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IL-23 protection against *Plasmodium berghei* infection in mice is partially dependent on IL-17 from macrophages

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Although IL-12 is believed to contribute to protective immune responses, the role played by IL-23 (a member of the IL-12 family) in malaria is elusive. Here, we show that IL-23 is produced during infection with *Plasmodium berghei* NK65. Mice deficient in IL-23 (p19KO) had higher parasitemia and died earlier than wild-type (WT) controls. Interestingly, p19KO mice had lower numbers of IL-17-producing splenic cells than their WT counterparts. Furthermore, mice deficient in IL-17 (17KO) suffered higher parasitemia than the WT controls, indicating that IL-23-mediated protection is dependent on induction of IL-17 during infection. We found that macrophages were responsible for IL-17 production in response to IL-23. We observed a striking reduction in splenic macrophages in the p19KO and 17KO mice, both of which became highly susceptible to infection. Thus, IL-17 appears to be crucial for maintenance of splenic macrophages. Adoptive transfer of macrophages into macrophage-depleted mice confirmed that macrophage-derived IL-17 is required for macrophage accumulation and parasite eradication in the recipient mice. We also found that IL-17 induces CCL2/7, which recruit macrophages. Our findings reveal a novel protective mechanism whereby IL-23, IL-17, and macrophages reduce the severity of infection with blood-stage malaria parasites.

Keywords: IL-17 · IL-23 · Macrophage · Malaria · *Plasmodium berghei*

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Introduction

Malaria, caused by protozoan parasites of the genus *Plasmodium*, is still one of the most life-threatening infectious diseases. Two hundred million people are infected annually with malaria parasites while about a million people are believed to die every year from the disease [1]. Despite the urgent need for effective vaccines against malaria, progress on vaccine development has been disappointing [2]. A major obstacle for vaccine development is that the immune responses crucial for eradication of malaria

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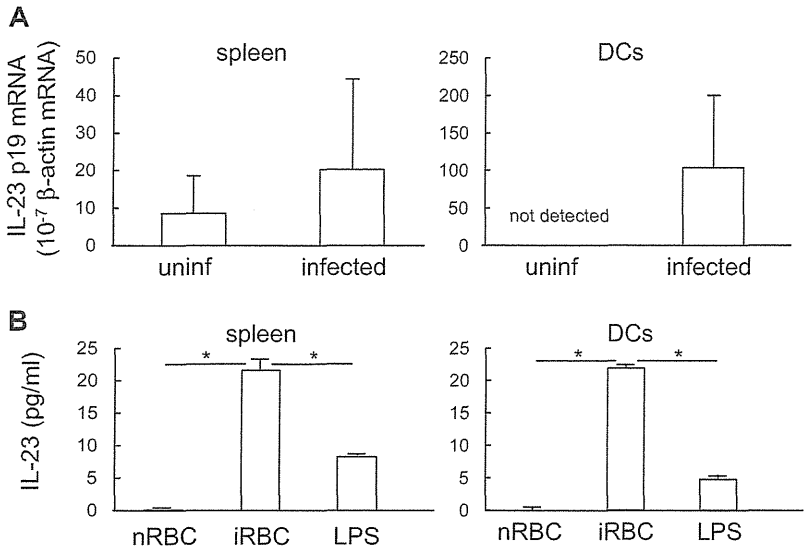

Figure 1. Induction of IL-23 during infection with *Plasmodium berghei* PbNK. (A) Spleen cells and purified splenic DCs from PbNK-infected mice were analyzed 8 days after infection for the expression of the IL-23 p19 subunit using quantitative real-time RT-PCR. (B) Spleen cells and purified splenic DCs were cultured with the indicated stimulants for 24 h, after which the IL-23 levels were measured by ELISA. Data are shown as mean + SD of three samples and are from one experiment representative of two performed. *$p < 0.01$, Student's t-test.

parasites are not fully understood. Another concern is that infection with *Plasmodium falciparum*, which causes the most severe form of the disease in young children and pregnant women, can lead to pathological inflammation resulting in severe complications, such as cerebral malaria. Thus, to succeed in developing an effective vaccine against malaria, it is crucial that the mechanisms underlying protective immunity as well as those that drive pathogenic responses are better understood.

IL-23 is a proinflammatory cytokine that belongs to IL-12 cytokine family. It is a heterodimeric molecule composed of p40 and p19 subunits [3]. The proinflammatory activities of IL-23 are responsible for the onset not only of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis [4, 5], but also infection-induced pathological consequences of infection with the pathogens causing Lyme disease and toxoplasma encephalitis, for example [6, 7]. The known biological roles for IL-23 in inflammation include induction of Th17-induced secretion of IL-17 [8] and suppression of $CD4^+CD25^+$ Treg [9]. In contrast, recent studies have reported that IL-23 confers resistance to infection, especially infection with extracellular pathogens [10]. IL-23-mediated induction of IL-17 from Th17 and γδ T cells activates granulocytes to kill bacteria and fungi [11, 12]. In addition, this cytokine restricts the growth of intracellular pathogens in an IL-17-dependent manner [13–15]. However, there are only a few reports about whether or not IL-23 plays a protective role against malaria parasites residing in red blood cells (RBCs).

IL-12, composed of two heterologous chains (p35 and p40), is known to confer protection against disease as well as causing host pathogenicity during infection with malaria parasites, which can be explained through the activation of IFN-γ-producing Th1 $CD4^+$ T cells [16–18]. These findings were observed in p40-lacking mice deficient in not only IL-12, but also IL-23 (the p40 subunit is shared between IL-12 and IL-23), raising a possibility that the phenomena attributed to IL-12 might in fact be due to IL-23. In the experiments described herein, we tested the role of IL-23 in malaria protection and pathogenesis using p19-deficient mice infected with the rodent malaria parasite *Plasmodium berghei* NK65 (PbNK). Our results show that IL-23 contributes, at least in part, to protection against malaria through the induction of IL-17 in macrophages.

Results

Protective roles for IL-23 during infection with PbNK

First, we investigated whether infecting mice with PbNK induced IL-23 expression. We found that whole spleen cells and splenic dendritic cells (DCs) obtained from mice infected with PbNK contained higher levels of the IL-23 p19 subunit mRNA than the uninfected control mice (Fig. 1A). Furthermore, splenocytes and splenic DCs from uninfected mice cultured with PbNK-infected RBCs produced substantial IL-23 protein in vitro (Fig. 1B), indicating that IL-23 is induced during infection with PbNK. To evaluate the ability of IL-23 to protect against malaria infection, we infected IL-23 p19-deficient (p19KO) mice with PbNK. Wild-type (WT) C57BL/6 mice infected with PbNK showed a gradual increase in their parasitemia and died after 1 month. PbNK infections in p19KO mice, however, resulted in higher parasitemia during the early phase of their infections, and their survival was significantly shorter than the WT mice (Fig. 2). These results show that lack of IL-23 partially impairs resistance to infection with PbNK.

Previous studies have reported that one of the proinflammatory effects of IL-23 is due to suppression of Treg [9]. Our previous findings that activation of Treg is required for immune evasion during malaria [19, 20] suggest that Treg suppression might be an important factor for IL-23-mediated protection. However, this appears unlikely because we found that activation of Treg was similar in both p19KO and WT mice following infection with PbNK

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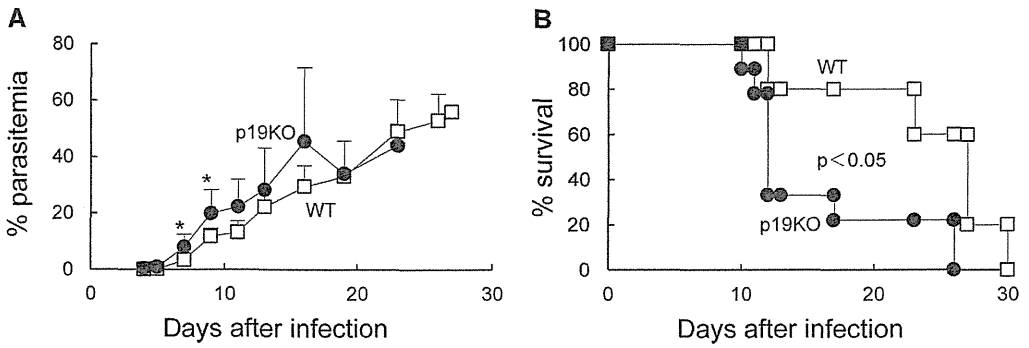

Figure 2. Course of infection of PbNK in p19KO mice. (A) Daily parasitemia and (B) survival rates were monitored in WT (open squares) and p19KO mice (closed circles). Parasitemia was calculated based on microscopic observations of Giemsa-stained thin blood films. WT and P19KO groups comprised five and nine mice, respectively. *$p < 0.05$, Student's t-test. Data are shown as mean + SD and are from one experiment representative of four performed.

(as assessed by the proportion of $CD25^+foxp3^+$ cells in $CD4^+$ cells, and by the degree of suppression of T-cell proliferation after TCR signaling (Supporting Information Fig. 1)).

Induction of IL-17 in macrophages in an IL-23-dependent manner

We next investigated the possibility that IL-23 plays a protective role in malaria infection via production of IL-17, as suggested by many studies that have described IL-17-dependent protection of IL-23 [8]. We found that PbNK-infected WT mice had increased levels of IL-17 transcripts and IL-17-producing cells in their spleens, compared with the uninfected controls (Fig. 3A and B and Supporting Information Fig. 2). In contrast, the PbNK-infected and uninfected p19KO mice had similar IL-17 transcripts levels and numbers of IL-17-producing cells (Fig. 3A and B and Supporting Information Fig. 2). These results clearly show that IL-17 is produced in an IL-23-dependent manner during PbNK infection, thus leading us to evaluate the contribution of this cytokine in protection against PbNK disease. IL-17-deficient (17KO) mice infected with PbNK had significantly higher parasitemia (Fig. 3C), and shortened survival rates than the WT mice (Fig. 3D), as was also observed in p19KO mice, suggesting that secretion of IL-17 could explain most of the protective role for IL-23. However, lack of IL-17 attenuated protection from death to a lesser degree compared with lack of IL-23 as statistical significance in survival was not observed in 17KO mice, suggesting that IL-23 plays a different role than IL-17. In sharp contrast, production of IFN-γ was not impaired in the absence of IL-23 (Supporting Information Fig. 3), indicating that this cytokine is not involved in IL-23-dependent protection.

Many types of immune cells including T cells, natural killer (NK) cells, γδ T cells, and macrophages are known to produce IL-17 [21–26]. Hence, we sought to investigate the identity of IL-17-producing cells induced in response to infection with PbNK. Neither IL-17-producing $CD4^+$ cells nor IL-17-producing $CD3^+$ cells were found even in the presence of IL-23 after infection (Supporting Information Fig. 4). NK and γδ T cells producing IL-17 could not be detected, regardless of infection (data not shown). IL-17-producing cells were found in the $CD3^-$ fraction (Supporting Information Fig. 4), suggesting that nonlymphoid cells were produced. Further investigations revealed that $CD11b^+$ myeloid cells produced IL-17 after infection with PbNK in WT, but not p19KO mice (Fig. 4A and B). Among the myeloid cells, $F4/80^+$ macrophages expressed the IL-23 receptor (Fig. 4C), although the IL-23 receptor was predominantly expressed on $CD11b^-$ cells, presumably T cells. Finally, splenic $F4/80^+$ macrophages isolated from uninfected mice produced a larger amount of IL-17 than whole spleen cells in the presence of recombinant IL-23 (Fig. 4D). These results clearly show that macrophages are responsible for IL-17 production in an IL-23-dependent manner in mice infected with PbNK.

Macrophages recruitment in response to macrophage-derived IL-17 production via the CCL2/7 pathway

We next explored the mechanisms by which IL-17 protects against malarial disease. As shown in Figure 4A and B, a dramatic reduction in the number of $CD11b^+$ cells in p19KO mice occurred irrespective of IL-17 production. To investigate the possibility that macrophages (which are crucial for eradication of malaria parasites [27, 28] via ingesting and digesting parasitized RBCs) are involved in IL-23-dependent protection, we conducted a detailed analysis of such cells. In the absence of infection, comparable numbers of macrophages within p19KO and 17KO mouse spleens were identified. Whereas splenic $CD11b^+$ and $CD11b^+$ $F4/80^+$ cells in the PbNK-infected WT mice were gradually increased, PbNK infection in the mutant mice caused a remarkable reduction in the numbers of $CD11b^+$ macrophages and $F4/80^+$ cells, especially at 8 days after infection (Fig. 5).

To assess the importance of macrophages and their production of IL-17 in resistance to malaria, mice previously treated with clodronate liposomes (C/L), a specific depletor of macrophages [29], received adoptive transfer of WT macrophages or those deficient in IL-17. Reduction of splenic macrophages by C/L treatment was about 70% in number (Fig. 6A), and recipient mice

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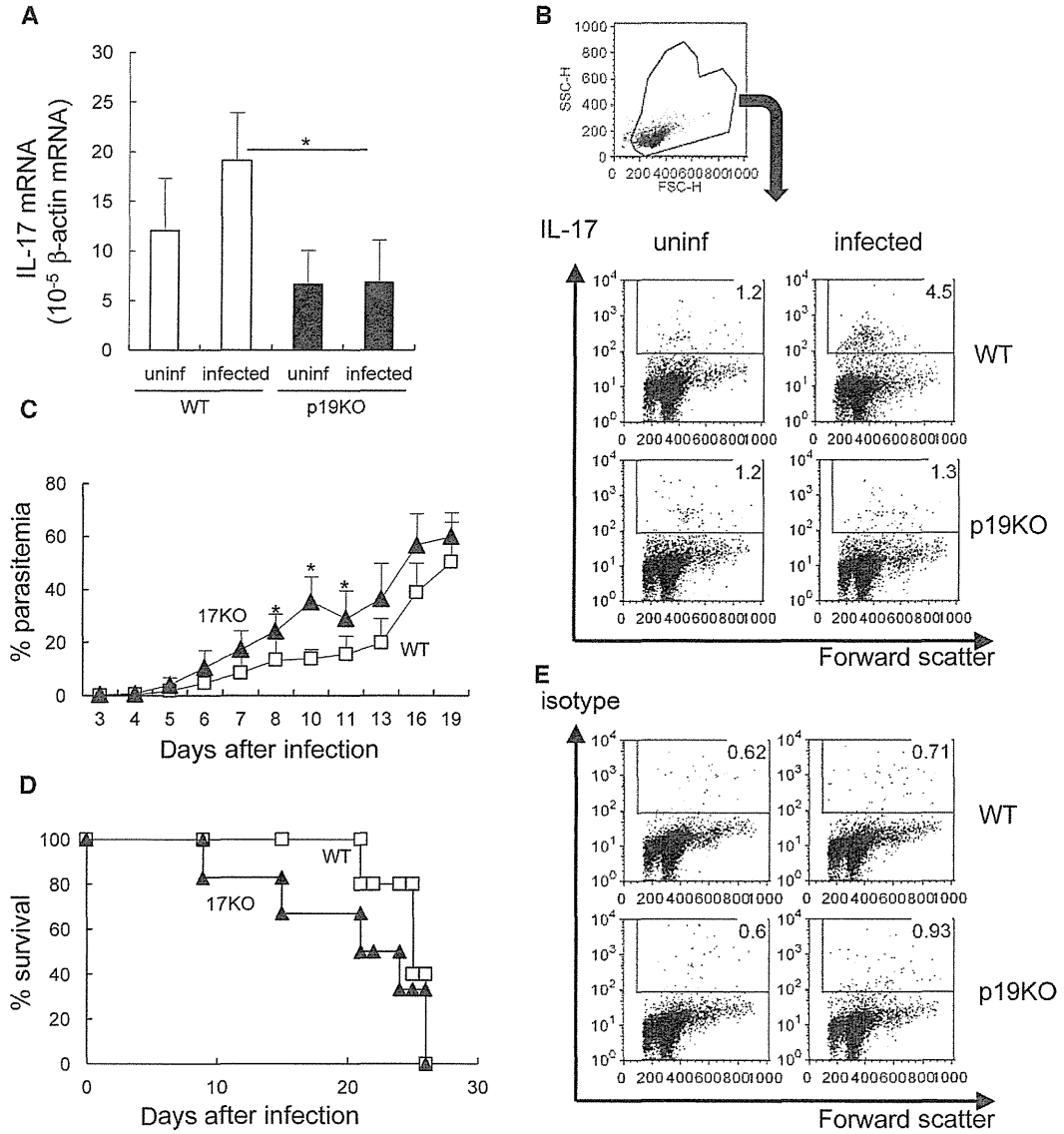

Figure 3. Contribution of IL-17 in the protective role of IL-23. (A) RNA samples extracted from spleen cells from WT and p19KO mice 8 days after infection with PbNK were analyzed for expression of IL-17 mRNA as described in Figure 1A. *$p < 0.05$, Student's t-test. (B) Production of IL-17 in response to PbNK infection was analyzed in spleen cells from WT and p19KO mice 8 days after infection. Cells were stained with anti-IL-17 (upper panels) or isotype control (lower panels). Numbers indicate the percentage of IL-17$^+$ cells in the total number of splenocytes. The gating strategy is shown (top panel). The (C) parasitemia and (D) survival ratio of the IL-17KO mice were evaluated as described in Figure 2. (A, C, and D) Data are shown as mean + SD of five to six mice and are from one experiment representative of two performed.

treated with C/L had higher parasitemia and significantly higher mortality rates (Fig. 6B and C) compared with the untreated control mice, confirming that macrophages contribute to protection against malaria. WT macrophage transfer into C/L-treated recipient mice enabled slight recovery of macrophages and conferred partial resistance to PbNK, as judged by the presence of comparable parasitemia and survival rates in the control mice (Fig. 6B and C). However, transfer with IL-17-deficient macrophages did not increase the macrophage, resulting in a failure to inhibit parasitemia and death in the C/L-treated mice (Fig. 6B and C). These results clearly indicate that recruitment or accumulation of macrophages during infection with PbNK plays a crucial role in protection against death and is dependent at least on macrophage-producing IL-17. We also evaluated the possibility that IL-23/IL-17 affects not only quantification but also quality of macrophages. Infection with PbNK activated macrophages in terms of expression of MHC class II molecules indifference of presence or absence of IL-17 or IL-23 (Supporting Information Fig. 5), suggesting that the number of macrophages is important for IL-23-dependent protection.

Finally, we addressed how IL-17 affects the number of macrophages present in the spleen. To increase macrophage

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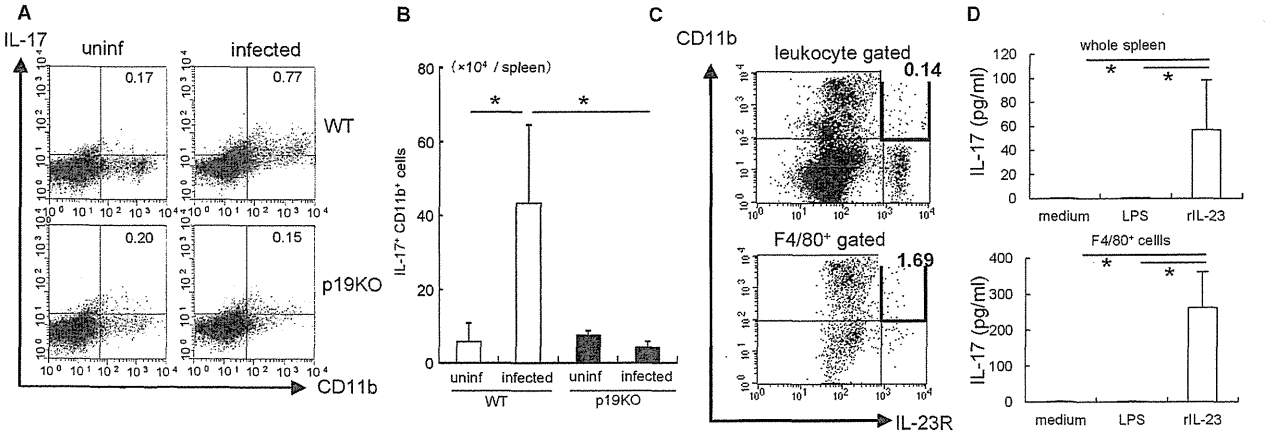

Figure 4. Splenic macrophages analyzed for production of IL-17 in response to IL-23 during PbNK infection. (A) Spleen cells from WT and p19KO mice 8 days after infection with PbNK were stained with anti-IL-17 and anti-CD11b, and the percentages of IL-17-producing CD11b$^+$ cells in whole spleen determined. (B) The number of IL-17-producing CD11b$^+$ cells in whole spleen was calculated based on flow cytometeric analysis. Data are shown as mean + SD of three mice from one experiment representative of two performed. *$p < 0.05$, Student's t-test. (C) Spleen cells from uninfected WT mice were stained with anti-CD11b, anti-F4/80, and anti-IL-23R. Percentages of CD11b$^+$IL-23R$^+$ cells in whole spleen (upper panel) and F4/80$^+$ cells (bottom panel). (D) Spleen cells (upper panel) and purified F4/80$^+$ cells (bottom panel) were cultured with LPS (100 ng/mL) or recombinant IL-23 (50 ng/mL) for 24 h, after which the supernatants were analyzed for IL-17. Data are shown as mean + SD from three cultures. All experiments were repeated at least twice with similar results.

accumulation during PbNK infection, generation and/or recruitment of macrophages must be enhanced. IL-17 is reported to induce production of GM-CSF and CCL2 and CCL7 chemokines [30–32]. The former chemokine stimulates progenitor cells to proliferate and differentiate into granulocytes and macrophages, while the latter attracts macrophages to the expression site. Thus, we analyzed the expression pattern of such molecules in mice infected with PbNK. We found that infection with PbNK decreased expression of GM-CSF mRNA, whereas IL-23- or IL-17-deficiency did not affect GM-CSF expression levels in infected mice (Fig. 7A). A striking induction in expression of CCL2 and its related chemokine CCL7 was observed in WT mice in response to infection with PbNK (Fig. 7B and C). This chemokine enhancement was never observed in the p19- and IL-17-deficient mice. Involvement of these macrophage-recruiting chemokines was assessed by using RS504393, a highly specific antagonist against the CCR2 receptor for CCL2 and CCL7 not the CXCR1, CCR1, or CCR3 [33]. Infection of vehicle-treated WT mice with PbNK increased the number of macrophages in their spleens (Fig. 7D). Mice treated with RS504393 exhibited a remarkable decrease in the number of macrophages after PbNK infection (Fig. 7D). Moreover, these mice suffered from higher parasitemia and mortality than the control mice (Fig. 7E and F). These results clearly show that CCL2/7 is critical for maintenance of macrophages, and is important, therefore, for IL-23- and IL-17-dependent protection against PbNK.

Discussion

This study has revealed the IL-17-dependent protection conferred by IL-23 against severe malarial disease in a rodent model of infection. Both p19KO and 17KO mice were more susceptible to the type of pathology associated with PbNK infection. In addition, a loss of splenic macrophages crucial for eradication of parasitized RBCs was observed in such mice. Our preliminary experiments have also shown that IL-23 contributes to protection against other rodent malaria parasite species and strains, such as the *P. berghei* XAT strain (derived from PbNK following irradiation) and *Plasmodium yoelii* 17XL (data not shown). In contrast, we reported that protection against severe pathology in *P. berghei* ANKA infections was independent of IL-23, which was not even produced during such infections [34], thus indicating that the role IL-23 plays in host immune responses might depend on the species and strain of malaria parasite.

IL-23 is presumably produced by DCs in response to parasitized RBCs. Although we did not address how DCs recognize such cells, Toll-like receptor (TLR) signals appear to be important for induction of IL-23 [35, 36]. It would be interesting to investigate whether the strain/species dependency of IL-23-mediated disease protection is attributable to quantitative and/or qualitative differences in TLR ligands, something we have previously suggested accounts for the difference in TLR9 ligands between an avirulent and virulent strain of *P. yoelii* [20].

From a pathological perspective, it is quite possible that IL-23 causes pathogenesis in malaria for the following reasons. First, the related cytokine, IL-12, contributes more toward disease pathology, liver injury specifically, than disease protection in mice infected with PbNK [17, 18]. Second, IL-23 is reported to induce liver damage from causes other than malaria such as hepatic ischemia/reperfusion injury or hepatitis B [37, 38]. However, our results showed that liver injury induced in mice infected with PbNK occurred even in the absence of IL-23. Infection of p19KO mice with PbNK increased the level of serum alanine

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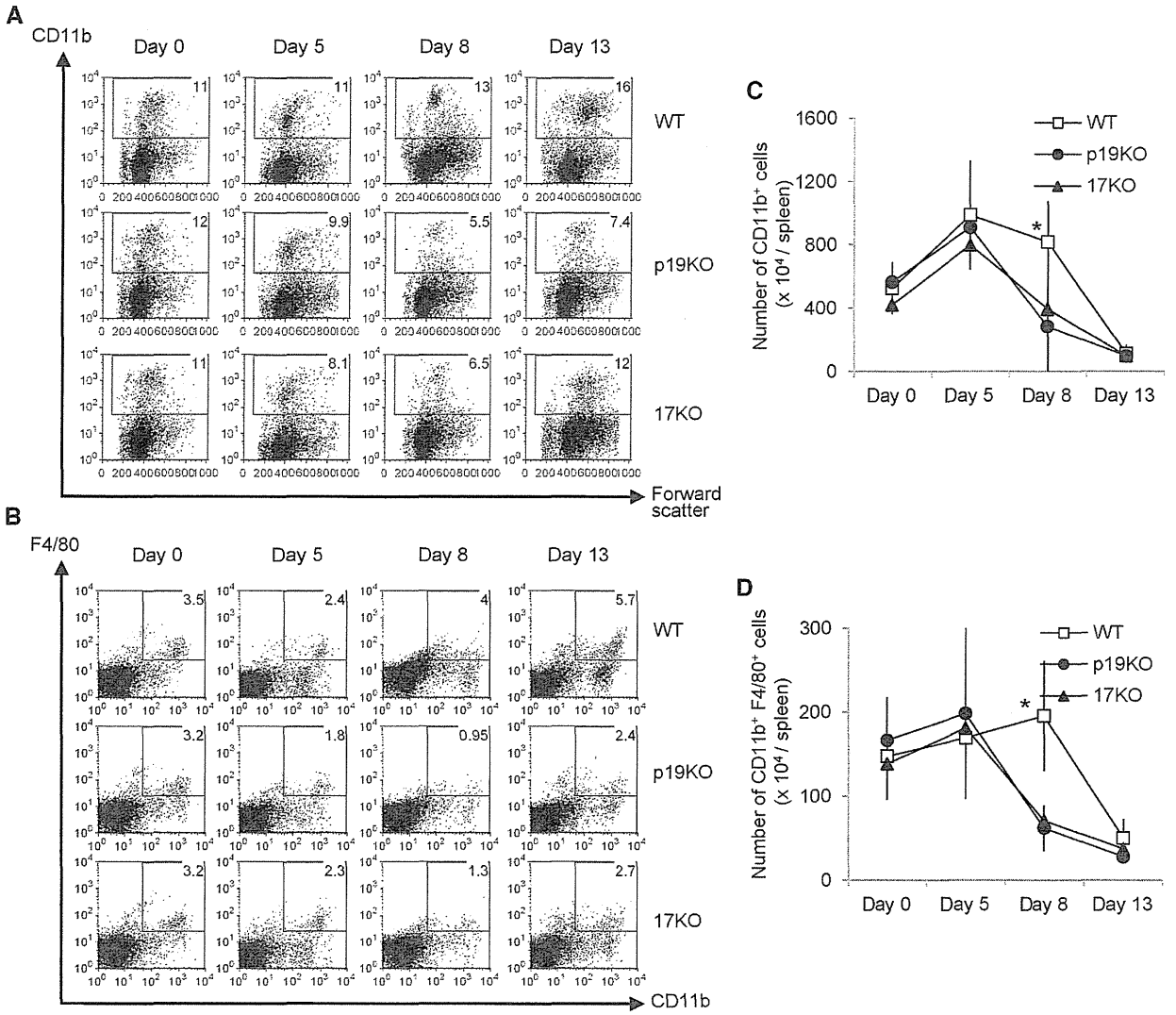

Figure 5. Macrophage numbers in IL-17KO and p19KO mice after infection with PbNK. Spleen cells from WT, IL-17KO, and p19KO mice were stained with anti-CD11b and anti-F4/80 antibodies after the indicated days after infection. The percentages of (A) $CD11b^+$ and (B) $CD11b^+F4/80^+$ cells in whole spleen are indicated. The numbers of (C) $CD11b^+$ splenic macrophage and (D) $CD11b^+$ $F4/80^+$ cells in whole spleen were calculated based on flow cytometry analysis. Data are shown as mean ± SD from three to six mice from one experiment representative of three performed. *$p < 0.05$, Student's t-test.

transaminase released from their hepatocytes, an indicator of liver injury (Supporting Information Fig. 6A). Furthermore, IL-23-deficient mice infected with PbNK had comparable numbers of cells secreting IFN-γ, crucial for IL-12-mediated liver injury, as their WT counterparts (Supporting Information Fig. 3). Thus, we suggest that IL-23 is not involved in malaria-related pathogenesis, unlike IL-12, which shares the p40 subunit. In addition, our previous study on experimental cerebral malaria revealed pathological consequences in C57BL/6 mice infected with *P. berghei* ANKA that developed in the absence of IL-23 [34].

The protective effects of IL-23 are largely attributed to induction of IL-17 and activation of Th17, γδ T, NK, and NK T cells to produce this cytokine [21, 23, 24]. In these experiments, production of IL-17 may account for much of the protection that IL-23 confers against malaria, as has been seen with other infectious diseases. However, this is the first report that macrophages produce IL-17 in response to IL-23. Indeed, macrophages expressed the IL-23 receptor and produced IL-17 when stimulated with recombinant IL-23 in vitro (Fig. 4B and C). Furthermore, the increase of IL-17-producing $CD11b^+$ cells after infection with PbNK was diminished in p19KO mice (Fig. 4A). While recent studies have reported that macrophages produce IL-17 [22, 25, 26], whether the IL-17-producing macrophages contribute to protection against infections remains to be determined. In this study, we clearly demonstrated that adoptive transfer with macrophages from 17KO

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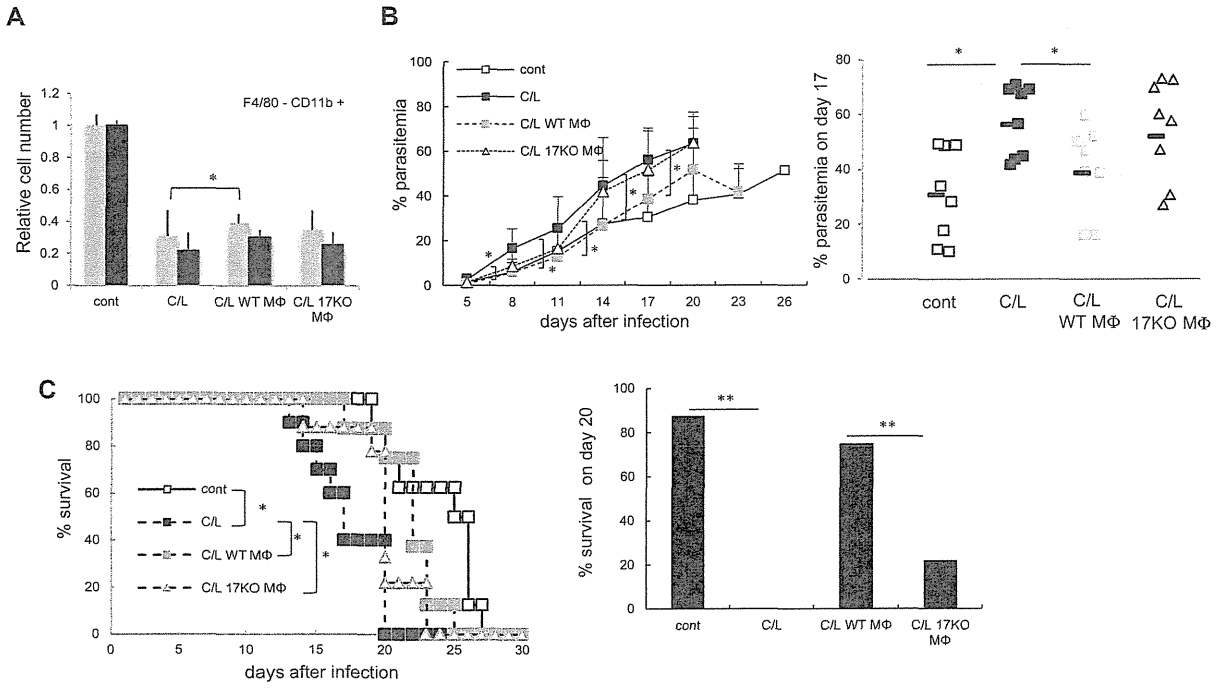

Figure 6. Reconstitution of macrophages in WT mice treated with clodronate-liposome (C/L). (A) Spleen cells from control mice treated with liposomes alone, mice treated with C/L, mice reconstituted with WT macrophages, and those reconstituted with IL-17-dificient macrophages were analyzed for macrophages as described in Fig. 5. Data are shown as mean + SD from three mice. (B) Mice in (A) were infected with PbNK and their parasitemia measured as described in Fig. 2 (left panel), and parasitemia of individual mice on day 17 was plotted (right panels). Each group consisted of eight to ten mice and data are shown as the mean ± SD from one experiment representative of two performed. $^{*}p < 0.05$, Student's t-test. (C) A survival curve (left panel) and percent survival on day 20 (right panel) after infection with PbNK in WT- and C/L-treated mice were indicated. $^{*}p < 0.05$, Kaplan–Meier log rank test (left) and $^{**}p < 0.001$ using a χ^2 test (right).

mice was not sufficient to recover the attenuated protection in mice treated with C/L (Fig. 6), thus determining the relative contribution of macrophage-derived IL-17 in protection.

Although macrophage-derived IL-17 seems a major effector of IL-23-mediated protection, it is suggested the existence of IL-17-independent protection because p19KO mice showed a worse course of infection than 17KO mice. One possibility is that IL-23 protects against liver pathology and/or early death. In agreement with serological results that tendency of higher ALT level in p19KO mice was observed, histopathological examinations revealed that infection of p19KO mice with PbNK developed obvious lesions in liver as early as 8 days after infection when no apparent lesions in WT mice were observed (Supporting Information Fig. 6B). This failure to protect against liver damage in p19KO mice could account for early death of these mice, as it is possible that IL-17 and/or IL-23 induce IL-22, which has recently been reported to be important for liver damage in rodent malaria [39]. Alternatively, liver injury is easily induced in these mice showing higher parasitemia due to attenuated protection because immunopathology is partially dependent on parasite burden [40]. It would be of great interest to investigate these issues in detail.

A major role for IL-17 is to recruit neutrophils that have protective roles against bacterial infections [10, 13]. The number of Gr-1$^+$ cell (mainly neutrophils) in both p19KO and 17KO mice before and after PbNK infection was much less than that observed in WT mice (Supporting Information Fig. 7). However, a protective role for neutrophils in malaria is supposed to be less important [41]; therefore, the decrease in neutrophils is hard to explain on the basis of the impaired resistance of these mice to malaria. In contrast, macrophages are known to be important for protection against blood-stage malaria as they phagocytose and digest parasitized RBCs [27, 28]. While there was increase or a slight loss of splenic macrophages in the WT mice after PbNK infection, splenic macrophages from mutant mice infected with PbNK were markedly reduced (Figs. 5 and 7D). This reduction might have been a result of host defense mechanisms against malaria, because some macrophage populations that are apt to phagocytose parasitized RBCs were immunologically eliminated in the spleen [42]. Thus, a dependable supply of macrophages must be critical for the eradication of blood-stage malaria parasites. Our results demonstrate that IL-17 from macrophages attracted macrophages in a CCL2/7-dependent manner, which promoted a continuous supply of macrophages to the spleen. Indeed, IL-17 has been reported to induce CCL2 in fibroblasts, thus recruiting monocytes [30]. Then, CCL2 may attract CCR2-expressing macrophages as a CCR2 antagonist affects macrophage number and protection (Fig. 7). This chemokine receptor has been reported to play an important role in control infection with *Plasmodium chabaudi* by recruiting

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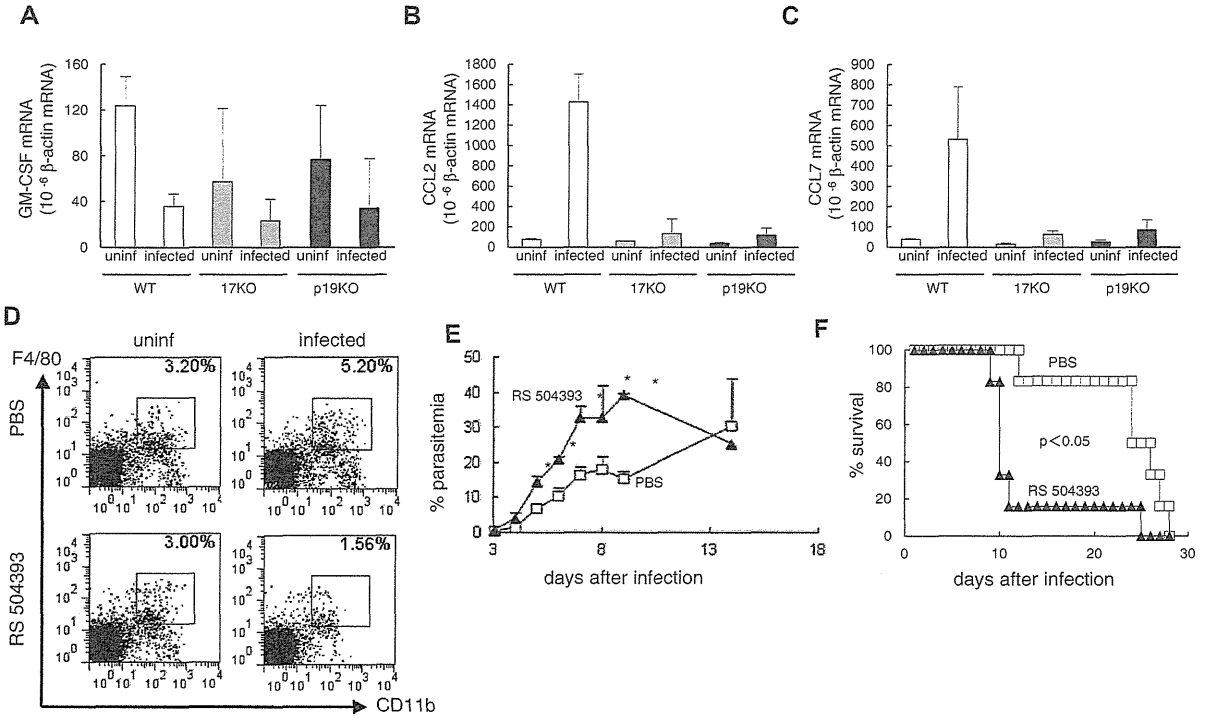

Figure 7. Involvement of macrophage chemokines in IL-23- and IL-17-dependent protection against PbNK. (A–C) Spleen cells from WT, IL-17KO, and p19KO mice were analyzed for mRNA encoding. (A) GM-CSF, (B) CCL2, and (C) CCL7 before and 8 days after infection. Data are shown as the mean + SD of three mice from one experiment representative of two performed. Mice administered with the CCL2-specific antagonist RS504393 (closed triangles) and with PBS (open squares) were (D) evaluated for splenic macrophages 8 days after infection and (E, F) the course of infection with PbNK as described in Figs. 2 and 5, respectively. Data are shown as mean + SD of eight mice from one experiment representative of two performed. *$p < 0.05$, Student's t-test.

monocytes [43]. In this report, CCR2-deficient mice showed higher parasitemia and transfer with WT CD11c$^+$Ly6C$^+$ monocytes reduces parasitemia. Thus, CCL2–CCR2 interactions on monocytes contribute to protection against malaria. Although we did not address what cells produce CCL2, macrophages from WT mice after infection with PbNK accumulated CCL2 mRNA (Supporting Information Fig. 8), suggesting that macrophages might produce CCL2 during malaria.

However, reduction of macrophages in mice treated with a CCR2 antagonist was less than in p19KO and 17KO mice, suggesting that IL-23/IL-17 maintains macrophage number via CCL2-independent fashion. In spite of less reduction of macrophages, mice treated with a CCR2 antagonist showed obvious shortening of survival and progression of parasitemia compared with the mutant mice, which might not deny importance of macrophage recruitment. This discrepancy is presumably due to lack of accumulation of other immune cells expressing CCR2 than macrophages, such as T cells [44]. Thus, the chemokine antagonism might affect the course of infection more than lack of IL-23 or IL-17 did.

Taken together, our data clearly demonstrate, for the first time, the protective role that IL-23 plays against the severity of malarial disease. IL-23 induces IL-17 production in macrophages, which in turn stimulates macrophages to produce CCL2/7 in a probable autocrine fashion. Thereafter, macrophage recruitment might work to effectively eliminate malaria parasites. This mode of protection is quite novel in the IL-23/IL-17 axis of inflammation. As human malaria parasites also induce IL-23 [45], our findings provide insight into the immune mechanisms that are important to take into account malaria vaccine development or therapeutic strategies based upon IL-23.

Materials and methods

Mice and parasites

Female C57BL/6 mice were purchased from SLC (Hamamatsu, Japan). Age- and sex-matched groups of WT, IL-23 p19 subunit (p19) KO, and IL-17A (17) KO mice were used for these experiments. Mutant mice on a C57BL/6 background had been backcrossed for at least 15 generations. All experiments that involved mice were reviewed and approved by the Committee for Ethics on Animal Experiments in the Graduate School of Gunma University (approved number 12–031), and were conducted under the control of the Guidelines for Animal Experiments in the Graduate School of Medicine, Gunma University, and the Low

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(No. 105) and Notification (No. 6) of the Japanese Government. Blood-stage PbNK parasites were a generous gift from Dr. M. Torii (Ehime University, Japan); the parasites were obtained after fresh passage through a donor mouse, 2–3 days after inoculation from frozen stock. Mice were infected with 50 000 parasitized RBC via i.p. injection.

Cell purification and culture

Single-cell suspensions were prepared from mouse spleens. To purify macrophage and DCs, spleen cells were stained with anti-CD16/32 (eBioscience, San Diego, CA, USA), then with fluorescein isothiocyanate (FITC) anti-F4/80, and FITC-anti-CD11c, respectively, followed by incubation with anti-FITC microbeads (Miltenyi Biotec K. K., Tokyo, Japan). The cell populations obtained had purities between 85 and 91% by flow cytometry. Such positively selected cells were cultured with purified parasitized RBCs (5×10^5/well), LPS (1000 or 100 ng/mL) (Sigma, St. Louis, MO, USA), or recombinant IL-23 (50 ng/mL) (R&D Systems, Minneapolis, MN, USA) in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 μg streptomycin (complete medium).

Real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA extracted from purified cells was reverse-transcribed into cDNA. mRNA encoding the genes of interest was quantified by evaluating SYBR Green dye incorporation (Takara, Tokyo, Japan) using a real-time PCR GeneAmp7000 thermal cycler system (Applied Biosystems, Foster City, CA, USA). PCR was performed according to the manufacturer's protocols. The expression level of each target gene was quantified from the difference in the cycle threshold (Ct) between the β-actin-encoding gene and the target genes using the following formula: Ct = Ct target gene – Ct β-actin gene. The PCR primer sequences used are as follows: IL-23p19, 5′-TGGCTGTGCCTAGGAAGTAGCA-3′ and 5′-TTCATC CTCTTCTTCTCTTAGTAGATTCATA-3′; IL-17, 5′-TCATCTGTGTC TCTGATGCTGTTG-3′ and 5′-TCGCT GCCTTCACTGT-3′; IFN-γ, 5′-AGCGGCTGACTGAACTCAGATTGTAG-3′ and 5′- GTCACAGTT TTCAGCTGTATAGGG-3′; GM-CSF, 5′-AGATATTCGAGCAGGGTC TAC-3′ and 5′- GGGATATCAGTCAGAAAGGTT-3′; CCL2, 5′-ACTG AAGCCAGCTCTCTCTTCCTC-3′ and 5′- TTCCTTCTTGGGGTCAG CACAGAC-3′; CCL7, 5′- GCATGGAAGTCTGTGCTGAA-3′ and 5′-AGAAAGAACAGCGGTGAGGA-3′ and β-actin, 5′-TGGAATCC TGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACA GTCCG-3′.

Cytokine ELISA

IL-23 and IL-17A cytokine levels in culture supernatants from macrophages and DCs were measured using standard sandwich ELISA accordingly to the manufacturer's protocols (IL-23, R&D Systems; IL-17A, eBioscience).

Flow cytometry

Spleen cells were stained with a combination of fluorochrome-labeled antibodies (eBioscience). The IL-23 receptor was stained with an anti-IL-23R rabbit antibody (Millipore, Temecula, CA, USA) and a FITC-labeled anti-rabbit IgG goat antibody (Rockland, Gilbertsville, PA, USA). For intracellular staining, spleen cells were stimulated with 50 ng/mL phorbal myristate acetate (PMA) and 500 ng/mL calcium ionophore in the presence of Golgi plug (BD Bioscience, San Jose, CA, USA) for 4 h. The surface of the cells was stained with antibodies, after which the cells were fixed and permeabilized with BD Cytofix/Perm (BD Bioscience). Finally, the cells were stained with PE-anti-IL-17 or PE-anti-IFN-γ antibodies then analyzed using a FACSCalibur cytometer and FACSAria flow cytometer (BD Bioscience). Data were analyzed using CellQuest Pro (BD Bioscience) and Flowjo software (Treestar, Ashland, OR, USA).

Depletion, reconstitution, and in vivo macrophage migration inhibition assays

Mouse macrophages were depleted by intravenously administering liposomes containing clodronate (Sigma) (1.5 mg/300 μL per mouse) as previously described [29,46]. Four days later, the same mice were transfused with 1×10^6 cultured macrophages derived from the BMs of WT and 17KO mice in the presence recombinant GM-CSF (Peprotech) and infected with PbNK. A highly selective chemokine antagonist for CCR2, RS504393 (Tocris, Ellisville, MO, USA), was administered orally to the mice (2 mg/kg, twice a day).

Histopathology

Livers were removed on 8 days after infection with PbNK and fixed in 4% paraformaldehyde. Fixed tissues were paraffin embedded and sectioned before being stained with H&E. Slides were analyzed using a BIOREVO BZ-9000 microscope (KEYENCE, Osaka, Japan). Data were analyzed with BZ-II software (KEYENCE).

Statistical analyses

Statistical differences between the different experimental groups were evaluated using a two-tailed unpaired Student's *t*-test. For survival curves, Kaplan–Meier plots and χ^2 tests were performed. p values < 0.05 were considered statistically significant.

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Abbreviations: **C/L:** clodronate liposomes · **PbNK:** *Plasmodium berghei* NK65

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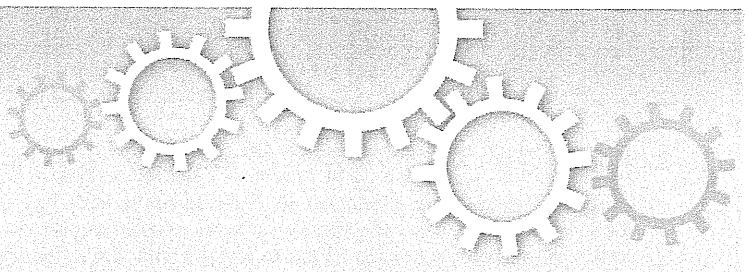

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CD8$^+$ T cell activation by murine erythroblasts infected with malaria parasites

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Recent studies show that some human malaria parasite species *Plasmodium falciparum* and *P. vivax* parasitize erythroblasts; however, the biological and clinical significance of this is unclear. To investigate further, we generated a rodent malaria parasite (*P. yoelii* 17XNL) expressing GFP-ovalbumin (OVA). Its infectivity to erythroblasts was confirmed, and parasitized erythroblasts were capable of initiating malaria infections. Experiments showed that MHC class I molecules were highly expressed on parasitized erythroblasts. As CD8$^+$ T cells recognize MHC class I and peptide complexes on target cells, and are involved in protection or pathology against malaria, we examined whether erythroblasts are targeted by CD8$^+$ T cells. Purified non-parasitized erythroblasts pulsed with OVA peptides were recognized by OVA-specific CD8$^+$ T cells. Crucially, parasitized erythroblasts isolated from GFP-OVA-, but not GFP- infected-mice, activated OT-I CD8$^+$ T cells, indicating that CD8$^+$ T cells recognize parasitized erythroblasts in an antigen-specific manner.

Malaria occurs throughout tropical and subtropical zones with 40% of the world's population at risk from the disease. Malaria affects approximately 300–500 million people, killing 1–3 million of them each year[1,2]. Malaria symptoms are caused by parasite multiplication within host erythrocytes and erythrocyte destruction causes anemia, one of the main clinical manifestations of the disease. Elimination of parasitized and non-parasitized erythrocytes can also occur via activation of the reticuloendothelial system[3,4] and acute malaria induces bone marrow suppression, resulting in a reduction in hematopoietic efficiency[5–7].

Malaria parasites normally parasitize mature erythrocytes and/or reticulocytes. Recently, however, both *P. falciparum* and *P. vivax* have been shown to invade erythroblasts *in vitro*[8,9], and parasitized erythroblasts were found within the bone marrow of patients with vivax malaria[10]. Infection of erythroblasts with malaria parasites is thought to cause anemia. However, the biological significance and pathological consequences of erythroblast infection by *Plasmodium* spp. remains unclear.

Little is known about erythroblast parasitism in rodent malaria parasites. We evaluated such parasitism using a mouse malaria model, through use of transgenic rodent malaria parasites that constitutively express GFP and OVA. Using fluorescence microscopy and flow cytometry, we confirmed that nucleated erythrocytes were parasitized in the bone marrow and spleen, where persistent hematopoiesis occurs in adult mice. The parasitized cells were infectious to malaria naive mice, and we found that the erythroblasts expressed substantial levels of MHC class I molecules, both before and after malaria infection. We also showed that erythroblasts pulsed with an antigenic epitope were recognized by specific CD8$^+$ T cells. Finally, we demonstrated that parasitized erythroblasts were recognized by CD8$^+$ T cells in an antigen-specific manner. These results are the first to demonstrate that rodent malaria parasites can parasitize erythroblasts and activate CD8$^+$ T cells. Our findings indicate that erythroblast parasitism might be a common feature of the host-parasite relationship in malaria.

Results

Generation of malaria parasites expressing GFP. We successfully generated a *P. yoelii* 17XNL line (PyNL) expressing GFP (PyNL-GFP) using a *Plasmodium* artificial chromosome (PAC) to investigate erythroblast parasitism in this rodent malaria line. GFP is expressed under the control of the elongation factor promoter

(Fig. 1a), which enables malaria parasites to express GFP during all of the erythrocytic stages of an infection. GFP expression in the transgenic parasite was detected by fluorescence microscopy in TER119$^+$ erythrocytes parasitized with mononuclear trophozoites and multinuclear schizonts (Fig. 1b, upper and lower panels, respectively). The parasite growth kinetics in mice infected with the transgenic parasite was similar to that observed for the wild-type parasite infections. The peak parasitemias and infection recovery times were comparable in both groups of mice (Fig. 1c), indicating that parasite pathogenicity was not adversely affected by genetic manipulation.

Detection of erythroblasts. Prior to examining PyNL parasitism of erythroblasts, we analyzed the phenotypic characteristics of erythroblasts to facilitate optimal detection. Almost all of the anucleate erythrocytes were removed after lysis in the peripheral blood, and the remaining lysis-resistant cells in the bone marrow were identified as nucleated cells. Thirty percent of the nucleated cell population consisted of TER119$^+$ cells (Supplementary Fig. 1a), which expressed CD71 (transferrin receptor), CD44 (adhesion molecule)[11] and MHC class I molecules. These molecules are typically expressed in erythroblasts, demonstrating that, after lysis, the TER119$^+$ cells were erythroblasts (Supplementary Fig. 1b).

Hematopoiesis occurs in both the bone marrow and spleen, even in adult mice. As the spleen plays important roles in eliminating parasitized erythrocytes and has pathological consequences for a malaria infection, we investigated the erythroblasts within this organ. CD44$^+$/MHC class I$^+$ erythroblasts were found within splenic tissue even after lysis, although their frequencies were low (Supplementary Fig. 1c, d). It should be noted that a substantial amount of TER119$^+$ nucleic acid$^+$ cells were present, even before lysis; these may have existed as lysis-susceptible cells and reticulocytes immediately after denucleation in the bone marrow or spleen. Induction of hemolytic anemia by administrating phenylhydrazine (PHZ), was found to increase the number of erythroblasts within the spleen possibly due to enhanced hematopoiesis (Supplementary Fig. 1c, d), suggesting that erythroblast numbers might increase during anemia-causing malaria attacks.

To confirm that the TER119$^+$ cells were erythroblasts and not reticulocytes (after lysis), fluorescence microscopic analysis was

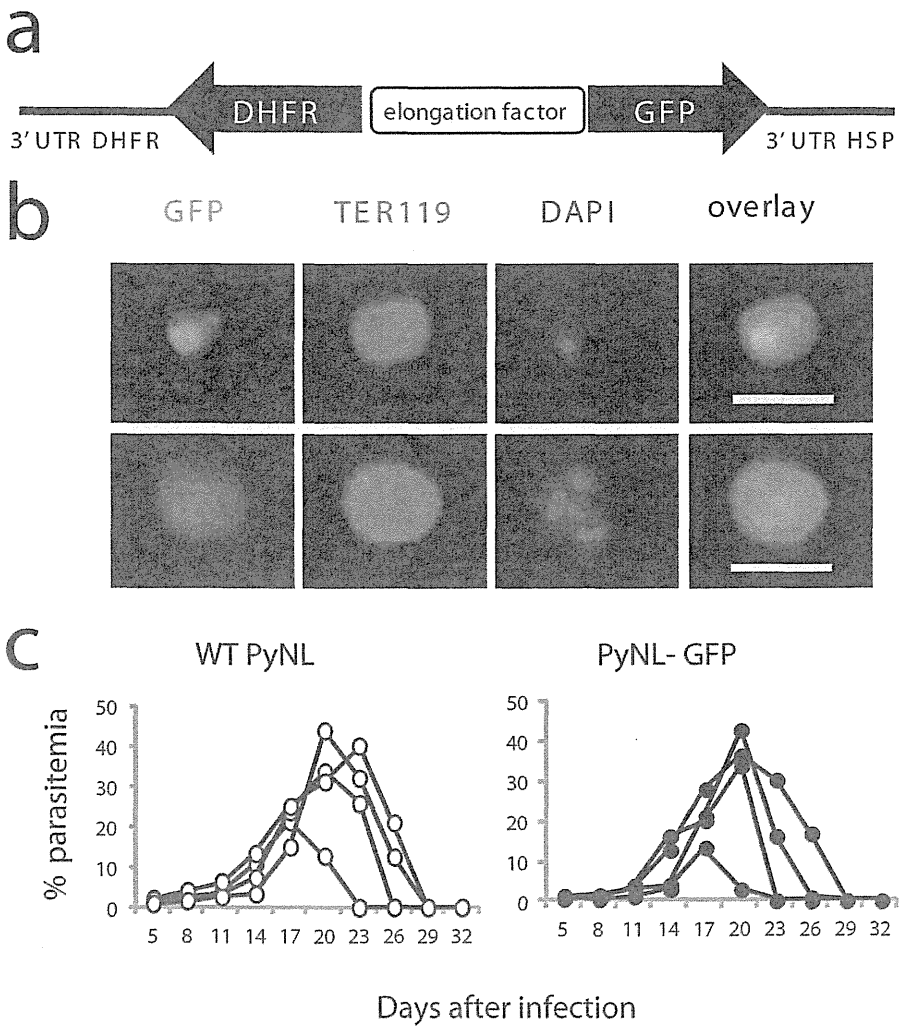

Figure 1 | Generation of recombinant *Plasmodium yoelii* 17XNL. (a) Construction of the *Plasmodium* artificial chromosome (PAC) containing GFP. PyNL parasites were transfected and recombinants were selected based on resistance to pyrimethamine conferred by exogenous expression of DHFR. (b) Detection of recombinant parasites using fluorescence microscopy. Peripheral blood from infected mice was stained with PE labeled anti-TER119 Ab and DAPI. Scale bars represent 10 μm. (c) Infectivity of recombinant parasites. C57BL/6 mice were intraperitoneally infected with WT or recombinant parasites (25000 parasitized erythrocytes). The time course of the parasitemia is shown. Each symbol indicates an individual mouse. Representative data from three independent experiments are shown.

performed. Although a few nucleated cells were observed in the sorted TER119$^+$ splenic cells from PHZ-treated mice before lysis, all TER119$^+$ cells were nucleated erythroblasts after lysis (Supplementary Fig. 1e). These nucleated TER119$^+$ cells expressed CD44 or CD71 (Supplementary Fig. 1f), and were consequently deemed to be erythroblasts.

Mouse malaria parasites parasitize nucleated erythroblasts. Using PyNL-GFP and the methods to distinguish erythroblasts described above, we were able to evaluate whether malaria parasites parasitize erythroblasts. TER119$^+$ cells were isolated from the peripheral blood, bone marrow, and spleens of mice infected (at around the peak parasitemia) with recombinant PyNL, and parasite GFP expression was detected. TER119$^+$ cells were nucleated erythroblasts after lysis. Although the majority of these cells lacked a GFP signal, TER119$^+$ GFP$^+$ cells were still clearly identified. TER119$^+$ GFP$^+$ cells contained parasite nuclei that co-localized with GFP and host cell nuclei, whilst the parasitized erythrocytes contained only parasite nuclei (Fig. 2a). These cells also expressed CD44 and CD71 (Fig. 2b, c), confirming that nucleated erythroblasts could be parasitized by malaria parasites. Although it was difficult to clearly distinguish the developmental stage of the parasites, multinucleated 'grape-like' parasites that were likely to be schizonts were observed (Fig. 2c). These observations indicate that the malaria parasites could multiply within the erythroblasts. Light microscopic analyses of Giemsa-stained preparations also indicated potential parasitism of the nucleated cells and some erythroblasts contained schizonts (Fig. 2d).

We next performed quantitative flow cytometric analyses. The percentage parasitemia of GFP$^+$ in TER119$^+$ cells within the peripheral blood was equivalent to that calculated based on microscopic examination of Giemsa-stained thin blood films (Fig. 3a, Fig. 3c), where 0.94 ± 0.54% or 20.41 ± 5.95% GFP$^+$ cells were detected 8 days or 18 days post-infection, respectively (Fig. 3b upper panel). The proportion of erythroblasts (TER119$^+$ cells/lysis-resistant cells) in the spleen increased dramatically (up to 30%) during the late stages of the infection (Fig. 3a), when mice suffered from high parasitemia and anemia (Fig. 3b lower panel). Such increases in the numbers of erythroblasts might be attributed to enhancement of erythropoiesis in the spleen to compensate for the anemia, as observed in the PHZ-treated mice (Supplementary Fig. 1c). GFP$^+$ erythroblasts were clearly detected within the spleen after lysis. Incidence of infection in splenic erythroblasts was 3.85 ± 0.96% in the early stage (Day 8), which was higher than that in the peripheral blood erythrocytes. Incidence in the late stage of the infection was 7.43 ± 2.55% (Day 18), which was lower than that in the peripheral blood (Fig. 3b upper panel).

We next analyzed the surface markers on the parasitized erythroblasts. In the peripheral blood, the parasitized GFP$^+$ erythrocytes expressed CD44 and were either negative or slightly positive for MHC class I (Fig. 3c). These phenotypes coincided with those of the reticulocytes, which are the preferred host cells for this parasite species[12]. The spleen and bone marrow contained GFP$^+$ cells with a similar phenotype to that observed in the peripheral blood before lysis. These cells were removed after lysis, and were thus considered to be contaminating reticulocytes from the peripheral blood. CD44$^+$/MHC class I^{hi} GFP$^+$ cells that were phenotypically distinct from the contaminating cells in the hemolyzed spleen and bone marrow were found to be parasitize erythroblasts (Fig. 3c). Expression of MHC class I molecules on parasitized erythroblasts was higher than that observed in non-parasitized erythroblasts in the infected mouse (mean fluorescence intercity [MFI], 1167 ± 261 vs 315 ± 46, day 20 or day 14 post infection, Fig. 3d, Fig. 4b). The GFP intensity in the infected erythroblasts was similar to that in infected reticulocytes and comprised two peaks (GFP^{hi} represents schizonts and GFP^{lo} represents trophozoites, Fig. 3c), suggesting that parasites grow in erythroblasts comparably in reticulocytes.

Parasitized erythroblasts are a potential source of infection. We observed parasite maturation and replication in erythroblasts; however, the number of schizonts appeared to be limited in number compared to what would normally be observed in reticulocytes. We next asked why erythroblast parasitism might be advantageous for malaria parasites. To confirm that new potentially invasive parasites had been generated within the parasitized erythroblasts, mice were transfused with GFP$^+$ MHC class I^{hi} parasitized erythroblasts isolated from the spleens of mice infected with PyNL-GFP (Fig. 4a, b). Infection of the mice with parasitized erythroblasts caused an elevation in parasitemia, and the kinetics of infection was similar to that seen using parasitized RBCs isolated from the peripheral blood of the same donor mice that the parasitized erythroblasts originated from (Fig. 4c). These data indicate that erythroblasts can support parasite growth and are a potential source of blood-stage infection.

Erythroblasts expressing MHC class I molecules are recognized by CD8$^+$ T cells. We and the others have previously reported that CD8$^+$ T cells are important for protection against blood stage malaria parasites[13–15]. On the other hand, there are some reports that CD8$^+$ T cells are pathogenic in murine experimental cerebral malaria[16,17]. In any case, CD8$^+$ T cells seem to be the important player in malaria infection. As shown above, the mouse malaria parasite could infect erythroblasts that have a high level of MHC class I molecules on their surface, suggesting that erythroblasts might be a target of CD8$^+$ T cells. To test this hypothesis, erythroblasts purified from PHZ-treated mouse spleens were pulsed with the OVA epitope (SIINFEKL), then co-cultured with CD8$^+$ T cells from the OT-I mice, before IFN-γ concentrations in the culture supernatants were measured (Fig. 5a). Expression level of MHC class I on erythroblasts was lower than that of splenocyte, higher than that of erythrocytes (Fig. 5b). We checked the expression level of H-2K^b and OVA CTL epitope complex on the surface of peptide pulsed erythroblasts or splenocytes (CD4 T cells). We found that H-2K^b and OVA CTL epitope complex positive cell in erythroblasts were fewer than that of CD4 T cells (Fig. 5c). Epitope-pulsed erythroblasts induced IFN-γ expression by CD8$^+$ T cells in a dose-dependent manner, although to a lesser extent than that exhibited by splenocytes (Fig. 5d). Response of OT-I CD8 T cells was abrogated in the presence of 25-D1.16 Ab that recognizes H-2K^b and OVA CTL epitope complex (Fig. 5e), clearly indicating requirement of interaction TCR and MHC class I/epitope complex for activation of CD8 T cells.

Next, we investigated whether erythroblasts were susceptible to the cytotoxicity exerted by CD8$^+$ T cells. Sorted erythroblasts were separated into two groups. One was pulsed with the OVA epitope and stained with green fluorescence (CFSE), while the other group was only stained with red fluorescence (PKH26). Cells were then cultured with OT-I CD8$^+$ T cells (effectors) as target cells or negative controls (Fig. 6a). Epitope-pulsed target cells were recognized by OT-I effector cells and were damaged as a result of the loss of membrane integrity; they were subsequently detected on the basis of PI-incorporation (Fig. 6b). These PI-stained cells were never observed in the presence of the CD8$^+$ T cells obtained from C57BL/6 mice (data not shown). In the absence of effector cells, the number of CFSE$^+$ erythroblasts was equal to the number of PKH26$^+$ cells in PI-excluded live cells. However, CFSE$^+$ erythroblasts decreased in a dose-dependent manner as the CFSE$^-$ PKH26$^-$ effector cells increased (Fig. 6c), indicating that target erythroblasts were susceptible to cytotoxicity. It should be noted that cytotoxicity against the erythroblasts was approximately two-fold lower than for the splenocytes (Fig. 6d). Response of OT-I CD8 T cells was abrogated by 25-D1.16 Ab (Fig. 6e). Together with the reduced ability of the

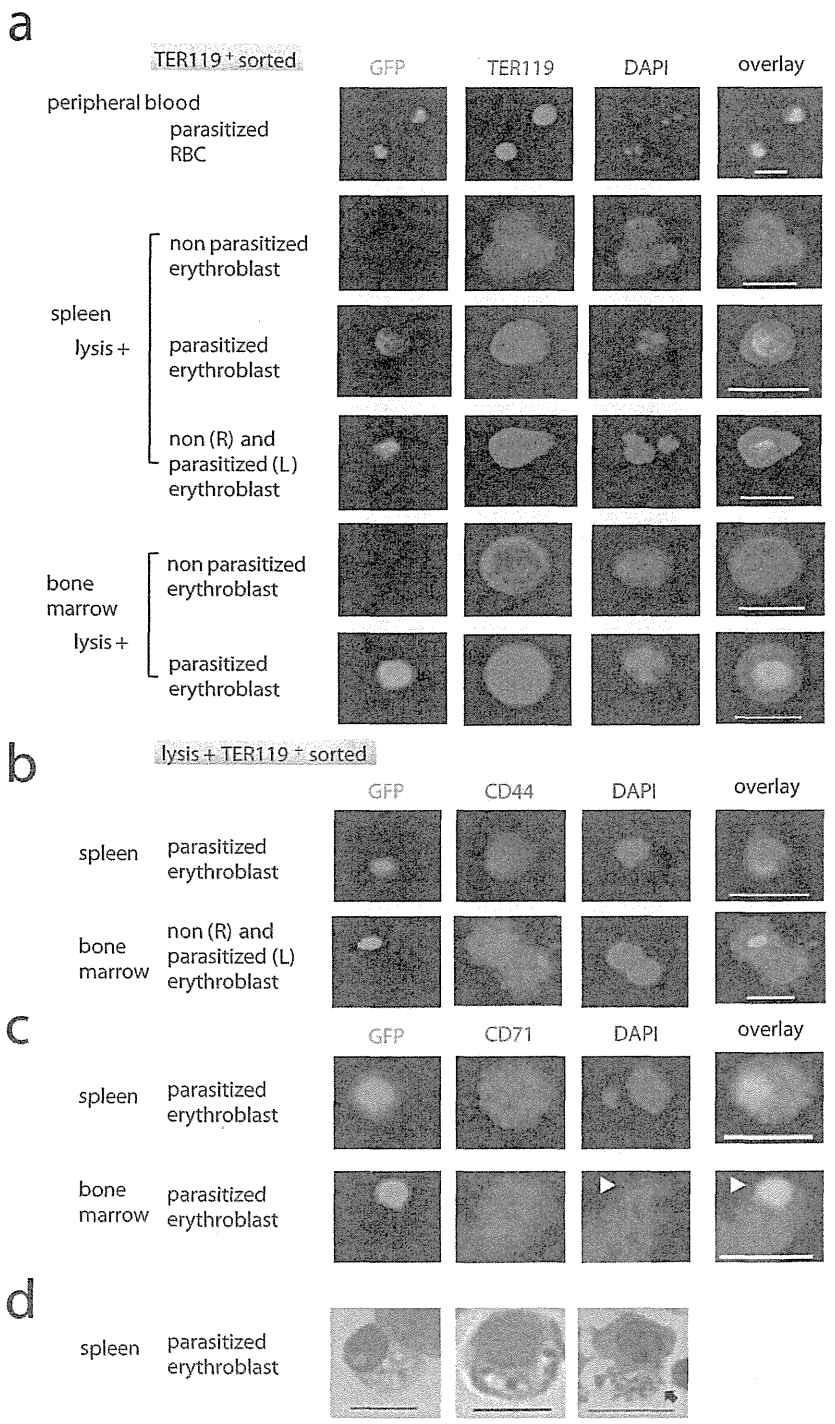

Figure 2 | *Plasmodium yoelii* parasitism of mouse erythroblasts. TER119$^+$ cells were sorted using MACS from the spleen and bone marrow samples from C57BL/6 mice 18 days after infection with PyNL-GFP. Cells stained with PE-conjugated anti-TER119 antibody (Ab) (a), PE-conjugated anti-CD44 Ab (b), anti-CD71 Ab (c), or DAPI, were analyzed by fluorescence microscopy. (d) Sorted cells were also stained with Giemsa solution. Scale bars represent 10 μm. Multi-nucleated 'grape-like' parasites likely to be schizonts (arrow head in c, arrow in d) were observed. Data comprise one representative of at least three independent experiments.

erythroblasts to induce IFN-γ expression by antigen-specific CD8$^+$ T cells, these results may explain the lower expression of MHC class I molecules when compared with splenocytes (Fig. 5b).

Parasitized erythroblasts activate CD8$^+$ T cells. Our data clearly indicate that erythroblasts are able to present antigen to CD8$^+$ T cells in conjunction with MHC class I molecules and induce IFN-γ production or cytotoxicity, thus prompting us to investigate antigen presentation by parasitized erythroblasts. To further utilize the OT-I CD8$^+$ T cells as an indicator for antigen presentation, the parasite lines PyNL-GFP and PyNL-GFP-OVA were generated. Expression of OVA protein within RBCs infected with the

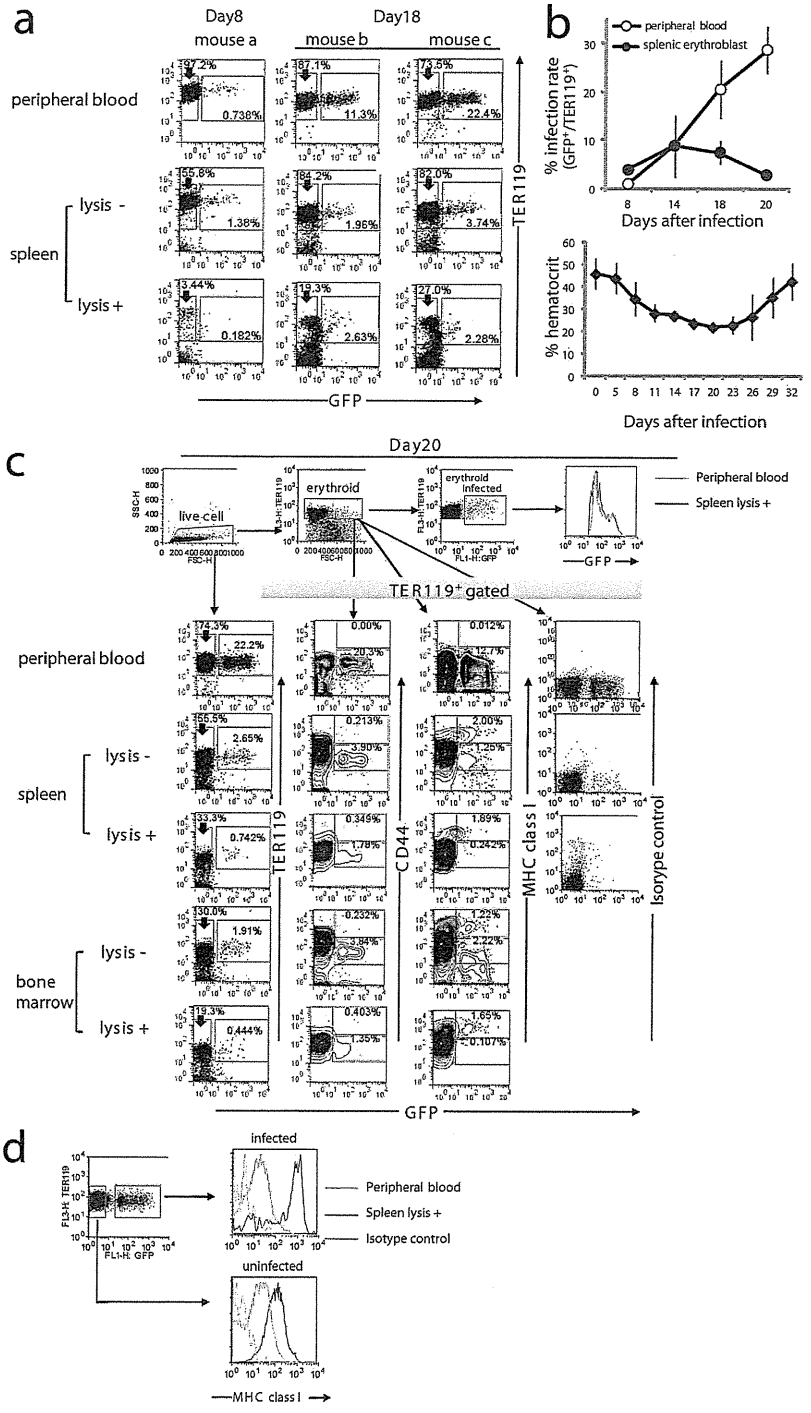

Figure 3 | Flow cytometric analyses of parasitized erythroblasts. C57BL/6 mice were infected with GFP-expressing malaria parasites. (a) Peripheral blood or spleen cells were stained with PE-Cy7 conjugated anti-TER119 Ab on day 8 and 18. Spleen cells were treated with or without lysis buffer before staining. Data are from one representative of at least four independent experiments. (b) The percentages of the GFP^+ cells within $TER119^+$ cells in the peripheral blood (parasitemia, open circles) and those in spleen cells after lysis (infected erythroblasts, closed circles) were calculated (upper panel). Data are mean ± SD from six mice. Hematocrits were evaluated to monitor anemia in mice infected with PyNL (lower panel). Data are ± SD from 10 mice. (c) Cells from the indicated organs on day 20 were stained with PE-Cy7 conjugated anti-TER119 Ab and PE-conjugated anti-CD44 Ab, or anti-MHC class I Ab. GFP expression in erythroblasts in mice infected with GFP-PyNL and the gating strategies (top panels). First, live cells were gated by forward scatter (FSC) and side scatter (SSC), then erythroid cells were gated as $TER119^+$ cells expression, and infected erythroid cells were gated as GFP^+ cells. A histogram shows GFP expression of erythrocytes (peripheral blood) and erythroblasts (lysed spleen cells) infected with GFP-PyNL. Cells after gating by FSC and SSC were analyzed for TER119 and GFP as in (a). Eythroid cells were analyzed for CD44, MHC class I, isotype control for MHC class I in right 3 columns. Numbers indicate percentage of boxed cells in gated population (not total cells). (d) Infected and uninfected erythroid cells were analyzed for expression of MHC class I. Data comprise one representative of three independent experiments.

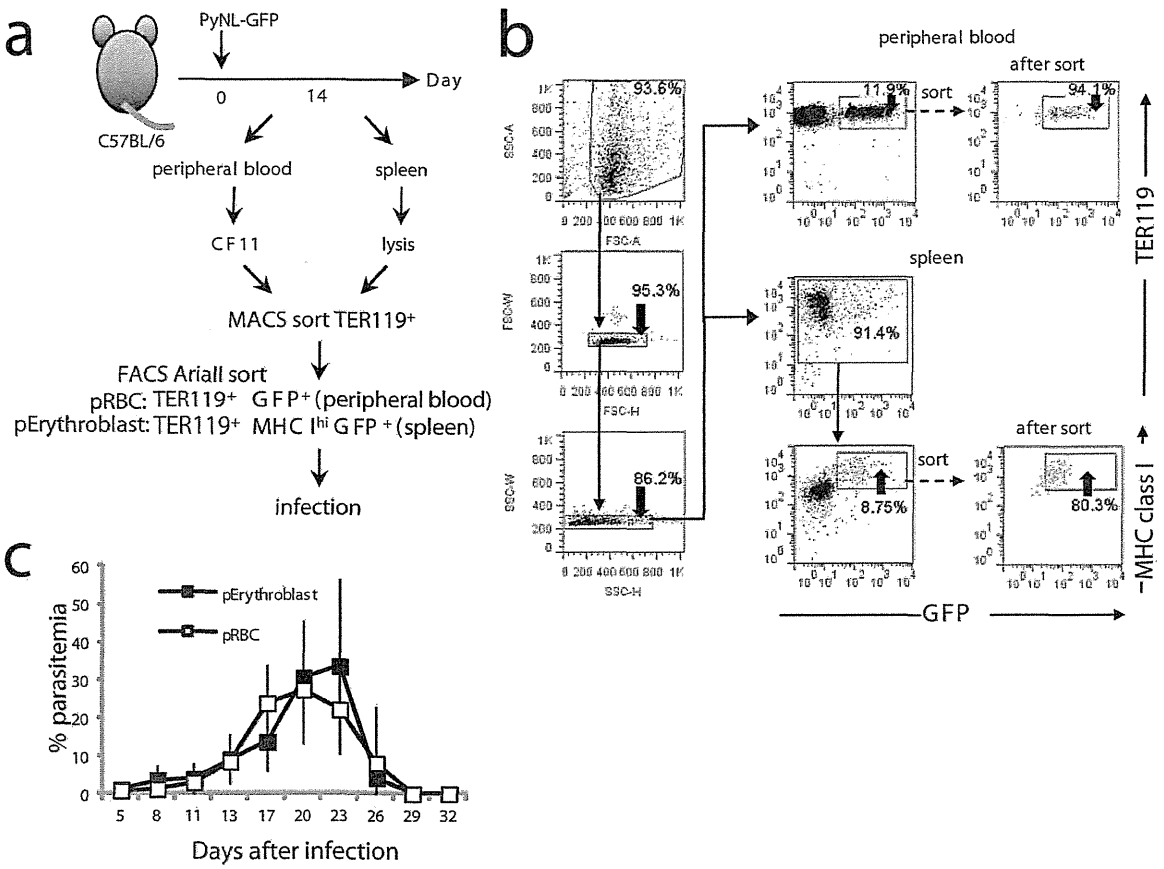

Figure 4 | Parasitized erythroblasts are a potential source of blood stage infection. (a) Protocol for challenge infection with parasitized erythroblasts (pErythroblasts). Peripheral blood was passed through a CF11 column to eliminate white blood cells then the TER119$^+$ RBCs were sorted. RBC lysed splenic TER119$^+$ erythroblasts were magnetically sorted 14 days after infection. (b) TER119$^+$ GFP$^+$ peripheral RBCs or TER119$^+$ MHC class I^{hi} GFP$^+$ splenic erythroblasts were purified by cell sorting. Mice were intravenously infected with 25,000 purified pRBCs or pErythroblasts. (c) Time course of parasitemia from pRBC injected mice (n = 9 mice, open square) or pErythroblast injected mice (n = 8 mice, closed square) (Mean ± S.D.). Representative data from two independent experiments are shown.

recombinant parasite was confirmed by western blot analysis (Fig. 7a, b). We first evaluated immunogenicity of the recombinant parasites. CD11c$^+$ DCs sorted from uninfected mice were cultured with OT-I CD8$^+$ T cells in the presence of soluble extracts from parasitized RBCs. Neither normal RBCs nor PyNL-GFP-parasitized RBCs induced production of IFN-γ from OT-I CD8$^+$ T cells. In contrast, addition of the PyNL-OVA-GFP-parasitized RBCs led OT-I cells to produce IFN-γ (Fig. 7c), indicating that exogenously expressed OVA in malaria parasites is immunogenic against OT-I T cells. Then, we investigated whether parasitized erythroblasts can present malarial antigens to CD8$^+$ cells. Mice were infected with PyNL-GFP or PyNL-GFP-OVA, and their splenic GFP$^+$ parasitized erythroblasts were purified and were fixed and co-cultured with OT-I CD8$^+$ T cells (Fig. 7d). GFP$^-$ uninfected erythroblasts could not induce IFN-γ production in OT-I cells. Parasitized erythroblasts isolated from mice infected with PyNL-GFP-OVA but not with PyNL-GFP could activate OT-I CD8$^+$ T cells (Fig. 7e). To exclude the possibility that contaminated DCs in erythroblast preparations activated OT-I cells, we voluntarily added DCs isolated from mice infected with PyNL-OVA-GFP. The addition of the DCs to erythroblasts did not affect the cytokine production of OT-I CD8$^+$ T cells (Fig. 7f). Thus, stimulatory features exerted by erythroblasts would not be affected if any DCs exist. These results clearly show that CD8$^+$ T cells recognize parasitized erythroblasts in an antigen-specific manner.

Discussion

Although human malaria parasites can parasitize erythroblasts, the biological and clinical significance of this phenomenon remains unclear. In this study, use of the PyNL-GFP transgenic line enabled us to demonstrate that rodent malaria parasites can also parasitize erythroblasts. Parasitized erythroblasts expressed MHC class I molecules on their surface that were recognized by antigen-specific CD8$^+$ T cells. Upon recognition, CD8$^+$ T cells secreted IFN-γ, which is essential for protection against malaria parasites.

Tamez and colleagues reported that the human malaria parasite, *P. falciparum* (Pf), parasitizes erythroblasts *in vitro*[8]. They found that as erythroblasts mature, they become more susceptible to Pf invasion[8]. Pf cell division was observed only in orthochromatic erythroblasts, the final precursor cells that form immediately before enucleation[8]. In our experiments, PyNL preferred to infect mature erythroblasts later than orthochromatic erythroblasts as judged by the CD44 expression patterns[11]. However, detailed analyses to define the relationship between the maturation stages of the erythroblasts and the susceptibility to infection are still required. The reason for parasite preference for the later stages of the erythroblast population might be due to the expression of receptor molecules on their surface ligated to merozoite molecules.

It is not clear if parasite invasion of erythroblasts could lead to formation of parasites that can re-infect RBCs. We confirmed that parasitized erythroblasts were able to parasitize RBCs to initiate new

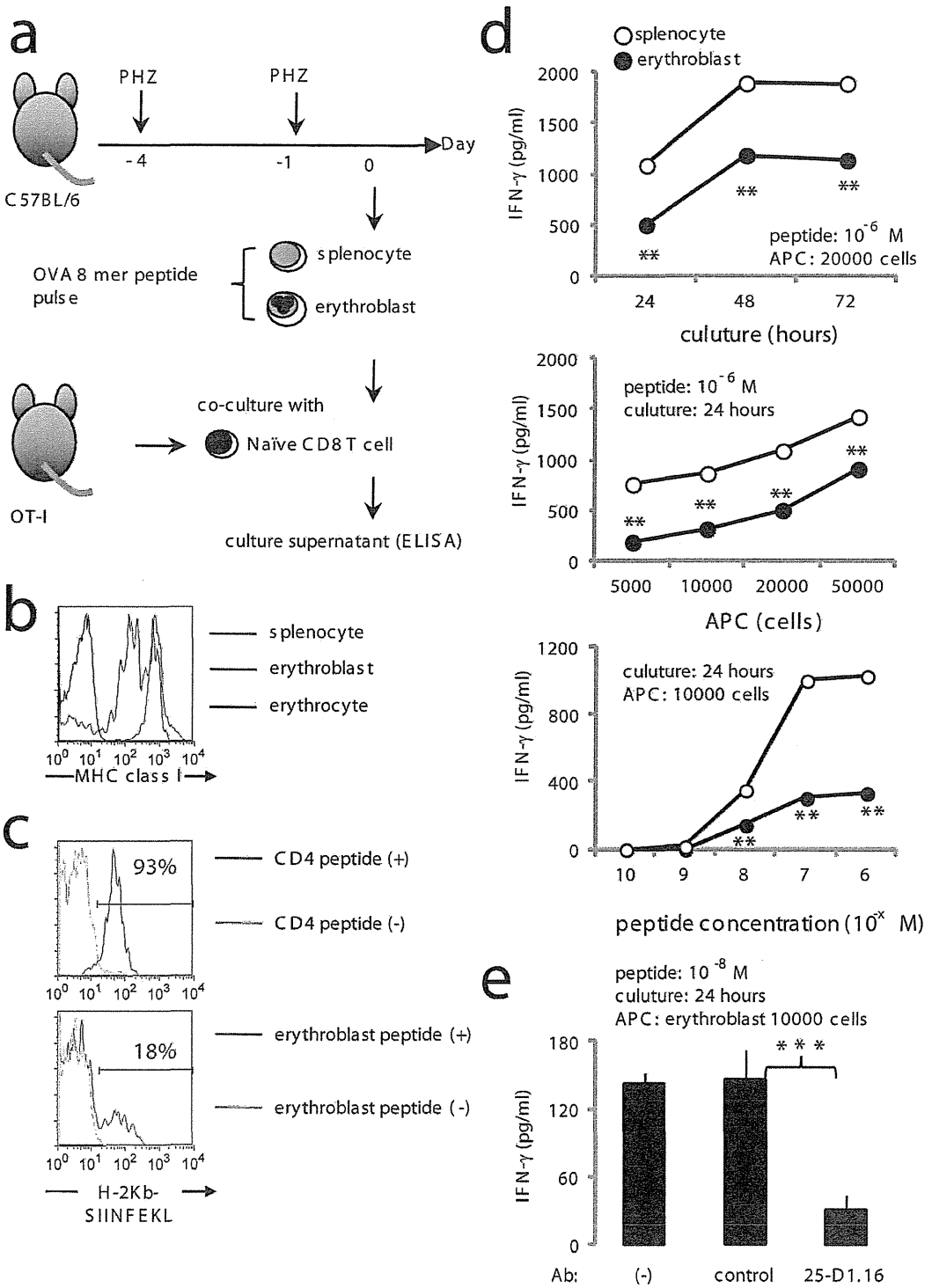

Figure 5 | CD8⁺ T cell recognition of erythroblasts. (a) Protocols for the antigen presentation assay. Erythroblasts were isolated from spleen cells from C57BL/6 mice treated with 50 mg/kg weight PHZ on days −1 and −4. Cells were pulsed with the OVA epitope for 1 h followed by three extensive washes with culture medium before culturing with CD8⁺ T cells from OT-I mice. Culture supernatants were collected for IFN-γ measurements using ELISA. (b) Expression of MHC class I on splenocytes, erythrocytes and erythroblasts are shown. (c) Expression of MHC class I-epitope complex on erythroblasts. Purified splenic CD4⁺ T cells and erythroblasts were pulsed with or OVA epitope (SIINFEKL) and analyzed using 25-D1.16 Ab. Numbers indicate percentage of H-2K^b and OVA epitope complex positive cells in gated population. (d) Ten thousand or the indicated number of erythroblasts (closed circles) or splenocytes (open circles) pulsed with the OVA epitope were cultured with 1×10^5 OT-I CD8⁺ T cells using the conditions indicated. (e) Abrogation of IFN-γ production due to blocking MHC/TCR interactions. OT-I cells and epitope-pulsed erythroblasts were cultured in the presence of 25-D1.16 or the control Ab. Concentration of IFN-γ in collected culture supernatants is shown. Data are shown as the mean ± S.D. from triplicate cultures of 3 mice. In d, e statistical significance is indicated as $^{**}P < 0.01$ and $^{***}P < 0.01$. Representative data from 4–8 independent experiments are shown (a–e).

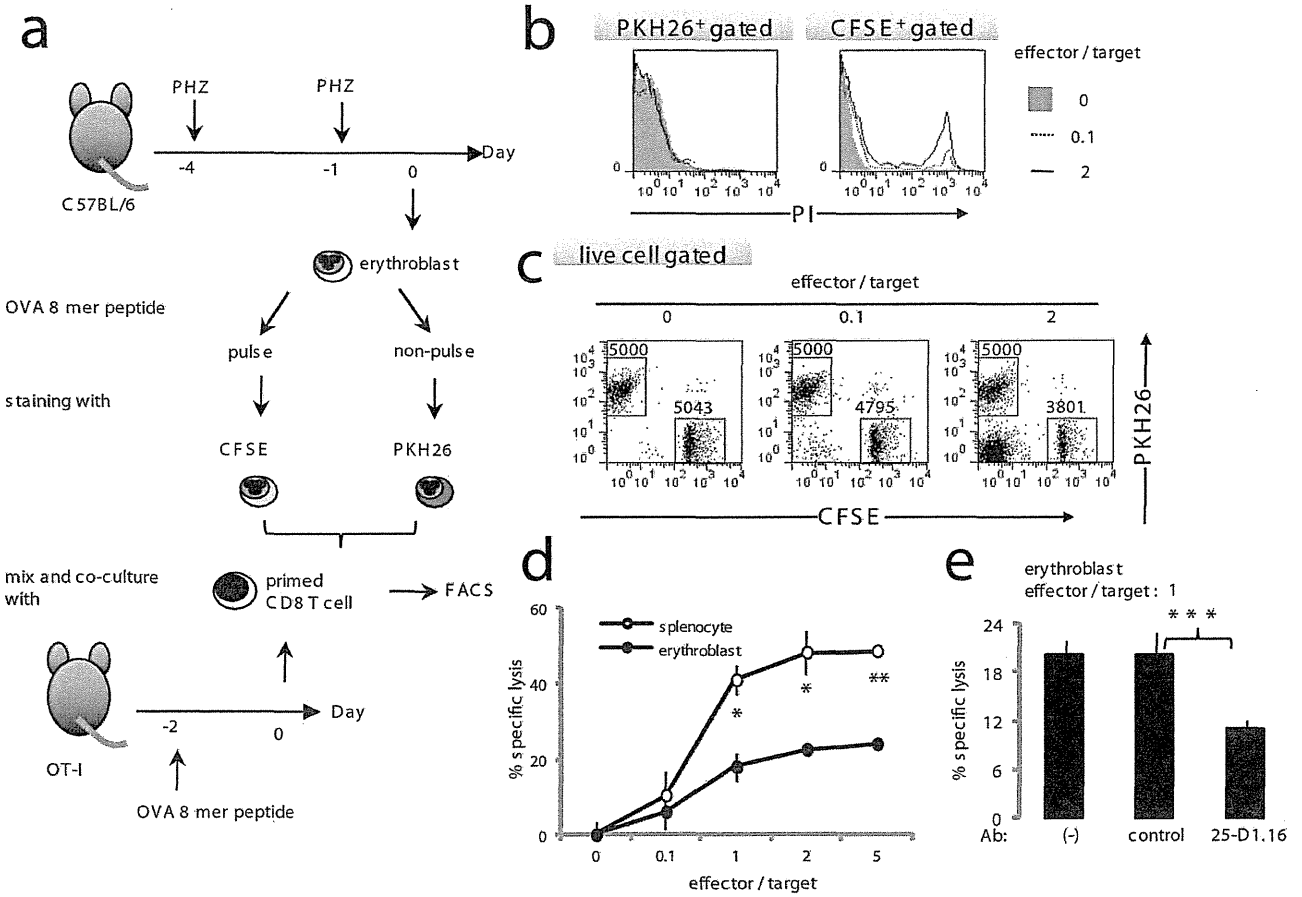

Figure 6 | Cytotoxicity by CD8$^+$ T cells against erythroblasts. (a) Protocols for the cytotoxicity assay. Erythroblasts or splenocytes were separated into two groups. One group was pulsed with 10^{-6} M OVA 8-mer peptide stained with CFSE. The other was only stained with PKH26. CFSE or PKH26 labeled cells were co-cultured for 4 h with variable numbers of primed CD8$^+$ T cells from the OT-I mice that had been injected with 20 nmol of the OVA 8-mer peptide without any adjuvants 2 days before the assay was commenced. After co-culturing at the ratio of OT-I cells indicated, the erythroblasts were stained with PI and analyzed using flow cytometry. (b) Incorporation of PI among CFSE$^+$ and PKH26$^+$ cells was demonstrated. (c) Cytotoxicity to erythroblasts by CD8$^+$ T cells was evaluated. OVA-pulsed CFSE$^+$ and OVA-free PKH26$^+$ cells were cultured with OT-I CD8$^+$ T cells at the effector/target ratio indicated. PI$^-$ live erythroblasts were plotted against CFSE and PKH26. The numbers of PKH26$^+$ or CFSE$^+$ cells are indicated in each panel. (d) OVA-specific cytotoxicity against splenocytes (open circles) or erythroblasts (closed circles) is represented. (e) Abrogation of cytotoxicity due to blocking MHC/TCR interactions. OT-I cells and 10^{-8} M OVA epitope-pulsed erythroblasts were cultured in the presence of 25-D1.16 or the control Ab. Cytotoxicity was calculated as the proportion of CFSE$^+$ surviving cells to PKH26$^+$ live cells. In d, e statistical significance is indicated as *$P < 0.05$, **$P < 0.01$ and ***$P < 0.01$. Representative data from 4 independent experiments are shown (a–e).

infections in mice. Infection of nucleated cells with malaria parasites is not as nonsensical as it might sound; avian malaria parasite species complete their whole blood-stage cycle in nucleated erythrocytes[18]. Thus, we suggest that erythroblast parasitism is not a 'dead-end' process but an important adaptation strategy for malaria parasites. Parasitized erythroblasts might confer resistance to host elimination mechanisms. It would be interesting to see what happens if we repeat these experiments using *P.chabaudi*, for example, where the murine host can control the primary peak parasitaemia, which is followed by the secondary appearance of the parasite[19]. It is quite possible that parasites remain in the erythroblast population post-peak when no parasites are visible in the peripheral blood using standard microscopy. Alternatively, parasitized erythroblasts may represent a dormant form of the parasite that has greater resistance to therapeutic agents[20], which might account for the recrudescence observed in individuals with Pf malaria after successful treatment with artemisinin-based drugs.

We confirmed that erythroblasts infected with PyNL expressed high levels of MHC class I molecules. However, the functional properties of these molecules on erythroblasts remain unknown, as no reports have directly demonstrated the antigen-presenting capacity of erythroblasts. We found that erythroblasts pulsed with an antigenic epitope were recognized by CD8$^+$ T cells, resulting in a lower induction of IFN-γ production and cytotoxic activities than for splenocytes. Furthermore, we demonstrated that parasitized erythroblasts activated CD8$^+$ T cells in an antigen-specific manner. Antigen presentation via class I molecules requires a supply of antigenic peptides resulting from proteolysis by proteasomes[21]. Experiments using proteasome inhibitors revealed protein degradation dependent on proteasomes in erythroblasts[22], suggesting that antigen processing does occur in erythroblasts. We speculate that parasite proteins are transported to the host cytosol or ER from the parasitophorous vacuole where they are processed and presented by the MHC class I.

We previously reported that CD8$^+$ T cells protect the host from blood-stage malaria primarily via secretion of IFN-γ, and the cytotoxic activity also contributes to protection[23]. One of the important functions of IFN-γ is up-regulation of MHC class I expression[24]. Although we did not evaluate whether or not erythroblasts have

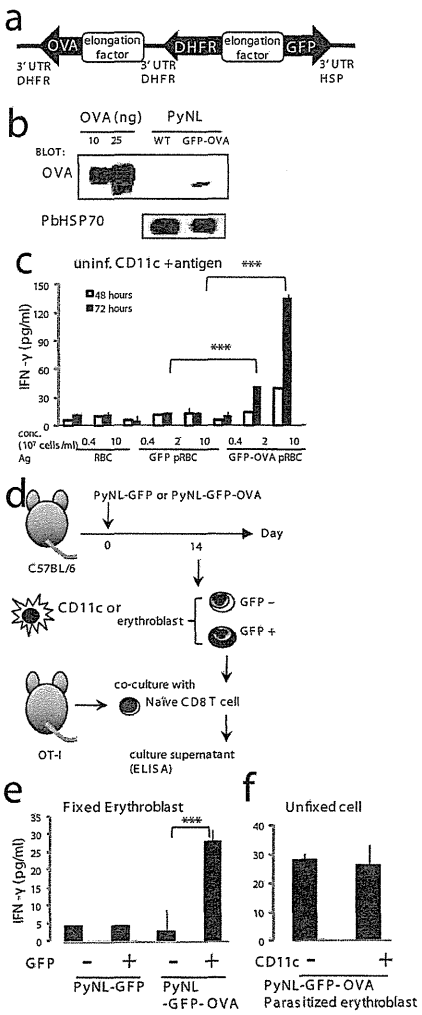

Figure 7 | Parasitized erythroblasts activate CD8$^+$ T cells.
(a) Construction of the *Plasmodium* artificial chromosome (PAC) containing GFP and OVA. PyNL parasites were transfected and the recombinants selected based on resistance to pyrimethamine conferred by exogenous expression of DHFR. (b) Expression of OVA in the recombinant parasite was detected by western blot analysis. Protein extracts from parasitized RBCs purified from mice infected with the recombinant and parental (WT) parasite and OVA protein were separated on SDS-PAGE and transferred to nitrocellulose membranes followed by blotting with an anti-OVA antibody. An antibody against *P. berghei* HSP70 was used as an internal control. (c) Immunogenicity of OVA-expressing recombinant parasites. Purified CD11c$^+$ cells (2×10^4) from uninfected mice were co-cultured with 10^5 OT-I CD8$^+$ T cells in the presence of antigens from the indicated cells. (d) Protocol for antigen-specific recognition of parasitized erythroblasts. GFP$^-$ parasitized or GFP$^-$ uninfected erythroblasts isolated from spleen samples of C57BL/6 mice infected with PyNL-GFP or PyNL-GFP-OVA as shown in Fig. 4b. (e) Purified CD11c$^+$ cells (2×10^4) from C57BL/6 mice infected with the indicated parasite were pulsed with or without OVA peptide and fixed, then were co-cultured with 10^5 OT-I CD8$^+$ T cells. (f) Purified PyNL-GFP-OVA parasitized erythroblasts (2×10^4 cells) were mixed with or without CD11c$^+$ cells (2×10^4) from PyNL-GFP-OVA infected mouse and co-cultured with purified CD8$^+$ CD11c$^-$ T cells (10^5 cells) from Rag2$^{-/-}$ OT-I mice. (c, e, f) Activation of OT-I CD8$^+$ T cells was assessed by production of IFN-γ. Culture supernatants were collected after 72 hours otherwise mentioned, and the concentration of IFN-γ was measured. Mean ± S.D. from triplicate cultures from 4 mice is shown. Representative data from two to six independent experiments are shown. Statistical significance is indicated as *$P < 0.05$ and ***$P < 0.001$.

receptors for IFN-γ, nevertheless, erythroblasts are affected by IFN-γ[25]. Furthermore, we confirmed that expression of MHC class I on erythroblasts were upregulated by recombinant IFN-γ *in vitro* (Fig. S2). Expression of MHC class I molecules on parasitized erythroblasts was much higher than that of non-parasitized erythroblasts in infected mice (Fig. 3c, d and Fig. 4b); this might enable parasitized erythroblasts to be effectively recognized by CD8$^+$ T cells. Thus, it is quite possible that parasitized erythroblast killing is involved in CD8$^+$ T cell-mediated protection against malaria. However, killing of parasitized erythroblasts might play limited roles in overall protection to blood-stage malaria as most of parasites are found in reticulocytes or mature erythrocytes that could not be targeted of CD8$^+$ T cells. We postulate that the significance of erythroblast recognition implies reactivation of the primed CD8$^+$ T cells to produce IFN-γ, which in turn activates macrophages to effectively phagocytose parasitized reticulocytes and mature erythrocytes indifferently of MHC class I expression.

From a pathological point of view, erythroblast parasitism may partly contribute to the development of anemia, one of the major symptoms of malaria. Erythroblast parasitism, for example, may suppress erythropoiesis. Indeed, Tamez et al. reported that hematopoietic suppression-associated genes, such as *JUN* and *RARA*, were induced in Pf-parasitized erythroblasts[26]. Another possibility is that the destruction of parasitized erythroblasts caused by parasite growth and host immune responses reduces erythropoiesis, which is observed in other pathological situations. Parvovirus B19 infects erythroblasts and is the causative agent of pure red cell aplasia (PRCA). The marked decrease in erythroblast number observed after infection is a result of erythroblast destruction due to viral replication and the cytotoxic activity by CD8$^+$ T cells as a defense mechanism. Destruction of erythroblasts by self-reactive CD8$^+$ T cells is assumed to be the etiology of autoimmune PRCA[27]. Furthermore, Friend virus[28] infects erythroblasts, inducing tumorigenesis and resulting in the development of erythroleukemia. Reduction in the enlargement of the spleen containing massive virally transformed erythroblasts depends on CD8$^+$ T cells.

Our findings show that parasitism of erythroblasts by malaria parasites is an important aspect of malaria infection. Better understanding of the pathological and immunological aspects of malaria infection should provide new approaches for the effective control strategies. The blood-stage malaria is thought mostly to be controlled by humoral immunity. Blood-stage vaccines have been developed targeting antigens that expressed on parasites' surface, which are recognized by antibodies. Such molecules exposed to humoral immunity tend to be polymorphic, hampering development of effective vaccines. In contrast, CD8 T cells could recognize intracellular conserved antigens after antigen presentation. Thus, development of a malaria vaccine that activates CD8$^+$ T cells against blood-stage malaria might be hopeful.

Methods

Generation of malaria parasite lines expressing GFP and GFP-OVA. Blood-stage *Plasmodium yoelii* 17XNL (PyNL) was a generous gift from Dr. M. Torii (Ehime University). Recombinant PyNL was generated using a *Plasmodium* artificial chromosome (PAC)[29]. The following elements were inserted into the PAC: 1) elongation factor promoter; 2) green fluorescence protein (GFP); 3) Pb hsp70 3′ untranslated region; 4) *Plasmodium berghei* (Pb) pyrimethamine resistant gene dihydrofolate reductase-thymidyltransferase-ts (DHFR-ts) gene; 5) Pb DHFR-ts 3′ untranslated region (Fig. 1A); and 6) GFP-OVA, cytosolic form without the ovalbumin (OVA) signal sequence open reading frame (Fig. 6A). The elongation factor promoter facilitated PyNL gene expression during all of the erythrocytic stages. Transfection and drug selection have been described previously[30].

Mice and PyNL infection. C57BL/6 mice were obtained from SLC (Hamamatsu, Japan). Rag2$^{-/-}$ OT-I mice[31] were generous gifts from Dr. K. Yui (Nagasaki University). All mouse experiments 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, Gunma University, Japanese Law (No. 105) and Notification (No. 6) of the Government. PyNL-GFP or PyNL-GFP-OVA were obtained after fresh passage through a donor