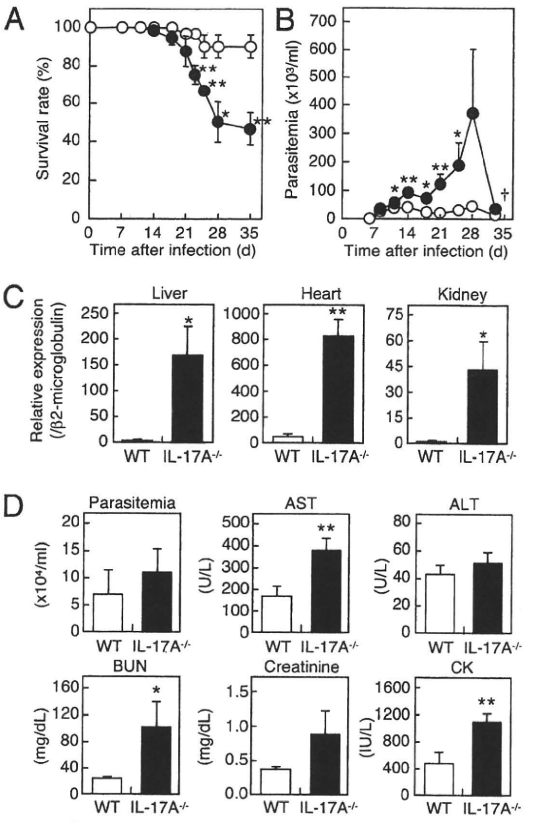

FIGURE 2. Increased mortality accompanied by sustained severe parasitemia and aggravated multiple organ failure in *IL-17A*$^{-/-}$ mice. *A*, WT (○) and *IL-17A*$^{-/-}$ (●) mice were infected i.p. with 2×10^3 blood trypomastigotes of *T. cruzi*. Mortality was monitored at the indicated days postinfection. Data are mean ± SEM from three independent experiments (n = 10–20 each). *B*, Parasitemia was monitored at the indicated days postinfection as in *A*. Data are mean ± SEM (n = 18–20). Experiments were repeated at least three times with similar results. *C*, Seventeen days postinfection, parasitism in liver, heart, and kidney was measured with a *T. cruzi* 195-bp-repeat DNA specific-PCR, as described in *Materials and Methods*. Data are mean ± SEM (n = 4). Experiments were repeated at least two times with similar results. *D*, Serum was prepared from WT and *IL-17A*$^{-/-}$ mice on day 21 of infection and analyzed for parasitemia and serologic marker concentrations, as described in *Materials and Methods*. Experiments were repeated at least four times with similar results. Data are mean ± SEM (n = 4). $*p < 0.05$; $**p < 0.01$ versus WT samples; †, Mean parasitemia in survivors at 33 d postinfection. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase.

survived >3 mo postinfection (data not shown). This may indicate that some factors, such as IL-17F, may compensate for IL-17A during the infection. Nonetheless, it was shown that *IL-17A*$^{-/-}$ mice were essentially more sensitive to *T. cruzi* infection than WT mice, and lethal expansion of the parasites occurred in more than half of the infected *IL-17A*$^{-/-}$ mice (Fig. 2*A*, 2*B*). These results indicated that IL-17A plays an important role in the successful resolution of *T. cruzi* infection.

In the acute phase of infection, *T. cruzi* replicates and infects various target organs, which leads to tissue injury. Therefore, we assessed the tissue parasitism and the degree of tissue damage by measuring serum concentrations, including aspartate aminotransferase and alanine aminotransferase for liver injury, blood urea nitrogen and creatinine for renal damage, and creatine kinase for heart failure. As shown in Fig. 2*C*, *IL-17A*$^{-/-}$ mice showed significantly greater parasitism in liver, heart, and kidney compared with WT mice. Furthermore, all markers measured were higher in *IL-17A*$^{-/-}$ mice compared with WT mice (Fig. 2*D*). These data suggested that *T. cruzi* infection leads to severe multiple organ failure resulting from physical damage by the parasites in *IL-17A*$^{-/-}$ mice, which was assumed to be a reason for the increased mortality.

Equivalent cellular infiltration in the liver of WT and IL-17A$^{-/-}$ *mice*

We then explored mechanisms of IL-17A resistance to *T. cruzi* infection. Liver is a secondary infection site of *T. cruzi*, and it is well known that IL-17A induces the recruitment of immune-related cells to infected sites. We initially assumed that the cellular accumulation in the infected liver was impaired in *IL-17A*$^{-/-}$ mice. However, cellular infiltration was unexpectedly equivalent between WT and *IL-17A*$^{-/-}$ mice (Fig. 3*A*). Also, microscopically discernible differences between WT and *IL-17A*$^{-/-}$ mice were not observed in the heart, which is another target organ of the infection (data not shown). As shown in Fig. 3*B*, the ratio of CD4$^+$ and CD8$^+$ T cells, NK and NKT cells, macrophages and B cells, and neutrophils in the infected liver of *IL-17A*$^{-/-}$ mice was similar to that of WT mice 21 d after the infection. Similar results were obtained ~14 d after the infection (data not shown). Total numbers of liver-infiltrated MNCs were also similar in WT and *IL-17A*$^{-/-}$ mice on days 14 and 21 (data not shown). To address whether IL-17A deficiency affected cell migration at very early phases of infection, cellular infiltration into the peritoneal

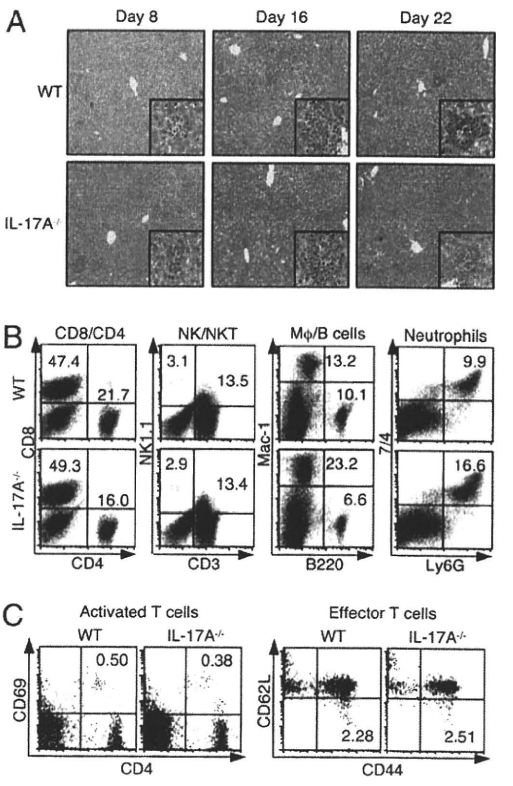

FIGURE 3. Equivalent cellular infiltration in the liver of WT and *IL-17A*$^{-/-}$ mice. *A*, Liver segments from WT and *IL-17A*$^{-/-}$ mice were stained with H&E for histological examination. Original magnification, ×200 (insets ×640). Experiments were repeated two times with similar results. *B* and *C*, Liver MNCs prepared from WT (*B, upper row*) and *IL-17A*$^{-/-}$ (*B, lower row*) mice on day 21 (*B*) or day 16 (*C*) of infection were stained for indicated surface markers and analyzed with FACS, as described in *Materials and Methods*. Numbers shown are the percentages of cells contained in each gated lineage. Experiments were repeated two times.

cavity was examined at 3 d after i.p. injection of *T. cruzi*. Despite our initial assumption, there was no significant difference between WT and *IL-17A*$^{-/-}$ mice with regard to the number of cells infiltrating the peritoneal cavity or the percentage of innate immune cells, such as NK/NKT (CD3$^-$NK1.1$^+$ or CD3$^+$NK1.1$^+$) cells, γδT (CD3$^+$γδTCR$^+$) cells, macrophages (CD11b$^+$Ly6G$^-$), and neutrophils (CD11b^{int}Ly6G$^+$) (Supplemental Fig. 1). Furthermore, expression levels of activation markers, such as CD69, CD44, and CD62L, on CD4$^+$ T cells in the infected liver were similar between WT and *IL-17A*$^{-/-}$ mice on day 16 (Fig. 3*C*).

In vitro assay of capacity of Th17 cells and IL-17A for macrophage and neutrophil activation

Next, we performed an in vitro assay to assess the helper function of IL-17A–producing CD4$^+$ T (Th17) cells to activate infected macrophages directly (Fig. 4*A*). Peritoneal exudate macrophages from thioglycolate-treated WT mice were infected with *T. cruzi* in vitro and then cocultured with spleen CD4$^+$ cells isolated from infected WT or *IL-17A*$^{-/-}$ mice. After 18 h of culture, the production of IL-6, TNF-α, and NO, which are mainly produced by activated macrophages, was enhanced by coculture with both CD4$^+$ cells compared with that in the absence of CD4$^+$ cells. However, there were no significant differences in the quantity of macrophage-derived factors between coculture with WT and *IL-17A*$^{-/-}$ CD4$^+$ cells (Fig. 4*A*). Therefore, a CD4$^+$ Th cell lineage other than Th17 was able to activate macrophages normally, even in the absence of IL-17A production. Furthermore, we examined the direct effect of IL-17A on macrophage activation; however, treatment with IL-17A did not enhance NO production by macrophages, even in the presence of costimulation with LPS and IFN-γ (Fig. 4*B*). In the thioglycolate-induced macrophages, STA could not induce NO production, whereas LPS and IFN-γ had a strong effect.

In addition, rIL-17A could not directly induce activation of neutrophils, as measured by upregulation of ROS production (Fig. 4*C*) and mRNA expression of MPO and 5-LOX (Fig. 4*D*). Interestingly, STA induced ROS production in the neutrophils more strongly than did 100 ng/ml LPS (Fig. 4*C*), and it was assumed that the *T. cruzi* infection immediately activated locally resident neutrophils or those recruited to the infection site. These results suggested that IL-17A produced from Th17 and other lineages in vivo is required for proper defense against *T. cruzi* by mechanisms other than the induction of cellular migration and direct activation of macrophages and neutrophils.

Cytokine production was impaired in IL-17A$^{-/-}$ *mice*

We then addressed the production of inflammatory cytokines, another important mechanism by which IL-17A plays a role during infection. As shown in Fig. 5*A*, cells from spleen, MLN, and liver of WT mice at 14 d postinfection produced substantial levels of IFN-γ and TNF-α in response to *T. cruzi* infection, which then decreased to 1/7 to 1/50 on day 21 (compare Fig. 5*A*, 5*B*), due to successful control of infection. At both time points, cytokine production by *IL-17A*$^{-/-}$ cells was impaired compared with that by WT cells derived from MLN and liver (Fig. 5*A*, 5*B*), demonstrating the influence of IL-17A on the production of inflammatory cytokines during *T. cruzi* infection. Of note, the high susceptibility of *IL-17A*$^{-/-}$ mice to infection was first apparent around day 14 and became more evident on day 21 and thereafter, as shown by greater mortality and more severe parasitemia (Fig. 2*A*, 2*B*). Similar results were obtained for mRNA expression in liver MNCs and MLN cells (i.e., expression of IFN-γ, TNF-α, and IL-6 were lower in *IL-17A*$^{-/-}$ mice than WT mice during *T. cruzi* infection) (Fig. 5*C*, data not shown). IL-4 expression was not affected by IL-17A deficiency. Interestingly, the impaired cytokine production was most evident in MLN cells and liver MNCs compared with splenocytes (Fig. 2*A*, 2*B*). These results indicate the differential requirement of IL-17A for cytokine production; IL-17A is required more in local sites, including draining lymph nodes and liver, rather than systemically, as shown for splenocytes. This might reflect observations

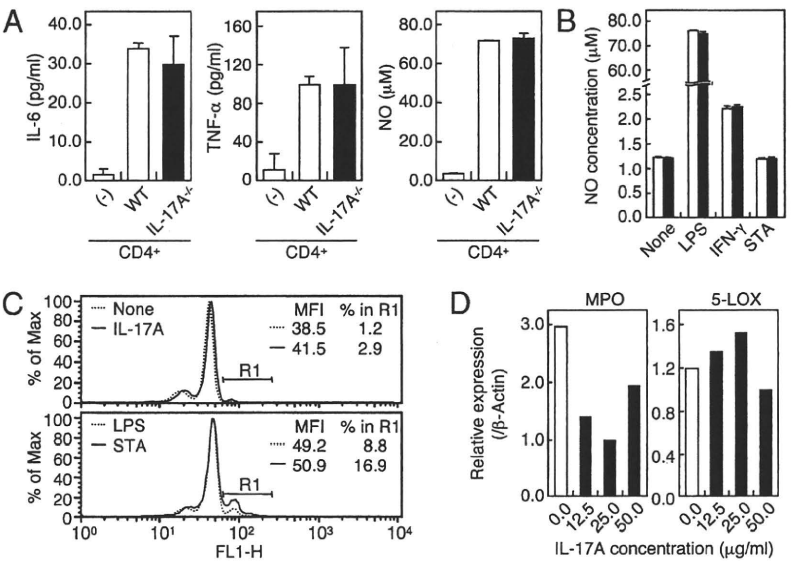

FIGURE 4. In vitro assays on function of Th17 and IL-17A in immune activation. *A*, Thioglycolate-induced macrophages (5 × 10^5 cells/well) infected with 2.5 × 10^4 *T. cruzi* trypomastigotes were cocultured for 18 h with spleen CD4$^+$ cells (1 × 10^5 cells/well) isolated from WT or *IL-17A*$^{-/-}$ mice at 14 d after *T. cruzi* infection. (-), Culture of infected peritoneal exudate macrophages without CD4$^+$ cells. After the culture, cytokines and NO levels in culture supernatant were measured. Data are mean ± SEM (n = 5–7). *B*, Thioglycolate-induced macrophages were treated with indicated stimuli for 18 h with or without 25 ng/ml IL-17A and then measured for NO secretion. Data are mean ± SEM (n = 6). *C*, Thioglycolate-induced neutrophils were incubated with none, 25 ng/ml IL-17A, 100 ng/ml LPS, or 1 × 10^6 STA. Two hours after the stimulation, ROS production was detected as described in *Materials and Methods*. Experiments were repeated two times with similar results. *D*, Thioglycolate-induced neutrophils were stimulated by the indicated concentrations of IL-17A for 18 h, and the mRNA expression of MPO and 5-LOX was measured. Experiments were repeated at least two times with similar results.

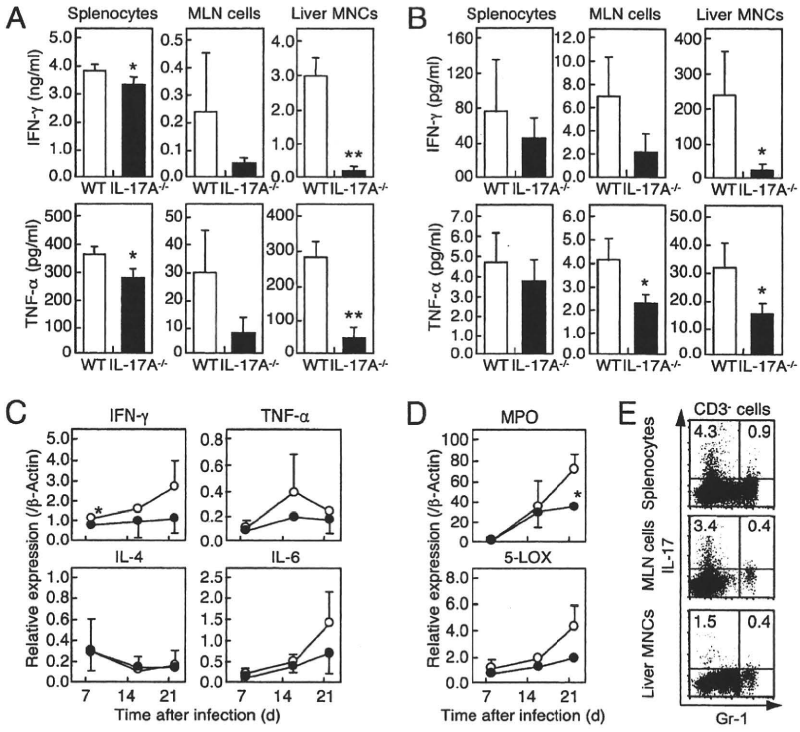

FIGURE 5. Attenuated cytokine production in *IL-17A*$^{-/-}$ mice during *T. cruzi* infection. Splenocytes, MLN cells, and liver MNCs prepared from WT and *IL-17A*$^{-/-}$ mice on day 14 (*A*) and day 21 (*B*) of infection were cultured for 64 h and analyzed for cytokine production, as described in *Materials and Methods*. Experiments were repeated at least three times with similar results. Data are mean ± SEM (n = 4). *C* and *D*, Liver MNCs were isolated from WT (○) and *IL-17A*$^{-/-}$ (●) mice at the indicated days postinfection and analyzed for mRNA expression by RT-PCR, as described in *Materials and Methods*. Experiments were repeated at least two times with similar results. Data are mean ± SEM (n = 2–4). *E*, Splenocytes (*top panel*), MLN cells (*middle panel*), and liver MNCs (*bottom panel*) collected from WT mice on day 21 of infection were stained for indicated surface markers and intracellular IL-17A. Numbers shown are the percentages of cells contained in each gated lineage. $*p < 0.05$; $**p < 0.01$ versus WT samples.

that producers of IL-17A, including NKT and γδT cells, exist and act in local sites, such as liver and intestinal tract. It is also known that marked pathologies of most autoimmune diseases caused by IL-17A are localized inflammation.

Furthermore, mRNA expression of neutrophilic enzymes, such as MPO and 5-LOX, was lower in *IL-17A*$^{-/-}$ mice compared with WT mice (Fig. 5*D*). Because MPO and 5-LOX participate in the biosynthesis of H_2O_2 and leukotrienes (34, 35), respectively, neutrophils in the infected *IL-17A*$^{-/-}$ mice are assumed to have weak trypanocidal activity compared with the WT neutrophils.

Neutrophils may also participate in the control of antiparasite immune responses as producers of IL-17A. As shown in Fig. 5*E*, CD3$^-$Gr-1$^+$ cells from spleen, MLN, and liver produced IL-17A in response to *T. cruzi* infection. Production of IL-17A by neutrophils was also observed in *Leishmania major* infection, as reported by Lopez Kostka et al. (36). However, the percentages of IL-17A$^+$ neutrophils were low, and production levels, as shown by intracellular cytokine staining, were also low compared with other types of cells (Fig. 1*B*). The contribution of neutrophil-derived IL-17A to defense against *T. cruzi* has not been examined in detail.

In conclusion, cytokine production and activation of lymphocytes, macrophages, and neutrophil were markedly impaired in the infected *IL-17A*$^{-/-}$ mice, whereas cellular migration was not affected by IL-17A deficiency. The attenuated immune activation in *IL-17A*$^{-/-}$ mice resulted in propagation of *T. cruzi* infection.

Discussion

Although IL-17A is known to participate in the induction of inflammation during infection of an intracellular protozoan parasite, *Toxoplasma gondii* (37), the pathogenic or protective roles of IL-17A in infection by other intracellular protozoan parasites are not well understood. In this study, we revealed that the production of IL-17A was induced against *T. cruzi* infection (Fig. 1*A*). As shown in Fig. 1*B*, although IL-17A was produced by a small population of CD4$^+$ T (Th17 cells) and CD8$^+$ T cells, its production was more potently induced in substantial numbers of NKT and γδT cells in response to *T. cruzi* infection (5–7). Some reports indicated that infection by *Leishmania amazonensis* and *L. braziliensis* induced the expression of IL-17A (38, 39), but there was no evidence for a relationship between IL-17A production and host protection in the protozoa infection. In this regard, we demonstrated, using *IL-17A*$^{-/-}$ mice, that IL-17A plays an important role in the successful resolution of *T. cruzi* infection (i.e., *T. cruzi*-infected *IL-17A*$^{-/-}$ mice showed prolonged, more severe parasitemia and exacerbated mortality compared with WT mice) (Fig. 2*A*, 2*B*).

Interestingly, the production of IFN-γ, which is a critical cytokine for achieving antitrypanosoma immunity (18, 19), was lower in *IL-17A*$^{-/-}$ mice during *T. cruzi* infection compared with WT mice (Fig. 5*A–C*). Therefore, the delay in parasite expulsion in *IL-17A*$^{-/-}$ mice might result from the weak IFN-γ responses against *T. cruzi* infection. Similar defects in IFN-γ production were observed in *IL-17A*$^{-/-}$ mice during *Mycobacterium* infection (30, 40). Furthermore, reduction of serum IL-17A by anti-IL-23p19 treatment also resulted in the attenuated production of IFN-γ, IL-6, and TNF-α in CNS during autoimmune encephalomyelitis (41). Unfortunately, the direct mechanisms for the decrease in IFN-γ production in *IL-17A*$^{-/-}$ mice have not been clarified. It was reported that TNF-α induces maturation of dendritic cells and the maturated dendritic cells drive IFN-γ production of CD4$^+$ T cells (42). Therefore, attenuated production of IFN-γ by IL-17A deficiency, as shown in Fig. 5, might result from the decrease in TNF-α production. Furthermore, activated production of TNF-α observed in steatohepatitis induced by fat/alcohol feeding with LPS injection was associated with increased expression of IFN-γ (43).

In addition to IFN-γ, IL-6 and TNF-α are well-known cytokines induced by IL-17A stimulation (8, 9). Production of these cytokines during *T. cruzi* infection was decreased in *IL-17A*$^{-/-}$ mice compared with WT mice (Fig. 5*A–C*). IL-6 and TNF-α were required for the successful resolution of *T. cruzi* infection (44–47). For example, IL-6 induces B cell terminal differentiation into plasma cells during *T. cruzi* infection, and *IL-6*$^{-/-}$ mice were

more susceptible to the infection (44, 45). TNF-α is known to act synergistically with IFN-γ on macrophages to augment killing of *T. cruzi* (22, 23), and a defect in TNF-α signaling by introduction of TNFR-Fc transgene or gene targeting of the receptor brought about increased susceptibility to *T. cruzi* infection (46, 47). Therefore, it is likely that poor production of IL-6 and TNF-α in *T. cruzi*-infected *IL-17A*$^{-/-}$ mice (Fig. 5*A–C*) was a factor contributing to the deviant expansion of the parasites and increase in mortality (Fig. 2*A*, 2*B*).

IL-17A induced during infection of bacteria and fungi and a protozoan parasite *Toxoplasma gondii* mobilizes neutrophils for elimination of the pathogens (28, 29, 31, 48). However, in the current study, deficiency in IL-17A did not affect the number of immune-related cells migrating into infected tissues (Fig. 3*A*, 3*B*). Therefore, IL-17A was not essential for the recruitment of neutrophils and other immune cells in *T. cruzi* infection. Presumably, cytokines, such as IL-17F, and other inflammatory cytokines produced during infection compensated for the lack of IL-17 for cell migration. Nevertheless, because neutrophilic enzyme activities were lower in *T. cruzi*-infected *IL-17A*$^{-/-}$ mice (Fig. 5*D*), IL-17A might be important for proper neutrophil activation required for killing of *T. cruzi* (34, 49). However, the activation of neutrophils by IL-17A seemed to be achieved indirectly, because IL-17A itself did not enhance the neutrophilic enzyme expression (Fig. 4*D*). Interestingly, neutrophils were activated for the enhanced ROS production by stimulation with *T. cruzi* Ags (Fig. 4*C*). Therefore, it was supposed that neutrophils participate in the protection against *T. cruzi* infection as effectors in host immune systems.

In conclusion, we demonstrated that IL-17A is induced in response to *T. cruzi* infection and results in efficient activation of the immune system critical for the killing of infected *T. cruzi*, mainly through sufficient production of INF-γ and other inflammatory cytokines. IL-17A is required for the elimination of bacteria, fungi, and *T. cruzi*; it controls cytokine production by T cells and macrophages, as well as neutrophil activation.

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Disclosures

The authors have no financial conflicts of interest.

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Development of experimental cerebral malaria is independent of IL-23 and IL-17

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ABSTRACT

Cerebral malaria (CM) is the most severe complication of *Plasmodium* infection. Although inappropriate immune responses to *Plasmodium falciparum* are reported as the major causes of CM, the precise mechanisms for development remain unclear. IL-23 and IL-17 have critical roles in the onset of autoimmunity and inflammatory diseases triggered by microbial infections. Thus, we investigated the influence of IL-23 and IL-17 on experimental CM (ECM) using *Plasmodium berghei* ANKA infection of C57BL/6 mice. Both IL-23 deficient mice and wild-type (WT) mice developed ECM. IL-17 deficient mice also developed ECM, while IL-17 producing cells other than $CD4^+$ T cells (Th17) were increased in WT mice that developed ECM. In conclusion, this study showed that IL-23 and IL-17 are not involved in ECM development.

1. Introduction

Malaria is a major life-threatening parasitic disease. Each year, 500 million cases and 2 million deaths are reported and 40% of the world's population is at risk of malaria infection [1]. Cerebral malaria (CM) associated with *Plasmodium falciparum* infection is responsible for almost all malaria deaths and is characterized by impaired consciousness/coma and generalized convulsions [2,3]. Approximately 1% of *P. falciparum* infected patients develop CM. The majority of these cases occur in young children in sub-Saharan Africa, of which, 10–20% are fatal and the remaining survivors acquire permanent neurological damage [4,5]. Histologically, multifocal capillary obstruction with parasitized red blood cells (RBC) and leukocytes is observed in brain tissue from patients that die of CM [6] and suggests *P. falciparum* infection may trigger vascular and immune system dysfunction. However, the precise mechanisms causing CM are not fully understood.

After the discovery of $CD4^+$ T cells producing IL-17 (Th17), a new subset of effector T helper cells, the immune responses involving IL-23 and IL-17 have been investigated in detail. IL-23 is a heterodimeric cytokine comprised of a p40 subunit and a unique p19 subunit [7]. Recently, it was revealed that IL-23 is critical for the development of Th17 [8] and for immune cell activation [9,10]. IL-17 is a proinflammatory cytokine secreted by immune cells and mediates granulopoiesis, infiltration of neutrophils and recruitment of T cells into peripheral tissues via the induction of chemokine and cytokine expression [11,12].

Many studies using *IL-23 p19*$^{-/-}$ mice (P19KO) and *IL-17*$^{-/-}$ mice (17KO) suggest that IL-23 and IL-17 play a critical role in the onset of autoimmune diseases, such as experimental autoimmune encephalitis, collagen induced arthritis and inflammatory bowel diseases [7,13–15]. Furthermore, as both cytokines contribute to the development of arthritis caused by *Borrelia burgdorferi* infection [16] and brain damage caused by *Toxoplasma gondii* infection [17], IL-23 and IL-17 may be associated with the immunopathology triggered by microorganism infection.

Infection of susceptible mouse strains with *Plasmodium berghei* ANKA (PbA) allows for the development of experimental CM (ECM) that shares some characteristics with CM. Immune cells, including T cells, antigen-presenting cells (APC), NK cells and neutrophils, play critical roles in the development of ECM [18–21]. During infection, these cells activate and cooperate with each other to allow T cell migration into brain. This results in blood–brain barrier (BBB) disruption and is a key pathological feature of ECM. IL-23 is

Abbreviations: IL-23, Interleukin-23; IL-17, Interleukin-17; RBC, red blood cell; Th, T helper cells; NK cells, natural killer cells; Treg, regulatory T cell.

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strongly associated with activation of APC and NK cells and also with development of Th17 that is capable BBB disruption [8–10]. IL-17 is involved in the activation and recruitment of neutrophils [11] and upregulation of CXCL 9, 10, and 11, which are ligands of CXCR3 essential for the migration of $CD8^+$ T cells into brain [12,22]. Given these findings, it is possible that IL-23 and/or IL-17 may contribute to development of ECM.

We therefore examined the role of IL-23 and IL-17 in ECM development using P19KO and 17KO mice. Our results demonstrated that P19KO mice developed ECM similarly to wild-type (WT) mice. 17KO mice also developed ECM while IL-17-producing cells other than $CD4^+$ T cells increased in WT mice during ECM. Thus, our results conclude that IL-23 and IL-17 may not be involved in ECM development.

2. Materials and methods

2.1. Mice and parasites

C57BL/6 mice were purchased from Kyudo (Tosu, Japan). Age and sex-matched groups of WT mice, P19KO mice and 17KO mice were used for experiments. All experiments that involved mice were reviewed by the Committee for Ethics on Animal Experiments in the Faculty of Medicine and were conducted under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government. Blood-stage parasites of *P. berghei* ANKA (PbA) were a generous gift from Dr. M. Torii (Ehime University), obtained after fresh passage through a donor mouse, 2–3 days after inoculation with frozen stock. Mice were infected with 50,000 parasitized RBC (pRBC) via intraperitoneal injection.

2.2. Determination of parasitemia

Blood samples were collected from the tail vein of experimental mice at the time indicated. Thin blood films were prepared and fixed with methanol before being stained with Giemsa solution (Sigma–Aldrich, St. Louis, MO, USA). Parasitemia was determined by counting the percentage of infected RBC (pRBC) using light microscopy.

2.3. Preparation of brain-sequestered leukocytes (BSL) and spleen cells

BSL were prepared according to the previously described methods [23,24]. Briefly, sacrificed mice were intracardially perfused with PBS to remove both circulating, nonadherent RBC and leukocytes from the brain. The brain was then removed and crushed in RPMI1640 supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin, 20 mM HEPES, 2 mM L-glutamine, 100 mM 2-mercaptoethanol and 10% inactivated fetal bovine serum (complete medium). The tissue suspension was centrifuged at 400 *g* for 5 min and the pellet was resuspended with HEPES buffer, supplemented with 0.05% collagenase (Roche Applied Science, Indianapolis, IN, USA) and 2 U of DNase (Sigma–Aldrich, St. Louis, MO, USA)/ml. The mixture was stirred at room temperature for 60 min, then passed through a sterile gauze and centrifuged at 80 *g* for 30 s to remove debris. The supernatant was deposited on a 30% Percoll (Sigma–Aldrich, St. Louis, MO, USA) to remove brain cells and centrifuged at 1400 *g* for 10 min. The pellet was resuspended with medium to form single-cell suspensions. Residual RBC in single-cell suspensions prepared from the brain and spleen were lysed with NH_4Cl. Cells were then washed twice with fresh medium before being used for experiments. $CD4^+$ cells were purified with positive selection using a MACS cell separation system (Miltenyi Biotech) according to the manufacturer's protocols. Separated cell purity was generally >95%.

2.4. Real-time RT-PCR

Total RNA was extracted from BSL, spleen cells and $CD4^+$ cells before being reverse-transcribed to cDNA. mRNA that encoded genes of interest was quantified from cDNA by evaluating SYBR Green dye incorporation (Takara, Tokyo, Japan) using a real-time PCR system GeneAmp7000 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR was performed according to the manufacturer's protocols. Expression levels of target genes were determined as the difference of Ct between the β-actin-encoding gene and target gene using the following formula: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$. PCR primers used were as follows: for IL-23p19, 5′-TGGCTGTG CCTAGGAGTAGCA-3′ and 5′-TTCATCCTCTTCTTCTCTTAGTAGATTC ATA-3′; for IL-17, 5′-TCATCTGTGTCTCTGATGCTGTTG-3′ and 5′-TCG CTGCCTTCACTGT-3′; for RORγt, 5′-AGCAGTGTAATGTGGCCTAC-3′ and 5′- GCACTTCTGCATGTAGACTG-3′; and for β-actin, 5′-TGGAAT CCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAG TCCG-3′.

2.5. Flow cytometry

BSL and spleen cells were stained with a combination of fluorochrome-labeled antibodies. For intracellular staining, cells were stimulated with 50 ng/ml PMA and 500 ng/ml calcium ionophore in the presence of Golgi plug (BD Bioscience, San Jose, CA, USA), in complete medium at 37 °C for 4 h. Cells were then incubated with anti-CD16/32 (Fc-block) and stained with FITC-anti-CD4 (eBioscience, San Diego, CA, USA) followed by fixation/permeabilization with BD cytofix/cytoperm (BD Bioscience, San Jose, CA, USA) according to manufacturer's protocols. Cells were stained with PE-anti-IL-17 or PE-anti-IFN-γ (BD Bioscience, San Jose, CA, USA) and analyzed using a FACS Caliber cytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed using CellQuest Pro software (Becton Dickinson).

2.6. Histology

For histological analysis of cerebral pathology, brains from mice developing ECM were perfused with PBS and carefully removed and fixed in formaldehyde solution (4 v/v%). The 5-μm tissue sections were prepared and stained with hematoxylin and eosin (HE).

2.7. Statistical analysis

Statistical differences between experimental groups were evaluated using a two-tailed unpaired Student's *t*-test. Survival analysis was determined using Kaplan Meier survival analysis. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. IL-23 is not involved in the onset of ECM

C57BL/6 mice infected with PbA developed ECM that was characterized by neurological manifestations, such as ataxia, paralysis (monoplegia, hemiplegia and tetraplegia) and coma as early as 7 or 8 days after infection, and death occurred between 7 and 10 days after infection [25]. We first investigated the expression of IL-23 during ECM to assess the involvement of IL-23. BSL and spleen cells were collected and p19 mRNA expression was analyzed by quantitative RT-PCR. No significant difference in the expression of p19 mRNA was observed between mice developing ECM and uninfected control mice (Fig. 1A and B). Thus, PbA infection of mice did not induce IL-23 in both tissues sampled. Furthermore, to directly investigate the contribution of IL-23 to the

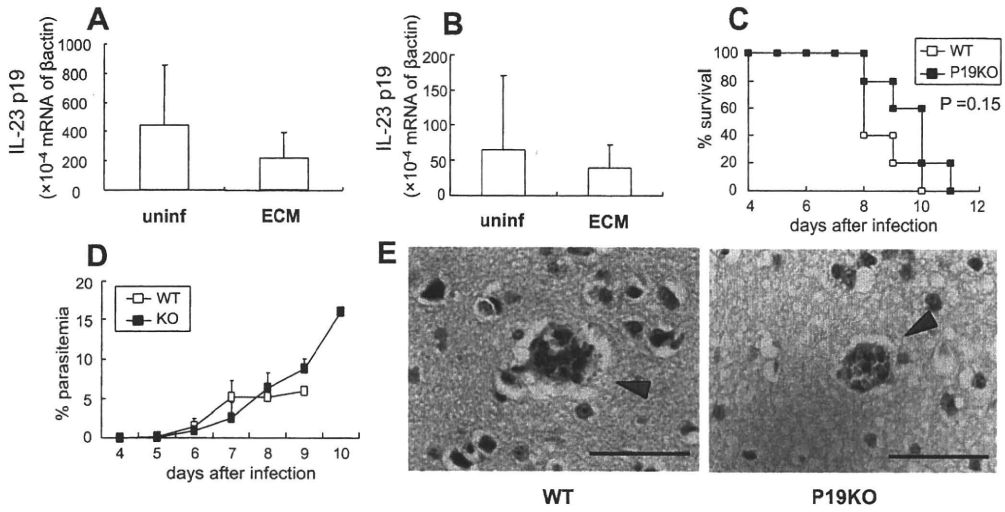

Fig. 1. ECM induced by PbA infection was independent of IL-23. mRNA encoding IL-23p19 in BSL (A) and spleen cells (B) isolated from mice developing ECM was quantified using RT-PCR. Values are relative to amounts of mRNA encoding β-actin. Data represent the mean ± SD of four mice. Survival rates (C) or parasitemia (D) of WT and P19KO mice infected with 50,000 PbA-pRBC were monitored. Each group is comprised of five mice. One representative of at least two repeated experiments is shown. Brains from WT (left panel) and P19KO mice (right panel) developing ECM were analyzed for histological examination (E). Representative histological sections from areas around the blood vessels of cerebrum with HE staining are shown (original magnification, ×1000). Arrowheads indicate accumulation of mononuclear cells and erythrocytes. Scale bars equal 50 μm.

development of ECM, P19KO mice were infected with PbA. Infected P19KO as well as WT mice showed severe neurological symptoms and died within 11 days when the parasitemia was lower than 10% (Fig. 1C, D), suggesting that P19KO mice developed ECM. To confirm development of ECM in these mutants, histological analyses of brains isolated from P19KO mice developing ECM were performed. Both P19KO and WT mice showed cerebral blood vessels sequestered with mononuclear cells and erythrocytes, a typical sing of ECM induced by PbA (Fig. 1E). No sequestered vessel was observed before infection (data not shown). These results clearly demonstrated that the development of ECM does not require IL-23.

3.2. IL-17 does not contribute to ECM development

To assess whether IL-17 contributes to ECM, we analyzed IL-17 producing cells using flow cytometry. IL-17 producing cells were increased in the brain and spleen of mice developing CM when compared to uninfected control mice (Fig. 2A and C). The emergence of Th17 during ECM was also investigated as an assessment of IL-17 production. Mice that developed ECM showed a higher frequency and absolute number of $CD4^+$ IFN-γ^+ Th1 cells (IFN-γ^+ $CD4^+$ cells (Th1)) in the brain and spleen when compared to uninfected control mice (Fig. 2B and C), which supported previous study [26]. By contrast, there were no differences in the frequency and absolute number of Th17 between mice that developed ECM and uninfected control mice (Fig. 2A and C). Furthermore, we analyzed the expression of mRNAs encoding IL-17 and a "master-regulator" transcription factor of Th17, RORγt, in $CD4^+$ cells purified from BSL and spleen cells. Mice that developed ECM showed lower mRNA expression levels compared to uninfected mice (Fig. 3A and B). These results demonstrate that IL-17-producing cells other than Th17 developed during ECM. Finally, to directly investigate the contribution of IL-17 to onset of ECM, 17KO mice were infected with PbA. Both infected 17KO mice and infected WT mice developed neurological symptoms and died (Fig. 4A) and produced no differences in parasitemia kinetics (Fig. 4B). Histological examination of brain sections revealed that 17KO as well as WT mice showed leukocyte-packed vessels (Fig. 4C). These results demonstrate that the development of ECM is independent of IL-17.

3.3. General discussion

As observed during our study, infection with PbA did not induce the expression of IL-23 and a lack of IL-23 did not affect the develop ECM. Moreover, IL-17 deficient mice also develop ECM, while IL-17 producing cells increased in WT mice affected with ECM. These results suggest that both cytokines are unnecessary for the development of ECM.

Th17 development was not observed during infection with PbA primarily because IL-23, which is critical for Th17 expansion, was not secreted. We confirmed that expression of mRNA encoding IL-17 or RORγt in $CD4^+$ T cells decreased in mice that developed ECM and that the frequency and cell number of Th17 were not increased. In addition, environments under malaria infection favor to induce Treg [29,30], consequently might disturb Th17 differentiation as a counter-regulatory action [31]. By contrast, we observed that the frequency of Th1 was increased in WT mice that developed ECM (Supplementary Fig. 1). Expression of mRNA encoding T-bet or IFN-γ in $CD4^+$ T cells from mice that developed ECM were higher than those from uninfected mice (data not shown). These findings suggested that Th1 rather than Th17 may be of importance in ECM development and supports previous suggestions that Th1 are dominant during ECM [26].

IL-17 is known to suppress pathogenic Th1 responses during inflammatory bowel disease [27,28], suggesting that augmented induction of Th1 covers or compensates lack of IL-17-mediated pathogenesis in ECM development. However, there was no difference between WT and 17KO mice in Th1 induced by infection with PbA (Supplementary Fig. 1). Given these results, it appears that IL-17 does not suppress Th1 responses during ECM and thus does not contribute to ECM development.

In addition to the ECM produced by PbA infection, hepatic injury observed in mice infected with *P. berghei* NK65 (PbNK) is also reported to be immunopathological [1,32]. Our preliminary results revealed that P19KO mice or 17KO mice infected with PbNK displayed elevated serum levels of enzymes released from the damaged hepatocytes, as was the case for WT mice (data not shown). Thus, these cytokines do not appear to be involved in ECM development or liver injury by infection with PbNK. Immunopathologies

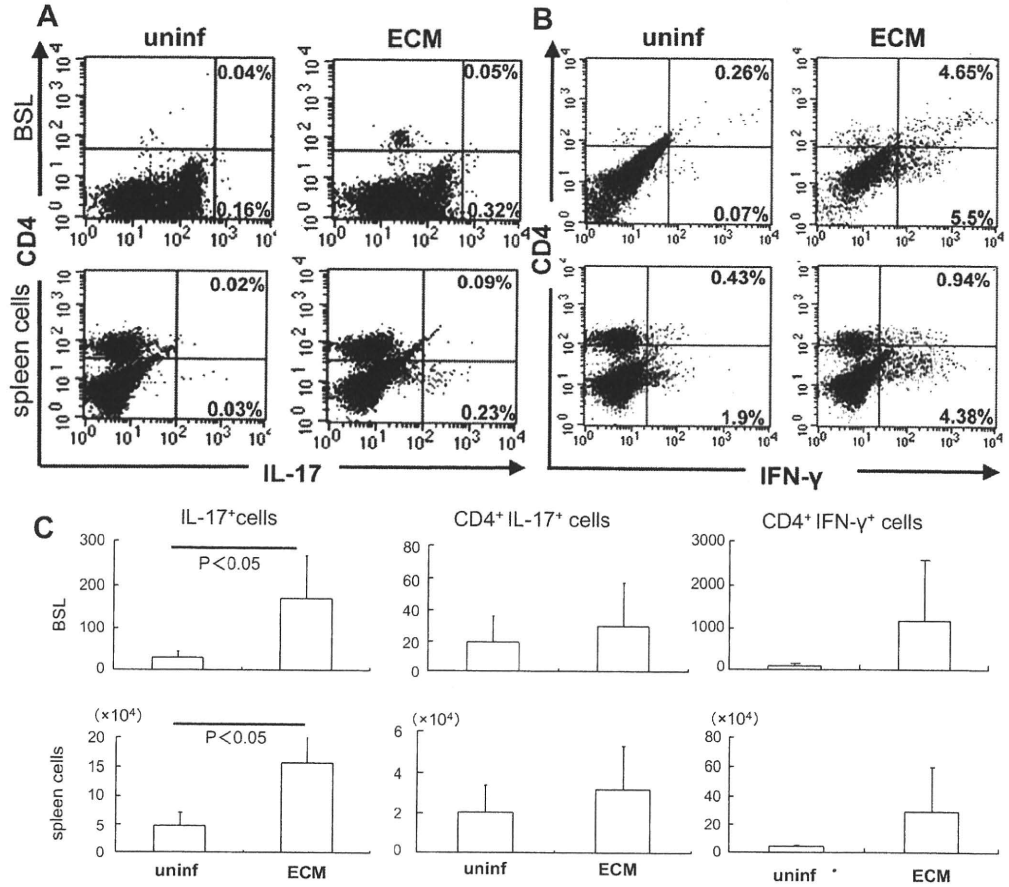

Fig. 2. Induction of IL-17-producing cells during infection with PbA. BSL (upper panel) or spleen cells (lower panel) isolated from mice that developed ECM were analyzed for IL-17 (A) and IFN-γ (B) production. Gated lymphocytes were plotted for CD4 and each cytokine. The numbers indicate the percentage of quadrants. Absolute numbers of the indicated cells were also shown (C). Data represent the mean ± SD of five mice. One representative of at least two repeated experiments is shown.

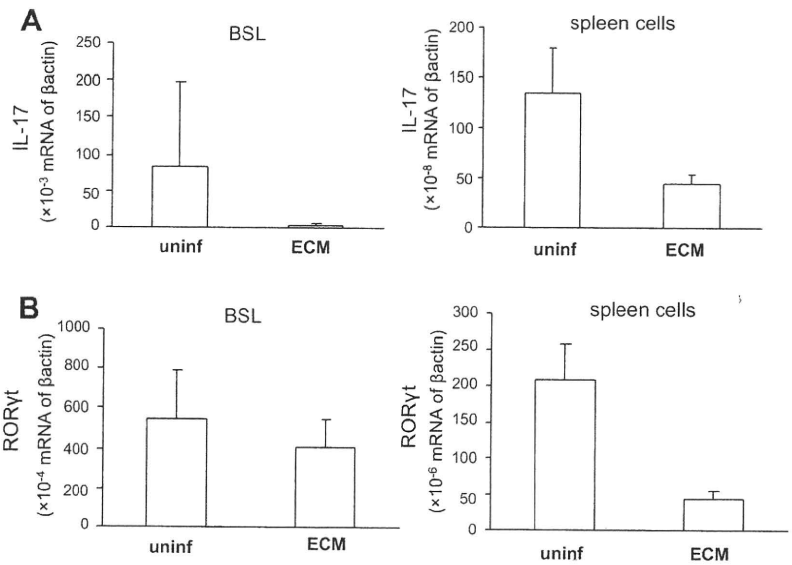

Fig. 3. Induction of Th17 in mice infected with PbA. CD4⁺ cells were purified from BSL and spleen cells of mice developing ECM and analyzed for the expression of mRNA encoding IL-17 (A) and RORγt (B) as in Fig. 1A. One representative of at least two repeated experiments is shown.

induced by malaria parasites may therefore be independent of IL-23 and IL-17, unlike other infectious diseases.

IL-23 and IL-17 are reported to be protective against some infections [33], yet are responsible for inflammatory pathologies

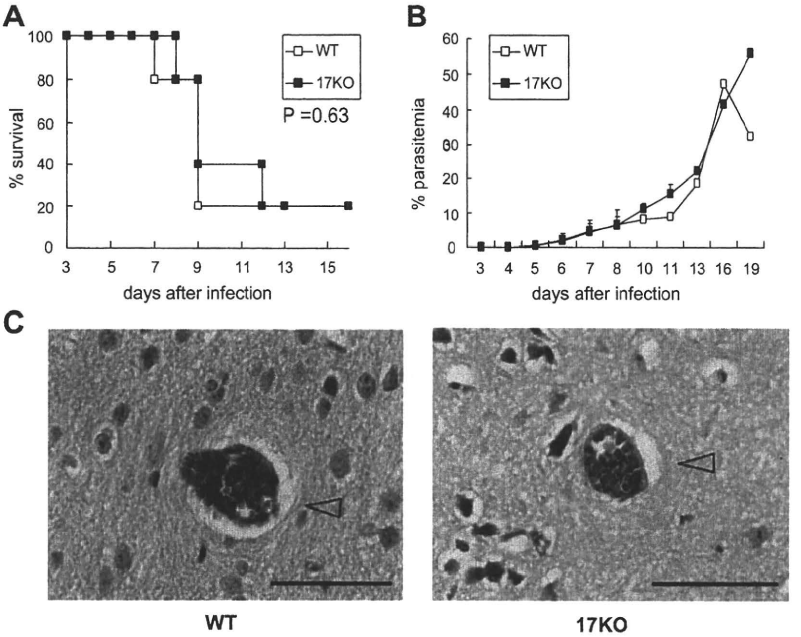

Fig. 4. ECM induced by PbA infection was independent of IL-17. Survival rates (A) or parasitemia (B) of WT and 17KO mice infected with 50,000 PbA-pRBC were monitored as in Fig. 1C. Histological analysis of brain sections of WT (left panels) and 17KO mice (right panels) developing ECM was performed as in Fig. 1E.

in other situations. Parasitemia in P19KO or 17KO mice infected with PbA was similar to that in infected WT mice. These findings suggest that IL-23 and IL-17 do not contribute to the reduction of parasite burdens in mice infected with PbA. However, those cytokines have protective effects against infection with PbNK65 and the lack of IL-23 shortened the survival of infected mice. IL-23 also has anti-protozoan effects against *Plasmodium yoelii* XL (manuscript in preparation). The protective effects of IL-23 and IL-17 during malaria therefore appear to dependent on the species and strain of malaria parasites.

In conclusion, IL-23 and IL-17 are not critical for the development of ECM, a complicated pathology that is produced in association with various immune cells, cytokines and chemokines. It would also be of interest to investigate whether similar results could be achieved using human patients. Such insights will potentially further our understanding CM pathogenesis and help establish new therapeutic strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.10.114.

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Involvement of CD8$^+$ T cells in protective immunity against murine blood-stage infection with *Plasmodium yoelii* 17XL strain

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When developing malaria vaccines, the most crucial step is to elucidate the mechanisms involved in protective immunity against the parasites. We found that CD8$^+$ T cells contribute to protective immunity against infection with blood-stage parasites of *Plasmodium yoelii*. Infection of C57BL/6 mice with *P. yoelii* 17XL was lethal, while all mice infected with a low-virulence strain of the parasite 17XNL acquired complete resistance against re-infection with *P. yoelii* 17XL. However, the host mice transferred with CD8$^+$ T cells from mice primed only with *P. yoelii* 17XNL failed to acquire protective immunity. On the other hand, the irradiated host mice were completely resistant to *P. yoelii* 17XL infection, showing no grade of parasitemia when adoptively transferred with CD8$^+$ T cells from immune mice that survived infection with both *P. yoelii* XNL and, subsequently, *P. yoelii* 17XL. These protective CD8$^+$ T cells from immune WT mice had the potential to generate IFN-γ, perforin (PFN) and granzyme B. When mice deficient in IFN-γ were used as donor mice for CD8$^+$ T cells, protective immunity in the host mice was fully abrogated, and the immunity was profoundly attenuated in PFN-deficient mice. Thus, CD8$^+$ T cells producing IFN-γ and PFN appear to be involved in protective immunity against infection with blood-stage malaria.

Key words: CD8 T cells · Immune responses · Infectious diseases · Malaria · Memory cells

Supporting Information available online

Introduction

Malaria is one of the main global infectious diseases, and results in 300–500 million clinical cases and one million deaths annually, mostly among young children in sub-Saharan Africa (http://rbm.who.int/wmr2005/[1]). The development of resistance to drugs in parasites and vectors poses one of the greatest threats to malaria control and has been linked to recent increases in malaria morbidity and mortality, so vaccine development is urgently required. To achieve this purpose, it is essential to elucidate the detailed protective mechanisms of the hosts against infection.

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Antibodies and T cells play crucial roles in protective immunity against malaria parasites. Antibodies specific for merozoites prevent invasion of those parasites into RBC [2–5]. These antibodies, attached to the parasites or parasitized RBC, lyse the parasites and RBC in a complement-dependent manner. Another function of these antibodies is to mediate phagocytosis and digestion by monocytes/macrophages [3–5]. $CD4^+$ T cells act as Th cells for B-cell differentiation and antibody production; on the other hand, Th cells activate phagocytosing macrophages and kill the parasites. Thus, $CD4^+$ T cells should play key roles in protective immunity against both liver and blood-stage malaria. Strangely, however, clinical vaccine trials using antigenic peptide for malaria have resulted in failure in some cases [6], despite the fact that activation of $CD4^+$ T cells and antibody production have been efficiently induced in vaccine trials.

$CD8^+$ T cells have been proposed to be essential for protective immunity against liver-stage malaria [2–5]. On the other hand, several studies have concluded that $CD8^+$ T cells do not contribute to protective immunity against blood-stage parasites, mainly because of the absence of MHC class I molecules on infected erythrocytes, by which antigens are presented to $CD8^+$ T cells [7–10]. For example, Vimetz *et al.* have reported that adoptive transfer of $CD8^+$ T cells from immune animals does not confer protective immunity to blood-stage infection in the host [7]. However, some studies have stressed the existence of $CD8^+$ T cells specific for blood-stage malaria parasites. First, $CD8^+$ T-cell clones that proliferate and produce IFN-γ in response to blood-stage malarial antigens in an HLA-restricted manner are generated in patients who live in a malaria-endemic area [11]. Second, experimental infections of non-immune volunteers with an ultra-low dose of infected RBC induce immunity to subsequent challenge in the absence of detectable antibody responses. Those who acquire immunity show proliferative responses in $CD8^+$ T cells [12].

There are few studies showing that $CD8^+$ T cells protect against blood-stage parasites. However, there are some reports in murine models that $CD8^+$ T cells contribute to the pathology of experimental cerebral malaria. Mice that are depleted in, or do not have, $CD8^+$ T cells, are protected against experimental cerebral malaria [13]. $CD8^+$ T cells might contribute *via* perforin (PFN)-dependent destruction of cerebral microvasculor endothelial cells [14]. These malaria Ag-specific $CD8^+$ T cells are induced by cross-presentation from dendritic cells [15]. In the present study, we evaluated the contribution of $CD8^+$ T cells in immunity against blood-stage parasites.

Results

$CD8^+$ T cells from *Plasmodium yoelii* 17XNL-recovered mice fail to transfer protection against PyL

We infected C57BL/6 mice with the blood-stage form of the rodent malaria parasite *P. yoelii* 17X, which has two substrains. One substrain, PyL, is highly virulent in mice and causes lethal infection; the other, PyNL, causes a self-limiting, non-lethal infection [16]. Mice infected with 25 000 PyNL-parasitized RBC (PyNL-pRBC) spontaneously recovered within 4 wk of infection (data not shown). Mice that recovered from infection with PyNL (PyNL-recovered mice) were then infected with PyL. These mice were highly resistant to the lethal infection and allowed no parasite growth, while control mice showed rapid parasite growth and died within 10 days (Fig. 1A). Thus, infection with PyNL functions as a live vaccination against lethal infection with PyL. To examine the involvement of $CD8^+$ T cells in protective immunity to blood-stage infection with PyL, we injected PyNL-recovered mice with anti-CD8 mAb to deplete $CD8^+$ T cells prior to infection with PyL. However, depletion of $CD8^+$ T cells, as well as $CD4^+$ T cells, did not attenuate protection in PyNL-recovered mice (data not shown). We next performed adoptive transfer experiments. $CD8^+$ T cells purified from PyNL-recovered mice were transferred to the syngeneic recipient mice. In order to exclude the influence of host lymphocytes, we irradiated the recipients with 5.5 Gy of γ radiation. One week after cell transfer, these mice were infected with PyL. These recipient mice failed to control infection with PyL, similar to those transferred with $CD8^+$ T cells from uninfected control mice (Fig. 1B). These results were very similar to those of previous studies [6–9], which indicates that $CD8^+$ T cells do not contribute to protection against blood-stage malaria parasites.

$CD8^+$ T cells from highly immunized mice transfer protection against PyL

PyNL-recovered mice showed sterile immunity against infection with PyL. However, $CD8^+$ T cells from these mice did not transfer protection to the recipient mice. Several factors, such as presence or absence of antibodies in the circulation, might account for this discrepancy. Among these factors, we speculate that $CD8^+$ T cells

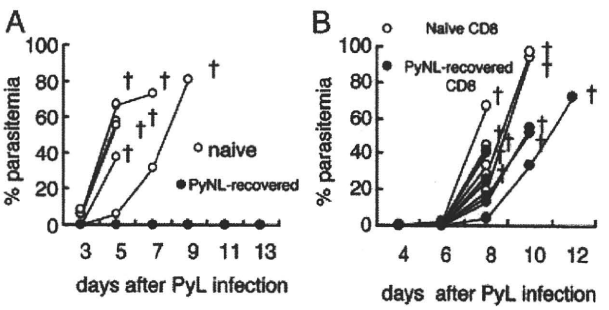

Figure 1. $CD8^+$ T cells from PyNL-recovered mice failed to transfer protection against PyL. (A) The kinetics of parasitemia in PyNL-recovered (closed circles) or naive (open circles) mice were monitored after infection with 25 000 PyL-pRBC. (B) $CD8^+$ T cells (8×10^6) purified from PyNL-recovered (closed circles) or naive (open circles) mice were transferred to the syngeneic irradiated recipient mice, followed by infection with PyL. Each symbol represents a value from an individual mouse. Daggers indicate death. Similar results were obtained from at least four experiments.

are hardly activated during primary infection, as prime-boost regimens are commonly used to fully activate antigen-specific $CD8^+$ T cells [17]. To assess this possibility, PyNL-recovered mice were further infected twice with PyL (immune mice, Fig. 2A). Although PyNL-recovered mice showed no signs of infection, or even parasite growth, when boosted with PyL as shown in Fig. 1A, serum antibodies specific for PyL were significantly increased after the boosts (Fig. 2B), which indicates successful reactivation of parasite-specific immunity. $CD4^+$ or $CD8^+$ T cells purified from immune mice were transferred to irradiated recipients, which were subsequently infected with PyL and monitored for the course of infection (Fig. 2C and D). All recipients transferred with $CD4^+$ or $CD8^+$ T cells from control mice died as parasites grew. Mice that received $CD4^+$ T cells from immune mice showed low parasitemia and survived with no parasite recrudescence (Fig. 2C). Surprisingly, $CD8^+$ T cells from immune mice transferred protection against infection with PyL as well as $CD4^+$ T cells (Fig. 2D). Furthermore, transfer of $CD8^+$ T cells from immune mice also conferred protection against PyL in RAG2 KO recipient mice (Supporting Information Fig. S1B). To exclude the possibility that small contaminating population of other cells was responsible for the protection, we sorted the three groups of cells from immune mice for adoptive transfer experiment, using MACS cell separation system; the first group was $CD8^+$ cells, the second was $CD4^-$ (CD4 depleted, negative selection) $CD8^+$ cells and last one was $CD4^-$ $CD8^-$ (CD4, CD8 depleted, negative selection) cells. Although $CD4^-$ $CD8^+$ T cells from immune mice transferred protection against infection with PyL, mice depleted of both CD4 and CD8 T cells failed to control the infection and died from high parasitemia (Supporting Information Fig. S1A), confirming that contaminants do not contribute to protection. These results clearly demonstrate that $CD8^+$ T cells play a protective role against blood-stage malaria parasites.

Immunophenotype characterization of $CD8^+$ T cells in immune mice

Mice that received $CD8^+$ T cells from immune mice showed low parasitemia and survived with no parasite recrudescence. This protective immunity against malaria parasites might induce activation of $CD8^+$ T cells and generate memory $CD8^+$ T cells. We analyzed the phenotype of $CD8^+$ T cells. Naive T cells express CD62L (L-selectin), which is known to be a homing receptor, and shed this molecule to migrate from lymphoid organs to inflammatory sites after activation [18]. CD44 appears to be the most reliable marker that is expressed at high levels in all memory T cells of mice, irrespective of their activation status [19]. $CD8^+$ T cells can be classified into three groups according to their expression patterns of CD62L and CD44: $CD62L^{hi}CD44^{lo}$, $CD62L^{hi}CD44^{hi}$ and $CD62L^{lo}CD44^{hi}$ patterns represent naive, central memory, and effector memory phenotypes, respectively [19]. Spleen cells from recipient mice transferred with the

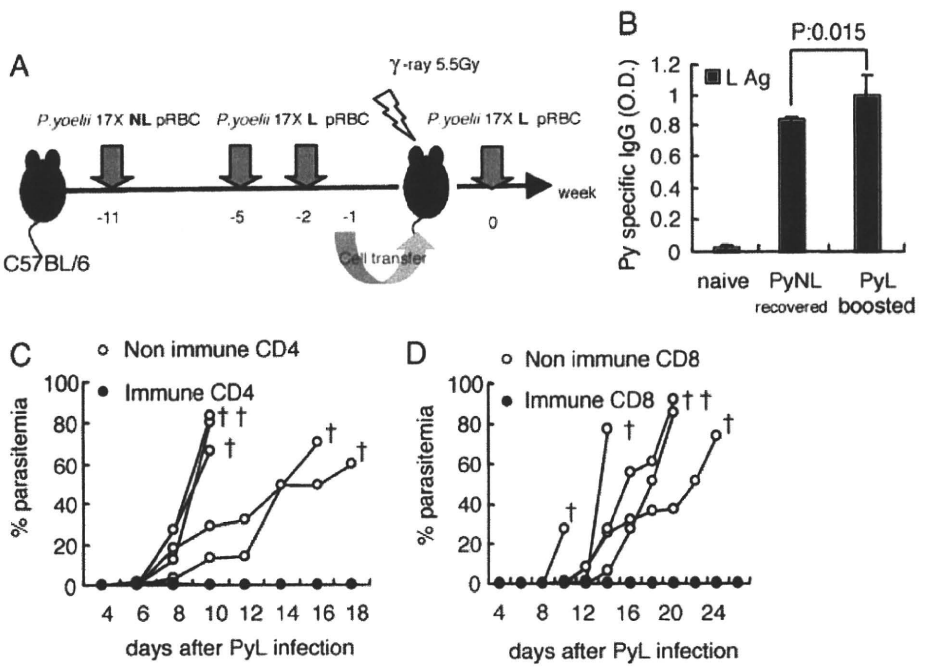

Figure 2. $CD8^+$ T cells from highly immunized mice transferred protection against PyL. (A) Protocol of immunization, cell transfer and challenge infection. (B) Antibody titers specific for malaria parasites in sera collected from mice were measured. Data are the means+SD of OD_{415} values of 200-fold-diluted sera from six mice in each group. Parasitemia of the recipients transferred with $CD4^+$ (C) or with $CD8^+$ (D) T cells purified from control (open circles) or immune (closed circles) mice were evaluated as in Fig. 1B. One representative of at least two repeated experiments is shown. Daggers indicate death.

indicated cells were analyzed 7 days after infection with PyL (Fig. 3A right panels). Cells from the recipients left uninfected were also analyzed (Fig. 3A left panels). Immune mice contained twice as many $CD62L^{lo}$ and effector memory $CD8^+$ T cells than naive control mice (Fig. 3A). Also, the numbers of $CD8^+$ T cells expressing CD30, which is known to be a marker of memory T cells, increased in immune mice by as much as 16.7% *versus* 5.8% in naive mice (data not shown). In response to infection with PyL, the numbers of effector memory $CD8^+$ T cells predominantly increased in recipients transferred with $CD8^+$ T cells from immune mice (Fig. 3A). Furthermore, infection of these mice markedly increased the numbers of $CD8^+$ T cells that express granzyme B, PFN or IFN-γ, all of which are important molecules for the protective role of $CD8^+$ T cells. In sharp contrast, infection of recipients transferred with $CD8^+$ T cells from non-immune mice did not affect the expression of these molecules (Fig. 3B), although $CD8^+$ T cells were slightly activated, as evaluated by an increase in the size of the $CD62L^{lo}$ population (Fig. 3A). These results demonstrate that the immunization protocol can effectively generate memory $CD8^+$ T cells that respond quickly to become effector cells.

IFN-γ from $CD8^+$ T cells is important to protect against infection with PyL

We evaluated the role of IFN-γ in protective immunity mediated by $CD8^+$ T cells. IFN-γ is a pro-inflammatory cytokine that is intimately involved in the innate and acquired immune responses. This cytokine has important roles in immunity against blood-stage malaria, because a previous study has demonstrated that IFN-γ KO mice are highly susceptible to infection with *Plasmodium chabaudi* AS-pRBC [20]. First, the irradiated recipients transferred with $CD8^+$ T cells from immune mice were treated with neutralizing antibodies to IFN-γ prior to infection with PyL. The recipients injected with anti-IFN-γ suffered from high parasitemia and died quickly, while those injected with irrelevant antibodies showed only a slight increase in parasitemia (Fig. 4A). To confirm the importance of IFN-γ secreted from $CD8^+$ T cells, we employed IFN-γ KO mice as immune donors, because the recipient-derived cells might have secreted the cytokine. IFN-γ KO mice were much more susceptible to infection with PyNL, and 25–50% of these mice survived (data not shown). However, once recovered from the infection, IFN-γ KO mice acquired sterile immunity to infection with PyL. $CD8^+$ T cells from immune IFN-γ KO mice were transferred to the irradiated recipients prior to infection with PyL. The recipients transferred with $CD8^+$ T cells from immune IFN-γ KO mice failed to control the infection similar to those transferred with $CD4^+$ T cells from immune IFN-γ KO mice (Fig. 4B). These results suggest that IFN-γ is important to $CD8^+$ T-cell-mediated protection against blood-stage malaria.

Macrophages play essential roles in $CD8^+$ T-cell-mediated protection against PyL

Among the pivotal roles of IFN-γ, activation of macrophages is supposed to be one of the major mechanisms for activating anti-malarial immunity. Macrophages play a critical role in innate immunity against malaria due to their ability to phagocytose pRBC in the absence of cytophilic or opsonizing parasite-specific antibodies [21]. In adaptive immunity, upon

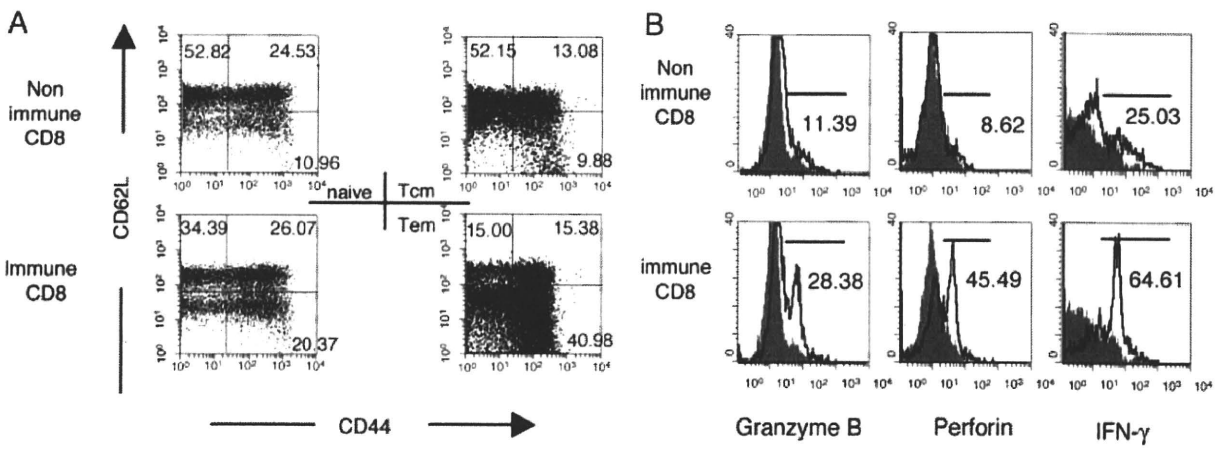

Figure 3. Immunophenotype and expression of effector molecules of protective $CD8^+$ T cells. (A) Spleen cells from recipient mice transferred with the indicated cells were analyzed 7 days after infection with PyL (right panels). Cells from the recipients left uninfected were also analyzed (left panels). Gated $CD8^+$ T cells from mice were classified into central memory (Tcm), effector memory (Tem) and naive cells by staining with CD62L and CD44. Numbers represent the percentage of cells in the corresponding quadrants. (B) Spleen cells from recipient mice transferred with non-immune (upper panel) or immune (lower panel) $CD8^+$ T cells were collected 7 days after PyL infection. Expression of granzyme B, PFN and IFN-γ in $CD8^+$ T cells from spleen was analyzed by intracellular staining with the corresponding antibodies. The expression profiles of $CD8^+$ T cells from the infected recipients (solid lines) were plotted over those of $CD8^+$ T cells from uninfected naive mice (shaded areas). Numbers indicate the percentages of $CD8^+$ T cells positive for the indicated marker in infected recipients. The percentages of $CD8^+$ T cells from uninfected mice positive for granzyme B, PFN, and IFN-γ were 4.6, 9.5 and 11.5 %, respectively.

stimulation with IFN-γ, macrophages function as effector cells that can mediate antibody-dependent cellular inhibition or the production of anti-parasitic molecules. Thus, we evaluated the role of macrophages in irradiated recipients transferred with $CD8^+$ T cells from immune mice. Carrageenan (CGN), a sulfate polygalactose preferentially phagocytosed but undigested in macrophages, was administered to the recipients to impair macrophages [22]. This manipulation completely abolished the protective ability of $CD8^+$ T cells (Fig. 4C). These results indicate that macrophages, presumably activated by IFN-γ secreted from $CD8^+$ T cells, are responsible for parasite eradication in recipient mouse transferred with immune $CD8^+$ T cells. Indeed, the potential of macrophages for phagocytosing pRBC was profoundly impaired in recipients transferred with $CD8^+$ T cells from immune IFN-γ KO mice compared with those from recipients transferred with immune WT mice (Fig. 4D).

PFN from $CD8^+$ T cells contribute partially to protection against PyL

$CD8^+$ T cells from immune mice expressed effector molecules related to cytotoxicity. We investigated the involvement of these molecules in the mechanism of protective immunity induced by $CD8^+$ T cells against blood-stage parasites. PFN KO mice were infected with PyNL. PFN KO mice were more susceptible to infection with PyNL than WT mice, and only half of them survived (Fig. 5A), which suggests that PFN has a protective role. Mice surviving infection with PyNL were resistant to boosting with PyL to the same degree as WT mice, and we employed these mice as immune mice. We compared the activation and phenotype of $CD8^+$ T cell from immune WT and immune PFN KO mice that were subsequently used in the adoptive transfer experiments (Supporting Information Fig. S2A). The activation maker (CD62L lo cells) was almost the same between WT and

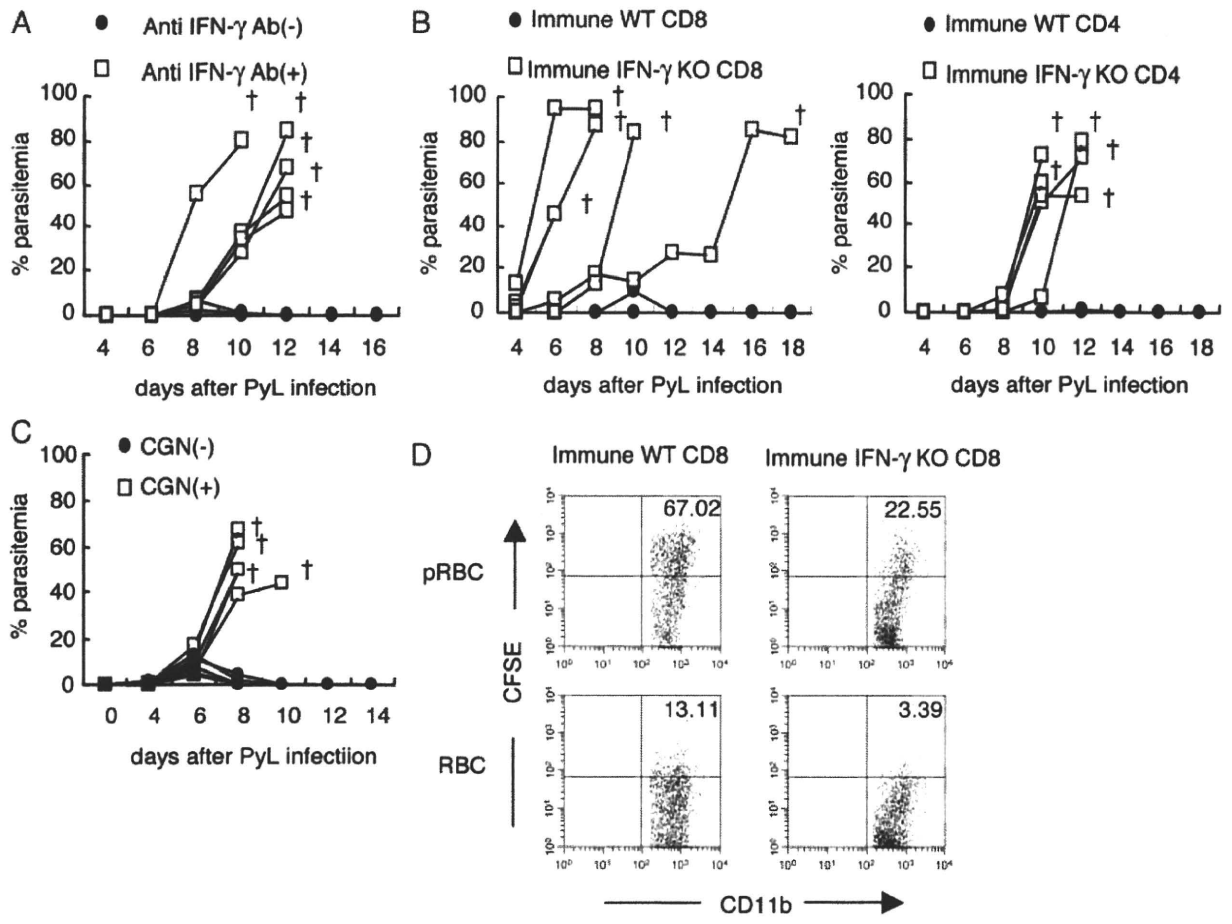

Figure 4. IFN-γ from $CD8^+$ T cells was essential for protection against blood-stage malaria. (A) Parasitemia in recipients transferred with immune $CD8^+$ T cells treated with anti-IFN-γ (open squares) or irrelevant antibodies (closed circles) was evaluated as in Fig. 1B. (B) $CD8^+$ (left panel) or $CD4^+$ (right panel) T cells from IFN-γ KO (open squares) or WT (closed circles) mice were transferred to the irradiated recipients, followed by infection with PyL. (C) Macrophages played an essential role in $CD8^+$ T-cell-mediated protection against PyL. The irradiated recipients transferred with $CD8^+$ T cells from immune mice were treated with CGN to impair macrophage function (open squares), and parasitemia was evaluated. Daggers indicate death. (D) Macrophage uptake assay. Splenic CD11b macrophages were removed from day 7 post-PyL-infected recipients that were transferred with $CD8^+$ T cells from immune WT or immune IFN-γ KO mice. Macrophages were co-cultured with CFSE-labeled pRBC or RBC. The percentage of $CD11b^+$ cells undergoing phagocytosis is presented. One representative of at least two repeated experiments is shown.

PFN KO (Supporting Information Fig. S2C). Furthermore, the number of transferred effector memory cells that are thought to be a key player in protection against infection was also the same between WT and PFN KO (Supporting Information Fig. S2B). These results indicated that activation and phenotype of transplanted immune PFN KO $CD8^+$ T cells are very similar to immune WT $CD8^+$ T cells even though PFN KO mouse are susceptible to PyNL infection. When infected with PyL, the irradiated recipients transferred with $CD8^+$ T cells from immune PFN KO mice showed higher parasitemia at an early stage of infection than those transferred with $CD8^+$ T cells from immune WT mice, and some mice in the former group failed to control the challenge infection (Fig. 5B). However, $CD4^+$ T cells from these donors protected recipients from infection with PyL, similar to those from immune WT mice (Fig. 5B). These results indicate that the cytotoxicity-related molecule PFN contribute, at least in part, to the protective immunity to blood-stage malaria infection conferred by $CD8^+$ T cells.

Discussion

In the present study, we found that $CD8^+$ T cells play roles in the protective immunity against blood-stage infection with highly virulent *P. yoelii* in C57BL/6 mice. Irradiated naive mice transferred with $CD8^+$ T cells from these mice were resistant to infection with PyL. IFN-γ and macrophages were essential for the protective immunity dependent on $CD8^+$ T cells. Additionally, cytotoxicity-related molecules expressed by $CD8^+$ T cells, such as PFN, appeared to also contribute to this protective immunity. It is noteworthy that the protective immunity mediated by $CD8^+$ T cells was not acquired when recipient mice were treated with CGN, a macrophage blocker, after challenge with the parasite. Based on those observations, the role of $CD8^+$ T cells in protective immunity would be exerted by a two-step mechanism. As a first step, these T cells would activate macrophages through the release of IFN-γ. These macrophages should potently phagocytize the infected erythrocytes and digest them. If the parasites were not completely eliminated by the first step mechanism, $CD8^+$ T cells might kill the infected macrophages *via* the PFN-granzyme system in the second step.

We used irradiated or RAG2-deficient mice as recipients for transfer with immune CD8 T cells. It is well established that T cells transferred into such immunodeficient hosts will expand in a non-physiologic manner (homeostatic proliferation) that may result in artificial activation [23]. Moreover, irradiated hosts likely represent a highly inflammatory environment, "hormesis effects" [24]. Similarly, RAG-deficient mice have robust innate immune systems to partially compensate for the lack of adaptive immunity [25]. It is quite possible that these unexpected effects are responsible for the protection observed here. However, only $CD8^+$ T cells from immune mice but not from naive mice (even after a single infection) were potent in transferring protection. These results clearly indicate that protection through the transfer of immune $CD8^+$ T cells is unlikely due to hormesis or homeostatic proliferation and is antigen-specific. In terms of antigen specificity, we determined whether immune $CD8^+$ T cells transfer protection against an irrelevant protozoan parasite, *Toxoplasma gondii*. Mice transferred with immune $CD8^+$ T cells were susceptible

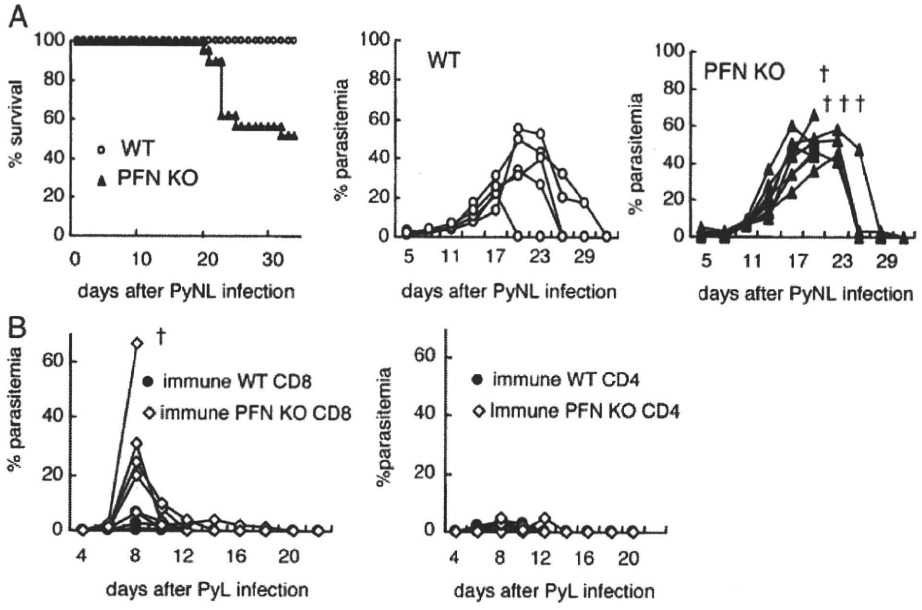

Figure 5. Molecules associated with CTL activity partially contributed to protection against blood-stage malaria. (A) Survival rates and parasitemia in PFN KO (closed triangles) mice were monitored after infection with 25 000 PyNL-pRBC. (B) The recipient mice transferred with $CD8^+$ or $CD4^+$ T cells from immune PFN KO mice were infected with PyL, and parasitemia was evaluated as in Fig. 1B. One representative of at least two repeated experiments is shown. Daggers indicate death.

to the infection similar to those with naive $CD8^+$ T cells (data not shown).

Malaria parasites reside within RBC to evade host immunity. Merozoite-specific antibodies in the circulation readily bind to free merozoites and may inhibit invasion of the parasites into RBC, but these antibodies cannot reach merozoites once they have invaded RBC. Furthermore, blood-stage parasites should escape recognition by $CD8^+$ T cells since RBC do not express MHC class I molecules on their surface, leading to the conclusion that $CD8^+$ T cells could not contribute to protective immunity against blood-stage parasites [6–9].

RBC have no MHC class I molecules. Nevertheless, $CD8^+$ T cells specific for malaria antigens are activated during blood-stage malaria [10, 11]. Taken together with our observation that the numbers of activated $CD8^+$ T cells increased after infection with PyL, antigens derived from malaria parasites must be presented on MHC class I molecules on APC including macrophages. The activation mechanism of $CD8^+$ T cells may be explained by cross-presentation of APC that have engulfed pRBC as other particle antigens [26].

$CD8^+$ T-cell-dependent protective immune responses have been shown to correlate well with IFN-γ production and cytotoxic activity. Our results showed important roles of IFN-γ in protection mediated by $CD8^+$ T cells. Macrophages were also important in this protection, strongly suggesting that $CD8^+$ T cells contribute to the elimination of blood-stage parasites by activating macrophages with IFN-γ, and that the activated macrophages would phagocytose and digest pRBC.

On the other hand, cytotoxic activity exerted by PFN might play some roles in the protective immunity in the late stage of the protective immunity. Macrophages activated with IFN-γ phagocytose antigen abundantly, and are exhausted after phagocytosis reaches its limit [27]. As a final step, $CD8^+$ T cells direct pRBC-phagocytosed monocytes/macrophages to undergo apoptosis *via* the PFN-granzyme B pathway, and completely eliminate them. A previous study reported that $CD8^+$ T cells mediate loss of macrophages in the spleen, which is the most important organ for removal of pRBC by macrophages [28]. Thus, cytotoxic activity exerted by PFN and granzyme B in $CD8^+$ T cells might be directed to these macrophages, resulting in the elimination of parasites and recruitment of fresh macrophages.

Although great efforts have been made to develop vaccines against malaria, practical vaccine strategies have not yet been established. One of the causes for this failure may be that host protective immunity and the immune evasion mechanisms of the parasites have not been fully understood. We clearly showed the involvement of $CD8^+$ T cells in protective immunity against blood-stage infection. In addition, blood-stage parasites seem to evade $CD8^+$ T cell immunity. That is, dendritic cells that interact with pRBC selectively impair the cell cycles of $CD8^+$ T cells, but not $CD4^+$ T cells [29]. In developing vaccines for blood-stage malaria, establishment of strategies for activating $CD8^+$ T cells specific for the parasites or infected erythrocytes may be essential.

Materials and Methods

Mice and parasites

C57BL/6 mice were obtained from Kyudo (Tosu, Japan), RAG2-deficient mice were obtained from Central Laboratory of Experimental Animals (Kawasaki, Japan), PFN-deficient mice were obtained from Jackson Immuno Research Laboratories (West Grove, PA, USA), IFN-γ-deficient mice were provided by Dr. A. Nakane (Hirosaki University), and Ly5.1C57BL/6 mice were obtained from Sankyo Lab Service (Tokyo, Japan) with the permission of Dr. H. Nakauchi (Tokyo University). Age- and sex-matched groups of WT and mutant mice were used. All experiments using mice were reviewed by the Committee for Ethics on Animal Experiments in the Faculty of Medicine, and carried out under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government. Blood-stage parasites of PyL or PyNL, generous gifts from Dr. M Torii (Ehime University) and Dr. K. Yui (Nagasaki University), were obtained after fresh passage through a donor mouse 2–3 days after inoculation with frozen stock. Mice were infected with 25 000 pRBC i.p., which were purified as previously described, and used for infection.

Determination of parasitemia

Blood samples were collected from the experimental mice by bleeding *via* the tail vein at the time indicated. Thin blood films were prepared and fixed with methanol followed by staining with Giemsa solution (Sigma-Aldrich, St. Louis, MO, USA). Parasitemia was determined by counting the percentages of pRBC under a microscope.

Reagents

FITC-, PE-, and PE-Cy5-anti-CD8, FITC-anti-CD4, FITC-anti-CD62L, PE-anti-CD30, PE-anti-granzyme B, and FITC-anti-PFN (eBioscience, San Diego, CA, USA) were used for flow cytometry. Purified anti-CD16/32 (2.4G2) antibodies were obtained from eBioscience. Anti-PE- and anti-FITC microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) were used for MACS cell purification. mAb to IFN-γ (R4-6A2) purified from the ascites fluid of hybridoma-injected athymic nude mice was used for *in vivo* treatment. CGN type IV (Wako, Osaka, Japan) was used for macrophage blockade.

Purification of cells and adoptive transfer

Spleens were aseptically removed from mice and prepared as single-cell suspensions. RBC were lysed with NH_4Cl, and the cells

were washed twice in fresh medium. $CD4^+$ or $CD8^+$ T cells were purified with positive selection using a MACS cell separation system (Miltenyi Biotech) according to the manufacturer's protocols; in some experiments negative and positive selection were done. The purity of the separated cells was usually >95%. Eight million purified cells were adoptively transferred i.v. to syngeneic recipients that had been irradiated with 5.5 Gy of γ-radiation (GammaCell) or to RAG2 KO mice.

Determination of antibodies specific for PyL

Serum antibodies specific for PyL were measured by ELISA. The 96-well microtiter plates were coated with 50 μL PyL antigen solution (5 μg/mL). Diluted serum samples were added to the wells, and then incubated at room temperature for 2 h. PyL-specific antibodies were detected by horseradish peroxidase-conjugated anti-mouse IgG (Zymed, San Francisco, CA, USA). Enzymatic activity was visualized using a substrate. Absorbance at 415 nm was measured using a spectrophotometer.

Flow cytometry

Spleen cell suspensions were stained with combinations of fluorochrome-labeled antibodies. For intracellular staining, cells were stimulated with 50 ng/mL PMA and 1 μg/mL calcium ionophore in the presence of 1 μg/mL brefeldin A, in complete medium at 37°C for 5 h. These cells were then incubated with anti-CD16/32 (Fc-block) and stained with surface markers, followed by fixation with 4% paraformaldehyde and permeabilization with 0.1% saponin. Then, the cells were stained with second antibodies. Stained cells were analyzed by a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA), and the data were analyzed using CellQuest Pro software (Becton Dickinson).

In vivo neutralization of IFN-γ and macrophage inhibition

To neutralize IFN-γ, mice received i.p. injection with 500 μg of anti-IFN-γ antibody 1 day prior to infection, and every 2 wk thereafter. To impair macrophages [22], mice were injected i.p. with 1 mg CGN on 2, 5, 8, 11 and 14 days after infection.

Macrophage uptake assay

The macrophage phagocytosis assay was a modification of a previous method [26]. To stain pRBC or normal RBC, cells (10^7 cells/mL) were incubated with 10 μM CFSE (Molecular Probes, CA, USA) in PBS for 15 min at 37°C. CFSE staining was stopped by adding excess complete medium and washing cells three times with medium. $CD8^+$ T cells from immune WT or immune IFN-γ KO mice were transferred to the recipients. Splenic CD11b macrophages were then removed from mice at day 7 post PyL infection for a macrophage uptake assay. Splenic $CD11b^+$ cells were purified with positive selection using an MACS cell separation system (Miltenyi Biotech) according to the manufacturer's protocols. Splenic CD11b macrophages (10^6 cells/well) were seeded with CFSE-labeled pRBC or normal RBC in a 1:20 ratio, at a final volume of 200 μL for 4 h at 37°C. Following co-culture, non-ingested RBC were removed by lysis with NH_4Cl lysing buffer, and the remaining macrophages were washed twice with PBS, FcR-blocked and then stained with PE-labeled anti-CD11b mAb, in sorting buffer consisting of PBS with 1% FBS and 0.05% sodium azide (Sigma-Aldrich, St. Louis, MO, USA). The capacity of macrophages to uptake CFSE-labeled pRBC or normal RBC was analyzed by a FACS Calibur cytometer (Becton Dickinson), and the data were analyzed using CellQuest Pro software (Becton Dickinson).

Statistical analysis

Statistical evaluation of differences between experimental groups was done by analysis of variance and two-tailed unpaired Student's *t*-tests; $p<0.05$ was considered statistically significant

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Abbreviations: **CGN:** carrageenan · **PFN:** perforin · **pRBC:** parasitized RBC · **PyNL:** *P. yoelii* 17XNL

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