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Murine Malaria Is Exacerbated by CTLA-4 Blockade

Thomas Jacobs, Sebastian E. B. Graefe, Sonja Niknafs, Iris Gaworski, and Bernhard Fleischer

Cytolytic T lymphocyte-associated Ag-4 (CD152) is a negatively regulating molecule, which is primarily expressed on T cells following their activation. In this study, we have examined the role of CTLA-4 expression in experimental blood-stage malaria. Similar to human malaria, CTLA-4 is expressed on CD4+ T cells of C57BL/6 mice after infection with Plasmodium berghei. A kinetic analysis revealed that CTLA-4 expression was increased on day 5 postinfection and reached a peak on day 9 postinfection, when almost 10% of splenic CD4+ T cells expressed CTLA-4. Blockade of CTLA-4 in vivo by a specific mAb and subsequent challenge with P. berghei caused neurological signs reminiscent of murine cerebral malaria and earlier death. Histologic examination of brain sections from anti-CTLA-4-treated mice revealed pathologic changes such as hemorrhages and edema, which were absent in control mice. Furthermore, treatment with anti-CTLA-4 also reversed the extensive loss of CD4+ T cells and the suppressed T cell response occurring during blood-stage malaria. Our data suggest that CTLA-4 expression prevents immune pathology by restricting T cell activation during malaria. They also indicate that the development of cerebral malaria is mediated by a failure to down-regulate T cell activation.


The role of T cells in human as well as in rodent malaria is still not completely understood. Blood-stage parasites are only briefly present extracellularly and mainly reside within erythrocytes, which lack the machinery to present Ag to T cells. Therefore, a direct interaction of T cells and parasites or infected cells is unlikely. However, the contribution of CD4+ T cells in acquired immunity to blood-stage malaria was demonstrated using several strains of Plasmodium parasites in mice (1–5). These studies suggested that malaria-specific T cells, especially cytokine-secreting Th1 cells, regulate other antiparasitic effector systems. IFN-γ was shown to play an important role in the elimination of parasites by activating macrophages to control peak parasitemia by Ab-independent mechanisms (6–9). In contrast, Th1-type cytokines such as IFN-γ and TNF-α are involved in the pathogenesis of cerebral malaria (CM), and the deletion of these cytokines abolished the development of CM (9–14). These data indicate that T cell responses in malaria are a double-edged sword and have to be tightly controlled.

We have previously shown that expression of the CTLA-4 (CD152) molecule is a very sensitive and highly dynamic marker for activation of T cells during the course of human malaria (15). Moreover, CTLA-4 expression on T cells was correlated with disease severity. CTLA-4 is highly homologous to the costimulatory molecule CD28 and binds to the same ligands. In contrast to CD28, however, CTLA-4 is induced mainly upon activation, and its binding to B7.1 or B7.2 was shown to deliver negative signals to T cells (16).

Several studies have demonstrated that a blockade of CTLA-4 by Abs improves the immune response against tumor cells and infectious agent (17–20), whereas under some circumstances, autoimmune diseases were enhanced (21, 22).

We used a murine malaria model with Plasmodium berghei to analyze the role of the CTLA-4 molecule in the development of malaria. A high percentage of CTLA-4-expressing CD4+ T cells was induced during blood-stage malaria very similar to the situation in human malaria. Furthermore, in vivo blockade of CTLA-4 by a mAb exacerbated the course of disease. Our data suggest that CTLA-4 expression restricts pathogen-specific T cell responses and thus prevents immune pathology during infection with P. berghei.

Materials and Methods

Mice and parasites

C57BL/6 mice, IL-12 p40−/− mice, and IFN-γ−/− mice on C57BL/6 mice were bred in the animal facility of the Bernhard Nocht Institute for Tropical Medicine. P. berghei ANKA parasites were obtained from a mouse previously infected with sporozoites kindly provided by the Parasitology Section, Bernhard Nocht Institute. Blood was taken from highly parasitemic C57BL/6 mice, and aliquots were stored in liquid nitrogen in 0.9% NaCl, 4.6% sorbitol, and 35% glycerol. Mice (6–8 wk old) were infected i.p. with 1 × 107 parasitized RBCs. Parasitemia was determined in Giemsa-stained blood smears from tail blood. Body weight was determined at different time points. For in vivo blockade of CTLA-4, mice received 500 μg anti-CTLA-4 (4F10) at the day of infection. For depletion of CD4+ T cells, mice were injected i.p. with 300 μg rat anti-mouse CD4 Ab (GK1.5) at days −3, 0, and +3. This regimen consistently depleted most CD4+ T cells. Until day 10, less than 1% of spleen cells were positive for CD4, as judged by flow cytometry.

Abs and reagents

Hybridoma cells producing neutralizing anti-CTLA-4 (UC10-4F10, hamster IgG) were obtained from J. Bluestone (University of Chicago, Chicago, IL). Abs were purified from supernatants or purified Ab subsequent to stimulation with Con A. Activity of the Ab was checked by staining spleen cells with supernatants or purified Ab subsequent to stimulation with Con A. Mice were injected with 500 μg anti-CTLA-4 i.p. at the day of infection. Control mice were treated with the same amount of hamster IgG. Anti-CD3 mAbs were purified from the hybridoma cell line 145-2C11. Rat anti-mouse CD4 used for depletion of CD4+ T cells was purified from the hybridoma cell line GK1.5, FITC-labeled anti-CD4, FITC-labeled anti-CD8, FITC-labeled anti-CD25, and PE-labeled anti-CTLA-4 Abs were purchased from BD Biosciences (Heidelberg, Germany). For immunohistochemistry, the following primary Abs from BD PharMingen (Hamburg, Germany) were used: anti-CD4 (L3T4, dilution 1/50), anti-CD8

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Materials and Methods (15). Flow cytometry revealed surface expression of CTLA-4 on 1% of CD4⁺ spleen cells in uninfected mice that increased to 4.6% on day 8 postinfection (p.i.) (Fig. 1A). Immunohistological staining intracellular and surface CTLA-4 rarely detected positive stained cells in uninfected mice, whereas up to 10% of spleen cells were stained on day 8 p.i. (Fig. 1B). This staining was restricted to CD4⁺ cells (Fig. 2), and all cells that expressed CTLA-4 intracellularly also expressed it at the surface, as seen by cap formation.

Apparently, at least in vivo, there is concomitant intracellular and surface expression of CTLA-4 on T cells. Because cytospin staining detects CTLA-4 in both localizations, this method is more accurate and more sensitive than flow cytometry and clearly has less background staining problems, although it is a simple technology. This notion is supported by the finding that in immunohistological staining of spleen sections, only very few CTLA-4-expressing cells were detected in uninfected mice, whereas after infection this number increased >20-fold (data not shown).

Induction of CTLA-4 expression during the course of malaria

A kinetic analysis revealed that CTLA-4 expression reached a peak on day 9 p.i. and declined thereafter (Fig. 3). Interestingly, maximum expression of CTLA-4 was found prior to the strong increase in parasitemia. In spleens from infected mice, an increase of CD25 expression by CD4⁺ T cells was detected, whereas no coexpression of CTLA-4 and CD25 was found (data not shown).

We also found a strong decrease of viable CD4⁺ cells in the spleens from infected mice. The proportion of splenic CD4⁺ T cells decreased from 24% in uninfected mice to 18% on day 5 p.i. (without overt parasitemia), to 12% on day 9, and to only 5% on day 12. In addition, both the ratio of CD4⁺ to CD8⁺ cells (2.5 on day 0 p.i. to 1.2 on day 8 p.i.) as well as the ratio of CD4⁺ to B220⁺ cells (0.45 on day 0 p.i. to 0.21 on day 8 p.i.) decreased, indicating a selective loss of CD4⁺ cells from the spleen. This decrease was accompanied by a small increase of apoptotic CD4⁺ T cells as judged by the TUNEL staining (0.3% in uninfected mice; 0.51% on day 5 p.i.; 1.58% on day 12 p.i.). However, no apoptotic cell positive for CTLA-4 could be detected (data not shown). A massive increase of propidium iodide-positive CD4⁺ T cells occurring after day 12 was detected by flow cytometry and precluded the analysis of CTLA-4 expression (data not shown).
After infection, mice were monitored daily for weight, parasitemia, and survival. Reproducibly, 20% of infected mice died between day 8 and 10 with a low parasitemia (80% of parasitized erythrocytes on day 20), suffered from severe anemia, and died between day 18 and 29 without neurological symptoms (Fig. 4A).

To study whether CTLA-4 blockade in vivo could influence the outcome of *P. berghei* blood-stage infection, mice were treated on day 0 with 500 μg anti-CTLA-4 Ab or hamster IgG as control and challenged subsequently with parasites. The mAb 4F10 has been shown to block ligand binding of CTLA-4 without agonistic activity (24).

Treatment with anti-CTLA-4 Ab dramatically altered the course of the disease. Essentially, all mice died between day 8 and 10 p.i., with low levels of parasitemia ranging from 5 to 8% (Fig. 4B). All anti-CTLA-4-treated mice were severely impaired, showing neurological symptoms reflecting CM. Infected mice treated with anti-CTLA-4 Ab also lost more weight than control mice did (weight of control mice, 17.9 g; anti-CTLA-4, 15.4 g on day 5 p.i.). The development of parasitemia did not differ between treated and control mice (Fig. 4B). Treatment of noninfected mice with anti-CTLA-4 did not induce weight loss or other observable disorders, and mice remained healthy for >100 days (data not shown). To further analyze the effect of CTLA-4 blockade, IL-12 p40−/− mice, IFN-γ−/− mice, and wild-type mice whose CD4+ T cells were depleted with the mAb GK1.5 were treated with anti-CTLA-4. Infection of these mice with *P. berghei* did not lead to the observed exacerbation of malaria as seen with anti-CTLA-4-treated wild-type mice (Table I), which indicates that IL-12 production, IFN-γ production, and CD4+ T cells are involved in the induction of CM.

**Ex vivo analysis of spleen cells from anti-CTLA-4-treated mice**

Spleen cells from treated and untreated mice were harvested and analyzed on day 5 p.i., when parasites were not yet detectable in either group, and on day 9 when both groups had comparable parasitemia (5–8%). Flow cytometry analysis showed that in anti-CTLA-4-treated animals, the selective decrease of CD4+ T cells in comparison with B cells, which was observed during the course of infection, was reversed (Fig. 5). However, the overall numbers of...
spleen cells isolated from mice of either group were comparable, and they were higher than in control mice (control, 0.9 × 10^6; day 9 p.i., 1.2 × 10^6; anti-CTLA-4 treated, day 9 p.i., 1.3 × 10^6).

Both the spontaneous and the anti-CD3-induced proliferation of spleen cells taken on day 5 p.i. were greatly enhanced in anti-CTLA-4-treated animals as compared with untreated, but infected mice (Fig. 6A). A specific proliferative response to *P. berghei* Ag, however, was detectable neither in control nor in anti-CTLA-4-treated mice (not shown). In the supernatants of anti-CD3-stimulated spleen cells from either group, neither IFN-γ nor NO was detectable (Fig. 6A).

In an identical set of experiments, spleen cells of infected mice were analyzed on day 8 p.i., when a fraction of the mice already had died. At this stage, the proliferative response was lower possibly due to the deceased state of the mice. In anti-CD3-stimulated spleen cells, IFN-γ and NO were detected in the supernatant (Fig. 6B). The levels of IFN-γ were not significantly different between untreated and anti-CTLA-4-treated mice, but a higher NO production was found in the treated group. In the sera from infected mice, which were either untreated or treated with anti-CTLA-4, we were unable to detect IFN-γ, TNF-α, IL-10, or IL-4 either on day 5 p.i. or on day 9 p.i. (data not shown).

These results show that anti-CTLA-4 treatment enhanced proliferation of splenic CD4^+ T cells in infected mice, although ex vivo cytokine production was not significantly altered.

**Histologic examination of brain sections and liver sections**

Because infected mice receiving anti-CTLA-4 treatment developed symptoms of CM and died early after infection, we compared the histological changes in the brain. At day 9 after infection, anti-CTLA-4-treated mice displayed severe vascular changes. Stasis in many capillaries and veins developed (Fig. 7A). Disruption of the vessel wall with petechial or more severe bleedings (Fig. 7, A–C) and swelling of endothelial cells (Fig. 7D) were common findings. Many erythrocytes were parasitized (arrow in Fig. 7, A–C). These changes were focal and located mainly in the white matter of the brain as well as in the cerebellum. Occasionally, histological signs of meningitis could be documented (Fig. 7E). In contrast, infected animals without CTLA-4 treatment revealed no bleedings, whereas perivascular edema without cellular infiltration were sometimes present (data not shown). Immunohistochemical analysis revealed that in infected mice that received anti-CTLA-4 treatment, perivascular cellular infiltrates containing macrophages, CD4^+ T cells, CD8^+ T cells (Fig. 7, F–H), and a few B cells (data not shown)
T cells, binds to B7 molecules with higher affinity than CD28. CTLA-4 may also antagonize CD28 function by competing for B7 molecules, or by inhibiting CD28-mediated TCR reorganization. The effect of CTLA-4 blockade in vivo was first demonstrated in a tumor model in which an enhanced antitumor immunity was observed. Several studies have shown that blocking of CTLA-4 ligation also improved adaptive immune response against various infectious agents, leading to an enhanced clearance of pathogens. However, in mycobacterial infections, an increased immune response was induced, but did not influence the course of infection. In other experimental systems, CTLA-4 blockade enhanced the severity of autoimmune diseases by increasing the pool of responding T cells. Taken together, these findings corroborate the notion that blocking of CTLA-4 in vivo leads to an enhanced T cell response.

We have previously described that CTLA-4 was strongly induced on T cells during acute human malaria. Furthermore, levels of CTLA-4 expression were positively correlated with disease severity and parasitemia, possibly due to activation of T cells by plasmodial Ags. Plasmodial extracts have been indeed shown to activate a large number of memory cells, possibly by cross-reactivity. To analyze the role of CTLA-4 on T cells in blood-stage malaria, we used the model of *P. berghei* infection of C57BL/6 mice that allows investigation of CTLA-4 function in vivo. This experimental infection has several similarities to human blood-stage malaria, such as the development of CM. Similar to human malaria, a strong increase of CTLA-4-positive T cells was found during the course of infection with *P. berghei*. Expression of CTLA-4 reached its maximum on day 9 p.i., when almost 10% of CD4+ T cells expressed CTLA-4, and this expression was almost exclusively confined to CD4+ T cells. Under our experimental conditions, the majority of mice developed high parasitemia of >60% and severe anemia, but survived the infection for >28 days without neurological signs. Only a minority of ~20% died early (day 8–10) with low parasite loads and symptoms of CM. Concomitantly, a suppression of the T cell system developed. In the spleen, the number of CD4+ cells decreased by >50%, and CD4+ cells analyzed ex vivo did neither proliferate spontaneously nor in response to anti-CD3. Treatment of such spleen cells from infected mice with anti-CTLA-4 did not restore immune suppression (data not shown), suggesting that TGF-β production by CTLA-4-positive cells is not linked to immune suppression in *P. berghei* malaria, as it was described for *Leishmania* infections.

In vivo treatment with the mAb 4F10 that blocks ligand binding of CTLA-4 dramatically changed the course of the disease. In contrast to untreated mice, essentially all anti-CTLA-4-treated mice developed clinical signs of CM and died between days 8 and 9 with parasitemia of only 5–8%. Ex vivo analysis of spleen cells from anti-CTLA-4-treated mice revealed that they showed a marked spontaneous proliferation and also responded strongly to anti-CD3 stimulation. They also produced more NO, although no differences in IFN-γ and TNF-α production in supernatants from stimulated spleen cells or in the sera were found.

Histologic analysis of brains from infected mice receiving anti-CTLA-4 treatment underlined that these mice suffered from CM, which was already suggested from the clinical course of the disease. Brain sections of these mice revealed severe vascular changes, with bleedings showing parasitized erythrocytes. In perivascular infiltrates, macrophages and CD4+ and CD8+ cells were detectable. In contrast, vascular changes in infected mice that had not received anti-CTLA-4 treatment were less prominent, and bleeding and parasite sequestration were virtually absent. Furthermore, we found that the observed exacerbation of pathology during CTLA-4 blockade was not only restricted to the brain, but was also present in the liver.

These findings demonstrate that CTLA-4 blockade exacerbated CM of *P. berghei*-infected mice, and thus suggest that a strong T cell response during blood-stage malaria induces immune pathology. Although an increased production of proinflammatory cytokines was neither detectable in the serum nor in the supernatant of stimulated spleen cells, IL-12/IFN-γ/IL-10 as well as IFN-γ/IL-10 were resistant to CM induced by anti-CTLA-4 treatment. The same was found when CD4+ T cells from C57BL/6 wild-type mice were depleted. This indicates that IFN-γ-producing Th1 cells promoted the observed exacerbation of the disease. Others have shown that during the onset of CM in mice, a coordinated increase of parasite mRNA and message of proinflammatory cytokines were only found in the brain.

**Discussion**

A role for CTLA-4 (CD152) in the negative regulation of T cell function is generally accepted. CTLA-4 is expressed on activated T cells, binds to B7 molecules with higher affinity than CD28, and is a critical negative regulator of T cell activation. The balance between the opposing signals elicited by CD28 and CTLA-4 is central in the regulation of T cell homeostasis. The fatal lymphoproliferative disease arising in *P. berghei*-knockout mice clearly demonstrates the essential role of this molecule to restrict T cell responses in vivo. Several molecular mechanisms have been proposed to mediate the inhibition of T cell responses by CTLA-4. Ligand-induced cross-linking of CTLA-4 leads to impaired IL-2 production and blocks cell cycle progression of activated T cells by inhibiting signals downstream of the TCR and/or CD28. CTLA-4 may also antagonize CD28 function by competing for B7 molecules, or by inhibiting CD28-mediated TCR reorganization. Finally, CTLA-4 triggering has been reported to induce the production of TGF-β.

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two different studies by Perez et al. (31) and Greenwald et al. (32) have shown that CTLA-4 is needed for the induction of anergy. Induction of anergy might be also important in the case of malaria, because T cells are confronted with enormous amounts of plasmodial Ags released by infected erythrocytes. High CTLA-4 expression during blood-stage malaria may be necessary for the induction and maintenance of anergic T cells. This might explain why infected mice, in our experimental system, did not show infiltration of lymphocytes into brain lesions unless CTLA-4 ligation was blocked.

Several reports have demonstrated that during the hepatic stage of P. berghei infection, responses of CD8+ and of CD4+ T cells conferred protective immunity (33-36). The role of CD4+ T cells during the blood stage of infection is not completely clear, but many examples demonstrate the importance of CD4+ T cells in the control of blood-stage malaria (1). However, it was shown that P. berghei-specific CD4+ T cells have the ability to promote disease (5, 11, 37). Symptoms were attenuated by immunosuppressive drugs (38, 39) or by removing the thymus (40). Elevated levels of cytokines (5, 11, 37). However, IL-12 production was also shown to induce immune pathology rather than protection using a more virulent pathogen in the pathogenesis of experimental murine cerebral malaria. J. Immunol. 157:1620.


