Cutting Edge: The Acquisition of TLR Tolerance during Malaria Infection Impacts T Cell Activation

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An effective immune response to infection requires control of pathogen growth while minimizing inflammation-associated pathology. During malaria infection, this balance is particularly important. Murine malaria is characterized by early production of proinflammatory cytokines, which declines in the face of continuing parasitemia. The mechanism by which this occurs remains poorly understood. In this study, we investigated the role of dendritic cells (DCs) in regulating pro- and anti-inflammatory cytokine responses. As malaria infection progresses, DCs become refractory to TLR-mediated IL-12 and TNF-α production, while increasing their ability to produce IL-10 and retaining the capacity for activation of naive T cells. IL-12-secreting DCs from early infection stimulate an IFN-γ-dominated T cell response, whereas IL-10-secreting DCs from later stages induce an IL-10-dominated T cell response. We suggest that phenotypic changes in DCs during Plasmodium yoelii infection represent a mechanism of controlling host inflammation while maintaining effective adaptive immunity. The Journal of Immunology, 2005, 174: 5921–5925.

Malaria infection results in the death of one million children each year (1). All of the disease manifestations occur during the blood stage of the parasite’s life cycle and immune-mediated disease is a contributing factor in this pathology. For example, abundant proinflammatory cytokine (IL-12, IFN-γ, TNF-α) production is associated with the occurrence of cerebral malaria in humans and in animal models (2, 3) and may contribute to anemia (3, 4). Infection of mice with Plasmodium chabaudi, Plasmodium yoelii, and Plasmodium berghei are used to study the control of inflammatory cytokines during malaria (5). The level of serum cytokines in mice infected with a variety of Plasmodium species suggests that proinflammatory responses (TNF-α and IFN-γ) peak at days 5–9 of infection, but diminish before a decrease in parasitemia (6–8). T cell cytokine responses reflect this change. IL-10 production has been associated with this decreased IFN-γ (9); however, the source of IL-10 has not been established.

Interactions between naive T cells and dendritic cells (DCs)3 determines the outcome of T cell responses (10). We therefore hypothesized that the DCs isolated from mice during acute infection would stimulate high levels of IFN-γ by responding T cells, but DCs isolated later would preferentially stimulate activation of anti-inflammatory T cells. In previous studies, we established the first part of this hypothesis by showing that DCs isolated from day 6 after infection with P. yoelii stimulate high levels of IFN-γ by naive T cells in an IL-12-dependent manner (11). In this study, we have investigated the function of DCs isolated from later stages of infection. We demonstrate that, when stimulated with TLR ligands, day 17 DCs produce low levels of IL-12 and TNF-α and do not stimulate IFN-γ from naive T cells. Such DCs produce increased levels of IL-10, however, and stimulate naive T cells to do the same.

Materials and Methods

Mice and P. yoelii infection
B10.D2, B6, and IL-10 knockout B6 mice were purchased from The Jackson Laboratory. B10.D2-D01110 (TCR-transgenic (Tg)) mice which express a TCR for OVA on CD4 T cells were purchased from The Jackson Laboratory and used as heterozygotes.

Purification of cellular subsets
DCs and T cells were purified from the pooled spleens of three to five mice by positive selection with anti-CD11c and anti-CD4, respectively, using magnetic beads as described elsewhere (11). For FACS purification, spleen cells were enriched for CD11c+ cells using magnetic beads and then stained for CD3/ CD19, CD11c, and CD11b. CD3/CD11c+ DCs were sorted based on differential expression of CD11b (MoFlo; DakoCytomation).

Cell culture conditions
T cell stimulation assays were conducted as described previously (11, 12). DC TLR ligation experiments were conducted by stimulating 3 × 10⁶ DCs with 1 µg/ml LPS or 0.5 µg/ml CpG (InVivoGen). Supernatants were collected at 24 and 72 h and analyzed for cytokines. All cultures were conducted in triplicate.

Cytokine assays
TNF-α, IFN-γ, IL-10, and IL-12p70 were analyzed by cytometric bead array and IL-12p40 by ELISA (BD Biosciences).

LPS treatment in vivo
Mice were bled via tail nick (~50 µl) and then given 1 mg Salmonella typhimurium LPS (Sigma-Aldrich) dissolved in PBS i.p. Three hours later, they were sacrificed and bled via cardiac puncture.

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Recombinant P. yoelii proteins

Merozoite surface protein (MSP)-N (330 aa starting at aa 20 of MSP-1) and MSP-C (182 aa starting at aa 1619) recombinant proteins were generated from our laboratory strain of P. yoelii by cloning and expression in Escherichia coli with a HIS tag, followed by purification with nickel chromatography.

Statistical analysis

Two-tailed Student t tests, assuming unequal variances, were used to calculate p values and to compare cytokine levels between treatment groups within individual experiments. Unless otherwise indicated, all experiments were conducted at least twice.

Results and Discussion

DCs support activation of naive T cells throughout infection

In previous work, we demonstrated that DCs isolated from day 6 postinfection (PI) were as efficient at activating naive CD4 T cells as DCs from uninfected mice (11). We extended these observations to DCs isolated at additional time points (days 3 and day 17 PI, Fig. 1A). DCs from day 3 and day 17 PI were cultured with TCR-Tg T cells and OVA. DCs from both days supported as much T cell IL-2 production as control DCs in 24-h cultures. T cells cultured with day 17 DCs however produced significantly less IL-2 at 72 h (65% of control, p = 0.007). To further explore this question, we measured the yield of T cells after 7 days of culture with DCs from each day of infection. Cultures with DCs from uninfected mice yielded a mean of 8.5 × 10⁵ (±3 × 10⁵) cells, day 3 DCs yielded 9.7 × 10⁵ cells (±2.1 × 10⁵), and day 17 DCs yielded 6.8 × 10⁵ (±2.5 × 10⁵) cells. None of these differences were statistically significant. Finally, we restimulated T cells that had been cultured for 5 days with DCs from naive or day 17 mice. IL-2 production was similar with the two different preparations of DCs (Fig. 1B). From these studies we concluded that, while DCs from late in infection support slightly less IL-2 production by 72 h, this does not appear to have significant consequences for T cell division. Additional studies also demonstrated that both CD11c⁺b⁻ and CD11c⁺b⁺ DC subsets maintained high levels of costimulatory proteins and class II MHC during the later stages of infection, consistent with their ability to activate naive T cells (data not shown).

DCs support less IFN-γ as infection progresses

Although DCs retain the ability to stimulate T cell division and IL-2 production throughout infection, their ability to elicit IFN-γ production by naive T cells increases during the acute stages of infection and then declines markedly (Fig. 1C). Previously we demonstrated that IFN-γ production in this system is dependent upon IL-12 (11). We asked whether IL-12 production paralleled that of IFN-γ, and found that, although IL-12p70 was below the limits of detection (~15 pg/ml) in all in vitro experiments, production of IL-12p40 in the presence of T cells and Ag was significantly lower in DCs from day 17 PI (mean IL-12 with DCs from uninfected mice 2449 ± 120 mean IL-12 with DCs from day 17 infected mice, 453 ± 12, < 0.05). It is important to note that at day 17 PI the mean parasitemia was 18%, whereas at day 3 the mean parasitemia was 0.5%. Thus, IL-12 production is not diminished because the parasite stimulus was removed.

DCs from the late stages of infection do not support the production of other proinflammatory cytokines

Perhaps the most crucial inducers of proinflammatory cytokines are signals transmitted by interaction of pathogen-derived molecules with TLRs (13). The failure of DCs to produce IL-12 late in infection is particularly intriguing because at this point in infection there is a substantial parasite burden. We therefore asked whether the response to TLR ligation by DCs was inhibited during the later stages of infection. Fig. 2 shows that DCs isolated at day 17 PI and stimulated with LPS or CpG produce significantly lower levels of proinflammatory cytokines than DCs from day 0 or day 3 (zymosan and R848 were tested with similar results). These results suggest significant inhibition of TLR-mediated responses during the late stages of malaria infection. A similar phenomenon is seen during endotoxin tolerance and sepsis, in which mice treated with sublethal doses of LPS are refractory to subsequent stimulation of proinflammatory cytokine production (14, 15).
DCs from late stages of infection produce high levels of IL-10 and induce its production in responding T cells

In contrast to proinflammatory cytokine production, DCs from late stages of infection produced increased levels of IL-10 following ligation with all TLR ligands tested (Fig. 2 and data not shown). This finding demonstrates that although DCs specifically down-regulate proinflammatory cytokine production, they are not globally refractory to stimulation. Furthermore, when cultured with naive T cells, day 17 DCs induced high levels of IL-10 and low levels of IFN-γ in responding T cells (Fig. 3). Neither IL-4 nor IL-5 was detected in these cultures. Our finding that DCs from infected mice make high levels of IL-10 is consistent with the idea that these mice experience a condition similar to endotoxin tolerance, since IL-10 production is not inhibited and may be enhanced during endotoxin tolerance (14, 16).

MSP-1-specific T cells in infected and recovered mice make IL-10 and not IFN-γ

Since we demonstrate that naive, TCR-Tg T cells produce IL-10 when stimulated by day 17 DCs, we asked whether such IL-10-producing T cells are generated in vivo during infection. We purified T cells from day 17-infected mice and stimulated them with two peptides representing the N and C termini of MSP-1. These T cells produced low levels of both IFN-γ and IL-10 (data not shown). This mixed pro/anti-inflammatory response might be explained by the presence of a mixture of effector T cells, some activated during the acute stage of infection and others activated later during infection. We further explored this question by looking at T cells from recovered mice. Approximately 60 days after mice had recovered from P. yoelii infection, they were reinfected to expand the Ag-specific T cell population. After 5 days of infection, we purified CD4 T cells and cultured them with MSP-1 peptides using spleen cells as APCs (Fig. 4). These T cells did not produce IFN-γ, IL-4, or IL-5, but did produce significant levels of IL-10. Together these results demonstrate that, although DCs retain their capacity for T cell activation throughout malaria infection, the phenotype of responding naive T cells changes as infection progresses. Naive T cells activated by DCs during the later parts of infection produce the anti-inflammatory cytokine IL-10, and little IFN-γ. Furthermore, malaria-specific T cells producing IL-10 but not IFN-γ are detectable in mice that have been infected and recovered.

**FIGURE 3.** Differential T cell cytokine activation by DCs from days 3 and 17 PI. TCR-Tg T cells were cultured for 7 days with DCs from the indicated day and 1 mg/ml OVA. The cells were then harvested and restimulated with (□) or without (▲) Ag for 24 h using naive spleen cells as APCs.

**FIGURE 4.** Malaria-specific IL-10 production by spleen cells and CD4 T cells. A, Spleen cells were isolated from mice that had cleared infection 2 mo previously, then reinfected 5 days before sacrifice. Cells were cultured with the indicated concentrations of MSP-1 N- and C-terminal peptides. B, CD4 T cells were purified from three different mice (treated as described in A), and cultured with naive spleen cells as APCs and MSP-N peptide. Cultures were stimulated for 24 h before harvest for cytokine measurement.

**FIGURE 5.** In vivo cytokine response to LPS by infected mice. A, Uninfected (▲, n = 3) or day 17 PI mice (□, n = 5) were treated with 1 mg of LPS i.p. Serum was taken 3 h after treatment and assayed for the indicated cytokines. All comparisons between day 0 and day 17 were statistically significant (p < 0.01) except IFN-γ, which did not achieve significance. B, Uninfected wild-type mice (▲, n = 5) or infected IL-10 knockout (□, n = 5) and wild-type mice (hatched □, n = 3) were given 1 mg of LPS i.p. Serum was taken 3 h post-treatment and assayed for the indicated cytokine. *, p < 0.05 between the indicated groups. All values are reported in nanograms per milliliter.

Late-stage malaria infection results in endotoxin tolerance in vivo

To determine whether the DC phenotype reflected the broader in vivo response to TLR ligation, we treated uninfected and day 17 PI mice with LPS and then measured serum cytokine levels. At baseline, there were no significant differences in IL-12p70, TNF-α, IFN-γ, or IL-10 between infected and uninfected mice (data not shown). After LPS administration, however, uninfected mice made between 5- and 50-fold more IL-12 and TNF-α than infected mice. In contrast, LPS provoked very high levels of IL-10 in infected mice compared with uninfected mice (Fig. 5A).

We then asked whether IL-10 was responsible for the down-regulation of TLR responses in vivo and in vitro. Fig. 5B shows that when LPS is given to infected IL-10 knockout mice, the IL-12 and IFN-γ responses were restored, but not TNF-α, demonstrating that IL-10 specifically down-regulates IFN-γ and IL-12 during malaria infection, as has been suggested by previous studies (6).

Finally, we determined that the behavior of DCs from wild-type and IL-10 knockout mice reflected the in vivo observations. DCs were purified by FACS into CD11c+ b+ , 11b medium, and 11b high populations. The rationale for this division...
was that with increasing expression of CD11b, DCs tend be increasingly phagocytic (17) and we reasoned that DCs with greater phagocytic capacity might be more dramatically affected by malaria infection. This was indirectly confirmed by the observation that cells expressing the highest levels of CD11b had the highest percentage of pigment-containing cells (Fig. 6D). The sorted subpopulations were cultured with CpG (Fig. 6). All DC subsets from day 17 PI mice exhibited a decrease in their ability to produce IL-12 after TLR ligation, although the CD11c^b m median population was the most affected. All DC subsets up-regulated IL-10 after TLR ligation as well, consistent with the in vivo observations.

Although IL-10 deficiency fully restored the ability of infected mice to make IL-12 and IFN-γ, it did not enhance TNF-α production after TLR ligation (Fig. 5 and data not shown). Anti-TGF-β administered along with anti-IL-10R Ab (but neither alone) enhanced TNF-α and IFN-γ production during P. yoelii 17XL infection (18), and anti-TGF-β marginally enhanced TNF-α levels in the serum of infected IL-10 knockout mice (19), suggesting that this cytokine may exert some additional control over TNF-α production. Recently, TGF-β was shown to play a central role in endotoxin tolerance (20), further supporting this idea.

Our studies offer several novel findings. First, we demonstrate that DCs from infected mice maintain their ability to activate naive T cells throughout infection, an issue that has been controversial. Although some studies show that DCs derived in vitro in the presence of parasitized RBC are dysfunctional (21, 22), other work, including our own, found that DCs derived from infected mice are fully mature with respect to expression of costimulatory proteins and T cell activation (11, 23, 24). One possible explanation for the discrepant results is the lack of T cell-DC interaction in systems where DCs are matured in vitro. Such DCs do not receive T cell-derived signals such as CD40-CD40 ligand interaction, which may contribute to their Ag-presenting capacity.

The second novel finding is that malaria-infected mice are refractory to proinflammatory cytokine production following TLR stimulation during a highly parasitic stage of infection. This state resembles endotoxin tolerance in which administration of LPS, a TLR4 ligand, renders cells unresponsive to subsequent stimulation through TLR4 and other TLRs (25). Hemozoin pigment is a ligand for TLR9 (26) and repeated stimulation through TLR9 can cause tolerance to signaling through TLR4 (27). We hypothesize that repeated signaling through TLR9 by hemozoin and other protozoan-derived ligands tolerizes or shunts the common MyD88 TLR signaling pathway such that anti-inflammatory cytokines are preferentially produced, resulting in the refractory state we and others (28) have observed.

Tolerance to TLR signaling by DCs may represent a mechanism by which the host can down-regulate innate proinflammatory cytokine responses and instruct the adaptive immune system to do the same. In order for TLR-tolerant DCs to transmit the down-regulatory signal to the adaptive immune system, they must maintain their ability to activate naive T cells as we demonstrate here. Maintenance of high levels of costimulatory proteins and class II MHC may be the result of TLR signaling through a pathway not tolerized by interaction with Plasmodium-derived molecules, such as the TLR4-dependent, MyD88-independent pathway (29), or by CD40-CD40 ligand interaction. Because they can activate naive T cells to become IL-10 producers, TLR ligand-tolerant DCs can generate an adaptive immune response that can aid in perpetuating an anti-inflammatory state. This could be one mechanism for generating antidiisease immunity in Plasmodium falciparum-infected people (30).

Disclosures
The authors have no financial conflict of interest.

References


