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Interaction of Mouse Dendritic Cells and Malaria-Infected Erythrocytes: Uptake, Maturation, and Antigen Presentation

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Consistent with their seminal role in detecting infection, both mouse bone marrow-derived and splenic CD11c+ dendritic cells (DCs) exhibited higher levels of uptake of Plasmodium chabaudi-parasitized RBCs (pRBCs) than of noninfected RBCs (nRBCs) as determined by our newly developed flow cytometric technique using the dye CFSE to label RBCs before coculture with DCs. To confirm that expression of CFSE by CD11c+ cells following coculture with CFSE-labeled pRBCs represents internalization of pRBC by DCs, we showed colocalization of CFSE-labeled pRBCs and PE-labeled CD11c+ DCs by confocal fluorescence microscopy. Treatment of DCs with cytochalasin D significantly inhibited the uptake of pRBCs, demonstrating that uptake is an actin-dependent phagocytic process. The uptake of pRBCs by splenic CD11c+ DCs was significantly enhanced after infection in vivo and was associated with the induction of DC maturation, IL-12 production, and stimulation of CD4+ T cell proliferation and IFN-γ production. These results suggest that DCs selectively phagocyte pRBCs and present pRBC-derived Ags to CD4+ T cells, thereby promoting development of protective Th1-dependent immune responses to blood-stage malaria infection. The Journal of Immunology, 2006, 176: 441–450.

Malaria, caused by the intracellular parasite Plasmodium, is the most important parasitic infection in humans. The global prevalence of malaria continues to rise at an alarming rate and is a major cause of high mortality in children and morbidity in infected adults living in the developing world (1). Despite extensive research, a vaccine is not yet available and many antimalarial drugs are increasingly ineffective due to widespread drug resistance. In areas where malaria is endemic, individuals acquire partial immunity against malaria only after repeated exposure to the parasite. It may take many years of frequent infection episodes to establish protective immunity capable of preventing clinical disease. Therefore, a better understanding of the mechanisms that induce protective immunity to malaria infection will help identify strategic targets for antimalaria vaccine development and immunotherapy.

Infections of mice with rodent Plasmodium species are useful tools for investigating the immunobiology of blood-stage malaria. In most rodent blood-stage malaria infections, survival depends on the ability to control parasite replication during the acute phase of infection. Immune effector mechanisms that mediate control of parasitemia involve macrophages, NK cells, CD4+ T cells, and IFN-γ production, while B cells and Th1-dependent Ab responses are required for elimination and resolution of the chronic stage of infection (reviewed in Ref. 2). Although several cytokines, such as IL-12 in synergy with IL-15 or IL-18, may result in Th1 cell development (3, 4), IFN-γ is the central effector cytokine mediating protective immunity to blood-stage malaria infection (5). These results highlight the importance of adaptive type 1 immune mechanisms for host resistance to blood-stage malaria and the need to identify critical components of the innate immune response that promote development of IFN-γ-producing CD4+ Th1 cells.

Early interactions between blood-stage parasites and cells of the innate immune system are thought to be important in shaping the adaptive immune response to blood-stage malaria. Compared with other APCs such as B cells and macrophages, dendritic cells (DCs) are primarily responsible for priming naïve CD4+ T cells and stimulating the production of proinflammatory cytokines, which in turn determine the phenotype of an ensuing immune response to invading pathogens. Several studies using mouse models of malaria have provided evidence for an important role of DCs in inducing protective immunity to blood-stage malaria. Plasmodium chabaudi chabaudi schizonts were found to induce bone marrow (BM)-derived DCs to express MHC class II and costimulatory molecules and to produce IL-12, TNF-α, and IL-6 (6). In mice infected with P. chabaudi, CD11c+ DCs were observed to migrate from the marginal zone of the spleen to the CD4+ T cell-rich periarteriolar lymphoid sheath and to exhibit up-regulated expression of costimulatory molecules as well as increased production of IFN-γ (7). In contrast, macrophage and B cell populations in the spleen expand but remain confined to the red pulp area (7) while CD11b+ macrophages produce a soluble factor(s) that inhibits CD4+ T cell proliferation and production of IL-2 (8, 9). Similarly, splenic CD11c+ DCs, but not CD11b+ macrophages or B220+ B cells, from Plasmodium yoelii 17X-infected mice stimulate high levels of IL-2, IFN-γ, and TNF-α production by naïve CD4+ T cells (8, 9). Importantly, the induction of T cell cytokine production was shown to critically require DC-derived IL-12 (9). Taken together, these results show that DCs activated in vitro and in vivo by rodent Plasmodium parasites are able to express costimulatory...
molecules and produce proinflammatory cytokines to stimulate adaptive type 1 immune responses that are protective against malaria infection.

To initiate an immune response against *Plasmodium* infection, DCs would be required to take up soluble malarial Ags or *Plasmodium*-parasitized RBCs (pRBCs). Because the *Plasmodium* parasite spends the majority of its erythrocytic life cycle within the RBC, DCs must be capable of recognizing and capturing pRBCs circulating in the blood or spleen and subsequently process and present pRBC-derived Ags to other immune cells. In addition, it would be more efficient if DCs internalized intact pRBCs rather than wait for malarial Ags to be released from rupturing pRBCs or delivered by other phagocytes such as macrophages and neutrophils. To date, most studies have focused on opsonic and nonopsonic phagocytosis of pRBCs by macrophages and demonstrated this process to be an important nonspecific immune defense mechanism in the early control of blood-stage parasite growth (10, 11). By contrast, the uptake of pRBCs by DCs and its role in the activation of downstream immune responses, including DC maturation and T cell stimulatory function, has not been investigated in detail.

It has been reported that DCs internalize *Plasmodium falciparum* or *P. yoelii*-infected RBC (12, 13). However, none of the previous studies compared the uptake of malaria-infected RBC by DCs to a physiologically relevant negative control nor measured the level of uptake by DCs before and after malaria infection in vivo. Because DCs have been shown to selectively endocytose apoptotic and allogeneic cells (14), we investigated here whether DCs take up pRBCs to a significantly greater extent than normal, noninfected RBCs (nRBCs). To do this, we developed a sensitive flow cytometry assay using the dye CFSE to measure the uptake of CFSE-labeled pRBCs or nRBCs by DCs. Using this technique, we compared the uptake of *P. chabaudi*-infected RBCs to that of nRBCs by BM-derived and splenic DCs and characterized the kinetics of pRBC uptake by splenic DCs following blood-stage *P. chabaudi* infection in vivo. Both BM-derived and splenic CD11c<sup>+</sup> DCs exhibited preferential uptake of pRBCs vs nRBCs, and this uptake was dependent in part on actin polymerization, a crucial event in the phagocytosis of large particles. We also hypothesized that the uptake of pRBCs by DCs would induce phenotypic and functional maturation as observed with splenic DCs isolated from mice infected with blood-stage *P. chabaudi* (7 or P. yoelii (8, 9)). Indeed, we observed that the selective uptake of pRBCs by splenic DCs was enhanced following infection in vivo and associated with increased expression of MHC class II and costimulatory molecules, IL-12 production, and stimulation of CD4<sup>+</sup> T cell proliferation and IFN-γ production. These results confirm the important role of DCs in initiating protective immunity to blood-stage malaria and also provide novel evidence that early interactions between mouse DCs and pRBCs result in DC activation and induction of protective Th1 cell-mediated immune responses.

Materials and Methods

**Mice, parasite, and pRBC purification**

Female C57BL/6 (B6) mice (Charles River Laboratories), ages 8–12 wk, were maintained in the animal facility of the Montreal General Hospital Research Institute in accordance with the guidelines and policies of the Canadian Council on Animal Care. *P. chabaudi* AS was maintained as previously described (15), and infections were initiated by i.p. injection of 10<sup>6</sup> *P. chabaudi* AS PRBCs. To purify pRBCs, heparinized blood was obtained via cardiac puncture from *P. chabaudi*-infected B6 mice with 30–50% parasitemia and washed twice with PBS. Blood was diluted with PBS (1–2 ml), loaded onto a 74% Percoll (Sigma-Aldrich) density gradient, and centrifuged at 5000 × g for 20 min at room temperature, and the top band was collected. Collected cells were washed twice with PBS and resuspended in cell culture medium.

**Generation of DCs from BM precursors**

DCs were cultured from BM precursors of naive B6 mice using a method adapted from Inaba et al. (16) and Lutz et al. (17) in the presence of GM-CSF supplied by supernatant from a hypoxanthine, aminopterin, and thymidine medium-sensitive Ag8653 myeloma cell line transfected with murine GM-CSF cDNA, which was generated by B. Stockinger (National Institute for Medical Research, Mill Hill, U.K.) (18) and generously provided by G. Carayannisiotis (Memorial University of Newfoundland, St. John’s, Canada). Ag8653 cells were cultured under strict G418 selection in RPMI 1640 medium (In vitro Life Technologies) with 5% FCS (HyClone), 0.2% L-glutamine (Invitrogen Life Technologies), 20 µg/ml gentamicin (Sabex), 2 mM L-glutamine (Invitrogen Life Technologies), and 0.5 µM 2-ME (Sigma-Aldrich). For the first 3 days of culture, complete medium was also supplemented with 20% Ag8653 culture supernatant. On days 3 and 5 of culture, the plates were swirled gently and 4 ml of supernatant, containing nonadherent cells, was discarded from each well and replaced with 5 ml of fresh complete medium with 10% Ag8653 culture supernatant. To enrich for growing DCs, the top 4 ml of supernatant from each well was discarded on day 7 of culture, and the cell clusters were collected for subculture. Cell aggregtes were gently dislodged from the plate by repeated pipetting with PBS and 1% FCS (HyClone). Cells were resuspended at 1.5 × 10<sup>6</sup> cells/ml in complete medium supplemented with 10% Ag8653 culture supernatant and cultured for an additional 20–24 h at 37°C. Resulting cells were routinely 84–88% positive for CD11c as determined by flow cytometry.

**Purification of CD11c<sup>+</sup> DCs and CD4<sup>+</sup> T cells from spleen**

Spleens from B6 mice were removed aseptically, perfused with PBS and 1% FCS (HyClone), teased gently apart, and pressed through a sterile fine wire mesh. To obtain DCs, cells were suspended in PBS and 5 mM EDTA (Sigma-Aldrich) and separated by loading onto Nycoprep (Axis-Shield) density gradient and centrifuging at 600 × g for 20 min at 4°C. Low-density pellets at the interface between PBS and lysate were washed twice with PBS and further purified by positive selection using anti-CD11c microbeads (Miltenyi Biotec). The resulting DCs were routinely ≥85–90% positive for CD11c as determined by flow cytometry. Spleen cells were enriched for CD4<sup>+</sup> T cells using anti-CD4 microbeads (Miltenyi Biotec) and were ≥85–90% positive for CD4 as determined by flow cytometry.

**DC uptake assay and flow cytometric analysis**

To stain pRBCs or nRBCs, cells (10<sup>7</sup> cells/ml) were incubated with 2 µM CFSE (Molecular Probes) in complete medium for 15 min at 37°C. CFSE staining was stopped by adding excess complete medium and washing cells twice with complete medium. BM-derived or splenic CD11c<sup>+</sup> DCs (10<sup>6</sup> cells/well) were seeded with CFSE-labeled pRBCs or nRBCs in a 1:20 ratio at a final volume of 200 µl for 4 h at 37°C unless otherwise indicated. DCs were pretreated with cytochalasin D (Sigma-Aldrich) or colchicine (Sigma-Aldrich) dissolved in DMSO (Fisher Scientific) at the indicated concentrations or with 0.2% DMSO control at 37°C for 30 min before the addition of pRBCs or nRBCs. Treatment with cytochalasin D or colchicine did not decrease DC viability as determined by trypan blue exclusion and 7-aminoactinomycin D (BD Biosciences) staining (data not shown). To determine the effect of cytokines on uptake by DCs, splenic CD11c<sup>+</sup> DCs isolated from naive mice were treated with mouse IFN-γ (25 ng/ml; R&D Systems) or rIL-10 (200 U/ml; BD Biosciences) in complete medium for 12 h before the addition of pRBCs or nRBCs. Following coculture, nonquantified red cells were digested by lysis with NH<sub>4</sub>Cl lysing buffer, and the remaining DCs were washed twice with PBS, FcR blocked, and then resuspended at 1.5–2 × 10<sup>6</sup> cells/ml in complete medium with 1% FCS (HyClone). 4 ml of supernatant, containing nonadherent cells, was discarded from each well and replaced with 5 ml of fresh complete medium with 10% Ag8653 culture supernatant. To enrich for growing DCs, the top 4 ml of supernatant from each well was discarded on day 7 of culture, and the cell clusters were collected for subculture. Cell aggregates were gently dislodged from the plate by repeated pipetting with PBS and 1% FCS (HyClone). Cells were resuspended at 1.5 × 10<sup>6</sup> cells/ml in complete medium supplemented with 10% Ag8653 culture supernatant and cultured for an additional 20–24 h at 37°C. Resulting cells were routinely 84–88% positive for CD11c as determined by flow cytometry.

**Staining of pRBCs or nRBCs**

Staining of pRBCs or nRBCs, cells (10<sup>7</sup> cells/ml) were incubated with 2 µM CFSE (Molecular Probes) in complete medium for 15 min at 37°C. CFSE staining was stopped by adding excess complete medium and washing cells twice with complete medium. BM-derived or splenic CD11c<sup>+</sup> DCs (10<sup>6</sup> cells/well) were seeded with CFSE-labeled pRBCs or nRBCs in a 1:20 ratio at a final volume of 200 µl for 4 h at 37°C unless otherwise indicated. DCs were pretreated with cytochalasin D (Sigma-Aldrich) or colchicine (Sigma-Aldrich) dissolved in DMSO (Fisher Scientific) at the indicated concentrations or with 0.2% DMSO control at 37°C for 30 min before the addition of pRBCs or nRBCs. Treatment with cytochalasin D or colchicine did not decrease DC viability as determined by trypan blue exclusion and 7-aminoactinomycin D (BD Biosciences) staining (data not shown). To determine the effect of cytokines on uptake by DCs, splenic CD11c<sup>+</sup> DCs isolated from naive mice were treated with mouse IFN-γ (25 ng/ml; R&D Systems) or rIL-10 (200 U/ml; BD Biosciences) in complete medium for 12 h before the addition of pRBCs or nRBCs. Following coculture, nonquantified red cells were digested by lysis with NH<sub>4</sub>Cl lysing buffer, and the remaining DCs were washed twice with PBS, FcR blocked, and then stained with PE-labeled anti-CD11c mAb (clone HL3; BD Biosciences) in sorting buffer consisting of PBS with 1% FCS (HyClone) and 0.05% sodium azide (Sigma-Aldrich). Control for transfer of CFSE from lysed RBCs to DCs during the staining procedure, DCs were added to pRBC or nRBC lysate and analyzed for CFSE expression. The uptake of CFSE-labeled pRBCs or nRBCs was determined by gating cells on SSC vs FL2
for the CD11c⁺ population and analyzing CFSE staining on FL1 using FACS Calibur equipped with CellQuest software (BD Biosciences). Uptake of pRBC by splenic DC subsets was determined by staining CD11c⁺ cells cocultured with pRBCs with PE-labeled mAb to B220 (clone R43-682), CD4 (clone GK1.5), and CD8 (clone 53.6.7) and analyzing CFSE expression by these CD11c⁺ subsets.

Light and confocal fluorescence microscopy

BM-derived DCs cocultured with CFSE-labeled pRBCs were analyzed by light and confocal microscopy. For light microscopy, cells were centrifuged at 600 rpm onto glass slides, fixed by 100% methanol, stained with Diff-Quik, dried, and photographed at ×1000 magnification using a Zeiss Axioskop 2 plus light microscope (Carl Zeiss). Images were acquired with Bioquant Nova prime software (version 6.710.10MT). For confocal fluorescence microscopy, cells were stained with PE-labeled anti-CD11c Ab and a small aliquot of cells at 10⁶ cells/ml were mounted onto glass slides with fluorescent mounting medium (DakoCytomation), allowed to dry overnight, and observed using a Zeiss Axioskop 2 plus fluorescent microscope. Confocal microscopy images were acquired and analyzed using Q capture software (version 1.60).

Splenic DC maturation, cytokine production, and Ag presentation to T cells

Following overnight coculture of splenic CD11c⁺ DCs with pRBCs or nRBCs, noningested red cells were removed by lysis with NH₄Cl lysing buffer, and DCs were FcR blocked and then stained with PE-labeled mAb (BD Biosciences) to CD11c, I-A⁺ (clone AF6-120.1), CD40 (clone 2/23), CD80 (clone 16-1041), and CD86 (clone GL-1) in sorting buffer. Cells were gated on the CD11c⁺ and CFSE⁺ population, and the percentages and mean fluorescence intensity (MFI) of cells expressing MHC class II and costimulatory molecules were determined by flow cytometry. To determine DC expression of maturation markers ex vivo before and after P. chabaudi infection, splenic CD11c⁺ DCs purified from naive and day 5-infected mice were stained with mAb as described above and analyzed by flow cytometry. Supernatants of splenic CD11c⁺ DCs (10⁶ cells/well) incubated with pRBCs or nRBCs in a 1:10 ratio for 48 h at 37°C were analyzed for cytokine production by ELISA as described previously (5, 19).

To determine Ag presentation function of malaria-pulsed DCs, splenic CD11c⁺ DCs were pulsed with pRBCs or nRBCs at the ratios indicated or incubated in complete medium only as nonpulsed controls. Following overnight culture, noningested DCs were removed by lysis with NH₄Cl lysing buffer, DCs were washed three times with complete medium, and 100 μl of DCs at 5 × 10⁶ cells/ml was plated with splenic CD4⁺ T cells, freshly purified from naive B6 mice, in varying ratios as indicated at a final volume of 200 μl in 96-well plates for 48 h at 37°C. Proliferation was determined by incorporation of [³H]thymidine during the last 6 h of incubation. Supernatants from DC and T cell cocultures were analyzed for IFN-γ production by ELISA as described previously (19). All pRBC and nRBC preparations used in this study tested negative for the presence of endotoxin using a Limulus amebocyte lysate gel clot test (Sigma-Aldrich) with a sensitivity limit of 0.015 EU/ml. In addition, to control for possible endotoxin contamination, pRBCs and nRBCs were treated with polymixin B (10 μg/ml; Sigma-Aldrich) before incubation with DCs. Results obtained with polymixin-treated pRBCs or nRBCs were identical to those obtained with untreated red cells (data not shown), confirming that the pRBC-induced DC responses observed in this study were not attributable to endotoxin contamination.

Statistical analyses

Data are expressed as mean ± SEM. Statistical significance of differences between experimental groups as indicated was analyzed by two-tailed, unpaired Student’s t test. Statistical significance was defined as follows: *, p < 0.05; **, p < 0.01; and ***, p < 0.001. All statistical analyses were performed using SAS/STAT software (SAS Institute).

Results

Preferential uptake of malaria-infected RBCs by BM-derived DCs

To better understand the early interactions between DCs and P. chabaudi-infected RBCs, we developed a flow cytometry technique to measure the uptake of pRBCs or nRBCs by DCs. The intraerythrocytic location of the P. chabaudi AS parasite enabled us to use the dye CFSE to label highly purified pRBCs, as well as nRBCs, and then detect CFSE fluorescence expressed by DCs following coculture. BM-derived DCs were incubated with CFSE-labeled pRBCs or nRBCs, and the percentages of CD11c⁺ DCs expressing CFSE, representing the population of DCs that had ingested the RBCs, were analyzed by flow cytometry. BM-derived DCs were able to ingest pRBCs to a considerably greater extent (4-fold) than nRBCs (Fig. 1, A and B). The possibility that CFSE inadvertently transferred from lysed RBCs to DCs during the RBC lysis and cell surface staining procedures was observed to be minimal (<1% for nRBCs and 5% for pRBCs; Fig. 1A, right panels). Lysis controls were routinely performed in all experiments, and the values obtained were subtracted from the percentages of uptake presented here. The preferential uptake of pRBCs by DCs was observed as early as 2 h of coculture and peaked at 6 h with no

FIGURE 1. DCs preferentially take up pRBCs vs nRBCs. BM-derived DCs were incubated with CFSE-labeled pRBCs or nRBCs in a ratio of 1:20 for 4 h at 37°C. Following coculture, noningested RBCs were lysed, DCs were stained with PE-labeled anti-CD11c mAb, and CFSE fluorescence was determined by flow cytometry. A, Percentages of cells expressing both PE-CD11c (FL2) and CFSE (FL1) following coculture of DCs with intact pRBCs or nRBCs (left panels) or lysis controls (right panels) in which DCs were cocultured with pRBC or nRBC lysate. B, Histograms show CFSE expression by CD11c⁺ DC control (dotted line) or by DCs cocultured with nRBCs (gray line) or with pRBCs (black line). C, Percentages of gated CD11c⁺ cells expressing CFSE after incubation with pRBCs or nRBCs for the time intervals as indicated. Data in A and B are representative of six to eight independent experiments. Data in C are expressed as mean ± SEM pooled from three independent experiments. Statistical analyses compared pRBCs vs nRBCs at each time point (***, p < 0.001).
notable increases thereafter at 20 h (Fig. 1C). Even after 20 h of coculture, the percentage of DCs that ingested nRBCs was significantly lower than the percentage that ingested pRBCs.

**DCs internalize malaria-infected RBCs via actin-dependent phagocytosis**

Phagocytosis of large particles (usually larger than 0.5 μm in diameter) by mammalian cells, including macrophages and DCs, requires actin polymerization at the site of entry and subsequent growth of new actin filaments that facilitate cell membrane motility (20). To determine whether the uptake of pRBCs by DCs was mediated by actin-dependent phagocytosis, BM-derived DCs were treated with cytochalasin D, a drug that inhibits actin polymerization and addition of actin monomers to the ends of growing filaments (21), before the addition of pRBCs or nRBCs. As shown in Fig. 2A, DCs treated with cytochalasin D had significantly lower uptake of both pRBCs (54 vs 13%) and nRBCs (7.5 vs 4%) than untreated DCs. This inhibition of uptake was observed at all ratios of DC cocultured with RBCs (Fig. 2B), as well as at all doses of cytochalasin D tested (Fig. 2C). There was no dose response using higher concentrations of cytochalasin D, and the observed reductions were independent of the DMSO used as a diluent, which resulted in similar levels of uptake of pRBCs and nRBCs as untreated DCs (Fig. 2C). Because treatment with cytochalasin D did not completely abrogate the uptake by DCs, some pRBCs may have been phagocytosed via nonactin-dependent mechanisms. Treatment with colchicine, a microtubule depolymerizing agent, did not impair uptake of pRBCs or nRBCs at various concentrations tested (Fig. 2D), suggesting that functional microtubules do not play a major role in the uptake of pRBCs by DCs.

To demonstrate internalization of pRBCs by DCs, cytopsins of Diff-Quik-stained BM-derived DCs cocultured with pRBCs were examined by light microscopy. Following 2–6 h of coculture and lysis of noningested red cells, intact pRBCs were observed inside cytoplasmic compartments of BM-derived DCs that resemble phagosomal vacuoles (Fig. 3A). To confirm that the expression of CFSE fluorescence by gated CD11c+ DCs truly represents internalization of pRBCs by DCs rather than only attachment of CFSE-labeled cells to the CD11c+ cell surface, we examined colocalization of CFSE-labeled pRBCs and PE-stained CD11c+ cells by confocal microscopy (Fig. 3, B–D). In representative confocal fluorescent microscopy images, CFSE-labeled pRBCs are shown to colocalize with three PE-labeled CD11c+ DCs (Fig. 3D). Collectively, these microscopy images provide strong evidence that flow cytometric analyses of CFSE expression by gated CD11c+ DCs using the assay we developed were an accurate and objective measurement of internalization by DCs of CFSE-labeled pRBCs or nRBCs.

**Uptake of malaria-infected RBC by splenic CD11c+ DCs is selectively enhanced following malaria infection in vivo**

Next, we used our flow cytometric technique to study the uptake of pRBCs by mouse spleen DCs before and during infection with *P. chabaudi* AS. Although we used BM-derived DCs to standardize our flow cytometric uptake assay and showed that these DCs exhibited preferential uptake of pRBCs, it is the DC population in the spleen that is of major interest because of the central role of this organ in filtering pRBCs from the blood and in generating a protective immune response to blood-stage malaria (22). As observed with BM-derived DCs, splenic CD11c+ DCs showed significantly higher (>4-fold) uptake of pRBCs than nRBCs (Fig. 4A) at all time points before and after *P. chabaudi* infection (Fig. 4B). Importantly, the percentage of splenic CD11c+ DCs ingesting pRBCs increased following *P. chabaudi* infection (47.7% at day 5 postinfection (p.i.) vs 18.6% at day 0; Fig. 4A). The uptake of pRBCs by splenic CD11c+ DCs increased markedly at days 2–5 p.i. and declined thereafter to preinfection levels by day 8 p.i. (Fig. 4B), indicating that maximal uptake of pRBCs by splenic DCs preceded peak parasitemia in *P. chabaudi*-infected mice. In contrast, the uptake of nRBCs increased only slightly after infection (5% higher at days 5 vs 0 p.i.) and was significantly lower than the uptake of pRBCs at all time points before and after infection. Uptake of pRBCs and nRBCs by splenic DCs from naive mice was...
FIGURE 3. Internalization of pRBCs by BM-derived DCs and colocalization of DCs and CFSE-labeled pRBCs. A, Diff-Quik-stained light microscopic images show pRBCs, indicated by arrows, internalized by DCs. B, Red fluorescence of PE-labeled CD11c+ DCs. C, Green fluorescence of CFSE-labeled cells, representing CFSE-labeled pRBCs inside DCs. D, Merged confocal microscopic image showing colocalization of CFSE-labeled pRBCs and CD11c+ DCs. Light microscopic images and confocal fluorescent images were acquired at \( \times 100 \) and \( \times 40 \) magnification, respectively. Data shown are representative of two independent experiments.

Effect of cytokines on uptake of malaria-infected RBCs by splenic CD11c+ DCs

Previous work in our laboratory showed that rIFN-\( \gamma \) activates macrophages and increases their phagocytosis of pRBCs while rIL-10 has the opposite effect (11). We questioned whether phagocytosis of pRBCs by DCs is similarly controlled by cytokines with opposing immunoregulatory effects. Splenic CD11c+ DCs from naive or day 5-infected mice were treated with rIFN-\( \gamma \), rIL-10, or not treated (medium) for 12 h before the addition of pRBCs or nRBCs as described previously (11). As shown above, splenic DCs from infected mice demonstrated higher levels of pRBC uptake than DCs from naive mice (Fig. 4E). However, treatment with rIFN-\( \gamma \) or rIL-10 did not significantly affect the uptake of pRBCs or nRBCs by DCs from either naive or infected mice, suggesting that uptake of pRBCs by DCs is not dependent on the cytokines present in the spleen microenvironment.

Uptake of malaria-infected RBC by splenic CD11c+ DCs induces maturation, cytokine production, and CD4+ T cell activation

The aforementioned flow cytometric analyses were conducted on total CD11c+ DC populations cocultured with pRBCs and included both phagocytic and nonphagocytic DCs. We then determined whether preferential uptake of pRBCs by DCs induces maturation and presentation of Ags to CD4+ T cells. Following an overnight DC uptake assay, splenic CD11c+ DCs that had captured pRBCs showed significantly higher expression of MHC class II and costimulatory molecules than DCs that had ingested nRBCs as demonstrated by the higher percentages of cells expressing these molecules (Fig. 5A) and by the higher levels of MFI (Fig. 5B). The ability of pRBCs, but not nRBCs, to stimulate maturation of splenic DCs in vitro was consistent with the observation of in vivo maturation after \( P. \) chabaudi infection (Fig. 5C). Because uptake of pRBCs by DCs was associated with phenotypic maturation, we assessed whether DCs that had phagocytosed pRBCs compared with DCs that had ingested nRBCs were also functionally more mature in terms of cytokine production and stimulation of CD4+ T cell responses. Splenic DCs cocultured with nRBCs or in medium alone did not produce detectable levels of IL-12p40 or, importantly, bioactive IL-12p70 compared with DCs pulsed with pRBCs (Fig. 6, A and B). In addition, splenic DCs pulsed with pRBCs produced significantly more IL-2 (Fig. 6C) and stimulated significantly higher levels of CD4+ T cell proliferation (Fig. 6D) and IFN-\( \gamma \) production (Fig. 6F) than did splenic DCs pulsed in medium alone (nonpulsed) or with nRBCs. Notably, stimulation of T cell proliferation and IFN-\( \gamma \) production increased in a dose-dependent manner when DCs were pulsed with a higher number of pRBCs (Fig. 6, E and F). The lower stimulation of T cell responses by DCs pulsed in medium alone or with nRBCs was not associated with the production of anti-inflammatory IL-10, which was significantly lower than the level produced by DCs.
FIGURE 4. Splenic CD11c<sup>+</sup> DCs preferentially take up pRBCs vs nRBCs at increasing levels in the early days following P. chabaudi infection in vivo. A. Splenic DCs enriched from naive (left panels) or day 5-infected (right panels) B6 mice were incubated with CFSE-labeled pRBCs or nRBCs, and the level of uptake by nongated cells (dot plots) or gated CD11c<sup>+</sup> cells (histograms) was determined by CFSE fluorescence as detected by flow cytometry. B, Kinetics of pRBC or nRBC uptake by splenic DCs enriched from mice before and after P. chabaudi infection and course of parasitemia in mice infected with P. chabaudi during the same experimental period. C, Splenic DCs preferentially take up pRBCs vs nRBCs at all ratios of DCs incubated with RBCs and uptake is inhibited by treatment with cytochalasin D (CyD, dotted lines). D, Uptake of pRBCs is mediated by CD4<sup>+</sup>, CD8<sup>+</sup>, and B220<sup>+</sup> subsets of splenic CD11c<sup>+</sup> DCs before and after infection. E, Pretreatment of splenic DCs from naive or day 5-infected mice with rIFN-γ or rIL-10 in vitro does not affect the uptake of pRBCs or nRBCs compared with untreated DCs. Data in B–D were pooled from three independent experiments each containing three to four mice per group and are presented as mean ± SEM. Data in E are representative of two independent experiments each containing three to four mice per group. Statistical analyses in B and C compared pRBCs vs nRBCs at each time point or DC:RBC ratio and in D compared days 0 vs 5 p.i. for each subset (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

pulsed with pRBCs (Fig. 6D). Several lines of evidence indicate that pRBC is a poor T cell mitogen in the absence of presentation by an APC such as the splenic DCs used in this study: 1) CD4<sup>+</sup> T cells incubated with pRBCs alone showed minimal proliferation and failed to produce detectable IFN-γ (Fig. 6, E and F); 2) DCs fixed with 1% paraformaldehyde before pulsing with pRBCs were unable to stimulate CD4<sup>+</sup> T cells (data not shown); and 3) Th2-type cytokines, IL-4 and IL-13, were not detected in any of the supernatants from DC single cultures or cocultures with T cells (data not shown). These results suggest that the selective interaction of splenic CD11c<sup>+</sup> DCs with pRBC induced DCs to mature, to produce IL-12, and to promote CD4<sup>+</sup> T cell proliferation and differentiation into IFN-γ-secreting cells.

Discussion

Early interactions between DCs and invading pathogens result in activation of DCs and thereby shape the development of adaptive immune responses. In mouse models of malaria, DCs are thought to play an important role in the induction of Th1-dependent immune responses that are protective against blood-stage malaria infection (2, 6, 9). In the present study, we describe a new flow cytometry technique to measure levels of phagocytosis of pRBCs and nRBCs by mouse BM-derived or spleen DCs in vitro. This is the first report showing that DCs take up and present malaria-infected RBCs in a highly selective manner that is enhanced significantly after infection in vivo. The uptake of pRBCs, but not of nRBCs, by splenic CD11c<sup>+</sup> DCs was associated with the induction of DC maturation, cytokine production, and stimulation of CD4<sup>+</sup> T cell responses. These results provide additional evidence that DCs are important APCs in the innate immune response to blood-stage malaria infection that activate T cells, particularly the CD4<sup>+</sup> Th1 cells that produce IFN-γ and mediate type 1 adaptive immune responses such as class switching of B cells to the protective Th1-dependent IgG subclasses during the chronic phase of infection (2, 22).

The uptake of pRBCs by splenic CD11c<sup>+</sup> DCs increased with acute P. chabaudi AS infection and peaked 2–3 days before peak parasitemia, which typically occurs at day 7 p.i. (5, 19, 23). This increased phagocytic activity may be due to an expansion of the DC population in the spleen following P. chabaudi infection (7, 24). As the parasite replicates, causing the parasite burden to increase in the blood and more pRBCs to be deposited in the spleen for removal, blood-borne DCs are recruited to the spleen in increasing numbers. These migratory DCs are likely to be immature and highly efficient in the recognition and capture of foreign Ags. Following peak phagocytic activity at day 5 p.i., the uptake of pRBCs by splenic DCs declined to baseline levels, which may reflect down-regulation of DC function and other innate responses once adaptive immunity becomes established. Alternatively, we considered the possibility that DC uptake, similar to macrophage
Phagocytosis (11), may be modulated by a balance of proinflammatory and anti-inflammatory cytokines. The kinetics of DC uptake of pRBCs closely coincided with the level of IFN-γ production observed in P. chabaudi-infected B6 mice (19, 23). Notably, the peak level of pRBC uptake by splenic DCs observed in this study also coincided with peak numbers of splenic CD11c+ DCs expressing IFN-γ at day 4–5 following P. chabaudi infection (7, 24). For macrophages, levels of nonopsonic phagocytosis also correlate with the ability of infected mice to produce IFN-γ and treatment of macrophages with IFN-γ in vitro enhances phagocytosis of pRBCs while treatment with IL-10 inhibits this activity. However, results presented here suggest that the uptake of pRBCs by DCs is not modulated by either IFN-γ or IL-10. Given that Ag capture by DCs is essential for DC activation and initiation of innate and adaptive immune responses, it is teleologically reasonable that the uptake of pRBCs by DCs is not influenced by immunoregulatory cytokines, which in turn are produced as a result of DC-parasite interaction. Therefore, we favor the view that the decreased uptake of pRBCs by DCs observed following P. chabaudi infection reflects an influx of immature DCs from the periphery into the spleen in response to increasing numbers of pRBCs deposited in the spleen during the period of rising parasitemia rather than an up-regulation by proinflammatory cytokines present in situ.

The development of a flow cytometry technique to measure uptake of pRBCs by DCs allowed us to investigate whether DCs selectively recognize and capture pRBCs in the absence of opsonization by Ab or complement. We used purified pRBCs to ensure that pRBC-specific DC uptake and responses were studied. Furthermore, to ensure that the expression of CFSE fluorescence by CD11c+ DCs represented internalization rather than attachment of pRBCs to the DC surface, we routinely used a RBC lysis procedure in each DC uptake assay to remove adherent, noningested pRBCs and confirmed colocalization of green (CFSE-labeled pRBCs) and red (PE-labeled CD11c+ DCs) fluorescence by confocal microscopy. As previously reported for macrophage phagocytosis (25), our flow cytometric analyses consistently showed that DCs ingest pRBCs at considerably higher rates than nRBCs. Moreover, the uptake of pRBCs by DCs required actin polymerization but not functional microtubules, although some pRBCs may be ingested by non-actin-dependent mechanisms. Light microscopic images suggested that internalized pRBCs are contained within phagosomal compartments in DCs that resemble those that form in macrophages (25, 26). Taken together, these data indicate that uptake of pRBCs by DCs occurs via actin-dependent phagocytosis and provide evidence that our flow cytometry assay offers an objective and accurate method to study DC-mediated phagocytosis of pRBCs in human and mouse malaria.

One potential application of our newly developed flow cytometric uptake assay is to investigate the specific components in the DC-parasite interaction in malaria. A key question is the identity of receptors and ligands involved in Ag capture and presentation. Consistent with mechanisms triggering nonopsonic phagocytosis, the process of pRBC internalization by DCs most likely occurs through engagement of pRBC-derived ligands with Ag uptake receptors (20). For macrophages, numerous studies have shown that CD36, a class B scavenger receptor, is the main receptor mediating nonopsonic phagocytosis of P. falciparum-infected RBCs (25–27). CD36 binds several ligands expressed on the surface of P. falciparum-infected RBCs, particularly the highly variable but structurally conserved P. falciparum erythrocyte membrane protein 1. Although CD36-mediated macrophage phagocytosis may be an important nonspecific defense mechanism for parasite clearance,
The interaction between *P. falciparum* erythrocyte membrane protein 1 and CD36 expressed by endothelial cells has been implicated in *P. falciparum* cytoadherence and sequestration, which are associated with severe malaria as well as cerebral malaria (26). Cytoadherent *P. falciparum* parasite lines bind to CD36 and CD54 (ICAM-1), whereas a nonadherent parasite line does not bind these receptors (12). *P. chabaudi*-infected RBCs also adhere to purified CD36 and to endothelial cells in an IFN-γ-dependent manner (28). However, these findings do not conclusively demonstrate that selective recognition and uptake of malaria-infected RBCs by DCs are mediated via CD36 or CD54. Other possible candidates include TLR9, which mediates the response of human and mouse plasmacytoid DCs to *P. falciparum*-infected RBCs (29), and receptors of the C-type lectin family (e.g., DC-SIGN and DEC-205) involved in receptor-mediated endocytosis (30, 31). Experiments are ongoing in our laboratory to address the role of these and other receptors in uptake of *P. chabaudi*-infected RBCs by mouse DCs.

Given the importance of the spleen in host resistance to murine blood-stage *Plasmodium* parasites, the expansion of the splenic DC population (7, 24) and migration of CD11c+ DCs to the T cell-rich zones of the spleen (7) following malaria infection suggest that splenic DCs are in a prime position to initiate protective immune responses against blood-stage malaria. DCs recognize foreign ligands through pattern recognition receptors, a process that could trigger their maturation as well as the production of proinflammatory cytokines. Indeed, the uptake of pRBCs by splenic CD11c+ DCs was linked to activation of DC responses that may play an important role in shaping adaptive T cell-dependent immune responses. Splenic CD11c+ DCs that phagocytosed pRBCs were able to present pRBC-derived Ags to CD4+ T cells as demonstrated by the significantly higher levels of proliferation and IFN-γ production induced by DCs pulsed with pRBCs compared with DCs incubated alone or with nRBCs. Although the extent of pRBC phagocytosis by splenic DCs was comparably lower than that by BM-derived DCs, our results suggest that massive uptake is not required to induce splenic DC activation nor for splenic DCs to present sufficient peptides for T cell activation. Rather, stimulation of T cell responses and, crucially, the induction of Th1 cell development were associated with increased expression of MHC class II and costimulatory molecules as well as production of IL-2 and IL-12. Although CD11c+ DCs enriched from the spleen comprise a heterogeneous population, our results suggest that the uptake of pRBC and subsequent DC activation were not restricted to a specific CD11c+ DC subset. However, because we did not investigate maturation and functional responses among distinct DC subpopulations, we cannot exclude the possibility that cytokine production and T cell stimulatory activity by pRBC-activated DCs were mediated by different DC subsets.

The findings presented here corroborate previous studies demonstrating that DC maturation and function are not impaired following interaction with *Plasmodium* parasites. We show that preferential uptake of *P. chabaudi*-infected RBCs by splenic CD11c+ DCs induced their maturation, cytokine production, and activation of CD4+ T cell responses. In vitro maturation of DCs exposed to pRBCs but not to nRBCs also reflected in vivo maturation following *P. chabaudi* infection. These results are in agreement with other studies showing that splenic CD11c+ DCs from mice infected with *P. chabaudi* exhibit up-regulated expression of CD40, CD54, and CD86 (7), as well as increased production of IFN-γ (7, 24). Similarly, splenic CD11c+ DCs from mice infected with *P. yoelii* 17X stimulate high levels of IL-2, IFN-γ, and TNF-α production by responding naive CD4+ T cells in an IL-12-dependent mechanism (8, 9). Activation of DCs is not limited to rodent models of malaria:
*P. falciparum* schizonts were found to stimulate human plasmacytoid DCs and mouse splenic B220⁺ DCs to up-regulate CD86 expression, to produce IFN-γ, and to promote γδ T cell proliferation and IFN-γ production through a TLR9-dependent pathway (29). However, these results as well as data presented here contrast those from other studies showing that some isolates of *P. falciparum* (12), as well as rodent *P. yoelii* (13, 32) and *P. chabaudi* (32), inhibit DC maturation in response to LPS stimulation in vitro. Cytoadherent *P. falciparum* parasites were also found to inhibit the ability of DCs to stimulate primary or secondary malaria-specific T cell proliferation (12), while modulation of DC functions by *P. yoelii*-infected RBCs was associated with suppressed protective CD8⁺ T cell responses against liver-stage malaria (13).

There are several possible explanations for the discrepancy among these findings. Studies showing inhibition of DC maturation by *Plasmodium* parasites were conducted with the use of LPS to mature DCs in vitro. However, following initial maturation with LPS, DCs may become exhausted or refractory to further stimulation with malarial Ags (reviewed in Ref. 2). Furthermore, LPS induces DC maturation in the absence of CD40 ligation, which has been shown to be required for maximal expression of a mature DC phenotype and for cytokine production (33, 34). Despite the lack of maturation in response to LPS, DCs pulsed with RBCs infected with *P. yoelii* or *P. chabaudi* were fully capable of inducing protective immunity against homologous challenge infection, suggesting that DC maturation is merely delayed rather than arrested in vitro (32). Studies showing inhibition of DC maturation and lower T cell stimulatory activity by *Plasmodium* parasites used DCs derived from human peripheral blood cells (12) or mouse BM precursors (13, 32), while other studies (7–9, 24), including the study presented here, showing up-regulated DC maturation and function used CD11c⁺ DCs purified from spleens of naive or infected mice. DCs grown in culture develop and interact with *Plasmodium spp.*, in the absence of the cytokine milieu provided by NK cells, T cells, and other cell types normally present in the spleen microenvironment that support and promote DC maturation. However, the different origins of DCs used among these studies do not fully explain the discrepancy because *P. chabaudi* schizonts were found to induce mouse BM-derived DCs to up-regulate expression of MHC class II, CD40, and CD86 and to produce IL-12, TNF-α, and IL-6 even in the absence of T cells, NK cells, or CD40 ligation (6).

In conclusion, there is growing evidence that DCs play an important role in the host defense against blood-stage malaria infection by linking innate and adaptive immunity. The results presented in this study support the contention that mouse splenic DCs interact with *P. chabaudi*-infected RBCs early after infection through preferential uptake, processing, and presentation of malarial-Ags to CD4⁺ T cells. The expression of costimulatory molecules as well as production of IL-2 and IL-12 following uptake of RBCs provide important costimulatory and cytokine signals to support the development and expansion of TH1 cells, which are critically required for protective immunity to blood-stage malaria. A better understanding of the early interactions of DCs with *Plasmodium* parasites will not only provide insights into the immunobiology of malaria infection but may also identify key targets for the future development of vaccines and immunotherapies.

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**Disclosures**

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