Rodent blood-stage *Plasmodium* survive in dendritic cells that infect naive mice

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*Plasmodium* spp. parasites cause malaria in 300 to 500 million individuals each year. Disease occurs during the blood-stage of the parasite’s life cycle, where the parasite is thought to replicate exclusively within erythrocytes. Infected individuals can also suffer relapses after several years, from *Plasmodium vivax* and *Plasmodium ovale* surviving in hepatocytes. *Plasmodium falciparum* and *Plasmodium malariae* also persist after the original bout of infection has apparently cleared. We observed that blood-stage parasites could infect, survive, and replicate within *CD31+* dendritic cells, and that these cells released parasites infectious for erythrocytes in vivo. These data have identified a unique survival strategy for blood-stage *Plasmodium*, which has significant implications for understanding the escape of *Plasmodium* spp. from immune-surveillance and for vaccine development.

**Immune evasion | Rodent malaria**

Malaria commences when an infected female anopheline mosquito bites and deposits, up to 125 *Plasmodium* sporozoites under the skin of the host (1). Studies using *Plasmodium berghei* sporozoites, showed that a proportion will remain in the skin and infect keratinocytes (2), others are drained by the lymphatic system and are trapped in lymph nodes, and a fraction of the deposited sporozoites enter blood vessels to migrate to the liver (3). In the liver, typically between 1 and 10 sporozoites invade hepatocytes. Other studies have shown that sporozoites can invade and migrate through other cell types, including macrophages (4), Kupffer cells (5, 6), epithelial cells, and fibroblasts (7).

The sporozoites within hepatocytes develop by a process of schizogony into merozoite forms, which escape from an infected liver cell into the sinusoid lumen (8) to invade RBC. Within RBC, the merozoites then develop into “ring” trophozoites, then mature trophozoites, and finally a schizont containing up to 32 new merozoites. These schizont-infected RBC then rupture to release merozoites that are able to invade new RBCs, resulting in an increase of parasite biomass. The *Plasmodium* life cycle continues when some merozoites develop into the sexual parasite stages, the male and female gametocytes, which can be taken up by mosquitoes during blood meals (9).

Some *Plasmodium* infections, such as *Plasmodium malariae* (10) and *Plasmodium inui* (11), persist for years and sometimes for the life of the host (12), and the reasons for this are not understood. To date, the blood-stage parasites of mammalian *Plasmodium* spp. are thought to survive and replicate only within RBC, but it has long been suspected that they may also have another survival strategy. The blood-stage-parasite of rodent *Plasmodium* spp. have been observed as sacks of merozoites within macrophages and polymorphonuclear leukocytes (13). The merozoites were associated with pigment that suggests that cells ingested parasitized RBC (pRBC) as opposed to invasion of the cells by merozoites. These merozoite-associated cells were principally in the spleen, thought to be formed by either phagocytosis or phagocytosis of parasites by macrophages (13). It is thus possible that this is a site of latency, but proof that the transfer of infection with these cells was not contaminated with infected RBC is lacking. Furthermore, blood-stage parasites have also been found inside platelets (14) but the intrathrombocytic environment did not support parasite growth or replication (15) and infectious studies were not undertaken. Finally, avian *Plasmodium* also has two stages in which the parasites invade macrophages: during the initial pre-erythrocytic stages of infection and later in the blood-stage (16). The infection starts when the sporozoites from the mosquito invade skin mononuclear cells where schizonts develop and release merozoites, which have three phases. These merozoites first invade mononuclear cells throughout the body, develop through schizogony, and are released to invade erythrocytes as part of the second phase. In the final phase, merozoites from erythrocytes invade many different endothelia where the parasite grows (second exo-erythrocytic stage) (16).

We originally suspected that blood-stage *Plasmodium* spp. might reside within an unidentified cell type and be protected from immunity, when passive transfer of *Plasmodia*-specific antibodies into immunocompromised mice could significantly reduce but not eliminate parasites in the blood (17). We then found that CD11c+ dendritic cells (DCs) from mice infected with...
Plasmodium yoelii transferred an infection to naive mice unless the donor mice were treated with antimalarial drugs, suggesting that some parasites might reside within the DCs. Here we demonstrate, predominantly using transgenic blood-stage *P. berghei* expressing GFP (PbGFP) (18), that the rodent malaria parasite *P. berghei* can survive and replicate within CD317+ DCs and that a small percentage of these DCs release parasites that are infectious for erythrocytes on transfer to naive mice.

**Results**

**Blood-Stage Plasmodium Has a Tropism for CD317+ DCs.** To determine if DCs could harbor *P. berghei*, we infected C57/B6 mice with 10^3 blood-stage, transgenic PbGFP (18, 19). Patent infection (first day of parasites visible on a blood smear) occurs around days 4 to 6 after inoculation (Fig. 1A), and the infection is usually lethal for mice by day 10 (Fig. 1B). Because mouse DCs are characterized by expression of CD11c (20), total CD11c+ DCs were isolated for mice by day 10 (Fig. 1B contrast with the termine if DCs could harbor infectious for erythrocytes on transfer to naive mice.

In multiple experiments, >20% of spleen CD11c+ DCs contained GFP (Fig. 1C) and >85% of CD11c+GFP+ DCs also expressed CD317 (Fig. 1D), labeled by monoclonal antibodies PDCA-1 and 120G8 (21). Further characterization of the GFP+CD317+ DCs found variable expression of other DC-associated markers (B220, Ly6G, Siglec-H, F4/80, Dec205, and CD8) (Fig. SL4). Data for DCs from naive mice are provided for contrast (Fig. S1B). Approximately 40% (range of 20–70%) of CD317+ DCs of the spleen contained GFP compared with <10% of CD317+ DC subpopulations by day 7 postinfection (Fig. S1C). Absolute numbers of CD317+ DCs were also significantly increased by day 7 (Fig. S1D).

All GFP+CD11c+ DCs were “hypermature,” as defined by high-level expression of MHC Class II, CD80, and CD86 (Fig. S1E). In contrast with the >20% of CD11c+ splenic cells, only ~1% of CD11c+ DC in the peripheral blood contained GFP (Fig. S1F).

**Blood-Stage Plasmodium Replicates Within CD317+ DCs.** To determine if the GFP seen in DCs represented pRBC, CD317+ DCs were also significantly increased by day 7 (Fig. S1D). All GFP+CD11c+ DCs were also GFP+ parasites that did not colocalize with these markers (Fig. S4A and B, arrows; and Movie S1, which is a 3D reconstruction of a PbGFP-infected CD317+ DCs).

**CD317+ DCs Are Infected in Vitro by *P. berghei* Schizont-Infected RBC in Culture.** In vitro culture followed by adoptive-transfer studies were undertaken to determine if CD317+ DC could be infected in vitro and transfer infection. Total CD11c DC or CD317+ DC were isolated from naive mice and multiple samples cultured with PbGFP schizont-infected RBC. In parallel, similar numbers of schizont-infected RBC were cultured alone without DCs under similar conditions for 42 to 48 h.

After 24 h, a sample of the CD11c+ DCs cultured with parasites was examined by flow cytometry and GFP was found to be predominantly associated with the CD11c+ population of DCs (Fig. 4A). After 42 to 48 h, aliquots of all parasite cultures with or without DCs were injected intravenously into naive mice (10^6 DC per mouse), which were monitored for infection (Fig. 4B). All mice given CD11c+ DC or CD317+ DC cultured with schizont-infected RBC developed an infection. In contrast, after the 42 to 48 h in culture, no infection was detected in mice given schizont-infected RBC cultured alone. This series of studies showed that the culture conditions used did not support survival of PbGFP schizont-infected RBC, but that CD317+ DCs could support the survival of PbGFP schizont-infected RBC in vitro and infect naive mice on transfer of the DCs. Furthermore, despite extensive attempts, we have never seen red cell membranes surrounding parasites within DCs, suggesting that membranes may be released by PbGFP schizont-infected RBC in culture and evade DCs.

**Demonstration That Infected RBC Were Not the Source of Infection in DC Preparations.** Although we could not detect contaminating pRBC by Ter119 labeling of our DC preparations, it could be ar-
gued that the infection was transferred as a result of a small number of contaminating infected RBC in the DC preparations. To analyze this further, we isolated CD317+ DCs from mice at day 7 after infection with PbGFP. These DCs were then labeled with CD11c and Ter119 for FACS sorting of Ter119+CD11c+CD317+GFP+ DCs or Ter119−CD11c−CD317−GFP− DCs, which were transferred to groups of naive mice. Although GFP+ DCs transferred the infection, no mouse given GFP− DCs developed infection (Table 1). This result demonstrated that our sorting method was highly sensitive in discriminating between infectious and noninfectious cells.

To further demonstrate that contaminating pRBC were not responsible for transferring the infection, CD317+ DCs were isolated from naive mice and mixed with infected RBC in a DC:pRBC ratio of 1:20 or 1:80. One-half of the sample was immediately treated with Gey’s solution to lyse RBC, CD317+ DCs reisolated by MACS (magnetic-activated cell sorting) columns and then labeled with antibody specific for CD11c. The CD11c+ DCs were cell-sorted and 10^6 DCs per mouse transferred to naive mice. The other half of the sample was not treated with Gey’s solution and adjusted to transfer an equal number of DCs (10^6 per mouse) to naive mice. In duplicate experiments, untreated DCs transferred the infection but all DC preparations treated with Gey’s solution and isolated failed to transfer infection, confirming our standard DC isolation procedure removed infected RBC.

To demonstrate that cell surface-associated merozoites, released following Gey’s lysis of pRBC, were not responsible for the transfer of infection by GFP+CD11c+ DCs, blocking studies were undertaken (Fig. 4C). For these studies, Ter119+GFP+ RBC and GFP−CD11c+Ter119− DCs were isolated from PbGFP-infected mice, treated with Gey’s solution (to free merozoites), followed by incubation with an equal volume of either hyperimmune serum specific for PbGFP (HIS) or naive mouse serum (NMS) before transfusion into naive mice. The treatment of lysed pRBC with HIS delayed the detection of parasitemia in recipient mice by 1 d, with nearly 10-fold lower parasitemia, compared with freed merozoites treated with NMS (Fig. 4C; Left). In contrast, HIS did not affect the infectivity of DCs (Fig. 4C; Right). These studies showed that the parasites were internalized into DCs and were protected from antibodies capable of neutralizing free merozoites.

**Plasmacytid DCs from PbGFP, P. yoelii 17XNL, and Plasmodium chabaudi-infected Mice Are Infectious to Naive Mice.** To demonstrate whether CD317+ DCs also supported parasite survival in vivo, GFP+CD317+CD11c+Ter119− DCs were isolated from spleens of mice infected with PbGFP. In particular, RBC in the spleen cell preparation were lysed by Gey’s solution and cell preparations were passed through a MACS magnet to deplete hemoglobin, residual schizonts, or any other cell containing hemozoin, before positive selection of GFP+CD317+CD11c+Ter119− DCs. Titrating numbers of these DCs (10^3–10^5) or infected RBC (10^10) were transferred to groups of naive mice (n = 10) and monitored for the development of blood-stage infections. Using a limiting dilution analysis, it was estimated that ~1 in 8,631...
These data show that during the subpatent RBC and only developed parasitemia by day 6, compared with spp. have a tropism for splenic CD317+ DCs, which can promote this study shows that rodent-infecting blood-stage asites were within DCs and not associated with the extracellular release infectious parasites even during subpatent infections. Confocal microscopy studies established that multinucleated parasites were within DCs and not associated with the extracellular membrane folds, as described for the Toxoplasma tachyzoites (23).

Discussion
This study shows that rodent-infecting blood-stage Plasmodium spp. have a tropism for splenic CD317+ DCs, which can promote their survival and replication and that ~360 to 580 infectious DCs per spleen, as the spleens of infected mice have 3 to 5 × 10^6 CD317+ DCs during a PbgFP infection (Fig S1D). Equivalent numbers (10^5 ) of GFG+CD317+CD11c+ DCs, GFG+CD317+CD11c- , or GFG+CD8+ DCs from infected mice did not transfer the infection (Table 1). In comparison, 1 in 22 PbGFP-infected RBC were infectious (Table 1). Similarly, when CD11c+ DCs isolated from mice infected with P. yoelii 17XNL and transferred to naive mice, 1 in 2193 DCs was infectious (Table 1 and Fig. S2) which, based on numbers of DCs per spleen, equates to 4,559 infectious DC per spleen (Table 1).

To determine if CD317+ DCs harbored Plasmodium after the apparent clearance of infection, cohorts of mice were infected with nonlethal P. chabaudi, which clears patent parasitemia within 20 d followed by recurrent bouts of low-level recrudescence parasitemia (Fig. 5A). Purified CD317+ DCs and infected RBC were taken after 8 d during peak parasitemia and after 20 d, when no parasitized RBC were observed by microscopy, and equal cell numbers (10^5 on day 8 and 10^6 on day 20) transferred to naive recipients. Recipient mice were smeared daily to detect the first appearance of parasites. All mice given RBC or CD317+ DCs taken from mice during peak parasitemia (day 8) developed infections, although the mice given RBC generated higher parasitemia on day 6 posttransfer compared with mice given DC (Fig. 5B). However, when RBC or CD317+ DCs were taken from mice after the apparent clearance of blood-stage infection (day 20), eight of eight mice given 10^6 CD317+ DCs developed ~0.67% parasitemia by day 6, compared with five of six mice that received RBC and only developed ~0.05% (P < 0.0007 ) parasitemia by the same day (Fig. 5B). These data show that during the subpatent stage of infection as measured by blood smears, CD317+ DC can harbor parasites and transfer infection more efficiently than the lower level of infection by RBC.

Cohorts of mice were infected with PbGFP and CD317+ DC isolated after 8 d for assessment by confocal microscopy. Representative confocal z-stack examples show (A) CD317+ DCs with whole (arrows) and degrading (arrowhead) PbGFP, immunostained with LAMP1 (red) and DAPI (blue), reconstructed to give a 3D view. Most Plasmodia are GFP+ but one, adjacent to a late endosome (red), has lost its GFP (arrowhead). (Scale bar, 5 μm.) (B) Cathepsin D-labeled sections show an inverse staining of a weak GFP+ schizont in cathepsin D+ lysosomes (arrowhead) and one with strong GFP that is not in a labeled lysosome (arrow). (Scale bar, 5 μm.) (C) Extracellular merozoites are not the source infection in DC preparations. Ter119+GFP− RBC (pRBC) or GFP+CD11c+Ter119− DCs were from blood or spleen of PbGFP-infected mice were treated with Gey's solution (to free merozoites) and an equal volume of either hyperimmune serum specific for P. berghei (HS) or serum from naive mice (NMS) was added immediately. Cohorts of naive mice were transfused with either 10^6 intact pRBC per mouse, lyzed-equivalent per mouse (ly pRBC, Left) or 10^4 DCs and all mice monitored daily for infection (Right). The data represent one of two experiments with groups of four mice, which had similar results. Error bars shown are Mean parasitemia ± SEM. (D) To calculate the frequency of CD317+ DC that support parasites in vivo, cohorts of mice were infected with PbGFP and GFP+CD317+ DC isolated after 8 d for transfer to naive recipients. Plots of the percentage of uninfected mice versus numbers of GFP+CD317+ DCs or pRBC from PbGFP-infected mice were used to calculate the frequency of infected DC or pRBC. (γ = mx + c was used to calculate the frequency of infectious DCs). The arrows indicate that all mice were infected.

Fig. 3. P. berghei and lyosomal markers within CD317+ DCs. Cohorts of naive mice were infected with PbGFP and CD317+ DC isolated after 7 d for assessment by confocal microscopy. Representative confocal z-stack examples show (A) CD317+ DCs with whole (arrows) and degrading (arrowhead) PbGFP, immunostained with LAMP1 (red) and DAPI (blue), reconstructed to give a 3D view. Most Plasmodia are GFP+ but one, adjacent to a late endosome (red), has lost its GFP (arrowhead). (Scale bar, 5 μm.) (B) Cathepsin D-labeled sections show an inverse staining of a weak GFP+ schizont in cathepsin D+ lysosomes (arrowhead) and one with strong GFP that is not in a labeled lysosome (arrow). (Scale bar, 5 μm.)
markers of PDC, and are heterogeneous in surface expression, but consistent with the infection, we were unable to reliably quantify cell cycle characteristics of infectious DCs in the spleen and following in vitro from naive mice could take up PbGFP from schizont-infected cell cycle with a slow development of trophozoites/schizonts; (a) normal cell cycle of 22 to 24 h comparable to asexual blood stages (26), splenectomy suffer an abrupt onset of malaria, often with no new infections for many years, following radical cure of the infection is the possible survival of parasites within 6 mo (12). One explanation for the effect of splenectomy on splenectomy, those that survived were able to clear the infection (12). Although some monkeys died from high parasitemia after that primate malaria with observations of malaria in primates, where it has been shown from immune surveillance. The observation of blood-stage parasites and trigger these relapses with malaria. Finally, we also suggest that arrested forms would have a significant advantage during peak parasitemia. Finally, we were able to show CD317+ DCs from naive mice could take up PbGFP from schizont-infected RBC, possibly through invasion as opposed to phagocytosis. These parasites within DCs survived for up to 48 h in vitro and transferred infection to naive mice. Unfortunately, because of the very low numbers of infectious DCs in the spleen and following in vitro infection, we were unable to reliably quantify cell cycle characteristics and determine if the parasites had: (i) a very prolonged cell cycle with a slow development of trophozoites/schizonts; (ii) “arrested forms” that can survive for prolonged periods after which a small percentage develop into infective merozoites; or (iii) a normal cell cycle of 22 to 24 h comparable to asexual blood stages in host RBC which constantly reinfect DC. Based on the low infectivity of DCs on transfer to naive mice and the observation that CD317+ DC can harbor parasites and transfer infection at similar levels during peak and subpatent infections, we hypothesize that these DCs may hold arrested forms of parasites that survive for extended periods. If DCs were constantly reinjected, then like RBC, their infectivity would have been higher during peak parasitemia. Finally, we also suggest that arrested forms would have a survival advantage over just having a prolonged cycle.

Table 1. Frequency of CD317+ DC that transfer infectious Plasmodium to naive mice

<table>
<thead>
<tr>
<th>Cell transferred</th>
<th>Frequency of infectious cells</th>
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<tbody>
<tr>
<td>GFP+ RBCs</td>
<td>1/22 Infectious</td>
</tr>
<tr>
<td>GFP+ CD317+ DC</td>
<td>1/8,631 Infectious (360 DC/spleen)</td>
</tr>
<tr>
<td>GFP+ CD317+ DC (10⁴)</td>
<td>No transfer of infection</td>
</tr>
<tr>
<td>GFP+ CD317+ DC (10⁵)</td>
<td>No transfer of infection</td>
</tr>
<tr>
<td>GFP+ CD8+ DC (10⁴)</td>
<td>No transfer of infection</td>
</tr>
<tr>
<td>P. yoelii pRBC</td>
<td>1/22 Infectious</td>
</tr>
<tr>
<td>P. yoelii CD317+ DC</td>
<td>1/2,193 Infectious (4,559 DC/spleen)</td>
</tr>
</tbody>
</table>

Naive mice were infected with PbGFP or P. yoelii 17XNL and CD317+ DCs isolated after 7 d. Titrating numbers (10⁶, 10⁵, 10⁴, 10³, 10²) of sorted GFP+CD317+ DCs, GFP+CD317- DCs, GFP+CD8+ DCs, GFP+ red cells (pRBC) from PbGFP infections or P. yoelii 17XNL pRBC and CD317+ DCs from P. yoelii 17XNL infections were transferred to multiple cohorts of naive mice (n = 5–10 per group), which were then smeared every 2 to 5 d to detect infection for up to 30 d. A plot of the log-percent uninfected mice versus number of transferred cells (Fig. 4D and Fig. S2) was used to calculate the frequency CD317+ DCs required to transfer infection. Data are representative of two to three independent experiments with similar results.

splectomies usually follow splenic trauma. We thus hypothesize that trauma to the spleen may disturb CD317+ DCs to release parasites and trigger these relapses with malaria.

In conclusion, we have found that CD317+ DCs can support the survival of the blood-stage of rodent malaria parasites, P. berghei, P. chabaudi, and P. yoelii 17XNL. Whether DCs are a reservoir for human Plasmodium spp. remains to be determined. Such findings could have important implications for the development of vaccine strategies and potentially new antimalarial therapeutics.

Materials and Methods

Please refer to the SI Materials and Methods for details, materials, and experimental rationale.

Animals. Specific pathogen-free, 6- to 8-wk-old female C57BL/6J mice were obtained from the Animal Resources Centre. All animal procedures were approved and monitored by the Queensland Institute of Medical Research Animal Ethics Committee. This work was conducted under Queensland Institute of Medical Research animal ethics approval number A0209-623M, in strict accordance with the "Australian code of practice for the care and use of animals for scientific purposes" (Australian National Health and Medical Research Council).

Preparation of Spleen Cells Depleted of RBC. Cohorts of 3 to 20 mice were infected intravenously with 10⁸ PbGFP or 10⁹ P. yoelii 17XNL, or P.chabaudi AS-infected RBC. The PbGFP line, as described by Franke-Fayard et al., was shown to express GFP at all previously known blood stages and successfully used to identify infected RBC by FACS (18). Spleens from naive or infected C57BL/6J mice were digested with Collagenase D (Roche Diagnostics) and DNase (Boehringer), as previously described (22). Approximately 1 × 10⁸ pelleted spleen cells were resuspended in 1 to 2 mL of Gey’s solution and incubated on ice for 1 to 3 min, shaking occasionally. The lysis was stopped with Iscove’s Modified Dulbecco’s Media (IMDM; Invitrogen) containing 5% FCS. The digested spleen cell suspension was then incubated with 100 μg/mL Rat IgG, to block FcR binding of subsequently used labeling antibodies.
Isolation of Spleen DCs. Total DC populations were enriched from digested spleen cells with Dynabeads Mouse DC enrichment kit (Dynal) and additional Dynal anti- IgM beads. These cell preparations were then passed through MACS columns on a magnet without any prior labeling to deplete hemozoin, residual schizonts, or any other cells containing hemozoin. The DC-enriched cells were then labeled with anti-CD11c or anti-POCA (CD317) MACS microbeads to isolate total CD11c+ DC or CD317+ DC, respectively (Milenyi Biotech Gmb). To isolate GFP−CD317+ DCs, the CD317+ DCs were labeled with anti-CD11c-APC and Ter119-PE, and using a narrow gate on the pulse-width settings (to exclude doublets), large GFP−CD317+CD11c−Ter119− DCs were sorted on a MoFlo (Beckman Coulter) or FACS Aria III cell sorter (BD Bioscience).

PbGFP Cell Transfer Studies. Titrating numbers of sorted GFP+Ter119 RBC (10−10) or GFP+CD317+CD11c+Ter119− DCs (10−10) per mouse were injected intravenously into cohorts of 5 or 10 naive mice per group. The development of blood-stage infections in these mice was monitored by analysis of Giemsa stained blood films made from tail blood every 1 to 2 d until infection was patent in control groups or up to 3 wk.

P. yoelii 17XL and P. chabaudi Cell Transfer Studies. To isolate CD317+CD11c+ Ter119− DCs, CD317+ DCs were isolated from spleens of infected mice using the method described in the previous sections and CD317+CD11c+Ter119− DCs were then cell-sorted.

Confocal Microscopy. GFP+CD11c+CD317+ DCs (day 7) from PbGFP-infected mice were immobilized on poly-lysine-coated slides (Sigma) and labeled to detect LAMP1 (BD Bioscience), LC3 (ubiquitin-like protein Atg8, which is a marker of autophagy; clone 5F10; Alexis Biochemicals), Cathepsin D, and exclude doublets), large GFP+CD317+CD11c+Ter119− DCs were sorted on a MoFlo (Beckman Coulter) or FACS Aria III cell sorter (BD Bioscience)

Electron Microscopy. DCs were isolated as for light microscopy and then processed for standard EM by fixation in glutaraldehyde, postfixation in potassium ferricyanide-reduced osmium tetroxide and uranyl acetate, and embedding in Epon resin following standard protocols.

Preparation of Schizonts. Mature schizonts were isolated from in vitro culture of synchronized ring forms as described by Janse et al. (19) using Nyodenz.
Supporting Information

Wykes et al. 10.1073/pnas.1108579108

SI Materials and Methods

**Infectious studies.** GFP+ dendritic cells are functionally compromised. To determine if splenic GFP+ dendritic cells (DCs) secreted cytokines relevant to DC function, total CD11c+ DCs were isolated from spleens of mice infected with *Plasmodium berghei* expressing green fluorescent protein (PbGFP) and separated into GFP+ or GFP− DCs using cell sorting. These, or total CD11c+ DCs from naive mice, were cultured unstimulated or stimulated with CpG alone or with CpG and poly IC (to induce secretion of IFN-α). After 36 h, culture supernatants were tested for cytokines. In all three groups, unstimulated DCs secreted low or undetectable levels of cytokines (Fig. S3). Following stimulation, DCs from naive mice secreted IL-12, IL-10, and abundant IFN-α. In comparison, the GFP− DCs did not secrete IL-12 (P < 0.0025), secreted 2- to 3.5-fold more IL-10 (P < 0.0053), and only half the level of IFN-α (P = 0.0544) compared with DCs from naive mice. GFP+ DCs from infected mice secreted lower amounts of all cytokines than DCs from naive mice. These data suggested that all DCs were functionally compromised during a PbGFP infection.

**Gey’s Stock Solution.** Stock A. For Stock A: 1,000 mL: NH₄Cl 35.0 g, KCl 1.85 g, Na₂HPO₄·12 H₂O 1.5 g (or Na₃HPO₄ 0.63 g), KH₂PO₄ 0.12 g, Glucose 5.0 g, Phenol red 50.0 mg. Stock B: For Stock B: 100 mL: MgCl₂·6 H₂O 0.42 g, MgSO₄·7 H₂O 0.14 g, CaCl₂ 0.34 g (or CaCl₂·2 H₂O 0.45 g). Stock C. For Stock C: 100 mL: NaHCO₃ 2.25 g.

**Gey’s working solution (1×).**

- Stock A: 20 parts
- Stock B: 5 parts
- Stock C: 5 Parts
- Distilled H₂O: 70 parts

**Preparation of Spleens Depleted of RBC.** Cohorts of 3 to 20 mice were infected intravenously with 10⁵ PbGFP or 10⁶ Plasmodium yoelii 17XNL or Plasmodium chabaudi AS-infected RBC. The PbGFP line we have used has been described by Franke-Fayard et al. (1) and it has been shown that all of the blood stages express GFP under the constitutive eef1a promoter. Reporter *P. berghei* lines expressing GFP under the eef1a promoter has been successfully used to discriminate all of the different blood stages and to FACS sort/select infected RBC based on GFP-fluorescence. Spleens from naive or infected C57BL/6J mice were digested with Collagenase D (Roche Diagnostics) and DNase (Boehringer), as previously described (2), and the cell suspension was then spun to pellet cells. Approximately 1 × 10⁶ spleen cells were resuspended in 1 to 2 mL of Gey’s solution and incubated on ice for 1 to 3 min, shaking occasionally. The lysis was stopped with Iscove’s Modified Dulbecco’s Media (IMDM; Invitrogen) containing 5% FCS, cells were pelleted and washed again with IMDM before use. The digested spleen cell suspension was then incubated with 100 μg/mL Rat IgG to block FcR binding of labeling antibodies.

**Details on Isolation of Spleen DCs.** Total DC populations were then enriched from total digested spleen cell suspensions by depletion of cells expressing IgM (B cells), CD2 (T cells, B cells, Natural Killer (NK) cells), CD3 (T cells), CD49b (T cells, B cells, macrophage/monocytes NK cells, NKT cells), and Ter119 (RBC) using Dynabeads Mouse DC enrichment kit (Dynal) and additional Dynal anti-IgM beads. This step was included to exclude any residual contaminating red cells and cells other than DC which might express CD317 (also known as BST2, as it is encoded by bone marrow stromal antigen-2 gene and is recognized by Mab PDCA and 120G8) during malaria.

The cell preparations were then passaged through MACS (magnetic-activated cell sorting) columns on a magnet without any prior labeling to deplete hemozoin, residual schizonts, or any other cells containing hemozoin. The DC-enriched cells were then labeled with anti-CD11c or anti-PDCA (CD317) MACS microbeads, as required to isolate total CD11c+ DCs or CD317+ DCs, respectively (Miltenyi Biotec Gmbh). Approximately 10⁹ enriched-DCs, were passed through a Miltenyi LS column, cluted with MACS buffer (PBS with 1% BSA and 20 mM EDTA) away from the magnet and then passed again through two or three more rounds of new columns until the purity was >95%.

To isolate GFP+ CD317+ DCs, the DCs were then labeled with anti–CD11c-APC (to identify DC expressing CD317) and Ter119-PE (binds mouse erythroid cells from early proerythroblast to mature erythrocyte stages). The Ter119 label and the use of a narrow gate on the pulse-width settings (to exclude doublets) on the cell sorter were used to exclude red cells that might be bound to the surface of the DCs. Furthermore, large GFP+CD317+Ter119+ DCs were then sorted on MoFlo (Beckman Coulter) or a FACSaria III cell sorter (BD Bioscience).

**Isolation of Blood DCs.** Blood was collected from mice infected with PbGFP, washed in PBS, and red cells depleted by Gey’s solution. The unlabeled sample was passed through MACS columns to remove hemozoin and residual schizonts. The remaining white cells were incubated with 100 μg/mL Rat IgG and then labeled with MACS anti-CD11c microbeads. Total CD11c+ DCs were then isolated using multiple columns for >95% purity.

**Isolation of GFP+ DCs from Spleens of PbGFP-Infected Mice for Measure of Cytokine Secretion.** Total CD11c+ DCs were enriched from the spleens of PbGFP-infected mice using Dynabeads Mouse DC enrichment kit (Dynal) and additional Dynal anti-IgM beads. This population was then labeled with MACS anti-CD11c microbeads and total CD11c+ DCs isolated using multiple columns for >95% purity. The cells were then sorted on the MoFlo cell sorter for GFP+ and GFP− DCs.

**Flow Cytometry of DCs.** DCs were routinely analyzed for CD11c (HL3), B220, Ly6G (Gr-1), 120G8, Siglec-H and CD8 (H35-17.2) expression using reagents purchased from PharMingen, e-Bioscience, or Biolegend, and PDCA (CD317) purchased from Miltenyi Biotec Gmbh. Samples were analyzed on FACS Calibur, gating on viable cells, using CELLQuest software (Version 3.3). Approximately 10⁷ to 2 × 10⁸ cells from each sample were analyzed.

**EdU Labeling.** To show parasite replication, mice were infected with PbGFP and after 7 d CD317+ cells were isolated by MACS and cultured with 20 μM EdU (5-ethyl- deoxyuridine, a nucleoside analog which is incorporated into replicating DNA; Molecular Probes) for 15 to 18 h in vitro, in IMDM with 5% FCS in polystyrene tubes (Greiner Bio-one) shown to maintain DC viability. The cells were then labeled for fluorescent Allophycocyanin (APC)-labeled EdU according to the instructions in the kit (BD Bioscience) and cell-sorted for GFP+ PE-CD11c+APC-EdU+ cells, as described above.


Fig. S1. Flow cytometry profiles of spleen DCs from mice infected with PbGFP. CD11c+ DCs were isolated from spleens of PbGFP-infected mice, 7 d post-infection. (A) Flow cytometry profiles of GFP+CD11c+DC317+ DCs (dot plot) labeled to show expression of various DC markers in the histogram (thick line) compared with labeling by isotype control antibody (dotted line). (B) Flow cytometry dot plots of DCs from naive mice. (C) Bar chart showing percentages of DC subpopulations that contained PbGFP. (D) Absolute numbers of CD317+ DCs, CD317− DCs, and total DCs per spleen in PbGFP-infected mice. (E) Flow cytometry profiles of total spleen CD11c+ DCs showing uptake of PbGFP and expression of MHC Class II or costimulatory molecules CD80 and CD86. (F) Flow cytometry profiles of total blood CD11c+ DCs showing uptake of PbGFP. Data represents one of multiple independent experiments.
Plots used to calculate the frequency of CD317+ DCs and pRBC that transfer *P. yoelii* 17XNL infections to naive mice. Cohorts of naive mice were infected with *P. yoelii* 17XNL and CD317+ DCs were isolated after 7 d. Titrating numbers (10^6, 10^5, 10^4, 10^3, 10^2, and 10) of CD317+ DCs and infected red cells were transferred to multiple cohorts of 5 to 10 naive mice per group, which were then smeared every 2 to 5 d to detect infection for up to 30 d. A plot of the log percentage of uninfected mice versus number of transferred cells was used to calculate the numbers of CD317+ DCs or infected RBC required to transfer infection. The arrows indicate that all mice were infected.

Secretion of IL-12, IL-10, and IFN-α by CD11c+ DCs from naive and PbGFP-infected mice. Groups of mice were infected with PbGFP and after 7 d total CD11c+ DCs were isolated from their spleens and cell-sorted to isolate GFP+ and GFP− DCs. GFP+ DCs, GFP− DCs, and DCs from naive mice were cultured either unstimulated, with CpG to stimulate secretion of inflammatory cytokines, or CpG and poly IC to stimulate secretion of IFN-α. After 36 h, the supernatants were tested for secretion of IL-12, IL-10, and IFN-α. All data are representative of two independent experiments, which had similar results. Error bars represents ± SEM (n = 3 or 5 independently processed mice per group).
Movie S1. Animated 3D reconstruction of multiple PbGFP parasites within CD317+ DCs. Cohorts of naive mice were infected with PbGFP, CD11c+CD217+GFP+ DCs isolated after 7 d for confocal microscopy after labeling the cells for LAMP1 (red) and DAPI (gray). Confocal z-stacks of DC with multiple PbGFP (green) immunostained with LAMP1 (red) and DAPI (gray) are reconstructed to give an animated 3D view.