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CD36-Mediated Nonopsonic Phagocytosis of Erythrocytes Infected with Stage I and IIA Gametocytes of *Plasmodium falciparum*

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Gametocytes, the sexual stages of malaria parasites (*Plasmodium* spp.) that are transmissible to mosquitoes, have been the focus of much recent research as potential targets for novel drug and vaccine therapies. However, little is known about the host clearance of gametocyte-infected erythrocytes (GEs). Using a number of experimental strategies, we found that the scavenger receptor CD36 mediates the uptake of nonopsonized erythrocytes infected with stage I and IIA gametocytes of *Plasmodium falciparum* by monocytes and culture-derived macrophages (Mϕs). Light microscopy and immunofluorescence assays revealed that stage I and IIA gametocytes were readily internalized by monocytes and Mϕs. Pretreating monocytes and Mϕs with a monoclonal antibody that blocked CD36 resulted in a significant reduction in phagocytosis, as did treating GEs with low concentrations of trypsin to remove *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1), a parasite ligand for CD36. Pretreating monocytes and Mϕs with peroxisome proliferator-activated receptor γ–retinoid X receptor agonists, which specifically upregulate CD36, resulted in a significant increase in the phagocytosis of GEs. Murine CD36 on mouse Mϕs also mediated the phagocytosis of *P. falciparum* stage I and IIA gametocytes, as determined by receptor blockade with anti-murine CD36 monoclonal antibodies and the lack of uptake by CD36-null Mϕs. These results indicate that phagocytosis of stage I and IIA gametocytes by monocytes and Mϕs appears to be mediated to a large extent by the interaction of PfEMP-1 and CD36, suggesting that CD36 may play a role in innate clearance of these early sexual stages.

Species of the protozoan genus *Plasmodium* are intraerythrocytic parasites that are the causative agents of malaria. Each year, there are 300 million to 500 million cases of malaria and 1.5 million to 2.7 million attributable fatalities (3). Many of these deaths occur in children and are the result of severe and cerebral malaria caused by *Plasmodium falciparum*, the most pathogenic of the four species that infect humans.

P. falciparum is unique among human malaria species in that erythrocytes infected with this parasite are believed to evade clearance by immune cells of the spleen by sequestering in the microvasculature of various tissues and organs, including the skin, lung, gut, muscle, heart, and brain (30). Sequestration is mediated by cytoadherence of parasitized erythrocytes (PEs) to microvascular endothelial cells (reviewed in reference 19). Trophozoites and schizonts of *P. falciparum* express ligands, including *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) (6, 7), on the surface of PEs. These ligands enable cytoadherence of PEs to various endothelial cell receptors, including the leukocyte differentiation antigen CD36 (32, 34, 35), intercellular adhesion molecule 1 (ICAM-1) (9, 33), thrombospondin (TSP) (36), integrin $\alpha_v\beta_3$ (42), chondroitin sulfate (16), and hyaluronic acid (8).

The scavenger receptor CD36, an 88-kDa integral membrane protein that is recognized by most natural isolates of *P. falciparum* as a major sequestration receptor (31, 33), has been implicated in the pathogenesis of severe malaria. However, since little CD36 is expressed on cerebral microvascular endothelial cells (1, 51), it is more likely that other receptors, including perhaps ICAM-1 that is upregulated by inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) (29), are responsible for the binding of PEs in the microvasculature of the brain. CD36 is also expressed on monocytes and monocyte-derived macrophages (Mϕs), phagocytic cells that are involved in the innate immune response and represent the first line of defense against malaria parasites. Recently, McGilvray and colleagues (28) described a novel mechanism of nonopsonic phagocytosis of trophozoites and schizonts of *P. falciparum* by monocytes and culture-derived Mϕs. Internalization of PEs was found to be mediated by an interaction between parasite ligands, including PfEMP-1, and CD36. This nonopsonic phagocytic mechanism may represent an important first line of defense against falciparum malaria in nonimmune individuals in which antibody-mediated opsonic uptake is expected to be less.

Treatment of monocytes and Mϕs with agonists of the peroxisome proliferator-activated receptor γ (PPARγ)–retinoid X receptor (RXR) complex upregulates CD36 expression in these cells (48). Recently, incubation of monocytes and Mϕs with PPARγ-RXR agonists, including 15d-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2), 9-*cis*-retinoic acid (9-*cis*-RA), and the thiazolidinedione class of drugs (25), was shown to increase the internalization of erythrocytes containing asexual stages of *P. falciparum* (40). This increase in phagocytosis of PEs was accompanied by a decrease in parasite-induced TNF-α production. These results indicate that specific upregulation of Mϕ

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CD36 by these compounds may represent a novel means for modulating host clearance of PEs and proinflammatory responses to *P. falciparum*.

P. falciparum undergoes an indeterminate number of cycles of asexual intraerythrocytic schizogony during an infection. After each cycle, a proportion of merozoites invade erythrocytes and differentiate into gametocytes, the sexual stages of the parasite (5). Mature male and female gametocytes undergo gametogenesis, fertilization, and sporogonic development in the midguts of mosquitoes of the genus *Anopheles* after these insects take a blood meal from an infected human. Gametocytes develop through five stages of gametocytogenesis from merozoite invasion of erythrocytes to elongated mature forms, a process that takes 8 to 10 days. A recent focus of research has involved the investigation of sexual differentiation of malaria parasites and the characterization of gametocyte proteins in order to determine potential targets for drugs and vaccines (24).

Mature stage V gametocytes circulate freely in the bloodstream, but stage I to IV gametocytes sequester in the microvasculature of various organs (37). Hayward and colleagues (22) reported that PfEMP-1 is the primary ligand responsible for binding of stage I and IIA gametocytes to CD36 expressed on C32 cells and that the mechanism of this cytoadherence is indistinguishable from that of asexual parasites. Rogers and colleagues (37) showed that later gametocytes (stages III and IV) cytoadhere to human bone marrow cell lines and that a PfEMP-1–CD36 interaction is not involved in this binding.

Based on these observations, we hypothesized that erythrocytes containing stage I and IIA gametocytes of *P. falciparum* (GEs) may be internalized by human monocytes and Mϕs via nonopsonic phagocytosis mediated by CD36. While not of direct benefit to the host, such a mechanism might represent a potential means of clearing these early transmission stages from a nonimmune host. In addition, we wished to test whether upregulation of CD36 after treatment with PPARγ-RXR agonists resulted in increased clearance of gametocytes. Such a strategy in a clinical setting may represent a novel means for decreasing transmission of malaria parasites.

MATERIALS AND METHODS

Media and reagents. Endotoxin-free RPMI 1640 culture medium, heat-inactivated fetal bovine serum, and heat-inactivated group AB human serum were obtained from Wisent (Mississauga, Canada). Dextran T500, Ficoll-Paque, and Percoll were obtained from Pharmacia (Peapack, N.J.). Triton X-100 was obtained from BDH (Toronto, Ontario, Canada), and 15d-PGJ2 was obtained from BIOMOL (Plymouth, Pa.). Sorbitol, HEPES, sodium bicarbonate, hypoxanthine, 0.9% saline, sterile water, trypsin-EDTA, dimethyl sulfoxide, 9-*cis*-RA, bovine serum albumin (BSA), propidium iodide, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG), 4′,6-diamidino-2-phenylindole (DAPI), and paraformaldehyde were obtained from Sigma-Aldrich (Oakville, Canada). Mouse anti-human monoclonal immunoglobulins (IgG1) used in phagocytosis assays included anti-CD36 clone FA6-152 (Beckman Coulter, Mississauga, Canada), anti-ICAM-1 clone 15.2 (Santa Cruz Biotech, Balthesa, Calif.), anti-$\alpha_v\beta_3$ clone 23C6 (Serotec, Raleigh, N.C.), anti-TSP clone C6.7 (Neomarkers, Union City, Calif.), and anti-CD45 clone T29 (Dako, Carpentaria, Calif.). Mouse anti-human monoclonal immunoglobulin (IgG1) clone HIT8a recognizes human CD8a and was obtained from eBioscience (San Diego, Calif.). Monoclonal anti-mouse CD36 IgA (clone 63) was generated from hybridomas of spleen cells taken from CD36-null mice after intravenous injection of recombinant adenovirus containing full-length murine CD36 cDNA (the adenovirus was a kind gift from Fred deBeer, University of Kentucky). Rat anti-mouse monoclonal immunoglobulin (IgG1) clone 30-F11 recognizes murine CD45 and was obtained from BD Pharmigen (Mississauga, Ontario, Canada). Human IgG Fc fragments were obtained from Calbiochem (San Diego, Calif.), and mouse IgG Fc fragments were obtained from Jackson Immunoresearch Laboratories (West Grove, Pa.). Mouse anti-parasite monoclonal immunoglobulin (IgG1) 93A3 (11), kindly provided by David Baker and Geoffrey Targett, targets Pfs16, a *P. falciparum* sexual stage-specific parasitophorous vacuole membrane protein that can be first be detected in gametocytes 35 h after merozoites invade erythrocytes (4, 12).

Cultures. *P. falciparum* clone 3D7 (53) was cultured in vitro by using standard procedures (49) and modifications outlined by Smith et al. (46). The methods of Carter and colleagues (14) were used to induce and synchronize gametocytogenesis in culture and to differentiate the various stages of gametocytes. Trophozoites and schizonts, the only other stages that are taken up by monocytes and Mϕs (23), were removed with an efficiency of 99% from gametocyte cultures by treating the cultures with 5% sorbitol at 150 and 30 min before phagocytosis assays were begun. The effectiveness of this purification method was evaluated by allowing gametocyte cultures to grow for 24 h after sorbitol treatment; in these cultures, rings were observed in very low numbers (maximum, 1% compared to gametocytes), indicating that very low numbers of asexual trophozoites were present in the gametocyte preparation. Cultures were washed three times in RPMI 1640 medium just prior to phagocytosis assays to remove free pigment. In experiments in which trypsinized GEs were used, cultures were treated with a 0.05% trypsin–EDTA solution, incubated at 37°C for 30 min, and washed twice in RPMI 1640 medium prior to phagocytosis assays.

Preparation of human monocytes and murine Mϕs. Monocytes were isolated from the venous blood of some of us (T.G.S., S.N.P., and K.C.K.) as described previously (28), by using a method that consistently yields a platelet-free population of nonactivated monocytes. About 2.5×10^5 purified human monocytes were adhered to circular glass coverslips (diameter, 18 mm) in 12-well culture plates. Monocytes, cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (RPMI-10) at 37°C in the presence of 5% CO_2, were aged for 5 days to derive Mϕs, which were assayed for phagocytic ability or surface levels of CD36.

Thioglycolate-derived Mϕs were collected from the peritoneal cavities of both wild-type and CD36-null mice (20). These Mϕs were plated directly onto circular glass coverslips in 12-well culture plates containing RPMI-10 and incubated at 37°C in the presence of 5% CO_2.

Phagocytosis assays. To study nonopsonic phagocytosis of GEs by human monocytes and Mϕs in culture plates, Fc receptors were first blocked by incubating cells for 30 min with 20 μg of human IgG Fc fragments per ml. For receptor inhibition experiments, some wells containing monocytes and Mϕs were also incubated with 10-μg/ml solutions of monoclonal antibodies to CD36, ICAM-1, integrin $\alpha_v\beta_3$, TSP, or CD45 (isotype control) or combinations of these antibodies. After the monocytes and Mϕs were washed twice with RPMI-10, 500-μl portions of a *P. falciparum* culture containing only ring stages and various stages of gametocytes were layered on the monocytes and Mϕs at a GE/phagocyte ratio of 2:1. The plates were rotated gently for 2 h at 37°C in the presence of 5% CO_2. Coverslips containing monocytes and Mϕs were then washed for 1 min in 4°C water to lyse parasitized erythrocytes that had bound to monocytes and Mϕs but were not internalized. After the coverslips were fixed with methanol and stained with Giemsa stain, the phagocytic index (the percentage of 500 monocytes and Mϕs that had completely internalized at least one GE) was determined for each coverslip. For the most part, the same protocol was used for studies of nonopsonic phagocytosis of GEs by murine Mϕs. However, Fc receptors on murine Mϕs were blocked by addition of 23 μg of murine IgG Fc fragments per ml, whereas CD36 was blocked by using a 1:10 dilution of anti-murine CD36. A concentration of anti-murine CD45 of 5 μg/ml was used as an isotype control for experiments with murine Mϕs.

In experiments in which PPAR-RXRγ activation was examined, human Mϕs were treated with RPMI-10 containing 5 μM 15d-PGJ2 and 1 μM 9-*cis*-RA or appropriate concentrations of dimethyl sulfoxide as a control and incubated at 37°C in the presence of 5% CO_2 for 48 h.

Immunofluorescence assays. After lysis of noninternalized GEs and uninfected erythrocytes, monocytes and Mϕs on coverslips were fixed and permeabilized in acetone for 10 s. Smears of asexual and gametocyte cultures were also made on coverslips and fixed in the same manner. Coverslips were first washed with block buffer (0.1% Triton X-100 and 3% BSA in 1× phosphate-buffered saline [PBS]) for 30 min and then incubated with 10 μl of monoclonal antibody 93A3 (diluted 1:10 in block buffer) for 45 min. The coverslips were washed in block buffer for 5 min and then incubated with 10 μl of FITC-conjugated anti-mouse IgG (diluted 1:50 in block buffer) containing the nuclear fluorochrome propidium iodide (1 μg/ml) (for phagocytosis assays) or DAPI (1 μg/ml) (for culture smears) for 30 min. The coverslips were washed with wash buffer

(0.1% Triton X-100 and 3% BSA in 1× PBS) for 5 min, and each coverslip was inverted onto a 10-μl drop of the anti-fade agent Vectashield that was applied to a glass slide and sealed at its edge with nail polish.

Detection of CD36 surface levels on Mϕs. Human Mϕs, including controls and Mϕs in which CD36 was upregulated, were assayed for surface levels of CD36. Cells gently scraped off wells of tissue culture dishes were stained with a 1:100 dilution (in 1% BSA in 1× PBS) of anti-CD36 monoclonal antibodies for 30 min, followed by a 1:50 dilution (in 1% BSA in 1× PBS) of a secondary FITC-conjugated anti-mouse IgG. The controls included secondary antibody-stained cells alone. Mϕs were then fixed in 0.5% paraformaldehyde in PBS and analyzed by using an EPICS ELITE flow cytometer and software (Beckman-Coulter, Marseille, France)

Statistical analysis. The data were expressed as means and standard deviations of *n* experiments. Statistical significance was determined by using the Student *t* test. Each mean represents the results of at least three equivalent and independent experiments, and each experiment was performed in duplicate. There was some variation between experiments due to the use of different monocyte donors; however, the results were consistent within each experiment. For Fig. 2 to 6, the phagocytic index values for coverslips incubated with various antibodies were normalized against controls (which were given a value of 100) for each experiment.

RESULTS

Phagocytosis of erythrocytes infected with gametocytes of *P. falciparum*. Erythrocytes containing stage I and IIA gametocytes of *P. falciparum* were internalized by monocytes and monocyte-derived Mϕs in the presence of Fc receptor blockade and in the absence of both complement and antibodies to *P. falciparum*. FITC-labeled monoclonal antibody 93A3, which specifically recognizes gametocytes (4, 12), revealed that several GEs could be taken up by a single monocyte or Mϕ (Fig. 1). This nonopsonic phagocytosis occurred under highly stringent conditions consisting of a ratio of GEs to monocytes or Mϕs of 2:1, which was lower than the 20:1 ratio of asexual PEs to monocytes or Mϕs that was used in previous studies of nonopsonic phagocytosis (28). Such stringency was designed to reflect the low numbers of gametocytes (compared to asexual parasites) present in natural *P. falciparum* infections (47).

Anti-CD36 antibodies reduce uptake of stage I and IIA gametocytes by monocytes and Mϕs. Prior to phagocytosis assays, monocytes or culture-derived Mϕs (day 5) were incubated with a panel of monoclonal antibodies to various Mϕ surface receptors that are involved in phagocytosis or are known to interact with PfEMP-1. For these experiments (Fig. 2) and the experiments whose results are shown in Fig. 3 to 5, typically 20 to 25% of the control monocytes and Mϕs internalized at least one stage I or IIA GE. Figure 2 shows that CD36 receptor blockade of culture-derived Mϕs with 10 μg of monoclonal antibody FA6 per ml caused a significant decrease (~50%) in the phagocytosis of nonopsonized erythrocytes containing stage I and IIA gametocytes. Treatment of Mϕs with 10 μg of monoclonal antibodies to other receptors (including ICAM-1, integrin $\alpha_v\beta_3$, TSP, and CD45) per ml did not result in a significant decrease in phagocytosis or had only a minor effect on phagocytosis. Phagocytosis of uninfected erythrocytes by monocytes or culture-derived Mϕs was not observed. Thus, CD36 is a major receptor mediating the binding and phagocytosis of GEs by Mϕs in vitro.

Treating GEs with trypsin reduced their uptake by monocytes and Mϕs. Trypsin has been shown to remove PfEMP-1, a ligand that mediates the binding of the parasite to CD36 (6). Parasite cultures were incubated with a low concentration of trypsin and then washed repeatedly before they were allowed to interact with monocytes and Mϕs. Treating nonopsonized GEs with trypsin resulted in a ~80% decrease in phagocytosis of stage I and IIA gametocytes compared to phagocytosis of control GEs (Fig. 3). Trypsin treatment in combination with antibody blockade of monocytes and Mϕs with anti-CD36 or anti-ICAM-1 did not result in a greater decrease in phagocytosis than treatment with trypsin alone. These results indicate that one or more trypsin-sensitive ligands, most likely including

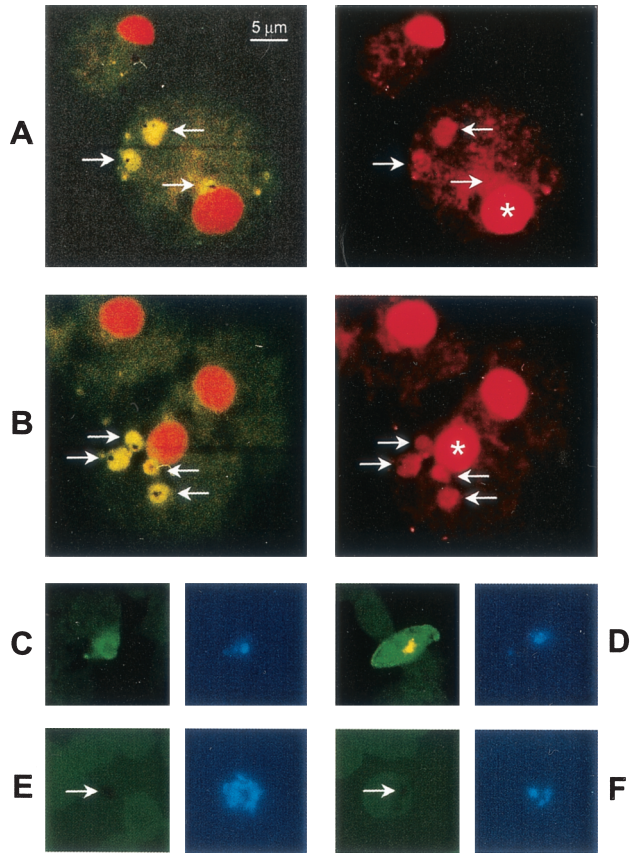

FIG. 1. Phagocytosis of GEs by Mϕs. Cultures of nonopsonized erythrocytes infected with stage I and IIA gametocytes of *P. falciparum* were incubated for 2 h with Fc receptor-blocked human Mϕs that were adhered to glass coverslips. After hypotonic lysis to remove noninternalized GEs, the monocytes on coverslips were fixed and prepared for monoclonal antibody immunofluorescence. Two pairs of fluorescence micrographs (A and B) show representative Mϕs that contain internalized nonopsonized erythrocytes containing stage I and IIA gametocytes. Internalized gametocytes (arrows) fluoresce green due to the presence of FITC-conjugated monoclonal antibody 93A3 (a mouse IgG1), which targets the gametocyte surface protein Pfs16 on GEs (left micrographs in panels A and B). Nuclei of gametocytes and Mϕs (asterisks) fluoresce orange due to the presence of propidium iodide, which targets DNA (right micrographs in panels A and B). FITC-conjugated 93A3 targets Pfs16 on various stages of gametocytes, including stage I (left micrograph in panel C) and stage III (right micrograph in panel D), in blood smears. When asexual parasites (one of which is indicated by an arrow) are incubated in blood smears with FITC-conjugated 93A3, they do not express Pfs16 (left micrograph in panel E). Gametocytes (one of which is indicated by an arrow) in blood smears do not react with FITC-conjugated monoclonal antibody HIT8a (a mouse IgG1 that targets human CD8a), which was used as an IgG1 isotype control (left micrograph in panel F). Nuclei of parasites fluoresce blue due to the presence of DAPI, which targets DNA (right micrographs in panels C to F). Bar = 5 μm.

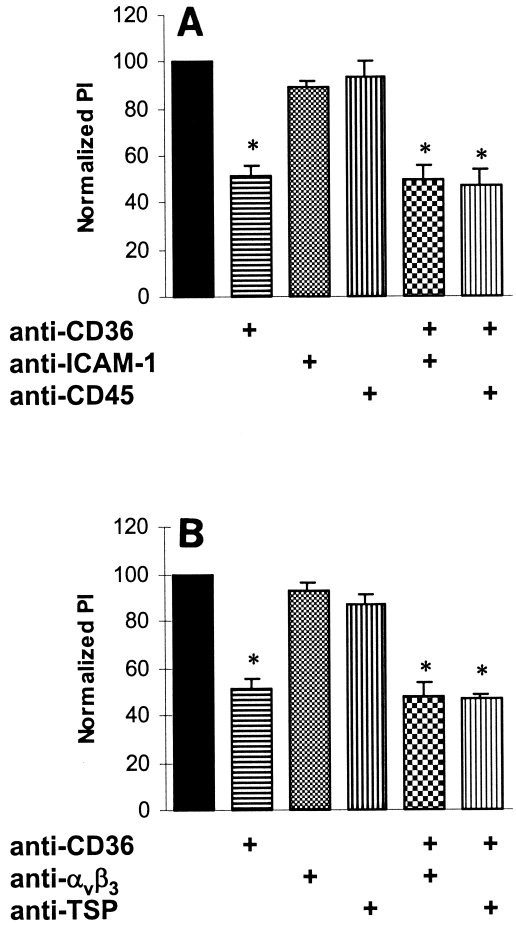

FIG. 2. Anti-CD36 antibody FA6 reduces phagocytosis of stage I and IIA gametocytes by monocytes and Mϕs. Prior to phagocytosis assays, monocytes or culture-derived Mϕs (day 5) were incubated with a panel of monoclonal antibodies to various monocyte and Mϕ surface receptors. Some of these receptors, including CD36 and ICAM-1, have been implicated in the cytoadherence of *P. falciparum*-infected erythrocytes via ligands on the surfaces of infected cells (see text), whereas CD36, integrin $\alpha_v\beta_3$, and TSP have been shown to be involved in the phagocytosis of apoptotic neutrophils (38). (A) Blocking CD36 on monocytes and Mϕs with 10 μg of FA6 (anti-CD36 monoclonal antibody) per ml resulted in a significant decrease in phagocytosis of nonopsonized erythrocytes infected with stage I and IIA gametocytes (mean inhibition, 49.0%; standard deviation, 4.4%). An asterisk indicates that the *P* value is <0.01 ($n = 5$). (B) Antibodies (10 μg/ml) to ICAM-1 and to CD45 (shown in panel A) or to integrin $\alpha_v\beta_3$ and TSP (shown in panel B) did not result in a significant decrease in phagocytosis or had only a minor effect on phagocytosis ($n = 3$). Blocking with FA6 in combination with antibodies to the receptors ICAM-1, integrin $\alpha_v\beta_3$, TSP, and CD45 caused a reduction in phagocytosis that was similar to that observed with FA6 alone ($n = 3$). Significant reductions in phagocytosis ($P < 0.01$) are indicated by an asterisk. PI, phagocytic index.

PfEMP-1, mediate the binding and internalization of GEs by human monocytes and Mϕs.

Phagocytosis of early- and late-stage gametocytes by monocytes and Mϕs. *P. falciparum* cultures were prepared so that at least 90% of all gametocytes were at stage I or IIA (early stages) or at least 90% of all gametocytes were at stages IIB to IV (late stages). In these assays, there was significantly greater uptake of early stages (~20 to 25%) than of late stages (~5 to

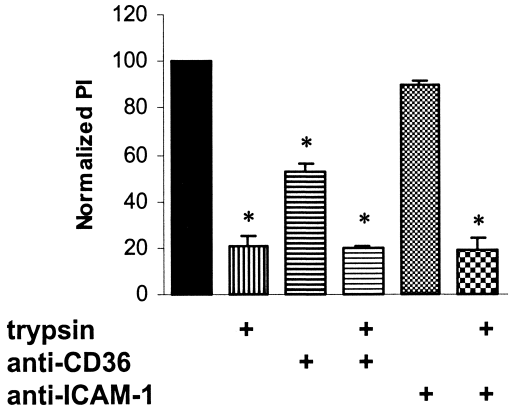

FIG. 3. Treating parasitized erythrocytes with trypsin reduces phagocytosis of gametocytes by monocytes and Mϕs. Cultures containing nonopsonized GEs were incubated with a low concentration of trypsin for 30 min and then washed repeatedly before they were allowed to interact with monocytes and Mϕs. Phagocytosis of trypsin-treated GEs was reduced by 79.2% ± 4.5% (mean ± standard deviation) compared to phagocytosis of control GEs. Combining trypsin treatment and antibody blockade of monocytes and Mϕs with 10 μg of either anti-CD36 or anti-ICAM-1 per ml did not result in a greater decrease in phagocytosis compared to the results obtained with trypsin alone. Significant reductions in phagocytosis ($P < 0.01$; $n = 3$) are indicated by an asterisk. PI, phagocytic index.

8%) ($P < 0.01$; $n = 4$). After incubation with 10 μg of anti-CD36 antibodies per ml for 30 min, monocytes and Mϕs were incubated with early- or late-stage gametocyte cultures for 2 h. Figure 4 shows that blocking CD36 on monocytes and Mϕs with FA6 reduced the phagocytosis of nonopsonized erythrocytes containing late-stage gametocytes by 21.2% (mean) compared to controls, which is significantly less that the 49.0% reduction in phagocytosis recorded with early-stage GEs ($P <$

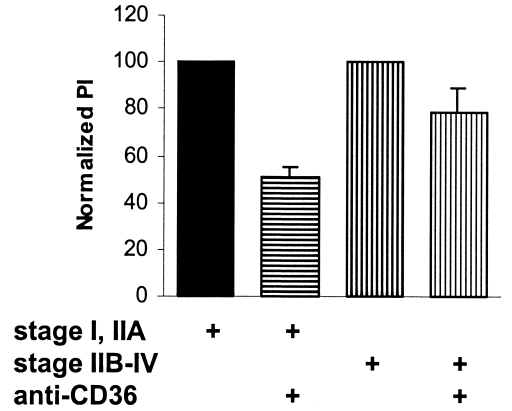

FIG. 4. Phagocytosis of early- and late-stage gametocytes by monocytes and Mϕs. Cultures were prepared so that at least 90% of all gametocytes were at stage I or IIA (early stages) or so that at least 90% of all gametocytes were at stages IIB to IV (late stages). After incubation with 10 μg of anti-CD36 antibody FA6 per ml, monocytes and Mϕs were incubated with early- or late-stage gametocyte cultures for 2 h. Blocking CD36 on monocytes and Mϕs with FA6 reduced the phagocytosis of late-stage GEs by 21.2% ± 10.1% (mean ± standard deviation) compared to the phagocytosis in the controls, which is significantly less ($P < 0.02$; $n = 4$) than the 49.0% ± 4.4% reduction in phagocytosis recorded with early-stage GEs. PI, phagocytic index.

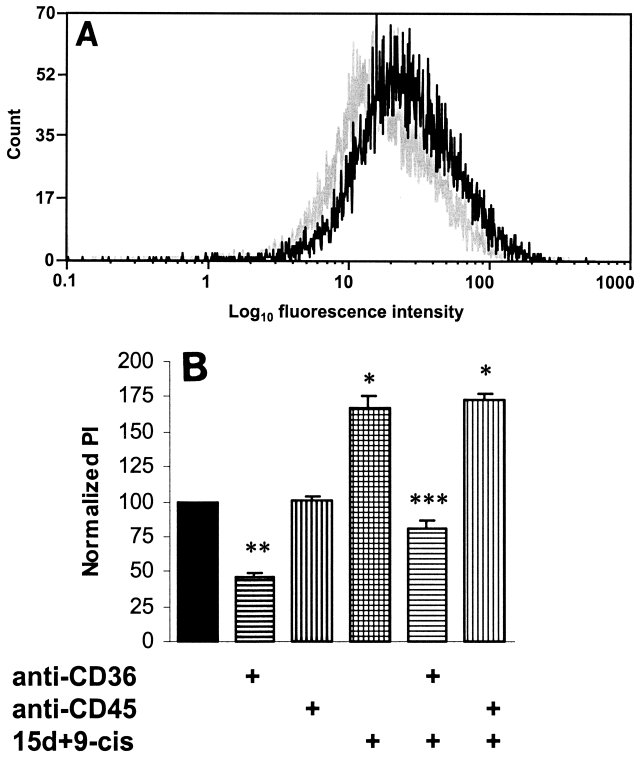

FIG. 5. Upregulating CD36 increases phagocytosis of stage I and IIA gametocytes. (A) Human Mϕs were incubated for 48 h with PPARγ-RXR agonists (5 μM 15d-PGJ2 and 1 μM 9-*cis*-RA) and processed for flow cytometry. In Mϕs that were incubated with PPARγ-RXR agonists (black lines), there was an increase in CD36 surface levels compared to the levels in untreated Mϕs (grey lines). (B) Phagocytosis assays were carried out by using adherent Mϕs that had been incubated for 48 h with PPARγ-RXR agonists. Phagocytosis of nonopsonized erythrocytes containing stage I and IIA gametocytes was increased in Mϕs treated with 5 μM 15d-PGJ2 and 1 μM 9-*cis*-RA (15d+9-cis) by 66.6% ± 9.4% (mean ± standard deviation) compared to phagocytosis in the controls ($P < 0.01$; $n = 3$) (one asterisk). Blocking CD36 with 10 μg of FA6 per ml in untreated Mϕs resulted in a mean reduction in phagocytosis of 53.9% ($P < 0.01$; $n = 3$) (two asterisks), whereas anti-CD36 blockade of treated Mϕs caused a mean reduction in phagocytosis of 52.0% ($P < 0.01$; $n = 3$) (three asterisks). PI, phagocytic index.

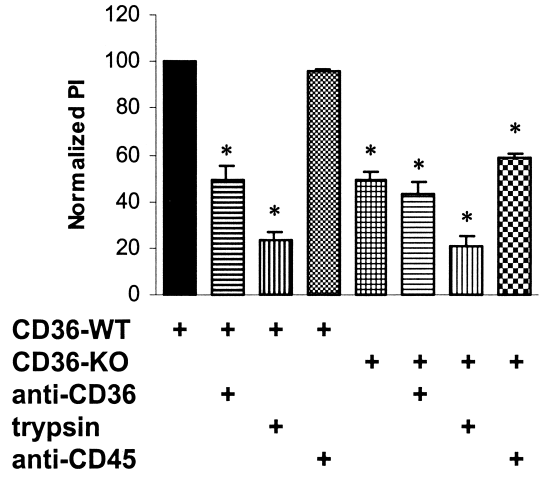

FIG. 6. Murine CD36 mediates the uptake of stage I and IIA gametocytes by murine Mϕs. Wild-type (WT) or CD36-null (KO) murine Mϕs were incubated in the presence or absence of anti-murine CD36 monoclonal antibodies prior to phagocytosis assays with untreated or trypsin-treated cultures. Phagocytosis of GEs by wild-type murine Mϕs was significantly reduced (mean ± standard deviation, 50.7% ± 5.6%) when it was blocked with anti-murine CD36 ($P < 0.01$; $n = 3$). Phagocytosis of GEs by CD36-null murine Mϕs was reduced by 51.0% ± 4.0% compared to phagocytosis by wild-type murine Mϕs ($P < 0.01$; $n = 3$), and treatment with anti-murine CD36 did not significantly reduce phagocytosis further. Treating cultures with 0.05% trypsin significantly reduced phagocytosis of GEs, by 77.1% ± 4.1% for wild-type murine Mϕs ($P < 0.01$; $n = 3$) and by 79.6% ± 4.3% for CD36-null Mϕs ($P < 0.01$; $n = 3$). Significant reductions in phagocytosis ($P < 0.01$) are indicated by an asterisk. PI, phagocytic index.

0.05; $n = 4$). If the reduction in uptake of stage IIB to IV gametocytes had been due in part to the internalization of contaminating stage I and IIA gametocytes in the late-stage gametocyte cultures, then there would have been an even greater difference in uptake between the two types of gametocytes. These data indicate that CD36 is a primary receptor involved in the phagocytosis of early-stage GEs by monocytes and Mϕs but plays a lesser role in the internalization of late-stage gametocytes.

PPARγ-RXR agonists upregulate CD36 in human Mϕs and increase their phagocytic capacity for GEs. Human Mϕs were incubated for 48 h with PPARγ-RXR agonists (5 μM 15d-PGJ2 and 1 μM 9-*cis*-RA) and processed for flow cytometry. Data from the flow cytometric analysis (Fig. 5A) revealed that CD36 levels were elevated on Mϕs that were incubated with PPARγ-RXR agonists. Phagocytosis assays were carried out by using Mϕs that had been incubated for 48 h with PPARγ-RXR agonists. Figure 5B shows that phagocytosis of nonopsonized erythrocytes containing stage I and IIA gametocytes was increased in treated Mϕs by ~70% compared to the controls ($P < 0.01$; $n = 3$). Blocking CD36 with 10 μg of FA6 per ml in both untreated and treated Mϕs resulted in significant reductions in phagocytosis (53.9 and 52.0%, respectively) compared to the phagocytosis in the controls ($P < 0.01$; $n = 3$).

Murine CD36 mediates the uptake of GEs by murine Mϕs. Erythrocytes containing asexual stages of *P. falciparum* have previously been shown to adhere to murine CD36 on the surfaces of COS-7 cells (39). As an alternative experimental strategy to examine the role of CD36 in mediating the uptake of GEs by Mϕs, we examined the phagocytosis of GEs by wild-type and CD36-null murine Mϕs. Wild-type or CD36-null thioglycolate-induced murine Mϕs were incubated with anti-murine CD36 prior to phagocytosis assays performed with untreated or trypsin-treated cultures. Typically, ~20% of wild-type murine Mϕs internalized at least one nonopsonized GE. Figure 6 shows that phagocytosis of GEs by CD36-null murine Mϕs was reduced by ~50% compared to phagocytosis of GEs by wild-type murine Mϕs. Furthermore, phagocytosis of GEs by wild-type murine Mϕs treated with anti-murine CD36 was reduced by ~50%, which is equivalent to the reduction observed in CD36-null Mϕs. CD36 blockade of CD36-null murine Mϕs did not significantly reduce phagocytosis compared to phagocytosis by control CD36-null murine Mϕs.

DISCUSSION

In this study, we obtained several lines of evidence demonstrating that the phagocytosis by human monocytes and cul-

ture-derived Mϕs of nonopsonized erythrocytes containing *P. falciparum* stage I and IIA gametocytes is mediated in large part by the scavenger receptor CD36. Fluorescent microscopy revealed that GEs are internalized by monocytes and Mϕs with relatively high frequency (~20 to 25% of phagocytes internalized at least one GE). Blocking CD36 with monoclonal antibodies specific to this receptor reduced phagocytosis of GEs by almost ~50%, whereas cleaving trypsin-sensitive ligands (including PfEMP-1), which are known to mediate binding to CD36, reduced uptake by ~80%. Thioglycolate-induced murine Mϕs were also able to internalize human erythrocytes containing *P. falciparum* stage I and IIA gametocytes, and this uptake was reduced by ~50% in CD36-null murine Mϕs. Finally, we show that treating Mϕs with PPARγ-RXR agonists increased by ~70% the phagocytosis of GEs via the pharmacological upregulation of CD36. This clearance mechanism and upregulation of phagocytosis are similar to those observed with asexual parasites (28, 40); however, internalization of erythrocytes infected with stage I and IIA gametocytes represents a previously unrecognized clearance pathway that may have transmission implications.

Little is known about the mechanisms by which gametocytes of *Plasmodium* species are cleared or destroyed by the human immune system. Most in vitro studies of the phagocytosis (opsonic or nonopsonic) or destruction (mediated by free radicals or cytokines) of *P. falciparum*-infected erythrocytes have focused primarily on asexual stages of the parasite (10, 15, 20, 50, 52, 56). Sinden and Smalley (43) described the in vitro phagocytosis by various leukocytes of extracellular mature gametocytes (stage V) that had been isolated from the guts of freshly fed mosquitoes. Lensen and colleagues (26, 27) attempted to correlate opsonic phagocytosis of gametes in the mosquito midgut by leukocytes with transmission-blocking activity. However, in neither of these studies did the researchers examine phagocytosis of early-stage GEs.

Healer and colleagues (23) investigated phagocytosis of various stages of *P. falciparum*, including schizonts, gametocytes, and gametes, by human leukocytes. Using in vitro phagocytosis assays, they found that nonopsonized or opsonized erythrocytes containing mature (stage V) gametocytes were not internalized to any significant degree by neutrophils or by monocytes and Mϕs. Based on this result, they surmised that mature gametocytes have few, if any, parasite-specific antigens expressed on the erythrocyte surface and thus that an antibody-mediated immune response could not be mounted against these stages. Based on our study, however, a lack of parasite-specific ligands might instead result in the inability of a monocyte or Mϕ to recognize and bind a nonopsonized erythrocyte infected with a mature gametocyte and subsequently to internalize it. In addition, since we have shown that immature gametocytes are readily ingested by monocytes and Mϕs, the conclusion of Healer et al. that phagocytosis of gametocytes does not play a role in blocking transmission of *P. falciparum* may now be modified.

We observed that phagocytosis of nonopsonized erythrocytes containing stage I and IIA gametocytes was significantly reduced by removing parasite ligands (including PfEMP-1) and by antibody blockade of CD36 expressed on monocytes and Mϕs. These results suggest that internalization of these early sexual stages was largely dependent upon an initial binding interaction between PfEMP-1 and CD36 and subsequent phagocytosis by a CD36-mediated pathway. Although uncharacterized at the time, such a nonopsonic mechanism was observed by Ferrante and colleagues (21), who reported that phagocytosis of asexual stages of *P. falciparum* by monocytes and Mϕs occurred with equal avidity in the absence and in the presence of opsonins (human immune sera), whereas phagocytosis of parasites by neutrophils was almost completely reliant on the presence of opsonins in human immune sera.

The potential for a beneficial role for CD36 in malaria, as suggested by this study and others (2, 40), contrasts with recent strategies to target the interaction between PEs and CD36 (6, 17). Based on our data, blocking the GE-CD36 interaction might be expected to reduce the number of gametocytes internalized by phagocytic cells, thus increasing the number of sexual stages present in the bloodstream and therefore transmission. These considerations, along with the observation that little CD36 is present on cerebral microvasculature and the observation that only a minority of infected nonimmune patients develop severe malaria (even though most wild isolates of *P. falciparum* adhere to CD36), suggest that the PE-CD36 interaction may represent a complex coevolutionary adaptation of the parasite and host (40).

The reduction in phagocytosis of GEs by monocytes and Mϕs by anti-CD36 monoclonal antibody blockade (~50%) was not as complete as that observed for asexual parasites (~70% inhibition by anti-CD36 antibodies). In addition, the increase in phagocytosis of GEs by Mϕs previously incubated with PPARγ-RXR agonists (~70%) was not as great as that observed for asexual parasites (~125%) (40). From these results, it appears that CD36 is the primary receptor mediating binding and phagocytosis of GEs by monocytes and Mϕs. However, one or more as-yet-unknown receptors are responsible for the other 50% of binding and internalization. Whether PfEMP-1 or other molecules, the ligands that mediate adherence to these unidentified receptors are largely trypsin sensitive, as indicated by the 80% inhibition of phagocytosis caused by protease pretreatment of cultures containing stage I and IIA gametocytes. At stage IIB, unidentified receptors were responsible for the vast majority of binding and phagocytosis of gametocytes, indicating that ligand-receptor complexes change greatly throughout the course of gametocytogenesis. Hayward and colleagues (22) observed that PfEMP-1 was expressed during stages I and IIA and bound to CD36 in a manner similar to that of asexual parasites. However, they found that PfEMP-1 expression abruptly ceased at stage IIB. Rogers and colleagues (37) showed that later gametocytes (stage III and IV gametocytes) cytoadhered to human bone marrow cell lines and that a PfEMP-1–CD36 interaction was not involved in this binding. Concomitant with this change in ligands expressed on GEs is a switch in sequestration sites from typical sites for asexual parasites to various cell types of the spleen and bone marrow (44, 45).

The CD36 promoter contains a binding site for the PPARγ-RXR transcriptional activator complex. When the PPARγ agonist 15d-PGJ2 and the RXR agonist 9-*cis*-RA bind to this receptor complex, CD36 gene expression in Mϕs is upregulated and CD36 surface levels increase (48). The increase in CD36 results in significantly increased phagocytic capacity of human monocytes and monocyte-derived Mϕs for asexual par-

asites (40) and gametocytes (this study). In the case of asexual parasites, PPARγ-RXR activation also caused a decrease in parasite-induced production of TNF-α (40).

Vitamin A supplementation has been shown to potentiate host resistance to malaria. Recent data indicate that the beneficial effects may be mediated, at least in part, by the action of 9-*cis* RA, a metabolite of vitamin A, on PPARγ-RXR, resulting in increased clearance of asexual PEs and decreased proinflammatory responses to infection (41). The findings of the present study suggest that vitamin A supplementation programs might be expected to have an impact on clearance of early sexual stages and malaria transmission; this question can now be addressed in vivo.

There are potential in vivo consequences of our in vitro data. Specifically, could the pharmacological modulation of the phagocytic capacity of monocytes and Mϕs represent a novel means to facilitate clearance of gametocytes and thus decrease transmission of *P. falciparum*? Theoretically, only one male gametocyte and one female gametocyte in a single mosquito blood meal are required to form an oocyst. The majority of human infections with *P. falciparum* are characterized by a gametocytemia, if it exists, that is at least 1 order of magnitude less than the level of asexual parasitemia (47). In addition, gametocytemia is generally correlated with the number of oocysts in mosquitoes that feed on infected humans (13). An argument in favor of pharmacological upregulation of phagocytosis is that since the numbers of gametocytes ingested by a mosquito are already very low, increasing the phagocytosis of early stages of gametocytes by 70% (as observed in this study) may be sufficient to block transmission. Gametes from male gametocytes must be highly efficient at finding females in a large blood meal in order to form ookinetes (18, 54). Nevertheless, a mathematical model that incorporates parameters such as gametocytemia in the blood and the size of a blood meal taken by a mosquito suggests that a threshold number of male and female gametocytes (i.e., more than 20 gametocytes) is required to ensure production of at least one viable oocyst (55). Reducing the gametocytemia to a level below this threshold may prevent the formation or at least greatly reduce the number of oocysts formed in a mosquito.

Recent research on the sexual stages of *P. falciparum* has concentrated on the development of gametocyte-specific drug therapies and the characterization of gametocyte and gamete antigens for the purpose of developing a transmission-blocking vaccine (24). However, it is evident from this study that innate immunity may also play a role in determining the number of circulating gametocytes available to be ingested by mosquitoes. The in vitro data presented here need to be confirmed by in vivo studies in order to determine the role of this clearance mechanism in decreasing transmission of *P. falciparum*.

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