CD47 regulates the phagocytic clearance and replication of the *Plasmodium yoelii* malaria parasite

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Several *Plasmodium* species exhibit a strong age-based preference for the red blood cells (RBC) they infect, which in turn is a major determinant of disease severity and pathogenesis. The molecular basis underlying this age constraint on the use of RBC and its influence on parasite burden is poorly understood. CD47 is a marker of self on most cells, including RBC, which, in conjunction with signal regulatory protein alpha (expressed on macrophages), prevents the clearance of cells by the immune system. In this report, we have investigated the role of CD47 on the growth and survival of nonlethal *Plasmodium yoelii* 17XNL (PyNL) malaria in C57BL/6 mice. By using a quantitative biotin-labeling procedure and a GFP-expressing parasite, we demonstrate that PyNL parasites preferentially infect high levels of CD47 (CD47^{hi})-expressing young RBC. Importantly, C57BL/6 CD47^{-/-} mice were highly resistant to PyNL infection and developed a 9.3-fold lower peak parasitemia than their wild-type (WT) counterparts. The enhanced resistance to malaria observed in CD47^{-/-} mice was associated with a higher percentage of splenic F4/80⁺ cells, and these cells had a higher percentage of phagocytized parasitized RBC than infected WT mice during the acute phase of infection, when parasitemia was rapidly rising. Furthermore, injection of CD47-neutralizing antibody caused a significant reduction in parasite burden in WT C57BL/6 mice. Together, these results strongly suggest that CD47^{hi} young RBC may provide a shield to the malaria parasite from clearance by the phagocytic cells, which may be an immune escape mechanism used by *Plasmodium* parasites that preferentially infect young RBC.

malaria | *Plasmodium yoelii* | RBC | CD47 | F4/80

Malaria, caused by *Plasmodium* parasites, remains a major cause of mortality and morbidity in the developing world. Among the four principal human *Plasmodium* species, *Plasmodium falciparum* is the most virulent, being responsible for more than 90% of malaria-associated deaths. Likewise, *Plasmodium* species that infect rodents and nonhuman primates also differ widely in their fulminant nature and in the mortality they cause (1–3). How different *Plasmodium* species have evolved to exhibit this wide array of virulence and disease severity remains one of the major unsolved questions in malaria biology and pathogenesis.

One important factor that is associated with *Plasmodium* parasite burden and disease severity is the age constraint of the host red blood cells (RBC) they infect. The age-based preference for restricted invasion of RBC by the *Plasmodium* parasite is characterized as young RBC (reticulocyte), aged RBC (mature), or both young and aged RBC. *Plasmodium* species that preferentially infect and grow inside young RBC generally cause a low-grade, self-resolving infection that is rarely fatal (e.g., *Plasmodium vivax* and *Plasmodium ovale*), whereas those that infect both young and aged RBC cause more fulminant infection that can be fatal in the absence of immunity (e.g., *P. falciparum*) (1, 4–6). Thus, along with host genetic background and immune response, restriction for age-specific RBC invasion is a major determinant of the severity and outcome of malaria infection.

Malaria parasites have evolved to use redundant receptors and pathways to invade the RBC. For example, sialic acid (7) and Duffy antigen (8) are the major RBC receptors for invasion of *P. falciparum* and *P. vivax*, respectively, although other receptors and invasion pathways are known to exist (9, 10). Although a redundancy in RBC receptor use would ensure successful invasion by mitigating the effects of polymorphism and immune targeting, the reasons behind the RBC age-based preference for invasion are not fully clear and remain a subject of debate.

Survival of normal cells through the course of their life cycle is essential to maintain homeostasis, and aberrant cells (e.g., senescent or foreign antigen-expressing cells) are eliminated through a sophisticated programmed cell removal system that relies on the recognition of self and nonself determinants (11). CD47, a cell surface molecule in the Ig superfamily, is ubiquitously expressed on many cell types, including RBC, and is a marker of self to avoid early clearance by phagocytic cells through ligation of signal regulatory protein alpha (12). In contrast, altered expression or conformational changes in CD47 may lead to a molecular switch that triggers a phagocytic signal to remove aged or damaged cells (11). Recent studies have shown that the level of CD47 expression is higher in progenitor cells and declines as they undergo maturation and are subsequently aged (13). This age-dependent difference in CD47 expression shields young cells but allows clearance of aging and damaged cells from the system.

CD47 is overexpressed in cancer cells (11, 14, 15), and the CD47–signal regulatory protein alpha interaction is considered a major pathway of immune evasion by tumor cells (15). Administration of anti-CD47 antibodies enabled the phagocytosis

Significance

Several *Plasmodium* species exhibit age-based preference for the red blood cells (RBC) they invade, with implications for virulence and disease severity. CD47, a marker of self, prevents the early clearance of cells by phagocytosis. We report here that in the absence of CD47, growth of a *Plasmodium* species that preferentially infects CD47^{hi} young RBC is highly attenuated, and the macrophages from CD47^{-/-} mice are more effective in phagocytizing the malaria-parasitized RBC than wild-type mice. We suggest that preferential invasion of young RBC is an evolutionary adaptation that shields the malaria parasite from phagocytic clearance and controls parasitemia, as fewer RBC are available for invasion. Modulation of CD47 levels by antibody treatment may have therapeutic value in patients with severe malaria.

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of tumor cells in vitro, reduced their growth, and prevented the metastasis of human patient tumor cells (14). In this article, using the murine *Plasmodium yoelii* nonlethal model, we provide quantitative evidence for age of RBC as the basis for the survival and growth of malaria parasites and provide supporting data that suggest that *P. yoelii* nonlethal parasites prefer to grow inside younger RBC, which allows them to evade immune clearance by phagocytic cells through a CD47-mediated process, and that CD47 modulates the clearance of malaria infection. To our knowledge, this is the first report that provides a molecular basis for the age-dependent preference for infection of RBC by a *Plasmodium* parasite and sheds light on its implications for the severity of malaria infection in a host.

Results

In Vivo Biotinylation Allows Discrimination of Young Versus Aged RBC and Measurement of Age-Based Preference for RBC Infection by GFP-PyNL Parasites. We used a quantitative, biotin-labeling-based procedure (16, 17) to examine the age of RBC as a factor for invasion, growth, and survival of green fluorescent protein (GFP) expressing *P. yoelii* 17XNL (GFP-PyNL) parasites in C57BL/6 mice. In vitro capturing of the biotin-RBC with streptavidin-allophycocyanin (APC) was used to differentiate the young (APC-negative) and aged (APC-positive) RBC by flow cytometry. Data showed that three i.v. injections of biotin on consecutive days transiently biotin-coated all of the existing RBC, and newly generated RBC released thereafter were free of biotin coat (Fig. S1). On the day after the third biotin injection, C57BL/6 mice were infected with the GFP-PyNL, and the differential infection in the young (APC-negative) versus aged (APC-positive) RBC was measured (Fig. 1A) on alternate days beginning from day 3 postinfection (p.i.) and then followed throughout the course of infection by flow cytometry; results were expressed as mean parasitemia (%) $\pm$ SEM (Fig. 1B). To determine whether biotin administration influenced the course of GFP-PyNL infection, parasitemias were compared in biotin-injected mice versus those mice that did not receive any biotin injection. Results showed that the ascending, peak, and clearance phases of infection were comparable between the two groups of mice (Fig. S2). As the infection progressed, the percentage of newly released RBC increased dramatically after day 5 p.i. and superseded the percentage of old RBC by day 9 p.i. (Fig. S1). The parasites consistently showed a tendency to infect young RBC even during the early phase of infection, which became more prominent and statistically significant as the parasitemia rose [$P < 0.005$; two-way analysis of variance (ANOVA), followed by Bonferroni post hoc comparison test] and remained so during the clearance phase (Fig. 1B). Thus, our results clearly demonstrated that GFP-PyNL

exhibits preferential infection of young RBC, particularly during the acute phase of infection (days 7–11), when the parasites almost exclusively prefer to infect the young RBC (Fig. 1*B*).

GFP-PyNL Parasites Preferentially Infect CD47^{hi} RBC. CD47 on RBC helps to avoid early clearance from circulation and maintain homeostasis (11, 12). To investigate the role of CD47 during malaria, we measured the expression of CD47 in young and aged RBC from biotin-injected C57BL/6 mice (Fig. 1C) during the course of GFP-PyNL infection by flow cytometry; the results are expressed as mean fluorescence intensity (MFI) $\pm$ SEM. We find that young RBC expressed a higher level of CD47 (Fig. 1D). After infection, the nonbiotin-tagged young and biotin-coated aged RBC have a comparable level of CD47 expression up to day 5 p.i. However, as the parasitemia rose (days 7–11), young RBC had significantly higher CD47 expression ($P < 0.05$; two-way ANOVA followed by Bonferroni test) than aged RBC (Fig. 1D), suggesting blood-stage PyNL infection induced the generation of young CD47^{hi} RBC from day 7 p.i. onward, when the parasitemia becomes well established.

To further understand the correlation between CD47 level on RBC and parasite burden, we investigated the CD47 levels on parasitized and nonparasitized RBC during the course of GFP-*PyNL* infection in C57BL/6 mice (Fig. 2A). Parasitized and nonparasitized RBC were discriminated on the basis of the expression of GFP by the *PyNL* parasite. Results demonstrated that compared with nonparasitized RBC, CD47 expression was significantly higher ($P < 0.0001$; two-way ANOVA followed by Bonferroni test) in parasitized RBC when the blood samples from the same mice were measured throughout the course of infection (Fig. 2B). We further differentiated the RBC populations as the CD47^{lo} and CD47^{hi}, on the basis of CD47 intensity on surface, and the parasite burden was measured in the two groups of RBC (Fig. 2C). We find that the CD47^{hi} RBC had significantly higher parasite burden than the population of CD47^{lo} RBC during the acute phase (day 9 and day 12 p.i.; $P < 0.001$, two-way ANOVA followed by Bonferroni test) of infection (Fig. 2D). Together, these results demonstrated that the highest infection and growth of *PyNL* parasites occurred in the young CD47^{hi} RBC.

CD47 Phenotype Is Necessary to Support PyNL Growth. Having established a direct relationship between the CD47 level of RBC and parasite density, we next wanted to determine the role of CD47 in the replication and survival of blood-stage parasites *in vivo*. To accomplish this, we determined the effect of loss of the CD47 phenotype on parasite growth by comparing the

Fig. 1. In vivo biotinylation separated young and aged populations of RBC that allowed determining the association between CD47 expression level and parasitemia. Three dosages of biotin were administered in WT C57BL/6 mice ($n = 5$) on consecutive days, and on the following day, mice were infected with GFP-PyNL pRBC. On the day of parasite challenge, 100% of RBC in all mice were biotin-coated, as determined by in vitro staining with streptavidin-APC (Fig. S1). A gradual accumulation of young RBC was observed as the population of the aged RBC decreased during a 13-d observation period. (A) A representative diagram showing the percentage GFP-PyNL parasitemia in young and aged populations of RBC in biotin-injected mice on day 9 p.i. (B) The parasite burden during the course of infection was determined in the young and aged populations of RBC and plotted as the mean $\pm$ SEM. (C) CD47 intensity in the young and aged populations of RBC in biotin-injected mice was assayed on day 9 after GFP-PyNL infection. (D) The CD47 levels on young and aged populations of RBC in biotin-injected mice were measured throughout the course of infection and expressed as MFI values (mean $\pm$ SEM, $n = 5$). Bonferroni comparison test was applied after two-way ANOVA.

Fig. 2. GFP-PyNL parasites prefer to infect CD47^{hi} RBC. (A) The parasitized RBC (pRBC) and nonparasitized RBC (npRBC) were differentiated on the basis of GFP expression by the GFP-PyNL, and the CD47 intensity was measured in the pRBC and npRBC groups assayed on day 9 post-GFP-PyNL infection. (B) The CD47 MFI of pRBC and npRBC from the same infected mouse ($n = 10$) were plotted throughout the course of infection. Statistically significant differences in the CD47 MFI values were noted between the two groups ($P < 0.0001$). (C) A representative diagram shows the GFP-PyNL burden in CD47^{hi} and CD47^{lo} RBC populations. (D) The percentage of parasitemia in the CD47^{lo} and CD47^{hi} group was plotted as mean $\pm$ SEM throughout the course of infection. Data were analyzed using the two-way ANOVA followed by Bonferroni test.

parasitemia during the course of infection with GFP-PyNL parasites in the wild-type (WT) and CD47^{-/-} mice by blood-film microscopy. In one experiment (representative of four independent experiments conducted), WT C57BL/6 mice ($n = 5$) developed an average parasitemia of $3.0 \pm 0.25\%$ on day 3 and reached a peak parasitemia of $28.0 \pm 5.8\%$ on day 11, and then the infection was self-resolved by day 17 p.i. In contrast, CD47^{-/-} mice developed a very low grade infection on day 3 ($0.02 \pm 0.02\%$) and maintained a lower parasitemia while reaching a peak parasitemia of $2.98 \pm 0.45\%$ on day 7 that was completely resolved by day 15 p.i. (Fig. 3). Thus, CD47^{-/-} mice reached an early peak parasitemia by day 7 p.i. that was 9.3-fold lower than the peak parasitemia of the WT mice that occurred on day 11 p.i. These results clearly show that absence of CD47 negatively regulates the growth of blood-stage GFP-PyNL parasites in mice.

In contrast to self-resolving PyNL, the *P. yoelii* YM (PyYM) strain parasite invades both young and mature RBC and causes uniformly fatal infection in mice. We also investigated the role of CD47 in parasite clearance against the virulent PyYM parasite in the C57BL/6 WT and CD47^{-/-} mice. The WT ($n = 5$) and CD47^{-/-} ($n = 5$) mice were infected with the PyYM parasite, and parasitemias were determined every day, beginning from day 3 p.i. After parasite challenge, the WT mice developed parasitemia of $14.3 \pm 0.95\%$ on day 3, which rapidly rose to $53.6 \pm 6.0\%$ on day 5 p.i., at which point all mice in this group became moribund and were killed. In contrast, CD47^{-/-} mice were highly resistant against this virulent parasite and developed only $0.02 \pm 0.008\%$ on day 3, reached a peak parasitemia of 0.06 ± 0.03 on day 4, and cleared their infection by day 7 p.i. (Fig. S3). Thus, absence of CD47 converted a highly virulent *P. yoelii* strain into a nonvirulent strain.

Modulation of CD47 Expression Affects the Parasite Burden and Host Survival. To further ascertain that CD47 phenotype is a determinant of malaria infectivity, we investigated the effect of induced generation of young RBC on the outcome of GFP-PyNL infection in the WT C57BL/6 mice. Phenyl hydrazine (PHZ), a known anemia inducer, causes oxidative damage to the RBC, followed by cell lysis that leads to the generation of new RBC to maintain the homeostasis (18). After two consecutive injections of PHZ in the WT mice, the packed cell volume of RBC dropped significantly ($60.4 \pm 1.2\%$ before PHZ treatment vs. $30.5 \pm 4.2\%$ after PHZ treatment; Fig. S4; $P = 0.0002$, Student's *t* test). Simultaneously, CD47 expression on RBC in PHZ-treated mice was significantly higher than in untreated mice (MFI: $2,585.4 \pm 71.8$, PHZ treated group, vs. $1,425.2 \pm 24.5$, PHZ untreated group; $P < 0.0001$, Student's *t* test), confirming that the percentage of young RBC is significantly higher in the anemia-induced model (Fig. S5). After GFP-PyNL infection, PHZ-treated mice developed uncontrolled parasitemia ($36.0 \pm 13.2\%$ in PHZ-treated vs. $1.74 \pm 0.25\%$ in untreated mice on day 3 p.i.;

Fig. 4A; $P = 0.0357$, Mann-Whitney *U* test). The non-PHZ-treated mice followed a course of parasitemia that was similar to that observed in Fig. 3, and these mice self-resolved their infection by day 17 p.i. In contrast, all PHZ-treated mice died of malaria by day 5 p.i.

We also investigated the effect of down-regulation of CD47 level by injecting an anti-CD47 neutralizing antibody and measured the parasitemia after GFP-PyNL infection in the WT C57BL/6 mice. A single administration of miap301, a mouse anti-CD47 neutralizing antibody, on the day of GFP-PyNL infection caused a significant reduction in parasite burden (neutralizing CD47 Ab-treated mice, $0.026 \pm 0.008\%$; isotype Ab-treated mice, $0.39 \pm 0.05\%$; Fig. 4B) on day 3 p.i. ($P = 0.0004$, Student's *t* test).

Together, these results clearly establish that CD47^{hi} RBC are more amiable to supporting the growth of PyNL parasite. Furthermore, a shift toward a population of CD47^{hi} RBC may convert a nonlethal malaria infection into a lethal one, or conversion toward a population of CD47^{lo} RBC may attenuate the parasite virulence.

Immunologic Basis Underlying CD47-Mediated Resistance from Malaria. We investigated an immunologic basis for the enhanced resistance from malaria observed in CD47^{-/-} mice. To accomplish this, we performed the flow cytometry analysis of splenic cells in the GFP-PyNL-infected (day 7) WT and CD47^{-/-} mice. In the splenic T-cell repertoire, in CD47^{-/-} mice, the percentage of CD4⁺ T cells was 1.4-fold lower, but it was 1.3-fold higher for CD8⁺ T cells than in the WT mice (Fig. S6). Simultaneously, the percentage of $\gamma\delta^+$ T cells was found to be twofold lower in the CD47^{-/-} mice, whereas the differences between the natural killer T (NKT) cell and B cell were not significant (Fig. S6). Interestingly, the percentage of F4/80⁺ tissue resident macrophages was 1.8-fold higher in the CD47^{-/-} mice than the WT mice (Fig. 5A). The analysis of absolute numbers of the studied cell populations showed no significant difference between the CD47^{-/-} and WT groups (Fig. 5B and Fig. S6). In addition, the serum cytokine profiling was performed to gain an understanding of the effect of cytokines toward the protection against PyNL infection in the CD47^{-/-} mice. The eight cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, GM-CSF, TNF- α , and IFN- γ) were measured in the serum of GFP-PyNL-infected CD47^{-/-} mice and WT mice on day 7 p.i. (Fig. S7). Among the cytokines measured, serum IL-10 level was 1.9-fold lower in CD47^{-/-} mice than WT mice (Fig. 5C); flow cytometry results had revealed a 7.5-fold-lower IL-10 producing F4/80⁺ cells in CD47^{-/-} mice (Fig. 5D). These results are in concordance with a previous report showing that IL-10^{-/-} mice were highly resistant to PyNL infection (19). No significant changes in serum levels of IL-2, IL-4, IL-5, IL-12, TNF- α , IFN- γ , and GM-CSF were observed (Fig. S7).

Fig. 3. Absence of CD47 confers resistance against *PyNL* malaria. Course of parasitemias in the CD47^{-/-} (*n* = 5) and the WT C57BL/6 mice (*n* = 5) after infection with GFP-*PyNL* parasite and percentage parasitemia were expressed as mean $\pm$ SEM. Data were compared using two-way ANOVA, followed by Bonferroni test.

Enhanced Phagocytic Activity by Splenic F4/80 Macrophages in GFP-*PyNL*-Infected CD47^{-/-} Mice. A major function of CD47 is to interact with the signal regulatory protein alpha receptor and to inhibit phagocytosis of young RBC by macrophages to maintain an adequate level of RBC in circulation. We investigated whether the GFP-*PyNL*-infected RBC were subjected to enhanced phagocytic clearance by F4/80⁺ macrophages from the CD47^{-/-} mice compared with those from the WT mice. F4/80⁺ is present on a wide distribution of resident macrophages in the lymphohematopoietic system, including in the red pulp area of spleen (20), a site known for the clearance of aged RBC and cells expressing foreign antigens, including infected RBC (20). GFP-*PyNL*-infected WT and CD47^{-/-} mice were killed on day 7 p.i., and the in vivo engulfment of GFP-*PyNL*-infected RBC was measured in spleen cell suspension by flow cytometry. We found that the percentage of F4/80⁺ macrophages that had internalized GFP-*PyNL*-infected RBC was significantly higher ($P = 0.0005$, Student's t test) in the spleen cells from the CD47^{-/-} mice than in WT mice ($13.59 \pm 1.2\%$ CD47^{-/-} vs. $3.69 \pm 0.7\%$ WT; Fig. 5E). This suggests the enhanced protection against malaria observed in the absence of CD47, at least in part, may be attributed to enhanced susceptibility to clearance of parasitized RBC by splenic macrophages during *PyNL* infection.

Discussion

The RBC age-based preference for invasion by merozoites of some *Plasmodium* species has important implications for malaria-induced disease severity and host survival (1). Evidence from human malarias and experimental models has established that *Plasmodium* species that invade and grow inside young RBC are generally benign in nature and cause a self-resolving infection. In a healthy host, reticulocytes are only 1% of circulating RBC (21), and even in the conditions of malaria anemia, the number of RBC available for invasion becomes a limiting factor that prevents an uncontrolled parasite growth, and infection is attenuated and self-resolved. Why certain *Plasmodia* have evolved to infect only young RBC is not fully understood.

CD47 is a marker of self on many cells, including RBC, and RBC that lack CD47 are rapidly cleared by splenic macrophages (12). In this article, we have quantitatively demonstrated that PyNL parasites preferentially invade and grow inside the young RBC and provide evidence for a direct relationship between the

CD47 intensity and parasite burden. We find that in the same mouse, parasitized RBC had higher CD47 levels than non-parasitized RBC, and CD47^{hi} RBC had a higher parasite burden than CD47^{lo} RBC (Fig. 2 *B* and *D*). As the level of CD47 declined in maturing RBC (Fig. S1), CD47^{lo} RBCs were less susceptible to GFP-*PyNL* infection. Importantly, we find that the CD47 phenotype is essential to support the optimal replication of the GFP-*PyNL* parasite (Fig. 3). CD47^{-/-} mice on the C57BL/6 background are healthy and exhibit normal RBC counts and hematocrit (22). Compared with WT mice, CD47^{-/-} mice maintained very low parasite burden, and they cleared their infection at an accelerated rate (Fig. 3). These results strongly suggest that the abundance of CD47^{hi} RBC exacerbates malaria infection, whereas CD47^{lo} RBC or RBC lacking CD47 were associated with less fulminant infection or resistance against malaria. Interestingly, the absence of CD47 was also detrimental for the growth of a highly virulent strain of *P. yoelii* that invades RBC of all ages. The CD47^{-/-} mice cleared their infection after reaching the peak average parasitemia of <0.1% on day 4 p.i., whereas their WT counterparts developed >50% parasitemia on day 5 p.i. (Fig. S3) and were killed. These results indicate the possible role of CD47 as a receptor of RBC used by the *P. yoelii* merozoites in the process of invasion, which is the subject of ongoing investigation in our laboratory.

Studies searching for a possible immunologic basis underlying the CD47-mediated enhanced immunity revealed that on day 7 p.i., infected CD47^{-/-} mice had reduced proportions of splenic CD4⁺T and $\gamma\delta^+$ T cells (Fig. S6), but an increased proportion of CD8⁺ T cells compared with WT mice. Notably, infected CD47^{-/-} mice had a significantly higher proportion of F4/80⁺ splenic cells (Fig. 5A); these F4/80⁺ cells also had a higher percentage of internalized GFP-PyNL-infected RBC than the F4/80⁺ cells from the WT mice (Fig. 5E). These results suggest that the absence of CD47 may render the infected RBC more susceptible to splenic clearance through phagocytic macrophages, which, at least in part, could be responsible for the enhanced resistance to malaria observed in

Fig. 4. Modulation of CD47 level affects the severity of GFP-PyNL infection. (A) Two dosages of PHZ were given on consecutive days to WT C57BL/6 mice ($n = 5$), and on the following day the PHZ-treated and PHZ-nontreated mice were infected with GFP-PyNL. Parasitemia was measured on day 3; results are shown as mean $\pm$ SEM values. (B) WT C57BL/6 mice ($n = 5$) were injected with either the neutralizing anti-CD47 mAb (miap301) or an isotype control antibody and were challenged with GFP-PyNL the same day. The parasite burden was measured on day 3 p.i.; results are shown as mean $\pm$ SEM. The percentage parasitemias between the neutralizing anti-CD47 antibody and isotype control mice were compared using the Student's *t* test (B), whereas comparisons between the PHZ-treated and PHZ-nontreated mice were made using Mann-Whitney *U* test (A).

Fig. 5. Immunologic analysis of resistance to GFP-PyNL infection in CD47^{-/-} mice. The WT and CD47^{-/-} C57BL/6 mice were infected with GFP-PyNL, and spleen cells from the day 7 p.i. mice were stained with anti-F4/80-PerCP antibody; results were analyzed by flow cytometry. (A) Percentage of F4/80⁺ cell population in GFP PyNL-infected WT and CD47^{-/-} mice are expressed as mean ± SEM. (B) Absolute number of F4/80⁺ cells in the WT and CD47^{-/-} mice on day 7 p.i. was calculated and presented as mean ± SEM. (C) Serum IL-10 levels in the WT and CD47^{-/-} mice on day 7 p.i. were measured by the bioplex assay and presented as picograms per milliliter. (D) Percentage of IL-10 cytokine producing F4/80⁺ cells was determined from the WT and CD47^{-/-} mice on day 7 p.i. (E) Percentage of F4/80⁺ cells that internalized the GFP-PyNL parasitized RBC was measured by flow cytometry. The statistical methods used were Student's *t* test (A, B, D, and E) and repeated measure ANOVA (C).

CD47^{-/-} mice; this is supported by an earlier report that absence of CD47 on RBC is associated with their phagocytic clearance (12).

We also find that F4/80⁺ cells from infected CD47^{-/-} mice had lower F4/80⁺ intracellular (Fig. 5D) and serum IL-10 levels (Fig. 5C) on day 7 p.i. Previously, IL-10 has been shown to suppress immune effector mechanisms during PyNL infection; IL-10^{-/-} mice were able to clear a PyNL infection at significantly lower parasitemia than their WT counterparts (19). The role of CD47 in driving malaria-specific immunity should be interpreted with caution, as CD47^{-/-} mice had a significantly lower antigenic load, which may have influenced the type of immune response generated. We propose that the preferential invasion of young, CD47^{hi} RBC by malaria parasites shields the parasitized RBC from an early clearance by the splenic phagocytic cells in the absence of immunity. However, the limited availability of young RBC limits the ability of reticulocyte-preferring plasmodia to allow uncontrolled growth, and such infections are resolved without turning fulminant. This argument is further supported by the observation that GFP-PyNL infection turns lethal when an unlimited supply of young, CD47^{hi} RBC was made available by inducing reticulocytosis by injections of PHZ (Fig. 4A). In the absence of malaria infection, PHZ-treated mice were able to slowly reconstitute their hematocrit and survived. Previously, it has been reported that up-regulation of CD47 expression on mouse hematopoietic cells can be achieved by external cytokine and inflammatory stimuli, and that the level of CD47 determined the fate of their phagocytic clearance (13).

CD47 was initially identified as a tumor antigen on human ovarian cancer cells (23); since then, it has been found to be overexpressed in several types of cancers (15). Many researchers considered this as an immune evasion mechanism to avoid clearance of cancer cells by phagocytic cells. Recent studies have shown that injection of anti-CD47 mAbs facilitated the phagocytosis of cancer cells by macrophages and initiated an anti-T-cell immune response. Thus, anti-CD47 antibody treatment is being pursued as a strategy to overcome the immune tolerance induced by high CD47 expression and permits the uncontrolled growth of malignant tumors (15).

The complete picture of the role of CD47 in parasite invasion and replication in RBC and its relative contribution in immune-driven clearance of *Plasmodium* parasites and host pathogenesis remain far from clear. However, we think that the preferential invasion of CD47^{hi} young RBC may be an evolutionary adaptation that benefits the parasite by avoiding early parasite clearance by macrophages and ensuring host survival, as the low reticulocyte count renders the infection avirulent. Further studies to understand the role of CD47 in the invasion, replication, and pathogenesis of the malaria parasites are ongoing. The potential benefits include the regulation of parasite burden and attenuation of severe disease by the injection of neutralizing anti-CD47 antibody in young children and naive adults during a virulent *Plasmodium* infection.

Materials and Methods

Mice, Parasite, and Infection. Age-matched female WT and CD47^{-/-} mice (B6.12957-Cd47tm1Fpl/J) mice on the C57BL/6 background were obtained from The Jackson Laboratory. Mice were maintained at the facilities at the Center for Biologics Evaluation and Research (CBER) at Kensington, MD. This study was done in accordance with the guidelines set forth for the care and use of laboratory animals by the Food and Drug Administration. The protocol under which these studies were performed was approved by the Institutional Animal Care and Use Committee of the CBER (Animal Study Protocol 2002-21). PyNL, GFP-PyNL (24), and PyYM were used as the parasite sources and delivered i.p. as 1×10^6 parasitized RBC to cause infection in the experimental mice. The details of these procedures are provided in the [SI Materials and Methods](#).

Parasitemia, Hematocrit, and RBC Count. The course of parasitemia in the WT and CD47^{-/-} C57BL/6 mice were determined by microscopy on Giemsa-stained thin blood films. Hematocrit determination was done by measuring the percentage of packed cell volume in whole blood, and results were compared among the test groups. RBC count was determined using a hemocytometer. The detail of parasitemia measurement is provided in the [SI Materials and Methods](#).

CD47 MFI Measurement. To determine the CD47 expression on RBC, whole blood was stained with anti-mouse CD47 antibody (miap301) tagged with

phycoerythrin cyanine 7 (PE-Cy7), and the MFI was measured by FACS Canto II, as described in the *SI Materials and Methods*.

Biotin Assay. To determine the age of the RBC, RBC were biotin-coated by three consecutive injections of biotin-X-NHS ester (Calbiochem), 1 mg each dose, i.v. Biotin-labeled RBC were detected by flow cytometry, using APC-streptavidin capturing in vitro. Mice were infected with 1×10^6 GFP-PyNL parasitized RBC on the following day after the last biotin injection, and parasitemia in young (biotin-streptavidin-negative) and aged (biotin-streptavidin-positive) populations of RBC was measured by flow cytometry. The detailed method is discussed in the *SI Materials and Methods*.

Induction of Anemia. Anemia in WT C57BL/6 mice was induced by i.p. injections of PHZ (60 mg/kg body weight; Sigma) on two consecutive days. The anemic condition was monitored by hematocrit assay and by determining the RBC count as described. On the day after the second PHZ injection, mice were infected with 1×10^6 GFP-PyNL parasitized RBC, and parasitemia was monitored by blood-film microscopy.

Phagocytosis Assay. The in vivo phagocytosis of parasitized RBC by the splenic F4/80⁺ population was measured on day 7 of GFP-PyNL p.i. WT and CD47^{-/-} C57BL/6 mice. Briefly, single-cell suspension of splenocytes was prepared as described previously (25), and after blocking with CD16/32 for 30 min (BD Biosciences), the splenic cells were stained for 30 min with perCP-anti-F4/80 antibody (Biolegend). The percentage of macrophages that had engulfed GFP-PyNL parasitized RBC was calculated on FACS Canto II.

In Vivo Neutralization of CD47. A single dose of anti-mouse CD47 neutralizing monoclonal antibody (clone: miap301, 100 μ g/dose; Biolegend) or rat IgG2a, κ isotype control antibody (Biolegend) was administered by i.p. route in the WT C57BL/6 mice and, on the same day, infected with the 1×10^6 GFP-PyNL parasitized RBC i.p., and parasitemias were determined by blood-film microscopy in blood samples taken on day 3 p.i.

Flow Cytometry. The biotin-labeling-based RBC age determination, CD47 expression level in RBC, and percentage GFP-PyNL infection in RBC were performed by flow cytometry, as described earlier, using a FACS Canto II and the FACS Diva software (BD Biosciences). In addition, flow cytometry was used for immunologic profiling. The intracellular cytokine assay was done in the F4/80⁺ population for IL-10 cytokine. The absolute number of cell population was also determined from the total live splenic cells. The details of the experimental procedure and information on the antibodies and their sources and methods used for data analysis are provided in the *SI Materials and Methods*.

Analysis of Serum Cytokines. Eight cytokines [IL-2, IL-4, IL-5, IL-10, IL-12 (p70), GM-CSF, IFN- γ , and TNF- α] were quantitated in serum samples collected from the WT and CD47^{-/-} C57BL/6 mice on day 7 p.i. with GFP-PyNL, using the Bio-Plex Pro Mouse Cytokine 8-plex Th1/Th2 assay kit (Bio-Rad Laboratories). Briefly, serum samples were incubated with beads coated with a capture antibody, biotinylated detection antibody, and streptavidin-phycoerythrin conjugate and three wash steps were performed between each step. Data were acquired using the Bio-Plex 200 reader, and cytokine quantity was determined using Bio-Plex Manager software version 6.0 (Bio-Rad Laboratories).

Statistical Analysis. Two-way ANOVA followed by Bonferroni post hoc comparison test was used for the percentage of parasitemia or CD47 intensity based on days postinfection. These comparisons were made between young RBC and aged RBC, parasitized RBC and nonparasitized RBC, CD47^{hi} and CD47^{lo}, or WT and KO mice. Repeated-measure ANOVA was applied to make comparisons of serum cytokine concentration level between WT and KO mice. The rest of the data were analyzed using either Student's *t* test or Mann-Whitney *U* test.

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