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Limited Role of CD4⁺Foxp3⁺ Regulatory T Cells in the Control of Experimental Cerebral Malaria

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Cerebral malaria (CM) is a life-threatening sequela of human infection with Plasmodium falciparum (1). Infection of susceptible mouse strains with Plasmodium berghei ANKA (PbA) is an experimental model of CM that shares several characteristics with the human disease (2, 3). During PbA infection, parasitized erythrocytes, but also leukocytes, are sequestered in the brain (4–6). Various studies clearly demonstrated a role for T cells secreting proinflammatory cytokines for the genesis of CM (7–10). We recently found that T cell activation during malaria is accompanied by an increased expression of the negative costimulators CTLA-4 (11, 12) and B and T lymphocyte attenuator (BTLA) (13). Both of these were shown to modulate T cell function during PbA infection. In PbA-infected C57BL/6 mice, we found that blockade of CTLA-4 leads to exacerbation of the immune pathology triggered by T cells producing proinflammatory cytokines, whereas an agonistic Ab against BTLA prevented the genesis of CM (13). This supports the idea that the induction of negative costimulators during PbA infection is a means of dampening the strong and polarized activation of the Th1 arm of the immune system to prevent immune pathology. To further analyze the role of T cell regulation, several studies have addressed the role of CD4⁺CD25⁻Foxp3⁻ natural regulatory T cells (Treg) during the pathogenesis of CM. However, until recently, no tools were available to deplete Treg specifically. In naive mice, CD4⁺Foxp3⁻ T cells express CD25. Thus, Abs against CD25 have been used to deplete CD4⁺CD25⁻ Treg (14, 15). Although this approach has provided novel information on the function of Treg, it is problematic because depletion is not complete in several organs (16) and may also interfere with activated T effector cells (Teff) expressing CD25, as recently shown during chronic Toxoplasma gondii infection (17). To unequivocally study the function of Treg during the genesis of CM, we used depletion of Treg (DEREG) mice (18). These mice are transgenic for a bacterial artificial chromosome expressing a diphtheria toxin (DT) receptor-enhanced GFP (eGFP) fusion protein under the control of the foxp3 gene locus. This method allows for a selective depletion of Foxp3⁺ Treg by DT injection and, also their specific detection, quantification, and purification during an ongoing infection. Using ex vivo analysis of purified Treg from either naive mice or PbA-infected mice, we found that both exhibit similar inhibitory capacity on T eff. The Journal of Immunology, 2009, 183: 7014–7022.

Materials and Methods

Mice and parasites

Male DEREG mice (18) were crossed with female C57BL/6 mice in the animal facility of the Bernhard Nocht Institute for Tropical Medicine. The resulting offspring was typed by PCR and flow cytometry. In each experiment, hemizygotic DEREG mice were used with sex- and age-matched wild-type littermates as controls.

PbA was maintained by alternating cyclic passage of the parasites in Anopheles stephensi mosquitoes and BALB/c mice at the mosquito colony of the Bernhard Nocht Institute for Tropical Medicine. Blood was collected from highly parasitemic mice, and aliquots were stored in liquid nitrogen.
in a solution of 0.9% NaCl, 4.6% sorbitol, and 35% glycerol. DEREG and C57BL/6 (5–8 wk old) were infected i.p. with $2 \times 10^6$ PbA-infected RBCs. Parasitemia was determined in Wright-stained blood smears from tail blood. C57BL/6 mice infected with blood stages of PbA have been shown to develop neurological behavioral changes, such as ataxia, convulsions, and coma, and usually die between days 6 and 8 postinfection (p.i.). Similarly, in our study, 90–100% of PbA-infected mice exhibited signs of cerebral involvement between days 6 and 8 p.i., characterized by weight loss, reduced locomotion, and marked ataxia. Mice developing cerebral malaria were scored according to the severity of the symptoms and subsequently euthanized to avoid unnecessary suffering between days 7 and 9 p.i. All experiments were in accordance with local Animal Ethics Committee regulations.

**Anti-CD3 mAb was purified from the hybridoma cell line 145-2C11. For flow cytometry, the following FITC-, PE-, PerCP-Cy5.5-, PerCP-, or allophycocyanin-labeled Abs and appropriate isotype controls were used: anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-CD45 (30-F11), and anti-CD62L (MEL-14) from BD Pharmingen. Anti-CD25 (PC61.5.3) was purchased from Cedarlane Laboratories; polyclonal anti-eGFP from Novus Biologicals; and anti-Foxp3 (FJK-16s) from eBioscience. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with the CellQuest program (BD Biosciences).

**Staining of blood and spleen lymphocytes**

A total of 30 μl of blood from the tail vein was collected in a heparinized capillary tube. RBC were lysed with 0.17 M ammonium chloride, and after blocking with anti-FcYR, cells were counted and stained with anti-CD4, anti-CD8, anti-CD62L, or anti-CD25. Spleen cells were homogenized and passed through a cell strainer. After lysis of RBC, $2 \times 10^6$ cells were stained with the indicated Abs. Intracellular staining for Foxp3 was conducted according to the manufacturer’s instruction (eBioscience).

**Staining of brain lymphocytes**

C57BL/6 or DEREG mice were infected with $2 \times 10^6$ PbA-infected RBC. On day 6 p.i., mice were sacrificed and brains were removed. For quantification of cell numbers, brains were weighed, homogenized, passed through a cell strainer, and stained with anti-CD45, anti-CD4 or anti-CD8, and anti-CD62L or anti-CD25. Cells prepared from one-tenth of the brain were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD62L, or anti-CD25. Spleen cells were homogenized and passed through a cell strainer. After lysis of RBC, $2 \times 10^6$ cells were stained with the indicated Abs. Intracellular staining for Foxp3 was conducted according to the manufacturer’s instruction (eBioscience).

**Inhibition assay**

Spleen cells from uninfected and PbA-infected mice at day 6 p.i. were sorted on a FACS Aria (BD Biosciences) into eGFP$^+$ T$_{reg}$ and eGFP$^+$ T$_{eff}$. Cells were mixed in different ratios and stimulated with 1 μg/ml anti-CD3 Ab for 48 h. IL-2 in the supernatants was analyzed in an ELISA (R&D Systems), and proliferation was measured by analyzing the incorporation of $[^{3}H]$thymidine after incubation of cells with 0.2 μCi/well for 16 h.

**Statistical analysis**

Statistical analyses were performed with unpaired Student’s t test. One-way ANOVA was used to compare parasitemia between different groups of mice. The log rank test was used to test the equality of survival functions across different groups of mice. All statistical analyses were performed with Prism software (GraphPad).

**Results**

**Quantification of Foxp3$^+$ T$_{reg}$ during blood-stage infection with PbA**

The blood stage of a PbA infection induces a strong activation of CD8$^+$ T cells as well as CD4$^+$ cells in spleen and blood, leading to an increased number of activated T cells, as indicated by the loss of CD62L expression on the surface (Fig. 1). To verify whether the expansion of T$_{eff}$ is also accompanied by an increasing number of Foxp3$^+$ T$_{reg}$, we made use of the recently described DEREG mice that allow for the detection of T$_{reg}$ by expression of eGFP and also their selective depletion by application of DT (18). Analysis of the absolute number of both T$_{eff}$ and T$_{reg}$ revealed only marginal changes in blood and spleen at day 7 p.i., which were of borderline significance or not statistically significant (Fig. 2, A and B). However, by comparing the percentage of GFP$^+$ T$_{reg}$ in the whole
CD25+ T cells in the spleen from uninfected control mice or PbA-naive mice. To further characterize these cells, we stained CD25 results, in which no increase in the numbers of Treg was found, we infection. CTLA-4 is expressed on Foxp3+ cells, suggesting that Treg might not be capable of controlling infection. We asked whether Treg also sequester in the brain, which would be the prerequisite for a local modulation of CM on inflamed endothelium in the brain of infected mice. Interestingly, hardly any CD4+ eGFP+ Treg or CD8+ eGFP+ Treg can be found in the fraction of T cells that were recovered from the brain (Fig. 4, B and C). The vast majority of sequestered cells are activated CD62Llow CD8+ or CD4+ T cells that were eGFP+ (Fig. 4, B and C) and Foxp3− (data not shown). Thus, the ratio between Treg and T eff in brain during PbA infection decreased compared with control mice, probably due to a rapid infiltration of T eff. These T eff can be recovered from brains of PbA-infected mice, whereas in this study, almost no Treg can be detected.

Effects of Treg depletion on the activation of T cells and the incidence of CM during PbA infection
To further study the role of Treg during the course of malaria, C57BL/6 wt and DEREG mice were infected with PbA and were injected with DT on days +1, +2, and +3. This protocol leads to a depletion of eGFP+ Treg in DEREG mice during the course of infection (Fig. 5A, upper panel). The absence of Treg in DEREG mice was also verified by intracellular staining of Foxp3 (data not shown). Interestingly, we found an increasing number of CD4+CD25+ T cells during infection in Treg-depleted DEREG mice (Fig. 5A, lower panel, and B). This further confirmed our observation that CD25 is not only expressed on Treg but also on activated T eff during PbA infection. Depletion of Treg does not alter the parasitemia of mice, and mice also showed a similar weight loss during the course of infection (Fig. 6, A–C). In addition, we observed no general differences in the incidence of CM. Also, when we used a more sensitive score to monitor early neurological symptoms occurring before the onset of CM (Fig. 6D), we observed no statistically significant increases in the clinical score in Treg-depleted DEREG mice between days 7 and 8 just before onset of CM. However, it is noteworthy that some Treg-depleted mice exhibit an increased clinical score. Depletion of Treg in DEREG mice resulted in an enhanced activation of CD4+ as well as CD8+ T cells upon PbA infection in spleen (Fig. 6, E and F) and blood (data not shown) when compared with DT-treated C57BL/6 wt mice. We also used a modified protocol for Treg depletion, in which DEREG mice were treated on days −3, −2, and −1 with DT. Similarly to our original protocol, no effect of Treg depletion on weight loss and incidence of CM was found (Fig. 6, G and H). Although the depletion of Treg was accompanied by an enhanced activation of T cells in the spleen (Fig. 6, E and F), the percentage and the absolute numbers of T cells recovered from brains of Treg-depleted and nondepleted mice were similar, which further supports the view that Treg are not capable of controlling infection.
the pathological T cell response in vivo and, hence, cannot prevent CM (Fig. 7).

**Ex vivo analysis of T<sub>reg</sub> function during the blood stage of malaria**

Numerous studies have been undertaken to characterize the inhibitory activity of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> from naive mice. Because these markers are not exclusively defining T<sub>reg</sub> during T cell activation, this strategy cannot be applied to analyze T<sub>reg</sub> function during an ongoing inflammation or infection. Based on our results, we hypothesize that during the course of the PbA infection, T<sub>reg</sub> function is either temporarily or irreversibly impaired. Thus, we asked whether PbA infection alters the function of T<sub>reg</sub>. To this end, we sorted eGFP<sup>+</sup>T<sub>reg</sub> from spleens of uninfected and infected DEREG mice. This method allows for the identification and purification of eGFP<sup>+</sup>T<sub>reg</sub> without staining for additional markers, which might interfere with their function. T<sub>reg</sub> that were sorted from naive and infected DEREG mice were highly pure (>95% were eGFP<sup>+</sup>Foxp3<sup>+</sup>; data not shown). To compare the regulatory function of T<sub>reg</sub> from naive mice with T<sub>reg</sub> from PbA-infected mice, these cells were sorted and added to eGFP<sup>+</sup>T cells from uninfected control mice that were stimulated with anti-CD3 (Fig. 8). Interestingly, eGFP<sup>+</sup>T<sub>reg</sub> from naive DEREG mice and eGFP<sup>+</sup>T<sub>reg</sub> from infected DEREG mice were both capable of inhibiting IL-2 production and proliferation. These data suggest that either T<sub>reg</sub> function is not impaired during the blood stage of PbA infection or these cells regain their regulatory capacity very fast upon cultivation in vitro. In addition, purified GFP<sup>+</sup>T<sub>eff</sub> from PbA-infected mice were also susceptible to inhibition by purified GFP<sup>+</sup>T<sub>reg</sub>. It is noteworthy that T<sub>eff</sub> from PbA-infected mice produce less IL-2 (and other cytokines) upon stimulation.

**Discussion**

CM during PbA infection critically depends on T cells producing proinflammatory cytokines. CD8<sup>+</sup>T cells and, to a lesser extent, also CD4<sup>+</sup>T cells sequester in the brain and produce proinflammatory cytokines (13, 19, 20). These cells can be found in direct

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**Figure 3.** Expansion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>T cells during PbA infection. C57BL/6 mice were infected with 2 × 10^6 PbA-infected RBCs at day 0. At day 7 p.i., spleen cells were stained with PerCP-labeled anti-CD4 and PE-labeled anti-CD25, and stained intracellularly for Foxp3 using allophycocyanin-labeled anti-Foxp3. In naive control mice, most Foxp3<sup>+</sup>T cells were CD25<sup>-</sup>, whereas during infection CD25<sup>-</sup> is induced on Foxp3<sup>-</sup>CD4<sup>+</sup>T cells (A). B, A summary of two experiments with n = 4 mice each is shown. Data are expressed as mean ± SD. C, DEREG mice were infected, as described. At day 7 p.i., cells were stained intracellularly using PE-labeled anti-CTLA-4 and FITC-labeled anti-Foxp3 (middle). As control, spleen cells from uninfected mice were stained (left). PE-labeled anti-hamster served as isotype control (right). This experiment was repeated twice with similar results. An identical experiment using C57BL/6 mice showed similar results (data not shown).
proximity to the brain endothelium. Several lines of evidence suggest that this process directly contributes to CM. Thus, immune regulatory pathways that control either the magnitude and/or the cytokine bias of this pathogenic T cell response are of particular interest. In general, such regulation can either take place: 1) in lymphoid organs during priming by influencing T cell activation, or 2) by the modulation of T cells that are already retained in the brain on activated endothelium. In the present study, we sought to determine whether Treg contribute to the control of pathogenic T cell responses during CM by either one of these mechanisms. Treg can be separated into natural Treg that are already present in naive mice and inducible Treg that arise during inflammation or infection (21), needing TGF-β for their induction, a cytokine that was already shown to influence pathology during malaria (22, 23). Both of these cell types express Foxp3; however, expression is only transient in the latter. Using the recently described DEREG mice, it is possible to specifically deplete both subtypes of Foxp3+ Treg by DT treatment during the whole course of PbA infection. In

**FIGURE 4.** Infiltration of T cells into the brain during PbA infection. DEREG mice were infected with $2 \times 10^6$ PbA-infected RBCs. On day 6 p.i., mice were sacrificed and cells from brains were isolated. Samples were stained with PerCP-labeled anti-CD45, allophycocyanin-labeled anti-CD4 or allophycocyanin-labeled anti-CD8, and PE-labeled anti-CD62L. Foxp3+ Treg were quantified by eGFP expression. Representative dot plots are shown for cells in the brain of uninfected control mice and PbA-infected mice. One-tenth of the brain was analyzed and gated on CD45+T cells (A). Flow cytometric analysis shows an increased number of cells that were CD4+/CD62Llow (D) or CD8+/CD62Llow (E) in PbA-infected mice compared with uninfected controls. Although an increased number of T cells was found in PbA-infected mice compared with control mice, almost none of these cells were CD4+ eGFP+ (B) or CD8+ eGFP+ T cells (C). Experiments were performed at least three times with n = 5 mice per group. A summary of this data expressed as mean ± SD is depicted in the lower panel.
addition, this technique does not only allow for the quantification of Treg in various organs, but also their purification by cell sorting due to their expression of eGFP. This enabled us to perform functional comparisons of Treg from naive and PbA-infected mice.

PbA infection is accompanied by an expansion of T cells that express activation markers such as CD69 and are CD62Llow. Although the absolute numbers of eGFP+Foxp3+CD4+ Treg in DEREG mice remained constant upon infection, due to a more rapid expansion of Teff, the Treg:Teff ratio declines. This decreased ratio of Treg might contribute to a strong antiparasitic T cell response with the eventual cost of pathology. By analyzing the cellular infiltrate of brains of PbA-infected DEREG mice, we found that Treg do not accumulate in the brain before or during the onset of CM, whereas activated conventional T cells massively accumulate in the brain during this time window. This supports recent findings using intracellular staining of Foxp3 as a marker for Treg in cells recovered from brains of PbA-infected mice (24), in which no enrichment of Treg in the brain was found. Therefore, a local regulatory activity of Treg in inflamed tissue in the brain is very unlikely. However, the principal ability of Treg to be retained in the brain was demonstrated in the experimental autoimmune encephalomyelitis model (25). This indicates that the homing capacities of Treg vary in different disease settings, which might also influence the outcome of the immune response (26).

To exclude the possibility that Treg are functionally impaired during PbA infection, we used the eGFP expression of Treg in DEREG mice to compare the regulatory effect of purified Treg from naive and PbA-infected mice. Functional impairment was plausible because malaria parasites trigger TLR-2 by GPI molecules and TLR-2 agonists have been shown to induce a temporary restriction of Treg function in vitro (27). However, Treg from both groups display a similar capacity to suppress T cell responses in vitro. This excludes a functional impairment of Treg during infection with PbA. An alternative explanation might be a fast recovery of Treg in the absence of TLR-2 agonistic molecules after purification in vitro.

Our results are in agreement with experiments in which CD25high cells purified from PbA-infected mice that are enriched for Foxp3+ Treg were even more active in vitro (24). Similarly to our results using the specific depletion of Treg in DEREG mice, the depletion of Treg using anti-CD25 did not alter the incidence of CM in this study (24). However, when we looked for subtle changes in the early development of neurological symptoms, we found that depletion of Treg leads to an increase in the clinical score. In addition, we found that Treg-depleted mice exhibit an increased number of activated T cells in the periphery, but not in the brain. This indicates that Treg can attenuate the activation of T cells during PbA infection to some extent, which might dampen several pathological responses occurring during infection, but are not capable of controlling the development of CM.

During the blood stage of malaria, the immune system is confronted with large amounts of TLR agonistic molecules. In particular, hemozoin stimulates cells of the innate immune system via TLR-9 (28), whereas GPI molecules of the parasite bind to TLR-2 (29). TLR-derived signaling contributes to the polarization of the adaptive immune response toward Th1, although its contribution to CM is controversial (30, 31). Recently, we have shown that TLR-2/4/9-deficient mice still develop CM during PbA infection (32). Even the increased parasitemia of MyD88-deficient mice in the Plasmodium yoelii model is due to the disturbed IL-1/IL-18 signaling rather than a disturbed TLR signaling (33).

Several studies using different models revealed that TLR-2-deficient signaling in particular may exert not only inhibitory effects on Treg, but may also activate Treg function. This might explain why TLR-2-deficient mice have a decreased Treg function and, as a consequence, suffer from an enhanced pathology during schistosoma infection (34). Interestingly, we found an increased number of activated T cells in TLR-2/4/9-deficient mice during P. yoelii infection, which may also suggest a decreased Treg activity (33). Further studies are needed to dissect the consequences of GPI-induced TLR-2 signaling on Treg function and on Th1 differentiation.

The development of the pathology of CM during PbA infection is a rapid and temporary highly coordinated process, and pathogenic CD8+ T cells accumulate in the brain just before the onset of symptoms. Thus, Treg that are often implicated in the control of chronic inflammatory processes might not be dynamic enough to control such a rapidly evolving pathology. Our data argue against a general restriction of Treg function during malaria, but may suggest that Treg lack a signature of homing molecules that allow their...
FIGURE 6. Effects of Treg depletion in DEREG mice during PbA infection. DEREG mice were infected with $2 \times 10^6$ PbA-infected RBCs at day 0. Mice received DT injection on days 1, 2, and 3. Parasitemia (A), incidence of CM (B), and body weight (C) were monitored at the indicated time points. To detect more subtle changes in the neurological outcome, mice were monitored twice per day and were scored blinded (D; see Materials and Methods for definition). To study the effects of Treg depletion on T cell activation, similar experiments were performed, but mice were sacrificed on day 7 p.i. and spleen cells were isolated subsequently. Samples were stained with allophycocyanin-labeled anti-CD4 or allophycocyanin-labeled anti-CD8, and PE-labeled anti-CD62L or PE-labeled anti-CD25. CD4+ or CD8+ T cells were analyzed for CD62L expression (E) or CD25 expression (F). The experiment was performed three times with each $n = 5$ mice. Data are expressed as mean ± SD. In a modified depletion protocol, DEREG mice received DT injection on days 3, 2, and 1. Mice were then infected with $2 \times 10^6$ PbA-infected RBCs at day 0. Body weight (G) and incidence of CM (H) were monitored at the indicated time points. This experiment was performed twice with each $n = 5$ mice.
homing to or retention in the inflamed brain, as observed for conventional T cells. Several studies have shown that during experimental malaria infection different regulatory mechanisms are triggered to avoid overwhelming pathology, such as production of IL-10 and TGF-β (35, 36). We have found that the negative co-stimulators CTLA-4 and BTLA expressed on T cells play a critical role during PbA infection by dampening the immune response (11–13). Recently, it was found that CTLA-4 is not only expressed by Foxp3+/CD4+ Treg constitutively, but is also critical for their function (37). In light of the present study and our previous work, in which we demonstrate that during experimental malaria many more cells are CTLA-4+CD4+Foxp3− T cells than CD4+Foxp3+ Treg, it is tempting to speculate that counterregulatory mechanisms on activated Teff such as CTLA-4 induction represent a more suitable mechanism to control a rapidly evolving pathology (11, 38). However, we cannot exclude the possibility that the exacerbation of pathology by anti-CTLA-4 treatment during PbA malaria (11, 12) is a consequence of a functional restriction of CTLA-4 on both activated Teff and Treg.

Interestingly, we found an increase of CD4+CD25+Foxp3− T cells upon infection that was even higher when Treg were depleted in DEREG mice during PbA infection. In contrast, in naive mice,
virtually all CD4+CD25+ T cells are Foxp3+. Currently, we do not know whether these CD4+CD25+ Foxp3+ T cells that are induced during infection are activated Treg or whether these cells represent an uncanonical population of Foxp3+ Treg. However, their presence indicates that previous studies addressing Treg function using anti-CD25 depletion must be interpreted with caution, because this strategy will not only have an effect on Foxp3+ Treg during infection. This is in agreement with previous studies showing that anti-CD25 deplete Treg during chronic T. gondii infection (17). This might explain the contrasting results obtained in previous studies, in which anti-CD25 treatment confers resistance to CM when applied during infection, but not when given 30 days before infection (15, 24). The effects of anti-CD25 treatment during PbA infection on different CD25+ T cell subsets, either being CD25+ Treg or CD25+ T eff, may critically depend on the available Ab concentration, and thus, on the time point of application.

Collectively, our data demonstrate that Treg defined by the expression of Foxp3, have only a moderate effect on general pathology and attenuate T cell activation in the periphery during PbA infection, but are not effective in the prevention of CM. In addition, our study suggests that data obtained using anti-CD25 application during malaria should be interpreted with caution because this may not only deplete Treg, but instead will also impair other T cell populations.

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Disclosures
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