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IL-2 Contributes to Maintaining a Balance between CD4+ Foxp3+ Regulatory T Cells and Effector CD4+ T Cells Required for Immune Control of Blood-Stage Malaria Infection

Floriana Berretta,*+,† Jessica St-Pierre,*+,‡ Ciriaco A. Piccirillo,*+,‡,§,∥ and Mary M. Stevenson*+,‡,§,∥

To investigate the role of CD4+CD25+Foxp3+ regulatory T (Treg) cells in blood-stage malaria, we compared Plasmodium chabaudi AS infection in wild-type (WT) C57BL/6 and transgenic mice overexpressing the transcription factor Foxp3 (Foxp3Tg) and observed that Foxp3Tg mice experienced lethal infection and deficient malaria-specific immune responses. Adoptive transfer of total CD4+ T cells from Foxp3Tg mice or CD4+CD25+ T cells from WT mice to naive WT recipients confirmed that high numbers of Treg cells compromised immune control of malaria. Transfer of GFP+CD4+CD25+ T cells to naive WT recipients together with immunohistochemical staining of spleens from infected WT mice demonstrated that Foxp3+ Treg cells localized in the T cell area of the spleen. Determination of CD4+Foxp3+ Treg cell responses in the spleen of infected WT mice revealed a significant but transient increase in CD4+Foxp3+ Treg cells early in infection. This was followed by a significant and sustained decrease due to reduced proliferation and apoptosis of CD4+Foxp3+ Treg cells. Importantly, the kinetics of IL-2 secretion by effector CD4+Foxp3+ T cells coincided with changes in CD4+Foxp3+ cells and the differentiation of CD4+T-bet+IFN-γ+ effector CD4+ T cells. Collectively, these data demonstrate that the ability to control and eliminate P. chabaudi AS infection is due to a tight balance between natural Treg cells and effector CD4+ Th1 cells, a balance regulated in part by IL-2.

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Successful control and resolution of blood-stage malaria infection requires coordinate and timely innate and adaptive immune responses involving dendritic cells, NK cells, effector CD4+ T cells, and B cells (1). Studies in individuals infected with Plasmodium falciparum, the most deadly and virulent Plasmodium species afflicting humans, and in experimental mouse models indicate that immunity to malaria is critically dependent on the type 1 cytokine IFN-γ (1, 2). A balance between proinflammatory and anti-inflammatory responses is also essential to limit the development of life-threatening immune-mediated pathology such as cerebral malaria and severe anemia (3). Although a clearer understanding of the underlying immune mechanisms involved in protection and immunopathology during blood-stage infection has emerged, our understanding of the regulatory mechanisms required to maintain a balance between beneficial and deleterious responses is limited.

Naturally occurring CD4+CD25+Foxp3+ T cells, a unique population of regulatory T (Treg) cells, play a pivotal role in maintaining peripheral tolerance to self and non-self Ags (4). Treg cells are also essential in determining the outcome of infection with various intracellular pathogens including protozoan parasites (5). Paradoxically, these cells may limit the magnitude of effector T cell responses during infection, resulting in inadequate control of pathogen replication and persistence of low-level infection. In contrast, the potent ability of natural Treg cells to modulate effector T cell responses may limit tissue damage caused by overexuberant immune responses.

Because of the critical importance of an appropriate balance between protective immunity and immune-mediated pathology to a favorable outcome of malaria, recent studies have investigated whether natural Treg cells play a role in regulating the immune response to Plasmodium parasites. Observations in humans infected with Plasmodium and in mice infected with various rodent Plasmodium species have revealed that there is important expansion of CD4+CD25+Foxp3+ Treg cells, the magnitude of which correlates with both high parasitemia and low proinflammatory responses (6–8). The recent findings of Walther et al. (9) provide evidence supporting a possible link between natural Treg cells and the clinical outcome of malaria. This study, in which human volunteers were experimentally challenged with...
P. falciparum sporozoites and followed longitudinally, showed that expansion of CD4\(^+\)Foxp3\(^+\) Treg cells in the peripheral blood of infected individuals is associated with enhanced parasite growth and reduced inflammatory responses.

Although these findings suggest that natural Treg cells are important in modulating immune responses and disease development during malaria, attempts to assess the impact of these cells in Plasmodium-infected mice by depleting CD25\(^+\) cells have led to inconsistent conclusions (6–8). This situation may be due to differences in the mouse–parasite strain combination and in the CD25 depletion strategy used in these studies (6). There is also the likelihood that Ab depletion of CD25\(^+\) cells may have resulted in the loss of effector as well as of activated T cells that express CD25 and the fact that some populations of Treg cells are CD25\(^+\) (reviewed in Ref. 6). A major concern regarding the inconsistency of studies on Treg cells in mouse models is the recent finding of Strauch et al. (10) indicating that the intestinal flora can influence the induction and expansion of Treg cells. Nevertheless, the results of these studies are consistent with the notion that CD25\(^+\) Treg cells suppress proinflammatory responses involved in both protective immunity and immunopathology during malaria (6–8).

IL-2 is a major T cell growth factor and is essential for the homeostasis, proliferation, and differentiation of CD4\(^+\) and CD8\(^+\) T cells as well as other effector cells such as NK cells (11). The IL-2R\(\alpha\) subunit (CD25) is expressed constitutively by natural Treg cells; indeed, an intact IL-2–IL-2R signaling pathway is crucial for Treg cell function and survival (12, 13). The administration of IL-2 as a complex of recombinant IL-2 and the anti–IL-2 JES6-1 mAb has been shown to promote the rapid and systemic proliferation of Foxp3\(^+\) Treg cells in naive mice (14). In vivo expansion of Foxp3\(^+\) Treg cells induced by this treatment prevents the development of diabetes, induces resistance to EAE, promotes long-term acceptance of islet allografts, and reduces the severity of allergen-induced inflammation (14–17). Recently, it was proposed that IL-2 might regulate the balance between effector Th1 cells and Treg cells during blood-stage malaria, but this issue has yet to be investigated (6, 7).

In this study, we investigated the role of CD4\(^+\)Foxp3\(^+\) Treg cells in blood-stage Plasmodium chabaudi AS infection by comparing the course and outcome of infection and malaria-specific immune responses during P. chabaudi AS infection in wild-type (WT) C57BL/6 (B6) and transgenic mice that overexpress the Foxp3 transcription factor (Foxp3Tg). To confirm that high numbers of natural Treg cells severely impaired the immune response to malaria, we performed adoptive transfer of Treg cells purified from Foxp3Tg or WT mice to naive WT recipient mice challenged with P. chabaudi AS. We also systematically analyzed the CD4\(^+\) Foxp3\(^+\) Treg cell response in the spleens of infected WT mice and investigated whether IL-2 plays a role in regulating the balance between these cells and CD4\(^+\)Foxp3\(^+\) T cells during blood-stage malaria. Taken together, our data indicate that the ability to control and eliminate P. chabaudi AS infection requires a tight balance between CD4\(^+\)Foxp3\(^+\) cells and effector CD4\(^+\)Th1 cells, a balance regulated in part by IL-2.

### Materials and Methods

#### Mice and parasites

Male WT mice, 6–8 wk old, were purchased from Charles River Laboratories (St. Constant, QC, Canada). Foxp3Tg (line 2826) and UBI-GFP/B6 mice, generated as previously described, were kindly provided by Dr. F. Ramsdell, currently at Celltech R&D (Bothell, WA), and Dr. B. Schaefer (Uniformed Services University of the Health Sciences, Bethesda, MD), respectively (18, 19). Transgenic mice were bred in the Lyman Dunn Animal Facility of McGill University under specific pathogen-free conditions, and male progeny were age-matched with male WT mice for all experiments. P. chabaudi AS was maintained as previously described (20). Mice were infected by i.p. injection of 1 × 10\(^5\) parasitized RBCs (pRBC). Parasitemia was monitored in the blood by microscopic examination of Diff-Quick (Fisher Scientific) stained blood smears. The experiments were conducted in accordance with the guidelines of the Canadian Council for Animal Care.

#### Cell preparation

Spleens from naive and infected mice were harvested at the indicated times postinfection (p.i.) with P. chabaudi AS. The spleen index for each individual mouse was calculated as the spleen weight (mg)/body weight (mg) × 100. In some experiments, lymph nodes (axillary, inguinal, and mesenteric) were also harvested. To prepare single-cell suspensions, tissues were perfused with PBS containing 1% FCS (HyClone Laboratories, Logan, UT), teased apart, and pressed gently through a sterile fine-wire mesh, and RBC were lysed with NH4Cl lysing buffer (Sigma-Aldrich, St. Louis, MO). The cells were washed and resuspended in RPMI 1640 (Invitrogen, Burlington, ON, Canada) supplemented with 10% heat-inactivated FCS (HyClone Laboratories), gentamicin (20 μg/ml), 2 mM L-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (all from Invitrogen-Life Technologies), and 50 μM 2-mercaptoethanol (Sigma-Aldrich). Cell viability was always >95% as determined by trypan blue exclusion (Invitrogen-Life Technologies).

#### CD4\(^+\) T cell purification and adoptive transfer

For adoptive transfer of CD4\(^+\)Foxp3\(^+\) T cells from Foxp3Tg mice, CD4\(^+\) T cells were purified from spleens harvested from naive mice with anti-CD25 magnetic beads (Miltenyi Biotec, A.Flam, CA) using an autoMACS (Miltenyi Biotec) according to the manufacturer’s instructions. The isolated cells were routinely >95% positive for CD4 with 80–85% expressing Foxp3 as determined by flow cytometry. The cells were washed, resuspended in PBS, and 5 × 10\(^5\) CD4\(^+\) T cells were adoptively transferred by i.v. injection to naive WT recipient mice. As a control, naive WT mice were injected i.v. with PBS. One day after adoptive transfer, recipient and control mice were infected i.p. with P. chabaudi AS as described above. For adoptive transfer of Treg and effector T cell populations from WT and UBI-GFP/B6 mice, CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\) T cells were purified from spleens harvested from naive mice with magnetic beads (Miltenyi Biotec). Briefly, single-cell suspensions were prepared as described above and depleted of CD4\(^+\) cells by negative selection, followed by positive selection for CD25\(^+\) cells. The purity of the isolated CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\) populations was routinely >85% as assessed by flow cytometry. Among the CD4\(^+\)CD25\(^+\) fraction ~85% of the cells also expressed Foxp3, whereas among the CD4\(^+\)CD25\(^-\) fraction <0.5% were Foxp3\(^+\) as determined by FACS analysis. Naive WT recipient mice were injected i.v. with 5 × 10\(^5\) CD4\(^+\)CD25\(^+\) or 5 × 10\(^5\) CD4\(^+\)CD25\(^-\) cells from WT or UBI-GFP/B6 mice, and adoptively transferred and PBS control WT mice were challenged with P. chabaudi AS 1 d later.

#### Phenotypic analysis of spleen cells

Single-cell suspensions of splenocytes, prepared as described above, were blocked with anti-CD16/32 mAb (clone 2.4G2; BD Pharmingen, San Diego, CA) and surface stained with FITC-conjugated anti-CD4 (clone GK.1; eBioscience, San Diego, CA) and biotinylated anti-CD25 (clone PC61; eBioscience) mAbs. Intracellular staining for Foxp3 was performed according to the manufacturer’s instructions using allophycocyanin-labeled anti-Foxp3 mAb (clone FJK-16s; eBioscience). To evaluate the proliferative capacity of CD4\(^+\) T cell populations, cells were stained intracellularly with PE-labeled anti-Ki67 mAb (clone B56; BD Biosciences, Mississauga, ON, Canada) according to the manufacturer’s instructions. In some experiments, cells were stained for the intracellular anti-apoptotic molecule Bcl-2 using PE-labeled anti–Bcl-2 mAb (clone 3F11; BD Pharmingen). For each mAb, staining was compared with cells stained with an appropriate isotype control mAb. Flow cytometry was performed using a FACSCalibur (BD Biosciences) for acquisition, and data were analyzed using FlowJo software (Tree Star).

#### Intracellular cytokine staining

Purified CD4\(^+\) T cells from the spleens of naive and infected mice were resuspended to 1 × 10\(^5\) cells/ml in complete RPMI 1640 medium and stimulated with 50 ng/ml PMA and 1 μM monomycin (Sigma-Aldrich) for 5 h at 37°C and 5% CO\(_2\) in the presence of 1 μM brefeldin A (BD Biosciences) to inhibit cytokine secretion. Cells were harvested and surface stained with FITC-conjugated anti-CD4 mAb as described above. After fixation and permeabilization using the intracellular fixation kit (eBioscience) according to the manufacturer’s instructions, the cells were
stained for intracellular cytokine expression using fluorochrome-conjugated mAbs from eBioscience against IFN-γ (clone XMG1.2), IL-2 (clone JES-514), and IL-10 (clone JES-5-16E3). Cells were also stained for intracellular Foxp3 as described above to identify natural Treg cells. Cells were gated on CD4+ cells for FACS analysis.

In vitro proliferation and IL-2 production

Single-cell suspensions of spleen cells were resuspended to 2.5 × 10^6 cells/ml in complete RPMI 1640 medium. Aliquots of 100 μl were seeded into 96-well flat bottom microtiter plates, and 100 μl pRB/C (25 × 10^6/ml) or complete RPMI 1640 medium as a negative control was added to triplicate wells. The cultures were incubated for 72 h at 37°C in 5% CO2 and pulsed with 0.5 μCi [3H]thymidine for the last 12 h. Cells were harvested onto filter paper and [3H]thymidine incorporation was assessed using a Perkin-Elmer 1450 MicroBeta liquid scintillation counter. For IL-2 production, spleen cells were stimulated for 18 h with 5 μg/ml anti-CD3 mAb (clone 145-2C11; eBioscience), and the supernatants were collected for measurement of IL-2 levels by ELISA.

Immunohistochemistry

Spleens from naive and infected WT mice were harvested at the indicated times p.i. and immediately embedded in Tissue-Tek OCT compound (Canemco-Merivac, St. Laurent, QC, Canada) and snap frozen. Frozen sections (5–7 mm) were fixed and permeabilized using the Fix/Perm and permeabilization buffers from eBioscience according to the manufacturer’s instructions. Subsequently, samples were incubated with 5% BSA and 5% goat serum in permeabilization buffer and stained with anti–Foxp3-Alexa Fluor 488 (clone FJK-16sl; eBioscience), anti–CD3-Alexa Fluor 647 (clone 17A2; eBioscience), and biotin-labeled anti-IgM (clone 1B4B1; SouthernBiotech, Birmingham, AL) mAbs. Staining for IgM was revealed by secondary streptavidin-Alexa Fluor 546 (eBioscience). Sections were analyzed by confocal microscopy (Zeiss Axiovert 200M).

Cytokine ELISAs

Cytokine levels in sera and supernatants of spleen cells were determined by sandwich ELISA specific for IFN-γ, IL-2, and IL-10 using paired capture and detection Abs and revealed by streptavidin-HP conjugate (R&D Systems, Minneapolis, MN) and anti–mouse IgM (clone 1B4B1; SouthernBiotech, Birmingham, AL) mAbs. Staining for IgM was revealed by secondary streptavidin-Alexa Fluor 546 (eBioscience). Sections were analyzed by confocal microscopy (Zeiss Axiovert 200M).

Treatment with IL-2/anti–IL-2 mAb complex

IL-2/anti–IL-2 mAb complex was prepared as previously described (14). Briefly, mouse recombinant IL-2 (R&D Systems) was mixed with anti-mouse IL-2 mAb (clone JES-514; Bio X Cell, West Lebanon, NH) at a 1:10 ratio and incubated at 37°C for 30 min. P. chabaudi AS-infected WT mice were treated i.p. for 3 consecutive days, beginning on the day of infection, with 200 μl IL-2/anti–IL-2 complex with the equivalent of 1 μg IL-2 and 10 μg anti–IL-2 mAb. As a control, infected WT mice were treated with an equal volume of PBS or 10 μg isotype control mAb following a similar treatment schedule.

Statistical analysis

Data are expressed as means ± SEM. The statistical significance of differences between the experimental and control groups was analyzed by a Student t test (two-tailed). For multiple comparisons, data were analyzed using ANOVA. All statistical analyses were performed using Prism 5 software (GraphPad Software, San Diego, CA). A p value of <0.05 was considered significant.

Results

High numbers of CD4+Foxp3+ Treg cells impair immune responses to blood-stage malaria

To investigate the role of natural Treg cells in blood-stage malaria, we examined the course and outcome of P. chabaudi AS infection in Foxp3Tg mice. Compared to WT mice, Foxp3Tg mice, which overexpress Foxp3 (Fig. 1A), experienced a severe course of P. chabaudi AS infection with significantly higher parasitemias early during infection and a significantly higher level of peak parasitemia on day 8 p.i. (Fig. 1B). By day 10 p.i., 100% of Foxp3Tg mice succumbed, whereas all WT mice survived until the experiment was terminated on day 30 p.i. (Fig. 1C). We also compared parameters that correlate with the development of immunity to P. chabaudi AS in WT and Foxp3Tg mice (21–23). As expected, spleen indices increased dramatically and significantly in infected WT compared with naive WT mice. In contrast, there was little or no change in spleen indices in infected Foxp3Tg mice, and spleen indices were significantly lower compared with infected WT mice on days 6 and 8 p.i. (Fig. 1D). Spleen cells from P. chabaudi AS-infected Foxp3Tg mice exhibited low to background proliferation in response to in vitro stimulation with pRB/C, whereas cells from infected WT mice displayed robust proliferation with significantly higher responses on days 4 and 6 p.i. (Fig. 1E). Proliferation of spleen cells from uninfected Foxp3Tg mice or cells recovered from these mice during infection were also significantly lower to Con A stimulation compared with cells from WT mice (data not shown). The serum levels of IFN-γ and IL-10 were significantly increased during infection in WT mice compared with naive WT mice, but the levels of both cytokines remained low in infected Foxp3Tg mice (Fig. 1F, 1G).

Adaptive transfer of natural Treg cells increases the severity of malaria in recipient mice

To confirm whether high numbers of natural Treg cells per se rather than deficient effector CD4+ T cell function were responsible for the increased severity of malaria infection in Foxp3Tg mice, we adoptively transferred total CD4+ T cells purified from the spleens of naive Foxp3Tg mice to naive WT recipient mice. One day later, the recipient mice and a group of control WT mice were challenged with P. chabaudi AS infection. Similar to infected Foxp3Tg mice, WT recipients of CD4+ T cells from these mice displayed significantly higher peak parasitemia, and 4 of 12 recipient mice (33%) succumbed to infection (Fig. 2A). We also adoptively transferred CD4+CD25+ or CD4+CD25– T cells from WT mice to naive WT mice. As before, the recipient mice and a control group of nontransfused WT mice were infected with P. chabaudi AS 1 d later. Adoptive transfer of CD4+CD25+ cells resulted in a more severe course of infection in recipient WT mice with significantly higher parasitemia levels early (day 5 p.i.) and at the peak (day 8 p.i.) than in control WT mice (Fig. 2B). Immune control and clearance of the parasites during the chronic phase of infection were also less efficient in recipients of CD4+CD25+ T cells as evidenced by significantly higher parasitemia levels after the peak and delayed clearance of P. chabaudi AS infection, up to 1 wk longer in some recipients, compared with parasite clearance on day 21 p.i. in control mice. In contrast, adoptive transfer of CD4+CD25– T cells resulted in significantly lower peak parasitemia levels and rapid clearance of the parasite in recipient compared with control mice.

To determine whether adoptive transfer of CD4+CD25+ T cells impaired immune responses to P. chabaudi AS infection in recipient mice, the levels of IFN-γ and IL-10 were determined in sera collected from recipients of CD4+CD25+ or CD4+CD25– T cells and in control WT mice. IFN-γ increased to similar levels by day 6 p.i. in all mice and declined thereafter with no significant differences among the three groups of mice at any time through day 21 p.i. (data not shown). Compared to their respective naive
controls, IL-10 levels were markedly increased in the three groups of mice on days 6 and 8 p.i. and declined to low levels by day 21 p.i. Significantly higher IL-10 levels were apparent on day 6 p.i. in the sera of WT mice adoptively transferred with CD4+CD25+ T cells compared with recipients of CD4+CD25− T cells or control mice (Fig. 2C). The cellular source of IL-10 during *P. cholabadi* AS infection was investigated. Spleen cells were recovered from naive and infected WT and Foxp3Tg mice on days 6 and 8 p.i. and stimulated in vitro with PMA and ionomycin in the presence of brefeldin A. The expression of intracellular Foxp3 and IL-10 was analyzed by FACS. Representative dot plots of the frequency of Foxp3−IL-10− and Foxp3−IL-10+ in gated CD4+ cells from naive (day 0, d0) and infected (day 8, d8) mice (A) and the frequency of Foxp3−IL-10− and Foxp3−IL-10+ during acute infection (D) were determined in naive and infected WT and Foxp3Tg mice at the indicated days p.i. Data are presented as the mean ± SEM of four mice per group from one of three replicate experiments. *p < 0.05, **p < 0.001, ***p < 0.001, infected WT compared with naive WT mice.

**FIGURE 2.** Adoptive transfer of Treg cells increases the severity of malaria in recipient WT mice. Course of parasitemia in WT mice adoptively transferred with 5 × 10^5 total CD4+ T cells purified from the spleens of naive Foxp3Tg mice (A) or 5 × 10^5 Treg (CD4+CD25+) or Teff (CD4+CD25−) cells purified from the spleens of naive WT mice (B). Control mice were injected i.v. with PBS. Mice were infected i.p. with 1 × 10^6 pRBC 1 d after adoptive transfer. *p < 0.05, compared with control WT mice. C, Levels of IL-10 in sera collected from adoptively transferred and control WT mice at the indicated days p.i. BLD, below level of detection. *p < 0.05, mice adoptively transferred with Treg cells compared with effector T (Teff) cells or control mice. Data are presented as the mean ± SEM of four mice per group from one of three replicate experiments. Spleen cells were recovered from naive and *P. cholabadi* AS-infected WT mice on days 6 and 8 p.i. and stimulated in vitro with PMA and ionomycin in the presence of brefeldin A. The expression of intracellular Foxp3 and IL-10 was analyzed in gated CD4+ cells by FACS. Representative dot plots of the frequency of Foxp3−IL-10− and Foxp3−IL-10+ in gated CD4+ cells from naive (day 0, d0) and infected (day 8, d8) mice (D) and the frequency of Foxp3−IL-10− and Foxp3−IL-10+ during acute infection (E) were determined in naive and infected WT and Foxp3Tg mice at the indicated days p.i. Data are presented as the mean ± SEM of three mice per group from one of three replicate experiments. *p < 0.05, infected compared with naive WT mice.
IL-2 REGULATES Foxp3+ AND Th1 CELL BALANCE DURING MALARIA

Previous studies have indicated that natural Treg cells localize to the draining lymph node or affected target tissue to mediate their suppressive and immunoregulatory functions (27, 28). To explore whether tissue-specific localization of these cells is important for modulation of immunity to malaria, CD4+CD25+ and CD4+CD25− T cells were purified from the spleens of reporter UBI-GFP/B6 mice and adoptively transferred to naive WT recipients. The two groups of recipient mice and a control group of nontransfused WT mice were infected with P. chabaudi AS 1 d later, and the in vivo tissue distribution of GFP+ cells was monitored by flow cytometry on day 7 p.i. Irrespective of CD25 expression, CD4+GFP+ cells were evident in the spleen but not in the blood or lymph nodes of P. chabaudi AS-infected recipients (Fig. 3A). Significantly higher numbers of GFP+ cells were detected in the spleen of recipients of CD4+CD25+ compared with recipients of CD4+CD25− T cells, suggesting that natural Treg cells rapidly localized to the spleen during P. chabaudi AS infection (Fig. 3B). The accumulation of natural Treg cells in the spleen of infected WT mice was confirmed by immunohistochemical staining (Fig. 3C). Foxp3+ Treg cells localized almost exclusively within the T cell areas of the white pulp starting from day 6 p.i. and were still evident in this site on days 8 and 10 p.i. These observations suggest that natural Treg cells localized to the spleen during malaria infection and are consistent with the importance of the spleen as the major site of immune responses during blood-stage malaria (29).

**Natural Treg cells transiently expand and then decrease in P. chabaudi AS-infected WT mice**

Expansion of natural Treg cells has been observed in the spleens of mice infected with various species and strains of *Plasmodium* (6–8). To investigate whether expansion of CD4+Foxp3+ Treg cells occurred in the spleen during P. chabaudi AS infection, we systematically analyzed spleen cells recovered from WT mice at various times postinfection. Consistent with previous findings, the absolute number but not the frequency of splenic CD4+Foxp3+ Treg cells was significantly increased beginning on day 4 (data not shown) and on day 6 in P. chabaudi AS-infected compared with naive WT mice (Fig. 4A–C). Unexpectedly, we observed that the frequencies and numbers of CD4+Foxp3+ T cells were significantly decreased around the time of peak parasitemia (days 8–9 p.i.), and Foxp3+ Treg cells remained significantly decreased during the time corresponding to the control of parasite replication (day 11 p.i.) (1). On day 33 p.i., when parasites were no longer visible in the blood smears, the frequency of Treg cells remained significantly decreased whereas the absolute numbers of Foxp3+ Treg cells were dramatically and significantly increased compared with naive WT mice.

The observation of a decrease in the frequency and number of CD4+Foxp3+ cells in the spleen of P. chabaudi AS-infected WT mice prompted us to investigate the basis of this contraction. To determine whether the decrease was due to impaired proliferation, we examined the expression of Ki67, a marker of actively cycling cells, in splenic natural Treg cells and effector CD4+ T cells by flow cytometry (30). The frequency of both CD4+Foxp3+Ki67+ and CD4+Foxp3−Ki67− cells increased significantly on days 6, 8, and 11 p.i. compared with cells from naive controls and returned to near normal levels when the infection was resolved (Fig 4D, 4E). However, proliferation of CD4+Foxp3+ T cells was significantly lower than that of CD4+Foxp3+ T cells between days 6 and 11 p.i.

Because apoptosis of splenic lymphocyte populations is an important feature of malaria infection, we also questioned whether...
increased apoptosis contributed to the decrease in natural Treg cells during *P. chabaudi* AS infection (23, 31, 32). To investigate this possibility, the expression of the anti-apoptotic marker Bcl-2 was determined in splenic CD4^+^ T cell populations during infection. The frequency of Bcl-2^+^ natural Treg cells decreased significantly in infected WT mice between days 6 and 33 p.i. compared with naive WT controls (Fig. 4F, 4G). The frequency of Bcl-2^+^-expressing CD4^+^Foxp3^+^ T cells was also significantly decreased during infection. There were no significant differences in Bcl-2 expression between the two populations except on day 11 p.i. when 60.8% of natural Treg cells expressed Bcl-2 compared with 45.5% of effector CD4^+^ T cells. Taken together, these data demonstrate that the decrease in CD4^+^Foxp3^+^ cells during *P. chabaudi* AS infection in WT mice was due in part to reduced proliferation of these cells compared with effector CD4^+^ T cells and that apoptosis occurred among both natural Treg cells and effector CD4^+^ T cells during infection.

**IL-2 production correlates with changes in natural Treg cells and effector Th1 cells during *P. chabaudi* AS infection**

IL-2, a cytokine produced by effector CD4^+^ T cells during pro-inflammatory responses, is required for the expansion, survival, and function of natural Treg cells as well as maintenance of Foxp3 expression in these cells (13, 33). Analysis of intracellular IL-2 expression by effector CD4^+^ T cells from *P. chabaudi* AS-infected WT mice revealed transient but significant increases in both the frequency and number of CD4^+^Foxp3^+^IL-2^+^ cells on day 6 p.i. (Fig. 5A–D). This was followed by a decrease to baseline levels around the time of peak of parasitemia (days 8–9 p.i.). Determination of IL-2 secretion by spleen cells stimulated in vitro with anti-CD3 mAb confirmed the kinetics of intracellular IL-2 expression by effector CD4^+^ T cells during infection (Fig. 5E). Remarkably, the kinetics of IL-2 production by effector CD4^+^ T cells coincided with changes in the frequency of splenic CD4^+^ Foxp3^+^ T cells in infected WT mice.

We next sought to determine whether the temporal changes in IL-2 production by effector CD4^+^ T cells influenced the differentiation of effector Th1 cells. To address this question, the expression of the transcription factor T-bet and intracellular IFN-γ by CD4^+^ Foxp3^+^ T cells was determined ex vivo during *P. chabaudi* AS infection in WT mice. There were significant increases in the frequency and number of cells expressing T-bet on day 8 p.i. compared with naive WT mice, with a further increase on day 11 p.i. (Fig. 5F–H). The increases in T-bet expression coincided with significant increases in intracellular IFN-γ expression by CD4^+^ Foxp3^+^ T cells beginning on day 8 p.i. through day 11 p.i. in infected compared with uninfected WT mice (Fig. 5I–K). Analysis of T-bet expression after stimulation in vitro with PMA and ionomycin confirmed that most IFN-γ^+^ T cells also expressed T-bet on day 8 p.i. (Fig. 5J). These data suggest that the ability of WT
mice to control and eliminate *P. chabaudi* AS infection is due to a tight balance between natural Treg and effector CD4+ Th1 cells and that IL-2 may be involved in regulating this balance. Treatment with IL-2/anti–IL-2 complex increases the severity of *P. chabaudi* AS infection in WT mice. Administration of a complex consisting of low doses of recombinant IL-2 and the anti–IL-2 clone JES6-1 mAb to mice infected with *Toxoplasma gondii* promotes the expansion of Foxp3+ Treg cells together with decreased immune-mediated pathology and morbidity (34). To determine the effects of this treatment on natural Treg cells and the outcome of blood-stage malaria infection, WT mice were infected with *P. chabaudi* AS and treated with this IL-2/anti–IL-2 mAb complex. As shown in Fig. 6A, the course of infection was more severe in infected WT mice treated with IL-2/anti–IL-2 mAb complex compared with control mice, either untreated or treated with isotype control Ab. Treated mice had significantly higher parasitemia levels as early as day 6 p.i. and a significantly higher level of peak parasitemia; there was also inefficient clearance of the parasites in treated mice, which experienced a marked and significantly higher recrudescent parasitemia on day 16 p.i. Coincidently, mice infected with *P. chabaudi* AS and treated with the IL-2/anti–IL-2 mAb complex had increased frequencies of CD4+CD25+Foxp3+ T cells in the spleen on days 7 and 9 p.i compared with control mice (Fig. 6B, 6C). The absolute numbers of natural Treg cells were also significantly increased in treated WT mice on day 6 p.i. (Fig. 6D).

Discussion

Previous studies in mice infected with *Plasmodium* have yielded inconsistent findings on the role of natural Treg cells in blood-stage malaria (6–8). To further address this issue, we investigated...
whether high numbers of CD4+Foxp3+ Treg cells affected the course of infection with *P. chabaudi* AS using two alternative approaches. First, we compared the course of parasitemia in *P. chabaudi* AS-infected Foxp3Tg mice that overexpress the Foxp3 gene and in WT mice (18). This strategy demonstrated that supernormal numbers of CD4+Foxp3+ Treg cells dramatically increased the peak parasitemia level and resulted in a lethal infection due to impaired immune control of parasite replication. These observations were confirmed and extended by our second approach whereby we performed adoptive transfer of total CD4+ T cells purified from the spleen of naive Foxp3Tg mice or of CD4+CD25+ T cells from naive WT mice. Additionally, the major T cell source of the immunoregulatory cytokine IL-10 in *P. chabaudi* AS-infected WT mice was identified as CD4+Foxp3+ T cells, an observation in agreement with previous findings in *P. yoelii*-infected WT mice and in individuals infected with *P. falciparum* (6, 25, 26). These observations suggest that IL-10 production might not be the mechanism whereby natural T reg cells suppress the immune response to malaria. Collectively, these findings provide compelling evidence that shifting the balance between CD4+Foxp3+ Treg cells and effector CD4+ T cells in favor of regulatory cells severely compromises immune control of blood-stage malaria infection. Although our data support the crucial importance of natural Treg cells in balancing Th1 responses during malaria, there is no evidence that this balance is perturbed in humans infected with *P. falciparum* (25).

We also analyzed the CD4+Foxp3+ T cell responses in the spleen of WT mice that are resistant to *P. chabaudi* AS infection (1, 21, 22). This approach is in contrast to other studies in which the role of natural Treg cells was investigated in mice with lethal *Plasmodium berghei* ANKA or *P. yoelii* 17XL infections (6–8). Adoptive transfer of CD4+GFP+ T cell populations from reporter UBI-GFP/B6 transgenic mice to WT recipients demonstrated that CD4+Foxp3+ T cells preferentially localized in the spleen and not in lymph nodes or blood during infection. Moreover, immunohistochemical staining of spleen sections from infected WT mice confirmed the accumulation of Foxp3+ Treg cells in the spleen and demonstrated their presence within the T cell area of the white pulp during infection. These findings are consistent with the critical role of the spleen in immunity to blood-stage malaria (29). Moreover, they suggest that localization of T reg cells in the white pulp may favor interactions between these cells and dendritic cells and/or effector CD4+ T cells leading to modulation of innate and adaptive immunity to malaria.

Consistent with previous observations of an important expansion of Treg cells during malaria infection in mice and humans, we observed a significant increase in the number of CD4+Foxp3+ T cells in the spleen of infected WT mice early during infection (6–8). This early increase was transient and was followed by a rapid and significant decrease that coincided with increasing blood parasitemia and was sustained for several days around the time of peak parasitemia (days 8–9 p.i.) and during the time of immune control and elimination of the parasite (day 11 p.i.). The absolute number of CD4+Foxp3+ T cells eventually returned to a higher than normal level on day 33 p.i. Total CD4+ T cells have been observed to increase in WT mice during the first week of *P. chabaudi* AS infection, followed by a sustained decrease as the infection progresses (23). To our knowledge, in this study we showed for the first time that the decrease in CD4+ T cells affected both the CD4+Foxp3+ Treg and effector CD4+ Foxp3+ T cell populations. Consistent with previous observations, we observed that both populations expanded early during infection as evidenced by intracellular Ki67 expression. Interestingly, proliferation and expansion of CD4+Foxp3+ T cells were found to be significantly lower than effector CD4+ T cells when parasitemia reached the peak level (day 8 p.i.). This observation suggests that during infection CD4+Foxp3+ T cells may be more vulnerable than CD4+Foxp3− T cells to changes in inflammatory cytokines in the microenvironment, especially the diminished availability of T cell growth factors.

Several lines of evidence indicate that apoptosis occurs among a significant proportion of CD4+ T and B cells in the spleen of *Plasmodium*-infected mice (23, 31, 32). Apoptosis of these lymphocyte populations, involved in adaptive immunity as well as in immune-mediated pathology during malaria, may limit the potentially harmful effects of Ag-specific expansion and activation of these cells. IL-2, a major T cell growth factor, is also responsible for upregulation of the anti-apoptotic molecule Bcl-2 (11, 35). Recent studies in humans indicate that CD4+CD25+ Foxp3+ T cells from individuals infected with *P. falciparum* express low levels of Bcl-2, suggesting that these cells may be highly susceptible to apoptosis following IL-2 deprivation, thus ensuring a more rapid turnover in vivo of natural Treg cells than conventional T cells (36–38). In areas of seasonal malaria transmission,
both natural Treg and effector memory T cells expand together when malaria exposure is high; both populations undergo apoptosis once exposure decreases (25). Our studies revealed that a significant proportion of CD4+Foxp3+ Treg and effector CD4+ T cells have reduced Bcl-2 expression during acute infection, indicating that both populations undergo apoptosis during P. chabaudi AS infection. The frequency of Bcl-2-Foxp3+ Treg cells was significantly lower than Bcl-2- effector CD4+ T cells through day 8 p.i. This suggests that CD4+Foxp3+ T cells may be more prone to apoptosis than conventional CD4+ T cells during acute malaria, an observation consistent with recent studies in malaria-exposed individuals (6, 25). Once control of parasite replication was ongoing (day 11 p.i.), natural Treg cells appeared to be more resistant to apoptotic cell death than did effector CD4+ T cells, a conclusion supported by our observation of more CD4+Foxp3+ Treg cells later during P. chabaudi AS infection.

In addition to regulating the expression of Bcl-2 in T cells, IL-2, which is produced mainly by effector CD4+Foxp3+ T cells, is critically important for the differentiation and function of several immune cell types (11, 39). Importantly, IL-2 is essential to promote Foxp3+ Treg cell expansion and to maintain expression of Foxp3 (12, 32, 39, 40). Because natural Treg cells constitutively express the high-affinity IL-2 receptor subunit CD25, they may be particularly susceptible to variations in the bioavailability of IL-2. We therefore questioned whether decreased IL-2 secretion by effector CD4+ T cells might explain our findings of significantly lower proliferation of Treg cells compared with effector CD4+ T cells and decreased numbers of CD4+Foxp3+ T cells in WT mice during P. chabaudi AS infection. Earlier studies demonstrated that IL-2 levels are decreased in Plasmodium-infected individuals (41–43). The notion that IL-2 might be important in regulating the balance between effector Th1 cells and natural Treg cells during malaria has been suggested, but the exact role of this cytokine remains unknown (6, 7, 25, 44).

To address this possibility, we determined the kinetics of IL-2 production by effector CD4+ T cells during P. chabaudi AS infection and observed that during acute infection there were significant but transient increases in the frequency and number of CD4+Foxp3+ T cells expressing intracellular IL-2 and in IL-2 secretion by spleen cells stimulated in vitro with anti-CD3 mAb. These increases paralleled increased proliferation and high Bcl-2 expression by CD4+Foxp3+ T cells. Secretion of IL-2 subsequently decreased around the time of peak parasitemia coinciding with reduced proliferation of natural Treg cells compared with effector CD4+ T cells and increased apoptosis of both populations. These changes were reflected in our observation of significant decreases in the frequency and number of CD4+Foxp3+ T cells and increases in the expression of T-bet and IFN-γ by effector CD4+ cells, indicating the differentiation of Th1 cells. These findings are consistent with the concept that IL-2 consumption by natural Treg cells may contribute to their suppressive effects and result in apoptosis of target T cells (45, 46). Although the exact mechanism is unclear, consumption of IL-2 by expanding natural Treg cells may play a previously unrecognized role in immunosuppression associated with blood-stage malaria in humans as well as in mice (23, 47–50).

To establish a causal relationship between increased IL-2 levels and expansion of Foxp3+ Treg cells in the context of a malaria infection, we treated P. chabaudi AS-infected WT mice with IL-2/anti–IL-2 mAb complex and examined the effects of this treatment on the course of infection. Immune complexes consisting of IL-2 and anti–IL-2 mAb induce marked and selective proliferation of different immune cell types depending on the epitope recognized by the mAb (21). In particular, administration of the IL-2/anti–IL-2 mAb (clone JES6-1) complex induces selective in vivo expansion of CD4+Foxp3+ Treg cells, resulting in a marked increase in these cells in several tissues, including the spleen, and little or no changes in other cells (14, 15). We observed that administration of this IL-2/anti–IL-2 mAb complex to P. chabaudi AS-infected WT mice resulted in significant increases in parasitemia levels early during infection, significantly higher peak parasitemia, and delayed clearance of parasites compared with control mice. Consistent with the known effects of the IL-2/anti–IL-2 mAb clone JES6-1 complex, CD4+Foxp3+ Treg cells expanded to a significantly higher proportion and number in the spleen of complex-treated mice compared with control mice treated with either isotype control Ab or PBS during P. chabaudi AS infection (14–16, 34). Importantly, these data provide novel evidence supporting a critical role for IL-2 in expansion of natural Treg cells during blood-stage malaria. These findings are consistent with our observations that Foxp3Tg mice or WT recipient mice adoptively transferred with Treg cells from Foxp3Tg or WT mice experienced a more severe course of P. chabaudi AS infection than did control WT mice. Taken together, these data provide strong evidence linking high numbers of CD4+Foxp3+ Treg cells and suppression of immune control of infection, resulting in a more severe and possibly lethal malaria infection. In conclusion, our findings demonstrate that a tight balance between natural CD4+Foxp3+ Treg cells and effector CD4+ T cells is required for immune control and elimination of blood-stage P. chabaudi AS infection and that IL-2 produced by CD4+Foxp3+ T cells is involved in regulating this balance.

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Disclosures
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References


