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Malaria Blood Stage Suppression of Liver Stage Immunity by Dendritic Cells

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Abstract

Malaria starts with *Plasmodium* sporozoites infection of the host's liver, where development into blood stage parasites occurs. It is not clear why natural infections do not induce protection against the initial liver stage and generate low $CD8^+$ T cell responses. Using a rodent malaria model, we show that *Plasmodium* blood stage infection suppresses $CD8^+$ T cell immune responses that were induced against the initial liver stage. Blood stage *Plasmodium* affects dendritic cell (DC) functions, inhibiting maturation and the capacity to initiate immune responses and inverting the interleukin (IL)-12/IL-10 secretion pattern. The interaction of blood stage parasites with DCs induces the secretion of soluble factors that inhibit the activation of $CD8^+$ T cells in vitro and the suppression of protective $CD8^+$ T cell responses against the liver stage in vivo. We propose that blood stage infection induces DCs to suppress $CD8^+$ T cell responses in natural malaria infections. This evasion mechanism leaves the host unprotected against reinfection by inhibiting the immune response against the initial liver stage of the disease.

Key words: *Plasmodium yoelii* • sporozoites • $CD8^+$ T cells • erythrocytes

Introduction

Malaria is transmitted through the bite of an infected mosquito, which introduces *Plasmodium* sporozoites into the mammalian host. Sporozoites rapidly reach the liver of the host where they develop and replicate into merozoites, the parasitic stage that infects erythrocytes and causes the pathology of the disease. It is unclear why natural infections do not induce protection against the initial liver stage of *Plasmodium* and generate only low cytotoxic $CD8^+$ T cell responses (1–3). In endemic areas, this lack of protection against liver stage infection results in constant malaria reinfections throughout life (2). Despite the lack of protection induced against the liver stage of the disease, the feasibility of vaccination was proven by immunization of mice and humans with irradiated *Plasmodium* sporozoites that induce protection against this stage of the parasite (4, 5). Protection is mediated by high $CD8^+$ T cell responses specific against infected hepatocytes (6).

Plasmodium is extremely well-adapted to its hosts. In humans, a malaria infection can be established by as few as 10 sporozoites (7). Another important factor in the success of this parasite is its ability to evade the host immune response by antigenic diversity, clonal antigenic variation, and T cell antagonism (8–10). In addition to these evasion mechanisms, the existence of malaria-induced immune suppression is suggested by the association of malaria with a higher incidence of other infectious diseases (11–13) and reduced immune responses to vaccination during malaria infections (14, 15). Impaired cell-mediated immunity in patients with acute blood stage malaria infections (16–18) and altered macrophage responses (19–22) have also been associated with malaria-induced immune suppression.

Dendritic cells (DCs)* are antigen-presenting cells that play a central role in both innate and adaptive immune responses. Immature DCs uptake and process antigens in the peripheral areas of the body. After detecting microbial products or proinflammatory cytokines, DCs mature and migrate to lymphoid organs to initiate immune responses (23). Maturation of DCs augments their antigen presentation capacity, as peptide loading, half-life, and delivery of MHC molecules to the cell surface are increased, as well as surface expression of T cell costimulatory molecules (23). Several pathogens interfere with the host immune response by targeting different

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*Abbreviations used in this paper: CFDA SE, succinimidyl ester; CS, circumsporozoite; DC, dendritic cell.

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functions of DCs, including the maturation and migration of these cells (24). In particular, *Plasmodium falciparum*–infected erythrocytes modulate the maturation of DCs and their capacity to activate T cells in vitro (25), suggesting a role for DCs in malaria-induced immune suppression.

Here, we show that *Plasmodium* blood stage infection suppresses $CD8^+$ T cell responses against the liver stage of the parasite. In this way, the blood stage infection inhibits the establishment of a protective response against the initial liver stage, leaving the host susceptible for the next infection. Our results provide an explanation for the lack of liver stage immunity that occurs in malaria and that is critical for the prevalence of this disease. We also found that *Plasmodium* blood stage infection affects DCs maturation, cytokine secretion, and the capacity to initiate new immune responses. After interaction with infected erythrocytes, DCs also acquire the capacity to suppress $CD8^+$ T cell responses in immunized mice. The incubation of infected erythrocytes with DCs induces the secretion of soluble factors that inhibit T cell activation. This mechanism probably plays an important role the suppression of liver stage immunity observed in malaria infections.

Materials and Methods

Parasites and Mice. *Plasmodium yoelii* (nonlethal parasite line 17 XNL) sporozoites were obtained from the dissection of infected *Anopheles stephensi* mosquito salivary glands. BALB/c mice (haplotype *H-2K*^{d}) were purchased from Taconic.

Immunization, Erythrocytes Isolation, Challenge with Sporozoites, and Artemisinin Treatment. Mice immunization was performed by intravenous injection of 10^5 radiation-attenuated sporozoites (γ source, 20 Krad). *P. yoelii*–infected erythrocytes used for induction of malaria blood stage were obtained from infected Swiss Webster mice with >25% parasitemia. *P. yoelii*–infected erythrocytes were washed three times with PBS and separated from white blood cells by centrifugation at 2,000 *g*. To induce blood stage infection, 4×10^6 *P. yoelii*–infected erythrocytes were injected intravenously into each mouse.

Challenge of mice was performed in groups of three mice by intravenous injection of 10^5 nonirradiated *P. yoelii* sporozoites. Real-time PCR using primers for *Plasmodium* 18s rRNA was used for quantification of parasite load in the livers of mice 44 h after challenge (26).

Groups of three mice were treated for 10 d with 50 mg/kg/day of artemisinin diluted in DMSO. Treatment started 1 d after infection with 10^5 sporozoites. Groups of control mice received the same volume of DMSO.

ELISPOT Assay and $CD8^+$ T Cell Proliferation. With spleen cells, determination of individual IFN-γ–secreting T cells specific for the *H-2K*^{d} CD8 epitope of the circumsporozoite (CS) protein SYVPSAEQI of *P. yoelii* was done by ELISPOT (27). T cells were enriched from spleen suspensions by the elimination of plastic adherent and IgG- and IgM-reactive cells. 1.5×10^5 A20.2J cells preincubated with a peptide spanning the CD8 epitope were used as antigen-presenting cells for enriched T cell suspensions. The numbers of antigen-specific T cells are calculated by subtracting the mean spot numbers in duplicate control wells where enriched T cells are incubated with A20.2J cells without peptide. With $CD8^+$ T cell clone (provided by F. Zavala, New York University, New York, NY), 10^5 A20.2J cells or bone marrow–derived DCs preincubated with uninfected or *P. yoelii*–infected erythrocytes and maturated as described below were pulsed with the specific CD8 epitope or infected with 10^6 PFU recombinant influenza virus expressing the same CD8 epitope (provided by A. Garcia-Sastre, Mount Sinai, New York, NY; reference 28). The pulsed or infected DCs were incubated with 100 or 300 cells per well of the $CD8^+$ T cell clone (29), which is specific for SYVPSAEQI epitope. The numbers of antigen-specific $CD8^+$ T cell clone cells are calculated by subtracting the mean spot numbers in duplicate control wells where $CD8^+$ T cell clone cells are incubated with A20.2J cells without peptide or virus. To test whether sporozoites would inhibit the activation of T cells by DCs, sporozoites and DCs were preincubated for 24 h at a ratio of 10:1 before pulsing DCs with the specific CD8 epitope and the addition of $CD8^+$ T cell clone. The incubation media of DCs and uninfected erythrocytes or *P. yoelii*–infected erythrocytes for 48 h were added to 10^5 DCs or 10^5 A20.2J cells loaded with the peptide spanning the CS epitope before the addition of $CD8^+$ T cell clone cells. Pulsed DCs were also incubated with $CD8^+$ T cell clone in the presence of 25 ng/ml of recombinant mouse IL-12 or 50 μg/ml of anti–mouse IL-10 monoclonal antibody (JES5-16E3; BD Biosciences). The concentrations of recombinant IL-12 and anti–IL-10 antibodies are at least 10^3 times greater than the concentration of these cytokines in the supernatant of DCs with LPS. The proliferation of antigen-activated $CD8^+$ T cell clone cells was measured using succinimidyl ester (CFDA SE) staining (Molecular Probes) with carboxyfluorescein diacetate. 10^5 CFDA SE-stained $CD8^+$ T cell clone cells were mixed with 10^6 DCs pulsed with the CD8 epitope and preincubated with uninfected or *P. yoelii*–infected erythrocytes. After 5 d CFDA SE fluorescence was analyzed by FACS®.

Preparation of DCs from Mouse Bone Marrow and Incubation with P. yoelii–infected Erythrocytes. Primary cultures of immature DCs from BALB/c mice were obtained by differentiation of bone marrow–derived precursors for 10 d (30) using the supernatant of the myeloma cell line Ag8653 expressing mouse recombinant GM-CSF (31). DCs were maintained in DMEM medium containing 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 50 β-M 2-mercaptoethanol, supplemented with 30% culture supernatant of Ag8653 that contained 10–20 mg/ml of GM-CSF. DCs were incubated for 20 h with uninfected or *P. yoelii*–infected erythrocytes at a 1:100 ratio (DCs/erythrocytes). Maturation of DCs was induced 20 h later with 1 μg/ml *Salmonella enterica* LPS (Sigma-Aldrich) for 24 h. Erythrocytes were lysed before DC transfer into mice or phenotypic characterization by FACS® analysis.

Isolation of DCs from Mice. $CD11c^+$ DCs were purified from the spleens of malaria-infected and noninfected mice using anti-CD11c antibodies bound to magnetic beads (Miltenyi Biotec). Isolated $CD11c^+$ DCs were incubated with or without 1 μg/ml LPS for 24 h before FACS® analysis.

Monoclonal Antibodies and Flow Cytometry. DCs were analyzed as previously described (32) with the following antibodies: PE anti-CD11c (HL3), FITC anti-CD40 (3/23), FITC anti-CD80 (16-10A1), FITC anti-CD86 (GL1), FITC anti–class II MHC (39-10-8), and FITC anti–class I MHC (SF1-1.1; all from BD Biosciences). Anti-CD16/CD32 (Fcγ III/II receptor; 2.4G2) was used to prevent the binding of antibodies to Fc receptors (BD Biosciences). Cell preparations were analyzed on a FACSCalibur® (Becton Dickinson).

Transfer of DCs Incubated with Uninfected or P. yoelii–infected Erythrocytes. Bone marrow–derived DCs were incubated with uninfected or *P. yoelii*–infected erythrocytes and maturated with

LPS as described above. Erythrocytes were lysed and 5×10^5 DCs were irradiated (γ irradiation, 20 Krad) and injected intravenously into BALB/c mice immunized with irradiated sporozoites 7 d before. The same number of irradiated *P. yoelii*–infected erythrocytes were transferred as control. ELISPOT assays were performed 7 d after injection.

Macropinocytosis and Phagocytosis. Macropinocytosis was quantified after incubation of bone marrow–derived DCs with 1 mg/ml FITC-dextran (40 kD; Molecular Probes) for 2 h at 4 and 37°C. FACS® analysis of $CD11c^+$ cells was performed. DCs were also analyzed using fluorescence microscopy. Phagocytosis was quantified after incubation of DCs with ethidium bromide–labeled *P. yoelii*–infected erythrocytes (1:100) and fluorescence-labeled latex beads (1:5; unpublished data) for 2 h at 4 and 37°C. To confirm that infected erythrocytes were phagocytosed by DCs and not only attached to the cell membrane, DCs were labeled with Cell Tracker green (Molecular Probes) before incubation with ethidium bromide–labeled *P. yoelii*–infected erythrocytes.

Quantification of IL-10 and IL-12p70 in DC Incubation Medium and in Mice Sera. Incubation media from bone marrow–derived DC cultures were collected 24 h after the addition or not of LPS. Sera from heparinized blood collected from mice was obtained by centrifugation at 2,000 *g*. The concentrations of IL-10 and IL-12-p70 were determined by ELISA (R&D Systems).

Results

Plasmodium Blood Stage Infection Suppresses $CD8^+$ T Cell Responses Against the Liver Stage of the Parasite. To investigate the cause of the apparent lack of $CD8^+$ T cell responses in malaria infections, we injected groups of BALB/c mice with *P. yoelii*–irradiated or nonirradiated sporozoites, or a mixture of both. $CD8^+$ T cell responses against an epitope of the CS protein of *P. yoelii* (29) were determined for each mouse 10 d after injection. Irradiated sporozoites induced a significant $CD8^+$ T cell response, whereas the same number of nonirradiated sporozoites did not (Fig. 1 a). When irradiated and nonirradiated sporozoites were injected together, a $CD8^+$ T cell response was not found (Fig. 1 a), suggesting that nonirradiated sporozoites inhibit the response induced by irradiated ones.

Both irradiated and nonirradiated sporozoites start a liver infection in the host, but irradiated sporozoites fail to progress into blood stage parasites (33). To determine whether the blood stage of the parasite is the cause for the observed lack of $CD8^+$ T cell response in normal infections, we immunized groups of mice with *P. yoelii*–irradiated sporozoites 10 d before inducing a blood stage infection by the transfer of *P. yoelii*–infected erythrocytes. We observed that blood stage infection inhibited the previously established $CD8^+$ T cell response induced by irradiated sporozoites (Fig. 1 b), indicating that blood stage malaria suppresses previously established $CD8^+$ T cell responses against a liver stage antigen.

Infections with sporozoites of the rodent malaria parasite *Plasmodium berghei* induce a protective response against the liver stage when blood stage development is inhibited by treatment with specific drugs (34, 35). We wanted to differentiate whether natural malaria infections do not induce a $CD8^+$ T cell response against liver stage antigens or this

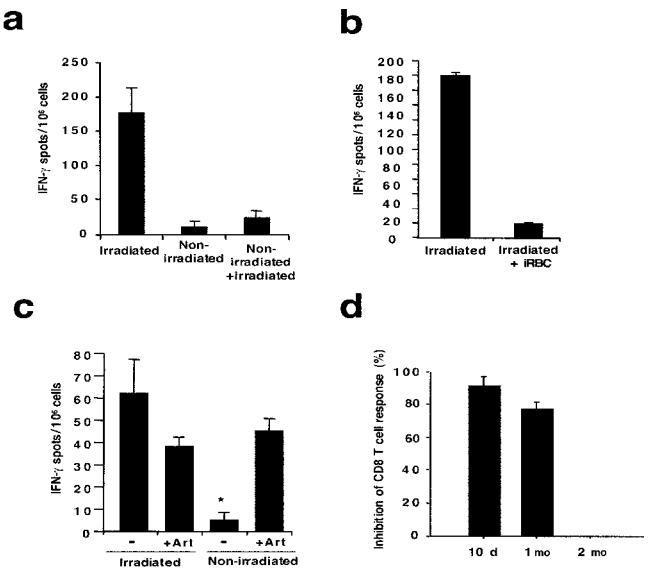

Figure 1. Liver stage *Plasmodium* infection induces $CD8^+$ T cell responses that are suppressed by blood stage parasites. ELISPOT results show IFN-γ–secreting $CD8^+$ T cells specific for the $CD8^+$ T cell *P. yoelii* CS epitope detected in the spleens of two mice. Results are expressed as average ± standard deviations for duplicates cultures. (a) Mice were immunized with 10^5 *P. yoelii*–irradiated sporozoites, 10^5 nonirradiated sporozoites, or a mixture of both. (b) Mice were immunized with 10^5 irradiated sporozoites followed by 4×10^6 *P. yoelii*–infected erythrocytes (iRBC) 7 d after immunization. (c) Mice were immunized with 10^5 irradiated or 10^5 nonirradiated sporozoites followed or not by treatment with artemisinin. *, significant difference ($P < 0.01$) in numbers of IFN-γ spots compared with aremisinin-treated group. (d) Mice were infected or not with 4×10^6 *P. yoelii*–infected erythrocytes (iRBC), followed by immunization with 10^5 irradiated sporozoites at different times after infection. Results are expressed as percentage inhibition of IFN-γ–secreting $CD8^+$ T cells as compared with noninfected immunized mice.

response is induced but is suppressed by the subsequent blood stage infection. Groups of mice were treated with a specific blood stage drug after infection with sporozoites to allow the development a liver stage–specific immune response while eliminating the potentially suppressive blood stage of the disease. When blood stage parasites were eliminated, a specific $CD8^+$ T cell response against a liver stage antigen was observed (Fig. 1 c). These results indicate that natural malaria infections generate $CD8^+$ T cells specific for liver stage antigens that are suppressed when the disease progresses into the blood stage.

Mice infected with blood stage *P. yoelii* are not able to initiate a $CD8^+$ T cell response when immunized with irradiated sporozoites (Fig. 1 d). Responses could not be initiated 1 mo after infection, even though the level of parasites in the blood at this time is very low (parasitemia ranged from 0.4 to 0.6%). The capacity to initiate immune responses is restored 2 mo after infection. Taken together, these results indicate that malaria blood stage infection not only suppresses existing $CD8^+$ T cell immune responses but also inhibits the initiation of new ones.

Plasmodium-infected Erythrocytes Inhibit the Maturation of DCs In Vitro and In Vivo. To investigate the role of DCs in blood stage suppression of liver stage responses, we first examined

whether *P. yoelii* could be used as a model for the modulation of DC maturation induced by erythrocytes infected with *P. falciparum* (25). Immature, bone marrow–derived mouse DCs increase the surface expression of T cell costimulatory and MHC molecules upon the addition of a maturation stimulus such as bacterial LPS (32). Preincubation of these DCs with *P. yoelii*–infected erythrocytes, but not with uninfected erythrocytes, prevents the increase of these surface molecules in response to LPS (Fig. 2 a), confirming the results obtained for *P. falciparum*–infected erythrocytes. This suggests that *P. yoelii* blood stage infection might be used as a model for the human *P. falciparum*, opening the possibility of in vivo experiments that are only feasible using animal models.

To investigate whether blood stage *P. yoelii* infection inhibits the maturation of DCs during malaria infections in vivo, we isolated DCs from the spleen of uninfected or blood stage malaria-infected mice. As previously described (36), we found that freshly isolated DCs from control mice already show a partially mature phenotype, as the purification process activates the maturation of these cells. The addition of LPS to these cells resulted in a fully mature phenotype (Fig. 2 b). In marked contrast, DCs obtained from mice infected with blood stage *P. yoelii* showed a significantly smaller up-regulation in the surface expression of T cell costimulatory and MHC molecules, even after the addition of LPS (Fig. 2 b). These results indicate that blood stage malaria infection inhibits the maturation capacity of DCs.

Plasmodium-infected Erythrocytes Affect DC Survival and Secretion of IL-12 and IL-10. DCs in vitro die within 48 h after the addition of a maturation stimulus (30). We observed that DCs preincubated with *P. yoelii*–infected erythrocytes, but not with uninfected erythrocytes, do not die in response to LPS stimulation (Fig. 3 a), confirming that these DCs are not able to mature. This also suggests that malaria blood stage infection may prolong the half-life of DCs in this atypical state.

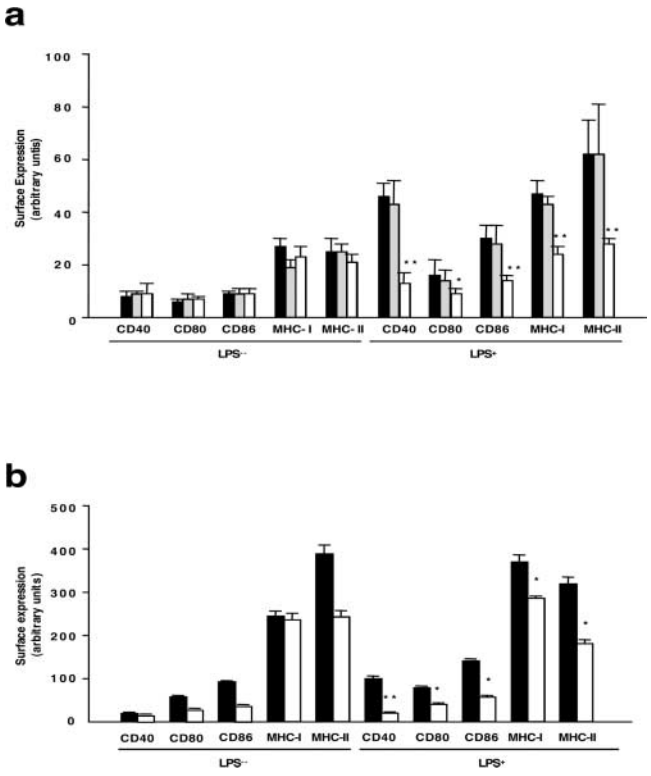

Figure 2. *P. yoelii*–infected erythrocytes inhibit the expression of costimulatory and MHC molecules on DCs in vitro and in vivo. (a) Expression of costimulatory and MHC molecules on DCs alone (black bars), preincubated with uninfected erythrocytes (gray bars) or *P. yoelii*–infected erythrocytes (white bars) 24 h after the addition or not of LPS. Results are expressed as mean fluorescence intensity as determined by FACS® analysis of gated $CD11c^+$ cells. Error bars indicate standard deviation of duplicated samples. Significant difference (*, $P < 0.05$; **, $P < 0.01$) in surface expression compared with expression on DCs preincubated with uninfected erythrocytes. Representative results from one of four independent experiments are shown. (b) Expression of MHC and costimulatory molecules on DCs isolated from the spleens of mice 7 d after infection with 4×10^6 *P. yoelii*–infected erythrocytes (iRBC, white bars) and uninfected mice (black bars) 24 h after the addition or not of LPS. Representative results from one of three independent experiments are shown. Error bars indicate standard deviation of duplicated samples. Significant difference (*, $P < 0.05$; **, $P < 0.01$) in surface expression compared with expression on DCs from uninfected mice.

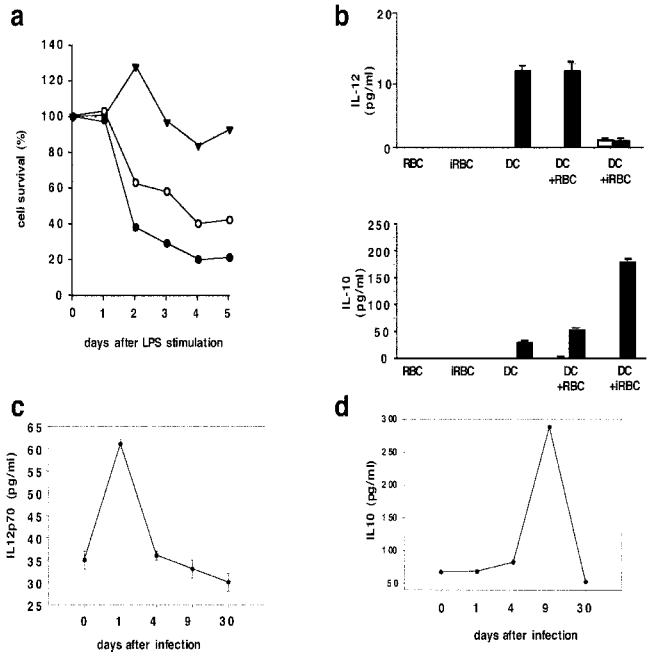

Figure 3. *P. yoelii*-infected erythrocytes prevent DC death and reverse the IL-12/IL-10 secretion pattern. (a) Percentage of survival of DCs (FACS® analysis of $CD11c^+$, propidium $iodide^-$) alone (●), preincubated with uninfected erythrocytes (○), or with *P. yoelii*–infected erythrocytes (▲) after the addition of LPS. (b) Concentration of IL-12p70 (top) and IL-10 (bottom) secreted by DCs alone, preincubated with uninfected erythrocytes (RBC), or *P. yoelii*–infected erythrocytes (iRBC) after the addition (black bars) or not (white bars) of LPS. Representative results from one of three independent experiments are shown. Error bars indicate standard deviation of duplicated samples. Concentration of (c) IL-12p70 and (d) IL-10 in sera of malaria-infected mice. Sera were obtained from heparinized blood at different time points after infection with 4×10^6 *P. yoelii*–infected erythrocytes. Representative results from one of three mice are shown. Error bars indicate standard deviation of duplicated samples.

IL-12 secreted by mature DCs is an important factor to initiate a cellular immune response. IL-10, on the other hand, has a suppressive effect on both DC maturation and cellular immune responses and can also be secreted by DCs (23). We found that the preincubation of DCs with *P. yoelii*–infected erythrocytes inhibits the secretion of IL-12 in response to a maturation stimulus such as LPS. Conversely, the secretion of IL-10 by DCs was increased under these conditions (Fig. 3 b). Interestingly, this pattern in the levels of the cytokines is also observed in vivo after the infection of mice with blood stage *P. yoelii* (Fig. 3, c and d).

Plasmodium-infected Erythrocytes Do Not Interfere with Antigen Internalization, but Inhibit Antigen-specific CD8$^+$ T Cell Activation by DCs. Immature DCs efficiently internalize exogenous antigens to use for antigen presentation associated with MHC class I and II molecules (23). We investigated the effect of *P. yoelii*–infected erythrocytes on DC macropinocytosis and phagocytosis, the two principal mechanisms DCs use to internalize exogenous antigens. DCs alone or preincubated with normal or *P. yoelii*–infected erythrocytes did not show any differences in their macropinocytic (Fig. 4, a and c) or phagocytic capacity (Fig. 4, b and d), indicating that these mechanisms of antigen up-take are not affected. We observed that *P. yoelii*–infected erythrocytes (Fig. 4 d) and fluorescent latex beads (not depicted) are efficiently phagocytosed by DCs preincubated or not with *P. yoelii*–infected erythrocytes.

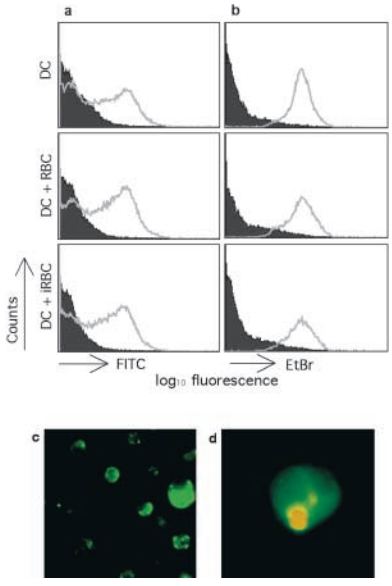

Figure 4. *P. yoelii*–infected erythrocytes do not affect antigen uptake by DCs. (a) Macropinocytosis of FITC-dextran and (b) phagocytosis of ethidium bromide–labeled *P. yoelii*–infected erythrocytes by DCs alone, preincubated with uninfected erythrocytes (RBC) or with *P. yoelii*–infected erythrocytes (iRBC). DCs were incubated for 2 h with FITC-dextran at 4°C (filled histogram) or 37°C (solid line). Histograms show CD11c$^+$ gated cells. (c) DCs after incubation with FITC-dextran at 37°C. Fluorescence shows dextran internalization and no staining was observed after incubation at 4°C (not depicted). (d) DCs labeled with Cell Tracker (green) after phagocytosis of a *P. yoelii*–infected erythrocyte labeled with ethidium bromide (red).

DCs are potent stimulators of naive and memory T cells (23). To investigate whether deficient T cell activation by DCs may play a role in the lack of CD8$^+$ T cell responses against *Plasmodium* liver stage antigens, we studied the capacity of DCs incubated with uninfected or *P. yoelii*–infected erythrocytes to activate CD8$^+$ T cells from a cloned line specific for a liver stage *P. yoelii* epitope (29). DCs were incubated with a peptide spanning the CD8 epitope or infected with a recombinant influenza virus expressing the same epitope (28). Preincubation of DCs with *P. yoelii*–infected erythrocytes inhibited antigen-specific T cell activation, measured as the capacity of CD8$^+$ T cell clone to secrete IFN-γ (Fig. 5, a and b) and proliferate (Fig. 5 c). Similar results were observed after the addition of LPS to the cocultures (not depicted). We also found that *P. yoelii* sporozoites, as opposed to infected erythrocytes, do not inhibit DC activation of T cells (not depicted).

To determine whether DCs from blood stage *Plasmodium*-infected mice also suppress CD8$^+$ T cell responses, we isolated DCs from uninfected or infected mice and determined their capacity to activate the CD8$^+$ T cell clone. We found that the activation of cells from the CD8$^+$ T cell clone is inhibited in DCs from infected mice (Fig. 5 d).

We wanted to distinguish whether the observed lack of activation of CD8$^+$ T cells is due to deficient antigen presentation by DCs or to an inhibitory effect on the T cells. Incubation medium from bone marrow–derived DCs and uninfected or *P. yoelii*–infected erythrocytes with or without LPS, was added to DCs or A20.2J B cell lymphoma cells loaded with the CD8-specific epitope. T cells from the specific CD8$^+$ T cell clone were added and activation was measured as secretion of IFN-γ by T cells. We observed that the presence of incubation medium from DCs and *P. yoelii*–infected erythrocytes inhibited the activation of specific T cells by A20.2J cells or DCs (Fig. 5, e and f). This inhibitory activity was not dependent on the presence of LPS in the DCs/erythrocytes cocultures. These results indicate that CD8$^+$ T cell activation is inhibited by soluble factors secreted by DC/*P. yoelii*–infected erythrocytes cocultures. These factors are probably secreted by DCs, as the inhibitory activity was not found in the incubation medium of infected erythrocytes alone (not depicted). A reduced inhibition (22%) of T cell activation was also observed in the presence of supernatants from DCs isolated from infected mice when compared with supernatants from cells of uninfected mice (not depicted).

We wanted to determine whether the reduced secretion of IL-12 or the increased secretion of IL-10 induced in DCs by *P. yoelii*–infected erythrocytes mediate the inhibitory activity on T cells. The addition of exogenous IL-12 or blocking antibodies against IL-10 did not interfere with the inhibition of T cell activation by DCs, suggesting that these cytokines do not mediate the inhibitory effect (Fig. 5 g).

DCs Incubated with Plasmodium-infected Erythrocytes Suppress Protective CD8$^+$ T Cell Responses Against Liver Stage Malaria. We found that after incubation with DCs preincubated with *P. yoelii*–infected erythrocytes, CD8$^+$ T cells are not responsive to antigen presentation (Fig. 6 a). We

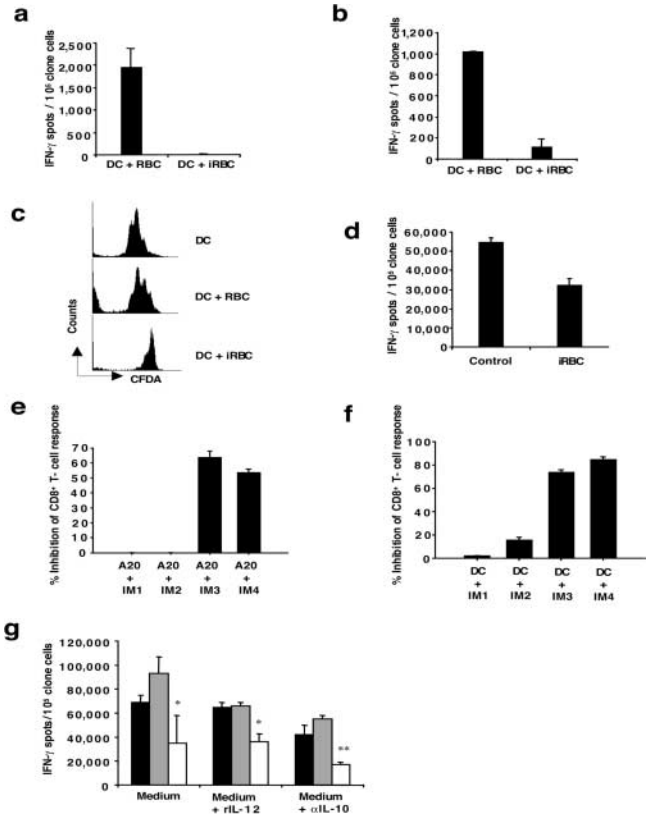

Figure 5. Blood stage infection induces DCs to secrete soluble factors that inhibit the activation of $CD8^+$ T cells. Results of ELISPOT showing IFN-γ–secreting cells from the $CD8^+$ T cell clone specific for *P. yoelii* CS epitope. Bone marrow–derived DCs preincubated with uninfected erythrocytes (RBC) or *P. yoelii*–infected erythrocytes (iRBC) were incubated with a peptide spanning the (a) CS CD8 epitope or (b) recombinant influenza virus expressing the same epitope before the addition of clone cells. As control, DCs without peptide or virus were incubated with clone cells. Representative results from one of three independent experiments are shown. (c) Proliferation of CFDA SE–stained $CD8^+$ T cell clone cells after 5 d of incubation with DCs pulsed with the CD8 epitope peptide and preincubated with uninfected erythrocytes (RBC) or *P. yoelii*–infected erythrocytes (iRBC). Representative results from one of two independent experiments are shown. (d) DCs were isolated from the spleens of mice 7 d after infection with 4×10^6 *P. yoelii*–infected erythrocytes (iRBC) or noninfected mice (control) before incubation with the CD8 epitope peptide and the addition of clone cells. Representative results from one of three mice are shown. (e and f) The incubation medium of cocultures of DCs and uninfected or *P. yoelii*–infected erythrocytes was added to (e) A20.2J cells or (f) DCs loaded with the peptide spanning the CS epitope before the addition of clone cells. Incubation medium (IM) from DCs and uninfected erythrocytes (IM1), DCs and uninfected erythrocytes with LPS (IM2), DCs and *P. yoelii*–infected erythrocytes (IM3), and DCs and *P. yoelii*–infected erythrocytes with LPS (IM3) are shown. As control, DCs or A20.2J cells without peptide were incubated with clone cells. Results are expressed as percentage inhibition of IFN-γ–secreting cells from $CD8^+$ T cell clone incubated with each IM as compared with those incubated with normal medium. Representative results from one of two independent experiments are shown. (g) DCs pulsed with the CD8 epitope and preincubated with medium (black bars), uninfected erythrocytes (gray bars), or *P. yoelii*–infected erythrocytes (white bars) were mixed with $CD8^+$ T cell clone cells in the presence of recombinant mouse IL-12 or anti–mouse IL-10 blocking monoclonal antibody. Significant difference (*, $P < 0.1$; **, $P < 0.05$) in IFN-γ spots compared with DCs preincubated with uninfected erythrocytes. Error bars indicate standard deviation of duplicated samples.

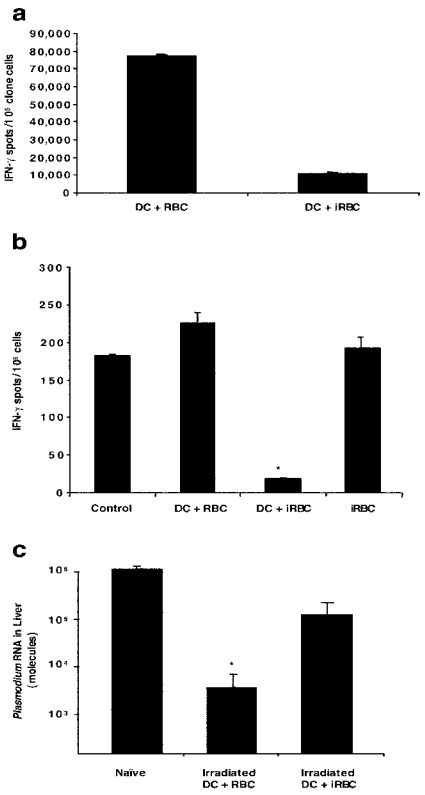

Figure 6. Protective immune responses against malaria liver stage are suppressed by the transfer of DCs incubated with *P. yoelii*–infected erythrocytes. (a) Results of ELISPOT showing the numbers of IFN-γ–secreting $CD8^+$ T cells from the $CD8^+$ T cell clone specific for *P. yoelii* CS epitope. DCs preincubated with uninfected erythrocytes (DC + RBC) or *P. yoelii*–infected erythrocytes (DC + iRBC) were incubated with clone cells at a 2:1 ratio for 7 d before the addition of A20.2J loaded with the peptide spanning the CD8 epitope. As control, A20.2J cells without peptide were incubated with cocultures of DCs/clone cells. Representative results from one of two independent experiments are shown. (b) Results of ELISPOT showing the numbers of IFN-γ–secreting $CD8^+$ T cells detected in the spleens of mice immunized with irradiated sporozoites and inoculated or not (control) 7 d later with DCs preincubated with uninfected erythrocytes (DC + RBC) or *P. yoelii*–infected erythrocytes (DC + iRBC) and stimulated with LPS. Another group of mice was inoculated with *P. yoelii*–infected erythrocytes alone (iRBC). All cells were irradiated before injection into mice to avoid the development of blood stage malaria infection. *, significant difference ($P < 0.01$) in IFN-γ spots compared with the other groups. ELISPOT was performed 7 d after the transfer of DCs. (c) *P. yoelii* development in the livers of mice after challenge with sporozoites. Mice were immunized with irradiated sporozoites, followed by inoculation 7 d later of DCs preincubated with uninfected erythrocytes (DC + RBC) or *P. yoelii*–infected erythrocytes (DC + iRBC), and challenged 7 d later. *, significant difference ($P < 0.01$) in the number of RNA molecules compared with the other groups. Representative results from one of three independent experiments are shown.

wanted to investigate whether DCs also inhibit $CD8^+$ T cell activation as a consequence of blood stage infection in vivo. DCs preincubated with uninfected or *P. yoelii*–infected erythrocytes were transferred into mice immunized with irradiated *P. yoelii* sporozoites. To avoid infection of mice with infected erythrocytes from the cocultures, erythrocytes were lysed and cultures were irradiated before injection into mice. We found that mice injected with DCs preincubated with *P. yoelii*–infected erythrocytes

loose the $CD8^+$ T cell response previously induced by irradiated sporozoites (Fig. 6 b). A control group of mice injected only with irradiated *P. yoelii*–infected erythrocytes did not show a decrease in the $CD8^+$ T cell response, confirming that the DCs and not residual infected erythrocytes are responsible for the suppressive effect on the T cell response.

To determine the importance of DC-induced suppression of $CD8^+$ T cell responses in protection against a malaria infection, we injected groups of mice immunized with irradiated sporozoites with DCs preincubated with uninfected or *P. yoelii*–infected erythrocytes. Development of *P. yoelii* in the liver was detected by real time PCR of *Plasmodium* ribosomal RNA in the livers of infected mice (26) after challenge with *P. yoelii* sporozoites. Mice receiving DCs preincubated with *P. yoelii*–infected erythrocytes presented a decreased level of protection (40 times higher parasite development in the liver) when compared with mice injected with DCs preincubated with normal erythrocytes (Fig. 6 c).

Discussion

Natural malaria infections induce much lower $CD8^+$ T cell responses against liver stage antigens than immunization with irradiated sporozoites (3). In endemic areas, the percentage of responder individuals and $CD8^+$ T cell precursor frequencies to liver stage malaria antigens are lower than those observed for other infectious agents. In addition, these T cell responses do not increase due to repeated infections (3). All these suggest that the generation or maintenance of $CD8^+$ T cell responses might be inhibited during natural malaria infections.

Our results show that *Plasmodium* infections induce liver stage–specific $CD8^+$ T cells, but these responses are inhibited during the blood stage of the disease. This evasion strategy is likely to have a major impact in the transmission of malaria because it confers a significant advantage to the parasite, leaving the host unprotected for the next infection. These results may explain the lack of protective immunity against liver stage parasites observed in populations living in malaria endemic areas, which results in constant reinfections throughout life.

Plasmodium-infected erythrocytes have been proposed as mediators of malaria-induced immune dysregulation through the modulation of DC maturation (25). The incubation of erythrocytes infected with the human parasite *P. falciparum* inhibits the maturation (25) and changes the cytokine secretion pattern of DCs (37) in response to LPS. We found that *P. yoelii*–infected erythrocytes induce the same in vitro responses as *P. falciparum*. Although another study using the rodent malaria parasite *Plasmodium chabaudi* found direct activation of DCs by infected erythrocytes in vitro (38), it is possible that this parasite also induces the suppression of DCs in vivo, as DCs isolated from *P. chabaudi*–infected mice fail to mature after stimulation with LPS (39).

The use of a rodent malaria model allowed us to study the role of DCs in malaria infections in vivo. As observed in the in vitro assays, we found that DCs isolated from malaria blood stage–infected mice are also unable to mature. Because maturation of DCs is required for the activation of naive T cells (23), a defect in this crucial step is likely to interfere with the initiation of new antigen-specific immune responses. This might be the cause for the inability to initiate immune responses against irradiated sporozoites that we observed in mice for 1 mo after infection with blood stage parasites and for the reduced responses to vaccination observed during malaria infections (14, 15).

In addition to the inhibition of maturation and initiation of T cell responses, we observed that DCs incubated with *P. yoelii*–infected erythrocytes actively suppress T cell responses that were established before. The inhibition of $CD8^+$ T cell responses is mediated by soluble factors that result in the inhibition of IFN-γ secretion by T cells. During malaria infections *Plasmodium*-infected erythrocytes encounter DCs in the blood and spleen. This interaction would lead to the functional dysregulation of DCs and the secretion of suppressive factors that inhibit the activation of circulating $CD8^+$ T cells. As IFN-γ secreted by $CD8^+$ T cells mediates protection against liver stage malaria (40), this inhibition might be the key factor mediating the lack of protection observed in natural infections.

IL-10 suppresses multiple activities in the immune system, including T cell activation (41). IL-10 concentrations are increased in malaria blood stage–infected mice and humans (37, 42), and probably contribute to the generalized immunosuppression that is observed in this stage of the disease. We observed that activation with LPS induced an increased secretion of this cytokine in DCs incubated with *P. yoelii*–infected erythrocytes. However, the inhibition of $CD8^+$ T cell activation that we observe in vitro cannot be attributed to IL-10, as neutralization of IL-10 with blocking antibodies did not interfere with the inhibition of T cell activation.

DCs maturation is followed by apoptotic death (23). We observed that DCs incubated with infected erythrocytes do not mature and also do not die in response to LPS. These DCs, which presumably secrete suppressive factors for T cell activation, would have a prolonged half-life in the body, enhancing the inhibitory effect over $CD8^+$ T cells. In fact, 1 mo after the initiation of a blood stage malaria infection when the levels of circulating infected erythrocytes are very low, we still found a profound inhibition of the $CD8^+$ T cell response against liver stage antigens.

Impaired phagocytosis of *P. falciparum*–infected erythrocytes by DCs was observed using electron microcopy (25). However, we found that phagocytosis and macropinocytosis, two of the main mechanisms for antigen uptake in DCs, are not altered after incubation with *P. yoelii*–infected erythrocytes. Therefore, the lack of T cell activation observed does not appear to be caused by deficient antigen internalization, but rather to the secretion of soluble factors that inhibit T cell activation.

LPS induces the maturation of DCs through the activation of the Toll-like receptor 4, which mediates the secretion of IL-12 and the up-regulation of costimulatory molecules in these cells (43). *Plasmodium*-infected erythrocytes presumably interfere with this signaling pathway, as DCs response to LPS is severely altered. The lack of secretion of IL-12 by DCs in response to LPS may explain the decreased levels of IL-12 observed during the blood stage of malaria infections, as these cells are the main source of this cytokine (44). IL-12 is not only involved in the initiation of T cell immune responses, but is also implicated in maintaining these responses (45). The decreased levels of IL-12 may also contribute to the disappearance of liver stage–specific $CD8^+$ T cells observed in vivo.

We conclude that DC functions that are essential for immune regulation are severely affected during *Plasmodium* blood stage infections. These include the inhibition of the expression of costimulatory and MHC class I and II molecules as well as the capacity to initiate new immune responses. In addition, DCs secrete soluble factors that inhibit the activation of $CD8^+$ T cells. This probably mediates the suppression of protective liver stage immune responses that are induced during the initial stage of the disease.

Understanding of the evasion mechanisms of *Plasmodium* is essential to develop effective strategies against malaria. Interfering with *Plasmodium*-induced suppression may lead to the establishment of effective protective immunity against the liver stage, opening new possibilities of treatment and prevention. Blood stage–induced immunosuppression should also be considered for vaccine design, as it reinforces the necessity of vaccinating against both liver and blood stages. Effective immunization against liver stage *Plasmodium* would overcome this suppressive strategy by avoiding the development of blood stage parasites. Immunization against blood stage would also be essential to minimize the suppressive effect of infected erythrocytes during infection.

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