Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen–specific T cells

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The capacity of splenic CD11c+ dendritic cell (DC) populations to present antigen (Ag) to T cells differs during malarial infection with *Plasmodium chabaudi* in mice. Both CD11c+CD8+ and CD8− DCs presented malarial peptides on their surface during infection. However, although both DC subsets expressing malaria peptides could induce interferon-γ production by CD4 T cells, only CD8− DCs isolated at the acute phase of infection stimulated Ag–specific T cell proliferation and interleukin (IL)–4 and –10 production from MSP1-specific T cell receptor for Ag transgenic T cells coincidental with our reported Th1 to Th2 switch at this stage in response to the pathogen. The timing of these distinct DC responses coincided with increased levels of apoptosis in the CD8+ population and an increase in the numbers of CD8− DCs in the spleen. Our data suggest that the switch in CD4 T cell responses observed in *P. chabaudi*–infected mice may be the result of the presentation by different DC populations modified by the malaria infection.

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Protective immunity in experimental malaria infections is dependent on CD4 T cells and B cells (1). However, the factors responsible for activation and regulation of these responses and the accompanying pathology (1) are poorly understood.

DCs provide a critical link between innate and adaptive immune responses (2). In addition to different populations such as conventional CD11c+CD8+ and CD8− DCs and plasmacytoid DCs (pDCs; reference 3), there are differences in how DCs are stimulated by pathogens (for review see reference 4). DC populations express distinct pattern recognition receptors such as Toll-like receptors (TLRs) and respond to different microbial products (5), resulting in different immune responses.

CD11c+ DCs are essential for the development of immunity to liver stages of malaria (6), but their role in the immune response to blood stages is not known. *Plasmodium chabaudi*–infected erythrocytes activate mouse BM–derived CD11c+ DCs in vitro (7), and *Plasmodium falciparum* has been shown to induce Flt-3 ligand–induced BM DCs to produce IFN-α (8) and up-regulate CD86 (9). In *P. chabaudi* infections

in mice, there is an increase in the number of CD11c+ DCs in the spleen (10), and the expression of CD80, CD86, and CD40 is up-regulated (11, 12). However, *P. falciparum*– and *P. yoelii*–infected erythrocytes also suppress DC maturation and T cell responses (13, 14). Blood-stage malaria infection stimulates a strong Th1 response that is down-regulated as the infection progresses, resulting in a later Th response that provides help for B cells (Fig. 1; reference 1). Because the T cell response is influenced by its interaction with DCs, it will be critical to determine those DCs that are able to present malaria antigens (Ags) in vivo.

In this study, we investigate the ability of two major subpopulations of splenic CD11c+ DC populations, CD8+ (CD11b−) and CD8− (CD11b+), to present malaria peptides and activate malaria-specific transgenic (Tg) CD4 T cells during a *P. chabaudi* infection. We focus on the Ag MSP1 (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20052450/DC1), which can stimulate protective immunity. CD8− DCs isolated at the infective peak present more MSP1 peptide than CD8+ DCs. Although both subsets of DCs can induce IFN-γ production, only CD8− DCs at this time induced the proliferation of MSP1-specific Tg

The online version of this article contains supplemental material.

CD4 T cells and considerable IL-4 and IL-10. This suggests that the switch from a Th1 to Th2 response later in infection with *P. chabaudi* (Fig. 1; reference 1) can be attributed to the activation of CD4 T cells by a specific subset of splenic DCs.

RESULTS AND DISCUSSION
Presentation of MSP1 T cell epitopes by CD8⁻ and CD8⁺ DCs in vivo during infection

We investigated in vivo processing and presentation by splenic CD11c⁺CD8⁺ and CD8⁻ DCs from *P. chabaudi*–infected mice at different days after a primary infection. Both CD8⁺ and CD8⁻ DC populations were activated in that CD40 and CD86 were rapidly up–regulated by day 5 (before peak infection; see Fig. 1) on both DC populations, whereas the level of MHC class II expression increased noticeably only on CD8⁺ DCs (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20052450/DC1).

To determine their capacity to capture, process, and present malaria Ags in vivo, isolated splenic DCs from mice at different stages of infection were compared for their ability to stimulate two MSP1-specific T cell hybridomas ex vivo. The B5 hybridoma recognizes an epitope (MSP1$_{1,157-1,171}$ located within the p39 fragment of the MSP1 protein), and B7 recognizes MSP1$_{1,690-1,709}$ in the MSP1$_{21}$ fragment (Fig. S1; reference 15). MSP1 peptides were present on both populations of DCs between days 5 and 14 of infection, as shown by their ability to activate the hybridomas (Fig. 2, A and B).

Although both CD11c⁺CD8⁺ and CD8⁻ DCs from *P. chabaudi*–infected mice presented MSP1 peptides to both hybridomas (Fig. 2), they did so with different efficacy and kinetics. At the peak of infection (days 7–11), CD8⁻ DCs were significantly more effective than CD8⁺ DCs (P < 0.05 for days 7–11; Fig. 2, A and B). The different presentation capacity of the DC cannot be explained by MHC class II expression because CD11c⁺CD8⁺ expressed higher levels of class II than CD8⁻ DCs (Fig. S2), and presented synthetic peptide added to the culture with equal efficiency (Fig. 2 A and Fig. S3 C, available at http://www.jem.org/cgi/content/full/jem.20052450/DC1).

Because the dose response of the hybridomas to their peptides is similar (15), a comparison can be made of the relative presentation of B7 and B5 peptides during the infection.
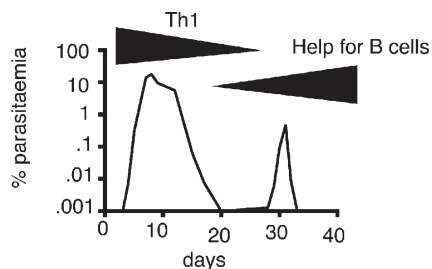

Figure 1. Course of *P. chabaudi* infection showing changes in T cell responses.

The B7 peptide was presented earlier in infection and for longer than the B5 peptide. A small response to the B7 peptide was observed at days 3 (not depicted) and 5 until day 14 (Fig. 2, A and B), whereas B5 was only detected on DCs during peak parasitaemia (days 7–11). This suggests that conditions for uptake, processing, or presentation of the different parts of MSP1 may be different during infection. Phagocytosis by DCs of the membrane-bound region of MSP1 containing the B7 peptide may be more effective than uptake of the B5 peptide, which is located within one of the cleaved fragments of MSP1 and may be more frequently endocytosed as soluble Ag. Alternatively, the fragment, which contains the B7 epitope, is more resistant to proteolysis than the B5 epitope and may be retained longer in the processing compartments, allowing a more effective presentation of the B7 epitope (16).

The difference in presentation of MSP1 peptides between CD8⁺ and CD8⁻ DCs were not intrinsic properties because CD8⁺ DCs isolated from uninfected mice were more effective at presenting MSP1 peptides than CD8⁻ DCs after coculture with schizonts (Fig. S3), suggesting that the malaria infection itself may have changed the relative Ag-presenting capacities of the two populations of CD11c⁺ DCs.

Both subsets of DCs induced IFN–γ production in malaria–specific TCR Tg T cells, but only CD8⁻ DCs from infected mice induced proliferation and high levels of IL–4 and IL–10 production

The presence of MSP1 peptides on DCs from infected mice detected by T cell hybridomas gives no indication of the ability of DCs to induce CD4 T cell responses, such as proliferation and cytokine production. Therefore, we determined whether the two CD11c⁺ splenic DC populations activated different T cell responses from CD4 T cells of Tg mice carrying a TCR specific for the B5 epitope of MSP1 (17). When Tg CD4 T cells were cultured with CD8⁺ and CD8⁻ DCs isolated from malaria-infected mice (7 d after infection), we found that only CD8⁻ DCs induced T cell proliferation (Fig. 3 A). CD8⁺ DCs failed to stimulate proliferation even in the presence of 1 μM of exogenously added B5 peptide (Fig. 3 B). Although CD8⁻ DCs from infected mice induced a >10-fold increase in T cell numbers, the numbers of Tg T cells did not increase during 6 d of coculture with CD8⁺ DCs from infected mice (Fig. 3). Both DC subsets induced IFN-γ production from the T cells, but only CD8⁻ DCs led to the development of IL-4 and IL-10 production in T cells. Importantly, when cell recovery was taken into account, CD8⁻ DCs induced considerably more of both IL-2 and IFN-γ (Fig. 3 A).

The more effective stimulation of Tg T cells by CD8⁻ DCs during infection is unlikely to be caused by intrinsic differences in their potential to present peptide to Tg T cells, as the differences were only apparent in DCs obtained from infected mice; both naive CD8⁺ and CD8⁻ DCs cocultured
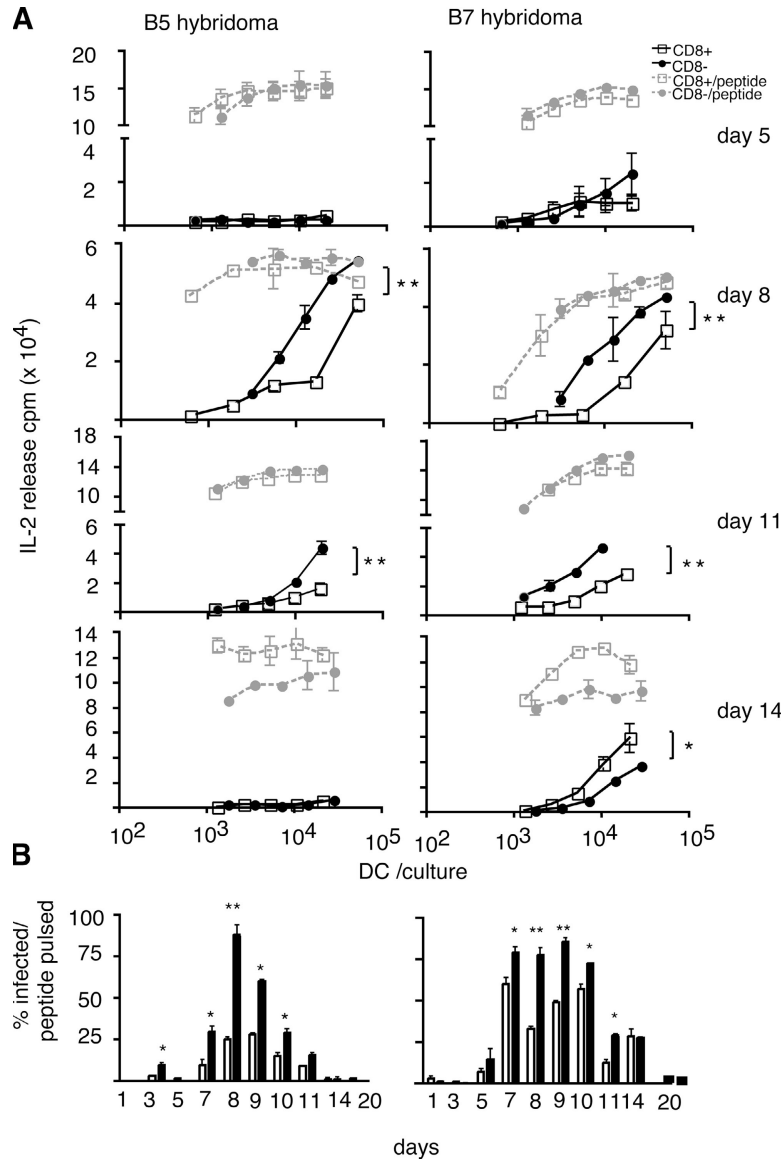
Figure 2. CD8⁻ DCs present MSP1 peptides more efficiently than CD8⁺ DCs in vivo at peak parasitaemia. (A) CD11c^high DCs were enriched from spleens of mice at 5, 8, 11, and 14 d after infection with 10⁵ *P. chabaudi*, sorted into CD8⁺ (open squares) or CD8⁻ DCs (closed circles), and cultured with 2 × 10⁴ B5 or B7 hybridoma cells for 24 h. IL-2 production was measured as described in Materials and methods. As a control for presentation capacity, the same number of DCs from infected mice were cultured in the presence of 1 μM of peptide (dotted lines). The data represent means and SEM (>10%; error bars) of triplicate samples. Statistical analysis was performed as described in Materials and methods. (B) The T cell hybridoma response to MSP1 peptides on splenic DCs from infected mice represented as a fraction of the maximal response to DCs that were pulsed with excess exogenous peptide. Sorted CD8⁺ (white bars) and CD8⁻ (black bars) DCs were cultured, and IL-2 was measured as described in A. Responses shown (at 10⁴ DCs) are the means of maximum responses to exogenously pulsed peptide with the SEM of triplicate samples. The data are from a representative experiment of three performed. *, P = 0.05–0.01; **, P = 0.01–0.001; and ***, P < 0.001 denote significant differences.

with 1 μM of schizonts or peptide in vitro induced the proliferation of Tg T cells (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20052450/DC1). The culture of either DC subset with schizonts but not peptide led to IL-4 production, possibly via additional signals to the DC provided by the infected RBC. However, naive CD8⁺ and CD8⁻ DCs induced equivalent amounts of IL-10 in the presence of peptide.

The effects seen in vivo were also unlikely to be caused by limiting peptide on CD8⁺ DCs because MHC class II expression on CD8⁺ DCs is greater than CD8⁻ (Fig S2). Both infected CD8⁺ and CD8⁻ DCs present to the B5 hybridoma (Fig. 2), and, most importantly, CD8⁺ DCs from infected mice pulsed with 1 μM of exogenous peptide were still unable to induce proliferation in the Tg T cells and induced considerably less cytokine (Fig. 3 B).
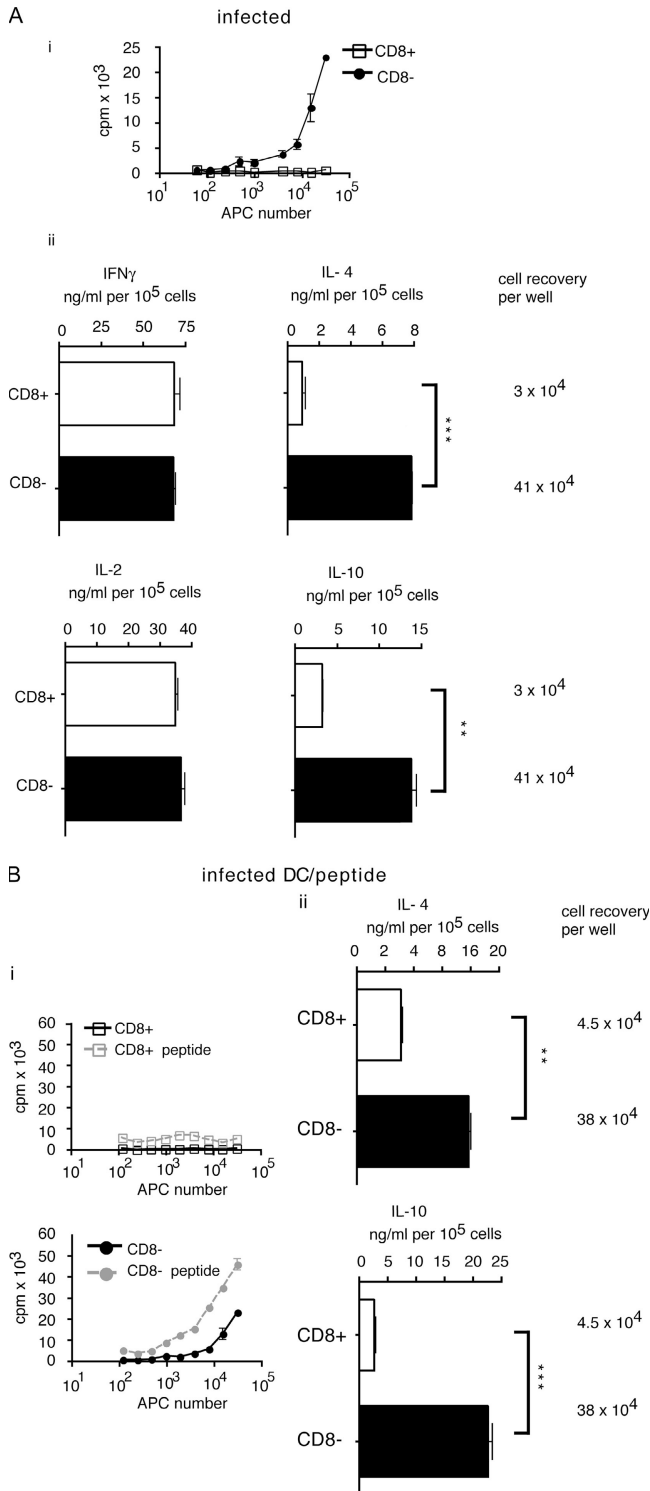
A

infected

i

ii

B

infected DC/peptide

Figure 3. CD8⁻ but not CD8⁺ DCs from infected mice induced proliferation and high levels of IL–4 and IL-10 production in specific Tg CD4 T cells. (A) CD11c⁺CD8⁺ (open squares) and CD8⁻ (closed circles) DCs from mice 7 d after infection with *P. chabaudi* (i) were cultured in the presence of 10⁵ Tg T cells for 3 d, and the proliferative response was determined. CD8⁺ (white bars) and CD8⁻ (black bars) splenic DCs from mice 7 d after inoculation with *P. chabaudi* (ii) were cultured in the presence of enriched CD4 B5 T cells. Graphs shown are the ELISAs of

Increase in splenic CD11c⁺CD8⁻ DC numbers during infection

At the peak of infection, the spleen contains a large number of CD11c⁺ cells (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20052450/DC1), which mostly comprise the CD8⁻ subpopulation. The increase in the number of CD8⁻ DCs was quantified by FACS analysis. During a *P. chabaudi* infection, there is a 20-fold increase in spleen cell numbers (18). In this study, we show a concomitant increase in the total number of CD11c⁺ DCs (Fig. 4 B), with the greatest increases observed in the CD8⁻ populations (which includes the population presenting MSP1; Fig. 4, A and B). The majority of the CD8⁻ DCs in the spleen during *P. chabaudi* infection expressed intermediate levels of CD11c (Fig. 4, A and B) and were distinct from pDCs, as >90% did not stain with the pDC-specific antibody (Ab) 120G8 (not depicted; reference 19). However, they expressed CD11b, intermediate levels of GR-1 and F4/80, as well as MHC class II (unpublished data) similar to inflammatory DCs, which migrate into the spleen in mice infected with *Listeria monocytogenes* (20). Lymphotoxin α1β2 produced in the spleen can induce the expansion of the CD8⁻ DC subpopulation from splenic DC precursors (21), suggesting that in addition to the influx of new DCs, the generation of CD8⁻ DCs may take place in the spleen during infection.

Apoptosis is greater in CD8⁺ than in CD8⁻ splenic DCs during infection

CD11c⁺CD8⁺ DCs have a shorter lifespan than CD8⁻ DCs (22). This could influence the ability of DCs to process and present Ags in vivo, with concomitant effects on the subsequent T cell response. To address this, we assessed cell death in the CD11c⁺CD8⁺ and CD8⁻ DC population during the course of infection using 7AAD as a marker of apoptosis (Fig. 4 C). The greatest increase in cell death was observed among CD8⁺ DCs; within 5 d of infection, >50% of the CD11c⁺CD8⁺ DCs were apoptotic. This number declined as parasite numbers decreased (day 20), suggesting that high parasitaemia was associated with the selective death of CD8⁺ DCs. In contrast, there was little increase in the number of apoptotic CD8⁻ DCs. The 7AAD⁺ CD8⁺ DC also expressed Annexin V on the cell surface (Fig. 4 D). This indicated that 7AAD staining was not caused by live CD8⁺ DCs endocytosing apoptotic cells. Therefore, it is possible that CD8⁺ DCs may have endocytosed Ags early in infection, undergone apoptosis, and been replaced by fresh DCs. Their inability to support proliferation or significant IL-4 (P < 0.001) or IL-10 (P = 0.01–0.001) production of CD4

the mean values of a representative experiment (of three performed) with the SEM (error bars) of triplicate samples. Asterisks denote significant differences. (B) CD8⁺ DCs from infected mice cocultured with peptide induce significantly lower levels of IL-4 and IL-10 and fail to induce proliferation in Tg T cells. Sorted DCs from infected mice were cultured in the presence of 1 μM B5 peptide and B5 T cells and tested for Ag-specific proliferation (i) and cytokine production (ii) as in A.
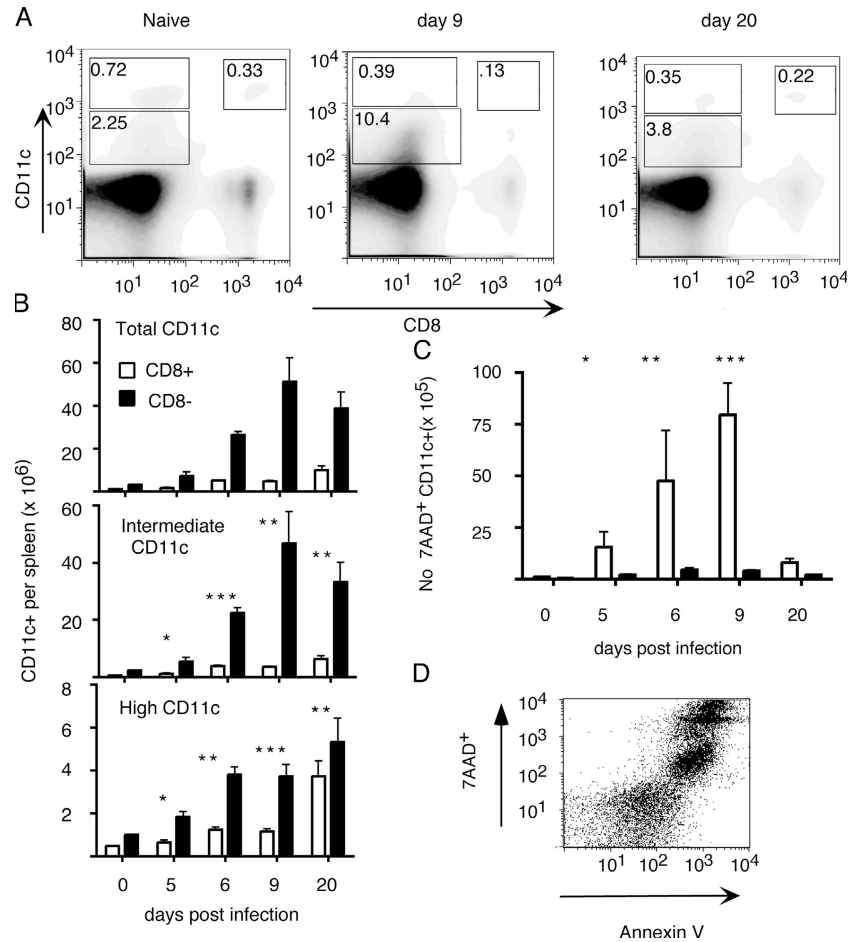
Figure 4. Increase in the numbers of CD11c⁺CD8⁻ DCs in spleens during infection with *P. chabaudi*. BALB/c mice were infected with 10⁵ *P. chabaudi*, killed on different days after infection, and stained with anti–CD11c and CD8 Abs. (A) Representative FACS plot of DCs from naive mice at day 9 and 20 after infection. Numbers in boxes refer to the percentages of cells in each gate. (B) CD11c⁺CD8⁺ DCs (white bars) and CD11c⁺CD8⁻ DCs (black bars) in spleens of naive mice and mice on days 5, 6, 9, and 20 after infection; total number of CD11c⁺ cells (top), cells with intermediate levels of CD11c⁺ (middle), and cells with high surface expression of CD11c (bottom). Isotype controls on both cell populations showed no staining at any time during infection. The numbers represent the means and SEM (>10%; error bars) of three mice. (C) Splenic CD11c⁺CD8⁺ DCs are more apoptotic than CD8⁻ DCs in spleens of mice infected with *P. chabaudi*. The number of 7AAD-positive CD11cʰⁱᵍʰ CD8⁺ (white bars) and CD11cʰⁱᵍʰ CD8⁻ DCs (black bars) in spleens of naive mice and mice infected for days 5, 6, 9, and 20. The data are means and SEM (>10%) of three mice and are representative of three experiments. Asterisks denote that the differences are significant. (D) Representative FACS plot of CD11c⁺CD8⁺ DCs stained with 7AAD and Annexin V from mice 9 d after infection.

T cells may be the result of apoptosis taking place before providing the signals for the induction of T cell proliferation and cytokine production. Engagement of TLR signaling pathways such as MyD88 have been shown to lead to apoptosis (23), and products of malaria-infected RBCs such as haemozoin and glycosyl-phosphatidylinositol may act through TLR9 and TLR2 (9). It remains to be directly demonstrated whether the engagement of these TLRs induces apoptosis in CD8⁺ DCs and, thus, the loss of stimulatory effects on T cells.

Interestingly, at day 14 after infection, the CD11c⁺CD8⁺ DC presented the B7 epitope and synthetic peptides better than the CD8⁻ DC. This may indicate that at this stage of infection, the spleen has been repopulated with fresh CD8⁺

DCs or that the cytokine environment at this time does not induce apoptosis in CD8⁺ DCs.

The most striking finding in our studies was that CD8⁻ DCs were the only conventional DC population in the acute stage of infection able to drive MSP1-specific Tg CD4 T cells into proliferation and production of high levels of IL-4 and IL-10. This is similar to observations in *Leishmania*-infected BALB/c mice in which CD8⁻ DCs were also responsible for Th2 cytokine induction (24). In this case, a nonprotective T cell response was observed. In malaria infection, however, it represents a change in the immune response, with time to allow both a protective Th1 response and B cell help from Th2 cytokines.

It has been previously proposed that different DC populations induce Th1 or Th2 cytokine production in T cells (25).

However, in our experiments, the capacity of DCs to activate and induce cytokines in T cells was associated with infection and was not an intrinsic property.

An effective Ag dose is a major factor influencing T cell responses. DCs incubated with low doses of peptide favor a Th2 response, whereas high doses of Ag induce a Th1 response (5). Therefore, variations in MSP1 peptide expression on DC subsets may be important for the differentiation of CD4 T cells. However, Ag dose in our case is unlikely to be the explanation, as the differences in T cell activation and cytokine production in response to the two DC populations from infected mice were observed at peak parasitaemia, when Ag dose is high. The responses of the T cell hybridomas suggested, in fact, that the amount of peptide was greater on CD8⁻ DCs than CD8⁺ cells despite the fact that they induced an IL-4 production in the Tg CD4 T cells. Furthermore, the addition of exogenous peptide to DCs from infected mice resulted in a greater IL-4 response of Tg T cells only with CD8⁻ DCs (Fig. 3 B). These cytokine responses induced by CD11c⁺CD8⁻ DCs are more in line with the idea that the changing environment in the spleen during infection, the cytokine milieu, and the activation state of DCs are the major contributing factors. It has been found that Th polarization correlated with the state of the DCs induced by different pathogens. BM DCs treated with *Proprionebacterium acnes* induces Th1 cytokines, but, on the other hand, the same populations stimulated with soluble egg Ag activate a Th2-type response (26, 27). Recently, a CD11c^low population with immune regulatory properties was identified in the spleens of mice infected with *Leishmania* (28). Indeed, the CD8⁻ DCs from malaria-infected spleens generated high levels of IL-10 production in the Tg T cells.

Our results suggest that populations of CD11c⁺ DCs have different but overlapping roles in malaria infection. Initial uptake and presentation to T cells is performed by CD8⁺ DCs, which then undergo apoptosis as part of a normal regulatory mechanism, limiting a potentially damaging immune response. Although both DCs can induce the production of IFN-γ, CD8⁻ DCs in the spleen could play a later role in Ag presentation and may support the Th2 and CD4 T cell–mediated B cell helper response observed in this infection (1). The increase in the CD8⁻ DC population in the spleen could also be important in the down-modulation of the immune response to malaria.

MATERIALS AND METHODS

Mice and parasites. Female BALB/c mice and B5 MSP-1–specific TCR Tg mice (6–12 wk; reference 17) were bred in the specific pathogen-free unit at the National Institute for Medical Research. *P. chabaudi chabaudi* (AS) infections were initiated by i.p. injection of 10⁵ of parasitized RBCs and monitored as described previously (7). All mouse studies were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. For the preparation of schizont-infected RBCs, blood was taken 7 d after infection and purified as described previously (>90% schizont- or late trophozoite-stage infected RBCs; reference 7).

Cell lines and peptides. CD4⁺ T cell hybridomas B5 and B7, which are specific for peptides of *P. chabaudi* MSP1, have been described previously (Fig. S1; reference 15).

Flow cytometry. The mAbs used were anti-CD8 FITC, CD8 PE, CD86 PE, CD40 PE, CD11c APC, H-2A^d FITC, and isotype control Abs (BD Biosciences). Annexin V (BD Biosciences) and 7-AAD (1 μg/2.5 × 10⁵ cells) were used to detect apoptotic cells.

Spleen cells from *P. chabaudi*–infected and naive mice were prepared, stained with Abs, and analyzed as described previously (18). Flow cytometry samples were acquired on a FACScalibur and analyzed with CellQuest software (BD Biosciences).

DC enrichment and cell sorting. Spleens from naive or *P. chabaudi*–infected mice were treated for 30 min at 37°C with 0.4 mg/ml Liberase CI (Boehringer). After RBC lysis, spleen cells were enriched using CD11c microbeads (Miltenyi Biotec) and labeled with anti-CD11c APCs and -CD8 FITC Abs (5) before sorting for viable CD11c^high DCs (>90% purity) on a MoFlo cytometer (DakoCytomation).

Ag presentation assays using B5 and B7 T cell hybridomas. Different numbers of sorted DCs from mice at different times of infection were incubated with 2 × 10⁴ B5 and B7 CD4 T cell hybridoma cells for 24 h in Iscove's modified Dulbecco's medium (Sigma-Aldrich), supernatants were added to CTLL-2 cells, and proliferation (as a measure of the IL-2 produced) was determined (15). As positive controls, 1 μM of the respective peptide was added to the cultures. The IL-2 response of the hybridomas reflected the amount of peptide presented after processing, and comparison with the maximum IL-2 response obtained when DCs are pulsed with a saturating dose of the synthetic peptide indicates the magnitude of the response.

Ag-specific proliferation. CD4⁺ splenic T cells were isolated (>95% purity) from spleens of B5 Tg mice using anti-CD4 beads (Miltenyi Biotec). 10⁵ cells were then cultured with sorted DCs for 3 d in 200 μl in 96-well round-bottom plates and pulsed with ³H-thymidine (GE Healthcare) as described previously (17).

Cytokine production. 5 × 10⁴ sorted DCs were cultured in 200 μl in 96-well round-bottom plates for 6 d with 10⁵ CD4⁺ T cells purified from the spleens of Tg B5 mice as described previously (17). The cells were washed, and 10⁵ cells were further cultured with 5 μg/ml of cross-linked anti-CD3 and 2 μg/ml anti-CD28 Ab in 250 μl in flat-bottomed 96-well plates as described previously (26). IL-2 was determined in the supernatant after 24 h, and IFN-γ, IL-4, and IL-10 was determined after 48 h of culture by ELISA as described previously (7).

Statistical analysis. Statistical analysis was performed using Student's *t* tests for unpaired samples (two tailed). *, P = 0.05–0.01; **, P = 0.01–0.001; and ***, P < 0.001.

Online supplemental material. Fig. S1 shows the B5 and B7 T cell epitopes of MSP1 protein. Fig. S2 shows the expression of MHC class II and co-stimulatory molecules CD86 and CD40 on CD11c⁺CD8⁺ and CD8⁻ DCs during a primary infection with *P. chabaudi*. Fig. S3 shows that CD8⁺ DCs from uninfected mice presented MSP1 peptides more efficiently than CD8⁻ DCs in vitro. Fig. S4 shows that both CD8⁺ and CD8⁻ DCs from uninfected mice induced proliferation and cytokine production in malaria-specific Tg T cells. Fig. S5 shows the location and increase in the numbers of CD11c⁺ DCs in the spleen of mice infected with *P. chabaudi*. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052450/DC1.

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