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Malaria Inhibits Surface Expression of Complement Receptor 1 in Monocytes/Macrophages, Causing Decreased Immune Complex Internalization

Cristina Fernandez-Arias,* Jean Pierre Lopez,[†] Jean Nikolae Hernandez-Perez,[†] Maria Dolores Bautista-Ojeda,[‡] OraLee Branch,* and Ana Rodriguez*

Complement receptor 1 (CR1) expressed on the surface of phagocytic cells binds complement-bound immune complexes (IC), playing an important role in the clearance of circulating IC. This receptor is critical to prevent accumulation of IC, which can contribute to inflammatory pathology. Accumulation of circulating IC is frequently observed during malaria, although the factors contributing to this accumulation are not clearly understood. We have observed that the surface expression of CR1 on monocytes/macrophages and B cells is strongly reduced in mice infected with *Plasmodium yoelii*, a rodent malaria model. Monocytes/macrophages from these infected mice present a specific inhibition of complement-mediated internalization of IC caused by the decreased CR1 expression. Accordingly, mice show accumulation of circulating IC and deposition of IC in the kidneys that inversely correlate with the decrease in CR1 surface expression. Our results indicate that malaria induces a significant decrease on surface CR1 expression in the monocyte/macrophage population that results in deficient internalization of IC by monocytes/macrophages. To determine whether this phenomenon is found in human malaria patients, we have analyzed 92 patients infected with either *P. falciparum* (22 patients) or *P. vivax* (70 patients), the most prevalent human malaria parasites. The levels of surface CR1 on peripheral monocytes/macrophages and B cells of these patients show a significant decrease compared with uninfected control individuals in the same area. We propose that this decrease in CR1 plays an essential role in impaired IC clearance during malaria. *The Journal of Immunology*, 2013, 190: 3363–3372.

Malaria is one of the most prevalent parasitic diseases in the world, causing >700,000 deaths each year, mostly in children in sub-Saharan Africa (1). Similar to other infectious diseases, malaria induces the formation of immune complexes (IC), which are detected in peripheral blood during infection (2, 3). IC have an inflammatory effect in the immune system, which is mediated by the Fc receptors that are present in most hematopoietic cells (4). In contrast, mononuclear phagocytes have a protective role against IC-mediated inflammation by removing circulating IC, which is required to avoid overstimulation of the system (5). The efficient handling of these complexes by the cells of the mononuclear phagocyte system contributes to their clearance, decreasing their deposition on other tissue sites such as renal glomeruli (6). Most of the IC uptake in the body takes place in the liver and the spleen (7), where complement receptor 1 (CR1

or CD35) is an important mediator in the clearance of IC (8). In addition to IC clearance, CR1 has an anti-inflammatory effect that is mediated by the inactivation of C3b and C4b, which attenuates complement amplification (9).

Different receptors recognize IC in different ways: Fc receptors bind directly to the Ig part of the IC, but CR1 recognizes complement factors that are bound to the IC, such as the C opsonins C4b, C3b, iC3b, and C1q (5). Therefore, the presence of a functional complement system is required for efficient clearance of IC (10).

CR1 is expressed in macrophages, B cells, neutrophils, and follicular dendritic cells in mice (11), but in humans it is also expressed in erythrocytes, where it contributes to the clearance of IC, transferring them to macrophages for degradation (12). In contrast, mouse erythrocytes do not express CR1; instead, they express a close homolog called Crry. This protein cannot act as C3 receptor and consequently does not contribute to IC clearance (13). Therefore, mice constitute an optimal model to study the role of CR1 in IC clearance mediated by phagocytes, because in humans it is difficult to differentiate between erythrocyte-mediated and macrophage-mediated CR1 clearance.

CR1 in the surface of human erythrocytes is also a receptor for *Plasmodium falciparum* invasion (14) and mediates adhesion of infected erythrocytes to uninfected ones (15), a phenomenon called rosetting, which is associated with cerebral malaria. Polymorphisms associated with low CR1 expression on erythrocytes are highest in the malaria-endemic regions of Asia and are thought to confer protection against severe malaria (16, 17). Importantly, note that these mechanisms do not play a role in the mouse model, as CR1 is not expressed in erythrocytes. Another point to consider is that the *Cr1* gene produces two splice variants, CR1 and CR2, but mouse monocytes/macrophages express very low levels of

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The online version of this article contains supplemental material.

Abbreviations used in this article: CR1, complement receptor 1; IC, immune complex; MSP-1, merozoite surface protein-1.

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CR2 (18). In this study we have focused on CR1 expressed on monocytes/macrophages and B cells, which had not been studied before in the context of malaria.

Although complement activation and IC formation are prominent features of malaria infection, the roles of complement regulatory proteins and IC in this infection remain unclear. In this study we have examined the role of CR1 on the surface of monocytes/macrophages in IC clearance during malaria infection. Using a rodent malaria model, *P. yoelii*, we found that CR1 expression on the surface of macrophages is strongly reduced during malaria infection. Monocytes/macrophages of *P. yoelii*-infected mice have reduced complement-mediated capacity to internalize IC caused by the decrease in CR1 levels, which probably contributes to the observed accumulation of circulating IC. Analysis of peripheral blood samples from human malaria patients in Iquitos, Peru, also present decreased levels of CR1 expression in the surface of monocytes/macrophages. Taken together, our results indicate that malaria induces a significant decrease on surface CR1 expression in the monocyte/macrophage population that results in deficient internalization of IC by monocytes/macrophages.

Materials and Methods

Ethics statement

This study has approval of the use of human subjects from the Internal Review Boards from the New York University School of Medicine and from the Peruvian National Institutes of Health. All studies involving human subjects were conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. All individuals and/or their legal guardians gave written informed consent. All personal information was removed from the files before handing this information to the laboratory team, in accordance with the Health Insurance Portability and Accountability Act.

This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of New York University School of Medicine, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Mice, parasites, and infections

Female Swiss Webster mice were purchased from the National Institutes of Health and The Jackson Laboratory. Nonlethal strain *P. yoelii* 17XNL-infected erythrocytes were harvested by cardiac puncture of infected, anesthetized Swiss Webster mice before the peak in parasitemia. Erythrocytes were washed twice with PBS and separated from WBCs by centrifugation at $2000 \times g$ for 3 min. Erythrocytes were then spun on an Accudenz (Accurate Chemical and Scientific) gradient to isolate schizonts and late trophozoite stage-infected erythrocytes. The collected infected erythrocytes were washed and resuspended in PBS. To start blood-stage infections, Swiss Webster mice were injected i.p. with 10^6 infected erythrocytes per mouse resuspended in PBS. To evaluate parasitemia, thin blood smears were made by bleeding mice from a nick in the tail. Smears were stained with KaryoMAX Giemsa (Life Technologies), and a minimum of 500 erythrocytes per smear were counted.

Histological and serological analysis

Histological examination of kidneys was done on H&E-stained paraffin sections. For immunofluorescence microscopy, 6- μ m frozen kidney sections were processed and stained with FITC goat anti-mouse IgG (BioLegend). For each condition, 10 images with constant time of acquisition and fluorescent light intensity were obtained randomly with a $\times 40$ objective lens in each of two different sections processed in parallel. Meta-morph imaging software was used to determine the average fluorescence intensity in each image. Mice anti-C1q and anti-merozoite surface protein-1 (MSP-1) Ab in serum were detected by ELISA. Plates were coated with anti-C1q (Abcam) or recombinant *P. yoelii* MSP-1 (obtained through MR4 [MRA-48] deposited by D.C. Kaslow) followed by incubation with serum and development with anti-mouse IgG labeled with HRP. Because C1q only binds to IC and not to individual Abs, development with anti-IgG results in specific detection of C1q bound to IC. For human detection of IC, an anti-C1q kit (Hycult Biotech) was used. Development was performed with anti-human IgG labeled with HRP to detect only C1q bound to IC.

J774 macrophage incubation with IC

J774 cells (10^6 /ml) were incubated with either IC (10 μ g/ml) purified by dialysis from serum of *P. yoelii*-infected mice at day 10 after infection or LPS (10 μ g/ml) in the presence of mouse control serum (1:1 serum/culture medium) for 30 min at 37°C, before analysis of surface markers by FACS.

Cell separation

For mice, single-cell suspensions were obtained by mechanical disruption of spleen or liver through a cell strainer and then osmotically lysing erythrocytes by incubation in an ammonium chloride/potassium hydrogen carbonate buffer. Peritoneal cells were extracted by aspiration and peripheral blood was processed as detailed for humans below. All spleen cell preparations were resuspended in PBS with 3% FBS (Life Technologies) and kept on ice.

For human peripheral blood, blood was incubated for 1 h with Fix/Lyse solution (BioLegend) at room temperature and centrifuged at $600 \times g$ for 5 min before staining.

Flow cytometry

All flow cytometry was performed on a FACSCalibur (Becton Dickinson) and analyzed with either CellQuest (Becton Dickinson) or FlowJo (Tree Star). All Abs for FACS were purchased from BioLegend or Becton Dickinson Biosciences.

Expression of PE F4/80, CD40, and MHC class II was analyzed on J774 macrophages stained with PE anti-F4/80 (BM8), PerCP anti-CD40 (3-23), and FITC anti-I-A^d (39-10-8).

Expression of CR1 was analyzed in mouse monocyte/macrophage CD11b⁺F4/80⁺ (stained with FITC anti-CD11b [M1/70]), PerCP anti-F4/80 (16-10A1), and PE anti-CD21/CD35 (CR2/CR1) (7E9) (BioLegend), as well as B cells stained with FITC-B220 (30-F11) and PerCP anti-CD19 (6D5). The anti-CD21/CD35 recognizes both CR2 and CR1; however, mouse monocytes/macrophages express very low levels of CR2 (18). For transfer experiments, splenocytes from control mice were labeled with DDAO (1 μ g/ml, 15 min at 37°C). Splenocytes (3×10^6) were injected i.v. into eight recipient mice and four of them were infected with *P. yoelii* 1 d later. Ten days after infection, the levels of CR1 were analyzed on monocytes/macrophages that were identified in recipient mice as cells positive for CD11b, F4/80, and DDAO. To measure the effect of inflammatory stimuli on CR1 expression *in vivo*, mice were treated with commercial FITC-labeled IC anti-OVA (4.5 mg/kg; Fc OxyBURST Green, Invitrogen), LPS (10 mg/kg), or both every 3 d during 10 d before analysis of CR1 expression on macrophages.

Expression of CR1 was analyzed in different populations of human PBMCs: monocytes/macrophages (CD16⁺, CD10⁻), B cells (B220⁺, CD19⁺), and neutrophils (CD10⁺), and CD35 expression was also analyzed on them. PBMCs were stained with either PE anti-human CD35, PerCP anti-human CD16, and FITC anti-human CD10 or PE anti-human CD35 (E11), PerCP anti-human CD45 (HI30), and FITC anti-human CD19 (HB19) (BioLegend). Data are expressed as percentage of CR1^{hi} cells in each cell population.

Phagocytosis quantification

Mouse splenocytes from control, *P. yoelii*-infected mice (day 10 of infection) or mice injected with anti-CR1 were incubated for 30 min at 37°C with commercial FITC-labeled IC anti-OVA (Fc OxyBURST Green [Invitrogen] at 120 μ g/ml) preincubated or not with uninfected mouse serum for 30 min at 4°C to allow for complement binding. Alternatively, splenocytes were incubated with control or *P. yoelii*-infected erythrocytes stained with DDAO (Invitrogen) for 1 h at 37°C. Cells were transferred to ice before staining with PerCP anti-CD11b and FITC anti-F4/80 and FACS analysis, as described above.

Injection of blocking anti-CR1 Ab, IC, and LPS

Groups of three mice were injected i.v. with 300 mg/mouse either anti-CR1 Ab (Abcam 7G9 rat IgG) or control rat IgG (Equitech-Bio). To study the effects on phagocytosis of IC, 5 d after inoculation, splenic monocytes/macrophages were analyzed for surface CR1 expression and phagocytosis of IC. To study deposition of IC in the kidneys, 5 d after anti-CR1 treatment, mice were inoculated with commercial IC (Fc OxyBURST Green [Invitrogen] at 4.5 mg/kg) and 10 d later kidneys were analyzed for IC deposition. To study the role of inflammation and IC in CR1 expression levels, groups of three mice were injected i.v. with commercial IC (Fc OxyBURST Green [Invitrogen] at 4.5 mg/kg), LPS (10 mg/kg), or both every 3 d during 10 d before analysis of CR1 expression on macrophages.

Field study site

The human samples were from participants in the Malaria Immunology and Genetics in the Amazon field study, including participants enrolled from

February 2008 to January 2009. The study sites are two networks of communities near Iquitos, Peru (the capital of the Amazon province of Loreto). The Zungarococha community is comprised of four villages: Zungarococha, Puerto Almendras, Ninarumi, and Llanhama. The Mazan community is located downstream along the Amazon River and is comprised of four villages: Mazan, Santa Cruz, Progreso, and Libertad. The transmission and endemicity are similar in both areas, and as such the data analysis does not separate the two sites.

Sample collection

Patients were followed actively, receiving a visit from the field team at regular intervals during the malaria season. During each visit, a blood smear was made. When the patient had a fever detected ($\geq 38.3^{\circ}\text{C}$) or reported by patient within 2 d, the blood slide was immediately observed for parasites by light microscopy. When the slide was positive for parasites, the nurse and physician drew one 8-ml Vacutainer of blood before treating the patient. Patients who were diagnosed with symptomatic *P. falciparum* or *P. vivax* malaria infections (defined as having a body temperature $\geq 38.3^{\circ}\text{C}$, a hematocrit of <30 packed cell volume, hemoglobin <10 g%, or a parasite density of >5000 parasites/ μl blood) were treated with artesunate and mefloquine for 3 d using a directly observed treatment strategy.

Passive cases also entered the study as individuals who went to the community-based health center reporting febrile illness and were diagnosed with malaria. These individuals were processed and followed up in a similar way as an active surveillance febrile patient. Medical histories of all patients, both active and passive, were documented, including recent symptoms of illness and drugs taken.

Healthy non-malaria-exposed controls were obtained from donations from healthy individuals living in the city of Iquitos with no prior malaria history.

Statistical analysis

Data were analyzed using Prism (GraphPad Software). The Mann–Whitney *U* test was used to identify statistical differences between groups of patients. A Student *t* test was used for all other studies.

Results

IC accumulate in the circulation during malaria in mice

To study IC accumulation during malaria, we first used groups of mice infected with *P. yoelii* 17X NL, a rodent malaria parasite frequently used as a model for the human infection. Infections with this parasite are not lethal and mice are able to clear the parasite and recover. The detection of IC was performed in the serum of *P. yoelii*-infected mice at different times of infection using an indirect ELISA to detect C1q bound to IC. The accumulation of IC in the circulation appears very early, raises with infection, and disappears after parasite is cleared (Fig. 1A, 1B). In contrast to the IC profile, Abs against a typical parasite Ag, MSP-1, increase during infection but remain at high levels after infection has been cleared (Fig. 1C). These results indicate that IC are formed and accumulate as a result of infection, but they are removed from the circulation after the parasite is controlled.

IC-induced activation, IC deposition, and glomerulonephritis in mice with malaria

To test whether circulating IC from mice infected with *P. yoelii* have an activatory effect on macrophages, we purified IC from infected mice and incubated them in vitro with J774 macrophages. We observed a pronounced increase in surface expression of F4/80, CD40, and MHC class II (Fig. 2A), which is indicative of macrophage activation (19). To determine whether the observed accumulation of IC in circulation results in deposition in the kidneys, as has been observed in other diseases (20, 21), we analyzed histological sections of kidneys from mice infected with *P. yoelii* at different times after infection. We observed IC deposition after 10 d infection, which is still detectable after 1 mo (Fig. 2B–D, 2I). Histological observation of kidney slides showed generalized inflammation with glomerular endocapillary proliferation and infiltration of inflammatory interstitial mononuclear cells after

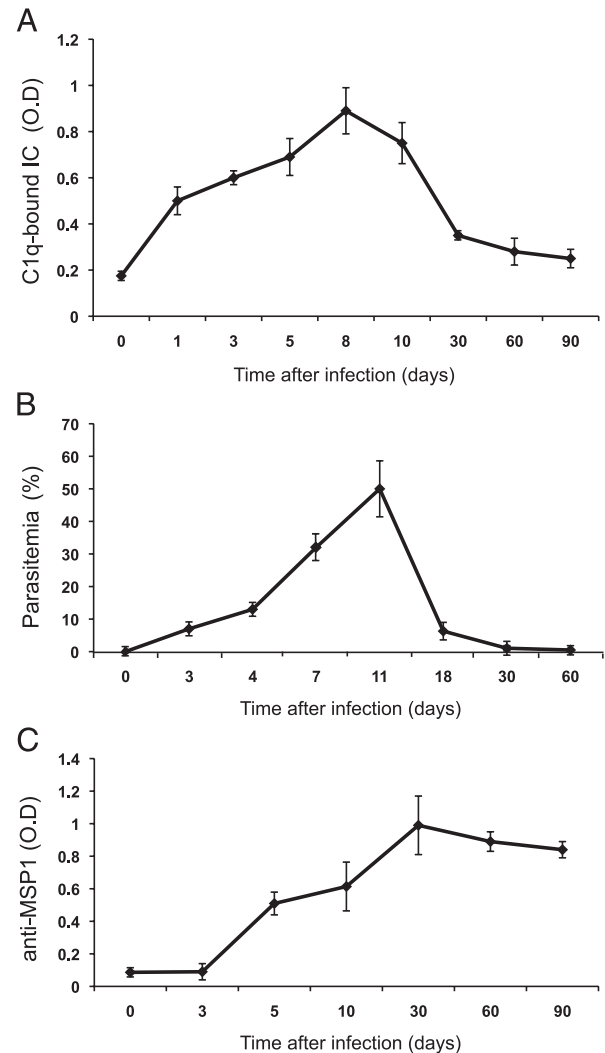


FIGURE 1. Circulating IC accumulation in *P. yoelii*-infected mice. Serum from mice infected with *P. yoelii* ($n = 5$) was used to determine: (A) IC (IgM and IgG), detected using an indirect ELISA coated with C1q; (B) parasitemia, quantified by Giemsa-stained blood smears; (C) anti-MSP-1 Abs (IgM and IgG), detected using a direct ELISA. Values are expressed as mean \pm SD.

10 d infection. One month after infection, when parasites have already been cleared, we observed a decrease in inflammatory infiltrates and an increase in interstitial and glomerular capillary permeability with erythrocytic glomerular generalized congestion (Supplemental Fig. 1). These observations coincide with malaria-induced pathology observed in human kidney samples from patients (22–24).

To confirm that CR1 is needed for IC clearance in our mouse model, we inoculated mice with high concentrations of IC and anti-CR1 blocking Abs. We found that blocking of CR1 results in IC deposition in the kidneys, which is not observed when IC are administered alone (Fig. 2E–H, 2J).

Surface expression of CR1 on splenic monocytes/macrophages and B cells is decreased in mice during malaria

Two different receptors play a major role in clearance of IC from circulation: Fc γ receptor, which binds to naked Abs, and CR1, which binds to C3b/iC3b already bound to IC. Because we had observed accumulation of IC in infected mice, we tested whether expression levels of these two receptors were altered during malaria.

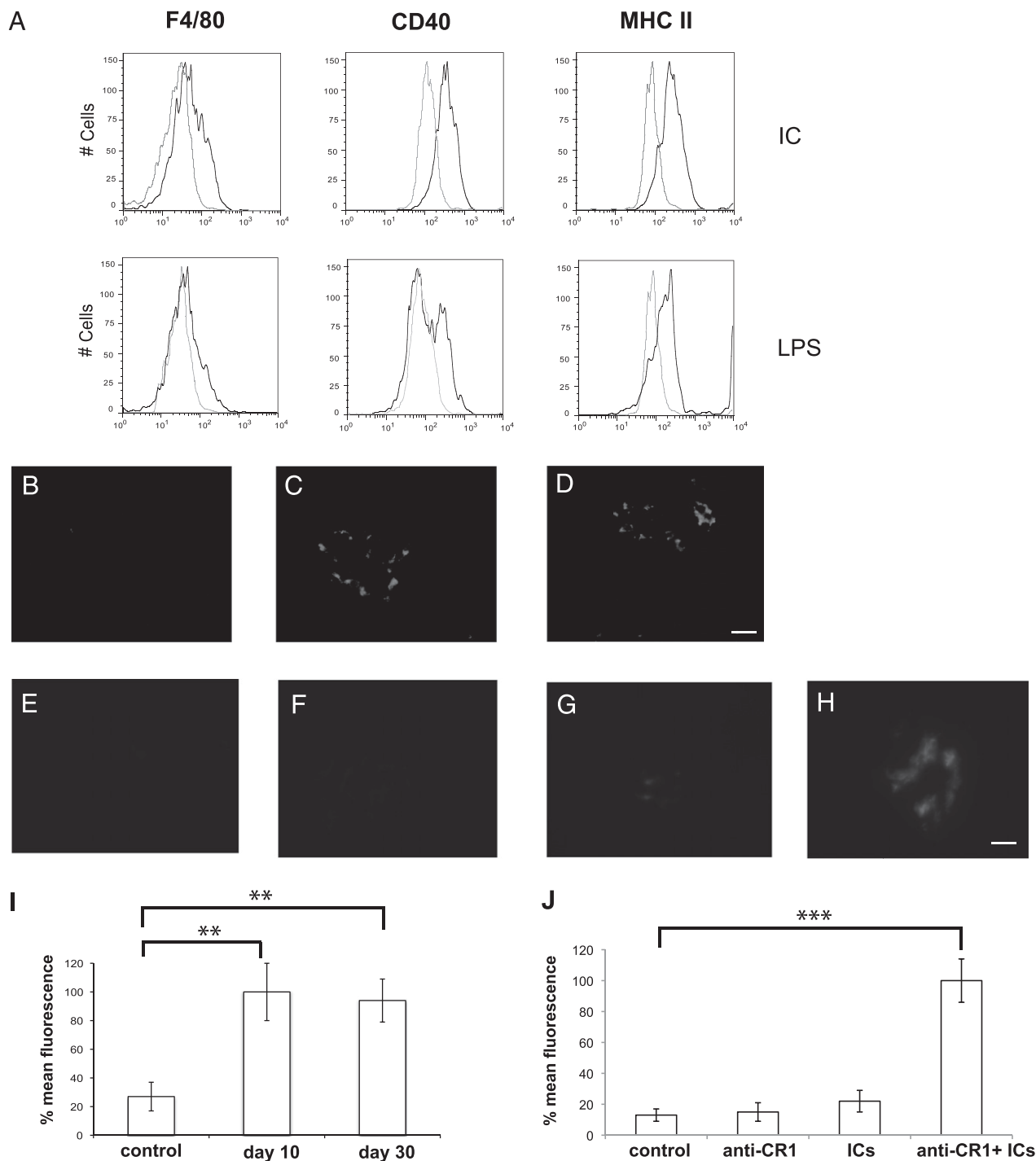


FIGURE 2. Macrophage activation and deposit of IC in the kidneys of *P. yoelii*-infected mice. **(A)** FACS plots show surface expression of F4/80, CD40, and MHC class II on J774 macrophages incubated with IC purified from *P. yoelii*-infected mice (10 μ g/ml) or LPS (10 μ g/ml). Light gray line shows control J774 cells with no stimulus. **(B–H)** Kidney histological sections of groups of five mice stained with anti-IgG Abs to show IgG deposits in glomeruli. **(B–D)** Uninfected control **(B)**; 10 d **(C)** or 30 d **(D)** afterinfection with *P. yoelii*. **(E–H)** Uninfected mice received different treatments: control **(E)**; injected with anti-CR1 **(F)**; injected with IC **(G)**; injected with anti-CR1 and IC **(H)**. **(I)** Quantification of IgG deposits by fluorescence in **(B)–(D)** *P. yoelii*-infected mice. **(J)** Quantification of IgG deposits by fluorescence in **(E)–(H)**. $**p < 0.01$, $***p < 0.001$, when each condition is compared with control. Representative images are shown. Scale bars, 20 μ m.

We found that *P. yoelii* infection induces a strong decrease of CR1 surface levels in splenic monocytes/macrophages (Fig. 3A, 3B), which are the major cell type that can internalize IC in circulation. The decrease in CR1 expression is recovered after parasite clearance (Fig. 3A, 3B) and follows an inverse pattern when compared with circulating IC (Fig. 1), suggesting that the accumulation of IC might be caused by a defect in IC clearance by

CR1. Levels of Fc γ IIIa receptor on the surface of monocytes/macrophages are increased during early infection and return to background levels at the peak of infection (day 10) (Fig. 3C, 3D). A gradual decrease of CR1 surface expression was also observed on B cells during the course of infection (Fig. 3E, 3F).

We also observed that the decrease in surface CR1 expression is found in monocytes/macrophages from spleen and liver, but

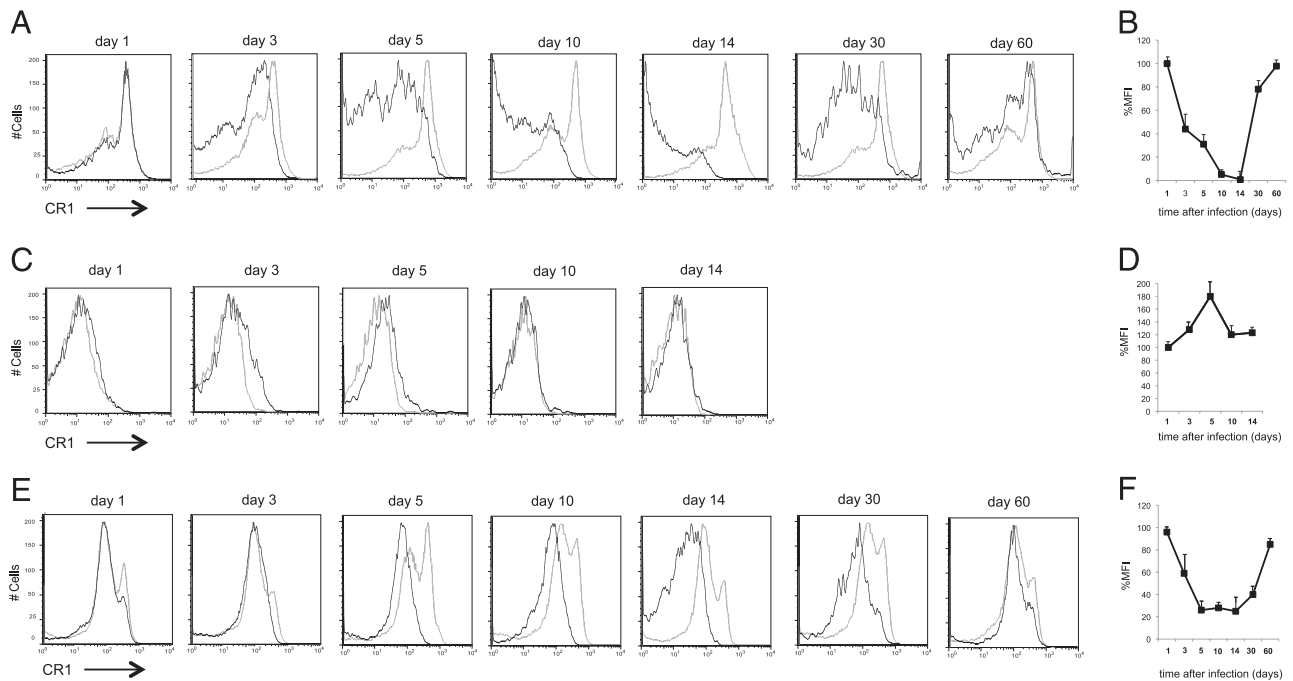


FIGURE 3. CR1 surface expression is decreased on mouse splenic monocytes/macrophages and B cells during *P. yoelii* infection. FACS plots show surface expression of (A) CR1 and (C) FcγRIII receptor on CD11b⁺F4/80⁺ cells and (E) CR1 on B200⁺CD19⁺ B cells from control (gray line) and *P. yoelii*-infected mice (black line) at different times after infection. The results are representative of three independent experiments with groups of four mice each. (B, D, and F) Percentage variation of average mean fluorescence intensity (MFI) of the surface expression of CR1 (B, F) and FcγRIII receptor (D) at the indicated days after infection compared with control uninfected mice processed on each day ($n = 12$ for each day and condition).

not in peritoneal ones (Fig. 4). This coincides with the localization of *Plasmodium*, which is found in the circulation and accumulates in organs such as liver and spleen, but does not invade the peritoneal cavity.

The observed decrease in CR1 expression on monocytes/macrophages may be caused by the binding of high concentrations of IC that induce internalization of this receptor, as a response to an inflammatory environment (25, 26) or through another parasite-induced mechanism. We first analyzed whether high concentrations of IC induce a decrease in macrophage CR1 surface expression *in vitro*. We incubated J774 macrophages with IC in the presence of mouse serum containing complement, and we

found that surface levels of CR1 do not decrease under these conditions, indicating that binding of IC do not induce a decrease of surface expression of CR1 *in vitro* (Fig. 5A). To study this question *in vivo*, mice were injected with either IC, a strong inflammatory stimulus (LPS), or both. We found that although IC can induce a decrease in CR1 surface expression on macrophages, LPS alone induces also a strong decrease (Fig. 5B, 5C), suggesting that inflammation is an important mediator of CR1 decrease. Interestingly, both stimuli together do not have an additive effect.

We wanted to determine whether the observed decrease in the levels of surface CR1 during malaria in the monocyte/macrophage population is caused by decreased expression of CR1 on the membrane of these cells or by selective disappearance of cells expressing this receptor. We transferred labeled splenocytes into mice that were infected with *P. yoelii* 1 d after the transfer. Ten days later, analysis of CR1 expression on monocytes/macrophages was performed. We found that the transferred monocytes/macrophages had strongly reduced their expression of surface CR1, compared control mice (Fig. 6). This result confirms that there is a decrease in surface expression levels of CR1 on monocytes/macrophages of *P. yoelii*-infected mice.

We have also analyzed the relative numbers of monocytes/macrophages in the spleen and peripheral blood of *P. yoelii*-infected mice and compared it to controls, finding no significant differences (in spleen: control, $4.2 \pm 1.7\%$; day 10 infected mice, $3.8 \pm 1.0\%$; in blood: control, $5.5 \pm 2.6\%$; day 10 infected mice, $5.7 \pm 1.9\%$), indicating that there is no selective disappearance of the monocyte/macrophage population during infection.

Malaria induces decreased internalization of complement-bound IC by monocytes/macrophages

We next wanted to determine whether the decreased levels of CR1 expression on monocytes/macrophages during malaria result in the inhibition of internalization of IC through this receptor.

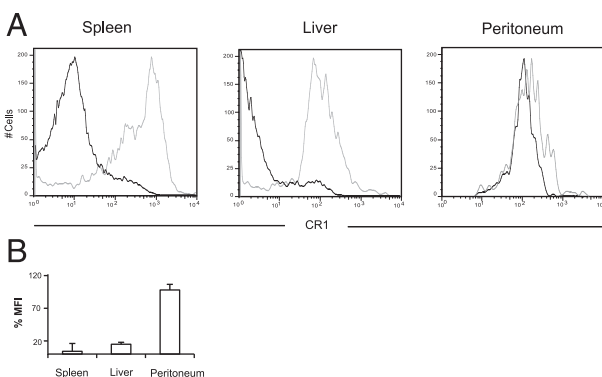


FIGURE 4. CR1 surface expression is decreased on monocytes/macrophages in spleen and liver. (A) FACS plots show the expression of CR1 on CD11b⁺F4/80⁺ cells in spleen, liver, and peritoneum in control (gray line) and *P. yoelii*-infected mice (black line) after 10 d infection. Results are representative of two independent experiments with three infected and three uninfected mice each. (B) percentage variation of average mean fluorescence intensity (MFI) of CR1 surface expression compared with cells from control uninfected mice processed in parallel ($n = 4$ for each condition).

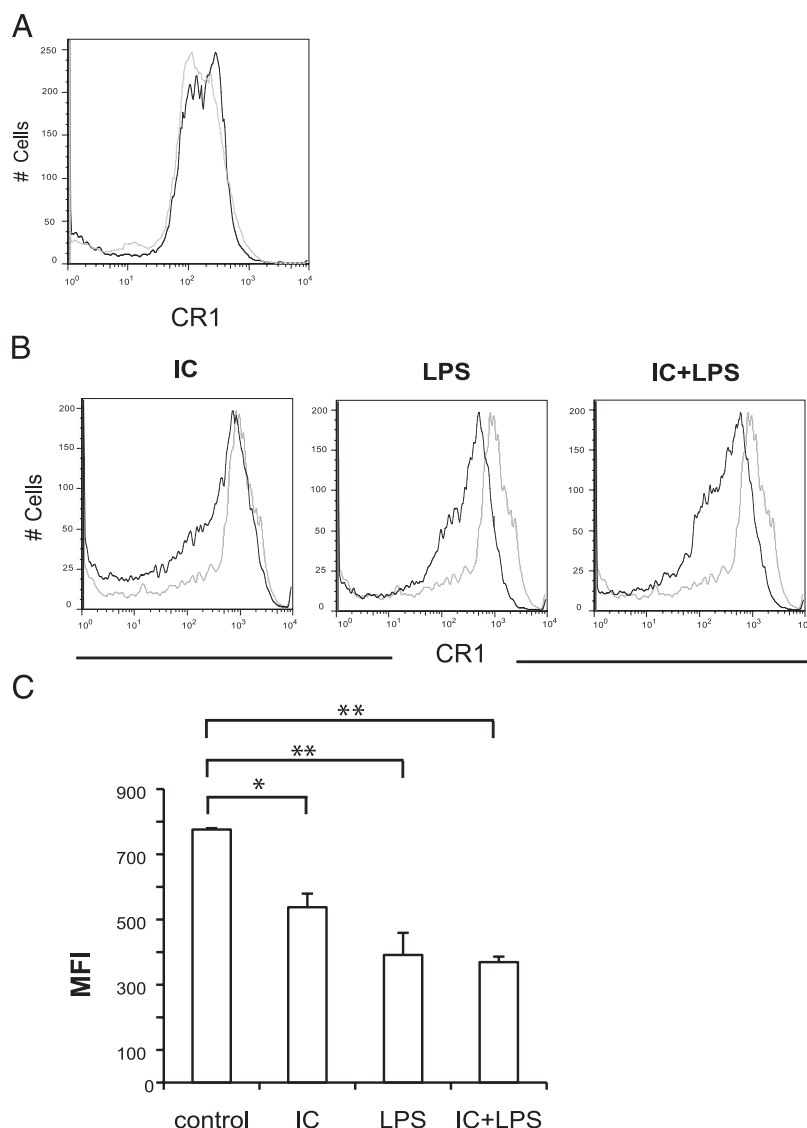


FIGURE 5. Effect of IC and LPS on macrophage surface CR1 expression. **(A)** J774 macrophages were incubated with commercial OVA-IC (120 μ g/ml) for 30 min before analysis of CR1 expression. Light gray line shows control J774 cells with no stimulus. **(B)** CR1 surface expression in CD11b⁺F4/80⁺ cells from mice treated with IC (4.5 mg/kg), LPS (10 mg/kg), or both every 3 d during 10 d. **(C)** Average mean fluorescence intensity (MFI) of CR1 surface expression from cells from each group of mice processed in parallel ($n = 3$ for each condition). ** $p < 0.01$, *** $p < 0.001$, when each condition is compared with control.

We first confirmed that monocytes/macrophages from *P. yoelii*-infected mice efficiently phagocytose control and infected erythrocytes. Similar results were described before for a different strain of *Plasmodium* (27, 28) and indicate that macrophages from infected mice present similar levels of phagocytosis compared with control mice (Supplemental Fig. 2).

To determine whether the capacity to clear IC in infected mice is specifically decreased during infection, we isolated splenocytes from infected mice at the peak of infection (day 10). Commercially available labeled IC were preincubated or not with mouse serum to allow for complement binding before addition to cells. This is important because IC are preferentially internalized through Fc γ in the absence of complement, but through CR1 in the presence of complement (29–31).

We found that internalization of IC preincubated with serum is strongly inhibited in monocytes/macrophages from mice with malaria compared with these cells isolated from control mice (Fig. 7). This inhibition was not found in the absence of serum, reflecting the intact capacity of monocytes/macrophages to internalize complement-free IC, most likely through Fc receptors.

Our results indicate that monocytes/macrophages from *P. yoelii*-infected mice present a specific inhibition of internalization of

complement-bound IC, probably contributing to the impaired clearance of IC in infected mice.

To confirm that this inhibition is mediated by the severe decrease in surface expression of CR1 on monocytes/macrophages, we inhibited CR1 in mice by inoculation of anti-CR1 blocking Abs. We confirmed that these mice had low levels of surface CR1 on immune cells (Fig. 8A), comparable to the levels found during malaria. Monocytes/macrophages from these mice were isolated and tested for their capacity of IC internalization. Similar to monocytes/macrophages from mice with malaria, we observed deficient IC internalization in the presence of serum and normal internalization in its absence (Fig. 8B, 8C), confirming that low expression of surface CR1 results in deficient internalization of IC in these cells.

Levels of surface CR1 in circulating monocytes/macrophages from human patients infected with P. falciparum and P. vivax in Peru

To study the relevance of our findings in a mouse malaria model to human disease, we analyzed patients infected with *P. falciparum* and *P. vivax* in Iquitos, Peru. Although most malaria infections occur in sub-Saharan Africa, studying immune responses to

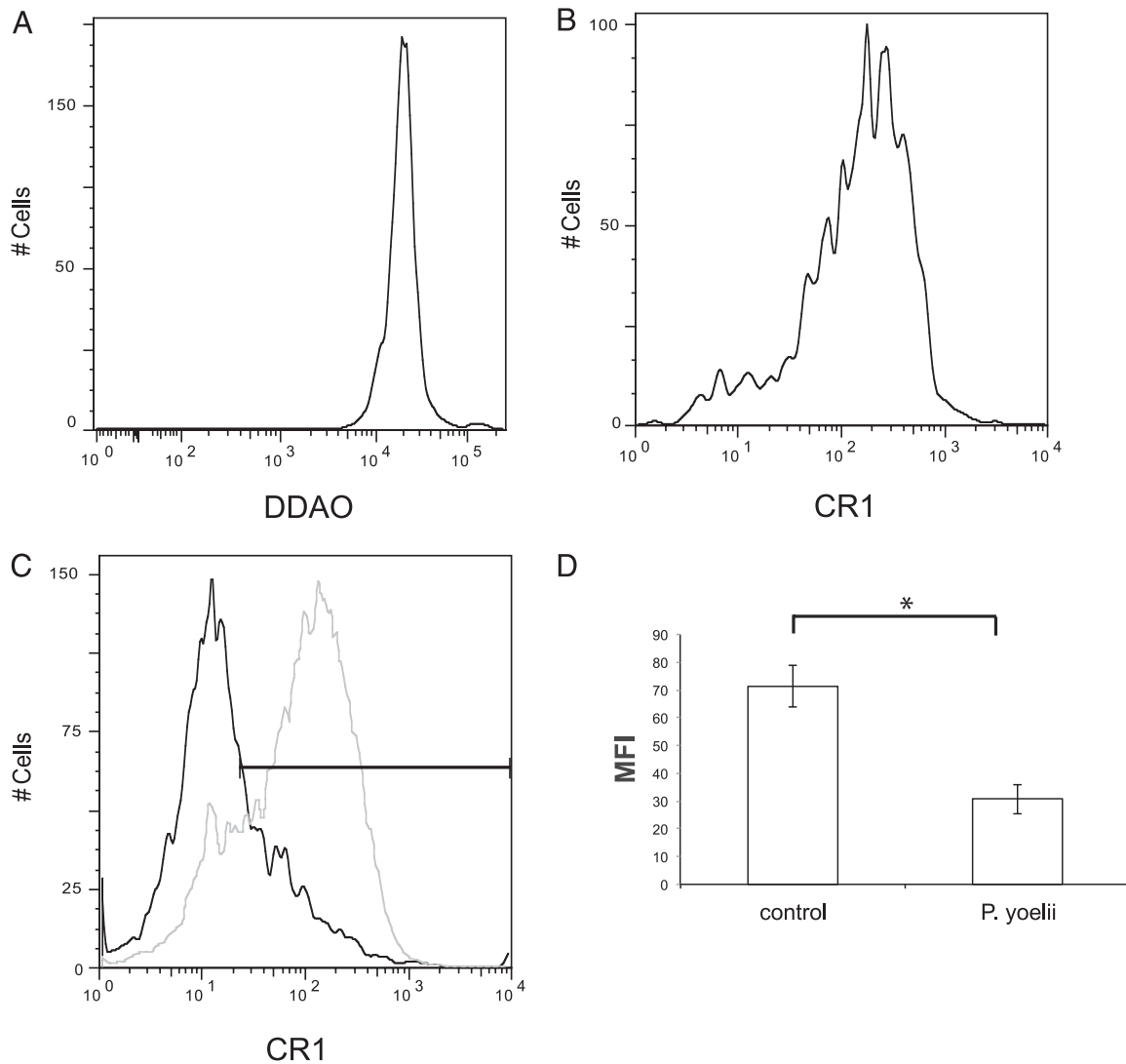


FIGURE 6. CR1 surface expression decrease in monocytes/macrophages transferred into *P. yoelii*-infected mice. Splenocytes isolated from a control uninfected mouse were labeled with DDAO and transferred to eight mice (3×10^6 each). DDAO (**A**) and CR1 (**B**) levels of CD11b⁺F4/80⁺ gated cells before transfer. One day after transfer, four of these mice were infected with *P. yoelii*. (**C**) CR1 levels of CD11b⁺F4/80⁺DDAO⁺ cells from control (gray line) and infected (black line) mice 10 d after infection. (**D**) Average mean fluorescence intensity (MFI) of CR1 surface expression of cells from infected and control mice processed in parallel ($n = 4$ for each condition). * $p < 0.05$.

Plasmodium in an African context is complicated by overlapping infections that occur as a result of the high transmission in these hyperendemic areas. In the Peruvian Amazon, *P. falciparum* transmission is low (0.49 infections per person per year) and as a result typically each case of *falciparum* malaria is a single, discrete infection. *P. vivax* is indeed more prevalent but is still transmitted at relatively low levels, with 1.21 infections per person per year (32, 33). Transmission is restricted to the wet season (January–July) and there is high prevalence of asymptomatic cases in this region (32).

For our study, we used remaining blood from samples taken from participants in a study regarding Malaria Immunology and Genetics in the Amazon, residing in several communities just south of Iquitos, Peru (see *Materials and Methods*). There were eight symptomatic individuals (fever $>38.3^{\circ}\text{C}$), from which samples were taken during an uncomplicated malaria infection. However, most samples were taken from asymptomatic individuals (temperature $<38^{\circ}\text{C}$) during a campaign for active detection of infection. Parasitemia in individuals infected with *P. vivax* ranged from 3 to 540/ μl and in those infected with *P. falciparum* ranged

from 2 to 30,000/ μl . As controls, there were nine samples taken from healthy individuals living in the area who had never reported a previous *Plasmodium* infection.

We determined the levels of circulating IC in these patients, finding that both *P. falciparum*- and *P. vivax*-infected patients show significant increases in the levels of IC (Fig. 9A).

To study CR1 expression on monocytes/macrophages during malaria infection, we first analyzed the percentage of CD16⁺CD10[−] monocytes/macrophages in PBMCs from participants. This population is decreased in peripheral blood of *Plasmodium*-infected individuals. We found that whereas healthy control individuals ($n = 9$) have $24.1 \pm 7\%$ of CD16⁺CD10[−] monocytes/macrophages in total PBMCs, *P. falciparum*- ($n = 24$) and *P. vivax*-infected individuals ($n = 68$) have 11.4 ± 7.3 and $10.5 \pm 8.4\%$, respectively. These results suggest that migration of monocytes/macrophages into other tissues, such as bone marrow and spleen, is taking place in infected individuals (34, 35).

Analysis of CR1 expression on the remaining CD16⁺CD10[−] monocytes/macrophages in peripheral blood showed a significant reduction of CR1 expression on these cells in patients with either

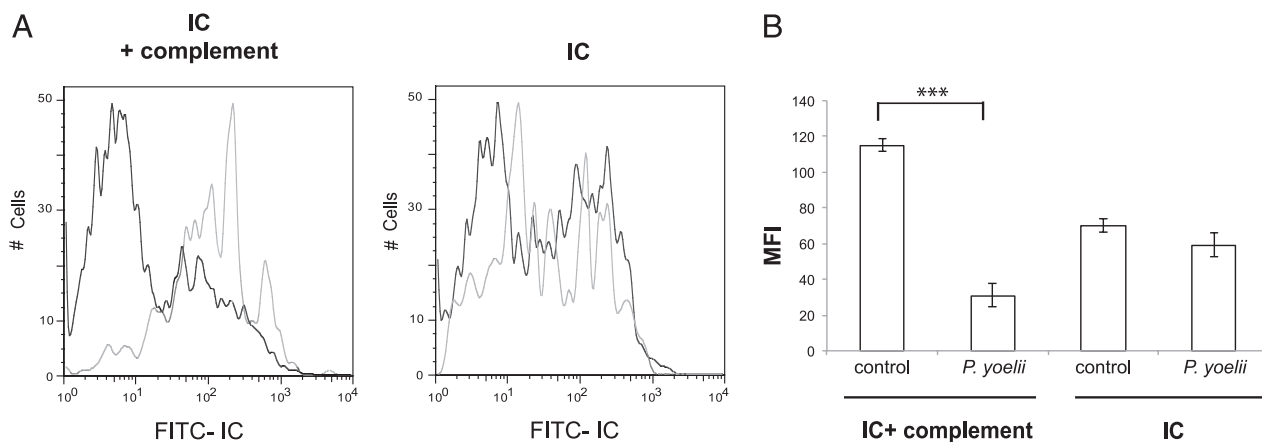


FIGURE 7. Phagocytosis of complement-bound IC by monocytes/macrophages is inhibited in *P. yoelii*-infected mice. Splenocytes from *P. yoelii*-infected or uninfected control mice were cultured for 30 min in vitro in the presence of FITC-labeled OVA-IC preincubated or not with mouse control serum (complement-bound IC). **(A)** Phagocytosis of IC by CD11b⁺F4/80⁺ cells from control mice (gray line) or infected mice (black line). **(B)** Average mean fluorescence intensity (MFI) of FITC-labeled IC in CD11b⁺F4/80⁺ cells from control and infected mice (n = 3). ***p < 0.001 when compared with control.

P. falciparum or *P. vivax* infections compared with controls (Fig. 9B). None of these patients showed symptoms of severe or complicated malaria, indicating that the decrease in monocyte/macrophage CR1 can take place in uncomplicated *Plasmodium* infections. It is possible that patients with severe disease may show even more drastic decreases in CR1 levels. We also analyzed the levels of CR1 surface expression in B cells (identified as CD19⁺, CD138⁺), another cell type where CR1 is highly expressed and its levels are regulated during infection and autoimmune diseases (13, 36, 37). Significantly decreased levels of CR1 were observed in B cells of patients with malaria, both in *P. falciparum* and *P. vivax* infections (Fig. 9C). Conversely, no differences were observed in CR1 surface levels of neutrophils (identified as CD16⁺, CD10⁺) in malaria patients compared with the control group (Fig. 9D).

Discussion

The complement cascade plays a key role in the modulation of inflammatory responses, and its activation is crucial to the pathogenesis of various diseases (38). In malaria, decreased levels of CR1 expression on erythrocytes are considered an important factor in the accumulation of IC during disease (39, 40), because of the contribution of erythrocyte CR1 in IC clearance. Erythrocytes transfer IC to macrophages for degradation, probably by transferring the IC from erythrocyte CR1 to macrophage CR1

(41). Macrophages also contribute to the clearance of IC by directly binding IC to CR1 expressed on their membrane. In any case, macrophage CR1 is the final destination of circulatory IC targeted for degradation; however, the role of CR1 expression and function on phagocytic cells in malaria had not been studied before.

Because mice do not express CR1 on the surface of erythrocytes, it constitutes an adequate model to study the role of CR1 on monocytes/macrophages in the IC clearance during malaria. We have demonstrated that monocytes/macrophages from mice with malaria do not internalize complement-bound IC, similar to macrophages from mice treated with blocking anti-CR1 Abs. Because monocytes/macrophages play a major role in the clearance of complement-bound IC (5), it is likely that the observed CR1 decrease contributes decisively to the accumulation of circulating IC during malaria.

It is well characterized that accumulation of IC is an essential contributor to inflammatory damage, which is in great part mediated by binding of IC to FcγRIII receptors (42). Expression of the FcγRIII receptor on mice monocytes/macrophages is not decreased; in fact, we found a transient, but significant, increase in its surface expression during the early days of infection. Expression of the equivalent receptor in humans (FcγRIIIA) is increased on the monocytes of malaria patients, especially in the ones with malaria-induced anemia (43). We confirmed that inflammatory stimuli decrease CR1 expression, as described before (13, 37),

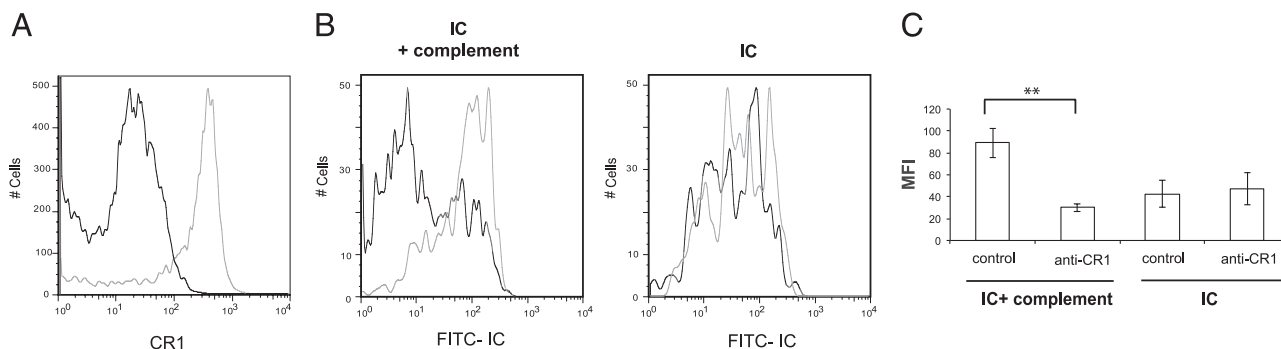


FIGURE 8. Blocking of CR1 in mice inhibits phagocytosis of IC by monocytes/macrophages. Mice were injected with CR1 blocking Ab (black line) or control Ab (gray line). Five days after injection, splenic CD11b⁺F4/80⁺ cells were analyzed for surface expression of CR1 **(A)** and also were cultured for 30 min in vitro in presence of FITC-labeled OVA-IC preincubated or not with mouse control serum (complement-bound IC) to determine phagocytosis of IC **(B)**. **(C)** Average mean fluorescence intensity (MFI) of FITC-labeled IC in CD11b⁺F4/80⁺ cells from control and anti-CR1 injected mice (n = 3). **p < 0.01 when compared with control.

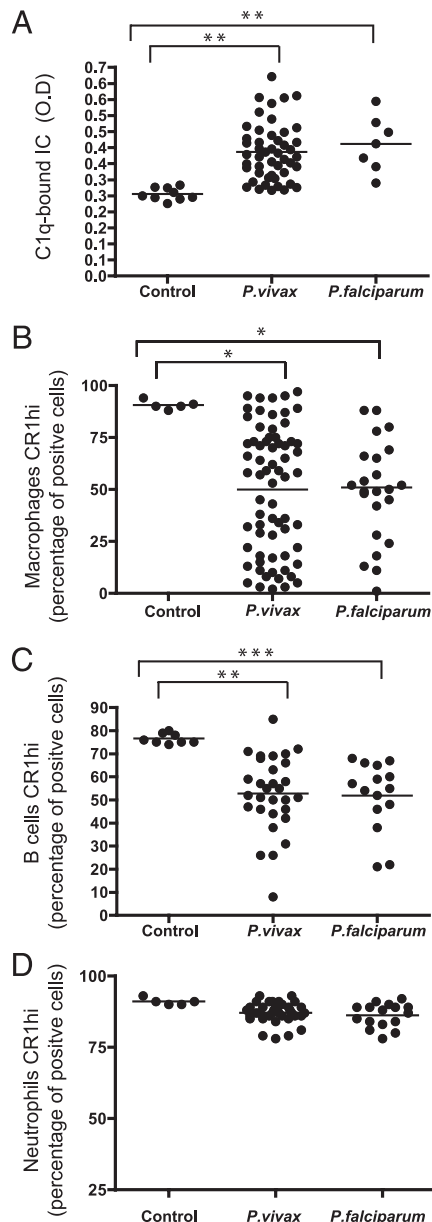


FIGURE 9. Circulating IC levels are increased and CR1 surface expression is decreased on monocytes/macrophages and B cells from Peruvian patients during *P. vivax* and *P. falciparum* infection. (A) IC (IgM and IgG), detected using an indirect ELISA coated with C1q. (B–D) CR1 surface expression levels on macrophages (CD16⁺CD11b⁺) (B), B cells (CD19⁺CD138⁺) (C), and neutrophils (CD16⁺CD10⁺) (D) were analyzed in peripheral blood from healthy controls or patients during *P. vivax* and *P. falciparum* infections. Data are expressed as percentage of cells expressing high levels of CR1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, when the control group is compared with either *P. falciparum*- or *P. vivax*-infected groups by Mann–Whitney *U* test.

which in turn would reduce IC clearance, contributing to the accumulation of inflammatory IC. Owing to increased FcγRIIIa expression during malaria, IC would induce a stronger inflammatory response to IC, closing a positive inflammatory feedback loop in the malaria patient.

CR1 surface expression is also reduced on B cells in mice and humans infected with *Plasmodium*. In these cells, CR1 plays an important role in differentiation and activation (44), and its decrease may have important consequences for B cell function during malaria.

It is unlikely that binding of IC to CR1 is a major contributor to the decreased expression found in vivo, as incubation of IC with macrophages in vitro does not have this effect. Additionally, an inflammatory stimulus, such as LPS, has a similar effect as malaria in decreasing CR1 surface expression, suggesting that an inflammation-triggered mediator is responsible for this effect. Injection of IC in addition to LPS has no additional effects in reducing CR1 expression, suggesting again that IC are not inducing a reduction in CR1 expression by receptor binding and internalization. Several studies have also observed decreased CR1 transcript in situations of infection and autoimmunity in the context of an inflammatory environment (25, 26). Because IC are also inflammatory, it is possible that the CR1 decrease observed in vivo is entirely caused by the inflammatory environment and not by internalization of CR1.

We found that the increased concentrations of circulating IC during malaria correlate approximately with parasitemia. The sole presence of IC in blood is not considered immune pathology, but is a normal response to infection; however, we have also observed deposition of IC in the kidneys of *P. yoelii*-infected mice, which is accompanied by glomerulonephritis. Although the relative importance of the different factors that may induce glomerulonephritis in malaria is not clear, IC deposition is probably one important contributor to kidney pathology (22).

Although CR1-mediated internalization of complement-bound IC is severely impaired, we and others (28) found efficient phagocytosis of control and infected erythrocytes by macrophages of malaria-infected mice. Internalization of IC by macrophages in the absence of complement was also very effective, suggesting that Fc receptor-mediated internalization is not inhibited during malaria. Taken together, these results indicate that the observed impaired CR1-mediated internalization of complement-bound IC is specific, because endocytic/phagocytic processes mediated by other receptors are fully functional in macrophages.

This study is focused on CR1 expression on monocytes/macrophages, which to our knowledge had not been previously addressed. In contrast, CR1 expression levels on erythrocytes in human malaria patients have been extensively studied. Decreased levels of CR1 expression on erythrocytes during malaria, especially in infections presenting severe anemia, have been characterized (45) and associations between specific CR1 polymorphisms that result in reduced erythrocyte rosetting and severe malaria have been found (16, 17). Interestingly, polymorphisms in the CR1 promoter that result in higher expression have been associated with protection against cerebral malaria (46, 47). These findings cannot be explained by the increase in rosetting, because this would result in increased cerebral malaria, but it is likely that this effect is mediated by the high expression of CR1 in erythrocytes and possibly in macrophages that would contribute to IC clearance and would decrease the inflammatory component of malaria. Indeed, IC are highly inflammatory (4) and are found at high levels in peripheral blood of malaria patients (24, 39, 48).

In our study, no differences were observed between *P. vivax*- and *P. falciparum*-infected individuals, suggesting that CR1-decreased expression is a common feature of *Plasmodium* infections.

The decrease in the levels of CR1 expression on peripheral monocytes/macrophages and B cells found in humans is not as pronounced as the decrease found in mice. This might be explained because of intrinsic differences, but also because experimental infections in mice are consistently very acute, compared with the natural variation in the inflammatory response in humans. In particular, our human sample is mostly composed of asymptomatic infected individuals. It is remarkable that such an evident decrease in the expression levels of CR1 in monocytes/macrophages is observed in this group.

Elevated levels of circulating IC are associated with malaria severity (49). Further studies will be performed to address whether CR1 expression in monocytes/macrophages is more significantly decreased during severe malaria.

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Disclosures

The authors have no financial conflicts of interest.

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