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Suppression of CD4$^+$ Effector Responses by Naturally Occurring CD4$^+$ CD25$^+$ Foxp3$^+$ Regulatory T Cells Contributes to Experimental Cerebral Malaria

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The role of naturally occurring CD4$^+$ CD25$^+$ Foxp3$^+$ regulatory T cells (nTreg) in the pathogenesis of cerebral malaria (CM), which involves both pathogenic T cell responses and parasite sequestration in the brain, is still unclear. To assess the contribution and dynamics of nTreg during the neuropathogenesis, we unbalanced the ratio between nTreg and naive CD4$^+$ T cells in an attenuated model of *Plasmodium berghei* ANKA-induced experimental CM (ECM) by using a selective cell enrichment strategy. We found that nTreg adoptive transfer accelerated the onset and increased the severity of CM in syngeneic C57BL/6 (B6) *P. berghei* ANKA-infected mice without affecting the level of parasitemia. In contrast, naive CD4$^+$ T cell enrichment prevented CM and promoted parasite clearance. Furthermore, early during the infection nTreg expanded in the spleen but did not efficiently migrate to the site of neuroinflammation, suggesting that nTreg exert their pathogenic action early in the spleen by suppressing the protective naive CD4$^+$ T cell response to *P. berghei* ANKA infection *in vivo* in both CM-susceptible (B6) and CM-resistant (B6-CD4$^{-/-}$) mice. However, their sole transfer was not sufficient to restore CM susceptibility in two CM-resistant congenic strains tested. Altogether, these results demonstrate that nTreg are activated and functional during *P. berghei* ANKA infection and that they contribute to the pathogenesis of CM. They further suggest that nTreg may represent an early target for the modulation of the immune response to malaria.

Cerebral malaria (CM) remains one of the most severe and intriguing complications associated with *Plasmodium falciparum* infection. In 2014, 97 countries and territories had ongoing malaria transmission. An estimated 3.3 billion people are at risk of malaria, of whom 1.2 billion are at high risk. In high-risk areas, more than one malaria case occurs per 1,000 population (1). Among these, up to 30% of the fatal cases are due to CM: annual deaths from malaria may in fact be twice as high (2). Although the pathogenesis of human CM is not completely elucidated, in addition to the sequestration of parasitized erythrocytes into the brain microvasculature and infiltration by leukocytes and platelets, this deadly syndrome involves an immunopathological T cell response promoted by an exacerbated inflammatory state. These events may also be preceded and amplified by the systemic production of proinflammatory cytokines favoring permeability of the blood-brain barrier (3, 4). However, the role of immune cells in the pathogenesis of human CM remains controversial.

While the host-parasite relationship in humans has been difficult to determine, murine experimental malaria models have enabled valuable contributions to the understanding of the pathogenesis of CM, even if they do not strictly replicate the pathophysiology of cerebral disease in humans (5). In experimental cerebral malaria (ECM), both CD4$^+$ and CD8$^+$ T cells are detected in the brain at the onset of neurological symptoms and play a role at both local and systemic levels by contributing to parasite tissue sequestration and high levels of tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) in circulation (6–13). Removal of CD4$^+$ T cells is protective around the time of challenge, whereas CD8$^+$ T cell depletion is effective immediately prior to the onset of neurological symptoms. Both CD4$^+$ and CD8$^+$ T cells are involved in the pathogenesis of ECM (14) but are also required for the effective control of malaria parasites (15), emphasizing the delicate balance that exists between host-mediated control and pathogenesis of infection. Since ECM is associated with an exacerbated immune response, it was hypothesized that naturally occurring regulatory T cells (nTreg), which are known for their intrinsic capacity to temper various immune responses to self or microbial antigens (16, 17), somehow fail to control the pathogenic response. However, to date, there is conflicting information on the role of nTreg in the development of this neuropathological syndrome, due to the difficulties of con-

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ducting human and animal studies (18–20), among which is the lack of specific reagents to target nTreg.

The main experimental approach used to evaluate the role of nTreg during ECM development has consisted of unbalancing the ratio between nTreg and effector T cells Teff). nTreg depletion prior to and/or during ECM pathogenesis, using various antibody treatments, has proven either beneficial (21–23) or neutral (24) in protecting *P. berghei* ANKA-infected mice against CM. Such discrepancies may be explained by the distinct depletion protocols used, which might have a variable impact on effector cells and/or sustained versus transient reduction of nTreg (25). In fact, a more selective Treg depletion protocol in DEREG mice (26) argued for a limited role of Treg in the control of ECM (27). Yet this depletion system requires the repeated injection of diphtheria toxin in the course of the infection, which might result in additional neurotoxicity and immune activation, as recently evidenced (28, 29), thereby modulating the disease.

Furthermore, a reverse experimental approach was concluded by Haque et al., consisting of enriching nTreg prior to *P. berghei* ANKA infection. The authors reported that interleukin-2 (IL-2)/anti-IL-2 systemic treatment, which notably drives nTreg expansion *in vivo*, prevents ECM (30), suggesting a protective role of nTreg. Altogether, the possible adverse side effects of the above protocols have made it difficult to decipher the role of nTreg in CM pathogenesis. Furthermore, the early (day 6 postinfection) and fulminant onset of CM in these murine models leaves perhaps a too short time window for effective manipulations. Therefore, to mitigate these issues, we applied a different strategy that consisted of selectively enriching either the regulatory or the naive $CD4^+$ T cell compartment by adoptive transfer prior to infection. This approach has ensured us a higher specificity of the cells manipulated and a single experimental intervention, in the absence of any drug treatment. In addition, we established a milder mouse model of *P. berghei* ANKA-mediated CM to improve the experimental readout, using a low-dose total-body-irradiation protocol before cell transfer.

In this sublethal irradiation model, we show for the first time that the specific increase of nTreg numbers by adoptive transfer before the infection worsens the neuropathogenesis in CM-sensitive mice, by inhibiting the protective $CD4^+$ T cell response. Moreover, our model provides a unique platform to study the role and plasticity of nTreg and other adoptively transferred cells as well as the residual endogenous immune response during ECM.

MATERIALS AND METHODS

Mice. Six- to 8-week old C57BL/6, C57BL/6-$CD4^{-/-}$, C57BL/6-$Foxp3^{EGFP}$, and WLA × C57BL/6 (F1) mice were used throughout the study and bred in our animal facility under specific-pathogen-free conditions. Additional C57BL/6 mice were purchased from Elevage Janvier (Le Genest-St-Isle, France). C57BL/6-$CD4^{-/-}$ and C57BL/6-$Foxp3^{EGFP}$ mice were on the C57BL/6 background for more than 20 generations.

Ethics statement. Animals were housed at the Institut Pasteur animal facility, which has been accredited by the French Ministry of Agriculture to perform experiments on live mice (accreditation no. B 75 15-01, 22 May 2008), in application of the French and European regulations on care and protection of Laboratory Animals (EC Directive 86/609, French Law 2001-486, 6 June 2001). The study was approved by the Animal Experimentation Ethics Committee of the Institut Pasteur and was performed in compliance with the NIH Animal Welfare Insurance (A5476-01, issued on 2 July 2007). All efforts were made to minimize animal suffering.

Parasite and infection. Mice were acclimatized for a week before any experiment. Mice were infected with *P. berghei* ANKA (clone 1.49L) by intraperitoneal (i.p.) injection of 10^6 infected erythrocytes. The parasite was maintained through limited passage in susceptible mice and stored as stabilates in liquid nitrogen.

Mice were judged as developing cerebral malaria if they displayed neurological signs such as ataxia, loss of reflex, hemiplegia, roll-over, or convulsions and died between 6 and 12 days postinfection with relatively low parasitemia. Each mouse was evaluated several times daily. Parasitemia was assessed from Giemsa-stained smears of tail blood, sampled at fixed times. For the determination of CM incidence, time course, and mortality, moribund animals were euthanized by CO_2 inhalation when they displayed severe clinical signs (paralysis, roll-over, convulsions, or coma). When clinical signs were not severe, CM death was used as an endpoint. Animals that did not develop CM were euthanized by CO_2 inhalation in the 2nd or 3rd week postinfection unless experiments were carried out later during the hyperparasitemia (HP) phase of *P. berghei* ANKA infection. In the latter case, animals were euthanized by CO_2 inhalation if they displayed severe weight loss, anemia, and weakness.

For investigations performed at the time of CM, animals were euthanized when they showed advanced signs of CM prior to blood, spleen, and brain collection, in this order. To avoid traumatic cerebral hemorrhage, cervical dislocation was not used.

Adoptive transfer. Spleens of naive C57BL/6-$Foxp3^{EGFP}$ mice (31) were prepared as single-cell suspensions, and $CD4^+$ $CD25^{+/-}$ cells were isolated by magnetic cell sorting using the $CD4^+$ $CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. Cell purity was controlled systematically and ranged between 80 and 90%. Recipient mice were irradiated at 300 rads, allowed to rest for 3 to 4 h, and then injected intravenously (i.v.) with 3×10^5 to 10×10^5 freshly isolated cells. Control mice received physiological serum. *P. berghei* ANKA infection was performed within 3 h.

Flow cytometry analysis. Purity of freshly isolated cells and recovery of transferred cells were analyzed by fluorescence-activated cell sorting (FACS). Blood, spleens, and brains were harvested in that order. Blood samples were immediately cleared up of red blood cells by ammonium-chloride-potassium (ACK) lysis. Spleens were gently mashed, homogenized, and filtered in phosphate-buffered saline (PBS) containing 3% fetal calf serum (FCS) before ACK treatment. Brain tissue homogenization was preceded by digestion in HEPES–0.05% collagenase (Sigma) for 30 min at 37°C. Infiltrated cells were obtained by centrifugation at 2,600 rpm for 20 min at 20°C in Dulbecco's modified Eagle's medium (DMEM)–35% Percoll (Pharmacia). All samples were resuspended in PBS–3% FCS for staining. Following FcγR blocking with purified anti-CD16/32 (2.4G2; BD Pharmingen), cell suspensions were labeled at 4°C with anti-CD25–phycoerythrin (PE) (7D4; BD Pharmingen), anti-CD4–allophycocyanin (APC) (RM4-5; BD Pharmingen), or anti-CD4–APC–Al750 (GK1.5; eBioscience) monoclonal antibodies. The expression of the nTreg and naive $CD4^+$ T cell activation marker LAG-3 (CD223) (32, 33) was also assessed using purified anti-LAG-3 (C9B7W; BD Pharmingen) monoclonal antibody or isotype control (IgG1κ [R3-34; BD Pharmingen]), followed by staining with anti-rat IgG1-biotin (RG11/39.4; BD Pharmingen) and streptavidin-APC (BD Pharmingen). Endogenous green fluorescent protein (GFP) was used to detect Foxp3 expression. Dead cells were gated out using propidium iodide. Data acquisition and analysis were performed using a FACSCanto (BD) and the FlowJo (Tree Star) software, respectively.

***In vivo* suppression assays.** Splenic nTreg ($CD4^+$ $CD25^+$ $Foxp3^{EGFP+}$) and naive $CD4^+$ T cells ($CD4^+$ $CD25^-$ $Foxp3^{EGFP-}$) were isolated from C57BL/6-$Foxp3^{EGFP}$ mice as described above. Suppression assays were performed *in vivo* by coinjecting intravenously (i.v.) a fixed number of naive $CD4^+$ T cells with titrated numbers of nTreg into irradiated syngeneic wild-type or C57BL/6-$CD4^{-/-}$ mice. Mice were infected i.p. with *P. berghei* ANKA within 3 h. Development of CM and parasitemia served as a readout of suppression activity.

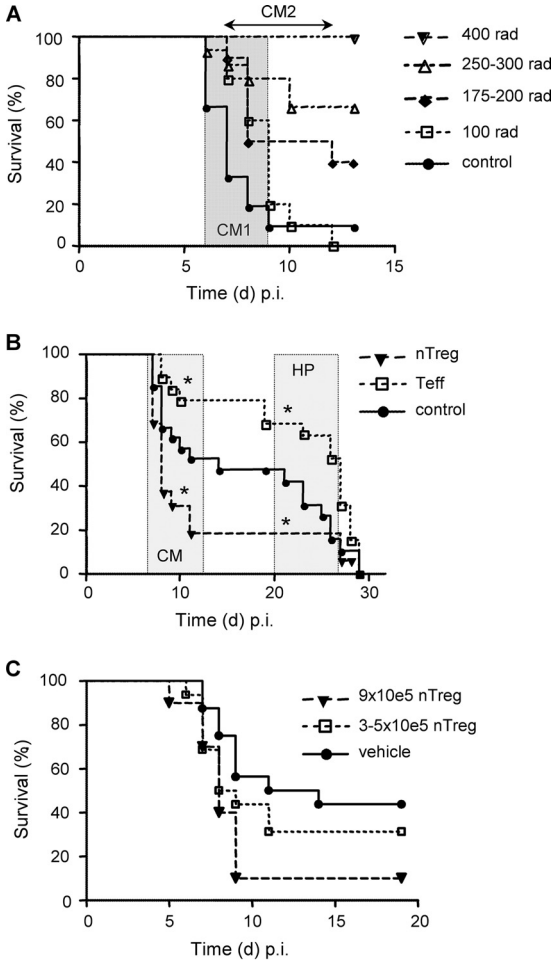

FIG 1 nTreg worsen experimental cerebral malaria. (A) Experimental model of cerebral malaria for adoptive cell transfer. C57BL/6 (B6) mice were irradiated at different doses and allowed to rest for a couple of hours prior to infection with 1×10^6 *P. berghei* ANKA-infected erythrocytes. Cumulative survival (Kaplan-Meier analysis) combined from 3 independent experiments is shown (400 rads, $n = 5$; 250 to 300 rads, $n = 15$; 175 to 200 rads, $n = 10$; 100 rads, $n = 10$; control, $n = 21$). The shaded zone indicates the CM window for the nonirradiated control group (CM1), and the arrow shows the new CM window following irradiation (CM2). Low-dose irradiation of 100 to 300 rads was then used throughout the study, and an irradiated control group was included in each experiment. (B and C) Naive B6-Foxp3^{EGFP} mice were used as donors for the adoptive transfer of various doses of freshly isolated nTreg (CD4$^+$ CD25$^+$ Foxp3^{EGFP+}) or CD4$^+$ T cells (CD4$^+$ CD25$^-$ Foxp3^{EGFP-}) to CM-susceptible B6 mice prior to standard infection. Transfer experiments were undertaken into irradiated WT mice only, not into unirradiated WT mice. Teff, effector T cells. (B) Cumulative survival by Kaplan-Meier analysis following transfer of 4.5×10^5 cells. Graphs show pools of 4 independent experiments ($n = 16$ to 21 animals per group). *, $P < 0.05$ by log-rank analysis over the shaded period considered compared to the control (physiological serum) group. (C) Cumulative survival by Kaplan-Meier analysis following transfer of increasing doses of nTreg and *P. berghei* ANKA infection. Three independent experiments were pooled ($n = 10$ to 16 per group).

Multiplex cytokine analysis. After 8 days of *in vivo* activation with or without *P. berghei* ANKA, spleens were removed, gently mashed into single-cell suspensions, and pooled by experimental group (5 to 8 animals each). Purification was performed in 2 steps. First, CD4$^+$ cells were magnetically enriched using the CD4$^+$ CD25$^+$ regulatory T cell biotin-antibody cocktail against CD8a, C11b, CD45R, CD49b, and Ter-119 (Miltenyi Biotec) and antibiotin microbeads. For infected samples, additional anti-Ter-119 microbeads were used to eliminate persisting infected red blood cells. Second, CD4$^+$ Foxp3^{EGFP+} nTreg cells were sorted using a MoFLo (Beckman Coulter) (purity of >97%) based on GFP expression. Cells were plated in complete RPMI medium (supplemented with 10% FCS) in a 96-well plate previously coated with purified anti-CD3 (5 μg/ml) and incubated at 37°C for 24 h. Control wells included unstimulated duplicates or plain medium. Supernatants were harvested, and cytokine and chemokine production of nTreg were simultaneously measured by a multiplexed bead assay, using the Milliplex Map Mouse cytokine/chemokine premixed 22 Plex (Millipore), according to the manufacturer's protocols. Cytokine concentrations were determined by the Luminex 100 system (Austin, TX) using the STarStation software (Applied Cytometry, Sheffield, United Kingdom) in two independent assays.

Statistical analysis. Data were analyzed with the Prism software package (GraphPad). Survival times were compared with a nonparametric log-rank test (Mantel-Cox). We used the Mann-Whitney test for the analysis of parasitemia data. *P* values of ≤0.05 were considered significant.

RESULTS

nTreg exacerbate cerebral malaria, while naive CD4$^+$ T cells are protective. To attenuate the sudden onset and development of ECM and favor cell engraftment, we first set up a CM permissive model of adoptive cell transfer by submitting CM-sensitive C57BL/6 (B6) mice to low-dose irradiation 4 h before *P. berghei* ANKA infection. As shown in Fig. 1A, in the nonirradiated control group, the first onset of neurological symptoms was observed at day 6 postinfection (p.i.), ranging from abnormal balance control to partial paralysis and sometimes convulsions. Ninety percent of the mice developed neurological symptoms, and all were dead by day 9 p.i. Interestingly after irradiation of the mice at 300 rads, the onset of CM was delayed by 1 day, and only 40 to 60% of them developed similar symptoms, with death extending to day 12 p.i. However, at 400 rads, no mice developed CM.

Using this experimental system endowed with a wider window of analysis, we then unbalanced the nTreg/naive CD4$^+$ T cell ratio in mice irradiated at 300 rads by injecting unactivated CD4$^+$ CD25$^+$ Foxp3$^+$LAG-3$^-$ (nTreg) (32) or CD4$^+$ CD25$^-$ Foxp3$^-$ LAG-3$^-$ (CD4$^+$ T cells) (33) splenic cells from naive C57BL/6-Foxp3^{EGFP} mice (31) (see Fig. S1 in the supplemental material) prior to *P. berghei* ANKA infection. As in previous experiments, 48% of irradiated control mice, which received physiological serum, died of CM between days 6 and 12 p.i., while the remaining mice succumbed from consequences of hyperparasitemia (HP) in the third week p.i. (Fig. 1B). Strikingly, recipients of 4.5 × 10e5 nTreg started to show typical neurological symptoms (abnormal walking, paralysis, roll-over) earlier than the control group. Furthermore, the incidence of CM and resulting death was higher (81%) after nTreg transfer. This effect was dependent on cell numbers, and the transfer of very high doses of nTreg (9 × 10e5) restored full CM susceptibility associated with a CM death rate of 90% (Fig. 1C). Surprisingly, the equivalent transfer of naive CD4$^+$ T cells significantly protected mice against CM as only 21% of the mice developed fatal neurological signs. In addition, protection consisted of full resistance to the neuropathy, since no abnormal neurological manifestations arose in naive CD4$^+$ T cell recipients. Yet, the transferred cells did not protect against HP-dependent death, and all remaining animals eventually succumbed in the third week p.i, following similar kinetics to the untreated group (Fig. 1B).

nTreg adoptive transfer does not affect parasitemia during *P. berghei* ANKA infection. Protection from CM following anti-

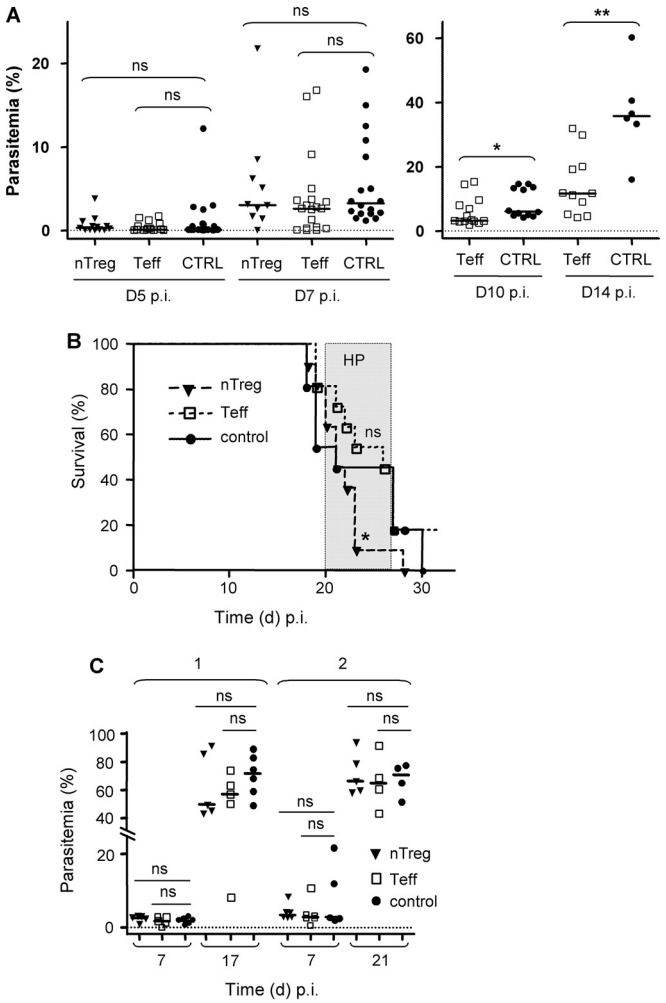

FIG 2 nTreg exacerbate pathology but not parasitemia in *P. berghei* ANKA-infected mice. (A) Course of parasitemia in CM-sensitive irradiated B6 mice following adoptive transfer of 4.5×10^5 nTreg ($CD4^+$ $CD25^+$ $Foxp3^{EGFP+}$) or naive $CD4^+$ T cells ($CD4^+$ $CD25^-$ $Foxp3^{EGFP-}$) prior to *P. berghei* ANKA infection. Graphs show pools of 4 independent experiments ($n = 16$ to 21 animals per group). Teff, effector T cells; CTRL, control. (B and C) CM-resistant (WLA × B6) F1 mice received 4.5×10^5 freshly isolated nTreg or naive $CD4^+$ T cells from naive B6 donors before standard *P. berghei* ANKA infection. (B) Cumulative survival was assessed by Kaplan-Meier analysis. The graph combines two independent experiments ($n = 10$ to 11 per group). The shaded zone indicates the HP phase. (C) Corresponding parasitemia in the course of infection. Two experiments are displayed ($n = 5$ to 6 per group). (A and C) ns, not significant; **, $P < 0.01$ by Mann Whitney analysis. Median bars are shown. (B) *, $P < 0.05$ by log-rank analysis during the HP phase compared to the control (physiological serum) group.

body-mediated Treg depletion has been shown to correlate with a reduced parasite burden in the peripheral vasculature (21, 22). To find out whether nTreg contribution to CM severity was associated with an increased parasitemia in our model, we assessed the rate of *P. berghei* ANKA erythrocyte infection following nTreg transfer. Interestingly, at the limiting neuropathogenic dose of 4.5 × 10e5 transferred nTreg, there was no significant impact on parasite growth during the blood stage at the early phase (days 5 and 7 p.i.) of infection (Fig. 2A). Similarly, the animals that received an equivalent number of neuroprotective naive $CD4^+$ T cells did not control their early parasitemia better than untreated mice (Fig. 2A). However, mice protected from CM in the naive $CD4^+$ T cell group had a lower parasitemia on days 10 and 14 after infection. Since very few mice escaped CM in the nTreg group, we could not evaluate the impact of nTreg on parasite development during the HP phase. Therefore, we used CM-resistant (CMR) (WLA × B6) F1 mice irradiated at 300 rads, which provide a model of *P. berghei* ANKA-mediated lethal hemolytic anemia associated with HP. This model provides excellent tool for analyzing the physiological pathways involved in disease processes to fatal syndromes during malaria and is totally compatible as F1 mice cannot reject the parental transferred cells (34). Donor B6 nTreg did not reverse the CMR phenotype of these mice but significantly decreased the frequency of mice that survived the HP phase, compared to those in the control and naive $CD4^+$ T cell groups ($P < 0.05$) (Fig. 2B). As in B6 mice, naive $CD4^+$ T cell transfer did not protect mice from death in the third week p.i. However, the differential effect of nTreg and naive $CD4^+$ T cell transfer on survival during the HP phase was neutral on parasite replication (Fig. 2C). Altogether, on one hand, these results show a dramatic deleterious effect of nTreg in CM development that was not associated with a higher rate of parasite replication. On the other hand, they also show that the naive $CD4^+$ T cell compartment, when conferring full resistance to the neurosyndrome, had a limited impact on the control of parasitemia before and during the CM phase. Finally, at later time points of *P. berghei* ANKA infection, naive $CD4^+$ T cell control of parasite growth was more significant in CM-susceptible mice (B6) than in CM-resistant mice (WLA × B6).

nTreg suppress the CM-protective naive $CD4^+$ T cell response *in vivo*. nTreg are defined by their intrinsic capacity to inhibit Teff responses (16, 17). Previous studies of Treg function in ECM models have shown that Treg maintain their capacity to suppress proliferative $CD4^+$ T cell proliferation to anti-CD3 (22, 27). However, nTreg function was never evaluated *in vivo* during CM progression.

To assess whether nTreg could inhibit the neuroprotective effect of naive $CD4^+$ T cell enrichment in B6 mice, we coinjected these populations at different ratios prior to *P. berghei* ANKA infection. Again the transfer of 5×10^5 naive $CD4^+$ T cells conferred full resistance to CM in 70% of the recipients (Fig. 3A). However, the cotransfer of nTreg counteracted this protective effect. Increasing the dose of nTreg along with naive $CD4^+$ T cells led to a gradual increased incidence, and high susceptibility to CM (80% death) was fully restored at a ratio of 1:1 of nTreg to naive $CD4^+$ T cells. In addition, nTreg also abrogated the control of parasitemia by naive $CD4^+$ T cells (Fig. 3B). Altogether, these data show that nTreg are functional during CM development *in vivo* and reveal one possible mechanism by which nTreg contribute to the immune-mediated neuropathology—that is by inhibiting protective $CD4^+$ effector T responses.

nTreg are activated in the spleen but do not migrate to the brain during ECM. To determine where nTreg exert their action, we tracked transferred $CD4^+$ $CD25^+$ $Foxp3^{EGFP+}$ cells (33) in the brain, blood, and spleen of infected animals by flow cytometry in the course of *P. berghei* ANKA infection (Fig. 4A). In nTreg-treated mice, significant brain infiltration of host $CD4^+$ cells was observed in all animals developing CM (Fig. 4B), and this was associated with an increased frequency of these cells in the blood compared to naive mice (Fig. 4C). In contrast, the presence of nTreg in both the brain and in circulation was always discrete and inconsistent (Fig. 4B and C). In addition, CM was not significantly

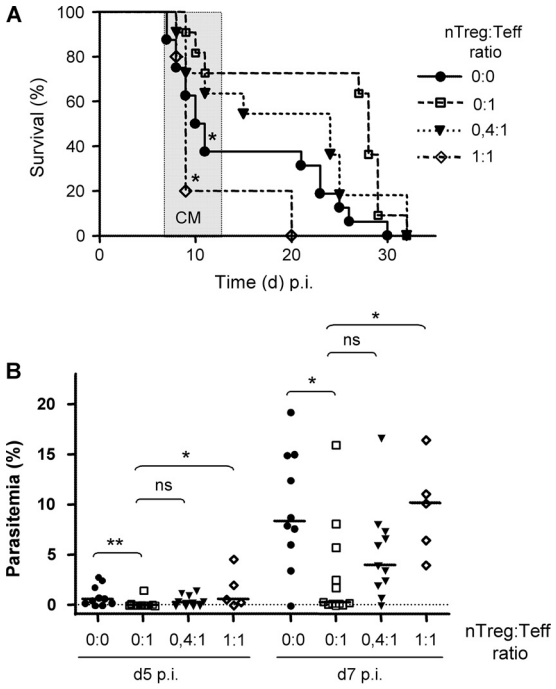

FIG 3 *In vivo* suppression assay of the protective naive CD4$^+$ T cell response in CM-sensitive mice. A total of 5×10^5 naive CD4$^+$ T cells (CD4$^+$ CD25$^-$ Foxp3^{EGFP-}) were cotransferred with titrated numbers of nTreg (CD4$^+$ CD25$^+$ Foxp3^{EGFP+}) to irradiated B6 mice prior to *P. berghei* ANKA infection. Cumulative survival by Kaplan-Meier analysis (A) and corresponding parasitemia (B) are shown. nTreg/naive CD4$^+$ T cell ratios are indicated. Graphs compile 2 to 3 independent experiments. For all groups, $n = 11$ to 16 except for at a 1:1 ratio, where $n = 5$ in panel A and $n = 5$ to 11 per group in panel B. (A) *, $P < 0.05$ by log-rank analysis over the shaded period (CM) considered compared to the 0:1 group. (B) ns, not significant; *, $P < 0.05$, and **, $P < 0.01$, by Mann-Whitney analysis. Median bars are shown.

associated with an increase of the ratio of nTreg and host CD4$^+$ in the brain (data not shown). Thus, there was no selective enrichment of nTreg over Teff at the site of neuroinflammation, as also stated by Steeg et al. in DEREG mice (27). This suggests that nTreg must exert their deleterious effect in the spleen where parasitized erythrocytes are sequestered. Indeed, as shown in Fig. 4D, transferred nTreg were readily detected in the spleen, and their number increased in parallel to that of host CD4$^+$ cells. *P. berghei* ANKA infection triggered a significant expansion of nTreg and CD4$^+$ cells during the pre-CM period (days 3 to 7 p.i.) followed by a contraction during CM (days 8 to 11 p.i.), whereas nTreg and CD4$^+$ numbers remained stable in uninfected animals (Fig. 4D).

Moreover, donor nTreg recovered from infected mice immediately before the onset of CM, had an increased capacity to produce cytokines *in vitro*. Strikingly, however, *P. berghei* ANKA-activated nTreg secreted large amounts of IFN-γ but small quantities of IL-4 and IL-10, whereas those recovered from noninfected recipients did not (Fig. 5). Other cytokines, such as Il-1β, IL-6, IL-17, and TNF-α, were not detected (see Fig. S2 in the supplemental material).

In conclusion, these experiments show that the spleen is a major site of nTreg activation in *P. berghei* ANKA-infected mice. Surprisingly, some of them were prone to secrete IFN-γ rather than anti-inflammatory cytokines, raising the possibility that they could also contribute to the systemic inflammation associated with ECM.

nTreg by themselves are not neuropathogenic. We thus investigated whether nTreg are neuropathogenic themselves. Using our low-irradiation protocol, B6-CD4$^{-/-}$ mice (which are resistant to ECM but which do develop HP) were injected with 5×10^5 nTreg prior to *P. berghei* ANKA infection. As shown in Fig. 6A, nTreg transfer did not induce CM nor alter survival in any recipient compared to the control animals. In addition, a similar course of parasitemia was observed in both groups (Fig. 6B).

In another set of experiments, B6-CD4$^{-/-}$ mice received naive CD4$^+$ T cells either alone or with nTreg. As previously observed in B6 mice, the transfer of naive CD4$^+$ T cells significantly delayed death by HP (Fig. 6C) by decreasing the rate of parasite replication in the blood (Fig. 6D). Both effects were partially inhibited by nTreg (Fig. 6C and D). In conclusion, transferred nTreg are not sufficient to induce ECM in B6-CD4$^{-/-}$ mice, but they inhibited the capacity of naive CD4$^+$ T cells to control parasitemia.

DISCUSSION

It remains critical to understand the mechanisms that could potentially regulate and counteract the development of cerebral malaria. This deadly syndrome involves an immunopathological T cell response promoted by an exacerbated inflammatory state. Thus, a possible curative treatment might be to modulate the activity of natural Treg in order to dampen the immune response. The aim of our work was to specifically address the behavior of nTreg and their role in ECM induced by *P. berghei* ANKA infection as their role during CM development has not yet been clarified.

Our new mouse model of *P. berghei* ANKA-mediated CM uses the classical model of syngeneic adaptive cell transfer in low-dose total-body-irradiated mice. This model was designed to bypass the syngeneic response, create a niche for engraftment of transferred cells, and potentiate enrichment relative to the lymphopenia induced (35–37) on one hand and to decrease the severity of ECM and thereby improve the experimental readout on the other hand.

Our data show that nTreg exacerbate ECM. To our knowledge, this is the first study using a nondepletion experimental approach to assess the role of nTreg in ECM. Our findings also contradict previous reports that showed a beneficial (30) or a neutral (24, 27) role of nTreg in CM pathogenesis. This discrepancy could be explained by the depletion or immunomodulation of activated pathogenic T cells and/or the alteration of the blood-brain barrier induced by the various conditioning regimens used (24, 27, 30). However, although the development of a neuropathological immune response and the contribution of host CD4$^+$ T and CD8$^+$ T cells to ECM were preserved, it is difficult to appreciate the whole systemic effects (endothelium, microglia, intestinal flora) in addition to the immunomodulatory effect of low-dose total-body irradiation (35–39) in our model. Similarly, we are not able to appreciate how the infection and neuropathogenesis could modulate the irradiation effect, which develops over time (35, 36).

It has been proposed that CM encephalopathy results from insufficient or impaired control of potentially neuropathogenic Teff responses by Treg (24, 27). So far, Treg function in the course of *P. berghei* ANKA infection had only been successfully tested *in vitro*, suggesting that Treg function was not abrogated. In addition, harmful *P. berghei* ANKA-activated naive CD4$^+$ T cells were not resistant to suppression *in vitro* (24, 27). Our data extend this observation and demonstrate that not only do nTreg exert their suppressive activity *in vivo* during the course of *P. berghei* ANKA

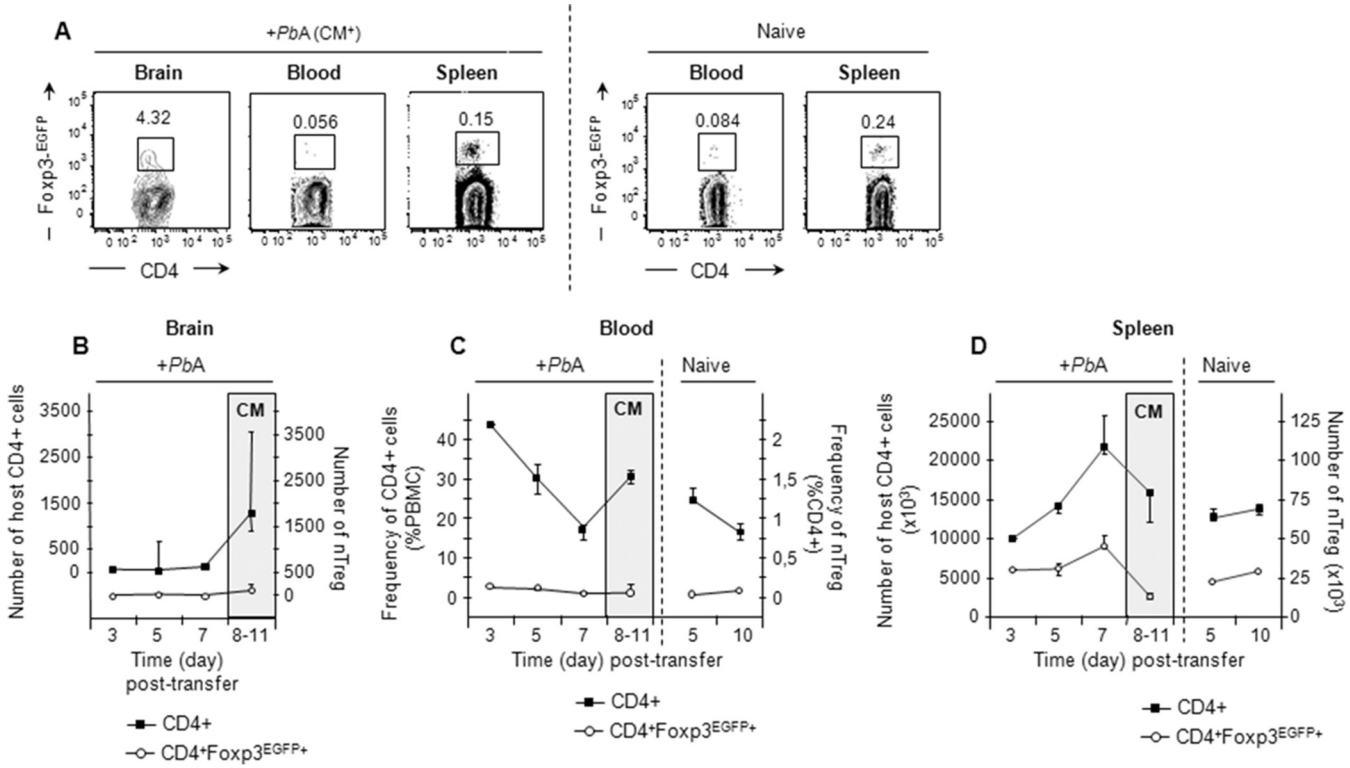

FIG 4 nTreg scarcely migrate to the site of neuroinflammation of CMS mice in the course of *P. berghei* ANKA (*PbA*) infection. Irradiated B6 mice received 5×10^5 freshly isolated splenic nTreg ($CD4^+$ $CD25^+$ $Foxp3^{EGFP+}$) from naive syngeneic donors i.v. prior to standard *P. berghei* ANKA infection. GFP was used to monitor the migration and expansion of transferred nTreg ($CD4^+$ $Foxp3^{EGFP+}$) versus host $CD4^+$ ($Foxp3^{EGFP-}$) cells in the course of the infection by flow cytometry. (A) Representative FACS plots of one infected animal at the time of CM (left panel) and one matching naive animal (day 10 posttransfer) (right panel) are shown. Gating is on $CD4^+$ cells. (B) Coevolution of the brain infiltrate by transferred nTreg (open symbols) and host $CD4^+$ (solid symbols) cells. Absolute numbers were calculated. (C) Relative evolution of transferred nTreg (open symbols) and $CD4^+$ (filled symbols) frequencies in the blood. PBMC, peripheral blood mononuclear cells. (D) Dynamics of transferred nTreg (open symbols) and host $CD4^+$ cell numbers in the spleen of infected and naive animals. CM or naive status is indicated. Medians are plotted, and the interquartile range is shown. Twenty animals were included in the study (naive, $n = 6$).

infection, but they also inhibit the $CD4^+$ T cell response associated with neuroprotection by interacting with the $CD8^+$ T cells' pathogenic response. On the other hand, increasing nTreg capacity *in vivo* did not prevent the migration to the brain of $CD4^+$ T cells potentially involved in the pathology at the onset of CM, reinforcing the idea of an inappropriate control of the immune response (24, 27). Since nTreg are functional both *in vitro* and *in vivo* during the course of CM, the reason for this unbalanced control of the pathogenic T cell response might be linked to naive $CD4^+$ T cell activation. $CD4^+$ T cells' function and migration are dependent on the level of cell activation, which can be suppressed by nTreg. However, it has been shown that hyperactivated T cells become resistant to suppression (40). Thus, one could hypothesize that the dual protective and pathogenic $CD4^+$ T cell response that develops during *P. berghei* ANKA infection could be the result of a differential state of activation within the spleen, which would lead to a selective escape of $CD4^+$ T cells from Treg control *in situ*. In this regard, previous work from our group showed that compartmentalized T cell receptor β (TCR-β) repertoire diversity is associated with experimental cerebral malaria (41; M. F. Encarnita, H. P. Pham, D. Sophie, G. Olivier, D. Klatzmann, P. A. Cazenave, S. Pied, and A. Six, unpublished data). Indeed, $CD4^+$ and $CD8^+$ T cells recovered from the blood and the brain of CM^+ B6 mice display a restricted oligoclonal TCR-β repertoire compared to splenic T cells (41; Encarnita et al., unpublished). This observation indicates that migration to and infiltration of the brain occur selectively. Altogether, these results tell us that unbalancing the nTreg/naive $CD4^+$ T cell ratio in favor of nTreg is not sufficient to promote the development of an appropriate immune response. This approach might be optimized by a combined treatment to restrain pathogenic naive $CD4^+$ T cell activation and favor their suppression by nTreg. The successful application of anti-CD25 antibody treatment (21–23), which also depletes activated Teff, on one hand and the exacerbation of pathology by anti-cytotoxic T lymphocyte-associated antigen 4 (anti-CTLA-4) treatment (42, 43) on the other support this conclusion.

The duality of nTreg action also applies to their cytokine production *in vitro*, which, though restricted, consists of both proinflammatory (IFN-γ) and regulatory (IL-10) cytokines. Our data also suggest that nTreg could contribute directly to the pathogenesis via the production of IFN-γ and to a lesser extent IL-4, both of which are associated with ECM (7, 44). Although never described in ECM, this observation is in line with two recent reports describing the polarization of Treg into IFN-γ-producing Th1 cells following acute infection by *Mycobacterium tuberculosis* (45) and *Toxoplasma gondii* (46) and in other inflammatory settings (47–51). In addition, nTreg plasticity was associated with a modulation of their transcription program (expression of T-bet and possible downregulation of Foxp3) (45–47, 51, 52). Our findings indicate that a fraction of nTreg might have acquired a Th1 inflammatory

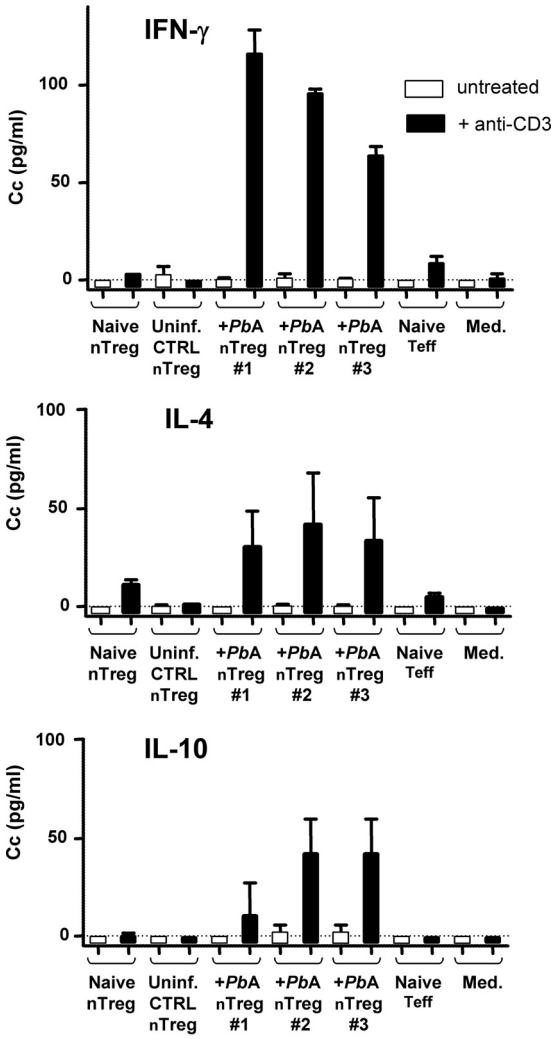

FIG 5 nTreg display a proinflammatory cytokine profile *in vitro*. Irradiated C57BL/6 mice received 4.5×10^5 naive CD4$^+$ CD25$^+$ Foxp3^{EGFP+} splenic cells intravenously and were subsequently infected (+*PbA*) or not (Naive) with *P. berghei* ANKA. Eight days later, splenic CD4$^+$ Foxp3^{EGFP+} cells were sorted (purity of >97%) and cultured in complete medium for 24 h, with (black bars) or without (white bars) anti-CD3. Freshly magnetically activated cell sorting-assisted (MACS) conMACS-isolated (Naive) nTreg and naive CD4$^+$ T cells were also included in the experiment, while plain medium (Med) served as an internal assay control. The supernatants were collected and analyzed by Luminex for production of IFN-γ, IL-4, and IL-10 (from left to right). Cc, cytokine concentration. Four independent experimental groups were enrolled in the study. *P. berghei* ANKA-infected mice (groups 1, 2, and 3, $n = 5$ to 6 per group) received different nTreg, whereas the uninfected control group (Uninf. CTRL; $n = 8$) received a corresponding pool of nTreg. Histograms represent means of replicates, and error bars show standard errors of the mean.

phenotype in the course of *P. berghei* ANKA infection, driven by the antigen stimulation in a favorable inflammatory environment (53). However, further investigation will be required to fully assess nTreg plasticity during ECM as well as quantify the impact of their production of IFN-γ on pathogenesis, in the midst of other IFN-γ-producing cell types (15).

Despite their remarkable expansion and activation in the spleen during infection, transferred nTreg did not significantly circulate in the blood nor emigrate in the brain at the onset of neurological symptoms, as did naive CD4$^+$ T cells. This differential migration behavior indicates that nTreg might be retained in the spleen. Indeed, it has been shown that nTreg can adapt their transcription program according to the microenvironment where they are stimulated (45, 54). This includes, for example, the acquisition of chemokine receptors and adhesion molecules that are also shared by effector T cells differentiating in the same environment. Thus, the failure of nTreg to efficiently migrate to the brain along with effector T cells at the time of CM raises the question of why in this experimental system of malaria, a coordinated migration response does not take place. This further suggests that key events in the regulation of the immune response impacting both protection and pathogenesis take place early in the spleen.

Interestingly, a recent study showed that the timely *in vivo* increase of nTreg numbers driven by IL-2/anti-IL-2 antibody complexes could prevent ECM (30). In striking contrast to our observations, this treatment led to a tremendous expansion of nTreg, which were further prone to mainly differentiate into IL-10-producing cells. In addition, a completely reverse migration pattern of CD4$^+$ T cells was elicited, with a selective infiltration of the brain by nTreg but not effector CD4$^+$ T cells at the onset of ECM. Because the direct effect of this treatment on the ability of the host to mount an effector CD4$^+$ response in an inflammatory setting was not characterized, one can only speculate that nTreg-mediated protection against cerebral malaria requires an overwhelming boost of this cell compartment, which compromises any therapeutic development in this line. However, the fact that neuroprotection and neuropathogenesis were, respectively, associated with opposite nTreg responses in these two experimental models reinforces the idea of a critical role of nTreg in the immunomodulation of the neuropathogenesis.

Given the fact that both CD4$^+$ and CD8$^+$ T cell populations are involved in the pathogenic responses associated with CM, it could be expected that a strong and appropriate nTreg response would prevent immunopathology caused by acute Th1 responses at the cost of interfering with parasite clearance (18–20). At odds with this idea, the dramatic neuropathological effect of nTreg in our model was not associated with an early and unrestrained HP, as it has been shown in nonencephalopathic malaria models (55). On the other hand, the inhibition of Th-mediated protection against CM did not prevent death by HP. Altogether these observations underline the fact that mechanisms leading to CM and to lethal HP are independent (56). We are therefore prone to hypothesize that, within a critical window after infection, nTreg favor parasite escape and thus sequestration in deeper vascular beds like the brain, rather than blood replication. This would explain how nTreg efficiently inhibit Th protective responses *in vivo* but fail to protect against CM. In this regard, the measure of parasite biomass might be more informative (57).

Besides, the fact that nTreg suppress early protective CD4$^+$ T cell responses that may control the pathogenic CD8$^+$ T cells in our CM model is in line with a recent report showing that Treg are involved in the regulation of early immunity to an acute viral infection. Interestingly, in this report Treg played a beneficial role by orchestrating the homing of protective effector T cells from the lymphoid tissue to the peripheral site of infection (58). However, such a strict partition does not occur during *Plasmodium* infection as the spleen constitutes a site of both lymphoid activation and parasite sequestration. Thus, based on the respective dynamics of infection and immune activation, Treg seem to play different roles in acute settings.

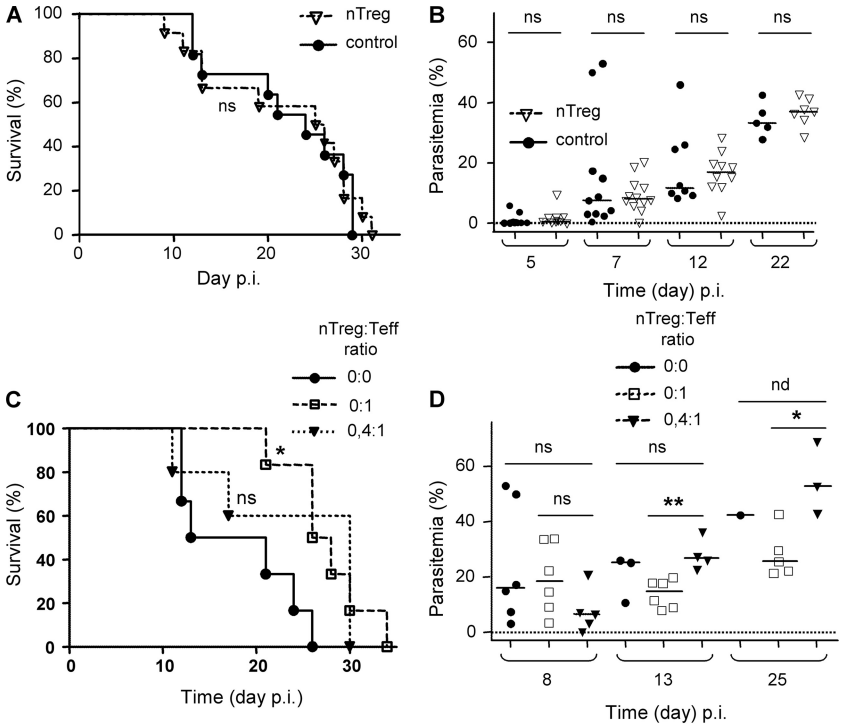

FIG 6 nTreg suppress the naive CD4$^+$ T cells' protective response to *P. berghei* ANKA infection in B6-CD4$^{-/-}$ mice but are not sufficient to induce CM. Cumulative survival (A) and parasitemia (B) of irradiated B6-CD4$^{-/-}$ mice following adoptive transfer of 5×10^5 freshly isolated naive splenic nTreg (CD4$^+$ CD25$^+$ Foxp3^{EGFP+}) or physiological serum (control). Each panel combines two independent experiments ($n = 11$ to 12 per group). (C and D) A total of 5×10^5 naive CD4$^+$ T cells (CD4$^+$ CD25$^-$ Foxp3^{EGFP-}) were transferred alone or with nTreg to irradiated B6-CD4$^{-/-}$ mice prior to *P. berghei* ANKA infection. (C) Cumulative survival was assessed by Kaplan-Meier analysis. (D) Corresponding parasitemia over time. Treg/naive CD4$^+$ T cell ratios are indicated ($n = 5$ to 6 per group). ns, not significant; nd, not determined; *, $P < 0.05$, and **, $P < 0.01$, by log-rank (A and C) or Mann-Whitney (B and D) analysis. Median bars are shown.

In summary, our work provides the proof of principle that, in a highly proinflammatory setting, increasing the systemic Treg/naive CD4$^+$ T cell ratio at the onset of an infection can be harmful to an immunocompetent host. In the face of the challenges encountered for the development of efficient therapeutic or vaccine strategies against malaria, not only does our study give new insight into the immune mechanisms involved in CM pathogenesis, but it also reinforces the idea that nTreg may represent an early immunomodulatory target for the control of the disease. However, our results strongly highlight the need for forethought on the use of Treg-mediated immunotherapy in the field.

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Anne-Laurence Blanc, Sylviane Pied, and Pierre-André Cazenave conceived and designed the experiments. Anne-Laurence Blanc, Sylviane Pied, Olivier Gorgette, and Pierre-André Cazenave performed the experiments. Anne-Laurence Blanc, Tarun Keswani, Sylviane Pied, Olivier Gorgette, Pierre-André Cazenave, and Antonio Bandeira analyzed the data. Bernard Malissen and Antonio Bandeira contributed reagents, materials, and analysis tools. Anne-Laurence Blanc, Tarun Keswani, and Antonio Bandeira wrote the manuscript. Sylviane Pied and Pierre-André Cazenave read and corrected the manuscript.

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REFERENCES

1. **World Health Organization** 2014. World malaria report 2014. World Health Organization, Geneva, Switzerland.
2. **Murray CJ, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, Haring D, Fullman N, Naghavi M, Lozano R, Lopez AD.** 2012. Global malaria mortality between 1980 and 2010: a systematic analysis. Lancet **379:**413–431. http://dx.doi.org/10.1016/S0140-6736(12)60034-8.
3. **van der Heyde HC, Nolan J, Combes V, Gramaglia I, Grau GE.** 2006. A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. Trends Parasitol **22:**503–508. http://dx.doi.org/10.1016/j.pt.2006.09.002.
4. **Renia L, Wu Howland S, Claser C, Charlotte Gruner A, Suwanarusk R, Hui Teo T, Russell B, Ng LF.** 2012. Cerebral malaria: mysteries at the blood-brain barrier. Virulence **3:**193–201. http://dx.doi.org/10.4161/viru.19013.

5. **Craig AG, Grau GE, Janse C, Kazura JW, Milner D, Barnwell JW, Turner G, Langhorne J, Participants of the Hinxton Retreat Meeting on Animal Models for Research on Severe Malaria.** 2012. The role of animal models for research on severe malaria. PLoS Pathog **8:**e1002401. http://dx.doi.org/10.1371/journal.ppat.1002401.
6. **Grau GE, Piguet PF, Engers HD, Louis JA, Vassalli P, Lambert PH.** 1986. L3T4+ T lymphocytes play a major role in the pathogenesis of murine cerebral malaria. J Immunol **137:**2348–2354.
7. **Yanez DM, Manning DD, Cooley AJ, Weidanz WP, van der Heyde HC.** 1996. Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. J Immunol **157:**1620–1624.
8. **Belnoue E, Kayibanda M, Vigario AM, Deschemin JC, van Rooijen N, Viguier M, Snounou G, Rénia L.** 2002. On the pathogenic role of brain-sequestered alphabeta CD8+ T cells in experimental cerebral malaria. J Immunol **169:**6369–6375. http://dx.doi.org/10.4049/jimmunol.169.11.6369.
9. **Bagot S, Nogueira F, Collette A, do Rosario V, Lemonier F, Cazenave PA, Pied S.** 2004. Comparative study of brain CD8+ T cells induced by sporozoites and those induced by blood-stage *Plasmodium berghei* ANKA involved in the development of cerebral malaria. Infect Immun **72:**2817–2826. http://dx.doi.org/10.1128/IAI.72.5.2817-2826.2004.
10. **Nie CQ, Bernard NJ, Norman MU, Amante FH, Lundie RJ, Crabb BS, Heath WR, Engwerda CR, Hickey MJ, Schofield L, Hansen DS.** 2009. IP-10-mediated T cell homing promotes cerebral inflammation over splenic immunity to malaria infection. PLoS Pathog **5:**e1000369. http://dx.doi.org/10.1371/journal.ppat.1000369.
11. **Amante FH, Haque A, Stanley AC, Rivera Fde L, Randall LM, Wilson YA, Yeo G, Pieper C, Crabb BS, de Koning-Ward TF, Lundie RJ, Good MF, Pinzon-Charry A, Pearson MS, Duke MG, McManus DP, Loukas A, Hill GR, Engwerda CR.** 2010. Immune-mediated mechanisms of parasite tissue sequestration during experimental cerebral malaria. J Immunol **185:**3632–3642. http://dx.doi.org/10.4049/jimmunol.1000944.
12. **Claser C, Malleret B, Gun SY, Wong AY, Chang ZW, Teo P, See PC, Howland SW, Ginhoux F, Rénia L.** 2011. CD8+ T cells and IFN-gamma mediate the time-dependent accumulation of infected red blood cells in deep organs during experimental cerebral malaria. PLoS One **6:**e18720. http://dx.doi.org/10.1371/journal.pone.0018720.
13. **Villegas-Mendez A, Greig R, Shaw TN, de Souza JB, Gwyer Findlay E, Stumhofer JS, Hafalla JC, Blount DG, Hunter CA, Riley EM, Couper KN.** 2012. IFN-gamma-producing CD4+ T cells promote experimental cerebral malaria by modulating CD8+ T cell accumulation within the brain. J Immunol **189:**968–979. http://dx.doi.org/10.4049/jimmunol.1200688.
14. **Hermsen C, van de Wiel T, Mommers E, Sauerwein R, Eling W.** 1997. Depletion of CD4+ or CD8+ T-cells prevents *Plasmodium berghei* induced cerebral malaria in end-stage disease. Parasitology **114:**7–12. http://dx.doi.org/10.1017/S0031182096008293.
15. **Weidanz WP, Melancon-Kaplan J, Cavacini LA.** 1990. Cell-mediated immunity to the asexual blood stages of malarial parasites: animal models. Immunol Lett **25:**87–95. http://dx.doi.org/10.1016/0165-2478(90)90097-A.
16. **Wing K, Sakaguchi S.** 2010. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol **11:**7–13. http://dx.doi.org/10.1038/ni.1818.
17. **Belkaid Y.** 2007. Regulatory T cells and infection: a dangerous necessity. Nat Rev Immunol **7:**875–888. http://dx.doi.org/10.1038/nri2189.
18. **Scholzen A, Minigo G, Plebanski M.** 2010. Heroes or villains? T regulatory cells in malaria infection. Trends Parasitol **26:**16–25. http://dx.doi.org/10.1016/j.pt.2009.10.004.
19. **Finney OC, Riley EM, Walther M.** 2010. Regulatory T cells in malaria—friend or foe? Trends Immunol **31:**63–70. http://dx.doi.org/10.1016/j.it.2009.12.002.
20. **Hansen DS, Schofield L.** 2010. Natural regulatory T cells in malaria: host or parasite allies? PLoS Pathog **6:**e1000771. http://dx.doi.org/10.1371/journal.ppat.1000771.
21. **Amante FH, Stanley AC, Randall LM, Zhou Y, Haque A, McSweeney K, Waters AP, Janse CJ, Good MF, Hill GR, Engwerda CR.** 2007. A role for natural regulatory T cells in the pathogenesis of experimental cerebral malaria. Am J Pathol **171:**548–559. http://dx.doi.org/10.2353/ajpath.2007.061033.
22. **Randall LM, Amante FH, McSweeney KA, Zhou Y, Stanley AC, Haque A, Jones MK, Hill GR, Boyle GM, Engwerda CR.** 2008. Common strategies to prevent and modulate experimental cerebral malaria in mouse strains with different susceptibilities. Infect Immun **76:**3312–3320. http://dx.doi.org/10.1128/IAI.01475-07.
23. **Wu JJ, Chen G, Liu J, Wang T, Zheng W, Cao YM.** 2010. Natural regulatory T cells mediate the development of cerebral malaria by modifying the pro-inflammatory response. Parasitol Int **59:**232–241. http://dx.doi.org/10.1016/j.parint.2010.02.007.
24. **Vigario AM, Gorgette O, Dujardin HC, Cruz T, Cazenave PA, Six A, Bandeira A, Pied S.** 2007. Regulatory CD4+ CD25+ Foxp3+ T cells expand during experimental Plasmodium infection but do not prevent cerebral malaria. Int J Parasitol **37:**963–973. http://dx.doi.org/10.1016/j.ijpara.2007.01.004.
25. **Couper KN, Blount DG, de Souza JB, Suffia I, Belkaid Y, Riley EM.** 2007. Incomplete depletion and rapid regeneration of Foxp3+ regulatory T cells following anti-CD25 treatment in malaria-infected mice. J Immunol **178:**4136–4146. http://dx.doi.org/10.4049/jimmunol.178.7.4136.
26. **Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, Hamann A, Wagner H, Huehn J, Sparwasser T.** 2007. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J Exp Med **204:**57–63. http://dx.doi.org/10.1084/jem.20061852.
27. **Steeg C, Adler G, Sparwasser T, Fleischer B, Jacobs T.** 2009. Limited role of CD4+ Foxp3+ regulatory T cells in the control of experimental cerebral malaria. J Immunol **183:**7014–7022. http://dx.doi.org/10.4049/jimmunol.0901422.
28. **Meyer Zu Horste G, Zozulya AL, El-Haddad H, Lehmann HC, Hartung HP, Wiendl H, Kieseier BC.** 2010. Active immunization induces toxicity of diphtheria toxin in diphtheria resistant mice—implications for neuroinflammatory models. J Immunol Methods **354:**80–84. http://dx.doi.org/10.1016/j.jim.2010.01.012.
29. **Westermark L, Fahlgren A, Fallman M.** 2012. Immune response to diphtheria toxin-mediated depletion complicates the use of the CD11c-DTR(tg) model for studies of bacterial gastrointestinal infections. Microb Pathog **53:**154–161. http://dx.doi.org/10.1016/j.micpath.2012.06.004.
30. **Haque A, Best SE, Amante FH, Mustafah S, Desbarrieres L, de Labastida F, Sparwasser T, Hill GR, Engwerda CR.** 2010. CD4+ natural regulatory T cells prevent experimental cerebral malaria via CTLA-4 when expanded in vivo. PLoS Pathog **6:**e1001221. http://dx.doi.org/10.1371/journal.ppat.1001221.
31. **Wang Y, Kissenpfennig A, Mingueneau M, Richelme S, Perrin P, Chevrier S, Genton C, Lucas B, DiSanto JP, Acha-Orbea H, Malissen B, Malissen M.** 2008. Th2 lymphoproliferative disorder of LatY136F mutant mice unfolds independently of TCR-MHC engagement and is insensitive to the action of Foxp3+ regulatory T cells. J Immunol **180:**1565–1575. http://dx.doi.org/10.4049/jimmunol.180.3.1565.
32. **Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, Hipkiss EL, Ravi S, Kowalski J, Levitsky HI, Powell JD, Pardoll DM, Drake CG, Vignali DA.** 2004. Role of LAG-3 in regulatory T cells. Immunity **21:**503–513. http://dx.doi.org/10.1016/j.immuni.2004.08.010.
33. **Workman CJ, Rice DS, Dugger KJ, Kurschner C, Vignali DA.** 2002. Phenotypic analysis of the murine CD4-related glycoprotein, CD223 (LAG-3) Eur J Immunol **32:**2255–2263.
34. **Bagot S, Idrissa Boubou M, Campino S, Behrschmidt C, Gorgette O, Guénet JL, Penha-Gonçalves C, Mazier D, Pied S, Cazenave PA.** 2002. Susceptibility to experimental cerebral malaria induced by Plasmodium berghei ANKA in inbred mouse strains recently derived from wild stock. Infect Immun **70:**2049–2056. http://dx.doi.org/10.1128/IAI.70.4.2049-2056.2002.
35. **Bogdándi EN, Balogh A, Felgyinszki N, Szatmári T, Persa E, Hildebrandt G, Sáfrány G, Lumniczky K.** 2010. Effects of low-dose radiation on the immune system of mice after total-body irradiation. Radiat Res **174:**480–489. http://dx.doi.org/10.1667/RR2160.1.
36. **Balogh A, Persa E, Bogdándi EN, Benedek A, Hegyesi H, Safrany G, Lumniczky K.** 2013. The effect of ionizing radiation on the homeostasis and functional integrity of murine splenic regulatory T cells. Inflamm Res **62:**201–212. http://dx.doi.org/10.1007/s00011-012-0567-y.
37. **Ito M, Shibamoto Y, Ayakawa A, Tomita N, Sugie C, Ogino H.** 2008. Effect of low-dose total-body irradiation on transplantibility of tumor cells in syngenic mice. J Radiat Res **49:**197–201. http://dx.doi.org/10.1269/jrr.07094.
38. **Lee KF, Weng JTY, Hsu PWC, Chi YH, Chen CK, Liu IY, Chen YC, Wu LH.** 2014. Gene expression profiling of biological pathway alterations by radiation exposure. BioMed Res Int **2014:**834087.
39. **Strauch UG, Obermeier F, Grunwald N, Gurster S, Dunger N, Schultz M, Griese DP, Mähler M, Schölmerich J, Rath HC.** 2005. Influence of intestinal bacteria on induction of regulatory T cells: lessons from a transfer model of colitis. Gut **54:**1546–1552. http://dx.doi.org/10.1136/gut.2004.059451.

40. **Billiard F, Litvinova E, Saadoun D, Djelti F, Klatzmann D, Cohen JL, Marodon G, Salomon BL.** 2006. Regulatory and effector T cell activation levels are prime determinants of in vivo immune regulation. J Immunol **177:**2167–2174. http://dx.doi.org/10.4049/jimmunol.177.4.2167.
41. **Collette A, Bagot S, Ferrandiz ME, Cazenave PA, Six A, Pied S.** 2004. A profound alteration of blood TCRB repertoire allows prediction of cerebral malaria. J Immunol **173:**4568–4575. http://dx.doi.org/10.4049/jimmunol.173.7.4568.
42. **Jacobs T, Graefe SE, Niknafs S, Gaworski I, Fleischer B.** 2002. Murine malaria is exacerbated by CTLA-4 blockade. J Immunol **169:**2323–2329. http://dx.doi.org/10.4049/jimmunol.169.5.2323.
43. **Jacobs T, Plate T, Gaworski I, Fleischer B.** 2004. CTLA-4-dependent mechanisms prevent T cell induced-liver pathology during the erythrocyte stage of Plasmodium berghei malaria. Eur J Immunol **34:**972–980. http://dx.doi.org/10.1002/eji.200324477.
44. **Belnoue E, Potter SM, Rosa DS, Mauduit M, Gruner AC, Kayibanda M, Mitchell AJ, Hunt NH, Rénia L.** 2008. Control of pathogenic CD8+ T cell migration to the brain by IFN-gamma during experimental cerebral malaria. Parasite Immunol **30:**544–553. http://dx.doi.org/10.1111/j.1365-3024.2008.01053.x.
45. **Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ.** 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nat Immunol **10:**595–602. http://dx.doi.org/10.1038/ni.1731.
46. **Oldenhove G, Bouladoux N, Wohlfert EA, Hall JA, Chou D, Dos Santos L, O'Brien S, Blank R, Lamb E, Natarajan S, Kastenmayer R, Hunter C, Grigg ME, Belkaid Y.** 2009. Decrease of Foxp3_ Treg cell number and acquisition of effector cell phenotype during lethal infection. Immunity **31:**772–786. http://dx.doi.org/10.1016/j.immuni.2009.10.001.
47. **Murai M, Krause P, Cheroutre H, Kronenberg M.** 2010. Regulatory T-cell stability and plasticity in mucosal and systemic immune systems. Mucosal Immunol **3:**443–449. http://dx.doi.org/10.1038/mi.2010.27.
48. **Zhao J, Zhao J, Fett C, Trandem K, Fleming E, Perlman S.** 2011. IFN-g and IL-10-expressing virus epitope-specific Foxp3(+) T reg cells in the central nervous system during encephalomyelitis. J Exp Med **208:**1571–1577. http://dx.doi.org/10.1084/jem.20110236.
49. **Koenecke C, Czeloth N, Bubke A, Schmitz S, Kissenpfennig A, Malissen B, Huehn J, Ganser A, Förster R, Prinz I.** 2009. Alloantigen-specific de novo induced Foxp3+ Treg revert in vivo and do not protect from experimental GVHD. Eur J Immunol **39:**3091–3096. http://dx.doi.org/10.1002/eji.200939432.
50. **Cao AT, Yao S, Stefka AT, Liu Z, Qin H, Liu H, Evans-Marin HL, Elson CO, Nagler CR, Cong Y.** 2014. TLR4 regulates IFN-γ and IL-17 production by both thymic and induced Foxp3 Tregs during intestinal inflammation. J Leukoc Biol **96:**895–905. http://dx.doi.org/10.1189/jlb.3A0114-056RR.
51. **Daniel V, Trojan K, Adamek M, Opelz G.** 2015. IFNγ+ Treg in-vivo and in-vitro represent both activated nTreg and peripherally induced aTreg and remain phenotypically stable in-vitro after removal of the stimulus. BMC Immunol **16:**45. http://dx.doi.org/10.1186/s12865-015-0111-2.
52. **Daniel V, Wang H, Sadeghi M, Opelz G.** 2014. Interferon-gamma producing regulatory T cells as a diagnostic and therapeutic tool in organ transplantation. Int Rev Immunol **33:**195–211. http://dx.doi.org/10.3109/08830185.2013.845181.
53. **de Kossodo S, Grau GE.** 1993. Profiles of cytokine production in relation with susceptibility to cerebral malaria. J Immunol **151:**4811–4820.
54. **Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu TT, Corcoran L, Treuting P, Klein U, Rudensky AY.** 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. Nature **458:**351–356. http://dx.doi.org/10.1038/nature07674.
55. **Hisaeda H, Maekawa Y, Iwakawa D, Okada H, Himeno K, Kishihara K, Tsukumo S, Yasutomo K.** 2004. Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. Nat Med **10:**29–30. http://dx.doi.org/10.1038/nm975.
56. **Curfs JH, Hermsen CC, Meuwissen JH, Eling WM.** 1992. Immunization against cerebral pathology in Plasmodium berghei-infected mice. Parasitology **105:**7–14. http://dx.doi.org/10.1017/S0031182000073625.
57. **Dondorp AM, Desakorn V, Pongtavornpinyo W, Sahassananda D, Silamut K, Chotivanich K, Newton PN, Pitisuttithum P, Smithyman AM, White NJ, Day NP.** 2005. Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. PLoS Med **2:**e204. http://dx.doi.org/10.1371/journal.pmed.0020204.
58. **Lund JM, Hsing L, Pham TT, Rudensky AY.** 2008. Coordination of early protective immunity to viral infection by regulatory T cells. Science **320:**1220–1224. http://dx.doi.org/10.1126/science.1155209.