

**Prevention of experimental cerebral malaria by Flt3 ligand
during infection with *Plasmodium berghei* ANKA**

Running title: Prevention of cerebral malaria by Flt3 ligand

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Abstract

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 which 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, lower parasitemia, and were protected from the development of lethal experimental cerebral malaria (ECM). Rag2 knock-out mice treated with Flt3 ligand also showed inhibition of parasitemia, suggesting that this protection was, at least in part, due to stimulation of innate immunity. However, it was unlikely that the inhibition of ECM was simply due to the reduction in parasitemia. In the peripheral T cell compartment, CD8⁺ T cells 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 between treated and untreated mice, while the proportion of CD8⁺ T cells that produce IFN- γ and granzyme B was significantly reduced in treated mice. In addition, sequestration of parasitized 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 change in the dendritic cell compartment is important for the pathogenesis of ECM.

Introduction

Malaria is one of the most serious infections in the world, and is responsible for more than one 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 of this important parasite (14, 29, 36). Infection with *Plasmodium berghei* ANKA (PbA), a rodent malaria parasite, induces neurological symptoms and death in C57BL/6 and CBA mice, and is widely used as a mouse model of experimental cerebral malaria (ECM) (8). Previous studies using the PbA-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 development of ECM, while reconstitution of CD8⁺ T cells in RAG deficient mice, which lack both T and B cells, resulted in the development of ECM after infection with PbA (3, 26). In addition, concomitant accumulation of parasitized RBCs in the brain is critical for the development of ECM (1). Recruitment of CD8⁺ T cells to the brain and the pathogenesis of ECM is dependent on chemokine receptor CCR5 (2, 26), and CXCR3 expressed on CD8⁺ T cells, as well as CXCR3 ligands CXCL9/CXCL10 (6, 24, 37). In the effector phase, production of proinflammatory cytokines such as IFN- γ and 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 (pRBC) during infection and can present malaria antigen in both MHC class I and class II pathways, activating malaria-specific CD8⁺ and CD4⁺ T cells, respectively, 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 was previously shown 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 a steady state (16). Administration of Flt3L induces a drastic increase of DCs in spleen and lymph nodes (21). In contrast, the lack of Flt3L leads to severe reductions in DC numbers in many tissues (22). It was previously shown that the number and phenotype of DCs in the spleen fluctuated during infection with malaria parasite (33, 39). However, it was unclear 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 stimulated the expansion of DCs *in vivo* by administration of Flt3L prior to infection with PbA, and examined its effects on the activation of the immune system as well as 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 the pathogenesis of ECM.

Materials and Methods

Mice and plasmid transduction

Rag-2^{-/-} mice of C57BL/6 background (31) were provided by Dr. 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 herein 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 from 1 to 189) of mouse Flt3L was obtained by PCR from cDNA prepared from mouse spleen using primer pairs 5'-GATCCACCATGACAGTGCTGGCGCCAGC-3' and 5'-GATCTACTGCCTGGGCCGAGGCTCTG-3'. After confirming the sequence, the PCR product was cloned into pCAGGS, resulting in pCAGGS-Flt3L. The plasmid was purified using PureYieldTM Plasmid Maxiprep System (Promega, Madison, WI), and its endotoxin level was 2.43 EU (equivalent to 0.55ng endotoxin) per µg DNA as determined by Limulus test (Wako, Osaka, Japan). A solution containing the plasmid (5µg in PBS) was injected into mice using the hydrodynamics method, as previously described (12).

GFP-expressing parasites and PbA infection

PbA was originally obtained from Dr. R.E. Sinden (Imperial College London, London, UK). Recombinant PbA parasites that constitutively express GFP (PbA-GFP) were engineered as previously described (25). The gene construct based on pBluescript KS(+) (Stratagene) contains a PbA dihydrofolate reductase-thymidyltransferase-ts (DHFR-ts) gene, PbA hsp70 5' untranslated region, its N-terminal coding sequence and coding

sequence of GFP. PbA merozoites were transfected with the DNA construct by electroporation and were selected in rats using pyrimethamine. Surviving parasites were cloned by limiting dilution in mice.

PbA was maintained by passing through BALB/c mice. For infection, mice were inoculated with parasitized RBC (pRBC) (1×10^6) *i.p.* unless the dose was specifically indicated. Parasitemia of infected mice was monitored by microscopic examination of Giemsa stained tail blood smears. Typically, five to six days after infection with PbA, mice began to show neurological signs, such as hunching, paralysis, ataxia, and succumbed to death within ten days of infection (6). PBS solution containing 2% Evans blue dye was injected into mice on day 6 after PbA infection. After one hour mice were euthanized, brains were removed and fixed in 3% paraformaldehyde solution and photographed.

Flow cytometry

To prepare DCs, spleens were cut into small fragments, incubated with RPMI1640 containing collagenase (100U/ml), mechanically disrupted and filtered to prepare single-cell suspensions. After lysis of RBC, cells were stained with FITC-anti-CD4, APC-anti-CD8, FITC-anti-MHC class II, FITC-anti-CD40, FITC-anti-CD80, FITC-anti-CD86, PE-anti-CD11c, PECy7-anti-CD3 ϵ , PECy7-anti-CD19, PECy7-anti-NK1.1, PECy7-anti-Ter119, FITC-anti-TCR β , FITC-anti-F4/80, PE-anti-Gr1, APC-anti-Ly6G, APC-anti-CD45, APCCy7-anti-Ly6C, APCCy7-anti-CD45, biotin-anti-DX5, biotin-anti-CD11c, biotin-anti-CD11b Abs and PECy7- or APC-streptavidin. Antibodies were purchased from BD Bioscience (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 anti-TCR mAb (H57; 2 μ g/ml) for 5 hrs with an addition of Golgi stop (BD Biosciences) during the final 4 hs. The cells were collected and stained with anti-IFN- γ or anti-granzyme B mAb according to the manufacturer's instruction. For intracellular staining of Foxp3, splenocytes were stained using anti-Foxp3 staining set (eBioscience) according manufacturer's instruction. Cells were analyzed using FACSCanto II (BD Bioscience) and data analysis was performed using CellQuest software. The following cell populations were defined; DCs, CD11c^{high}CD3 ϵ ⁻CD19⁻DX5⁻; NK cells, NK1.1⁺ TCR β ⁻; macrophages, F4/80^{high}CD11b^{low}CD11c⁻; granulocytes, Gr1^{high}Ly6G⁺CD11b⁺.

Cytokine quantification

Serum levels of IFN- γ and Flt3L were quantified using a cytometric bead array (CBA) assay (BD Bioscience) and mouse Flt3 ligand Quantikine ELISA kit (R&D systems, Minneapolis, MN), respectively. Splenic CD4⁺ and CD8⁺ T cells were purified using anti-CD4 or anti-CD8 IMag (BD Biosciences), respectively. Splenic DCs were purified using anti-CD11c MACS microbeads and autoMACS (Miltenyi Biotech, Gladbach, Germany) or by cell sorting of CD11c⁺/MHC class II⁺ cells using FACSARIA (BD Biosciences)(Fig. 1D). CD4⁺ or CD8⁺ T cells (1x10⁵ cells/well) were cultured for 48 hr on 96-well plates coated with anti-TCR mAb (H57, 2 μ g/ml) or with DCs (3x10⁴ cells/well) pulsed with RBC lysate (1mg/ml) for two hours. The levels of IFN- γ in the supernatant were determined by ELISA as described (25).

Preparation of brain-sequestered leukocytes and RBCs

Mice were sacrificed 6 days after PbA-infection, and brain-sequestered leukocytes were prepared as previously described with slight modification (24). Briefly, euthanized mice were perfused intracardially with PBS, and brains were removed. Brains were crushed and treated with collagenase (100U/ml) at 37°C for 15 min. The brain extract was centrifuged at 1,500 rpm for 20 min in 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 pRBCs, total numbers of RBCs were counted after Percoll centrifugation of brain extract without RBC lysis. Cells were stained with PECy7-anti-Ter119 and APC-anti-CD45 mAbs and were analyzed using FACSCanto II. The proportion of pRBC was determined by microscopic examination in a manner similar to standard blood films. The number of pRBC in brains was calculated by multiplying the number of RBCs with the proportion of pRBC.

Depletion of neutrophils

The hybridoma cell line secreting RB6-8C5 mAb (anti-Gr1) was provided by Dr. H. Asao (Yamagata University, Yamagata, Japan) (7). Cells were cultured using CELLline system (BD Biosciences) and the supernatant was purified using a HiTrap protein G HP column (GE healthcare). To deplete neutrophils, mice received *i.p.* injection of mAb (50 µg) two 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, that depleted >99% Ly6G⁺ granulocytes, while maintaining the majority of T cells (>85%) two 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 \pm SD. Statistical analysis was performed using Mann-Whitney test between two experiment groups or the logrank test for survival, and the data were calculated using GraphPad Prism software (version 4.0). Differences with $P<0.05$ were considered significant.

Results

Prevention of cerebral malaria by Flt3L-treatment

We introduced a plasmid encoding mouse Flt3L into C57BL/6 mice using the hydrodynamics method to stimulate expansion of DCs *in vivo*. The serum concentration of Flt3L was significantly increased one week after Flt3L-treatment (Fig. 1A). The numbers of DCs and granulocytes in the spleen of treated mice were significantly increased as previously reported (38), while numbers of NK cells and macrophages were not increased (Fig. 1B). The number of RBCs in peripheral blood was also unchanged, consistent with the previous report that megakaryocyte/erythrocyte progenitors in the bone marrow do not express Flt3 and do not expand after Flt3L administration (16). We next examined DC sub-populations and their expression of MHC and co-stimulatory molecules (Fig. 1C). The proportion of CD8⁺ DCs was increased while that of CD4⁺ DCs was reduced in the spleen of Flt3L-treated mice when compared with those of untreated mice. The expression levels of MHC class II, CD80 and CD86 were not significantly different from those of 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 of malaria-specific CD8⁺ T cells much better than control DCs, which is consistent with the increase in the proportion of CD8⁺ DCs in Flt3L-treated mice (9, 19).

To determine the effect of Flt3L on the pathogenesis of PbA-infection, Flt3L-treated and untreated mice were infected with PbA, and their survival was monitored (Fig. 2A). Most PbA-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 in some of the Flt3L-treated mice within ten 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 PbA, 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 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 (Supplemental Fig. 1). In addition, mice that were transferred with Flt3L-induced DCs showed reduced parasitemia levels, suggesting that these DCs were directly involved in anti-malaria effects of Flt3L-treatment during early period of the infection (Supplementary Fig. 2). To visualize the integrity of the blood brain barrier in infected mice, the leakage of dye in the brain was examined after *i.v.* injection of Evans blue (Fig. 2B). In control mice, brains were stained with the dye, suggesting the blood brain barrier was impaired. However, the brains of Flt3L-treated mice showed little leakage, indicating that the integrity of 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 PbA (Fig. 2C). The levels of parasitemia in Flt3L-treated Rag2^{-/-} mice were lower than parasitemia levels in untreated mice during the early period of the infection, suggesting that enhancement of innate

immunity by Flt3L-treatment effectively limited the expansion of PbA during this period. However, this effect was transient and was not sufficient for protection as 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 using flow cytometry (Fig. 2D). In both Flt3L-treated and untreated mice an equivalent proportion of DCs phagocytosed PbA-GFP. 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 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 levels of IFN- γ in Flt3L-treated and untreated mice during infection (Fig. 2F). The level of IFN- γ was slightly increased in Flt3L-treated mice when compared with untreated mice after PbA infection. To exclude the possibility that Flt3L directly inhibited parasite growth, we examined the effect of Flt3L on the growth of PbA *in vivo* prior to the expansion of DCs. Thus, we treated mice with Flt3L 4 days after the infection (Fig. 2G). Two days after

Flt3L-treatment, when serum Flt3L levels significantly increased (1.71 ± 0.79 ng/ml in treated vs. 0.32 ± 0.02 ng/ml in untreated mice), the parasitemia level was not reduced, suggesting that Flt3L did not directly inhibit parasite growth. Collectively, we concluded that it was unlikely that inhibition of ECM development in Flt3L-treated mice was simply due to the inhibition of parasitemia or due to the change in systemic IFN- γ response.

Expansion and activation of CD8⁺ T cells in Flt3L-treated PbA-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 PbA. The number of CD4⁺ and CD8⁺ T cells did not change significantly after PbA infection in untreated mice, but increased dramatically in both spleen and peripheral blood after PbA infection in Flt3L-treated mice (Fig. 3A). In particular, the increase in CD8⁺ T cells was prominent. To investigate the function of these CD8⁺ T cells, they were stimulated with DC pulsed with 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 was 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 PbA, while CD11c was not significantly induced in CD4⁺ T cells upon PbA infection (Fig. 4A). The majority of CD11c⁺CD8⁺ T cells co-expressed 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 spleen of PbA-infected mice, and stimulated them with anti-TCR mAb *in vitro*. CD11c⁺CD8⁺ T cells produced IFN- γ at a level much higher than 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 chemokine receptor, CXCR3, which was previously reported to be instrumental to ECM-pathogenesis (Fig. 4B, C) (6, 24, 37). The expression of CD44 and CXCR3 on CD11c⁺CD8⁺ T cells was similarly up-regulated in both Flt3L-treated and untreated 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 PbA.

Next, cells sequestered in the brain of PbA-infected mice 6 days after infection with PbA were examined (Fig. 4D). The number of both CD8⁺ and CD4⁺ T cells dramatically increased in both Flt3L-treated and untreated mice after PbA 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 level of CD44 expression was similar in both Flt3L-treated and untreated mice, while CD11c expression levels were slightly lower in brain-sequestered CD8⁺ T cells from Flt3L-treated mice when compared with those from untreated mice. To gain insight on the function of these brain-sequestered CD8⁺ T cells, expression of IFN- γ and granzyme B was evaluated by

intracellular staining after stimulation with anti-TCR mAb (Fig. 4E). The proportion of CD8⁺ T cells that produce IFN- γ or both IFN- γ and granzyme B were lower in Flt3L-treated mice when compared with the control group. Taken together, these results suggest that the activation status of brain-sequestered CD8⁺ T cells in Flt3L-treated mice is diminished when compared with those from untreated mice. Since sequestration of both CD8⁺ T cells and pRBCs are required for the pathogenesis ECM, we also examined the number of pRBCs in the brain of Flt3L-treated mice (Fig. 4F). The number of brain-sequestered pRBCs 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 cells (Fig. 5A). The proportion of Treg in the spleen of Flt3L-treated mice was higher than the proportion in untreated mice, consistent with the previous report (35). However, the proportion of Treg did not significantly change after infection with PbA in both control and Flt3L-treated mice. Therefore, Treg did not proliferate preferentially in Flt3L-treated mice during PbA-infection. We next examined IFN- γ production of CD4⁺ T cells, and found that CD4⁺ T cells from Flt3L-treated and untreated mice produced a similar level of IFN- γ in response to anti-TCR Ab. Since CD4⁺ T cells from Flt3L-treated and untreated mice contained similar levels of FoxP3⁺Tregs after infection with PbA, it is unlikely that the function of Treg in these two groups of mice is 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 malaria pathogenesis was previously evaluated; 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 evaluated the role of granulocytes in the inhibition of ECM and parasitemia (Fig. 6). Depletion of granulocytes with anti-Gr1 mAb (>99%) prior to the infection did not significantly affect the levels of parasitemia in both control and Flt3L-treated mice, and did not alter the inhibitory effect of Flt3L on the development of ECM. Therefore, we concluded that inhibition of ECM by Flt3L-treatment was not due to the increase in the number of granulocytes.

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 initiation of protective immune responses. To better understand the role of DCs during malaria infection, we have taken an approach to expand DCs with Flt3L *in vivo* prior to infection with PbA. Flt3L was effective in augmenting protective innate immune responses during the early phase of PbA 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 previous report that used adenoviral vector expressing recombinant Flt3L (23). These DCs expressed MHC and co-stimulatory molecules at levels similar to 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 PbA, a significant reduction of parasitemia levels 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 mainly due 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 contribute to reduced parasitemia through DC phagocytosis of pRBC. Granulocytes may be less involved, since the parasitemia levels were not significantly increased in mice depleted of granulocytes. This protection, however, was not long-lasting, and parasitemia 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 spleen of Flt3L-treated mice after infection with PbA. Since CD8⁺ DCs preferentially increased 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 PbA 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 was not impaired in Flt3L-treated mice. However, phenotypic study of the CD8⁺ T cells in the spleen showed some interesting features. First, we unexpectedly found that most CD8⁺ T cells in PbA-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 parasite, *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 stimulation of TCR *in vitro* (data not shown). To our knowledge, this is the first report of CD11c expression in CD8⁺ T cells during parasite infection. CD11c, forming a heterodimer with β 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 cellular infiltration and demyelination (5). CD11c may be involved in the activation or effector function of CD8⁺ T cells during PbA-infection via 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 PbA-infected BALB/c mice expressed CD11c at levels similar to those in C57BL/6 mice, yet PbA-infected BALB/c mice do not develop ECM (data not shown).

The phenotypic characterization of CD8⁺ T cells from Flt3L-treated mice during PbA-infection suggests that their activation status is distinct from CD8⁺ T cells from control mice. We used CD11c as a general activation marker of CD8⁺ T cells and examined cells that co-express activation-induced molecules with CD11c. Among CD11c⁺CD8⁺ T cells from PbA-infected mice, the expression of CD44 and CXCR3 was similar between 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 for the pathogenesis of ECM (10). The differential activation of CD8⁺ T cells in Flt3L-treated mice may reflect the difference in the type of DCs that were expanded by Flt3L treatment. In support of this possibility, it was 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 PbA 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 PbA 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 anti-TCR mAb. Thus, regulatory CD4⁺ T cells are unlikely to be involved in the observed phenotypic differences in CD8⁺ T cells.

Interestingly, the number of T cells recovered from the brain was not significantly different between Flt3L-treated and untreated mice after infection with PbA, although Flt3L-treated mice did not develop lethal ECM. Expression of CD11c 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, 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 pRBCs in the brain was severely reduced in Flt3L-treated mice, which is consistent with the lack of ECM in these mice. The results clearly indicate that accumulation of CD8⁺ T cells in the brain is not sufficient for the sequestration of pRBC in the brain. We suspect that recruitment of PbA-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 sequester in the brain.

ECM is a complex process resulting from the intricate interplay of PbA-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 PbA. In addition, the present study indicates that the number of brain-sequestered T cells does not directly correlate 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.

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Figure legends

Figure. 1.

Expansion of splenic DCs by expression of exogenous Flt3L.

(A) Mice were inoculated (4 mice) or not (5 mice) with the 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. (B) Splenocytes were stained with antibodies and the number 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 with the ratio of each cell type. The number of RBCs (Ter119⁺CD45⁻) in peripheral blood was also determined. *A significant difference was observed between untreated and Flt3L-treated mice ($p < 0.05$, Mann-Whitney test). (C) Splenocytes from mice untreated (solid line) or treated (dotted line) with pCAGGS-Flt3L were stained with antibodies and flow cytometric analysis of MHC class II, CD80, CD86, CD40 (upper panel) and CD4 vs. CD8 (lower panel) on DCs are shown. (D) DCs (3×10^4) were prepared from untreated and Flt3L-treated mice, pulsed with and without pRBC lysate (1 mg/ml) and were cocultured with splenic CD4⁺ or CD8⁺ T cells (1×10^5) from PbA-infected mice. After 48 hrs, the levels of IFN- γ in the supernatant were determined by ELISA. The experiments were repeated twice and the representative data are shown.

Figure. 2.

Flt3L-treatment enhanced innate immunity against malaria infection, and prevented the development of lethal ECM by mechanisms independent of the reduction of parasitemia.

(A) Mice were untreated (blue line, 6 mice) or inoculated with Flt3L-expressing plasmid (green line, 5 mice) or empty vector (red line, 5 mice) on day-7, and were infected with pRBC on day0. Survival was monitored daily (left). The shaded area (day6-10) indicates dates that ECM symptoms appear in this disease model in general. *A significant difference was observed between untreated or empty vector-treated vs pCAGGS-Flt3L-treated mice ($p<0.05$, logrank test). Parasitemia was monitored every 2-3 days in surviving mice and the mean \pm SD in each group was indicated (right). * A significant difference was observed between untreated or empty vector-treated vs Flt3L-treated mice ($p<0.05$, Mann-Whitney test).

(B) 6 days after infection with PbA, the integrity of the blood-brain barrier was evaluated by injection of Evans blue solution.

(C) Rag2^{-/-} mice were untreated (blue line, 6 mice) or Flt3L-treated (green line, 5 mice) and infected with pRBC 7 days later. Parasitemia level was monitored every 2-3 days and the mean \pm SD in each group was shown. * A significant difference was observed between untreated and Flt3L-treated mice ($p<0.05$, Mann-Whitney test).

(D) Untreated or Flt3L-treated C57BL/6 mice were infected with PbA-GFP. Five days after infection, spleen cells were analyzed using flow cytometry. Numbers in the upper right corner indicate percentage of GFP⁺ cells within CD11c^{high} DCs (upper panel). The expression of Ly6C within GFP⁺CD11c⁻ cells is shown (lower panel).

(E) Mice (3 mice) were infected with the indicated number of pRBC. Survival was monitored daily (left). * $p<0.05$, logrank test. The levels of parasitemia 5 days after

infection are expressed as mean \pm SD (right).

(F) Sera were collected from untreated or Flt3L-treated mice (3 mice/group) five days after PbA-infection, and were subjected to CBA assay. The mean \pm SD in each group was shown. (G) Mice (3 mice/Flt3L and pCAGGS groups, 5 mice/untreated group) were infected with PbA and were untreated (blue line) or inoculated with Flt3L-expressing plasmid (green line) or empty vector (red line) on day 4. The levels of parasitemia were monitored and the mean \pm SD in each group was determined. The experiments (A-D, F) were repeated twice and the representative data are shown.

Figure 3

The number of CD8⁺ T cells was markedly increased in Flt3L-treated mice after infection with PbA.

(A) Mice were untreated or Flt3L-treated and were infected or not infected with PbA for 5 days. Spleen cells and PBL were stained for CD4, CD8 and TCR, and the number of CD4⁺ and CD8⁺ T cells was calculated. * $p < 0.05$, Mann-Whitney test. (B) CD8⁺ T cells were cultured in triplicate wells in the presence of DCs pulsed with nothing (open bar), pRBC lysate (filled bar) or RBC lysate (gray bar) for 48 hrs, and the levels of IFN- γ were determined by ELISA. The experiments were repeated twice and the representative data are shown.

Figure 4

The activation phenotype of CD8⁺ T cells from Flt3L-treated mice after PbA-infection was distinct from that of untreated mice.

(A) B6 mice were uninfected (solid line) or infected (dotted line) with PbA. 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, C) Mice were untreated or treated with Flt3L, uninfected or infected with PbA, and splenocytes were stained with mAbs 5 days after infection. The dot plots demonstrate the expression of CD11c, CD44, CXCR3 (B) and CD25 (C, upper) on CD8⁺ T cells. The number in each quadrant represents the proportion of cells within the region. The histograms demonstrate expression of CD25, CD137 and granzyme B on splenic CD11c⁺CD8⁺ T cells from untreated (green) and Flt3L-treated (red) mice 5 days after PbA-infection, or from untreated mice without infection (black).

(D, left) Brain-sequestered cells were isolated from uninfected and infected mice (3 mice/group) and their total cell number was 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 expressed as mean \pm SD. (D, right) The expression of CD44 and CD11c on brain-sequestered CD8⁺ T cells from untreated (green) and Flt3L-treated (red) B6 mice after 6 days of PbA-infection. The black line represents control unstained brain-sequestered CD8⁺ T cells. N.S.= not significant, Mann-Whitney test between Ctrl and Flt3L groups. (E) Brain-sequestered CD8⁺ T cells were stimulated with anti-TCR mAb, and the expression of IFN- γ and granzyme B was determined using intracellular staining. * $p < 0.05$, Mann-Whitney test. (F) Mice (3 mice/group) were treated and untreated with Flt3L and were infected with PbA. After 6 days,

brain-sequestered pRBCs were counted. * $p < 0.05$, Mann-Whitney test. The experiments were repeated twice and the representative data are shown.

Figure 5

Treg are unlikely to be involved in inhibition of CD8⁺ T cell activation in Flt3L-treated mice after PbA infection.

(A) Untreated (3 mice/group) and Flt3L-treated (4 mice/group) mice were uninfected or infected with PbA. After 5 days, splenocytes were stained for CD4, CD25 and Foxp3. The ratio of Tregs (CD4⁺CD25⁺Foxp3⁺) within CD4⁺ T cells was expressed as mean \pm SD in each group. * $p < 0.05$, Mann-Whitney test.

(B) Five days after infection, CD4⁺ splenic T cells were stimulated with plate-bound anti-TCR mAb, and their IFN- γ production was determined by ELISA in quadruplicate assay. The experiments were repeated twice and the representative data are shown.

Figure 6.

The role of granulocytes on Flt3L-mediated effects in PbA infection.

Mice were untreated or treated with Flt3L 7 days prior to infection and received anti-Gr1 mAb (50 μ g) *i.p.* 2 days prior to infection. The levels of parasitemia 5 days after infection are expressed as mean \pm SD (left). N.S.= not significant, Mann-Whitney test. Survival of infected mice was monitored daily (right). * $p < 0.05$, logrank test. The experiments were repeated twice and the representative data are shown.

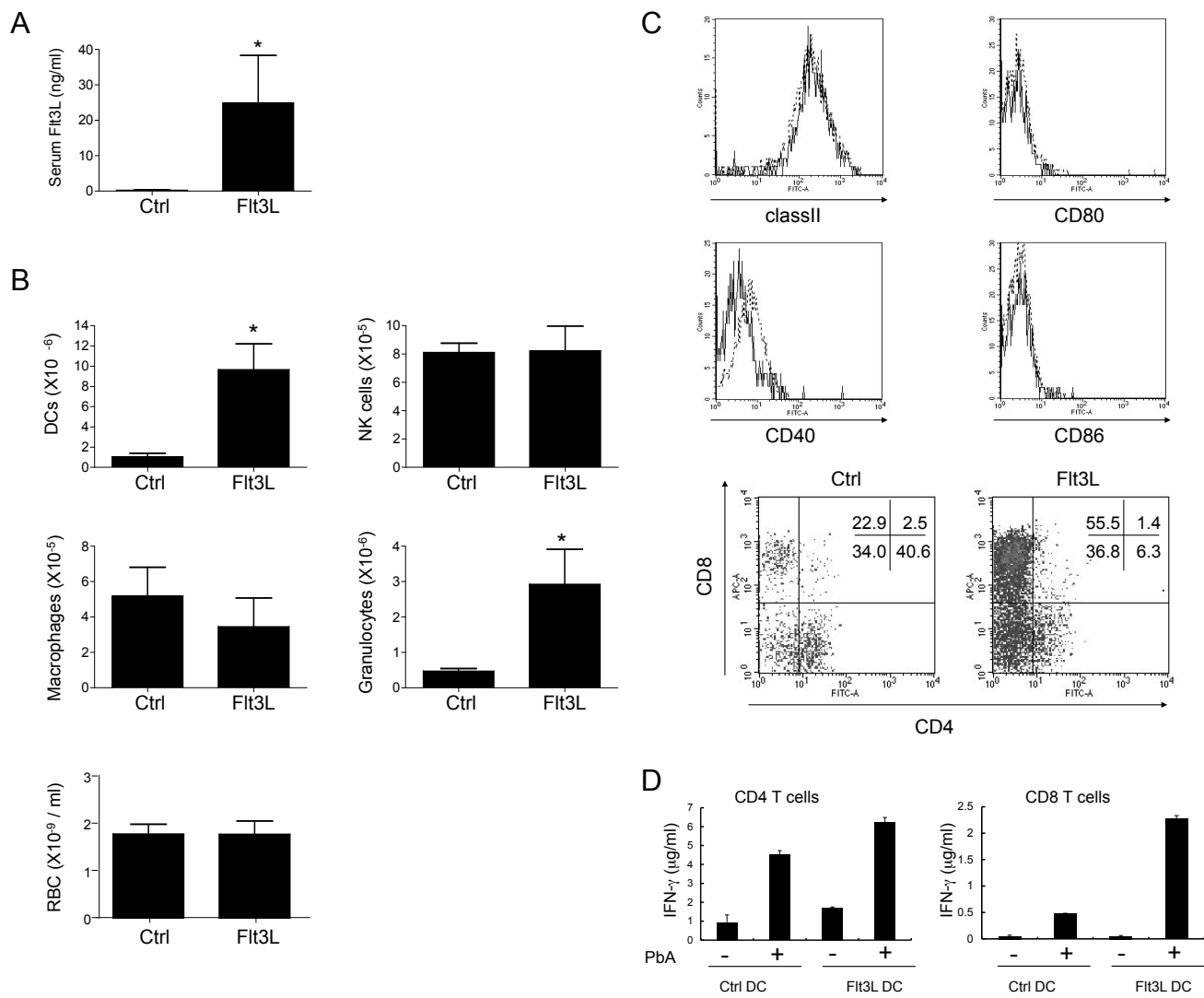


Figure1

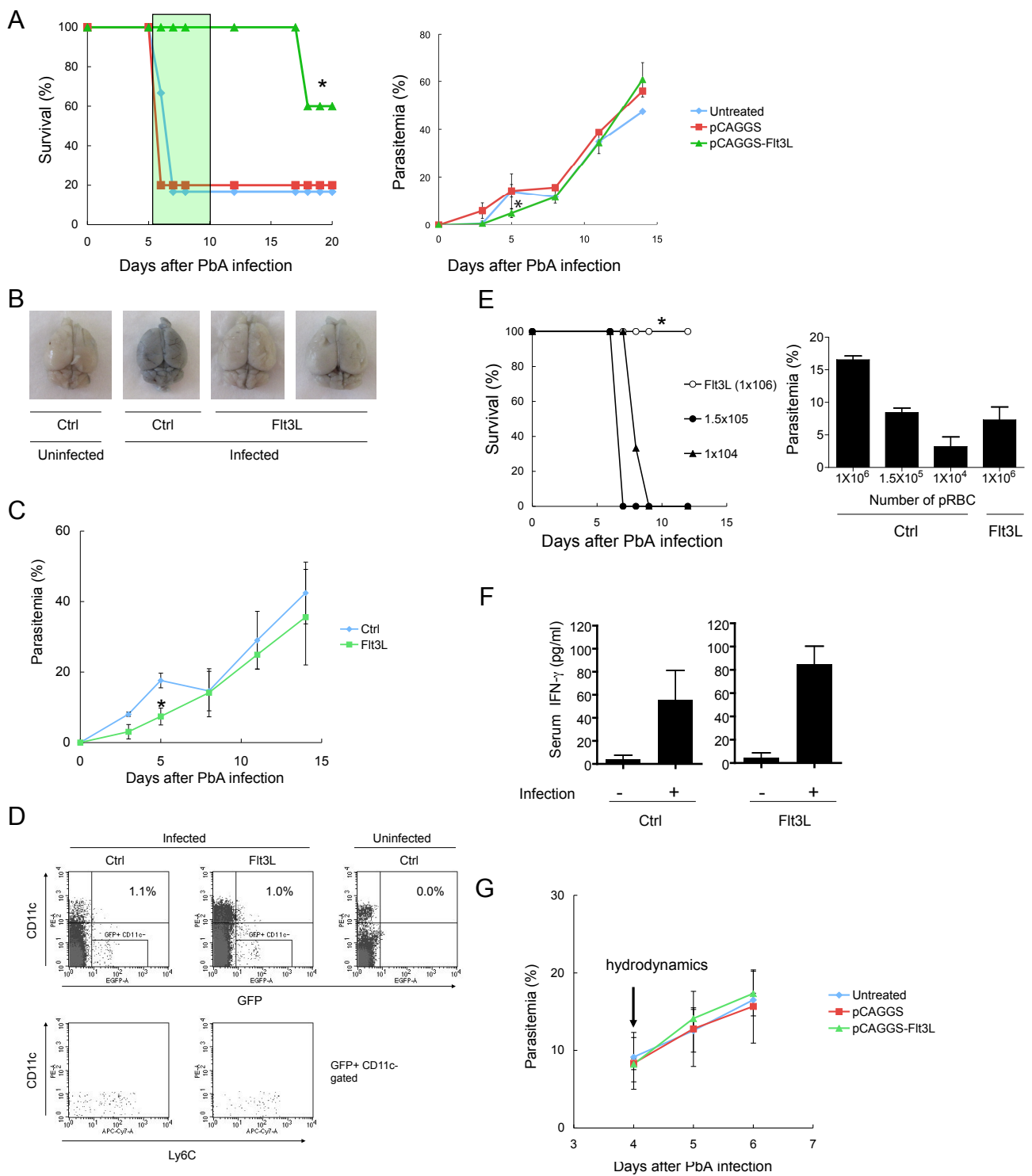
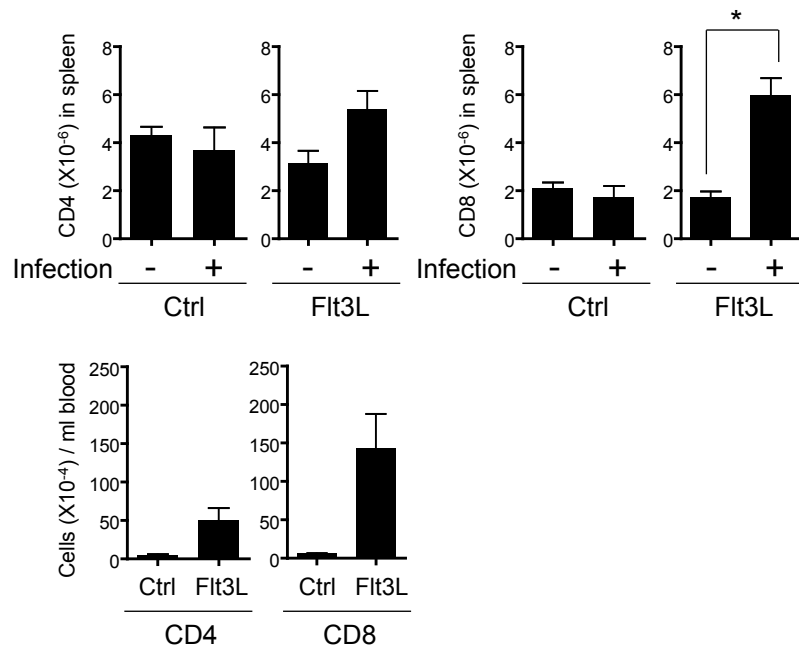


Figure2

A



B

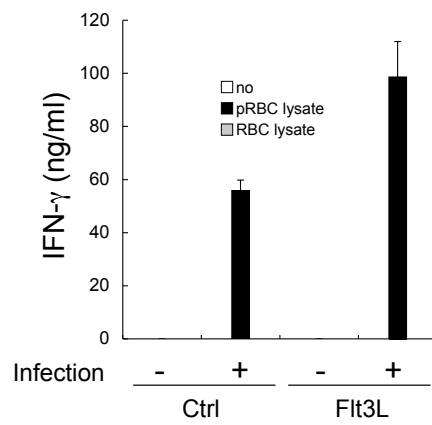


Figure3

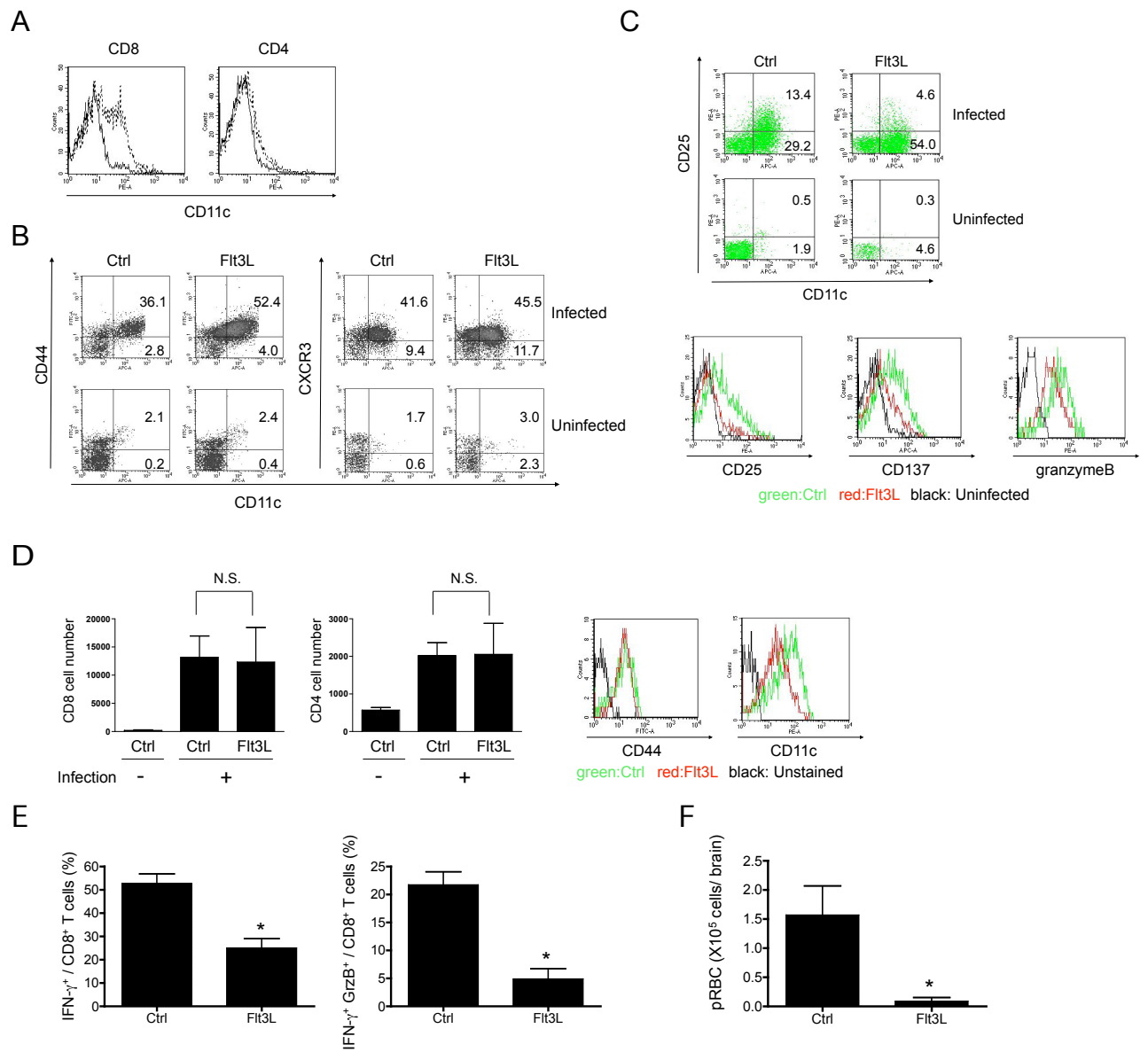
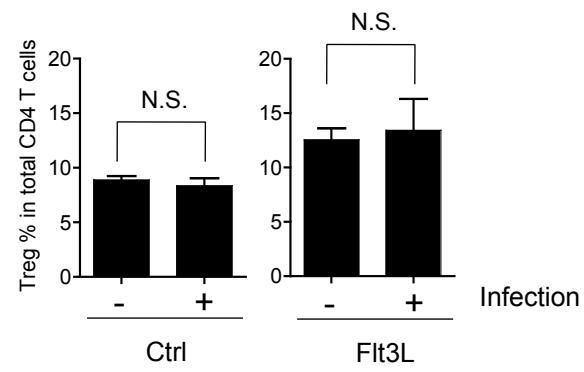


Figure4

A



B

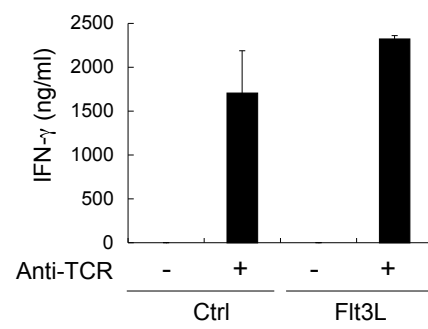


Figure5

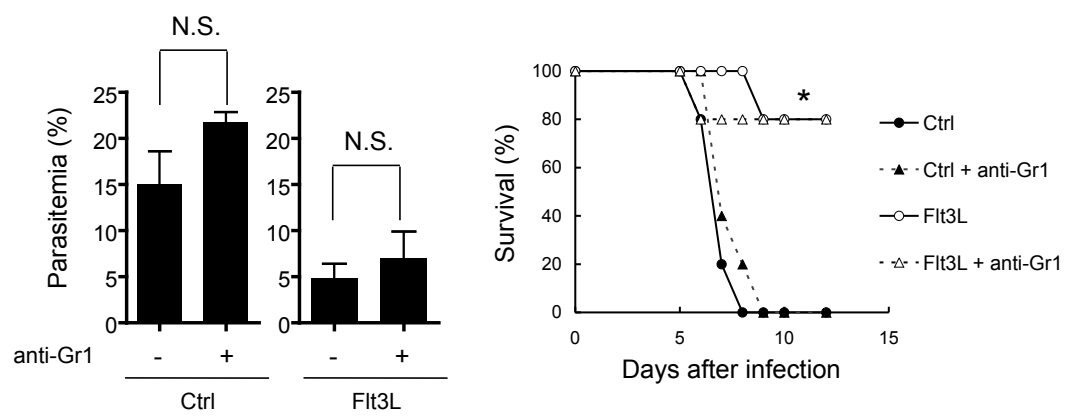
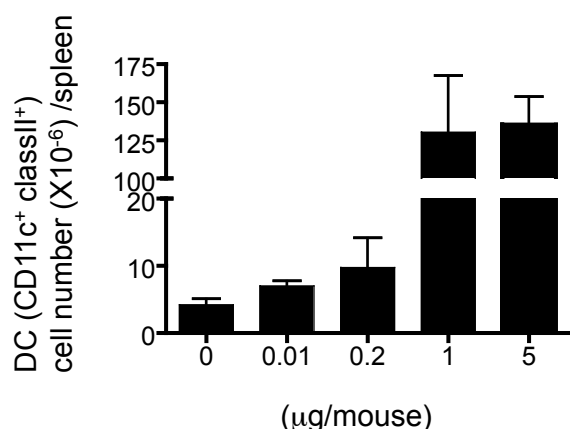
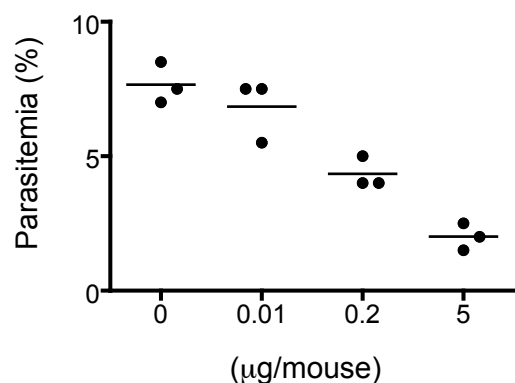


Figure6

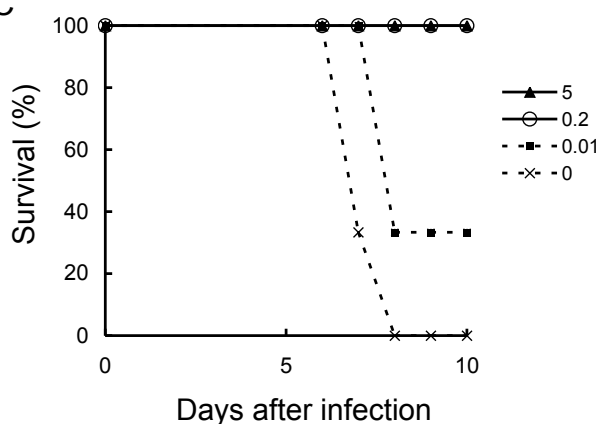
A



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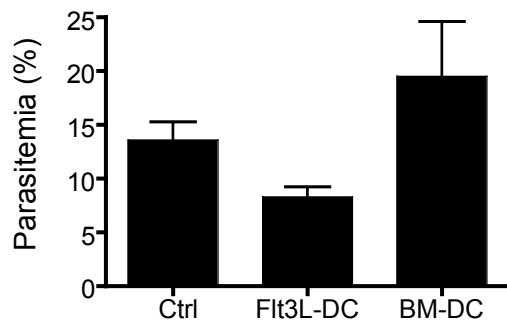


C



Supplemental Figure 1:

The correlation between the number of DCs and anti-malaria effect in mice treated with Flt3L. (A) A different doses (0-5 µg/mouse) of the plasmid encoding Flt3L were injected into C57BL/6 mice (3 mice/group) by the hydrodynamics method. After seven days, spleens were collected, and analyzed using flow cytometry. The number of DCs was calculated by multiplying the total spleen cell number with the ratio of CD11c⁺MHC class II⁺ cells. (B, C) The indicated doses of the plasmid were introduced into C57BL/6 mice by hydrodynamics method. After seven days, the mice were infected with pRBCs (2X10⁵ cells) by *i.p.* inoculation. The levels of parasitemia (%) were determined five days after infection (B), and survival of the mice was monitored (C).



Supplemental Figure 2:

Inhibition of the parasitemia by inoculation with Flt3L-induced DCs.

Flt3L-DCs were purified from C57BL/6 mice that were inoculated with Flt3L-plasmid (5µg/mouse) by hydrodynamics method. Seven days later, splenic DCs were purified by cell sorting using FACS Aria as described in Materials and Methods. BM-DCs were obtained using standard method by culturing bone marrow cells in media containing GM-CSF (1). C57BL/6 mice were inoculated with (Flt3L-DCs, BM-DCs, 3 mice/group) and without (Ctrl, 5 mice) these DCs (2×10^6 cells) *i.p.*, and were infected with PbA by *i.p.* inoculation with pRBCs (1×10^6 cells). Five days after infection, the levels of parasitemia were determined.

Reference:

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