

# CD47-SIRP $\alpha$ Interactions Regulate Macrophage Uptake of *Plasmodium falciparum*-Infected Erythrocytes and Clearance of Malaria *In Vivo*

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**CD47 engagement by the macrophage signal regulatory protein alpha (SIRP $\alpha$ ) inhibits phagocytic activity and protects red blood cells (RBCs) from erythrophagocytosis. The role of CD47-SIRP $\alpha$  in the innate immune response to *Plasmodium falciparum* infection is unknown. We hypothesized that disruption of SIRP $\alpha$  signaling may enhance macrophage uptake of malaria parasite-infected RBCs. To test this hypothesis, we examined *in vivo* clearance in CD47-deficient mice infected with *Plasmodium berghei* ANKA and *in vitro* phagocytosis of *P. falciparum*-infected RBCs by macrophages from SHP-1-deficient (*Shp-1*<sup>-/-</sup>) mice and NOD.NOR-*Idd13.Prkdc*<sup>scid</sup> (NS-*Idd13*) mice, as well as human macrophages, following disruption of CD47-SIRP $\alpha$  interactions with anti-SIRP $\alpha$  antibodies or recombinant SIRP $\alpha$ -Fc fusion protein. Compared to their wild-type counterparts, *Cd47*<sup>-/-</sup> mice displayed significantly lower parasitemia, decreased endothelial activation, and enhanced survival. Using macrophages from SHP-1-deficient mice or from NS-*Idd13* mice, which express a SIRP $\alpha$  variant that does not bind human CD47, we showed that altered SIRP $\alpha$  signaling resulted in enhanced phagocytosis of *P. falciparum*-infected RBCs. Moreover, disrupting CD47-SIRP $\alpha$  engagement using anti-SIRP $\alpha$  antibodies or SIRP $\alpha$ -Fc fusion protein also increased phagocytosis of *P. falciparum*-infected RBCs. These results indicate an important role for CD47-SIRP $\alpha$  interactions in innate control of malaria and suggest novel targets for intervention.**

Macrophages can recognize altered cell surface ligands on aged, malignant, or infected cells, triggering their phagocytic uptake. CD47 is a glycoprotein “marker of self” that is expressed on cell membranes in humans and mice (1, 2). Decreased levels of CD47 are present on senescent and apoptotic cells and are associated with increased macrophage clearance. Conversely, high levels of CD47 expressed on cancer cells and leukemic stem cells may prevent the cells from being efficiently cleared *in vivo* (2, 3).

CD47 exerts its inhibitory effect on phagocytosis through its interaction with macrophage signal-regulatory protein alpha (SIRP $\alpha$ ). SIRP $\alpha$  and CD47 constitute a cell-cell communication system that plays essential roles in hematopoietic and immunological regulation. Following CD47 engagement, SIRP $\alpha$  cytoplasmic-region immunoreceptor tyrosine-based inhibitory motifs (ITIMs) recruit Src homology-2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2, resulting in decreased macrophage uptake and reduced production of proinflammatory mediators (2, 4–7). A critical role of CD47-SIRP $\alpha$  signaling in negatively regulating erythrophagocytosis has been demonstrated by the rapid macrophage clearance of CD47-deficient red blood cells (RBCs) when transfused into wild-type animals (2).

CD47 has been implicated in mediating the outcome of several infectious processes. CD47-deficient mice succumb to bacterial peritonitis (8) but are less susceptible to *Staphylococcus aureus*-induced arthritis (9) and are protected from lipopolysaccharide (LPS)-induced acute lung injury and *Escherichia coli* pneumonia (10). SIRP $\alpha$  is polymorphic and highly expressed on hepatic Kupffer cells and splenic red pulp macrophages, cell populations important in the innate control of malaria (11, 12). A recent study of *Plasmodium yoelii* malaria in a nonlethal murine model re-

ported that CD47 was an important determinant of age-specific RBC invasion and parasite burden (13). Although *Plasmodium falciparum* is responsible for the majority of cases of severe and cerebral malaria (CM), the role of CD47-SIRP $\alpha$  in falciparum malaria has not been reported. In this study, we used a combined genetic and functional approach to investigate the contribution of CD47-SIRP $\alpha$  interactions to innate clearance of *P. falciparum*-infected RBCs *in vitro* and in a lethal model of experimental cerebral malaria (ECM). We show that CD47 on infected RBCs and SIRP $\alpha$  on macrophages are important determinants of the outcome *in vivo* and of macrophage phagocytosis of *P. falciparum*-infected RBCs *in vitro*. A direct role for SIRP $\alpha$  was established by functional disruption of receptor signaling using anti-SIRP $\alpha$  antibodies and recombinant SIRP $\alpha$ -Fc fusion proteins.

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## MATERIALS AND METHODS

**Ethics statement and *Plasmodium berghei* ANKA ECM infection.** The animal use protocols were reviewed and approved by the Faculty of Medicine Advisory Committee on Animal Services at the University of Toronto according to the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care (53).

C57BL/6J-Ptpn6<sup><me-v></sup>/J (*Shp1*<sup>-/-</sup>), C57BL/6 129-cd47<sup><tm1Fpl></sup>/J (*Cd47*<sup>-/-</sup>), and wild-type counterpart C57BL/6J mice (6 to 9 weeks old) were obtained from Jackson Laboratories (West Grove, PA). *P. berghei* ANKA parasites (MR4) were cultivated by passage through C57BL/6J mice, and experimental infections were performed as described previously (14).

**Reagents.** Endotoxin-free RPMI 1640 culture medium was obtained from Life Technologies (Burlington, Canada). Fetal calf serum (FCS) was obtained from Wisent (Mississauga, Canada) and was heat inactivated at 56°C for 30 min before use. Mouse anti-human CD47 antibodies (clone B6H12) with isotype control mouse IgG1, anti-CD47 monoclonal antibody (MAb) (clone miap301) with isotype control, and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD47 antibodies (clone B6H12) with FITC-conjugated mouse IgG isotype control were from BD PharMingen, San Diego, CA. Ficoll-Paque and Percoll were obtained from Pharmacia (Peapack, NJ). Monoclonal anti-SIRP $\alpha$ /CD172a and anti-mouse IgG conjugated to alkaline phosphatase were from Sigma (St. Louis, MO). All other reagents were obtained from Sigma-Aldrich (Oakville, Canada).

**Cytokine ELISA.** Gamma interferon (IFN- $\gamma$ ), tumor necrosis factor (TNF), von Willebrand factor (vWF), angiopoietin-1 (Ang-1), and soluble intracellular adhesion molecule-1 (sICAM-1) were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Briefly, plates were coated with the respective cytokine capture antibodies in phosphate-buffered saline (PBS) overnight at 4°C and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. Plasma from infected and uninfected mice was diluted 1:50, added to the plates, and incubated for 2 h at room temperature (RT). Detection antibody (1:10,000) and streptavidin-horseradish peroxidase (HRP) (1:200) were then incubated at RT, followed by substrate solution, stop solution, and reading at 450 nm.

**RBC CD47 assays.** Percoll-washed RBCs were infected with *P. falciparum*. Uninfected and ring-stage- and mature-stage-infected RBCs were purified with sorbitol and Percoll as described previously (15). The purified cells were washed with PBS supplemented with 0.5% BSA and 0.1% sodium azide. Five million cells were incubated at 4°C with mouse anti-human CD47 (clone B6H12), as well as the isotype control mouse IgG1. Fifty million RBCs from wild-type or CD47-deficient mice were incubated with anti-CD47 MAb (clone miap301) or with an isotype control. All the antibodies were diluted 1:500 in PBS–0.1% BSA. RBCs were washed and incubated with anti-mouse IgG conjugated to alkaline phosphatase. After three washings, the RBC membranes were extracted with Tris-EDTA buffer (1 mM Tris, 5 mM EDTA, pH 8) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF) and then solubilized in PBS–0.1% Tween 20 at 37°C. CD47 levels were analyzed with the alkaline phosphatase-conjugated substrate nitroblue tetrazolium chloride–5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt (NBT-BCIP) and expressed as the optical density at 450 nm (OD<sub>450</sub>). The values of CD47 were brought to 100% parasitemia using the following calculation, as described previously (15):  $I = (\text{Tot} - N \times n) / (1 - n)$ , where  $I$  is the concentration of CD47 on 100% infected RBCs, Tot is the anti-CD47 bound on uninfected RBCs and infected RBCs,  $N$  is the anti-CD47 bound on uninfected RBCs, and  $n$  is the fraction of uninfected RBCs.

**Flow cytometric analysis of CD47 on RBCs.** Direct fluorescent staining was measured on uninfected or ring-stage- or mature-stage-*P. falciparum*-infected RBCs in darkness at 4°C for 20 min with anti-human CD47-FITC (clone B6H12) or isotype control. CD47 on murine RBCs was analyzed using anti-mouse CD47-FITC (clone miap301) or isotype control diluted 1:25 (vol/vol). To detect membrane glycoprotein, RBCs were stained with phycoerythrin anti-human CD235ab antibody (clone

HIR2; BioLegend) for 30 min at RT. The RBCs were washed and resuspended in 500  $\mu$ l 1 $\times$  PBS containing 2% formaldehyde supplemented with Vibrant (Life Technologies) diluted 3:1,000 (vol/vol) to detect parasite DNA. All samples were analyzed by flow cytometry (FACSCalibur; BD), and the data were analyzed using FlowJo (Tree Star).

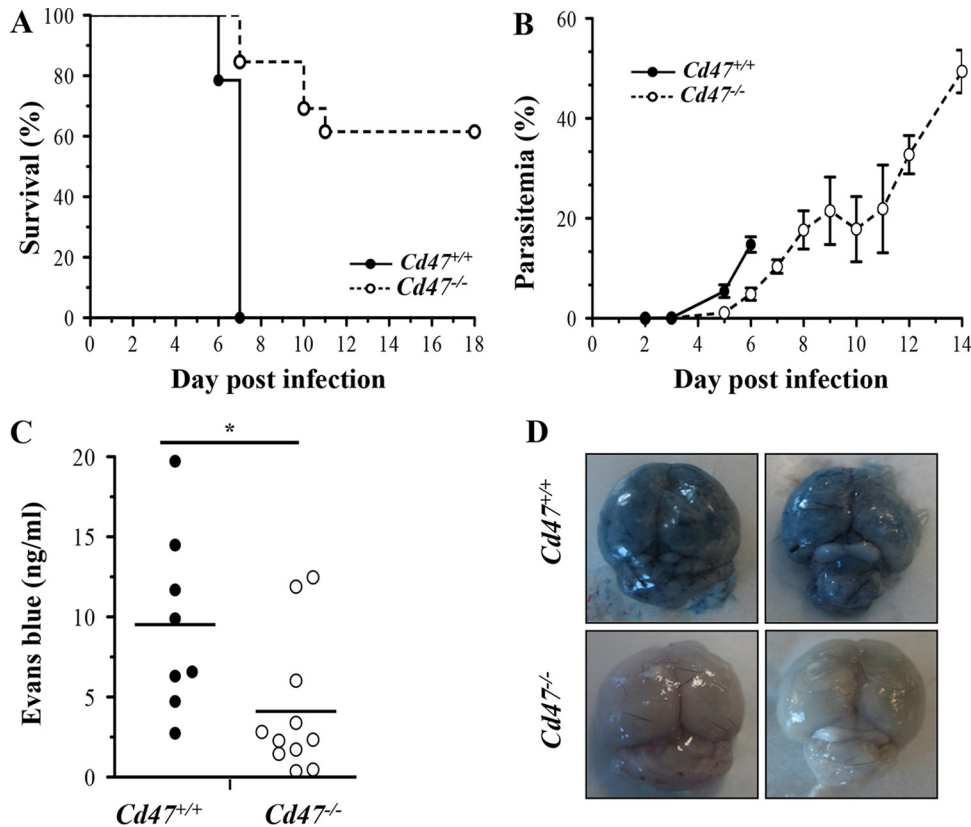
**Phagocytosis assays with *P. falciparum*-infected RBCs.** Human peripheral blood mononuclear cells were isolated and purified from the peripheral blood of healthy donors as previously described (16). Resident peritoneal macrophages from *Shp1*<sup>-/-</sup>, NOD.NOR-*Idd13.Prkdc*<sup>scid</sup> (NS-*Idd13*), and wild-type mice were harvested as described previously (17, 18). Macrophages used in phagocytosis assays were examined with and without prestimulation with LPS and IFN- $\gamma$  (17). Briefly, seeded macrophages were incubated with 100 ng/ml IFN- $\gamma$  (R&D Systems) for 24 h and 0.3  $\mu$ g/ml LPS (Sigma-Aldrich) for 1 h and washed three times with RPMI 1640. *P. falciparum* clones ITG and 3D7 (mycoplasma free) were maintained in continuous culture, synchronized, and purified as described previously (15). Uninfected and *P. falciparum*-infected RBCs were incubated with 50% fresh autologous serum for 30 min at 37°C; resuspended in RPMI 1640 with 20  $\mu$ g/ml anti-SIRP $\alpha$  antibody (Sigma-Aldrich), 10  $\mu$ g/ml SIRP $\alpha$ -Fc (human SIRP $\alpha$ -Fc fusion protein) (17), or the respective isotype control antibodies at 10% hematocrit; and incubated with macrophages (adhered to glass coverslips at a target/effector ratio of 40:1 [ring stage] or 20:1 [mature stage]) at 37°C for 120 min. Phagocytosis assays were conducted as described previously (16). All experiments were performed in duplicate and repeated at least 3 times.

**Statistical analysis.** Statistical significance for the survival study was assessed by a log-rank test and parasitemia and cytokine levels by two-way repeated-measures analysis of variance (ANOVA). Other comparisons were assessed by a Mann-Whitney test. Statistical analysis was performed using GraphPad (La Jolla, CA) Prism software.

## RESULTS

**CD47 deficiency is associated with improved survival in a pre-clinical model of ECM.** To determine whether CD47 participates in the innate control of malaria *in vivo*, *Cd47*<sup>-/-</sup> mice and their wild-type *Cd47*<sup>+/+</sup> counterparts were inoculated intraperitoneally with 1  $\times$  10<sup>6</sup> *P. berghei* ANKA-infected RBCs and monitored for survival and parasitemia in the ECM model (Fig. 1). Compared to *Cd47*<sup>+/+</sup> mice, *Cd47*<sup>-/-</sup> mice developed significantly lower parasite burdens over the course of infection (Fig. 1B) and displayed improved survival (Fig. 1A). To rule out a preferential invasion of RBCs from *Cd47*<sup>+/+</sup> mice, we conducted *in vitro* assays of *P. berghei* ANKA invasion of RBCs from *Cd47*<sup>+/+</sup> mice and *Cd47*<sup>-/-</sup> mice. We did not observe significant differences in invasion in these experiments (see Fig. S2 in the supplemental material). However, in other ongoing studies, we have observed significantly impaired invasion of *Cd47*<sup>-/-</sup> RBCs by *Plasmodium chabaudi chabaudi* AS (see Fig. S3 in the supplemental material), suggesting species-specific differences in mechanisms of invasion. All *Cd47*<sup>+/+</sup> mice developed ECM and succumbed to infection, whereas the majority of *Cd47*<sup>-/-</sup> mice survived without developing signs of ECM until the completion of the experiment. In addition, the rapid murine coma and behavior scale (RMCBS) for quantitative assessment of murine ECM (19) showed that *Cd47*<sup>-/-</sup> mice are less susceptible to ECM and neurological injury (see Fig. S1 in the supplemental material).

To assess whether improved survival in *Cd47*<sup>-/-</sup> mice was associated with preservation of the blood-brain barrier (BBB), we assessed vascular integrity at day 6 postinfection using the Evans blue dye assay (14). Compared to *Cd47*<sup>+/+</sup> mice, *Cd47*<sup>-/-</sup> mice had significantly reduced cerebral microvascular leakage and preserved BBB function, as assessed by decreased dye extravasation into the brain parenchyma (Fig. 1C and D). Although *P. berghei*



**FIG 1** *Cd47*<sup>-/-</sup> mice are less susceptible to *P. berghei* ANKA ECM. (A) Survival curves following challenge with 1 million *P. berghei* ANKA-infected erythrocytes by intraperitoneal (i.p.) injection for *Cd47*<sup>+/+</sup> mice (solid line) versus *Cd47*<sup>-/-</sup> mice (dashed line).  $P < 0.0001$  by log-rank test;  $n = 13$  mice/group. (B) Parasite burdens as assessed by peripheral parasitemia were significantly decreased in *Cd47*<sup>-/-</sup> versus *Cd47*<sup>+/+</sup> *P. berghei* ANKA-infected mice but increased progressively.  $P < 0.001$  by two-way ANOVA;  $P < 0.05$  by Sidak's multiple test comparing *Cd47*<sup>+/+</sup> versus *Cd47*<sup>-/-</sup> on day 5 and day 6;  $n = 10$  mice/group. The parasitemia and survival curves are from one representative experiment out of the three performed. The error bars represent standard errors of the mean (SEM). (C and D) An Evans blue dye extravasation assay was performed on days 6 and 7 postinfection to assess microvascular leakage into the brain parenchyma. Shown are photographs of representative brains from *P. berghei* ANKA-infected *Cd47*<sup>+/+</sup> mice (top) and *Cd47*<sup>-/-</sup> mice (bottom) (D) and quantification of Evans blue dye in the brains (C) (\*,  $P = 0.0232$ ; Mann-Whitney test;  $n = 8$  for *Cd47*<sup>+/+</sup> mice and  $n = 11$  for *Cd47*<sup>-/-</sup> mice). The points represent individual animals, and the lines are group means.

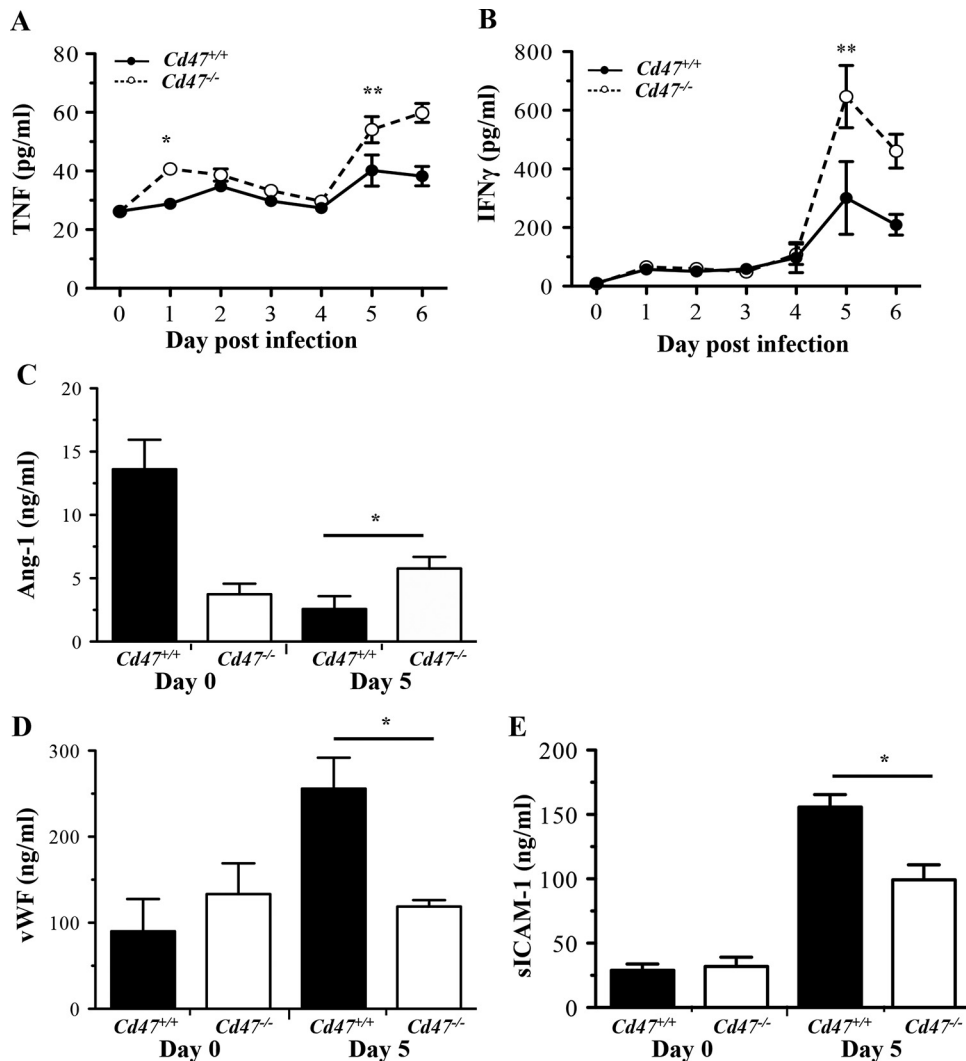
ANKA-infected *Cd47*<sup>-/-</sup> mice did not develop ECM, a proportion did succumb to anemia during the 2nd and 3rd weeks of infection.

**CD47-SIRP $\alpha$  signaling modulates malaria-induced inflammation and endothelial activation.** Increased proinflammatory cytokines are associated with poor clinical outcomes in both murine malaria models and human malaria parasite infection (12, 20, 21). To determine whether CD47-SIRP $\alpha$  interactions modify malaria-induced inflammation, we assessed levels of proinflammatory cytokines and endothelial activation biomarkers in plasma following *P. berghei* ANKA infection (Fig. 2). Of note, at days 5 and 6 postinfection, when *Cd47*<sup>+/+</sup> mice began showing signs of ECM, *Cd47*<sup>-/-</sup> mice had significantly higher levels of TNF and IFN- $\gamma$  in plasma (Fig. 2A and B) than their wild-type counterparts. Although high levels of proinflammatory cytokines are characteristic of cerebral malaria, production of these mediators early in the course of infection (~24 h postinfection) is associated with subsequent protection from ECM (22). Increased levels of circulating TNF were observed as early as 24 h after infection.

Endothelial activation is also a central feature of both human CM and murine ECM (14, 23). Ang-1 functions to maintain a quiescent endothelium, while elevated circulating levels of vWF

are associated with endothelial activation and severe malaria parasite infections (24, 25). Despite higher levels of proinflammatory cytokines, *P. berghei* ANKA-infected *Cd47*<sup>-/-</sup> mice had significantly higher levels of Ang-1 in plasma than infected *Cd47*<sup>+/+</sup> mice (Fig. 2C), and significantly lower levels of vWF and sICAM-1 in plasma (Fig. 2D and E) at day 5 postinfection. Collectively, these data indicate that disruption of SIRP $\alpha$  signaling is associated with preserved endothelial quiescence and BBB integrity (Fig. 1D) despite increased circulating levels of proinflammatory cytokines during malaria parasite infection.

**CD47 levels decrease on *P. falciparum*-infected RBCs compared to uninfected RBCs.** To extend our observations to human malaria, we investigated the role of CD47-SIRP $\alpha$  interactions in *P. falciparum* malaria. To determine whether CD47 expression is altered on malaria parasite-infected RBCs, we measured CD47 on human uninfected RBCs (uRBCs) and *P. falciparum*-infected RBCs (PfrRBCs) (Fig. 3). As a control, we examined levels of CD47 on uninfected *Cd47*<sup>-/-</sup> and *Cd47*<sup>+/+</sup> murine RBCs (Fig. 3A, left, and B, bottom). As determined by an immunoenzymatic assay (15), we observed decreased levels of CD47 on ring-stage (PfrRBCs) and mature-stage (PfmRBCs) *P. falciparum*-infected human RBCs compared to uninfected RBCs (Fig. 3A). Similarly,



**FIG 2** *P. berghei* ANKA infection of *Cd47<sup>-/-</sup>* mice is associated with increased systemic inflammation but decreased endothelial activation. The levels of inflammatory and endothelial markers in plasma were determined by ELISA in *Cd47<sup>+/+</sup>* and *Cd47<sup>-/-</sup>* mice at day 0 (prior to infection) and following infection with *P. berghei* ANKA. (A and B) TNF (A) and IFN- $\gamma$  (B) (\*,  $P < 0.001$ ; \*\*,  $P < 0.0001$ ; two-way ANOVA comparing infection and CD47 genotype with Sidak posttest comparisons; means and SEM;  $n \geq 4$ ). (C to E) Ang-1 (C), vWF (D), and sICAM-1 (E) (\*,  $P = 0.0379$ ,  $P = 0.0006$ , and  $P = 0.0059$ , respectively; Mann-Whitney test; means and SEM for groups;  $n \geq 7$ ).

flow cytometric analysis demonstrated decreased levels of anti-CD47 bound on PfrRBCs compared to uninfected RBCs and PfrRBCs (Fig. 3B, top). CD47 levels measured with anti-CD47 antibody (clone B6H12) decreased proportionally to the degree of maturation of the parasite in the RBCs (uninfected > ring-infected > mature-infected RBCs).

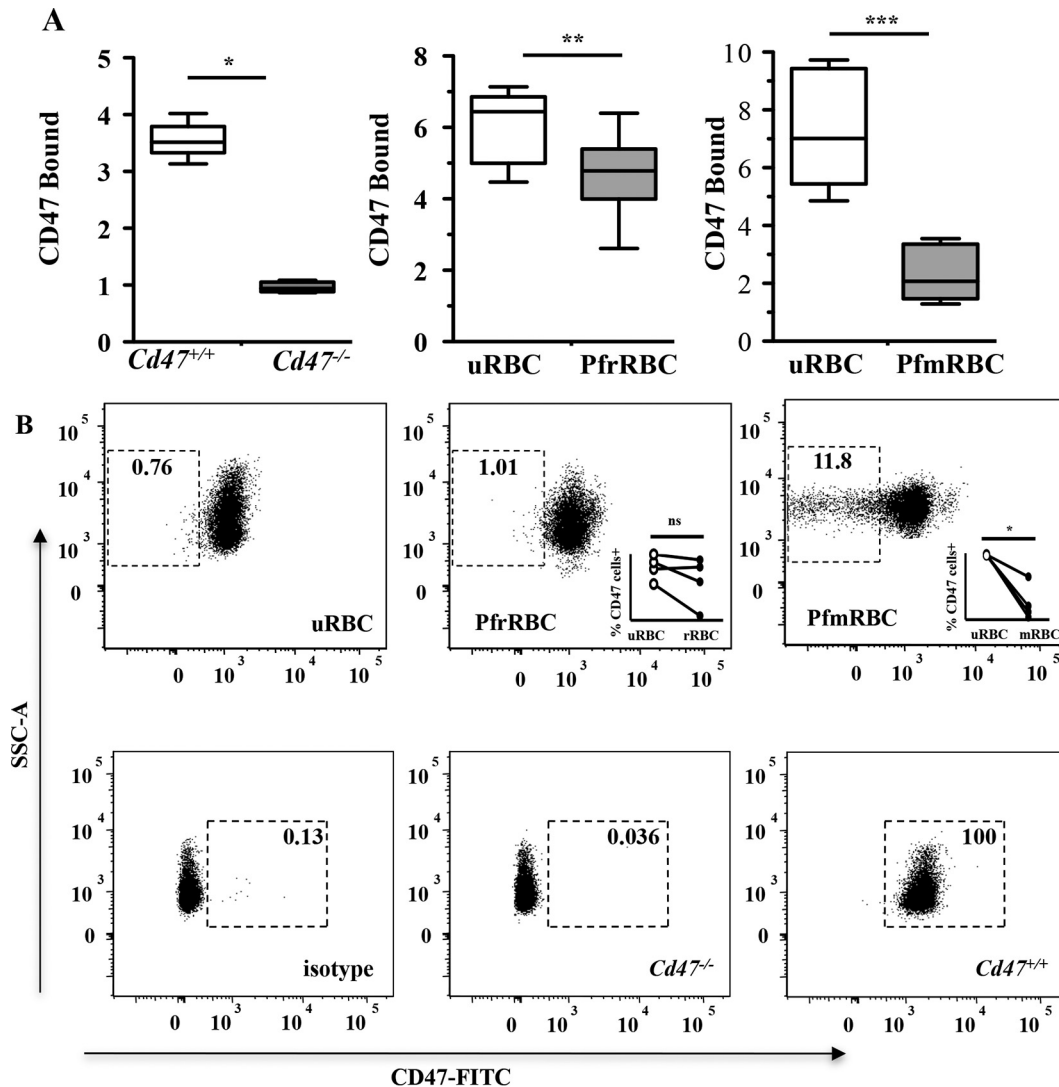
**Disrupted CD47-SIRP $\alpha$  interactions enhance phagocytosis of *Plasmodium falciparum*-infected RBCs.** Based on the *in vivo* observations that parasite burdens were lower in *P. berghei* ANKA-infected *Cd47<sup>-/-</sup>* mice than in their wild-type counterparts, we examined the impact of disruption of the CD47-SIRP $\alpha$  pathway on macrophage uptake of PfrRBCs.

Following ligation of CD47, SIRP $\alpha$  recruits and activates SHP-1, an important negative regulator of the macrophage effector function (5–7, 26). We examined macrophage uptake of both PfrRBCs and PfmRBCs using macrophages from SHP-1-deficient mice (*Shp1<sup>-/-</sup>* [*me<sup>v</sup>/me<sup>v</sup>* mice]) (27, 28). Compared to wild-type

control macrophages, *Shp1<sup>-/-</sup>* macrophages showed significantly increased phagocytosis of both PfrRBCs and PfmRBCs (Fig. 4A).

In order to obtain evidence of a direct role for SIRP $\alpha$  in PfrRBC uptake, we performed PfrRBC phagocytic assays (Fig. 4B) with bone marrow-derived macrophages from NOD.NOR-*Idd13.Prkdc<sup>scid</sup>* (NS-*Idd13*) mice, which express a SIRP $\alpha$  variant that does not bind to human CD47, or NS mice as controls (29). NS-*Idd13* mice were previously shown to eliminate xenotransplanted human hematopoietic stem cells via altered CD47-SIRP $\alpha$  interactions (29). We hypothesized that if macrophages also clear PfrRBCs via a SIRP $\alpha$ -dependent pathway, NS-*Idd13* variants would display increased phagocytotic uptake of PfrRBCs compared to NS control macrophages (17, 29). Indeed, macrophages from NS-*Idd13* mice showed significantly enhanced phagocytosis of both PfrRBCs and PfmRBCs compared to NS macrophages (Fig. 4B), supporting a direct role for SIRP $\alpha$  in mediating uptake of malaria parasite-infected RBCs.





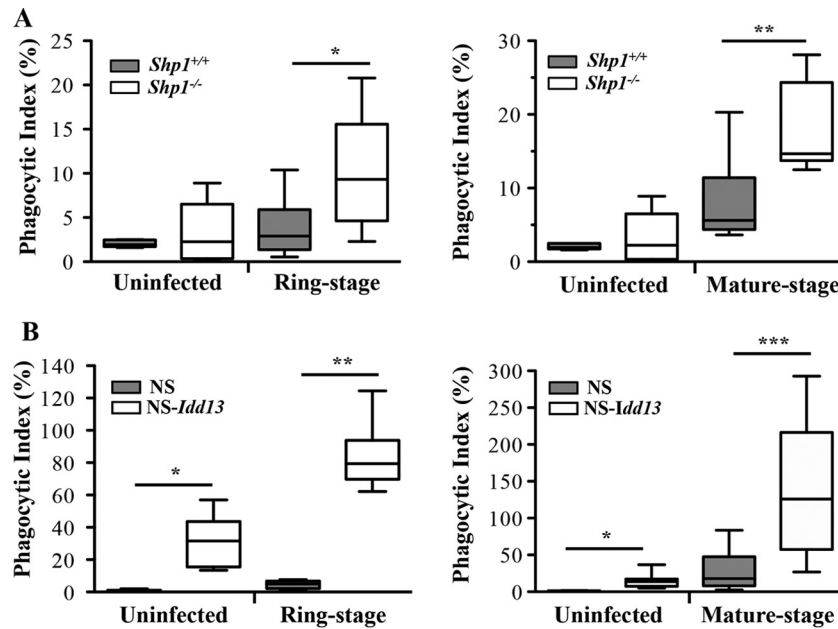
**FIG 3** *P. falciparum* infection of RBCs alters CD47 levels. (A) An immunoenzyme assay was used to measure mouse CD47 (clone miap-301) on uninfected RBCs from *Cd47<sup>+/+</sup>* and *Cd47<sup>-/-</sup>* mice (left) and human CD47 (clone B6H12) on uRBCs and PfrRBCs (middle) and PfmRBCs (right). The data are expressed as the ratio of anti-CD47-stained cells to isotype control-stained cells and are presented as the medians and ranges of two independent experiments with miap-301 (\*,  $P < 0.0001$ ,  $n = 10$  *Cd47<sup>+/+</sup>* mice versus  $n = 6$  *Cd47<sup>-/-</sup>* mice; Mann-Whitney test) and of eight experiments with B6H12 (\*\*,  $P = 0.022$ , and \*\*\*,  $P = 0.0001$ ; Mann-Whitney test). (B) Flow cytometry was also used to measure the levels of CD47 on uRBCs, PfrRBCs, PfmRBCs, *Cd47<sup>+/+</sup>* RBCs, and *Cd47<sup>-/-</sup>* RBCs. Uninfected RBCs were identified as vibrant negative, while infected RBCs (ring and mature stages) were identified as vibrant positive. Representative dot plots showing CD47 versus side scatter (SSC-A) are shown. The gates were based on isotype controls. The numbers indicate the percentages of cells within the CD47-negative gate (top) or the percentages of cells within the CD47-positive gate (bottom). The data are representative of the results of 4 experiments. Statistical comparisons were by Mann-Whitney matched-pair tests; \*,  $P = 0.013$ ; ns, not significant.

Importantly, the phagocytic index for infected RBCs was significantly higher than that for uninfected RBCs for both *Shp1<sup>-/-</sup>* macrophages (Fig. 4A) and NS-*Idd13* macrophages (Fig. 4B), demonstrating preferential uptake of malaria parasite-infected RBCs over uninfected, normal RBCs by macrophages upon disruption of CD47-SIRP $\alpha$  signaling. These data, combined with the observation of uptake when CD47 is altered (see Fig. S4 in the supplemental material), establish the relevance of CD47-SIRP signaling for the uptake of malaria parasite-infected RBCs by macrophages.

**Disruption of CD47-SIRP $\alpha$  engagement by SIRP $\alpha$ -Fc and anti-human SIRP $\alpha$  antibody induces phagocytosis of *P. falciparum*-infected RBCs.** As a complementary strategy to establish a

role for CD47 on PfrRBCs and SIRP $\alpha$  on murine or human monocyte-derived macrophages (MDMs) in the uptake of infected RBCs, we examined the consequences of functional blockade of SIRP $\alpha$  signaling in phagocytosis assays using human SIRP $\alpha$ -Fc protein (SIRP $\alpha$ -Fc) and anti-human SIRP $\alpha$  antibodies (anti-SIRP $\alpha$ ). SIRP $\alpha$ -Fc is a fusion protein composed of the CD47-binding IgV domain of human SIRP $\alpha$  fused to a human IgG4-Fc moiety and has been previously shown to induce preferential macrophage uptake of acute myeloid leukemia (AML) stem cells (17).

Human MDMs incubated with infected RBCs in the presence of anti-SIRP $\alpha$  or SIRP $\alpha$ -Fc displayed significantly increased uptake of ring-stage (Fig. 5), but not mature-stage, PfrRBCs. When human MDMs were prestimulated with LPS and IFN- $\gamma$ , uptake



**FIG 4** Genetic disruption of CD47-SIRP $\alpha$  interactions increases phagocytosis of *P. falciparum*-infected RBCs by murine macrophages. Uninfected RBCs and *P. falciparum*-infected ring-stage (left) and mature-stage (right) RBCs were incubated with macrophages from mice with SHP1 deficiency (*Shp1*<sup>-/-</sup>) (A) and variant SIRP $\alpha$  mice (NS-*Idd13*) (white boxes) compared to their wild-type counterparts (gray boxes) (B). Differential uptake of parasitized RBCs at ring stage and mature stage by macrophages from wild-type mice versus *Shp1*<sup>-/-</sup> mice (A) (\*,  $P = 0.022$ , and \*\*,  $P < 0.0001$ ; Mann-Whitney test) and macrophages from NS versus NS-*Idd13* mice (B) (\*,  $P = 0.005$ ; \*\*,  $P = 0.0022$ ; \*\*\*,  $P = 0.0006$ ; Mann-Whitney test) was observed. The results are the combined data from three experiments and are shown as box-and-whisker plots, representing interquartile and complete ranges, with the horizontal line in each box indicating the median level of phagocytic index.

increased 2-fold at ring stage and mature stage (Fig. 6A); however, similar to unprimed human MDMs, only ring-stage PfrRBCs were significantly different from isotype controls. In contrast, macrophages from NS mice primed with LPS and IFN- $\gamma$  exhibited significantly increased uptake of both ring- and mature-stage PfrRBCs in the presence of SIRP $\alpha$ -Fc compared to the isotype control (Fig. 6B). Notably, and in contrast to mouse MDMs, human MDMs incubated with uninfected RBCs in the presence of either SIRP $\alpha$ -Fc or anti-SIRP $\alpha$  did not show increased phagocytosis. Two *P. falciparum* lines (ItG and 3D7) were used in this study and displayed the same phenotype in macrophage uptake assays, indicating that our findings were not limited to a single parasite clone.

Thus, as shown using genetic approaches, functional disruption of SIRP $\alpha$  signaling by SIRP $\alpha$ -Fc fusion protein or anti-SIRP $\alpha$  antibodies resulted in significant and preferential phagocytosis of malaria parasite-infected RBCs over uninfected RBCs.

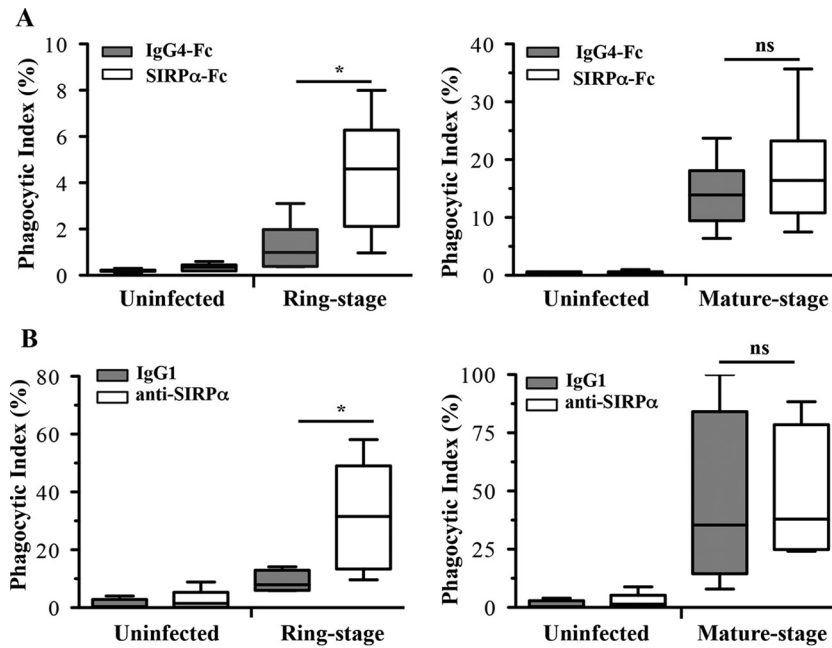
## DISCUSSION

This study provides direct evidence for the involvement of the CD47-SIRP $\alpha$  pathway in the host innate immune response to falciparum malaria. We show that CD47 deficiency is associated with decreased parasite burdens and improved survival in a preclinical model of experimental cerebral malaria. Despite increased levels of proinflammatory mediators, endothelial integrity was preserved in *Cd47*<sup>-/-</sup> infected mice, as indicated by higher circulating levels of Ang-1, lower levels of vWF and sICAM-1, and enhanced BBB integrity. Experiments using *P. falciparum*-infected RBCs suggest that the mechanism of protection is via enhanced macrophage phagocytosis of both ring-stage and mature-stage malaria parasite-infected RBCs. *P. falciparum* infection of RBCs

was associated with altered CD47 expression, and evidence for a direct role for SIRP $\alpha$  in regulating macrophage phagocytosis was demonstrated by disrupting SIRP $\alpha$  signaling using both genetic (e.g., NS-*Idd13*) and functional (e.g., SIRP $\alpha$ -Fc) strategies.

Contrary to our data, Hempel et al. (30) recently showed no alteration of infected-RBC CD47 expression. In this study, Albumax (Life Technologies, Paisley, United Kingdom) was used to grow the parasites. Compared to the use of human serum, parasites grown with Albumax display altered expression of over 500 genes (31). For example, the cytoadhesion of infected RBCs to various endothelial receptors is greatly reduced in the presence of Albumax compared to human serum. Therefore, the results obtained with Albumax use may not represent the actual state of CD47 expression on infected RBCs.

As part of homeostasis, senescent and modified RBCs are recognized as “altered self” and removed by phagocytes of the reticuloendothelial system. Several molecules on the RBC membrane may be modified during aging, including band 3 and phosphatidylserine (32–35), targeting RBCs for macrophage uptake. In addition, CD47 on RBCs also interacts with SIRP $\alpha$  on macrophages to regulate erythrophagocytosis. Alterations in CD47 or SIRP $\alpha$  abundance, structure, or function may disrupt signaling through SIRP $\alpha$ , resulting in enhanced macrophage clearance of RBCs (2, 36). Previous studies have linked modification of CD47 on senescent RBCs to increased phagocytic uptake (13, 37–39). *P. falciparum* infection exerts substantial oxidative stress on host RBCs, resulting in membrane alterations similar to those observed on senescent RBCs (40, 41). Moreover, malaria parasite infection induces RBC rigidity that has recently been implicated in modifying CD47-SIRP $\alpha$  interactions (42). Consequently, we hypothesized



**FIG 5** Functional disruption of CD47-SIRP $\alpha$  interactions with human SIRP $\alpha$ -Fc proteins and anti-SIRP $\alpha$  antibodies increases phagocytosis of *P. falciparum*-infected RBCs by human macrophages not LPS or IFN- $\gamma$  prestimulated. Shown are human SIRP $\alpha$ -Fc recombinant protein treatment (white boxes) or control IgG4-Fc treatment (gray boxes) (A) and anti-SIRP $\alpha$  antibody treatment (white boxes) or control IgG1 treatment (gray boxes) (B) of uninfected RBCs and *P. falciparum*-infected RBCs at ring stage (left) and mature stage (right) incubated with human monocyte-derived macrophages. Comparisons of phagocytic indexes in control IgG4-Fc- versus SIRP $\alpha$ -Fc-treated macrophages (A) (\*,  $P = 0.026$ ; Mann-Whitney test) or control IgG1- versus anti-SIRP $\alpha$  antibody-treated macrophages (B) (\*,  $P = 0.019$ ; Mann-Whitney test) for ring-stage *P. falciparum*-infected-RBC phagocytosis are shown. The results are the combined data from three experiments and are shown as box-and-whisker plots, representing interquartile and complete ranges, with the horizontal line in each box indicating the median level of phagocytic index. ns, not significant.

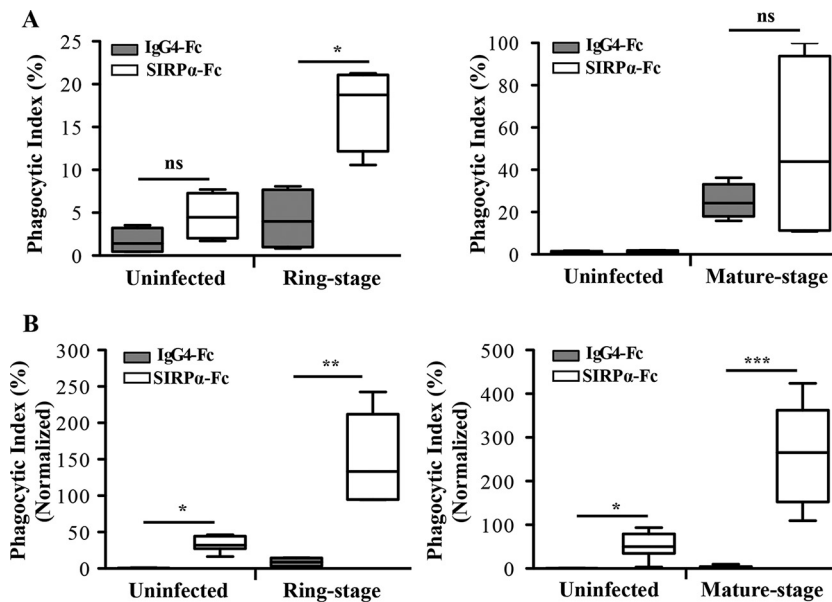
that *P. falciparum* infection may result in an alteration in the levels or conformation of CD47 and its interaction with macrophage SIRP $\alpha$ , resulting in preferential innate clearance of malaria parasite-infected RBCs.

We initially investigated this hypothesis *in vivo* in the ECM model using CD47-deficient mice. *Cd47*<sup>-/-</sup> mice are viable and have normal RBC parameters (8). However, when *Cd47*<sup>-/-</sup> RBCs are transfused into *Cd47*<sup>+/+</sup> mice, they are rapidly cleared by splenic macrophages, whereas *Cd47*<sup>+/+</sup> RBCs transfused into *Cd47*<sup>-/-</sup> mice circulate normally (2). In our experimental system, challenge of *Cd47*<sup>+/+</sup> mice with *P. berghei* ANKA resulted in high parasite burdens and 100% lethality due to ECM. In contrast, *Cd47*<sup>-/-</sup> mice displayed progressively increased parasitemia, and the majority survived without developing ECM (neurological manifestations between days 5 and 10 postinfection), consistent with our hypothesis that impaired CD47-SIRP $\alpha$  signaling would result in enhanced phagocytosis of malaria parasite-infected RBCs.

Endothelial activation and loss of vascular integrity play important mechanistic roles in the pathophysiology of CM, and vascular leakage has been shown to directly correlate with the onset of neurological signs in ECM (43). Of interest, *P. berghei* ANKA-infected *Cd47*<sup>-/-</sup> mice were able to maintain relative endothelial quiescence despite circulating levels of proinflammatory cytokines higher than those observed in *Cd47*<sup>+/+</sup> mice. While high levels of proinflammatory cytokines may contribute to severe malaria (20, 21), early proinflammatory cytokine production (as observed here) has been shown to be protective in murine malaria models (22). TNF has anti-parasitic activity, and TNF-deficient

mice have increased parasite burdens (44, 45). Of note, SIRP $\alpha$  signaling negatively regulates cytokine production, with CD47 engagement by SIRP $\alpha$  attenuating production of interleukin-12 (IL-12), TNF, and IL-6 (7, 46). Collectively, these data support the hypothesis that CD47 deficiency confers a survival advantage by enhancing preferential clearance of malaria parasite-infected RBCs and by permitting early proinflammatory cytokine responses to malaria parasite infection.

Murine preclinical models, while informative in genetic studies, may have limitations with respect to translation to human disease. Moreover, CD47 may interact with multiple different ligands *in vivo*, including integrins, thrombospondin, and G proteins (17). Therefore, to extend these observations to human malaria and to establish a direct role for SIRP $\alpha$ , we utilized genetic and functional approaches with *P. falciparum*-infected RBCs and murine and human MDMs. CD47 binding to SIRP $\alpha$  results in the recruitment of SHP1 in order to induce intracellular signaling. Therefore, to investigate the role of SIRP $\alpha$  signaling in malaria parasite-infected-RBC uptake, we used *Shp1*<sup>-/-</sup> macrophages in *P. falciparum*-infected-RBC phagocytosis assays. Disruption of SIRP $\alpha$  signaling in *Shp1*<sup>-/-</sup> macrophages resulted in increased phagocytosis of both ring- and mature-stage-parasite-infected RBCs. Since a number of receptors utilize SHP1, we used an alternative genetic approach to directly implicate SIRP $\alpha$ . A number of SIRP $\alpha$  variants with differing capacities to bind human CD47 have been identified in mice (17, 29). We used bone marrow-derived macrophages from NS-*Idd13* mice, which express a SIRP $\alpha$  variant that does not bind to human CD47, to specifically implicate disruption of CD47-SIRP $\alpha$  interactions in mediating en-



**FIG 6** Functional blockade of SIRP $\alpha$  signaling by human SIRP $\alpha$ -Fc in phagocytosis assays. LPS- and IFN- $\gamma$ -prestimated macrophages were incubated with uninfected or ring-stage- or mature-stage-*P. falciparum*-infected RBCs in the presence of SIRP $\alpha$ -Fc (white boxes) and the isotype IgG4-Fc control (gray boxes). (A) Human MDMs prestimulated with LPS and IFN- $\gamma$  showed increased uptake of both ring-stage- and mature-stage-*P. falciparum*-infected RBCs. Comparisons of phagocytic indexes between control IgG4-Fc- versus SIRP $\alpha$ -Fc-treated macrophages with ring-stage-*P. falciparum*-infected RBCs are shown. \*,  $P = 0.005$ ; ns, not significant;  $n = 4$ ;  $t$  test. The data are the combined results from two experiments. (B) Mouse SN macrophages showed a significant increase of both ring-stage and mature-stage parasites. The values of the phagocytic indexes were normalized to 100% infected RBCs as described previously (15) using the following calculation:  $I = (Tot - N \times n) / (1 - n)$ , where  $I$  is the concentration of CD47 on 100% infected RBCs, Tot is the anti-CD47 bound on uninfected RBCs and infected RBCs,  $N$  is the anti-CD47 bound on uninfected RBCs, and  $n$  is the fraction of uninfected RBCs. Comparisons for uninfected-RBC and infected-RBC uptake for ring-stage- and mature-stage-*P. falciparum*-infected RBCs are shown (\*,  $P = 0.005$ ; \*\*,  $P = 0.0022$ ; \*\*\*,  $P < 0.0001$ ). The data were analyzed by the Mann-Whitney test and are the combined results of at least three experiments. The results are shown as box-and-whisker plots, representing interquartile and complete ranges, with the horizontal line in each box indicating the median level of phagocytic index.

hanced uptake of malaria parasite-infected RBCs. Compared to NS controls, NS-*Idd13* macrophages displayed increased phagocytosis of both ring- and mature-stage *P. falciparum*-infected RBCs. For NS-*Idd13* macrophages, increased phagocytosis of infected RBCs *in vitro* was observed only when macrophages were activated (i.e., LPS and IFN- $\gamma$  prestimulated), indicating that disruption of CD47-SIRP $\alpha$  alone is insufficient to enhance uptake of infected RBCs. This is in agreement with recent models of AML, where prestimulation of macrophages, through TLRs or IFN- $\gamma$  receptor, is required for phagocytosis of AML target cells (17). Our data are also consistent with reports that other activating signals through Fc $\gamma$  or complement receptors may also be required to induce phagocytosis when SIRP $\alpha$ -inhibitory signals are absent (2, 47). Overall, these data fit a model where the cellular commitment to phagocytose a target is determined by integrated signals generated from both activating receptors and inhibitory receptors, such as SIRP $\alpha$  (47).

To generate an independent line of evidence, we also employed functional strategies to disrupt CD47-SIRP $\alpha$  signaling using anti-SIRP $\alpha$  antibodies and a SIRP $\alpha$ -Fc fusion protein. SIRP $\alpha$ -Fc is a fusion protein designed to bind and block CD47 on target cells and to enhance their phagocytic clearance. This product is currently under evaluation as a therapeutic in AML (17). Blockade of CD47-SIRP $\alpha$  interactions with either of these reagents resulted in increased phagocytosis of *P. falciparum*-infected RBCs by activated human and murine macrophages.

Notably, treatment with SIRP $\alpha$ -Fc did not increase human macrophage uptake of uninfected RBCs, but rather, conferred

preferential macrophage phagocytosis of infected RBCs over uninfected RBCs. These results are consistent with those observed in AML models and support the development of therapeutics that antagonize SIRP $\alpha$  signaling.

During *P. falciparum* invasion and maturation, parasite proteins are deposited on and exported to the RBC surface, inducing structural, morphological, and functional changes in RBC proteins and membranes (48, 49). We investigated whether *P. falciparum* RBC infection might modify CD47 on the RBC membrane. Using ELISA and flow cytometry, we showed decreased levels of anti-CD47 binding on *P. falciparum*-infected RBCs. Although these decreases in antibody reactivity may indicate decreased surface levels of CD47, it is also possible that they are the result of conformational changes in CD47 induced by parasite invasion and growth within the RBC. How malaria might alter CD47 levels or conformation is unknown. Recently, it has been reported that experimental aging of erythrocytes induces a conformational change in CD47 (39). However, the mechanism proposed to explain CD47 involvement in clearance of experimental RBC aging is unclear (50). *Plasmodium* infection may induce a form of accelerated RBC aging by the generation of reactive oxygen species that oxidize RBC hemoglobin to hemichrome. Hemichrome has a high affinity for band 3 proteins (32) that form complexes with CD47 (51). Damage, mutations, or deficiency in band 3 can alter CD47 levels and/or conformation (51). Taking these data together, we propose a model where malaria parasite infection induces oxidant stress, with the deposition of hemichromes on RBC band 3, thereby altering CD47 levels and/or conformation, resulting in



disrupted SIRP $\alpha$  interaction and enhanced phagocytosis of infected RBCs (52). Alternatively, malaria-induced changes in RBC rigidity may also alter CD47-SIRP $\alpha$  interactions (42). These putative mechanisms warrant further study.

Notably, SIRP $\alpha$  is polymorphic and displays geographic variation without a clear mechanistic explanation for this heterogeneity. Takenaka and colleagues sequenced the SIRP $\alpha$  domain encoding IgV from 37 individuals of Caucasian, African, Chinese, and Japanese descent and identified 4 distinct SIRP $\alpha$  IgV-encoding alleles (V1 to V4), with V1 and V2 being the most common (29). These observations suggest that SIRP $\alpha$  variants with altered CD47 binding may have been retained or selected in areas where malaria is endemic if they confer enhanced innate clearance of infected RBCs, a hypothesis that can now be formally tested.

In summary, our data suggest that the CD47-SIRP $\alpha$  pathway may play a role in the innate immune response to malaria by regulating prophagocytic and proinflammatory processes essential to the control of blood-stage malaria parasite infection. Disrupting SIRP $\alpha$  signaling represents a potential and rational pharmacological target, and current therapeutics being developed for cancer may inform the choice of agents with potential utility as adjunctive treatments for malaria.

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We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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