cells (NK1.1⁺ TCRβ⁻), macrophages (F4/80^{high} CD11b^{low} CD11c⁻), and granulocytes (Gr1^{high} Ly6G⁺ CD11b⁺).

Cytokine quantification. Serum IFN-γ and Flt3L levels were quantified using a cytometric bead array (CBA) assay (BD Biosciences) and a mouse Flt3 ligand Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN), respectively. Splenic CD4⁺ and CD8⁺ T cells were purified using an anti-CD4 or anti-CD8 IMag cell separation system (BD Biosciences), respectively. Splenic DCs were purified using anti-CD11c magnetic cell sorting (MACS) microbeads and an autoMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) or by sorting of CD11c⁺ MHC class II⁺ cells using a FACSaria flow cytometer (BD Biosciences). CD4⁺ or CD8⁺ T cells (1×10^5 /well) were cultured for 48 h on 96-well plates coated with an anti-TCR MAb (H57; 2 µg/ml) or with DCs (3×10^4 /well) pulsed with an RBC lysate (1 mg/ml) for 2 h. The levels of IFN-γ in the supernatant were determined by ELISA as described previously (25).

Preparation of brain-sequestered leukocytes and RBC. Mice were sacrificed 6 days after *P. berghei* ANKA infection, and brain-sequestered leukocytes were prepared as previously described with slight modifications (24). Briefly, euthanized mice were perfused intracardially with PBS, and brains were removed. Brains were crushed and treated with collagenase (100 U/ml) at 37°C for 15 min. The brain extract was centrifuged at 1,500 rpm for 20 min in a 30% Percoll solution to remove debris, and the cell pellet was collected. Cells were treated with Gey's solution to remove RBC, stained with antibodies, and analyzed by flow cytometry. After gating for CD45⁺ Ter119⁻ cells, CD4⁺ and CD8⁺ T cells were defined as TCRβ⁺ CD4⁺ and TCRβ⁺ CD8⁺ cells, respectively. For analysis of pRBC, total numbers of RBC were counted after Percoll centrifugation of brain extracts without RBC lysis. Cells were stained with PE-Cy7-anti-Ter119 and APC-anti-CD45 MAbs and were analyzed using a FACSCanto II flow cytometer. The proportion of pRBC was determined by microscopic examination in a manner similar to that of standard blood films. The number of pRBC in the brain was calculated by multiplying the number of RBC by the proportion of pRBC.

Depletion of neutrophils. The hybridoma cell line secreting MAb RB6-8C5 (anti-Gr1) was provided by H. Asao (Yamagata University, Yamagata, Japan) (7). Cells were cultured using the CELLLine system (BD Biosciences), and the supernatant was purified using a HiTrap protein G HP column (GE Healthcare). To deplete neutrophils, mice received an i.p. injection of the MAb (50 µg) 2 days before the infection. Anti-Gr1 MAb RB6-8C5 is specific for both Ly6C and Ly6G (11). Since Ly6C is expressed on some T cells, we used 50 µg MAb, which depleted >99% of Ly6G⁺ granulocytes while maintaining the majority of T cells (>85%) 2 days after the treatment. Under the conditions used, we estimated that the effect on the Ly6C⁺ population was minimal.

Statistics. Results are expressed as means ± standard deviations (SD). Statistical analysis was performed using the Mann-Whitney test for the comparison of two experimental groups or the log rank test for survival, and the data were calculated using GraphPad Prism software (version 4.0). Differences with a *P* value of <0.05 were considered significant.

RESULTS

Prevention of cerebral malaria by Flt3L treatment. We introduced a plasmid encoding mouse Flt3L into C57BL/6 mice by using the hydrodynamics method to stimulate the expansion of DCs *in vivo*. The serum Flt3L concentration was significantly increased 1 week after Flt3L treatment (Fig. 1A). The numbers of DCs and granulocytes in the spleens of treated mice were significantly increased as previously reported (38), while the numbers of NK cells and macrophages were not increased (Fig. 1B). The number of RBC in peripheral blood was also unchanged, consistent with the previous report that megakaryocyte/erythrocyte progenitors in the bone marrow do not express Flt3 and that their populations do not expand after Flt3L administration (16). We next examined DC subpopulations and their expression of MHC and costimulatory molecules (Fig. 1C). The proportion of CD8⁺ DCs was increased, while that of CD4⁺ DCs was reduced, in the spleens of Flt3L-treated mice compared with those in untreated mice. The

FIG. 1. Expansion of splenic DCs by expression of exogenous Flt3L. (A) Mice were inoculated (4 mice) or not (5 mice) with plasmid pCAGGS-Flt3L (5 μ g) by the hydrodynamics method. After 7 days, serum was collected, and the level of Flt3L was determined by ELISA. Ctrl, control. (B) Splenocytes were stained with antibodies, and the numbers of DCs (CD11c^{high} CD3⁻ CD19⁻ DX5⁻), NK cells (TCR β ⁻ NK1.1⁺), macrophages (CD11c⁻ F4/80^{high} Gr1⁻), and granulocytes (CD11c⁻ Gr1^{high} F4/80⁻) were determined by multiplying the total number of spleen cells by the ratio of each cell type. The number of RBC (Ter119⁺ CD45⁻) in peripheral blood was also determined. Asterisks indicate that a significant difference was observed between untreated and Flt3L-treated mice (P , < 0.05 by the Mann-Whitney test). (C) Splenocytes from mice that were either left untreated (solid lines) or treated with pCAGGS-Flt3L (dotted lines) were stained with antibodies, and flow cytometric analysis of MHC class II, CD80, CD86, CD40, and CD4 versus CD8 on DCs was conducted. (D) DCs (3×10^4) were prepared from untreated or Flt3L-treated mice, pulsed with or without pRBC lysates (1 mg/ml), and cocultured with splenic CD4⁺ or CD8⁺ T cells (1×10^5) from *P. berghei* ANKA-infected mice. After 48 h, the levels of IFN- γ in the supernatant were determined by ELISA. The experiments were repeated twice, and representative data are shown.

expression levels of MHC class II, CD80, and CD86 were not significantly different from those in untreated mice, while that of CD40 was slightly increased. The ability of these DCs to present malaria antigen was evaluated *in vitro* (Fig. 1D). DCs from Flt3L-treated mice stimulated IFN- γ production by malaria-specific CD8⁺ T cells much better than control DCs, a finding consistent with the increase in the proportion of CD8⁺ DCs in Flt3L-treated mice (8, 19).

To determine the effect of Flt3L on the pathogenesis of *P. berghei* ANKA infection, Flt3L-treated and untreated mice were infected with *P. berghei* ANKA, and their survival was

monitored (Fig. 2A). Most *P. berghei* ANKA-infected control mice died within 8 days with severe symptoms of ECM, such as coma. In contrast, Flt3L-treated mice were clearly protected from lethal CM. Mild symptoms of ECM, such as fur ruffling or hunching, were observed for some of the Flt3L-treated mice within 10 days, but none succumbed to death during this period. The level of parasitemia was significantly reduced in Flt3L-treated mice 5 days after infection with *P. berghei* ANKA but continued to increase to more than 50% within 2 weeks of the infection. The few control mice that survived the critical period of ECM also showed a similar increase in the level of

FIG. 2. FIt3L treatment enhanced innate immunity to malaria infection and prevented the development of lethal ECM by mechanisms independent of the reduction of parasitemia. (A) Mice were either left untreated (6 mice) (blue line) or were inoculated with an FIt3L-expressing plasmid (5 mice) (green line) or an empty vector (5 mice) (red line) on day -7 and were infected with pRBC on day 0. (Left) Survival was monitored daily. The green shaded area (days 6 to 10) indicates the dates on which ECM symptoms appear in this disease model in general. The

parasitemia. We tested various doses of the Flt3L plasmid and found an inverse correlation between the number of DCs in the spleen and the levels of parasitemia 5 days after infection (see Fig. S1 in the supplemental material). In addition, mice to which Flt3L-induced DCs were transferred showed reduced parasitemia levels, suggesting that these DCs were directly involved in the antimalaria effects of Flt3L treatment during the early period of the infection (see Fig. S2 in the supplemental material). To visualize the integrity of the blood-brain barrier in infected mice, the leakage of dye in the brain was examined after intravenous (i.v.) injection of Evans blue (Fig. 2B). In control mice, brains were stained with the dye, suggesting that the blood-brain barrier was impaired. However, the brains of Flt3L-treated mice showed little leakage, indicating that the integrity of the blood-brain barrier was maintained. These results suggest that Flt3L treatment effectively prevented the development of ECM.

Since parasitemia levels were inhibited during the early period of the infection in Flt3L-treated mice, we suspected that innate immune responses might be responsible for controlling the parasitemia. To examine this possibility, Rag2^{-/-} mice, which lack both T and B cells, were treated with Flt3L and were infected with *P. berghei* ANKA (Fig. 2C). The levels of parasitemia in Flt3L-treated Rag2^{-/-} mice were lower than those in untreated mice during the early period of the infection, suggesting that enhancement of innate immunity by Flt3L treatment effectively limited the expansion of *P. berghei* ANKA during this period. However, this effect was transient and was not sufficient for protection, since the parasitemia levels continued to rise in infected Rag2^{-/-} mice. Since DCs can phagocytose *Plasmodium*-infected RBC (15), we examined the possibility that Flt3L-expanded DCs phagocytosed pRBC. Flt3L-treated and untreated wild-type C57BL/6 mice were infected with PbA-GFP, and splenocytes were examined 5 days later by using flow cytometry (Fig. 2D). Equivalent proportions of DCs phagocytosed PbA-GFP in Flt3L-treated and untreated mice. In addition to DCs, phagocytosis of PbA-GFP was mediated by a population of CD11c⁻ Ly6C⁺ cells, which are similar to recently described inflammatory monocytes (34).

It is possible that the inhibition of ECM development in Flt3L-treated mice was due to the reduction in parasitemia levels during a critical window for the disease, the early period of infection. To closely examine the relationship between ECM

development and parasitemia levels during the early infection period, mice were infected with lower doses of pRBC (Fig. 2E). Although mice infected with 1.5×10^5 pRBC showed parasitemia levels similar to those of Flt3L-treated mice infected with 1×10^6 pRBC, the untreated mice infected with the lower pRBC dose did develop lethal CM. Since IFN- γ plays a critical role in the pathogenesis of ECM (40), we determined serum IFN- γ levels in Flt3L-treated and untreated mice during infection (Fig. 2F). The level of IFN- γ was slightly higher in Flt3L-treated mice than in untreated mice after *P. berghei* ANKA infection. To exclude the possibility that Flt3L directly inhibited parasite growth, we examined the effect of Flt3L on the growth of *P. berghei* ANKA *in vivo* prior to the expansion of DCs. Thus, we treated mice with Flt3L 4 days after infection (Fig. 2G). Two days after Flt3L treatment, when serum Flt3L levels had significantly increased (1.71 ± 0.79 ng/ml in treated mice versus 0.32 ± 0.02 ng/ml in untreated mice), the parasitemia level was not reduced, suggesting that Flt3L did not directly inhibit parasite growth. Taking these data together, we concluded that it was unlikely that inhibition of ECM development in Flt3L-treated mice was due simply to the inhibition of parasitemia or to the change in the systemic IFN- γ response.

Expansion and activation of CD8⁺ T cells in Flt3L-treated *P. berghei* ANKA-infected mice. Since ECM is a T cell-dependent disease, we next examined the number and phenotype of T cells 5 days after infection with *P. berghei* ANKA. The numbers of CD4⁺ and CD8⁺ T cells did not change significantly after infection of untreated mice with *P. berghei* ANKA but increased dramatically in both the spleen and peripheral blood after infection of Flt3L-treated mice with *P. berghei* ANKA (Fig. 3A). In particular, the increase in the number of CD8⁺ T cells was prominent. To investigate the function of these CD8⁺ T cells, they were stimulated with DCs pulsed with a pRBC lysate. CD8⁺ T cells from Flt3L-treated mice produced IFN- γ in response to malaria antigen at levels similar to or higher than those from untreated mice, suggesting that the priming and IFN- γ production of CD8⁺ T cells specific for malaria antigen were not impaired in Flt3L-treated mice (Fig. 3B).

We next examined the phenotypes of CD8⁺ T cells, since they are critical for the pathogenesis of ECM. Interestingly, a large population of CD8⁺ T cells was found to express CD11c in mice infected with *P. berghei* ANKA, while CD11c was not

asterisk indicates a significant difference between untreated or empty-vector-treated mice and pCAGGS-Flt3L-treated mice (P , <0.05 by the log rank test). (Right) Parasitemia was monitored every 2 to 3 days in surviving mice, and the mean $\pm$ SD for each group is shown. The asterisk indicates a significant difference between untreated or empty-vector-treated mice and Flt3L-treated mice (P , <0.05 by the Mann-Whitney test). (B) Six days after infection with *P. berghei* ANKA, the integrity of the blood-brain barrier was evaluated by injection of Evans blue solution. (C) Rag2^{-/-} mice were either left untreated (6 mice) (blue line) or treated with Flt3L (5 mice) (green line) and were infected with pRBC 7 days later. The parasitemia level was monitored every 2 to 3 days, and the mean $\pm$ SD for each group is shown. The asterisk indicates a significant difference between untreated and Flt3L-treated mice (P , <0.05 by the Mann-Whitney test). (D) Untreated or Flt3L-treated B6 mice were infected with PbA-GFP. Five days after infection, spleen cells were analyzed using flow cytometry. (Top) The percentage of GFP⁺ cells within CD11c^{high} DCs is given in the upper right corner. (Bottom) The expression of Ly6C within GFP⁺ CD11c^{high} cells is shown. (E) Mice (3 mice/group) were infected with the indicated number of pRBC. (Left) Survival was monitored daily. The asterisk indicates a significant difference (P < 0.05) by the log rank test. (Right) The levels of parasitemia 5 days after infection are expressed as means $\pm$ SD. (F) Sera were collected from untreated (Ctrl) or Flt3L-treated mice (3 mice/group) 5 days after *P. berghei* ANKA infection and were subjected to a CBA assay. The mean $\pm$ SD for each group is shown. (G) Mice (3 mice each in the Flt3L and pCAGGS groups; 5 mice in the untreated group) were infected with *P. berghei* ANKA and were either left untreated (blue line) or inoculated with an Flt3L-expressing plasmid (green line) or an empty vector (red line) on day 4. The levels of parasitemia were monitored, and the mean $\pm$ SD for each group was determined. Experiments for which results are shown in panels A to D and F were repeated twice, and representative data are shown.

FIG. 3. The number of CD8⁺ T cells was markedly increased in Flt3L-treated mice after infection with *P. berghei* ANKA. (A) Mice were either left untreated or were treated with Flt3L; then they were either left uninfected or infected with *P. berghei* ANKA for 5 days. Spleen cells and peripheral blood lymphocytes were stained for CD4, CD8, and TCR, and the numbers of CD4⁺ and CD8⁺ T cells were calculated. *, $P < 0.05$ by the Mann-Whitney test. (B) CD8⁺ T cells were cultured in triplicate wells in the presence of DCs pulsed with nothing (open bars), a pRBC lysate (filled bars), or an RBC lysate (shaded bars) for 48 h, and the levels of IFN- γ were determined by ELISA. The experiments were repeated twice, and representative data are shown.

significantly induced in CD4⁺ T cells upon *P. berghei* ANKA infection (Fig. 4A). The majority of CD11c⁺ CD8⁺ T cells coexpressed CD44, suggesting that they were recently activated effector T cells (Fig. 4B). CD11c is a well-known marker of DCs, but it was previously reported that some activated CD8⁺ T cells express CD11c (4, 13, 18). We sorted CD11c⁺ CD8⁺ and CD11c⁻ CD8⁺ T cells from the spleens of *P. berghei* ANKA-infected mice, and we stimulated them with an anti-TCR MAb *in vitro*. CD11c⁺ CD8⁺ T cells produced IFN- γ at a level much higher than that of CD11c⁻ CD8⁺ T cells, confirming that they were primed T cells (data not shown).

Focusing on CD11c⁺ CD8⁺ T cells, we examined their expression of other activation-associated molecules and the chemokine receptor CXCR3, which was previously reported to be instrumental to ECM pathogenesis (Fig. 4B and C) (6, 24, 37). The expression of CD44 and CXCR3 on CD11c⁺ CD8⁺ T cells was similarly upregulated in both Flt3L-treated and un-

treated mice. However, the expression of CD25, CD137, and granzyme B in CD11c⁺ CD8⁺ T cells from Flt3L-treated mice was lower than their expression in CD11c⁺ CD8⁺ T cells from untreated mice (Fig. 4C). These results suggest that the activation status of CD8⁺ T cells in Flt3L-treated mice was distinct from that in untreated mice during infection with *P. berghei* ANKA.

Next, cells sequestered in the brains of *P. berghei* ANKA-infected mice 6 days after infection were examined (Fig. 4D). The numbers of both CD8⁺ and CD4⁺ T cells increased dramatically in both Flt3L-treated and untreated mice after *P. berghei* ANKA infection. No significant difference in the number of brain-sequestered T cells between Flt3L-treated and untreated mice was found. The majority of brain-sequestered CD8⁺ T cells expressed CD44 and CD11c, indicating that they were activated T cells. The levels of CD44 expression were similar in Flt3L-treated and untreated mice, while CD11c expression levels were slightly lower in brain-sequestered CD8⁺ T cells from Flt3L-treated mice than in those from untreated mice. To gain insight into the function of these brain-sequestered CD8⁺ T cells, the expression of IFN- γ and granzyme B was evaluated by intracellular staining after stimulation with an anti-TCR MAb (Fig. 4E). The proportions of CD8⁺ T cells that produced IFN- γ alone or both IFN- γ and granzyme B were lower in Flt3L-treated mice than in the control group. Taken together, these results suggest that the activation status of brain-sequestered CD8⁺ T cells in Flt3L-treated mice is lower than that for untreated mice. Since sequestration of both CD8⁺ T cells and pRBC is required for the pathogenesis of ECM, we also examined the number of pRBC in the brains of Flt3L-treated mice (Fig. 4F). The number of brain-sequestered pRBC was strikingly reduced in Flt3L-treated mice, consistent with protection from lethal ECM.

Roles of Treg and granulocytes in Flt3L-treated mice. To investigate the possible role of regulatory CD4⁺ T cells (Treg) in the differential activation of CD8⁺ T cells in Flt3L-treated mice, we first examined the number of Foxp3⁺ Treg (Fig. 5A). The proportion of Treg in the spleens of Flt3L-treated mice was higher than the proportion in untreated mice, consistent with a previous report (35). However, the proportion of Treg did not change significantly after infection with *P. berghei* ANKA in either control or Flt3L-treated mice. Therefore, Treg did not proliferate preferentially in Flt3L-treated mice during *P. berghei* ANKA infection. We next examined IFN- γ production by CD4⁺ T cells and found that CD4⁺ T cells from Flt3L-treated and untreated mice produced similar levels of IFN- γ in response to an anti-TCR Ab (Fig. 5B). Since CD4⁺ T cells from Flt3L-treated and untreated mice contained similar levels of Foxp3⁺ Treg after infection with *P. berghei* ANKA, it is unlikely that the functions of Treg in these two groups of mice are significantly different. Therefore, it is unlikely that the differences in CD8⁺ T cells between Flt3L-treated and untreated mice are due to differences in Treg.

Treatment of mice with Flt3L increased the number of granulocytes and DCs in the spleen (Fig. 1B). The role of granulocytes in the pathogenesis of malaria has been evaluated previously; two studies reported that the depletion of granulocytes by antibody treatment did not significantly affect the level of parasitemia but prevented the development of ECM (7, 30), while one study reported that granulocyte depletion in the effector phase did not affect ECM pathogenesis (3). Thus, we

FIG. 4. The activation phenotype of CD8⁺ T cells from Flt3L-treated mice after *P. berghei* ANKA infection was distinct from that of untreated mice. (A) B6 mice were either left uninfected (solid lines) or infected with *P. berghei* ANKA (dotted lines). Five days later, spleen cells were stained for CD11c, TCR, CD8, and CD4, and the expression of CD11c on CD8⁺ and CD4⁺ T cells was examined. (B and C) Mice were either left untreated or treated with Flt3L, after which they were either left uninfected or infected with *P. berghei* ANKA, and splenocytes were stained with MAbs 5 days after infection. The dot plots demonstrate the expression of CD11c, CD44, CXCR3 (B), and CD25 (C) on CD8⁺ T cells. The number in each quadrant represents the proportion of cells within the region. The histograms (C) demonstrate the expression of CD25, CD137, and granzyme B on splenic CD11c⁺ CD8⁺ T cells from untreated (green) and Flt3L-treated (red) mice 5 days after *P. berghei* ANKA infection or from uninfected, untreated mice (black). Ctrl, control. (D) (Left) Brain-sequestered cells were isolated from uninfected and infected mice (3 mice/group), and total cell numbers were quantified. Cells were stained for CD45, Ter119, CD4, CD8, and TCR, and the numbers of CD8⁺ and CD4⁺ T cells (CD45⁺ Ter119⁻ TCR⁺) were calculated and are shown as means $\pm$ SD. N.S., no significant difference between the Ctrl and Flt3L groups by the Mann-Whitney test. (Right) Expression of CD44 and CD11c on brain-sequestered CD8⁺ T cells from untreated (green) and Flt3L-treated (red) B6 mice after 6 days of strain ANKA infection. The black line represents control unstained brain-sequestered CD8⁺ T cells. (E) Brain-sequestered CD8⁺ T cells were stimulated with an anti-TCR MAb, and the expression of IFN- γ and granzyme B (GrzB) was determined using intracellular staining. *, $P < 0.05$ by the Mann-Whitney test. (F) Mice (3 per group) were either left untreated or treated with Flt3L and were infected with *P. berghei* ANKA. After 6 days, brain-sequestered pRBC were counted. *, $P < 0.05$ by the Mann-Whitney test. The experiments were repeated twice, and representative data are shown.

evaluated the role of granulocytes in the inhibition of ECM and parasitemia (Fig. 6). Depletion of granulocytes with an anti-Gr1 MAb (>99%) prior to infection did not significantly affect the levels of parasitemia in either control or Flt3L-

treated mice and did not alter the inhibitory effect of Flt3L on the development of ECM. Therefore, we concluded that the inhibition of ECM by Flt3L treatment was not due to an increase in the number of granulocytes.

FIG. 5. Treg are unlikely to be involved in the inhibition of CD8⁺ T cell activation in Flt3L-treated mice after *P. berghei* ANKA infection. (A) Untreated (3 per group) and Flt3L-treated (4 per group) mice were either left uninfected or infected with *P. berghei* ANKA. After 5 days, splenocytes were stained for CD4, CD25, and Foxp3. The ratio of Treg (CD4⁺ CD25⁺ Foxp3⁺) within CD4⁺ T cells was expressed as the mean $\pm$ SD for each group. N.S., no significant difference. (B) Five days after infection, CD4⁺ splenic T cells were stimulated with a plate-bound anti-TCR MAb, and their IFN- γ production was determined by a quadruplicate ELISA. The experiments were repeated twice, and representative data are shown.

DISCUSSION

DCs are critical immune cells in both innate and adaptive immunity. During infection with malaria parasites, DCs take up *Plasmodium*-infected RBC and can induce the initiation of protective immune responses. To better understand the role of DCs during malaria infection, we have taken the approach of expanding DCs with Flt3L *in vivo* prior to infection with *P. berghei* ANKA. Flt3L was effective in augmenting protective innate immune responses during the early phase of

P. berghei ANKA infection, and it induced altered activation of CD8⁺ T cells, culminating in prevention of ECM.

We employed the hydrodynamic method to induce Flt3L *in vivo*, and we observed an increase in the number of DCs in the spleen. The proportion of CD8⁺ DCs was increased to more than 50% of all DCs, consistent with the report from a previous study that used an adenoviral vector expressing recombinant Flt3L (23). These DCs expressed MHC and costimulatory molecules at levels similar to those of controls and were superior in cross-presenting malaria antigens to specific CD8⁺ T cells, as previously reported (19). After infection of Flt3L-treated mice with *P. berghei* ANKA, a significant reduction in the level of parasitemia was observed during the initial period of the infection, suggesting that Flt3L-treated mice were more protected than untreated mice. We speculate that this initial protective response was due mainly to enhancement of innate immunity, since we observed a similar reduction in parasitemia levels in Flt3L-treated Rag2^{-/-} mice, which lack both T and B cells. We suspect that the expansion of DCs contributes to reduced parasitemia through DC phagocytosis of pRBC. Granulocytes may be less involved, since parasitemia levels were not significantly increased in mice depleted of granulocytes. This protection, however, was not long-lasting, and parasitemia levels rose in Flt3L-treated mice until they succumbed to death.

To investigate the mechanisms that lead to the prevention of ECM by Flt3L treatment, we studied the effects of Flt3L treatment on T cells, since T cells play an instrumental role in the pathogenesis of ECM. We first observed the marked increase in the number of CD8⁺ T cells in the spleens of Flt3L-treated mice after infection with *P. berghei* ANKA. Since CD8⁺ DCs increased preferentially in Flt3L-treated mice, we speculate that these DCs cross-presented and activated malaria-specific CD8⁺ T cells. These CD8⁺ T cells were found to produce IFN- γ in response to strain ANKA antigens at levels equal to or even higher than those from untreated mice (Fig. 3B), indicating that priming and IFN- γ production of malaria-specific CD8⁺ T cells were not impaired in Flt3L-treated mice. However, phenotypic study of the CD8⁺ T cells in the spleen showed some interesting features. First, we found, unexpectedly, that most CD8⁺ T cells in *P. berghei* ANKA-infected mice expressed CD11c, an integrin molecule that is often considered a marker of DCs. CD11c expression was also observed on CD8⁺ T cells after infection with another rodent malaria par-

FIG. 6. Role of granulocytes in Flt3L-mediated effects on *P. berghei* ANKA infection. Mice were either left untreated or treated with Flt3L 7 days prior to infection and received an anti-Gr1 MAb (50 μ g) i.p. 2 days prior to infection. (Left) The levels of parasitemia 5 days after infection are expressed as means $\pm$ SD. N.S., not significant by the Mann-Whitney test. (Right) The survival of infected mice was monitored daily. *, $P < 0.05$ by the log rank test. The experiments were repeated twice, and representative data are shown.

asite, *Plasmodium yoelii* (data not shown), and was previously reported in intraepithelial lymphocytes (IEL) and recently activated CD8⁺ T cells in virus-infected mice (4, 13, 18). IEL from germ-free mice did not express CD11c, and bacterial colonization induced CD11c in IEL (13). However, antigen-driven activation of CD8⁺ T cells alone is not sufficient for the induction of CD11c, since we and others failed to induce CD11c on CD8⁺ T cells through TCR stimulation *in vitro* (data not shown). To our knowledge, this is the first report of CD11c expression in CD8⁺ T cells during parasite infection. CD11c, which forms a heterodimer with $\beta 2$ integrin (CD18), is involved in the adhesion of cells via ligands, including ICAM-1, ICAM-2, and VCAM-1 (28). The absence of CD11c significantly attenuated the severity of experimental autoimmune encephalomyelitis and reduced the levels of cellular infiltration and demyelination (5). CD11c may be involved in the activation or effector function of CD8⁺ T cells during *P. berghei* ANKA infection through its role in strengthening the CD8⁺ T cell–target cell interaction, thus participating in the pathogenesis of ECM. Alternatively, CD11c may be involved in the preferential accumulation of activated CD8⁺ T cells in the inflamed brain. However, the expression of CD11c on CD8⁺ T cells is not sufficient for the onset of ECM, because CD8⁺ T cells in *P. berghei* ANKA-infected BALB/c mice expressed CD11c at levels similar to those in B6 mice, yet strain ANKA-infected BALB/c mice do not develop ECM (data not shown).

The phenotypic characterization of CD8⁺ T cells from Flt3L-treated mice during *P. berghei* ANKA infection suggests that their activation status is distinct from that of CD8⁺ T cells from control mice. We used CD11c as a general activation marker of CD8⁺ T cells and examined cells that coexpress activation-induced molecules with CD11c. Among CD11c⁺ CD8⁺ T cells from *P. berghei* ANKA-infected mice, the expression of CD44 and CXCR3 was similar for Flt3L-treated and untreated mice, while the expression of CD25, CD137, and granzyme B was diminished in Flt3L-treated mice. Conventional DCs play critical roles in the pathogenesis of ECM (10). The differential activation of CD8⁺ T cells in Flt3L-treated mice may reflect a difference in the type of DCs that were expanded by Flt3L treatment. In support of this possibility, it has been reported that DCs that expanded by *in vivo* administration of Flt3L produced altered cytokine profiles and had tolerogenic effects on T cells (23). DCs are composed of multiple subsets, and the combined action of DC stimulation with Flt3L and *P. berghei* ANKA infection may have resulted in the altered phenotype of the activated CD8⁺ T cells (27). An alternative possibility is the involvement of other T cells, such as helper or regulatory CD4⁺ T cells, that were differentially activated in Flt3L-treated mice. However, Flt3L treatment resulted in similarly increased proportions of regulatory CD4⁺ T cells both with and without *P. berghei* ANKA infection, consistent with the previous report (35). In addition, CD4⁺ T cells from Flt3L-treated and untreated mice produced similar levels of IFN- γ in response to an anti-TCR MAb. Thus, regulatory CD4⁺ T cells are unlikely to be involved in the observed phenotypic differences in CD8⁺ T cells.

Interestingly, the numbers of T cells recovered from the brain were not significantly different for Flt3L-treated versus untreated mice after infection with *P. berghei* ANKA, although Flt3L-treated mice did not develop lethal ECM. CD11c ex-

pression in brain-sequestered CD8⁺ T cells was reduced in Flt3L-treated mice, which might influence the strength of the interaction between CD8⁺ T cells and their target or local tissue. Functionally, levels of CD8⁺ T cells that can produce both IFN- γ and granzyme B were significantly reduced in Flt3L-treated mice, supporting the altered activation status of these CD8⁺ T cells. Finally, the number of pRBC in the brain was dramatically reduced in Flt3L-treated mice, a finding consistent with the lack of ECM in these mice. The results clearly indicate that the accumulation of CD8⁺ T cells in the brain is not sufficient for the sequestration of pRBC in the brain. We suspect that recruitment of *P. berghei* ANKA-specific activated CD8⁺ T cells to the brain might condition brain blood vessels for the sequestration of pRBC. In Flt3L-treated mice, CD8⁺ T cells might not be sufficiently activated to achieve this conditioning, while they themselves were able to be sequestered in the brain.

ECM is a complex process resulting from the intricate interplay of *P. berghei* ANKA infection and the host immune response. We have shown that the development of ECM can be effectively prevented by the administration of Flt3L, which stimulates the innate immune system, including dendritic cells, and indirectly alters the activation status of CD8⁺ T cells after infection with *P. berghei* ANKA. In addition, the present study indicates that the number of brain-sequestered T cells does not correlate directly with the pathogenesis of ECM. Further detailed analysis of this system may reveal the critical function of the immune system in the pathogenesis of ECM.

ACKNOWLEDGMENTS

We appreciate Y. Yoshikai for providing mice, H. Asao for providing the hybridoma cell line, Y. Yamato and Y. Tsuchiya for technical assistance and secretarial work, M. Masumoto for cell sorting, and R. Kamei for help in animal care. We also thank the members of our laboratory for helpful discussions and advice.

This study was supported by the Global COE Program at Nagasaki University and by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture to K.Y. (21390125).

The authors declare no competing financial interests.

REFERENCES

- Baptista, F. G., et al. 2010. Accumulation of *Plasmodium berghei*-infected red blood cells in the brain is crucial for the development of cerebral malaria in mice. *Infect. Immun.* **78**:4033–4039.
- Belnoue, E., et al. 2003. CCR5 deficiency decreases susceptibility to experimental cerebral malaria. *Blood* **101**:4253–4259.
- Belnoue, E., et al. 2002. On the pathogenic role of brain-sequestered $\alpha\beta$ CD8⁺ T cells in experimental cerebral malaria. *J. Immunol.* **169**:6369–6375.
- Beyer, M., et al. 2005. The $\beta 2$ integrin CD11c distinguishes a subset of cytotoxic pulmonary T cells with potent antiviral effects *in vitro* and *in vivo*. *Respir. Res.* **6**:70.
- Bullard, D. C., X. Hu, J. E. Adams, T. R. Schoeb, and S. R. Barnum. 2007. p150/95 (CD11c/CD18) expression is required for the development of experimental autoimmune encephalomyelitis. *Am. J. Pathol.* **170**:2001–2008.
- Campanella, G. S., et al. 2008. 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.* **105**:4814–4819.
- Chen, L., Z. Zhang, and F. Sando. 2000. Neutrophils play a critical role in the pathogenesis of experimental cerebral malaria. *Clin. Exp. Immunol.* **120**:125–133.
- den Haan, J. M., S. M. Lehar, and M. J. Bevan. 2000. CD8⁺ but not CD8[−] dendritic cells cross-prime cytotoxic T cells *in vivo*. *J. Exp. Med.* **192**:1685–1696.
- de Souza, J. B., J. C. Hafalla, E. M. Riley, and K. N. Couper. 2009. Cerebral malaria: why experimental murine models are required to understand the pathogenesis of disease. *Parasitology* **137**:755–772.
- deWalick, S., et al. 2007. Conventional dendritic cells are the critical APC required for the induction of experimental cerebral malaria. *J. Immunol.* **178**:6033–6037.

11. Fleming, T. J., M. L. Fleming, and T. R. Malek. 1993. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J. Immunol.* **151**:2399–2408.
12. Herweijer, H., and J. A. Wolff. 2007. Gene therapy progress and prospects: hydrodynamic gene delivery. *Gene Ther.* **14**:99–107.
13. Huleatt, J. W., and L. Lefrancois. 1995. Antigen-driven induction of CD11c on intestinal intraepithelial lymphocytes and CD8⁺ T cells *in vivo*. *J. Immunol.* **154**:5684–5693.
14. Idro, R., N. E. Jenkins, and C. R. Newton. 2005. Pathogenesis, clinical features, and neurological outcome of cerebral malaria. *Lancet Neurol.* **4**:827–840.
15. Ing, R., M. Segura, N. Thawani, M. Tam, and M. M. Stevenson. 2006. Interaction of mouse dendritic cells and malaria-infected erythrocytes: uptake, maturation, and antigen presentation. *J. Immunol.* **176**:441–450.
16. Karsunky, H., M. Merad, A. Cozzio, I. L. Weissman, and M. G. Manz. 2003. Flt3 ligand regulates dendritic cell development from Flt3⁺ lymphoid and myeloid-committed progenitors to Flt3⁺ dendritic cells *in vivo*. *J. Exp. Med.* **198**:305–313.
17. Langhorne, J., et al. 2004. Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection. *Immunol. Rev.* **201**:35–47.
18. Lin, Y., T. J. Roberts, V. Sriram, S. Cho, and R. R. Brutkiewicz. 2003. Myeloid marker expression on antiviral CD8⁺ T cells following an acute virus infection. *Eur. J. Immunol.* **33**:2736–2743.
19. Lundie, R. J., et al. 2008. Blood-stage *Plasmodium* infection induces CD8⁺ T lymphocytes to parasite-expressed antigens, largely regulated by CD8 α ⁺ dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **105**:14509–14514.
20. Lundie, R. J., et al. 2010. Blood-stage *Plasmodium berghei* infection leads to short-lived parasite-associated antigen presentation by dendritic cells. *Eur. J. Immunol.* **40**:1674–1681.
21. Maraskovsky, E., et al. 1996. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J. Exp. Med.* **184**:1953–1962.
22. McKenna, H. J., et al. 2000. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* **95**:3489–3497.
23. Miller, G., V. G. Pillarisetty, A. B. Shah, S. Lahrs, and R. P. DeMatteo. 2003. Murine Flt3 ligand expands distinct dendritic cells with both tolerogenic and immunogenic properties. *J. Immunol.* **170**:3554–3564.
24. Miu, J., et al. 2008. Chemokine gene expression during fatal murine cerebral malaria and protection due to CXCR3 deficiency. *J. Immunol.* **180**:1217–1230.
25. Miyakoda, M., et al. 2008. Malaria-specific and nonspecific activation of CD8⁺ T cells during blood stage of *Plasmodium berghei* infection. *J. Immunol.* **181**:1420–1428.
26. Nitcheu, J., et al. 2003. Perforin-dependent brain-infiltrating cytotoxic CD8⁺ T lymphocytes mediate experimental cerebral malaria pathogenesis. *J. Immunol.* **170**:2221–2228.
27. Pulendran, B., H. Tang, and S. Manicassamy. 2010. Programming dendritic cells to induce T_H2 and tolerogenic responses. *Nat. Immunol.* **11**:647–655.
28. Sadhu, C., et al. 2007. CD11c/CD18: novel ligands and a role in delayed-type hypersensitivity. *J. Leukoc. Biol.* **81**:1395–1403.
29. Schofield, L., and G. E. Grau. 2005. Immunological processes in malaria pathogenesis. *Nat. Rev. Immunol.* **5**:722–735.
30. Senaldi, G., C. Vesin, R. Chang, G. E. Grau, and P. F. Piguet. 1994. Role of polymorphonuclear neutrophil leukocytes and their integrin CD11a (LFA-1) in the pathogenesis of severe murine malaria. *Infect. Immun.* **62**:1144–1149.
31. Shinkai, Y., et al. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68**:855–867.
32. Shortman, K., and S. H. Naik. 2007. Steady-state and inflammatory dendritic-cell development. *Nat. Rev. Immunol.* **7**:19–30.
33. Sponaas, A. M., et al. 2006. Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells. *J. Exp. Med.* **203**:1427–1433.
34. Sponaas, A. M., et al. 2009. Migrating monocytes recruited to the spleen play an important role in control of blood stage malaria. *Blood* **114**:5522–5531.
35. Swee, L. K., N. Bosco, B. Malissen, R. Ceredig, and A. Rolink. 2009. Expansion of peripheral naturally occurring T regulatory cells by Fms-like tyrosine kinase 3 ligand treatment. *Blood* **113**:6277–6287.
36. Taylor, T. E., et al. 2004. Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nat. Med.* **10**:143–145.
37. Van den Steen, P. E., et al. 2008. CXCR3 determines strain susceptibility to murine cerebral malaria by mediating T lymphocyte migration toward IFN- γ -induced chemokines. *Eur. J. Immunol.* **38**:1082–1095.
38. Waskow, C., et al. 2008. The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat. Immunol.* **9**:676–683.
39. Wykes, M. N., et al. 2007. *Plasmodium* strain determines dendritic cell function essential for survival from malaria. *PLoS Pathog.* **3**:e96.
40. Yanez, D. M., D. D. Manning, A. J. Cooley, W. P. Weidanz, and H. C. van der Heyde. 1996. Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. *J. Immunol.* **157**:1620–1624.