Dendritic Cells Induce the Expansion of Regulatory Cells during Plasmodium Infection and Endotoxic Shock

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Plasmodium Infection and Endotoxic Shock Induce the Expansion of Regulatory Dendritic Cells

Kurt A. Wong and Ana Rodriguez

During an acute Plasmodium infection, uncontrolled proinflammatory responses can cause morbidity and mortality. Regulation of this response is required to prevent immunopathology. We therefore decided to investigate a recently characterized subset of regulatory dendritic cells (DCs) that expresses low levels of CD11c and high levels of CD45RB. During a Plasmodium yoelii infection, these regulatory CD11clowCD45RBhigh DCs become the prevalent CD11c-expressing cells in the spleen, overtaking the conventional CD11chigh DCs. Furthermore, the regulatory CD11clowCD45RBhigh DCs induce IL-10-expressing CD4 T cells. A similar change in splenic DC subsets is seen when mice are injected with sublethal doses of LPS, suggesting that shifting the splenic DC subsets in favor of regulatory CD11clowCD45RBhigh DCs can be triggered solely by a high inflammatory stimulus. This is the first time regulatory DCs have been observed in a natural immune response to an infectious disease or endotoxic shock. The Journal of Immunology, 2008, 180: 716–726.

The encounter of dendritic cells (DCs) with microbes or microbial products results in activation and maturation of the DCs, leading to optimal Ag-presenting function and activation of T cells. Part of this process involves the induction of a proinflammatory cytokine response that helps to activate T cells in appropriate ways to counter the pathogen. As the immune response progresses, there is a need to inhibit the inflammatory response to prevent immunopathology (1). Although there has been a lot of research demonstrating the involvement of anti-inflammatory cytokines, such as IL-10, in the dampening of the inflammatory response, little is known about the host cells involved in this process.

Malaria is a devastating disease caused by the Plasmodium parasite, killing one person every 30 s and causing between 300 and 500 million clinical cases every year. Malaria creates a parasite, killing one person every 30 s and causing between 300 and 500 million clinical cases every year. Malaria creates a parasite, killing one person every 30 s and causing between 300 and 500 million clinical cases every year. Malaria creates a parasite, killing one person every 30 s and causing between 300 and 500 million clinical cases every year. Malaria creates a parasite, killing one person every 30 s and causing between 300 and 500 million clinical cases every year. Malaria creates a parasite, killing one person every 30 s and causing between 300 and 500 million clinical cases every year. Malaria creates a parasite, killing one person every 30 s and causing between 300 and 500 million clinical cases every year. 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The splenic DC compartment is a heterogeneous population, with subsets of cells differing across a spectrum of functions and morphologies (23). However, malaria researchers have largely treated splenic DCs as a single, homogeneous population. Only recently, the possibilities of DC subsets playing different roles during the course of a murine Plasmodium infection have been explored (13, 14, 16, 18), yet these reports have only considered conventional DCs which are identified by their high expression of the integrin α-chain CD11c. To attempt to reconcile the different DC phenotypes as seen by different research groups, we chose the context of a nonlethal murine Plasmodium infection that we had previously characterized in detail (8–10) and decided to turn our attention to CD11clow-expressing DCs, a largely neglected subset of DCs.

Recently, several reports have discussed the potential regulatory function of a DC subset characterized by its particular CD11clowCD45RBhigh surface marker expression (24–27). Although originally derived by the addition of IL-10 to in vitro-differentiation cultures of bone marrow-derived DCs, CD11clowCD45RBhigh DCs were also observed to be resident in the spleens of mice (24, 27). They display a characteristic cytokine profile, secreting high levels of IL-10 without IL-12 when stimulated. They also express lower levels of CD86 and class II MHC than their CD11chigh counterparts (24, 27). The CD11clowCD45RBhigh DCs earned their regulatory label through their capacity to induce regulatory T cells in immunity. Moreover, there are also numerous studies that describe immune suppression as a result of Plasmodium infections (3).

DCs play an integral role in immunity by bridging the gap between the innate and adaptive immune systems. As the most potent APC, DCs are the primary cell responsible for launching and coordinating appropriate immune responses to pathogens and priming memory so that the organism is protected against future infections (1). In playing such a central role in immunity, it is not surprising that the function of DCs is targeted by the parasite. Indeed, several studies in both humans (5, 6) and mice (7–13, 21) have documented changes in DC function in response to Plasmodium. In contrast, other reports have found fully functional DCs when exposed to parasites (14–20). Therefore, a consensus is lacking on how DCs are regulating T cell responses during an infection (22).

Abbreviations used in this paper: DC, dendritic cell; iRBC, infected erythrocyte.
vitro and in vivo (24–26). Moreover, when regulatory CD11c<sup>low</sup> CD45RB<sup>high</sup> DCs are differentiated in vitro and transferred into mice, they induce Ag-specific tolerance (24–26) and suppress LPS-induced host inflammatory responses (27). Based on these properties, regulatory CD11c<sup>low</sup>CD45RB<sup>high</sup> DCs have been proposed as potential therapeutic tools for the treatment of inflammatory diseases (26, 27).

We have found that this regulatory CD11c<sup>low</sup>CD45RB<sup>high</sup> DC subset becomes the predominant CD11c<sup>+</sup>-expressing cell population in the spleen during an acute *Plasmodium yoelii* infection, overtaking the conventional CD11c<sup>high</sup> DCs. Furthermore, a similar rapid expansion of the regulatory DCs and contraction of the conventional DCs occurs after the administration of high doses of LPS to mice. This is first time regulatory CD11c<sup>low</sup>CD45RB<sup>high</sup> DCs have been observed over the course of a natural immune response to infection.

Materials and Methods

**Mice**

Female BALB/c, Swiss Webster, and C57BL/6 mice were purchased from the National Institutes of Health. Female DO11.10 TCR-transgenic mice were purchased from The Jackson Laboratory. All mice were housed in the Medical Parasitology animal facility, and all experiments were approved by the Institutional Animal Care and Use Committee.

**Parasites, infections, and endotoxin injection**

Nonlethal strain *P. yoelii* 17XNL-infected erythrocytes were harvested by cardiac puncture of infected, anesthetized Swiss Webster mice before the peak in parasitemia. Erythrocytes were washed twice with PBS and separated from white blood cells by centrifugation at 2000 × g for 3 min. Erythrocytes were then spun on an Accucentr (Accurate Chemical & Scientific) gradient to isolate schizonts and late trophozoite stage-infected erythrocytes. The collected infected erythrocytes were washed and resuspended in PBS. Infected erythrocytes of lethal strains *Plasmodium berghei ANN* and *P. yoelii* YM were obtained by bleeding mice from the tail and then diluting to the appropriate infected erythrocyte (IREC) concentration based on parasitemia counts. To start blood-stage infections, BALB/c and C57BL/6 mice were injected i.p. with infected erythrocytes (10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> cells/mouse) resuspended in PBS. Uninfected erythrocytes as controls were obtained in a similar manner from naive Swiss Webster mice. *P. yoelii* 17XNL sporozoites were obtained from the dissection of infected *Anopheles stephensi* mosquito salivary glands. To infect BALB/c mice, sporozoites (10, 100, or 1000/mouse) resuspended in RPMI 1640 medium were injected i.v. in the tail vein. Injection of RPMI 1640 medium was used as controls, respectively. In the group that received 10 sporozoites, only four of six mice became infected, indicating that we were infecting mice with a very low numbers of sporozoites.

To evaluate parasitemia, thin blood smears were made by bleeding mice from the tail. Smears were stained with KaryoMAX Giemsa stain (Invitrogen Life Technologies) and all experiments were approved by the Institutional Animal Care and Use Committee.

**Results**

Sorted DCs were cultured in various ratios with DO11.10 CD T cells (8 × 10<sup>5</sup>) and OVA<sub>233–241</sub> peptide (10 μg/mL; Peptides International) in 96-well U-bottom plates and incubated at 37°C in 5% CO<sub>2</sub>. After 80 h, culture media were harvested and analyzed using the BD Cytometric Bead Array (BD Biosciences) as instructed by the manufacturer.

**Flow cytometry**

All flow cytometry, outside of the cell sorting, was performed on a FACS Calibur (BD Biosciences) and analyzed with either CellQuest (BD Biosciences) or FlowJo (Tree Star). All Abs for FACS were purchased from Biolegend or BD Biosciences, unless otherwise indicated.

For analysis of spleen DCs, single-cell suspensions of splenocytes were first blocked with anti-CD16/CD32 (FcγRII/II receptor; 2.4G2), then stained using a combination of FITC anti-CD40 (3/23), FITC anti-CD80 (16-10A1), FITC anti-CD86 (GL1), PE anti-CD45RB (16A), and allophycocyanin anti-CD11c (HL3) and N 48/14). Isotype-matched Abs were used as staining controls. mPDCA-1 staining was performed using biotinylated anti-mPDCA-1 (JF05-1C2.4.1, Miltenyi Biotec) followed by Cy5-PE-conjugated streptavidin (Biolegend).

For T cell surface marker analysis, cells from 7-day DC-T cell cocultures were harvested, blocked with anti-CD16/CD32, then stained using a combination of FITC anti-CD44 (IM7), FITC anti-CD25 (PC61), FITC anti-CD69 (H1.2F3), PE anti-LFA-1 (2D7), PE anti-CD62L (MEL-14), PE anti-CTLA-4 (UC10-4F10-11), Cy5PE anti-CD4 (GK1.5), and allophycocyanin anti-CD3ε (145-2C11). For intracellular cytokine staining of T cells, cells from 7-day DC-T cell cocultures were restimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) alongside monensin (10 μg/ml) (all from Sigma-Aldrich) for 5 h. After harvesting, cells were blocked with anti-CD16/CD32, then stained using Cy5PE anti-CD4 and allophycocyanin anti-CD3ε. After fixation and permeabilization using Foxp3 staining buffer reagents (eBioscience) as instructed by the manufacturer, cells were incubated with FITC anti-IFN-γ (XMGl1.2), PE anti-IL-10 (JES5-16E3), and Alexa Fluor 488 anti-IL-4 (11B11). Isotype-matched Abs were used as staining controls.

**Statistical analysis**

Data were analyzed using Prism (GraphPad). Student’s t tests and one- or two-way ANOVA were performed as mentioned. All statistics were considered significant if p < 0.05.
the marker, although it is indeed present on 10% of the CD11clowCD45RBhigh cells from naive mice (Fig. 1E).

Because of the splenomegaly induced by P. yoelii infections, which results in an increase of 10- to 15-fold in the number of total cells in the spleen (Fig. 1F), we calculated the absolute number of these two subpopulations in the spleen. The number of regulatory CD11c<sup>hi</sup>CD45RB<sup>hi</sup> DCs increased over 30-fold during the 14 days of acute infection. In contrast, the number of conventional CD11c<sup>hi</sup> DCs only increased 3-fold by day 10 (Fig. 1G). This indicates that the ratio of regulatory vs conventional DCs has changed from 3:1 in control mice to 28:1 at day 6 after infection, before the peak of parasitemia at day 10 (Fig. 1H).

Regulatory DCs do not up-regulate surface expression of CD40 over the course of an acute P. yoelii infection

We also determined the maturation phenotype of the splenic DCs as a whole or analyzed separately in the subpopulations described above in Fig. 1A. Cells were assayed for their expression of costimulatory molecules CD40, CD80, and CD86 since up-regulation of these molecules is considered an indicator of DC maturation (1). CD11c<sup>hi</sup>
DCs expressed increased levels of all three costimulatory markers over the course of infection (Fig. 2A, left panels), confirming that this subset correlates with the conventional DC population (24). Regulatory CD11c\textsuperscript{low}CD45RB\textsuperscript{high} DCs, although increasing their CD80 and CD86 early in the acute infection, only had CD80 up-regulated on day 10. More striking was that regulatory DCs did not up-regulate CD40 at any point during the acute infection. In fact, expression of CD40 on this subset dropped below the levels found in the uninfected control mice late in infection (Fig. 2, A, center panels, and B). When analyzing the entire DC population (CD11c\textsuperscript{+} cells), their expression of costimulatory molecules appeared to mirror that of the regulatory CD11c\textsuperscript{low}CD45RB\textsuperscript{high} subpopulation, with the reduction of CD86 to background levels by day 10, and with very poor up-regulation of CD40 at any point during the infection (Fig. 2A, right panels). This is a consequence of the fact that the regulatory subset is the dominant DC population in the spleen (Fig. 1B).

**FIGURE 2.** Regulatory CD11c\textsuperscript{low}CD45RB\textsuperscript{high} DCs up-regulate CD80 and CD86, but not CD40. Mice were injected i.p. with 10\textsuperscript{6} control or infected erythrocytes. At the indicated days postinjection, spleen CD11c\textsuperscript{+} DC subsets were analyzed for their expression of the costimulatory molecules. A, CD40, CD80, and CD86 expression on DCs from control (RBC, ●) or infected (iRBC, □) mice, expressed as mean fluorescence intensity. Error bars represent SD within groups of five mice (*, p < 0.05; **, p < 0.01; ***, p < 0.001 when comparing iRBC to RBC by two-way ANOVA). B, FACS plots showing expression of CD40 on DCs from control (gray histogram) and infected (open histogram with heavy line) mice. An isotype-matched control was also analyzed (thin gray line). Parasitemia were similar to those indicated in Fig. 1. The results are representative of two independent experiments.

Regulatory CD11c\textsuperscript{low}CD45RB\textsuperscript{high} DCs induce proliferation and IL-10 expression in Ag-specific CD4 T cells

To functionally characterize the subsets of DCs analyzed, we determined the capacity of each subset to activate Ag-specific CD4 T cells in vitro. The two subpopulations of DCs as described above in Fig. 1A were FACS sorted from the spleens of mice at day 10 postinfection and cocultured with OVA-specific CD4 T cells. These CD4 T cells, purified from the spleens of DO11.10 mice, express a specific TCR that recognizes the CD4 OVA\textsuperscript{323–339} epitope. We also sorted the two DC subpopulations from naive littermates to determine whether they acted differently within the context of a *Plasmodium* infection. When assayed for proliferation after 4 days of peptide-induced stimulation, both DC subsets from naive and infected mice were equally capable of inducing proliferation from the OVA-specific T cells (Fig. 3A). Indeed, similar levels of T cell proliferation are induced by conventional and regulatory DCs in response to peptide (24).
T cells were also assayed for their cell surface expression of T cell activation molecules after coincubation with either the conventional CD11c<sup>high</sup> or the regulatory CD11c<sup>low</sup>CD45RB<sup>high</sup> DC subsets in the presence of the OVA epitope. T cells demonstrated similar activation phenotypes when looking at the activation markers CD25, CD44, CD69, and LFA-1 after 24 h or 7 days of coinoculation with DCs sorted from infected (B) or naive (C) mice. T cells were collected and assayed via flow cytometry for markers of T cell activation. FACS histograms show the expression of the surface marker when T cells are cocultured with CD11c<sup>high</sup> DCs (thick line) or CD11c<sup>low</sup>CD45RB<sup>high</sup> DCs (thin line). Stainings with isotype control Abs are shown in the gray histograms. Each FACS plot is representative of one of three individual mice. D, After 7 days of coculture with sorted DCs from day 0 (naive) or day 10-infected mice, cells were restimulated with PMA and ionomycin for 5 h. CD4 T cells were stained intracellularly for cytokine expression. Isotype controls for the cytokine-specific Abs are shown as FACS histograms, as detailed in B. Each FACS plot is representative of one of three DC-T cell cocultures and two independent experiments.

To get a better functional phenotype of the T cells being activated by conventional and regulatory DCs, we looked at the intracellular cytokine expression of the T cells after 7 days coculture with the separate DC subsets from infected and naive mice. When the OVA-specific CD4 T cells were stained for expression of intracellular cytokines after 7 days incubation, a large proportion of T cells cocultured with conventional DCs from day 10-infected mice expressed high amounts of IFN-γ without IL-10 (33.1 ± 2.4% of CD4 T cells). If instead the CD4 T cells were cocultured with regulatory DCs, the proportion of T cells expressing IFN-γ without IL-10 was decreased by half (15.3 ± 0.1%, p < 0.0005). In contrast, regulatory DCs from day 10-infected mice induced four times as many T cells to express IL-10 without IFN-γ (35.1 ± 1.3% of CD4 T cells by regulatory DCs vs 8.1 ± 2.4% by conventional DCs, p < 0.00001; Fig. 3D, day 10, top panels). Moreover, coculture with regulatory DCs induced twice
the number of CD4 T cells expressing IL-10 without IL-4 (52.0 ± 0.9% by regulatory DCs vs 21.9 ± 3.0% by conventional DCs, *p* < 0.0001; Fig. 3D, day 10, bottom panels). Cocultures with DCs isolated from naive mice (day 0) demonstrated similar trends, however in a much more polarized fashion. Conventional DCs induce 15 times as many CD4 T cells to express IFN-γ without IL-10, whereas regulatory DCs induce IL-10 without IFN-γ from nearly 70% of the T cells (Fig. 3D, day 0, top panels). Notably, naive regulatory DCs induced coexpression of IL-4 and IL-10 from a good proportion of CD4 T cells (15.3 ± 1.3% of CD4 T cells) which was not the case when the T cells were cocultured with regulatory DCs from infected mice (2.6 ± 0.3%; Fig. 3D, day 0, bottom panels), suggesting that regulatory DCs, particularly those from infected mice, may induce a regulatory phenotype in T cells as characterized by expression of IL-10 but not IL-4 or IFN-γ (29). In summary, these results appear to indicate that although both DC subsets can induce CD T cells to proliferate, conventional CD11clow DCs induce classical activation of IFN-γ-expressing T cells, while the regulatory CD11clowCD45RBhigh DCs preferentially induce CD62LhighCTLA-4+ T cells that express IL-10 without IL-4. Interestingly, prolonged CD62L and CTLA-4 expression has been correlated to a regulatory T cell phenotype (30–32).

Conventional DCs induce an inflammatory cytokine milieu

We looked at the cytokine milieu induced by the coculture of each DC subset with OVA-specific CD4 T cells. After sorting DCs from naive or day 10-infected mice, DCs were cocultured with OVA-specific CD4 T cells in the presence of OVA peptide for 80 h. The culture media from the wells were then assayed for their cytokine contents. Culture medium from the cocultures with conventional DCs from either naive or infected mice contained high levels of the inflammatory cytokines IL-6, TNF-α, and IL-12p40. Culture media from cocultures with regulatory DCs contained 50–75% less IL-6, 25–60% less TNF-α, and virtually no IL-12p40 (Fig. 4). IFN-γ was also elevated in the coculture of CD4 T cells with conventional DCs from naive mice when compared with regulatory DCs; however, this difference is not as evident in the cocultures with DCs from infected mice. We had found comparable results when analyzing intracellular cytokine staining of T cells from similar cocultures (Fig. 3D). Regulatory DCs from naive mice induced more IL-10 in the cocultures when compared with their conventional DC counterparts; however, the trend is less pronounced when considering the cocultures of DCs from infected mice (Fig. 4). It is likely that both DCs and T cells contribute to the cytokines detected in this assay and in particular IL-10 since both the regulatory DC and the malaria literature document the expression of IL-10 by DCs (9, 18, 24, 25).

Higher infection doses result in a faster progression toward a regulatory DC phenotype in the spleen

Since infection of humans with low, subphysiological doses of blood-stage parasites results in effective immunity against the disease (33), we were curious as to whether the shifting of the DC subsets could be dependent on the dose of parasite inoculum.

We infected mice with 10⁶ infected erythrocytes, similar to our previous experiments, and 10-fold dilutions to the subphysiological amount of 1000 infected erythrocytes. Splenic DC subsets (Fig. 5A) and parasitemia (Fig. 5B) were assessed at different points up until day 10 postinfection.

When mice were infected with the high dose of 10⁶ infected erythrocytes, the regulatory CD11chighCD45RBhigh DCs had already begun to expand by day 3. By day 6, >85% of the splenic DCs were comprised of this population. In contrast, expansion of the regulatory DC subset was delayed to day 10 in mice that received the lower doses of 10⁴ or 10³ infected erythrocytes (Fig. 5A, right panel). The levels of CD86 mirror this temporal delay between high and low parasite doses, with up-regulation seen earlier on day 3 on the DCs from mice that had received 10⁶ infected erythrocytes and later (day 6) on the DCs from mice that had received 10⁴ and 10³ infected erythrocytes (Fig. 5C, bottom right panel).

The conventional CD11chigh subset had declined to its minimum proportion by day 10 in all groups of mice. Interestingly, on day 3 the three lower doses had induced an expansion of this population.

**FIGURE 4.** Conventional DCs induce a stronger inflammatory cytokine environment. Mice were injected i.p. with 10⁶ infected erythrocytes. At days 0 (naive mice) and 10 postinfection, DC subsets were sorted by FACS. DC subsets were then cocultured at various ratios with naive CD4 T cells isolated from D011.10 mice, with or without OVA peptide. After 80 h of coculture, the culture medium was assayed for cytokine content using the BD Cytometric Bead Array. Error bars represent SD within cocultures of cells sorted from three individual mice (*p* < 0.05; **p** < 0.01; ***p** < 0.001 when comparing CD11chigh cocultures to CD11clowCD45RBhigh cocultures by Student’s *t* test).
This is in contrast to mice infected with the highest dose of $10^6$ infected erythrocytes, which on day 3 already showed lower proportions of this subset than the uninfected controls (Fig. 5A, left panel). We therefore questioned whether there had been an earlier expansion of the conventional DC subpopulation in mice that had received $10^6$ infected erythrocytes. We analyzed the splenic DC subsets at 24 and 48 h postinfection. Neither of the early time points demonstrated any significant changes in the proportions (Fig. 5D) or absolute numbers (data not shown) of either of the two DC subpopulations analyzed. Moreover, regulatory DCs maintained low levels of CD40 even at these early time points (Fig. 5E).

Malaria starts with an average of 15–123 sporozoites inoculated into the skin by an infected mosquito. A fraction of these sporozoites will reach the liver where they infect hepatocytes. After vigorous asexual replication, each infected hepatocyte produces thousands of merozoites (34). *P. yoelii* can produce $>8,000$ merozoites per infected hepatocyte (35), compared with 40,000 merozoites produced by *P. falciparum* (36). This initial step guarantees that blood-stage infection starts with a high dose of merozoites.
delivered to the bloodstream. We also investigated whether infection started by sporozoites would change the proportions of splenic DC subsets. We therefore injected 10, 100 and 1,000 sporozoites i.v. into mice and assayed their splenic DC subsets on day 12 postinjection. As *P. yoelii* sporozoites take 2 days to develop in the liver and start a blood-stage infection (35), day 12 postinjection of sporozoites correlates to day 10 of a blood-stage infection. By day 10 of the blood-stage infection, even the mice that had received only 10 sporozoites demonstrated the maximum regulatory DC phenotype in their spleens (Fig. 6A). Parasitemia curves from infections started with 10⁶ and 10⁵ infected erythrocytes from Fig. 5B for comparison (gray symbols). The differences between the doses of 10⁶ and 10⁵ infected erythrocytes on day 3 become apparent because of the logarithmic scale used. The graph is normalized to account for the 2 days of development required by sporozoites before starting blood-stage infections. Parasitemia counts below the level of detection are plotted below the black dashed line.

FIGURE 6. Blood-stage infections started with sporozoites are similar to those started with 10⁶ infected erythrocytes. Mice were injected i.v. with 1000, 100, or 10 sporozoites (spz, black and gray bars) or the vehicle RPMI 1640 (white bars). A, At the indicated days postinjection, splenocytes from groups of three mice were analyzed for the two subpopulations, expressed as the proportion of CD11c⁺ cells (DCs). Error bars represent SD within groups of three mice (***, p < 0.001 when comparing iRBC to RBC by one-way ANOVA). B, Parasitemia were counted via Giemsa-stained slides (□, △ and ○) and are overlaid here with the parasitemia curves from infections started with 10⁶ and 10⁵ infected erythrocytes from Fig. 5B for comparison (gray symbols). The differences between the doses of 10⁶ and 10⁵ infected erythrocytes on day 3 become apparent because of the logarithmic scale used. The graph is normalized to account for the 2 days of development required by sporozoites before starting blood-stage infections. Parasitemia counts below the level of detection are plotted below the black dashed line.

We were curious whether the predominance of regulatory CD11c⁺CD45RB⁺ DCs observed during infection was unique to *P. yoelii* blood-stage infection, even if started with subphysiological doses (10³, 10⁴, and 10⁵) of infected erythrocytes, which will result with the emergence of the regulatory CD11c⁺CD45RB⁺ DCs in the spleen. The results also demonstrate that subphysiological doses of infected erythrocytes induce an early expansion of the conventional DC population that is not observed at the higher physiological doses of parasites.

Injection of LPS also induces the expansion of the regulatory CD11c⁺CD45RB⁺ DCs

We were curious whether the predominance of regulatory CD11c⁺CD45RB⁺ DCs observed during infection was unique to malaria. Indeed, the loss of conventional CD11c⁺DCs in the spleen has been documented as part of the tolerance response to endotoxin (37). We therefore used a high, but sublethal, dose of LPS to investigate changes in splenic DC subsets after an acute inflammatory response.

Mice were injected i.v. with a sublethal dose of LPS (15 mg/kg) and their splenic DC subsets were assayed at different points up until day 10 postinjection. Surprisingly, as early as 24 h after LPS administration, the regulatory DC subset had rapidly expanded to represent >75% of the splenic DCs (Fig. 7A, left panel). Over the
same period, the conventional DCs had reduced to half their starting proportion (Fig. 7A, right panel). Both of these trends continued and peaked at 48 h postinjection when the conventional DCs are reduced to <1% of splenic DCs. On day 3, the regulatory DC shift began to resolve and was back to control levels by day 6. Notably, unlike during a Plasmodium infection (Fig. 2), regulatory CD11c-low CD45RB-high DCs had up-regulated their CD40 at 24 h after LPS administration, similarly to the conventional DCs (Fig. 7, B and C). In conclusion, it appears that the expansion of the regulatory DC population also occurs in response to LPS and is not distinctive of the immune response to malaria.

Discussion

We have analyzed the prevalence and function of regulatory DCs, a recently characterized subset, identified by their low expression of CD11c and the high expression of CD45RB. These CD11c-low CD45RB-high DCs have been characterized primarily in vitro, where their regulatory characteristics were initially observed (24). Their regulatory function has also been shown in vivo, where regulatory CD11c-low CD45RB-high DCs were transferred into mice in order induce tolerance by inducing regulatory T cells (24–26) or to suppress inflammatory immune responses (27). Although it has been demonstrated that this regulatory DC subset exists naturally in vivo (24, 27), we are the first to document their expansion during an inflammatory immune response, either in response to endotoxic shock or malaria. During an acute P. yoelii infection, we found that the regulatory CD11c-low CD45RB-high DCs increase in proportion, making up >85% of the splenic CD11c+ DC population. This is accompanied by a concurrent decrease in proportion of the conventional CD11c-high DC subset. This change in the composition of the splenic DC population is maintained during the course of the disease. Moreover, these changes are irrespective of whether the blood-stage infection was started directly with the injection of infected erythrocytes or by first going through the liver stage via the injection of sporozoites. Because the same switch is seen in both nonlethal (P. yoelii 17XNL) and lethal malaria infections (P. berghei ANKA and P. yoelii YM), it seems that the capacity to expand the regulatory DC population is not related to the lethality of the parasite strain. However, it is likely that different parasite strains may induce differential cytokine and T cell responses from the regulatory DC population leading to differences in parasite clearance mechanisms.

Regulatory CD11c-low CD45RB-high DCs differentiate as a result of a high IL-10 environment (24, 27). Since malaria is known to induce high amounts of IL-10 in mice (9, 38) as well as in humans (39), such an environment could foster the development and expansion of regulatory CD11c-low CD45RB-high DCs. We have observed the increase in proportion of the regulatory DC subset in response to LPS and P. yoelii infection, suggesting that this expansion could be the result of the inflammation induced by these two stimuli; however, further study would be required to substantiate this hypothesis. Indeed, early in Plasmodium infections there are large amounts of IFN-γ, TNF-α, and IL-12 found in the serum (2). Although an early proinflammatory response appears to be essential for the survival of a lethal infection (40–42), the complications associated with inflammation cause most of the pathology associated with malaria (43). IL-10 is an important anti-inflammatory mediator that can prevent death due to LPS- or malaria-induced inflammation (44–46). Notably, regulatory CD11c-low CD45RB-high DCs induce IL-10-expressing T cells (24–26). We too have seen a similar functional role for the regulatory CD11c-low CD45RB-high DCs isolated from P. yoelii-infected mice since they do not induce T cells to express high amounts of IFN-γ. Instead, the regulatory DCs induce IL-10-expressing T cells and reduce the level of inflammatory cytokines in the medium. Thus, by eliciting the regulatory CD11c-low CD45RB-high DC subset, the immune response may be seeking to stem the inflammation, saving the organism from an uncontrolled inflammatory response. It must be emphasized that the mechanisms by which regulatory DCs are induced, either in response to LPS or P. yoelii, are not yet known and could differ between the two systems.

The similarities between bacterial endotoxin tolerance and malaria have been discussed in detail (47). As in malaria, the regulatory CD11c-low CD45RB-high DC subset expands rapidly in response to LPS injection. This is matched by a quick contraction of the conventional CD11c-high subset that is a contributor of proinflammatory mediators during an immune response (1). Indeed, a decrease in the conventional CD11c-high DCs has already been documented as part of the endotoxin tolerance response (37). Moreover, conventional DCs have recently been shown to be key mediators of inflammation in the experimental murine model for cerebral malaria (48), and our results indicate that this subset in particular induces high amounts of inflammatory cytokines when cocultured with T cells. Considering the regulatory effects of the CD11c-low CD45RB-high DCs on T cells, the changes in relative abundance of regulatory DCs will likely influence the course of the adaptive immune response during high inflammatory conditions. It is interesting to point out that in malaria, it seems to be the balance between the regulatory and conventional DCs that provides the tolerogenic response rather than the induction of a refractory DC phenotype. This is suggested by the fact that both conventional and regulatory DCs extracted from infected appear to act in a similar way to those taken from naive mice. Moreover, conventional DCs during malaria do not appear to be refractory, since even in late infection they are found in a mature state and are able to stimulate T cells to produce IFN-γ.

There is a notable difference between the regulatory CD11c-low CD45RB-high DCs induced by a Plasmodium infection and those induced by endotoxic shock: while LPS administration causes regulatory DCs to up-regulate all of their costimulatory molecules including CD40, at no point during a P. yoelii infection is CD40 up-regulated on this DC subset. CD40-CD40L interactions are very important both in their involvement in priming T cells as well as in their role in fully licensing DCs to become professional APCs (49). Failure to provide this signal inhibits CD4 T cell activation (50), confers tolerance (49), and induces regulatory T cells (51). Indeed, our results demonstrate that regulatory DCs do not activate T cells as well as their conventional DC counterparts. Recently, it has been shown that induction of CD4 memory T cells requires CD40; moreover, CD40 is also necessary to elicit effector functions (IFN-γ secretion) from CD4 memory T cells (52). Thus, by retaining low surface expression of CD40 on the regulatory DCs, the parasite may be interfering with the induction of effector and memory T cell responses.

There has been much debate in trying to understand how DCs function during a Plasmodium infection. On one side, several groups have demonstrated that DCs mature in response to the malaria parasite, up-regulating costimulatory and MHC molecules, secreting Th1-inducing and proinflammatory cytokines, and initiating Ag-specific T cell responses (14–20). In contrast, other groups have also documented seemingly opposite results, where the DC maturation response is modulated by Plasmodium, resulting in the inability to up-regulate costimulatory and histocompatibility molecules, secretion of anti-inflammatory mediators, and inhibition of T cell responses (5–13, 21). More recently, there has been a renewed attempt to understand these differences, looking at the time of infection (14, 18), CD11c-high DC subpopulations (13,
16, 18), and the different Plasmodium species being used as model infections (13, 21).

In this study, we show that there are several factors that will dictate the phenotype of the DC population during a Plasmodium infection: the definition of the DC population based on CD11c expression, the time during infection at which the cells are assayed, and the dose of infected erythrocytes used to begin the blood-stage infection in the murine malaria model.

CD11c is an integrin receptor used as a marker to define DCs in mice (23). Classically, the analysis of DCs isolated from infected mice or humans typically involves the use of flow cytometry that will lead to different results depending on whether only CD11c<sup>high</sup> cells (14–16) or all CD11c<sup>+</sup> cells are considered for the analysis (8–10, 18, 19). We found that when considering all the CD11c<sup>+</sup> cells (and not CD11c<sup>low</sup> cells alone), the low costimulatory molecule expression pattern resembled that of the CD11c<sup>low</sup> cells because of the dominance of this subset in the spleen, especially late in infection. Considering exclusively the CD11c<sup>high</sup> cells shows DCs with up-regulated levels of costimulatory molecules. And so it is likely that the discrepancy in the maturation state of DCs during malaria, as reported by different groups, may have simply been the result of the differential gating of the CD11c<sup>+</sup> population. Moreover, consideration must be given to the method of ex vivo enrichment of DCs, as certain magnetic bead protocols can preferentially select for the CD11c<sup>high</sup> population.

We have also analyzed costimulatory molecules surface expression changes over the course of the acute blood-stage infection. We observed that the day postinfection at which cells are harvested also determined the level of maturation seen on DCs. This is not so much a result of temporal changes in DC maturation as it is a shift in the balance toward the regulatory DC subset later in infection.

Finally, the dose of infected erythrocytes used to start a blood-stage infection can influence the phenotype of the immune response, especially early in infection. We have shown that subphysiological doses of infected erythrocytes appear to induce a different immune response, particularly within the first 3 days of infection. There is an expansion of the conventional DCs on day 3 that is not seen with the higher physiological dose of infected erythrocytes and which could be relevant for the downstream immune effector functions.

Disclosures

The authors have no financial conflict of interest.

References


