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IL-27 Promotes IL-10 Production by Effector Th1 CD4+ T Cells: A Critical Mechanism for Protection from Severe Immunopathology during Malaria Infection

Ana Paula Freitas do Rosário,* Tracey Lamb,*‡ Philip Spence,* Robin Stephens,*‡ Agathe Lang,*† Axel Roers, † Werner Muller,‡ Anne O’Garra,§ and Jean Langhorne*

Infection with the malaria parasite, Plasmodium, is characterized by excessive inflammation. The establishment of a precise balance between the pro- and anti-inflammatory responses is critical to guarantee control of the parasite and survival of the host. IL-10, a key regulatory cytokine produced by many cells of the immune system, has been shown to protect mice against pathology during acute Plasmodium chabaudi chabaudi AS model of malaria. However, the critical cellular source of IL-10 is still unknown. In this article, we demonstrate that T cell-derived IL-10 is necessary for the control of pathology during acute malaria, as mice bearing specific deletion of Il10 in Th1 cells fully reproduce the phenotype observed in Il10−/− mice, with significant weight loss, decline in temperature, and increased mortality. Furthermore, we show that IFN-γ+ Th1 cells are the main producers of IL-10 throughout acute infection, expressing high levels of CD44 and ICOS, and low levels of CD127. Although Foxp3+ regulatory CD4+ T cells produce IL-10 during infection, highly activated IFN-γ+ Th1 cells were shown to be the essential and sufficient source of IL-10 to guarantee protection against severe immune-mediated pathology. Finally, in this model of malaria, we demonstrate that the generation of protective IL10+IFN-γ+ Th1 cells is dependent on IL-27 signaling and independent of IL-21. The Journal of Immunology, 2012, 188: 1178–1190.

The blood stages of the malaria parasite, Plasmodium, induce a pro-inflammatory response in the host, which although important for the clearance of the parasite, can lead to severe immune-mediated pathology. In humans and in mice, high levels of the proinflammatory cytokines IFN-γ, TNF-α, IFN-γ–induced protein (IP)-10 (CXCL10), and IL-1β have been shown to correlate with complications during malaria, such as severe anemia, hypoglycemia, and cerebral malaria (1–3).

A successful response must strike a balance between protection from the parasite and immunopathology, and IL-10 appears to be one of the means by which this balance is established (4, 5). Downregulation of the protective immune response by IL-10 during infection with a variety of pathogens, including Leishmania spp., Candida spp., Mycobacterium tuberculosis (6), Borrelia burgdorferi spp. (7), and HIV (8), is detrimental, promoting pathogen survival. Conversely, the absence or low levels of IL-10 have been shown to correlate with severe or fatal outcome in infections with P. falciparum (9), Toxoplasma gondii (10), and Trypanosoma spp. (11).

IL-10 can play either a positive or negative role in protecting the host from infectious disease or the associated immune-driven pathology. During a blood-stage Plasmodium chabaudi chabaudi AS infection in mice, the requirement of this cytokine for downregulating the pathology-inducing inflammatory response is evident as Il10−/− mice have greater amounts of plasma IFN-γ, TNF-α, and IL-12, higher mortality, and more pronounced pathology than wild-type (WT) animals (12, 13). Inactivation of IFN-γ reduces the mortality (13) and pathology is ameliorated by anti–TNF-α treatment (2), confirming that IL-10 regulation of inflammatory responses is crucial in preventing severe immunopathology in this model of malaria.

IL-10 is produced by different immune cell types, including B cells, macrophages, dendritic cells (DCs) (4, 14), and several T cell subsets, such as CD8+ (15) and CD4+ T cells, including Th1 (16, 17), Th17 (18), as well as Foxp3+ regulatory T cells (Tregs) (19) and regulatory type 1 (Tr1) cells (20). IL-10 from CD4+ T cells (16), CD8+ T cells (15), and myeloid cells such as DCs (14) have all been shown to play important roles in regulating immunopathology in different infection models. Furthermore, within the CD4+ T cell subset, IL-10 from effector (16) or Tregs (21) can have similar roles in different infections, or different roles in the same infection. For example, in Leishmania major infections, IL-10 from effector Th1 cells is necessary for suppression of inflammatory response during acute infection (16), whereas IL-10–producing Ag-specific Foxp3+ CD4+ T cells (Tregs) suppress the clearance of the parasite by the effector CD4+ T cells (21, 22).
Despite intensive research on the mechanisms responsible for regulation of immunopathology in malaria, the cellular source of protective IL-10 is not known. During P. falciparum infection, greater frequencies of effect Th1 CD4+ T cells producing IL-10 have been observed in children with uncomplicated malaria (23), although their protective function has not been established. In a mouse model of nonlethal malaria, P. yoelli, IL-10 is primarily produced early in the infection by CD4+CD25 Foxp3+ adaptive Tregs (24). However, a role for IL-10 from these cells in reducing proinflammatory responses, pathology, or parasite clearance has not been directly demonstrated. Therefore, a clear definition of the critical protective source of IL-10 during malaria infection and the mechanisms of its induction are still to be determined. Several mechanisms have been proposed to regulate IL-10 production, including TGF-β, IL-6, IL-21, and IL-27, which can mediate suppression of immune responses via the induction of IL-10 (25–30). However, the exact mechanism involved in regulating production of protective IL-10 in many infections and inflammatory diseases including malaria is not known.

In this study, we show that CD4+ T cells are the major source of IL-10 throughout infection, and that mice carrying a deletion of the Il10 gene specifically in T cells fully reproduce the phenotype previously observed in Il10−/− mice (13). Importantly, immunocompromised mice reconstituted with WT, but not Il10−/− CD4+ T cells, are rescued from death and present substantially ameliorated pathology. Although Foxp3+ regulatory CD4+ T cells also produce IL-10 during this infection, adoptive transfer studies revealed that IL-10 from effector Th1 CD4+ T cells, but not from Foxp3+ Tregs, is both essential and sufficient to regulate the immunopathology of a P. chabaudi chabaudi AS infection. We further demonstrate that the induction of IL-10 in Th1 cells requires direct IL-27 signaling but is independent of IL-21. These findings are of the real importance for the malaria field because they define the critical cellular source of protective IL-10 and the pathways leading to its production. This knowledge has significant impact in this field and might be important for the establishment of protocols for manipulation of these pathways to prevent severe malaria in humans.

Materials and methods

Animals and infections
Female C57BL/6 (CD45.2 and CD45.1), C57BL/6 Il10−/− (31), Rag1−/− (32), IL21−/− (33), and Foxp3-enhanced GFP reporter mice (34) (8–12 wk) were bred in the specific pathogen-free facilities at the Medical Research Council National Institute for Medical Research (NIMR, London, U.K.). Il10−/− mice, which have loxP sites flanking exon 1 of Il10 (35), crossed with CD4-Cre (35, 36), and LysM-Cre (37, 38) mice were backcrossed onto NIMR (GL113) (42) was injected i.p. into C57BL/6 mice 2 d after i.v. infection. Weight loss and temperature, as well as survival rate, parasitemia, and total numbers of iRBC, were assessed.

Cell preparations
Single-cell suspensions obtained from spleens were incubated with FcR block (43) followed by specific Abs (BD Pharmingen, BioLegend, or eBioscience) using appropriate combinations of fluorochromes (FITC, PE, PerCP, PE Texas Red, PerCP-Cy5.5, PE-Cy5.5, PE-Cy7, Pacific Blue, allophycocyanin, and allophycocyanin-Cy7). CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), CD45RB (16A), CD45L (MEL-14), CD44 (IM7), CD45.1 (A20), CD45.2 (104). The samples were acquired on CyAn (DAKO) using Summit (Cytomation). FlowJo (Tree Star) software was used for analysis of the data.

Intracellular staining
Intracellular cytokines were detected as described previously with few modifications (44). After stimulation and surface labeling, the cells were fixed with 2% paraformaldehyde (PFA; Sigma) in PBS (Life Technologies, Invitrogen), followed by permeabilization with FACS buffer (PBS supplemented with 2% FBS and 0.1% sodium azide) containing 0.5% saponin (Sigma). To detect intracellular cytokines, we used anti-IFN-γ–PECy7 (XM1G1.2; eBioscience) and anti-II–10–allophycocyanin (JES5-16E3; BD Pharmingen, or the respective isotype controls. Transcription factor expression was assessed with anti–Foxp3–Alexa Fluor 488 (150D; BioLegend), Foxp3-PE (FJK-16s; eBioscience), and T-bet–Pacific Blue (4B10; BioLegend); Abs were used according to the manufacturers’ instructions.

Cell sorting
Single-cell suspensions from spleens were incubated with anti-CD4, -CD19, -CD11b, or -CD11c magnetic beads (Miltenyi Biotec). CD4+ and CD19+ cells were purified (>97%) using autoMACS (Miltenyi Biotec). CD3+ CD8−, CD5−, CD4−, CD11c−, and CD11b− cells were purified (>95%) on a MoFlo cell sorter (Cytometry) using specific Abs anti-CD3 (145-2C11) or anti-CD4 (M48-145) and anti-CD11b–PECy7, and CD11c (N418) allophycocyanin (BD Pharmingen and eBioscience), 7-Aminoactinomycin D (Sigma) or propidium iodide (Sigma) was used to exclude dead cells. To obtain Foxp3-GFP+ cells, we incubated splenocytes from Foxp3-EGF reporter mice with anti-CD4 magnetic beads and purified them using autoMACS. EGFP+ or EGFP− cells were sorted on a MoFlo cell sorter (purity >95%).

Quantitative RT-PCR
Purified and sorted cells were placed in TRizol (Invitrogen), RNA extracted, and RT-PCR assay was performed as described previously (43). Target gene mRNA expression was quantified using SYBR green (Fisher Scientific) and normalized to ubiquitin mRNA levels, using the 2ΔΔCt method (45). IL-10 primers: forward, 5′-TTTGAATCTCCTGCGGTAAG-3′; reverse, 5′-GCCAATCGTCTGAGTCA-3′; ubiquitin primers: forward, 5′-TGCCATTATATTGGCTGCAT-3′; reverse, 5′-GCCAAGTGCTAAGAGTCAAGTA-3′.

Adoptive transfer
Spleenic CD4+ cells from naive C57BL/6 and Il10−/− mice were purified (>97%) using anti-CD4 microbeads, and naive Rag1−/− mice received 107 CD4+ T cells i.v. Mice were infected with 105 iRBC and pathology was monitored.

For adoptive transfer of effector T cells and Tregs, splenocytes from naive C57BL/6 (CD45.2 or CD45.1) and Il10−/− mice were obtained. CD25+ and CD25− cells were purified using the CD4+CD25+ Treg isolation kit (Miltenyi Biotec) and sorted (>95% purity) on a MoFlo cell sorter. CD25+ (0.5 × 106) and CD25− (3.5 × 105) were injected i.v. into Rag1−/− mice. After 3 wk, the reconstituted mice were infected with 104 iRBC. Rag1−/− mice receiving 4 × 106 WT CD4+ cells were used as controls.

Generation of bone marrow chimeric mice
Rag1−/− mice were sublethally irradiated with 5 Gy using a [137Cs] source and reconstituted i.v. with 1.7 × 108 BM cells from WT CD45.1+ mice, 129/C57−/− CD45.2+ (TCCR−/−) mice (40), or a mixture of both at ratios 90:10, 10:90, and 50:50. Reconstitution was assessed by flow cytometry 7
Regarding the frequency of IL-10+ cells in the spleen during this infection, as shown in the pie charts (Fig. 1C), these frequencies do not change significantly (data not shown). Interestingly, IL-10+ IFN-γ+ double-producing cells expressed higher levels of IFN-γ than single IFN-γ+ cells at the beginning of infection (day 5, mean fluorescence intensity [MFI]: 1072 ± 154.1 and 509.5 ± 48.15, respectively; p < 0.0002; t test). The induction of these IL-10+ Th1 CD4+ T cells has been shown to require high Ag load (48). In Fig. 2A, we also observe this correlation, as double-producing cells are induced after the peak of parasitemia, when the greatest amounts of Ag are available, and later, when parasitemia drops, the frequency of those cells also decreases.

Interestingly, IL-10+ IFN-γ+ double-producing cells expressed higher levels of IFN-γ than single IFN-γ+ cells at the beginning of infection (day 5, mean fluorescence intensity [MFI]: 1072 ± 154.1 and 509.5 ± 48.15, respectively; p = 0.028; t test; Fig. 2C).

Similarly, at the time of maximum numbers of double IL-10+ IFN-γ+ cells (day 15), MFI of IL-10 was greater in these cells
compared with the single IL-10 producers (138.5 ± 3.0 and 102.5 ± 2.86, respectively; \( p = 0.028; \) \( t \) test).

The higher levels of IFN-γ and IL-10 in the double-positive cells may indicate that they are highly activated or more differentiated. Indeed, IL-10+ IFN-γ+ CD4+ T cells expressed more CD44 (MFI: 125.2 ± 6.3) than either IFN-γ (66.8 ± 0.9; \( p = 0.0022; \) \( t \) test) or IL-10 (28.6 ± 0.9; \( p = 0.005; \) \( t \) test) single-producing CD4+ T cells (Fig. 2D). These double producers also expressed the highest levels of the ICOS and the lowest levels of IL-7Rα (CD127) compared with IFN-γ and IL-10 single producers (Fig. 2F), demonstrating an increased activation state.

Critically, the expression of the transcription factor T-bet in IL-10+ IFN-γ+ Th1 cells was at similar levels to those of IFN-γ+ Th1 cells, demonstrating that highly activated effector Th1 cells are the major source of IL-10 during \( P. \) chabaudi infection (Fig. 2E).

**Mice with \( I l 10 \) deletion specifically in \( T \) cells reproduce the phenotype observed in \( I l 10^{−/−} \) mice**

As effector Th1 cells were the major source of IL-10 after the peak of infection, the next step was to determine whether IL-10 from T cells was important to control immune-mediated pathology in vivo. We therefore asked whether mice carrying a deletion of \( I l 10 \) specifically in T cells, CD19+ B cells, or macrophages and neutrophils (LysM+ cells) would reproduce the phenotype observed in completely deficient \( I l 10^{−/−} \) mice.

For these experiments, we used \( I l 10^{−/−} /flox\) mice (35) crossed to CD4-Cre (35, 36), CD19-Cre (39), or LysM-Cre (37, 38), and the appropriate \( I l 10^{−/−} /flox\) littermate controls. The mice were infected with 10⁵ \( P. \) chabaudi, and survival rates, weight loss, temperature decline, and anemia (RBC/ml) were monitored for 20 d. As shown in Fig. 3A, only those mice unable to secrete IL-10 specifically from T cells (\( I l 10^{−/−} /flox\) CD4Cre+ mice) fully reproduced the pathological features of the infection in the \( I l 10^{−/−} \) mice, in that they exhibited substantial weight loss, hypothermia, and anemia, and only 30–40% of mice survived the acute infection, despite the fact that in these mice other cells are competent in secreting IL-10. Therefore, not only are T cells the major source of IL-10 during \( P. \) chabaudi infection, but they are the critical source of protective IL-10, which promotes protection from severe immunopathology. Importantly, lack of IL-10 in T cells did not affect the duration or magnitude of the low/subpatent chronic \( P. \) chabaudi AS infection that follows the acute infection (data not shown).
IL-10 FROM Th1 CD4 CELLS AMELIORATES PATHOLOGY IN MALARIA

Interestingly, mice carrying B cells unable to produce IL-10 (Il10\(^{-}\)/CD19\(^{+}\)) also presented partial signs of pathology, with 15% mortality rate during the infection, suggesting that IL-10-producing B cells may contribute to control of malaria disease. These Il10\(^{-}\)/CD19\(^{+}\) mice have significantly lower frequencies and total numbers of IL-10+ IFN-\(\gamma\) double-producing and IL-10+ single-producing CD4\(^{+}\) T cells compared with Il10\(^{-}\)/CD19\(^{-}\) controls (Supplemental Fig. 2A, 2B), yet comparable numbers of IFN-\(\gamma\) single-producing CD4\(^{+}\) T cells and Foxp3\(^{+}\) CD4\(^{+}\) T cells (Supplemental Fig. 2B, 2C). Thus, the increase in the severity of immunopathology observed in mice unable to secrete IL-10 specifically from B cells (Il10\(^{-}\)/CD19\(^{+}\) mice) during \(P.\) chabaudi infection may result from their inability to generate sufficient numbers of IL-10-secreting CD4\(^{+}\) T cells.

IL-10 from monocytes and neutrophils did not appear to be required for the control of immunopathology, as there was no mortality, and weight loss, anemia, or hypothermia in Il10\(^{-}\)/LysMCre\(^{+}\) mice similar to their respective Cre\(^{-}\) controls and WT C57BL/6 mice. Similar to infections in Il10\(^{-}\)/ mice (12, 13), the effects of the deletion of Il10 in any of the cell populations could not readily be ascribed to differences in parasitemias because these were similar between the Il10\(^{-}\)/Cre\(^{-}\) mice and their respective Cre\(^{-}\) controls, and not significantly different from those of WT C57BL/6 (Supplemental Fig. 2D).

Similarly to Il10\(^{-}\)/ mice (12, 13), infected Il10\(^{-}\)/CD4Cre\(^{+}\) mice exhibited greater amounts of the proinflammatory cytokines IFN-\(\gamma\), TNF-\(\alpha\), and IP-10 compared with infected WT and Il10\(^{-}\)/Cre\(^{-}\) control mice (Fig. 3B), indicating the more inflammatory nature of the host response to \(P.\) chabaudi when IL-10 was lacking in T cells. Taken together, these results demonstrate a major protective role for IL-10 produced by T cells, most likely CD4\(^{+}\) T cells, against immune-mediated pathology during an acute blood-stage \(P.\) chabaudi infection.

**IL-10 from WT CD4\(^{+}\) T cells rescues immunodeficient mice from death**

Because CD4\(^{+}\) T cells were found to be the major producers of IL-10 during a \(P.\) chabaudi infection, and T cell-derived IL-10 is important for the regulation of pathology in this infection, we wanted to verify directly that IL-10 from CD4\(^{+}\) T cells prevented pathology. Therefore, WT C57BL/6 or Il10\(^{-}\)/CD4\(^{+}\) T cells were adoptively transferred into Rag1\(^{-}\)/ mice, which were then infected with \(P.\) chabaudi and the acute-stage pathology monitored. In these experiments, the aim was to observe a direct protective role for CD4-derived IL-10 in the acute stage of infection when immunopathology is most evident. Rag1\(^{-}\)/ mice infected with \(P.\) chabaudi normally exhibit severe weight loss, hypothermia, and anemia, and ~40% die already within the first 15 d of infection (Fig. 4A). As shown in Fig. 4A and 4B, all Rag1\(^{-}\)/ mice that received WT CD4\(^{+}\) T cells survived for the duration of the analysis, exhibited significantly less weight loss, less hypothermia, and less anemia than Rag1\(^{-}\)/ mice receiving Il10\(^{-}\)/CD4\(^{+}\) T cells or unreconstituted Rag1\(^{-}\)/ mice, demonstrating that IL-10–sufficient CD4\(^{+}\) T cells controlled pathology in this model. By contrast, recipient mice transferred with Il10\(^{-}\)/CD4\(^{+}\) T cells showed equivalent pathology to unreconstituted Rag1\(^{-}\)/ mice and, in fact, required longer to recover.

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**FIGURE 2.** IFN-\(\gamma\)^+ CD4\(^{+}\) CD4\(^{+}\) T cells are the main source of IL-10 during acute infection. Spleen cells from C57BL/6 mice were harvested on different days postinfection and stimulated in culture with PMA and ionomycin, which was followed by intracellular staining for cytokines. A, Representative dot plots show gated CD4\(^{+}\) T cells. Production of IL-10 and IFN-\(\gamma\) was assessed throughout acute infection and mean percentages are presented. B, Total numbers of IL-10\(^{-}\) and IFN-\(\gamma\)^+ IL-10\(^{-}\)/CD4\(^{+}\) T cells per spleen (mean ± SEM; \(n = 7–9\) per time point; \(t\) test). C, MFI for IFN-\(\gamma\) (day 5) and IL-10 (day 15) observed for the different cytokine-producing cell populations. D, Representative histograms show CD44 surface staining and (E) expression of transcription factor T-bet on CD4\(^{+}\) T cells from day 15-infected mice. F, CD4\(^{+}\) T cells from day 15-infected mice were stained for CD45RB, CD127, ICOS, and CD62L, which was followed by intracellular staining for IFN-\(\gamma\) and IL-10. Representative histograms are presented, and the bar graphs show the MFI for each cell population and the respective marker. Data are representative of three (A–D, F) and two independent experiments (E). Mean ± SEM; \(n = 6–9\); \(t\) test; \(*p < 0.05\), \(**p < 0.01\), \(***p < 0.001\).
The differences in pathology in Rag1−/− mice receiving either WT or Il10−/− CD4+ T cells could not be explained by the differences in parasitemia, because they both showed similar numbers of parasitized RBC/L (Fig. 4C). Therefore, these data confirm that IL-10 from CD4+ T cells is critical to limit the immunopathology during P. chabaudi infection but is not important for the control of parasitemia.

As it has been demonstrated that transfer of CD4+ T cells into lymphopenic hosts, such as Rag1−/− mice, can result in a preferential expansion of Foxp3+ Tregs (49), we assessed the numbers of Foxp3+ CD4+ T cells in the reconstituted Rag1−/− mice. Before transfer, purified cells were assessed for the expression of Foxp3, and both WT and Il10−/− CD4+ T cells presented similar frequencies of Foxp3+ cells (∼14%; data not shown). Surprisingly, after transfer and infection, Rag1−/− mice receiving WT CD4+ T cells had significantly higher percentages and greater numbers of Foxp3+ CD4+ T cells per spleen compared with those mice receiving Il10−/− CD4+ T cells (Fig. 4D), suggesting that Il10−/− Foxp3+ CD4+ T cells are not able to expand postinfection, or that the effector cells outgrew the regulatory cells. Thus, it is possible that the transferred WT CD4+ T cells were better able to ameliorate immunopathology through increased Foxp3+ Treg frequencies, which may, in turn, regulate the outcome of disease directly through IL-10 production or via other suppressor mechanisms. Therefore, the next step was to determine whether Foxp3+ CD4+ Tregs were the critical source of IL-10 for the control of pathology in this model.

Foxp3+ Tregs produce IL-10 during infection but effector Th1 cells are the major source

We have shown that CD4+ T cells are the critical source of IL-10 for the attenuation of immunopathology in P. chabaudi infection, and that IFN-γ+ IL-10+ CD4+ T cells are the major producers of IL-10 and represent highly activated effector Th1 cells. However, they may not necessarily be the critical source regulating pathology; the single IL-10+ CD4+ T cell subset may be important and could represent a small population of suppressive Foxp3+ Tregs.

To examine whether IL-10–producing Tregs could be a source of protective IL-10 during this P. chabaudi infection, we used mice harboring the EGFP sequence inserted into the Foxp3 gene (Foxp3-EGFP reporter mice) (34) to determine the contribution of Foxp3− and Foxp3+ CD4+ T cells to protective IL-10 production. It is important to point out that there was a significant increase in the total numbers of Foxp3+ Tregs in the spleen postinfection (Fig. 5A), which could be observed already from day 5. Moreover, analysis of IL-10 transcription in sorted EGFP-Foxp3+ Tregs confirmed a large upregulation of IL-10 transcription in these cells during infection (Fig. 5B). CD4+ Foxp3+ and Foxp3− splenic T cells were sorted based on EGFP protein expression, and the two populations were then restimulated in vitro and stained for intracellular cytokines. Fig. 5C clearly shows that Foxp3− effector CD4+ T cells are the main source of IL-10 during acute infection, reaching nearly 2 × 106 secreting cells on day 15, compared with only 0.2 × 106 Foxp3+ CD4+ Tregs producing this cytokine.

The majority of IL-10+ Foxp3− effector cells coproduced IFN-γ, whereas the majority of IL-10+ Foxp3+ cells did not produce IFN-γ,
as observed in the representative dot plots (Fig. 5D). The bar graphs showing the total numbers of cytokine-producing cells confirm Foxp3+ effector cells as the major source of IL-10 (Fig. 5D). Therefore, although the total number of Foxp3+ Tregs increases during infection and IL-10 production is upregulated by these cells, IFN-γ+ IL-10+ double-producer Foxp3+ effector CD4+ T cells remain the prevailing source of IL-10. Unexpectedly, our intracellular analysis also revealed a minor population of IFN-γ+ single-producing Foxp3+ cells (Fig. 5D), which have also been observed in other parasitic infections (50, 51). Their function is currently unknown.

IL-10 from Foxp3+ CD4+ T effector cells is essential and sufficient to control immune-mediated pathology during acute P. chabaudi infection

It remained to be determined whether IL-10 from Foxp3+ Tregs was required to limit immunopathology or whether IL-10 from
effector Th1 cells was sufficient. To address this question, we sorted Foxp3+ CD4+ Tregs from naive C57BL/6 WT mice (CD45.1 or CD45.2) or Il10−/− mice (CD45.2) based on their high-level expression of CD25, whereas effector cells were sorted as CD25−. These 2 populations were cotransferred at a ratio of 7 effector CD25− CD4+ T cells (3.5 × 10^6) to 1 regulatory CD25+ CD4+ T cell (0.5 × 10^6) into Rag1−/− mice 3 wk before infection with P. chabaudi. Before transfer, the sorted cells were assessed for Foxp3 expression to verify that the majority of both WT and Il10−/− CD25+ CD4+ T cells were Foxp3+ (>86%), and that CD25− CD4+ cells were almost entirely Foxp3− (Fig. 6A). These results confirm that Foxp3+ Tregs can develop in the absence of IL-10 (52). Using the allotypic markers CD45.1 and CD45.2, we were able to determine that in mice receiving WT effector and WT regulatory CD4+ T cells, the percentage of Foxp3+ regulatory CD25+ cells after reconstitution (day 0) and postinfection (day 10), and their ratio to effector CD25− cells, remained constant (Fig. 6B). However, in mice receiving WT effector and Il10−/− regulatory CD4+ T cells, the percentage of regulatory CD25+ cells expressing Foxp3 had decreased after 3 wk of reconstitution (day 0); in addition, their ratio to effector CD25− cells decreased significantly postinfection (day 10; p = 0.05; t test; Fig. 6B).

Infected Rag1−/− mice reconstituted with the mixture of WT effector and WT regulatory CD4+ T cells presented equivalent pathology to those reconstituted with total WT CD4+ T cells (Fig. 6C), verifying the transfer model. Rag1−/− mice reconstituted with WT effector and Il10−/− Tregs recovered better from the pathology, with less hypothermia and lower anemia, compared with those reconstituted with total WT CD4+ T cells (Fig. 6C), demonstrating that despite a lower ratio of Foxp3-expressing CD25+ cells to effector CD4+ T cells, these mice were nevertheless protected from severe disease. Furthermore, these two groups present similar total numbers of effector cells, which is unlikely to be the reason for less severe disease in mice receiving WT effector and Il10−/− Tregs when compared with those reconstituted with total WT CD4+ T cells (53.87 ± 10.48 × 10^5 and 44.70 ± 7.94 × 10^5 cells, respectively; p = 0.55). Therefore, IL-10 derived from Foxp3+ Tregs is not required for protection from severe immunopathology during P. chabaudi infection. Rag1−/− mice reconstituted with Il10−/− effector and WT regulatory CD4+ T cells

**FIGURE 6.** IL-10 from Foxp3+ CD4+ T cells is essential and sufficient to limit pathology. Rag1−/− mice were reconstituted with naive cells: 0.5 × 10^6 CD4+CD25+ and 3.5 × 10^6 CD4+CD25− T cells either from C57BL/6 WT (CD45.1 or CD45.2) or Il10−/− (CD45.2) mice. After 3 wk of reconstitution, the mice were infected with 10^5 infected RBC, and analysis of pathology was performed for 15 d. A, Using intranuclear staining, representative histograms show Foxp3 expression in CD4+CD25+ and CD4+CD25− T cells before transfer into immunocompromised mice. B, On days 0 and 10 postinfection, spleens from reconstituted mice were harvested, and the percentages of Foxp3+ cells in the CD45.1 and CD45.2 cell populations were assessed, as well as the ratio between them. Dotted lines represent the original frequency of Foxp3+ cells in the CD25 population before transfer. C, Postinfection, signs of pathology were detected using several parameters: temperature, weight loss, and RBC count. Data are representative of two independent experiments. Mean ± SEM; n = 3–4 (B); n = 5–7 (C); *p < 0.05, **p < 0.01; t test.
displayed increased hypothermia, weight loss, and anemia compared with control mice receiving total WT CD4+ T cells (Fig. 6C), and showed little sign of recovery from the infection. Furthermore, the survival curves clearly confirm the severe immunemediated pathology in mice receiving Il10−/− effector and WT regulatory CD4+ T cells because they present significantly higher mortality (Supplemental Fig. 3). Regarding the parasite burden, no significant difference in peripheral parasitemia was observed between the groups. These data clearly demonstrate that IL-10 derived from Foxp3+ Tregs is not required for protection from severe immunopathology. IL-10 from effector CD4+ T cells is the critical source and is sufficient to limit immunopathology during P. chabaudi infection without contributing to an increase in parasite burden.

**IL-27, but not IL-21, is necessary to induce optimal IL-10 production by IFN-γ+ Th1 cells**

IL-27 has recently been implicated in the regulation of IL-10 production by T cells (53), and IL-21 has been shown to amplify the IL-27 signal for IL-10 production by Tr1 cells (54). We evaluated the role of these pathways in inducing IL-10 production by IFN-γ+ Th1 cells in this model of malaria. Il21−/− mice were infected with P. chabaudi, and their cytokine profile was analyzed by intracellular staining at day 15 postinfection. Interestingly, despite having lower total numbers of spleen cells (data not shown), IL-21−/− deficient mice showed greater frequencies and numbers of IL-10+ IFN-γ+ double-producing Th1 cells and IL-10+ single-producing CD4+ T cells compared with WT mice (Fig. 7A), clearly demonstrating that IL-21 is not required for IL-10 production in CD4+ T cells during P. chabaudi infection.

To address the role of IL-27 for the production of IL-10 by IFN-γ+ Th1 cells, we generated mixed bone marrow chimeras in which WT CD45.1+ and Il27ra−/− CD45.2+ (40) bone marrow cells were transferred to sublethally irradiated Rag1−/− mice at various ratios. As controls, chimeras receiving 100% WT CD45.1+ or 100% Il27ra−/− CD45.2+ bone marrow cells were also generated. After 7 wk, the chimerism was determined. We selected those mixed chimeric mice with ~50% reconstitution with Il27ra−/− T cells (Supplemental Fig. 4) for enumeration of IL-10+ IFN-γ+, IL-10+, and IFN-γ+ CD4+ T cells postinfection with P. chabaudi. Pathology was also analyzed. Using the gating strategy shown in Supplemental Fig. 4, we gated the WT and Il27ra−/− CD4+ T cells based on the expression of their allotypic marker. IL-27−deficient IFN-γ+ CD4+ T cells were significantly impaired in their ability to produce IL-10, with reduced frequencies and numbers of IL-10+ IFN-γ+ CD4+ T cells in chimeric mice reconstituted with 100% Il27ra−/− bone marrow compared with 100% WT bone marrow, and in Il27ra−/−, compared with WT, CD4+ T cells that coexisted in mixed chimeras (Fig. 7B). In addition, there was defective induction of IL-10+ single-producer CD4+ T cells in the Il27ra−/−, compared with WT, chimeras, whereas the number of IFN-γ+ single-producer CD4+ T cells per spleen was not affected by the absence of IL-27R (Fig. 7B).

As IL-10+ IFN-γ+ Th1 cells are necessary to control immunemediated pathology induced during acute P. chabaudi infection, it would be expected that chimeric mice reconstituted with only Il27ra−/− bone marrow cells, which generated the lowest number of IL-10+ IFN-γ+ Th1 cells, would present significantly increased symptoms of pathology, including hypothermia and weight loss, compared with chimeras reconstituted with 100% WT bone marrow cells. This was observed (Fig. 7C). In addition, mixed bone marrow chimeric mice also presented with a partial increase in the severity of pathology (Fig. 7C). In conclusion, our results clearly demonstrate that the induction of IL-10+ IFN-γ+ CD4+ T cells, which provide critical protection from immunopathology during P. chabaudi infection, requires signaling through the IL-27 pathway.

**Discussion**

The ability to establish a precise balance between the strength of the proinflammatory response triggered by infection and the consecutive immune-regulatory mechanisms can be crucial to guarantee efficient clearance of the pathogen without excessive tissue damage. In this article, we show that IL-10 production increases during acute P. chabaudi as infection, and that IFN-γ+ Th1 CD4+ T cells are the main source throughout acute phase. As a consequence, mice with specific deletion of Il10 in T cells develop severe disease, and a significant proportion succumbs to infection, reproducing the phenotype observed in infected Il10−/− mice. We demonstrate that, although Foxp3+ regulatory CD4+ T cells also secrete IL-10 during this malaria infection, IL-10 produced by activated effector Th1 cells co-producing IFN-γ is essential and sufficient to rescue mice from fatal immunopathology, and that this mechanism is highly dependent on IL-27.

Several studies have described CD4+ T cells as the critical source of IL-10 in different infections, contributing either to protection or chronicity (16, 17, 24). In *L. major* infection, IL-10 from myeloid cells did not seem to play a major role, but T cell-derived IL-10 was sufficient to establish the chronic infection (16, 21, 22). However, IL-10 from CD4+ T cells is not the crucial source in all infections. In acute influenza and corona virus infections, IL-10–producing CD8+ T cells were shown to be protective, despite the fact that CD4+ T cells also constituted a significant proportion of the IL-10–producing cells (15, 55), and IL-10 produced primarily by DCs has been reported to contribute to persistence of lymphocytic choriomeningitis virus infection (46). Therefore, the cellular origin of the essential IL-10 and its role seem to depend on the pathogen and its location (56).

B cells constituted a significant proportion of IL-10–secreting cells in the spleens of naive mice, as described previously for other peripheral lymphoid tissues, where it has been proposed that they play a role in immune homeostasis (57). Our data show that mice unable to secrete IL-10 specifically from CD19+ B cells present significantly reduced total numbers of splenic IL-10+ IFN-γ+ CD4+ cells, suggesting that B cell–derived IL-10 is important for IL-10 production by CD4+ T cells. It has been demonstrated that chronic activation of murine CD4+ cells in the presence of IL-10 can generate IL-10–producing Tr1 cells (58). However, the requirement of IL-10 to generate IL-10+ cells is not so clear, as recent data show thymic precursor cells giving rise to peripheral IL-10–expressing Tregs in the absence of IL-10 (52). In our model of malaria, the precise mechanism by which IL-10 is defectively induced in CD4+ T cells from Il10+/CD19Cre+ mice has to be further addressed. We cannot yet dismiss the possibility that B cell–derived IL-10 would also contribute to the control of pathology by other mechanisms.

A lack of IL-10 production by T cells during P. chabaudi infection results in a greater inflammatory response, similar to that observed in infected Il10−/− mice, with high plasma levels of IFN-γ, TNF-α, and IP-10. Because IFN-γ and TNF-α have both been shown to be involved in the pathology during *P. chabaudi* infection of Il10−/− mice (2, 13), this would suggest that the concomitant production of IL-10 by IFN-γ effector Th1 cells in this infection may represent an important self-limiting mechanism as proposed by Trinchieri (56) and Saraiva et al. (48), providing a highly regulated feedback loop that helps limit the collateral damage caused by exaggerated inflammation, as well as allowing a protective response to different pathogens. In line with this
hypothesis, it has been observed that PBMCs of children with uncomplicated \textit{P. falciparum} malaria contain significantly greater proportions of IL-10+ IFN-\(\gamma\) double-producing CD4+ T cells than PBMCs from children with severe malarial disease (23), although it remains to be demonstrated whether these double-producing cells are able to directly downregulate immunopathology.

Recently, IL-10–producing Th1 cells have been identified in human blood, which can suppress proliferation of naive and memory T cells via IL-10 secretion (59), and similar to our results, these cells were highly activated, expressing high levels of ICOS, which originally was claimed to drive IL-10–secreting cells (60) and low levels of the IL-7 receptor (CD127), suggesting recent activation (61). In the \textit{P. chabaudi} infection described in this article, these highly activated IL-10+ IFN-\(\gamma\)+ Th1 CD4+ T cells are present in greatest numbers after the peak of the acute infection and decline with the subsequent decrease in parasitemia, agreeing with the idea that persistence of IL-10–producing Th1 cells depends on chronic stimulation of the TCR and high Ag dose (48, 59).

IL-27 and IL-21 have been recently demonstrated to be involved in the induction of IL-10 (26, 54). In this article, we demonstrate

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**FIGURE 7.** Generation of IL-10+ IFN-\(\gamma\)+ CD4+ T cells is independent of IL-21 but requires IL-27 signaling. A, WT and Il21\(^{-/-}\) mice were injected with \(10^5\) \textit{P. chabaudi}-infected RBC, and 15 d later the splenocytes were harvested for intracellular analysis. Representative plots show IL-10 and IFN-\(\gamma\) production by gated CD4+ T cells. Mean percentages of cytokine-producing cells are shown. Total numbers of CD4+ T cells per spleen producing only IL-10 or IL-10 in combination with IFN-\(\gamma\) are presented (each dot represents an individual mouse; mean ± SEM; \(n = 5\); t test). Data are representative of three independent experiments. B, After sublethal irradiation, Rag1\(^{-/-}\) mice were reconstituted with \(1.7 \times 10^6\) total bone marrow cells from WT (CD45.1\(^+\)), Il27ra\(^{-/-}\) (CD45.2\(^+\)) mice, or a mixture of both at a ratio of 50:50, and they were designated as 100% WT, 100% Il27ra\(^{-/-}\), and 50%:50% WT:Il27ra\(^{-/-}\) chimeras, respectively. Nine weeks after the transplantation, all mice were infected with \(10^5\) \textit{P. chabaudi}, and 8 d later, they were culled for analysis. Representative contour plots show IL-10 and IFN-\(\gamma\) production by gated CD4+ T cells. These cells were selected based on their expression of the allotypic markers CD45.1 or CD45.2. The total numbers of IL-10+ IFN-\(\gamma\) double producers, IL-10+ single producers, and IFN-\(\gamma\)+ single producers per spleen are shown for WT and Il27ra\(^{-/-}\) cells. C, The graphs for temperature and weight loss represent data from days 8 and 7, respectively, when the major differences were observed between the groups (mean ± SEM; \(n = 6–11\); t test).
that IL-27 signaling is critical for optimal production of IL-10 by IFN-γ-producing Th1 cells during acute *P. chabaudi chabaudi* infection and, consequently, loss of IL-27 signaling increased the severity of immunopathology. A similar role for IL-27 has been described during autoimmune inflammation (62) and *L. major* infection (28). Moreover, our data on the requirement for the IL-27R in induction of IL-10 in Th1 cells would offer an explanation for the observation that IL-27R−deficient mice are highly susceptible to a *P. berghei* infection with exacerbated Th1 immune responses (29). IL-27 has been shown to induce IL-21, and that loss of IL-21 signaling in this situation resulted in inhibition of IL-10 production by T cells in vitro (54). The coordinated role of IL-27 and IL-21 in regulating IL-10 production is supported by the observation that elevated levels of these cytokines correlate with IL-10 responses in human visceral leishmaniasis (30). However, in this *P. chabaudi* infection, there was no requirement for IL-21 in the production of IL-10 by CD4 T cells; indeed, IL-21−deficient mice presented increased numbers of IL-10−IFN-γ CD4+ T cells.

Foxp3+ Tregs are known to suppress immune responses using several mechanisms, which include those targeting the T cells directly (via suppressor cytokines, IL-2 consumption, cytokolysis), or indirectly by suppressing APC function (via reduction of co-stimulation or Ag presentation) (63). Although some studies have shown that secretion of IL-10 by Tregs can constitute an important component of their suppressive capacity (21, 22, 64, 65), our results in this study provide strong evidence that IL-10 from Foxp3+ Tregs is not required to limit immunopathology elicited during acute *P. chabaudi* malaria. Lymphopenic mice receiving effector CD4+ T cells lacking IL-10 and Foxp3+ CD4+ Treg are able to produce IL-10 experience severe disease, whereas reciprocal cotransfer of Tregs lacking IL-10 together with IL-10−sufficient effector CD4+ T cells did reduce pathology. However, a role for Tregs in the modulation of immunopathology in this model of malaria through a mechanism independent of IL-10 production cannot be dismissed. Such a role for Tregs in malaria is supported by the observations that Foxp3−CD4+ T cells transferred protection against *P. berghei*-induced experimental cerebral malaria in mice is dependent upon CTLA-4 and not IL-10 (66), and that IFN-γ production by human PBMC stimulated with *P. falciparum* blood-stage Ags can be suppressed in vitro by CD25+ Foxp3+ Tregs independently of IL-10 (67). Interestingly, we observed that after adoptive transfer of Foxp3+ CD4+ T cells following infection, the loss of Foxp3 was more pronounced when IL10−/− Foxp3+ CD4+ T cells were transferred. Foxp3 expression can be lost after adoptive transfer into lymphopenic mice; however, it has been shown that IL-2 produced by co-transferred conventional T cells prevents Foxp3 downmodulation (68). Thus, in our case, loss of Foxp3 expression because of lack of IL-2 is unlikely as conventional CD4+ T cells were cotransferred. Tregs require IL-10R signaling to maintain Foxp3 expression (69). It is possible that the observed decrease of Foxp3 frequencies in our model is due to the lack of IL-10 in Foxp3+ T cells themselves.

In summary, our work has identified IL-10−IFN-γ−Th1 cells as the prevalent source of IL-10 during infection with *P. chabaudi*. These effector cells are extremely activated, expressing high levels of CD44 and ICOS, and low levels of CD127. In the beginning of infection, IL-10−IFN-γ−effector CD4 T cells secrete more IFN-γ than single producers, and later on, they secrete more IL-10 than the respective single producers, pointing to a possible self-feedback regulatory mechanism, to minimize immunopathology. Using cell transfer model into lymphopenic mice, we show clearly that IL-10 from highly activated Th1 cells is necessary and sufficient to regulate immune-mediated pathology elicited during this infection. Moreover, we demonstrate that IL-27, but not IL-21, signaling is required for induction of IL-10 by IFN-γ−producing CD4+ T cells, a critical mechanism for the control of immune-mediated pathology in mice. The next obvious step will be to validate these observations in human malaria in endemic areas. Given the already existing evidence for a protective role of IL-10 in severe malaria in humans, our identification of the potential cellular source of this cytokine and the requirement of the IL-27R for its induction in this malaria model may aid in the development of intervention strategies to promote this pathway of regulation, and thus potentiate immune responses that are protective against both disease and infection.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplemental Data

Figure S1. Direct signalling through the IL-10 receptor is crucial to guarantee protection during primary infection. C57Bl/6 WT resistant mice were treated i.p. with one dose of anti-IL10R (1B1.3a) or the IgG control (GL113) mAb (0.82 mg/mouse) 2 days after i.v. infection with $10^5$ iRBC. Pathology measurements (weight loss and hypothermia) along with survival rate, peripheral parasitemia and total numbers of iRBC were assessed during primary infection (Mean ± SEM; n = 4-5; t-test. *p<0.05 comparison between anti-IL10R1 and IgG control treatment; &p<0.05 comparison between Il10-/− and WT mice).

Figure S2. Mice bearing B cells unable to produce IL-10 present less IL-10+ IFN-γ+ CD4+ T cells and there is no alteration in the level of parasitemia in none of the conditional knockout mice. Il10fl/flCD19Cre+ and their Cre− littermate controls were infected with $10^5$ iRBC and 15 days later their splenocytes were harvested for analysis. (A) Representative plots exhibit IL-10 and IFN-γ production by CD4+ T cells with the mean frequencies of cytokine- producing cells. (B) The graphs show the total numbers of those cells per spleen. (each dot represents an individual mouse) (C) Representative plots show frequencies of Foxp3+ cells within the CD4+ population and the graph presents their total numbers per spleen. [Mean ± SEM; (B: n=14-20; C: n= 8-10); t-test]. ns= not significant. (D) Mice carrying specific deletions of Il10 in T cells (Il10fl/flCD4Cre+), CD19+ cells (Il10fl/flCD19Cre+) or monocytes/neutrophils (LysM+) cells (Il10fl/flLysMCre+) along with their correspondent Cre− littermate controls were infected with $10^5$ iRBC and their peripheral parasitemia followed for 13 days. (Mean ± SEM; n= 5-10).

Figure S3. Mice receiving Il10−/− CD25− and WT CD25+ cells present significant mortality. Rag1−/− mice were reconstituted as described in Figure 6. Survival curves are represented for the time of analysis.

Figure S4. Gating strategy used to selectively analyse Il27ra−/− and WT CD4+ T cells in chimeric mice. Rag1−/− mice were sub-lethally irradiated and reconstituted with 1.7 x $10^6$ total bone marrow cells from WT (CD45.1+) mice, Il27ra−/− (CD45.2+) mice or a mixture of both at various ratios. After seven weeks the chimerism was determined by flow citometry. Mice receiving either WT or Il27ra−/− cells were designed 100% WT and 100% Il27ra−/−, respectively. Those presenting approximately 50% reconstitution with Il27ra−/− cells were defined as 50%: 50% WT:Il27ra−/− chimeras. Representative plots show the gating strategy used to precisely determine the level of reconstitution by WT and Il27ra−/− cells based on their expression of the allotypic markers CD45.1 or CD45.2. (Mean ± SEM; n= 6-11; t-test).