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Inhibition of Dendritic Cell Maturation by Malaria Is Dose Dependent and Does Not Require *Plasmodium falciparum* Erythrocyte Membrane Protein 1[▽]

Salenna R. Elliott,[1]* Timothy P. Spurck,[2] Joelle M. Dodin,[1] Alexander G. Maier,[3] Till S. Voss,[3]† Francisca Yosaatmadja,[1] Paul D. Payne,[1] Geoffrey I. McFadden,[2] Alan F. Cowman,[3] Stephen J. Rogerson,[1] Louis Schofield,[3] and Graham V. Brown[1]

Department of Medicine, The University of Melbourne, Royal Melbourne Hospital, Melbourne, Victoria, Australia[1]; School of Botany, The University of Melbourne, Melbourne, Victoria, Australia[2]; and The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia[3]

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Red blood cells infected with *Plasmodium falciparum* (iRBCs) have been shown to modulate maturation of human monocyte-derived dendritic cells (DCs), interfering with their ability to activate T cells. Interaction between *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) and CD36 expressed by DCs is the proposed mechanism, but we show here that DC modulation does not require CD36 binding, PfEMP1, or contact between DCs and infected RBCs and depends on the iRBC dose. iRBCs expressing a PfEMP1 variant that binds chondroitin sulfate A (CSA) but not CD36 were phagocytosed, inhibited lipopolysaccharide (LPS)-induced phenotypic maturation and cytokine secretion, and abrogated the ability of DCs to stimulate allogeneic T-cell proliferation. CD36- and CSA-binding iRBCs showed comparable inhibition. *P. falciparum* lines rendered deficient in PfEMP1 expression by targeted gene knockout or knockdown also inhibited LPS-induced phenotypic maturation, and separation of DCs and iRBCs in transwells showed that inhibition was not contact dependent. Inhibition was observed at an iRBC:DC ratio of 100:1 but not at a ratio of 10:1. High doses of iRBCs were associated with apoptosis of DCs, which was not activation induced. Lower doses of iRBCs stimulated DC maturation sufficient to activate autologous T-cell proliferation. In conclusion, modulation of DC maturation by *P. falciparum* is dose dependent and does not require interaction between PfEMP1 and CD36. Inhibition and apoptosis of DCs by high-dose iRBCs may or may not be physiological. However, our observation that low-dose iRBCs initiate functional DC maturation warrants reevaluation and further investigation of DC interactions with blood-stage *P. falciparum*.

Dendritic cells (DCs) are specialized antigen-presenting cells that regulate both innate and adaptive immune responses and play a critical role in the initiation of primary T-cell responses. To function effectively as antigen-presenting cells, they undergo a process of maturation, characterized by increased expression of costimulator, major histocompatibility complex and adhesion molecules, and secretion of proinflammatory cytokines (reviewed in reference 35). DC maturation is usually activated by pathogens through ligation of pattern recognition receptors, such as Toll-like receptors, but may also be initiated by inflammatory cytokines and endogenous signals of cellular damage (reviewed in references 29 and 34).

It has been suggested that modulation of DC function by the malaria parasite *Plasmodium falciparum* contributes to both the delayed acquisition of antimalarial immunity as well as immunosuppression associated with acute malaria infection. Urban et al. (50, 52) showed that red blood cells infected with *P. falciparum* (iRBCs) at 100 iRBCs per DC inhibit maturation of human monocyte-derived DCs and interfere with their ability to activate T-cell responses. Interaction between the parasite protein *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), expressed on the surfaces of iRBCs, and the DC scavenger receptor CD36, was proposed to be the mechanism for this effect. PfEMP1 is a variant protein (3) that mediates adhesion of iRBCs to multiple host receptors (2, 12, 44), the most common ligands being CD36 and intercellular adhesion molecule 1 (4, 28). CD36-adherent iRBCs were shown to bind to and inhibit DC maturation in the presence of lipopolysaccharide (LPS), tumor necrosis factor alpha, or CD40 ligand (50, 52). A role for CD36 was supported by the observation that a monoclonal antibody targeting CD36 inhibited DC maturation (52). Antibodies against CD51 (α_V integrin) and exposure to apoptotic cells, which interact with both CD36 and CD51, also modulated DC responses to maturation stimuli (52).

Not all variants of PfEMP1 adhere to CD36, and in pregnant women, infected RBCs that accumulate in the placenta often bind instead to the proteoglycan molecule, chondroitin sulfate A (CSA) (11). In this study, we compared a CSA-binding laboratory *P. falciparum* line, a CD36-binding line, and lines deficient in PfEMP1 expression for their ability to modulate DC function.

DC modulation by *P. falciparum* was not dependent upon interaction with CD36 or the presence of PfEMP1. CSA-binding iRBCs (CSA-iRBCs) and PfEMP1-deficient iRBCs inhib-

* Corresponding author. Present address: The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3050, Australia. Phone: (613) 9345 2555. Fax: (613) 9347 0852. E-mail: elliott@wehi.edu.au.

† Present address: Swiss Tropical Institute, Basel, Switzerland.

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ited the phenotypic and functional maturation response of DCs to LPS stimulation to the same extent as CD36-binding iRBCs (CD36-iRBCs). Further investigation showed separation of DCs and iRBCs in transwells could not prevent inhibition of DC maturation. DCs cultured in the presence of high-dose iRBCs were apoptotic, a possible reason for their failure to mature or respond to LPS. Inhibition and apoptosis of DCs were dependent on the dose of iRBCs, and at lower doses, functional DC maturation was induced by iRBCs without exogenous stimulation.

Taken together, these data indicate that interactions of human myeloid DCs with blood-stage *P. falciparum* are more complex than was previously understood and may result in a spectrum of outcomes from activation to inhibition of DC function.

MATERIALS AND METHODS

Ethical approval. Use of blood products from Australian blood donors in this project, including RBCs, buffy coats, and serum, was approved by the Melbourne Health Research Directorate Human Research Ethics Committee.

Parasite lines. ItG and FAF-EA8 (E8B) (derived by selection of ItG on human umbilical endothelial cells) (5, 6) both bind to CD36. CS2 was obtained by repeated selection of FAF-EA8 on Chinese hamster ovary cells and purified CSA (8). 3D7/upsC (derived from the laboratory strain 3D7) is transfected with the plasmid pHBupsC, containing the blasticidin deaminase and human dihydrofolate reductase drug resistance genes, as well as the UpsC promoter region (53). Growth of this transfectant in the presence of the antifolate drug WR99210 activates the episomal UpSC *var* promoter and silences endogenous *var* gene expression, resulting in an absence of PfEMP1 on the iRBC surface. In the skeleton binding protein 1 knockout (SBP-1 KO) line (derived from CS2), PfEMP1 transport to the RBC surface is disrupted (23).

Parasite culture. *P. falciparum* lines were maintained in continuous culture in human group O-positive RBCs (kindly supplied by the Australian Red Cross Blood Service) at 2 to 3% hematocrit in RPMI 1640 medium, supplemented with 25 mM HEPES, 50 μg/ml hypoxanthine, 24 mM $NaHCO_3$, 2.5 μg/ml gentamicin, and 10% (vol/vol) heat-inactivated pooled human serum (Australian Red Cross Blood Service) or 0.5% Albumax II (Gibco) (49). Transfectant lines were grown in the presence of drugs as follows: 3D7/upsC was grown with blasticidin S (2 μg/ml) in the presence or absence of the antifolate drug WR992210 (4 nM) (53), and SBP-1 KO was grown with WR992210 (4 nM). Gelatin enrichment of knob-expressing iRBCs (14) and sorbitol synchronization (19) were performed every 1 to 2 weeks. Parasite lines were regularly tested for *Mycoplasma* contamination by PCR. Lines were also periodically treated with mycoplasma removal agent (ICN) as an added precaution. The adhesion phenotypes of the *P. falciparum* lines ItG, FAF-EA8 (E8B), and CS2 were checked repeatedly throughout the course of the study and were stable in continuous culture. As previously described (2, 38), ItG and FAF-EA8 adhered to CD36, whereas CS2 bound CSA, but not CD36 (results not shown).

Characterization of adhesion phenotype. Adhesion assays were performed as described previously (4). Receptors were diluted in phosphate-buffered saline (PBS) and coated in triplicate spots on the base of 25-ml plastic petri dishes by overnight incubation at 4°C (CD36 purified from platelets [a gift from M. Berndt, Baker Institute, Australia] or CD36-immunoglobulin [R&D Systems] used at 15 to 20 μg/ml; CSA [from bovine trachea [Sigma] used at 20 to 100 μg/ml]). After the reaction was blocked for 30 min with PBS containing 1% bovine serum albumin or casein blocker (Pierce), the plates were washed with RPMI 1640 medium supplemented with 25 mM HEPES (RPMI-HEPES), and trophozoites were resuspended in adhesion medium (RPMI 1640 medium supplemented with 25 mM HEPES, 50 μg/ml hypoxanthine, 2.5 μg/ml gentamicin, and 10% pooled human serum). Thirty-five microliters of this suspension was added per receptor spot and incubated at 37°C for 45 min. Unbound cells were removed by washing with RPMI-HEPES. Bound cells were fixed by incubation in 2% glutaraldehyde in PBS, then stained with Giemsa, and counted.

Generation of DCs. Peripheral blood mononuclear cells were separated from buffy coats (kindly supplied by the Australian Red Cross Blood Service) by density centrifugation over Ficoll-Paque Plus (Pharmacia). Monocytes were isolated by positive selection (human CD14 microbeads; Miltenyi Biotec) (≥97% CD14 positive) or negative selection (StemSep human monocyte enrichment kit) (≥85% CD14 positive). Monocytes were cultured for 6 days at 5×10^5 cells/ml in RPMI 1640 medium (Invitrogen) supplemented with 1% GlutaMAX-I supplement (Invitrogen) and either 10% heat-inactivated pooled human serum (same ABO blood group as the buffy coat donor) (supplied by Australian Red Cross Blood Service), kanamycin (50 μg/ml), granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng/ml), and interleukin 4 (IL-4) (50 ng/ml) (Peprotech or R & D Systems) by the method of Urban et al. (50, 52) or 10% heat-inactivated fetal calf serum (JRH Biosciences), penicillin (100 U per ml) and streptomycin (100 μg/ml) (Invitrogen), and GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) (Peprotech) (as described previously [18]). Monocyte-derived DCs harvested at 6 days were nonadherent, CD14 low/negative, and CD11c positive with typical morphology (18, 40).

DC-*P. falciparum* coculture. Nonadherent immature monocyte-derived DCs were harvested and resuspended in the same conditioned medium at 5×10^5 cells/ml in 24-well plates (2 ml per well). In some experiments, DCs were resuspended in fresh DC medium containing GM-CSF (50 ng/ml) and IL-4 (50 ng/ml) with similar results. Percoll-enriched trophozoites (mean purity [standard error of the mean {SEM}], 93.5% [±1.3%]) or uninfected RBCs (cultured overnight in RPMI-HEPES with 10% human serum and passed through a Percoll gradient) were added at the iRBC/DC ratio of 10:1 or the RBC/DC ratio of 100:1. Lysates of RBCs or iRBCs generated by three or four rounds of freeze-thawing of known concentrations of uninfected RBCs or iRBCs isolated with Percoll gradients were added at equivalent cell concentrations. In some studies, cocultures were performed in transwells (6.5-mm diameter, polycarbonate membrane with 0.4-μm pore size [Corning Incorporated] with DCs in the lower chamber and iRBCs in the upper chamber [total volume, 1 ml]). Bacterial LPS (*Escherichia coli* 026:B26 or *Salmonella enterica* serovar Typhimurium [Sigma], 100 ng/ml or 1 μg/ml) was added to some cultures after 4 to 20 h. After a further 48 h, supernatants were collected and stored at −70°C, and DC phenotypes were assessed by immunofluorescence staining. Cytospins were performed on cells sampled at indicated times, fixed in methanol, and Giemsa stained. In separate experiments, uninfected RBCs or trophozoites were labeled with carboxyfluorescein succinimidyl ester (CFSE) (resuspended at 10^7 cells/ml in 5 μM CFSE [Invitrogen] in PBS, 10 min at 37°C), ethidium bromide (resuspended at 10^7 cells/ml in 20 μg/ml ethidium bromide in PBS, 20 min at room temperature), or fluorescein isothiocyanate (FITC) (resuspended at 20% hematocrit in 4 μg/ml FITC [Invitrogen] in RPMI-HEPES, 10 min at room temperature). Labeled cells were washed three times in RPMI-HEPES and cocultured with DCs at the indicated ratios as described above. After 4 h, RBCs were lysed (BD FACS lysing solution; Becton Dickinson), and DCs were washed and analyzed by flow cytometry.

Scanning electron microscopy. DCs cultured with iRBCs (100 iRBCs per DC) for 4 h were washed in PBS and then fixed for 1 hour with 2.5% glutaraldehyde (ProSciTech) in PBS. Cells were washed three times in PBS, and a drop of cell suspension was placed on a circular coverslip coated with 1% polyethylenimine (Sigma) for several minutes. Affixed cells were rinsed in distilled H_2O (dH_2O) and slowly dehydrated in a graded series of ethanol solutions. Samples in 100% ethanol were critical point dried using a Baltec CPD 030 and gold coated using an Edwards S150B sputter coater. Samples were observed at 20 kV with a Philips XL30 FEG field emission scanning electron microscope. Captured images were further processed using Photoshop.

Transmission electron microscopy. A semisimultaneous fixation protocol was used (47). DCs cultured with iRBCs (100 iRBCs per DC) for 4 h were washed in RPMI 1640 medium and then fixed for 5 to 10 seconds with 0.5% glutaraldehyde (ProSciTech) in RPMI 1640 medium; about 10 seconds later, an equal volume of 1% OsO_4 in RPMI 1640 medium was added for 20 min. Samples were washed three times in RPMI 1640 medium with dH_2O (first wash with 2:1 RPMI:dH_2O, second wash with 1:1 RPMI:dH_2O, and third wash with pure dH_2O) and then stained overnight at 4°C with 2% aqueous uranyl acetate (ProSciTech) in dH_2O. Samples were slowly dehydrated in a graded ethanol series followed by three final exchanges of 100% ethanol. Following dehydration, samples were infiltrated with increasing concentrations of Spurr's resin up to 100% including three final changes of 100% resin. Samples were flat embedded on polytetrafluoroethylene-coated coverslips (37) and polymerized overnight at 70°C. Cells were selected by using a light microscope and mounted for sectioning. Sections (90 nm) were cut using a Leica Ultracut R ultramicrotome (Leica Microsystems) and Diatome diamond knife for collection onto single-slot pioloform (Alltech)-coated copper grids (ProSciTech). Sections were poststained with 2% aqueous uranyl acetate for 10 min, washed three times in dH_2O, and stained using a triple-lead solution for 5 min. Samples were examined using a Philips BioTwin CM 120 transmission electron microscope (Philips), and images were captured with a Gatan 791 multiscan camera. Images were further processed using Photoshop.

Immunofluorescence staining and flow cytometry. The phenotype of DCs was characterized using the following monoclonal antibodies: CD14-FITC, clone

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MØP9; CD11c-phycoerythrin (PE)-Cy5, clone B-ly6; CD80-PE, clone L307.4; CD86-PE, clone IT2.2; CD83-PE, clone HB15e; CD40-PE, clone 5C3; HLA-DR-PE or HLA-DR-PE-Cy5, clone TÜ36; CD36-PE, clone CB38 (NL07) and appropriate isotype controls (Becton Dickinson/Pharmingen). Cells were harvested, washed (cold PBS with 1 mM EDTA and 1% fetal bovine serum) and stained with appropriate monoclonal antibodies for 30 min on ice. RBCs were lysed (BD FACS lysing solution), and DCs were washed and analyzed on a Becton Dickinson FACSCalibur using Cell Quest software. Cell death was quantified by staining unlysed cells with Alexa Fluor 488-annexin V and propidium iodide using Vybrant apoptosis assay kit 2 (Invitrogen) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assays (ELISAs). Supernatants collected at 24 and 48 h were assayed for concentrations of IL12p70 and IL-10 according to the manufacturer's instructions (Mabtech). Tetramethylbenzidine substrate reagent set (Becton Dickinson) or 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) was used as substrate.

T-cell proliferation assays. Total $CD3^+$ T cells were isolated from cryopreserved allogeneic or autologous peripheral blood lymphocytes by negative selection using the Miltenyi Biotec pan T-cell isolation kit II according to the manufacturer's instructions (≥97% CD3 positive). For allogeneic T-cell stimulation, DCs were cultured alone or with uninfected RBCs, CD36-iRBCs, or CSA-iRBCs for 20 h and then cultured for an additional 48 h in the presence or absence of LPS (100 ng/ml) as described above. For autologous T-cell stimulation, DCs were cultured alone or with uninfected RBCs or CD36-binding iRBCs for 20 h with no exogenous stimulation. After centrifugation over Ficoll-Paque Plus (Pharmacia) to remove RBCs and parasite debris, DCs were γ-irradiated (3,000 rads), added to T cells at the ratios indicated (see Fig. 3 and 9) in triplicate wells (10^5 T cells per well, round-bottom 96-well plates), and incubated for 5 days. For the final 18 h, 0.5 μCi [^{3}H]thymidine (GE Health Care) was added per well.

Statistical analyses. Student's paired *t* tests and one-way analysis of variance were used for comparisons of log-transformed data. Analyses were performed with the software package GraphPad Prism.

RESULTS

CD36 expression on monocyte-derived DCs. We examined expression of CD36 on immature monocyte-derived DCs and found that a sizeable proportion (mean ± SEM, 40.8% ± 4.5% [$n = 10$]) did not express CD36 above the background level. The percentage of DCs expressing CD36 did not increase during 3 days of culture, and in the presence of LPS, there was a modest reduction in the percentage of CD36-positive cells. This observation was consistent with previous reports (1, 57) and suggested that receptors other than CD36 might mediate interactions of DCs with iRBCs.

Interaction of CD36- and CSA-binding *P. falciparum* with immature DCs. DCs cultured with uninfected RBCs, CD36-iRBCs, or CSA-iRBCs were examined by light microscopy and scanning and transmission electron microscopy. After 4 h of culture, uninfected RBCs and intact iRBCs of both the CD36- and CSA-binding *P. falciparum* lines were observed in close apposition with DCs (Fig. 1A and B). iRBCs and pigment granules were observed in the cytoplasm of DCs cocultured with either *P. falciparum* line, indicating phagocytic uptake of iRBCs. The majority of iRBCs were phagocytosed or underwent schizogony during coculture. While some iRBCs died (culture conditions being optimal for DCs rather than iRBCs), viable iRBCs matured, and rupture of schizonts within cocultures was observed by real-time microscopy (data not shown). Light microscopy revealed loss of ≥90% of iRBCs by 48 h.

To quantify DC interactions with iRBCs, CFSE-labeled uninfected RBCs, CD36-iRBCs, or CSA-iRBCs were cocultured with DCs for 4 h, RBCs were lysed, and DCs were analyzed for CFSE signal (Fig. 1C). iRBCs of both the CD36- and CSA-binding lines were taken up by DCs in a dose-dependent manner. Even though uninfected RBCs stained less brightly than

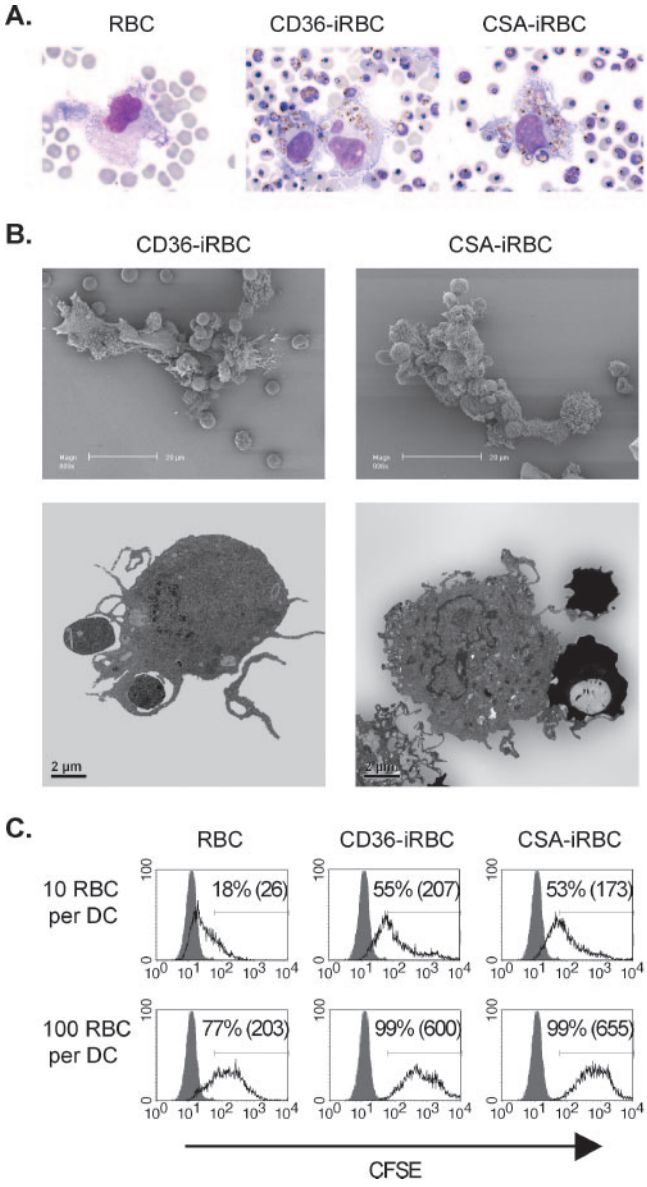

FIG. 1. CD36-iRBCs and CSA-iRBCs interacted with and were phagocytosed by DCs. Uninfected RBCs, CD36-iRBCs, or CSA-iRBCs were cultured with monocyte-derived DCs for 4 h. (A) Cytospins (Giemsa stained, viewed with a 100× objective). (B) Scanning and transmission electron microscope images. (C) Phagocytic uptake of CFSE-labeled uninfected RBCs, CD36-iRBCs, or CSA-iRBCs by DCs (black line) (DCs cultured alone are shown in gray). After 4 h of coculture, RBCs were lysed, and fluorescence-labeled DCs were quantified by flow cytometry (percentage positive and geometric mean fluorescence intensity are indicated on each plot). These data are representative of the data from four similar experiments.

iRBCs with CFSE (results not shown), it was also evident that uninfected RBCs were taken up by DCs in a dose-dependent manner (Fig. 1C). There was no consistent difference in uptake of CD36- and CSA-iRBCs. Similar results were found using ethidium bromide or FITC to stain iRBCs (results not shown). These data demonstrate that monocyte-derived DCs express a receptor(s) that binds antigens on the surfaces of intact CSA-

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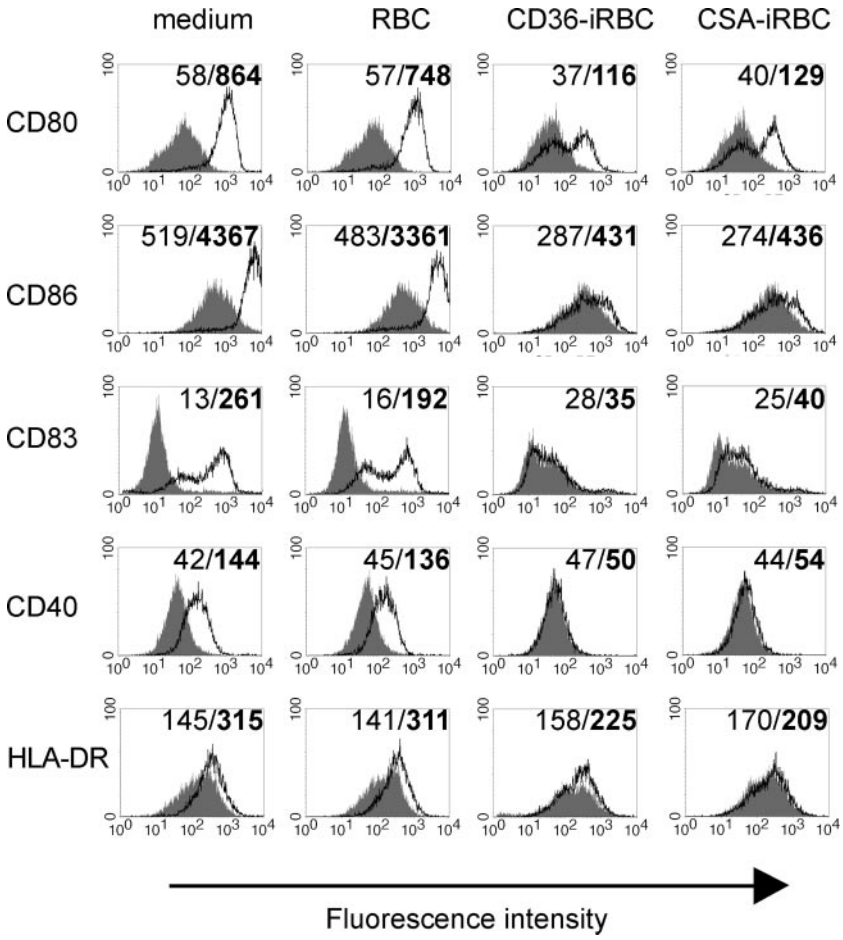

FIG. 2. LPS-induced phenotypic maturation of DCs was inhibited by CD36-iRBCs and CSA-iRBCs. DCs were cultured alone or with uninfected RBCs, CD36-iRBCs, or CSA-iRBCs (100 RBCs per DC) for 20 h, followed by 48 h of incubation in the presence or absence of LPS (100 ng/ml). The expression levels of maturation markers (geometric mean fluorescence intensity values) are indicated in each plot for unstimulated (gray-shaded histograms) and LPS-stimulated (black-outlined histograms) DCs, in regular font/boldface, respectively. All markers were expressed at significantly lower levels on LPS-stimulated DCs cultured with CD36-iRBCs or CSA-iRBCs compared to DCs cultured alone or with uninfected RBCs ($P < 0.01$ to $P < 0.0001$). These data are representative of the data from nine similar experiments.

iRBCs and mediate their phagocytic uptake independently of CD36.

Inhibition of LPS-induced phenotypic DC maturation by CD36- and CSA-binding *P. falciparum*. As reported previously (50), LPS-induced upregulation of CD80, CD86, CD83, CD40, and HLA-DR was significantly inhibited by prior exposure of DCs to high doses (100 iRBCs per DC) of CD36-iRBCs ($P < 0.0001$ for CD80 and CD83 [$n = 9$], $P < 0.001$ for CD86 [$n = 9$] and CD40 [$n = 7$], and $P < 0.01$ for HLA-DR [$n = 9$]) compared with unexposed LPS-stimulated DCs) (Fig. 2). CSA-iRBCs showed a similar capacity to inhibit LPS-induced phenotypic maturation of DCs ($P < 0.0001$ for CD80, CD86, and CD83, $P < 0.001$ for CD40, and $P < 0.01$ for HLA-DR compared with unexposed LPS-stimulated DCs) (Fig. 2). Inhibition by CSA-iRBCs was not significantly different from inhibition in the presence of CD36-iRBCs for any of the markers examined. We also observed modest but significant reductions in expression of CD80, CD86, and CD83 (but not CD40 or HLA-DR) in the presence of uninfected RBCs ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively); however, these markers were still expressed at significantly higher levels than on DCs cultured with CD36- or CSA-iRBCs.

To ensure comparability between studies and to exclude the possibility of protocol-dependent effects, monocyte-derived DCs were generated by either of two commonly used protocols (see Materials and Methods). DCs derived from monocytes by either method were inhibited in the presence of both CD36- and CSA-iRBCs at 100 iRBCs per DC. Inhibition of monocyte-derived DCs by *P. falciparum* iRBCs was observed regardless of whether positive or negative selection was used to isolate CD14-positive monocytes. Inhibition of phenotypic DC maturation occurred after coculture with iRBCs for either 4 or 20 h prior to the addition of LPS (results not shown).

Inhibition of LPS-induced DC cytokine secretion by CD36- and CSA-binding *P. falciparum*. LPS-induced IL12p70 production measured in supernatants collected after 48 h was significantly inhibited by both CD36- and CSA-iRBCs ($P < 0.01$ for each, compared to LPS stimulation alone, $n = 7$; 100 iRBCs per DC), but not uninfected RBCs ($P = 0.074$) (Fig. 3A). LPS-induced IL-10 production was also significantly inhibited

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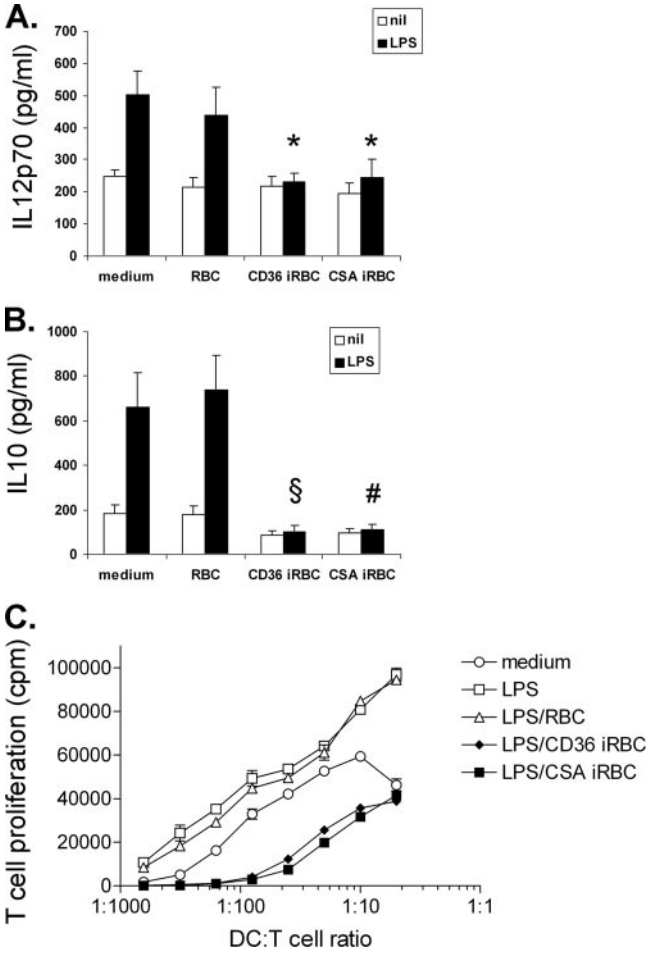

FIG. 3. Functional impairment of DCs cultured with CD36- and CSA-binding iRBCs. DCs were cultured alone or with uninfected RBCs, CD36-iRBCs, or CSA-iRBCs (100 RBCs per DC) for 20 h, followed by 48 h of incubation in the presence or absence (nil) of LPS (100 ng/ml). (A and B) Levels of IL12p70 (A) and IL-10 (B) were measured by ELISAs in supernatants collected at the end of this incubation. Data represent means plus SEMs (error bars) for seven experiments. Values that were significantly different from the value for LPS-stimulated DCs cultured alone are indicated as follows: *, $P < 0.01$; §, $P < 0.001$; #, $P < 0.0001$. (C) DCs exposed to CD36- or CSA-iRBCs were poor stimulators of T cells. DCs were cultured as described above. After removal of RBCs and parasite debris by density centrifugation, viable DCs were irradiated and added to allogeneic T cells at the indicated ratios. Proliferation was measured by uptake of tritiated thymidine after 5 days. The means ± SEMs (error bars) of triplicate wells are shown. T-cell proliferation was significantly lower in the presence of LPS-stimulated DCs cultured with CD36- or CSA-iRBCs compared to unexposed LPS-stimulated DCs ($P < 0.05$ to $P < 0.0001$ for all ratios). Data are representative of the data from two similar experiments.

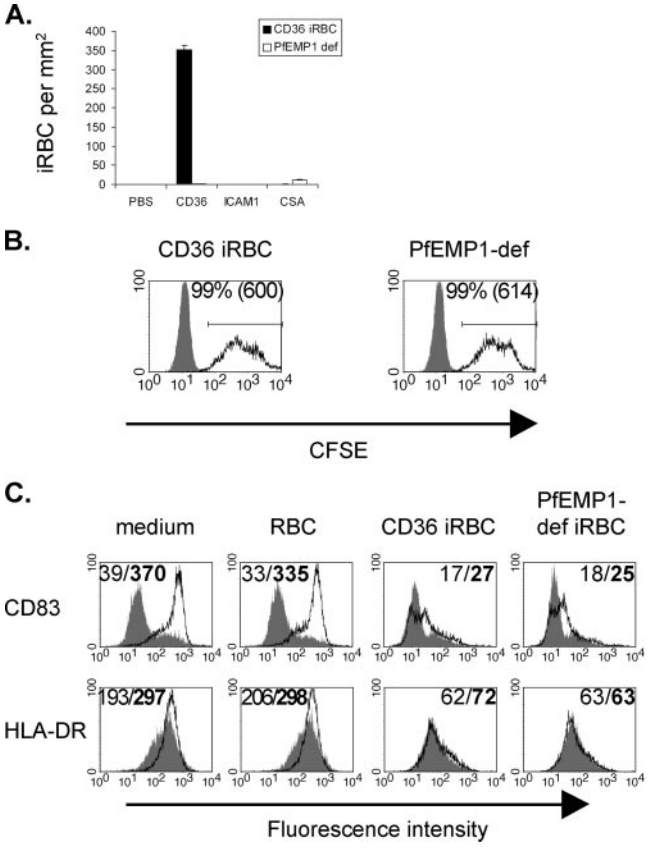

FIG. 4. PfEMP1-deficient iRBCs were phagocytosed by DCs and inhibited LPS-induced phenotypic maturation. (A) Adhesion phenotypes of the *P. falciparum* Ituxi lines, ItG (CD36-iRBCs), and the SBP-1 KO line (targeted disruption of SBP-1 prevents expression of PfEMP1 on the iRBC surface [23]). This representative experiment shows the mean number (± SEM) of iRBCs bound per mm^2, adjusted to 2% parasitemia. def, deficient; ICAM1, intercellular adhesion molecule 1. (B) Phagocytic uptake of CFSE-labeled PfEMP1-deficient iRBCs by DCs (100 iRBCs per DC) at 4 h was similar to uptake of CD36-iRBCs (representative of two similar experiments). (C) Inhibition of phenotypic DC maturation by PfEMP1-deficient iRBCs and CD36-iRBCs was comparable. DCs cultured alone or with uninfected RBCs, CD36-iRBCs, or PfEMP1-deficient iRBCs (100 RBCs per DC) were stimulated with LPS (100 ng/ml) at 20 h, and their phenotype was assessed after an additional 48 h. These data are representative of the data from four similar experiments. The expression levels (geometric mean fluorescence intensity values) of maturation markers are indicated in each plot for unstimulated (grey-shaded histograms) and LPS-stimulated (black-outlined histograms) DCs, in regular font/boldface, respectively.

by both CD36 and CSA-binding *P. falciparum* lines ($P < 0.001$ and $P < 0.0001$, respectively [$n = 7$]; uninfected RBCs [$P = 0.18$]) (Fig. 3B). Similar results were obtained for supernatants collected from DCs cultured for 24 h in the presence or absence of LPS (results not shown).

Functional impairment of DCs cultured with CD36- and CSA-binding *P. falciparum*. DCs cultured with high doses (100 iRBCs per DC) of either CD36- or CSA-iRBCs and subsequently matured with LPS were poor stimulators of allogeneic T-cell responses (Fig. 3C). Levels of T-cell proliferation were significantly lower in the presence of LPS-stimulated DCs cultured with CD36- or CSA-iRBCs compared to unexposed LPS-stimulated DCs ($P < 0.05$ to $P < 0.0001$ for all ratios of DCs to T cells in two similar experiments). In contrast, uninfected RBCs did not significantly inhibit allogeneic T-cell proliferation induced by LPS-matured DCs. T-cell stimulatory activity was reduced to a comparable degree for DCs exposed to CD36- or CSA-iRBCs.

Inhibition of phenotypic DC maturation by PfEMP1-deficient *P. falciparum* lines. Under the same conditions, DCs were

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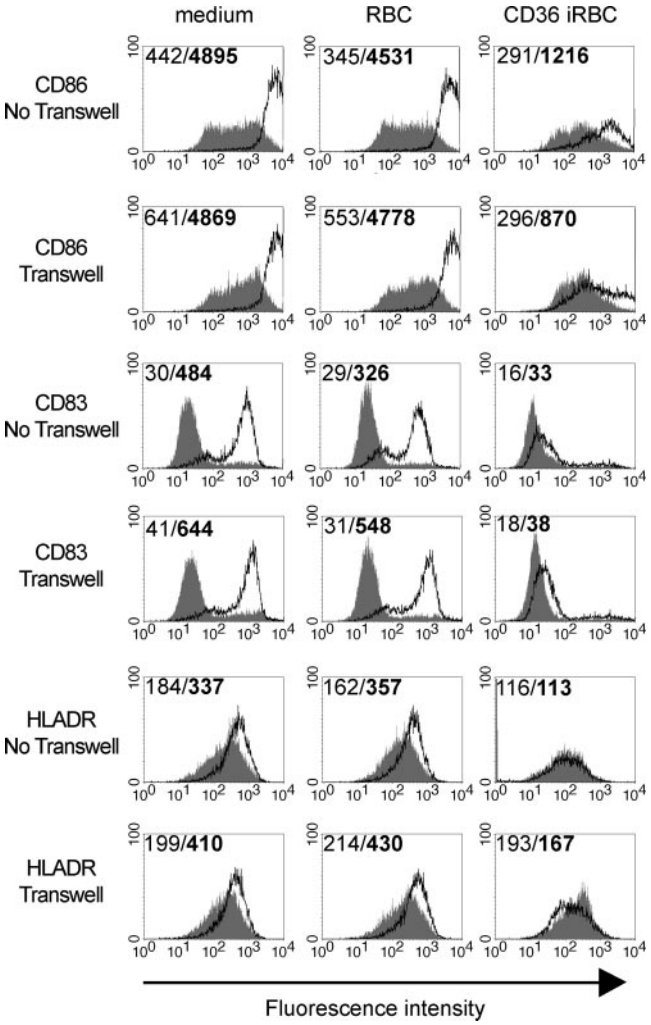

FIG. 5. iRBCs inhibited LPS-induced DC maturation across a transwell membrane. DCs were cultured in medium alone or with uninfected RBCs or CD36-iRBCs (100 RBCs per DC) either in the same well or separated from RBCs/iRBCs in transwells (0.4-μm membrane). At 20 h, DCs were stimulated with LPS (100 ng/ml or 1 μg/ml), and their phenotype was assessed after an additional 48 h. Data are representative of the data from three similar experiments. The expression levels (geometric mean fluorescence intensity values) of maturation markers are indicated in each plot for unstimulated (grey-shaded histograms) and LPS-stimulated (black-outlined histograms) DCs, in regular font/boldface, respectively.

cultured with *P. falciparum* lines deficient in PfEMP1 expression, either due to disrupted expression of SBP-1 leading to abrogation of PfEMP1 transport to the RBC surface (ItG background) (23) (Fig. 4A) or to silenced transcription of *var* genes encoding PfEMP1 expression (3D7 background) (53) (results not shown). Phagocytic uptake of CFSE-labeled PfEMP1-deficient iRBCs by DCs was comparable to that of CD36-iRBCs (Fig. 4B). At 100 iRBCs per DC, PfEMP1-deficient lines and CD-36 iRBCs inhibited LPS-induced phenotypic maturation of DCs to similar degrees (Fig. 4C).

Inhibition of phenotypic DC maturation by iRBCs across a transwell membrane. In three independent experiments, LPS-induced upregulation of CD86, CD83, and HLA-DR was inhibited on DCs exposed to CD36-iRBCs (at 100 iRBCs per DC), regardless of whether or not DCs and iRBCs were separated in transwells during coculture (Fig. 5). Levels of expression of CD86, CD83, or HLA-DR on LPS-stimulated DCs cultured with iRBCs were similar to levels on LPS-stimulated DCs separated from iRBCs in transwells.

LPS-induced phenotypic DC maturation in the presence of iRBC lysate. DCs incubated with CD36-iRBC lysate (equivalent to 100 iRBCs per DC) accumulated pigment in their cytoplasm (results not shown) without associated inhibition of LPS-induced maturation (Fig. 6). In fact, lysate alone induced partial phenotypic maturation, stimulating upregulation of CD86 but not CD83 or HLA-DR (Fig. 6). Similar results were obtained with CSA-iRBC lysate (results not shown). Consistent with our observations, previous studies have shown that iRBC lysate does not interfere with LPS-induced DC maturation (50). Taken together, these data suggest that inhibition of DC maturation in this system is not caused by *P. falciparum* pigment.

Dose-dependent inhibition of LPS-induced DC maturation by iRBCs. The inhibitory effect of *P. falciparum* on LPS-induced DC maturation has been reported for ratios of iRBCs to DCs between 10:1 and 100:1 (50). However, in our experiments, exposure of DCs to *P. falciparum* at 10 iRBCs per DC had no effect on the subsequent ability of DCs to mature and secrete IL12p70 in response to LPS stimulation (Fig. 7A and B). These data indicate that inhibition of LPS-stimulated DC maturation by *P. falciparum* is dose dependent. DCs exposed to low doses of iRBCs showed accumulation of *P. falciparum* pigment (results not shown) but normal LPS-induced phenotypic maturation, providing further evidence that *P. falciparum* pigment is unlikely to be the mechanism responsible for inhibition.

Cell death associated with exposure to high doses of iRBCs. DCs cultured with high doses of iRBCs were stained with annexin V-Alexa Fluor 488 and propidium iodide to assess viability. In the absence of LPS stimulation, there was a significant increase in the proportion of apoptotic DCs (annexin positive, propidium iodide negative) in cultures with high-dose CD36-iRBCs ($P < 0.001$ compared to DCs alone [$n = 5$]), but not uninfected RBCs or low-dose iRBCs (Fig. 8). Percentages of apoptotic cells were also significantly increased in LPS-stimulated cultures exposed to high-dose iRBCs (results not shown). There was a trend for higher proportions of necrotic (propidium iodide-positive) DCs in cultures with high-dose iRBCs, although this was variable and not statistically significant. Comparable observations were made with high-dose PfEMP1-deficient iRBCs (Fig. 8) and CSA-iRBCs (results not shown). These data suggested that cell death might account for the failure of DCs exposed to high-dose iRBCs to respond to LPS stimulation.

Dose-dependent activation of DCs by iRBCs. To investigate whether both tolerance to LPS stimulation and apoptosis in the presence of high-dose iRBCs might be explained by DC activation and subsequent exhaustion, we conducted a time course experiment. In two independent experiments, there was no evidence of parasite-induced phenotypic DC maturation in the presence of 100 iRBCs per DC at time points prior to addition of LPS (6 h and 20 h) (Fig. 9A), suggesting activation-

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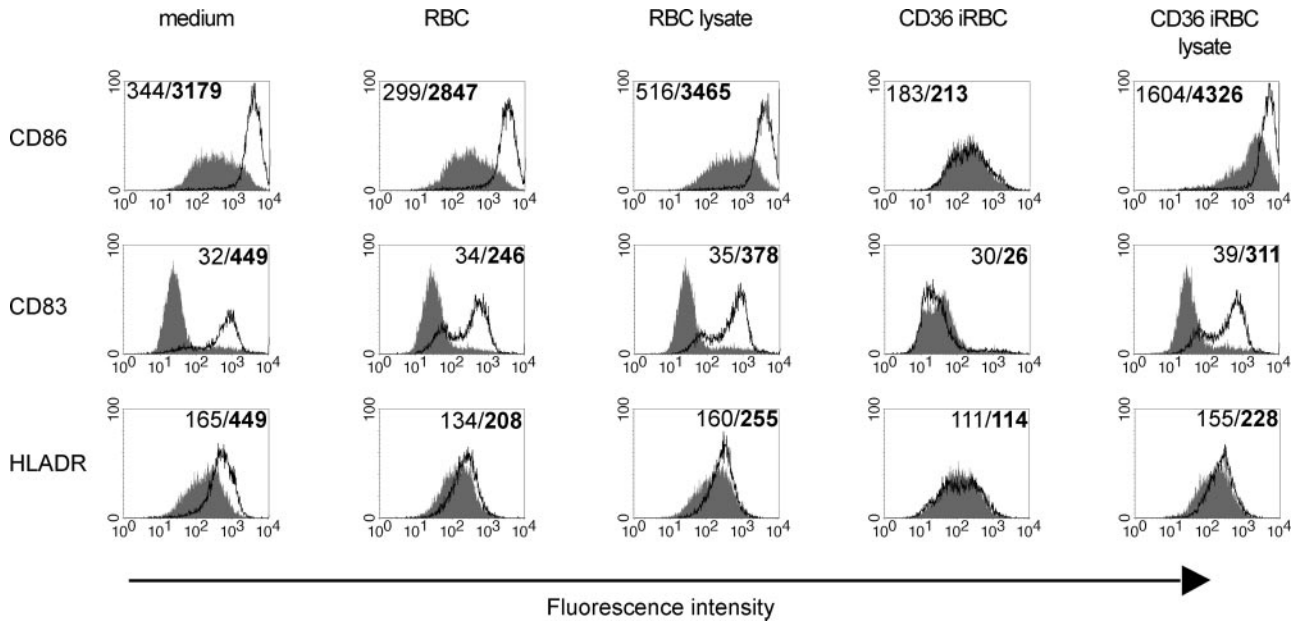

FIG. 6. iRBC lysate did not inhibit LPS-induced DC maturation, and alone, it induced partial DC maturation. DCs were cultured alone or with uninfected RBCs or CD36-iRBCs (100 RBCs per DC) or with the lysate of RBCs or iRBCs (equivalent to 100 RBCs per DC) for 20 h, followed by 48 h of incubation in the presence or absence of LPS (1 μg/ml). The expression levels (geometric mean fluorescence intensity values) of maturation markers are indicated in each plot for unstimulated (grey-shaded histograms) and LPS-stimulated (black-outlined histograms) DCs, in regular font/boldface, respectively. These data are representative of the data from three similar experiments.

induced DC exhaustion was not the reason DCs failed to respond to LPS.

In contrast, modest upregulation of maturation markers was observed in the presence of 10 iRBCs per DC by 20 h (Fig. 9A). Similar results were obtained with CSA-iRBCs (results not shown).

To investigate whether DCs activated by low-dose iRBCs could stimulate T cells, DCs were harvested after 20 h culture alone or with uninfected RBCs or CD36-iRBCs (10 RBCs/iRBCs per DC) and added to autologous T cells at the indicated ratios. DCs cultured with low-dose iRBCs induced proliferation of autologous T cells (Fig. 9B).

DISCUSSION

In this study, intact iRBCs interacted closely with DCs and were phagocytosed to a similar extent, regardless of the level or variant of PfEMP1 expressed. This finding demonstrates that interaction of PfEMP1 with CD36 is not necessary for phagocytic uptake of iRBCs by DCs. In contrast, nonopsonic phagocytosis of CSA-iRBCs by rodent and human macrophages is significantly reduced compared to phagocytosis of CD36-iRBCs (42).

Our observations that high-dose CD36-iRBCs (100 iRBCs per DC) inhibited LPS-induced upregulation of maturation markers (CD80, CD86, CD83, CD40, and HLA-DR), IL12p70 secretion, and the capacity of DCs to stimulate allogeneic T-cell responses are similar to those of Urban et al. (50, 52). It was postulated that inhibition of DC maturation is mediated by interaction between CD36 and the parasite ligand PfEMP1 (50–52). However, we found that iRBCs expressing a CSA-adherent PfEMP1, VAR2CSA (9), which does not bind to CD36 and is structurally distinct from other PfEMP1 molecules (39), inhibited DC responses to LPS stimulation to an extent similar to that observed with CD36-iRBCs. These observations suggested that the mechanism of DC inhibition by iRBCs might be independent of PfEMP1 and CD36. Phenotypic maturation of the whole DC population was inhibited by exposure to iRBCs, despite the fact that a large subpopulation of monocyte-derived DCs did not express CD36, also suggesting that alternative mechanisms are responsible for inhibition.

There was a modest reduction in the levels of phenotypic maturation markers on DCs stimulated with LPS in the presence of high-dose uninfected RBCs, although this was much less marked than the inhibition observed in the presence of iRBCs. This might be explained by the large numbers of RBCs preventing close apposition between DCs and interfering with paracrine signaling required for optimal response to LPS (13), a factor that could also contribute to the effect of iRBCs on DC maturation.

To further explore the role of PfEMP1 in inhibition of DCs by iRBCs, we used two different transfected *P. falciparum* lines in which PfEMP1 expression was specifically disrupted (23, 53; see Materials and Methods). Inhibition of LPS-induced DC maturation was comparable in wild-type and PfEMP1-deficient lines, indicating that inhibition of DC maturation by high-dose iRBCs is not PfEMP mediated.

To investigate whether other surface receptor-ligand interactions might mediate inhibition of DC maturation, we cultured DCs and high-dose iRBCs in transwells. iRBCs inhibited LPS-induced DC maturation even when separated from DCs by a 0.4-μm membrane, indicating that the phenomenon is not contact mediated and excluding a role for erythrocyte

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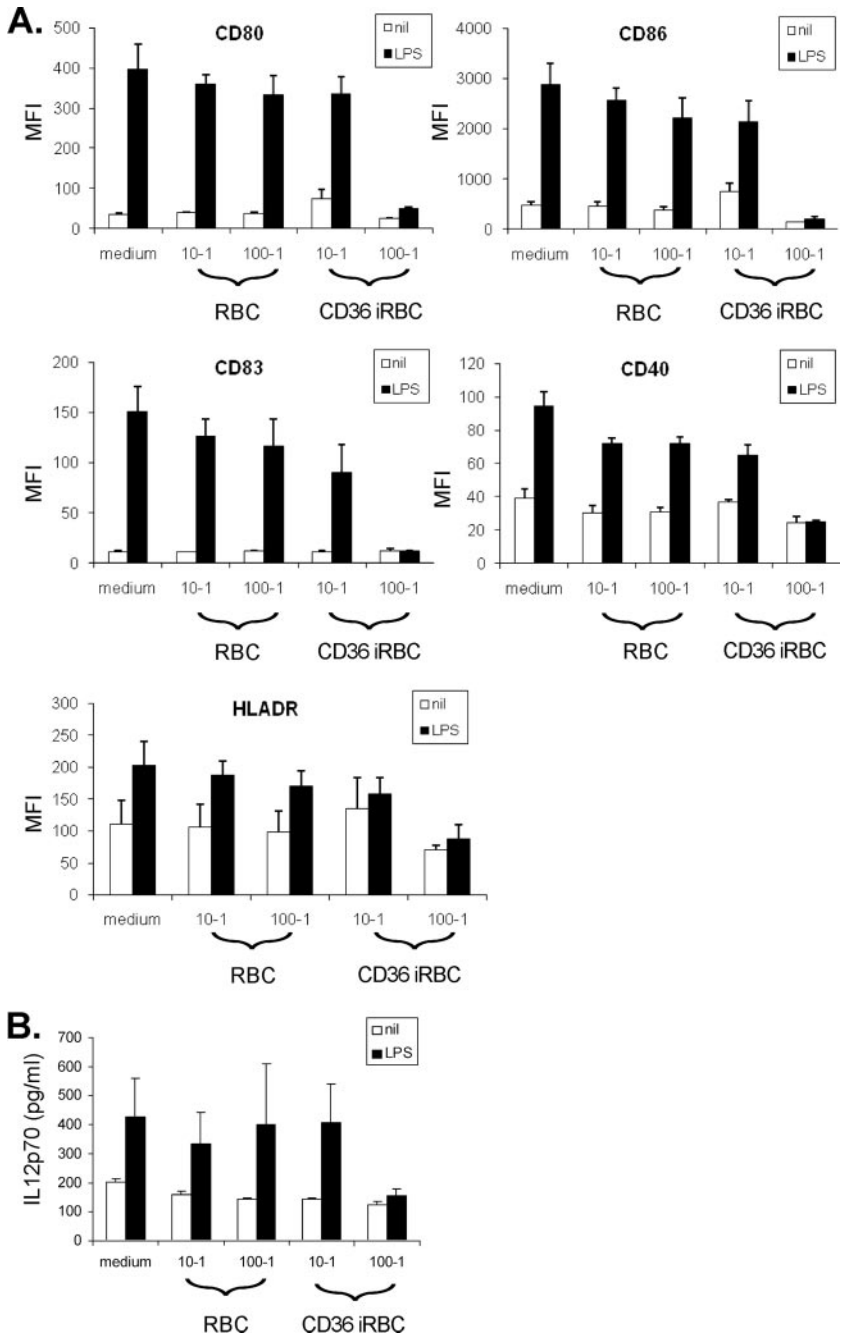

FIG. 7. Dose-dependent inhibition of DC maturation by iRBCs. (A) DCs were cultured alone or with uninfected RBCs or CD36-iRBCs (10 RBCs or 100 RBCs per DC) for 20 h, followed by 48 h of incubation in the presence or absence (nil) of LPS (100 ng/ml). The geometric mean fluorescence intensity (MFI) of expression of maturation markers is indicated. Data represent means plus SEMs (error bars) for three experiments. (B) Levels of IL12p70 were measured by ELISAs in supernatants collected from the above experiments 48 h after addition of LPS. Means plus SEMs (error bars) are shown for three experiments.

proteins or interaction of nonimmune immunoglobulin with Fc receptors.

We observed no inhibition of DCs by iRBC lysate, supporting previous observations in this system (50). Preparations containing the *P. falciparum* pigment hemozoin have been shown to inhibit LPS-induced DC maturation by others (24, 43). However, in the present study, *P. falciparum* pigment was observed to accumulate in the cytoplasm of DCs cultured with lysate without affecting their response to LPS stimulation. In our study, lysate actually induced upregulation of CD86 expression by DCs. DCs cultured with lower ratios of iRBCs also contained pigment granules but responded appropriately to LPS. In contrast, DCs separated from iRBCs in transwells contained no visible pigment but failed to mature in response to LPS. Taken together, these observations suggest that hemozoin is unlikely to be responsible for inhibition of DCs exposed to iRBCs in this system.

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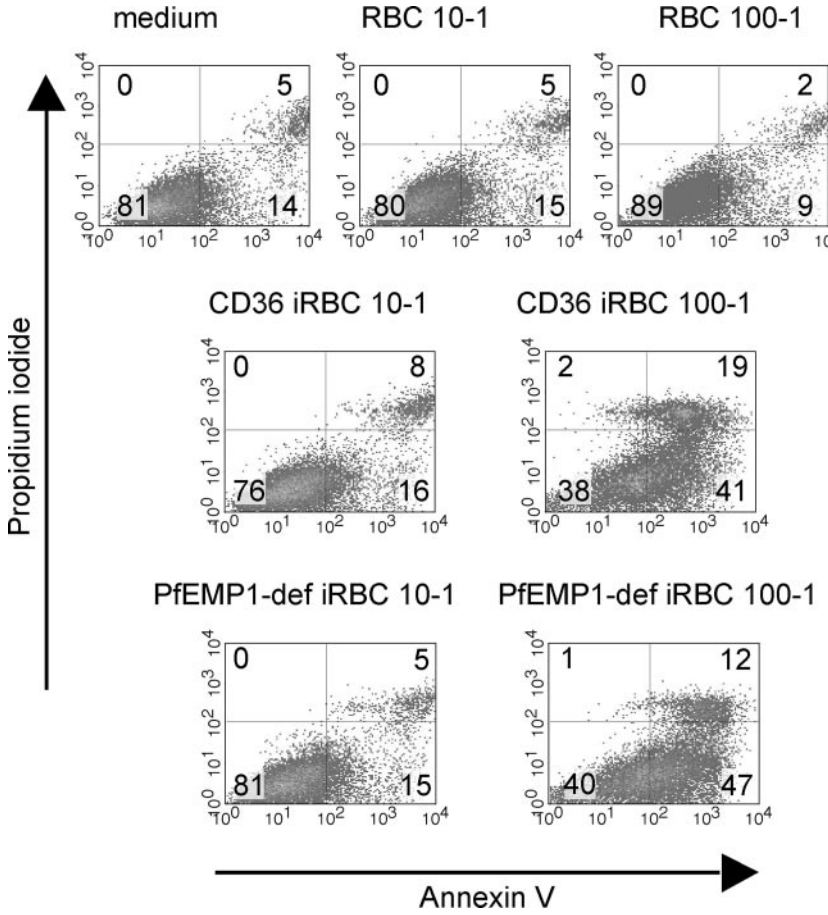

FIG. 8. Dose-dependent apoptosis of DCs cultured with iRBCs. DCs were cultured alone or with uninfected RBCs, CD36-iRBCs, or PfEMP1-deficient iRBCs (10 or 100 RBCs per DC) for 20 h, followed by 48 h in the presence or absence of LPS (100 ng/ml). DCs were harvested and stained with Alexa Fluor 488-annexin V and propidium iodide. Density plots of unstimulated DCs are shown (results for LPS-stimulated DCs were similar). These data are representative of the data from five similar experiments for CD36 iRBCs and two for PfEMP1-deficient (PfEMP1-def) iRBCs.

Inhibition of DC maturation by iRBCs was shown to be dose dependent. While inhibition was observed at ratios of 100 iRBCs per DC, lower ratios of iRBCs to DCs (10 iRBCs per DC) did not affect LPS-induced maturation of DCs. Compatible with the observed dose-related inhibition of the DC maturation response, we found an increased proportion of apoptotic DCs in cultures with high-dose, but not low-dose, iRBCs. It has been suggested that activation of DCs by iRBCs might trigger negative-feedback mechanisms that make them unresponsive to subsequent maturation stimuli (21). Cytokine production by DCs is known to be transient and refractory to further stimulation (20, 36). Although DC exhaustion was a plausible explanation for tolerance of malaria-exposed DCs to LPS stimulation, and possibly also for apoptosis, we found no evidence of activation of DCs by high-dose iRBCs.

Conceivably, a soluble factor produced by live iRBCs might be responsible for dose-dependent inhibition and apoptosis of DCs. iRBCs have been shown to cause apoptosis of B cells, T cells, macrophages, and endothelial cells in human in vitro studies and in animal malaria models (10, 15, 16, 33, 48, 55, 56), and a recent study found that the majority of $CD8^+$ splenic DCs harvested from *Plasmodium chabaudi*-infected mice were apoptotic (45). Apoptosis of DC subsets could interfere with developing antimalarial immune responses, and further studies are required to establish the physiological relevance of our observation and elucidate the mechanism. Given that there was DC apoptosis in the presence of iRBCs expressing two structurally distinct PfEMP1 variants, as well as PfEMP1-deficient iRBCs, a role for PfEMP1 in this process seems unlikely.

The possibility that apoptosis of DCs in the presence of large numbers of iRBCs might be attributable to exhaustion of the culture medium should also be considered. The concentration of iRBCs used in the original reports (10^8 purified iRBCs per 2-ml culture for 100 iRBCs per DC) (50, 52) and reproduced for the purpose of this study is much higher than would be sustainable in standard *P. falciparum* culture. This concentration of iRBCs might also be higher than occurs in vivo; however, it is difficult to establish what would be a physiological ratio of iRBCs to DCs in the spleen, where iRBCs sequester and therefore might be highly concentrated.

In this study, human monocyte-derived DCs showed a modest increase in expression of maturation markers in the presence of low-dose iRBCs and were subsequently able to stimulate

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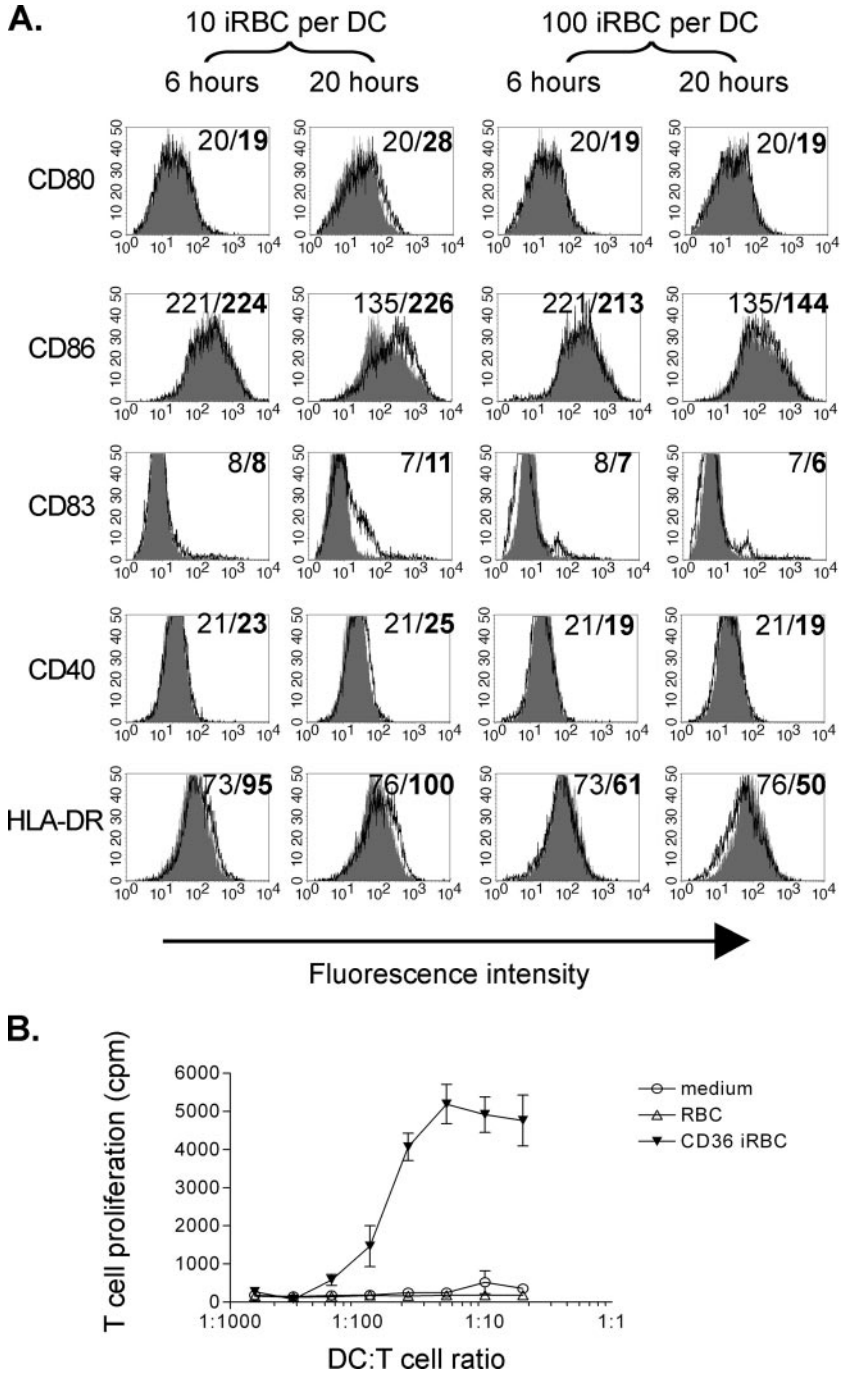

FIG. 9. Dose-dependent activation of DCs by iRBCs in the absence of exogenous stimulation. (A) DCs were cultured alone or with CD36-iRBCs (10 or 100 iRBCs per DC). DCs were harvested, and their phenotype was assessed at 6 h and 20 h. The geometric mean fluorescence intensity values of maturation marker expression are indicated on each plot for DCs cultured alone (gray histograms) or in the presence of iRBCs (black-outlined histograms) in regular font/boldface, respectively. These data are representative of the data from two similar experiments. (B) T-cell proliferation induced by DCs cultured with low-dose iRBCs. DCs were cultured alone, with uninfected RBCs or CD36-iRBCs (10 RBC per DC) for 20 h. After removal of RBCs and parasite debris by density centrifugation, viable DCs were irradiated and added to autologous T cells at the indicated ratios. Proliferation was measured by uptake of tritiated thymidine after 5 days. Means ± SEMs (error bars) of triplicate wells are shown. These data are representative of the data from two similar experiments.

proliferation of autologous T cells, consistent with activation of a primary antigen-specific T-cell response. These observations support those of a recent study reporting phenotypic maturation of peripheral blood myeloid DCs following short-term stimulation of peripheral blood mononuclear cells with live iRBCs (27). Another study showed that the CD36-binding cysteine-rich interdomain region 1α of PfEMP1 stimulates human peripheral blood myeloid DCs to secrete IL12p70, IL-10,

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and IL-18 (26). iRBCs have also been found to activate human plasmacytoid DCs (32), possibly through the action of hemozoin (7), which appears to target *P. falciparum* DNA to intracellular Toll-like receptor 9 receptors (30). Although data from animal models of malaria have been conflicting, there are several reports of activation of DCs during *Plasmodium* infection or after in vitro exposure to iRBCs (17, 22, 31, 41, 45, 54). Differences in *Plasmodium* species, DC subsets, or experimental methodology may account for apparent *Plasmodium*-induced DC suppression in some studies and activation in others (reviewed in references 21 and 46); however, our data suggest that parasite dose is one important factor regulating DC responses.

A further consideration is that many in vitro studies of DC responses to blood-stage *Plasmodium* have used purified DCs. There is increasing evidence that interactions between DCs and other cells of the innate immune system, including natural killer cells, natural killer T cells, and gamma delta T cells, are important for DC maturation (reviewed in reference 25). A recent study suggests that NK cells are required to maintain DC maturation status but are not necessary for the response to iRBCs (27); however, the role of other cell types in regulating DC responses to malaria has not been investigated. Further studies are now required to establish the optimal conditions and define the mechanisms of activation of human DCs by *P. falciparum*.

In conclusion, we found that phagocytosis of iRBCs by DCs was not affected by their adhesion phenotype or level of expression of PfEMP1. Second, we showed a dose-dependent effect of live intact iRBCs on DCs, with low doses initiating partial phenotypic maturation and high doses resulting in inhibition of LPS-induced phenotypic and functional maturation. Inhibition of DC maturation by iRBCs was unrelated to the PfEMP1 variant expressed and occurred in the presence of PfEMP1-deficient iRBCs or when cocultures were performed in transwells, indicating that inhibition was not mediated by interaction of surface receptors on DCs and iRBCs. The majority of DCs cultured in the presence of high-dose iRBCs were apoptotic, a likely explanation for their failure to mature or respond to LPS. Apoptosis might result from in vitro culture conditions or represent a programmed response to parasite-derived signals. The physiological counterparts of in vitro high- and low-dose exposure will need to be elucidated before the significance of modulation of DCs by exposure to high-dose iRBCs is apparent. Our observation that low-dose iRBCs initiate functional DC maturation justifies reconsideration and further investigation of DC interactions with blood-stage *P. falciparum*.

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