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IFN-γ–Induced Priming Maintains Long-Term Strain-Transcending Immunity against Blood-Stage Plasmodium chabaudi Malaria

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The mechanism by which protective immunity to Plasmodium is lost in the absence of continued exposure to this parasite has yet to be fully elucidated. It has been recently shown that IFN-γ produced during human and murine acute malaria primes the immune response to TLR agonists. In this study, we investigated whether IFN-γ–induced priming is important to maintain long-term protective immunity against Plasmodium chabaudi AS malaria. On day 60 postinfection, C57BL/6 mice still had chronic parasitemia and efficiently controlled homologous and heterologous (AJ strain) challenge. The spleens of chronic mice showed augmented numbers of effector/effector memory (T Eff) CD4+ cells, which is associated with increased levels of IFN-γ–induced priming (i.e., high expression of IFN-inducible genes and TLR hyperresponsiveness). After parasite elimination, IFN-γ–induced priming was no longer detected and protective immunity to heterologous challenge was mostly lost with >70% mortality. Spontaneously cured mice had high serum levels of parasite-specific IgG, but effector T/Eff cell numbers, parasite-driven CD4+ T cell proliferation, and IFN-γ production were similar to noninfected controls. Remarkably, the priming of cured mice with low doses of IFN-γ rescued TLR hyperresponsiveness and the capacity to control heterologous challenge, increasing the T Eff cell population and restoring the CD4+ T cell responses to parasites. Contribution of TLR signaling to the CD4+ T cell responses in chronic mice was supported by data obtained in mice lacking the MyD88 adaptor. These results indicate that IFN-γ–induced priming is required to maintain protective immunity against Plasmodium chabaudi and aid in establishing the molecular basis of strain-transcending immunity in human malaria. The Journal of Immunology, 2013, 191: 5160–5169.

Malaria remains a major health issue, especially in the tropical and subtropical areas of the world. Despite efforts to develop vaccines and antimalarials, the Plasmodium species that cause malaria persist, with an increase in the incidence of the disease in endemic regions, spreading to areas where control or eradication had been achieved previously (1). Parasite resistance to antimalarial drugs, inefficiency of vector control policies, and absence of successful vaccination explain the failure to reduce the risks of infection. A complete understanding of the mechanisms underlying the acquisition of protective immunity is crucial to outline new strategies to eradicate malaria. For children living in holoendemic areas, naturally acquired immunity appears to be achieved after successive rounds of infection, protects against clinical manifestations of the disease in a strain-specific way, and does not elicit sterile protection against the parasite (2). In adults, acquired immunity can be achieved after a few rounds of infection and elicits strain-transcending, but still not sterile, clinical protection (3). In any case, protective immunity is usually lost in the absence of continued exposure to the parasites (4). This observation raises two important questions: What are the effector mechanisms of the immune system that are lost after removal of the residual parasitemia and why does this loss occur?

Mouse models of Plasmodium infection are widely used to investigate the protective immune response to malaria. Among them, Plasmodium chabaudi infection is a feasible model to study strain-specific and strain-transcending immunity because of the variety of well-characterized parasite clones and the similarities to the human disease caused by Plasmodium falciparum (5). Infection with homologous P. falciparum parasites in humans or P. chabaudi in mice results in significant parasite control, whereas limited protection to heterologous secondary infections has been observed in both cases (3, 6–8). A polymorphism in the merozoite surface protein-1 (MSP-1) gene mediates, to a large degree, strain-specific immunity in blood-stage P. chabaudi malaria (9, 10). This gene is also polymorphic among P. falciparum strains, possibly as a result of host immune selection pressure (11). In addition, as observed in P. falciparum infection (4), resistance to P. chabaudi malaria is optimized by an existing infection (12), particularly for strain-transcending immunity that is maintained by the low levels of IFN-γ–induced priming.
of subpatent parasitemia encountered in chronic mice (13). On the basis of this finding, it has been suggested that sustained exposure to malarial Ags is required not only for the generation of memory and effector cells but also for their maintenance (14). In fact, the partial loss of protective immunity to homologous challenge observed after elimination of reminiscent parasitemia is related to a decline in the CD4+ memory T (T EM) cell response to parasites (15).

Although much is known about how innate immune recognition affects adaptive immune responses, the role of adaptive immune cells in shaping the innate immune system is largely unexplored. Dendritic cells (DCs) and macrophages are tightly regulated by cytokines to rapidly respond to infections and also to avoid the undesirable effects of excessive activation. The priming of DCs and macrophages by low concentrations of IFN-γ is a potent mechanism by which the innate immune system is optimized, allowing an increased response to several extracellular stimuli, including TLR agonists (16, 17). IFN-γ–induced priming does not actually activate cells but ensures rapid and strong responses to stimuli, which in excess can eventually cause deleterious consequences. The mechanisms underlying macrophage priming involves a complex network of IFN-inducible genes, whose understanding is still limited (18). Recently, it has been shown that the innate immune system is primed by IFN-γ during acute P. falciparum and P. chabaudi infections and, in consequence, displays an enhanced response to TLR agonists (19), establishing a crucial role for this cytokine in immunity to malaria. However, the contribution of IFN-γ–induced priming to the maintenance of acquired immunity to Plasmodium is still unclear. We wondered whether low levels of IFN-γ produced by effector T (T E) cells and/or effector memory T (T EM) cells in response to chronic parasitemia in infected individuals could prime the innate immune system and, together with parasite-specific Ab, ensure strain-transcending immunity. To investigate this possibility, we evaluated the correlation between the spleen cell response to TLR agonists (LPS and CpG oligonucleotides) and protection against homologous (AS) strain and heterologous (AJ strain) challenge in C57BL/6 mice infected with P. chabaudi AS. Our data indicate that IFN-γ–induced priming is required to maintain long-term strain-transcending protective immunity to P. chabaudi malaria, a process that optimizes TLR signaling and guarantees the generation/maintenance of T E/EM cells.

Materials and Methods

Mice and parasites

Six- to 8-wk-old C57BL/6, RAG knockout (KO), and MyD88KO (with a C57BL6 background) female mice (originally from The Jackson Laboratory) were bred under specific pathogen-free conditions at the Isogenic Mouse Facility of the Instituto de Ciências Biomédicas at the Universidade de São Paulo. P. chabaudi (PcAS and PcAJ strains) was maintained as described elsewhere (20). Because the schizogenic cycle of these parasites depends on the host circadian rhythm (21), the mice were maintained under an inverted light/dark cycle for at least 15 d before infection to access the period adjacent to erythrocyte invasion.

Infections and clinical analysis

For the primary infections, the mice were inoculated i.p. with 1 × 10^6 infected RBCs (iRBCs). The mice were challenged i.v. with 1 × 10^6 iRBCs on days 60 and 200 postinfection (p.i.) and monitored daily to determine survival curves. To evaluate subpatent parasitemia, 100 μl blood from 60–200– or 260-d infected mice were transferred i.v. into RAGKO mice. Parasitemias were quantified by microscopic examination of Giemsa-stained blood smears. Body weight, temperature, and hemoglobin concentration (Hemoglobin kit; Doles) also were assessed in the infected mice.

Ethics statement

All procedures were in accordance with the national regulations of the Conselho Nacional de Saúde and Colégio Brasileiro em Experimentação Animal, with respect to their ethical guidelines for mouse experimentation and welfare. The protocols were approved by the Comissão de Ética no Uso de Animais of the Instituto de Ciências Biomédicas at the Universidade de São Paulo (São Paulo, Brazil), with permit numbers 0019/2005 and 0036/2007.

Purification of mature iRBCs

Mature iRBCs were obtained from mice with 40–60% parasitemia and predominantly late trophozoites and schizonts. The pellets from 500 μl heparinized blood were resuspended in 1 ml PBS, pipetted over 5 ml 74% Percoll (GE Healthcare) and centrifuged (2500 × g, acceleration/break of 5/0) for 30 min at room temperature. The top cell layers were collected and washed three times with complete RPMI 1640 medium (supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 2 mM L-glutamine, and 1 mM sodium pyruvate). All supplements were purchased from Life Technologies. This purification technique yielded >95% of the purity of mature iRBCs.

In vivo erythrocyte reinvasion assay

Mice were infected i.v. with 5 × 10^6 mature iRBCs. Blood samples were collected at 1 h intervals and stained with SYTO16 (Molecular Probes, Life Technologies) as described previously (22). Parasitemias were determined by flow cytometry (FACSCalibur; BD Biosciences) with FlowJo software (Tree Star) starting at 30 min p.i. (t0), when nearly all parasites were late trophozoites or schizonts. The reinvasion index was calculated as the ratio between the parasitemia percentages at the different time points and the t0 values.

Parasite-specific ELISA

PcAS-specific IgG1 and IgG2a serum levels were quantified by ELISA as described elsewhere (15). Briefly, 96-well flat-bottom microtiter plates (Costar) were coated overnight (4°C) with a total PcAS extract (10 μg/ml). Plates were saturated with 1% BSA for 1 h. After washing, 100 μl of the mouse serum samples (diluted from 1/50 to 1/12,800) were added and left for 2 h at room temperature. The assays were developed by adding a goat anti-mouse IgG1 or IgG2a peroxidase-conjugated Abs (Southern Biotechnological Associates) for 1 h. After washing, 100 μl tetramethylbenzidine (Invitrogen Life Technologies) was added to each well, and 15 min later, the absorbance values were quantified using a Spectra Max 190 spectrophotometer ( Molecular Devices) with a 650-nm wavelength filter. The Ab level in each serum sample is expressed as the reciprocal of the end-point titer, which we defined as the lowest dilution that equals the background OD.

Phenotypic analysis of spleen cells

Splenic cell suspensions were incubated in lysis buffer (40 mM NH4Cl and 4.2 mM Tris [pH 7.4]) for 5 min (4°C) to eliminate RBCs. The splenocytes were washed three times with complete RPMI 1640 medium (supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 2 mM L-glutamine, and 1 mM sodium pyruvate). Our molecular biology facility (or medium alone for 72 h at 37°C in a 5% CO2 atmosphere). IFN-γ was quantified in the cell supernatants with the exception of F4/80 mAb, which was purchased from eBioscience. The cells were analyzed by flow cytometry (FACSCanto; BD Biosciences) with FlowJo software.

CFSE proliferation assay

The proliferative CD4+ T cell response to iRBCs was measured as described previously (15). Briefly, 3 × 10^5 cells/ml (resuspended in PBS with 0.1% BSA) were incubated with CFSE (Molecular Probes) at a final concentration of 5 μM for 20 min at 37°C. The cells (1 × 10^6) were then cultured in 96-well plates (Costar) with 3 × 10^5 iRBCs, 10 μg/ml of the recombinant 19-kDa fragment of MSP-1 from PcAS (MSP-19, produced in our molecular biology facility) or medium alone for 72 h at 37°C in a 5% CO2 atmosphere. After incubation, the cells were stained with an allophycocyanin-labeled mAb against CD4 and analyzed by flow cytometry (FACSCanto) with FlowJo software.

IFN-γ detection

Splenocytes (10^6) were cultured in 96-well plates (Costar) with 3 × 10^6 iRBCs, 10 μg/ml MSP-19, 1 μg/ml LPS (strain 0111:B4 from Escherichia coli, Sigma-Aldrich), 10 μg/ml CpG oligodeoxynucleotide (ODN) 1826 (Coley Pharmaceutical Group), or medium alone for 72 h at 37°C in a 5% CO2 atmosphere. IFN-γ was quantified in the cell supernatants using the OptEIA IFN-γ kit (BD Biosciences).
Anti–IFN-γ treatment

Four doses (0.5 mg/mouse) of depleting mAbs against IFN-γ (H22; eBioscience) or Armenian hamster IgG isotype control (eBioscience) were injected i.v. into C57BL/6 mice every 2 d starting 52 d p.i. This depletion strategy was based on a previous study (23).

DC purification

Spleen cells (1 × 10^6) were incubated with anti-CD11c and anti-Pan DC microbeads (Miltenyi Biotec) diluted in PBS with 0.5% BSA and 2 mM EDTA (Invitrogen) for 30 min at 4°C. The cells were then sorted using LS columns (Midi MACS; Miltenyi Biotec). The positive fraction showed >90% CD11c+I-A^d+ cells.

Quantitative PCR for IFN-inducible genes

RNA was extracted from DCs (5 × 10^6) with TRizol (Invitrogen) (24) and quantified with a NanoDrop device (Eppendorf). cDNA was prepared with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). The quantitative PCR (qPCR) reactions were performed in an ABI 7500 Real-Time PCR device (Applied Biosystems) using the SyBr Green kit from Applied Biosystems. The expression of the stat3, irf7, ifnar, ifnr2, ifnr3, il6, and β-actin genes was analyzed using the 2^-ΔΔ threshold cycle algorithm. The primers used (25, 26) were: stat3 forward, 5'-GGCGCAGGCA-CAAATGAAGC-3' and reverse, 5'-TCTCAGGATCC CGCAATAGC-3'; irf7 forward, 5'-TGAGGACCAACGCAATAGC-3' and reverse, 5'-TGGAGAAGCACACGATAGC-3'; ifnar forward, 5'-GGCCCTGCGTGA-ATAAGAGCAGC-3' and reverse, 5'-GTTGGGAAGCACACGATAGC-3'; ifnr2 forward, 5'-TGGTTCTGCTGTGAGATT-3' and reverse, 5'-TGTAAACGACAGCTTACG-3'; il6 forward, 5'-GGTTTACCTC'TGCCTTCAC-3' and reverse, 5'-GAAACTGCCATGTTTGAGCA-3'; β-actin forward, 5'-CAGGCAGGCAG-TGCCTTCAC-3' and reverse, 5'-TCTCCATAGAGTGCGGCA-3'.

IFN-γ–induced priming

Four doses (15 ng/kg; 10,000 U/mouse) of rIFN-γ (mouse rIFN-γ expressed in E. coli; PeproTech) were injected i.v. into C57BL/6 mice every 2 d starting 192 d p.i. This IFN-γ priming dose was established in a previous study (25).

Statistical analysis

Statistical significance was analyzed with Prism 5 software (GraphPad) using ANOVA and a Tukey's multiple comparison test or Student t test where appropriate. The existence of a normal distribution was confirmed using the Kolmogorov–Smirnov test. Differences were considered statistically significant at p < 0.05.

Results

The clearance of residual parasitemia leads to partial loss in protective immunity to heterologous PcAJ parasites

The infection of C57BL/6 mice with PcAS led to acute parasitemia and its subsequent control (Fig. 1A). After 30 d p.i., parasites were no longer detected in the blood smears, and sub- patent parasitemia was assessed by blood transfer to RAGKO mice. Low levels of chronic parasitemia remained up to day 60 p.i., but parasites were not detected in the bloodstream on days 200 and 260 p.i. (Fig. 1B). To evaluate whether these mice maintained protective immunity to homologous and heterologous parasites, they were challenged i.v. with a high inoculum (1 × 10^7 iRBCs) of PcAS or the lethal PcAJ strain (10). Although mice on day 60 p.i. (miceC60d) efficiently eliminated both parasite challenges and survived, mice on day 200 p.i. (mice200d) developed low parasitemias over the first 3 d following PcAS reinfection and failed to control the growth of PcAJ, resulting in >70% mortality (Fig. 1C, 1D and 1F, 1G). To evaluate whether long-term acquired immunity requires de novo activation of the immune system, we also analyzed the capacity of mice to inhibit the first round of erythrocyte invasion when challenged i.v. with purified late trophozoites and schizonts (5 × 10^6 iRBCs). For both PcAS and PcAJ, the generation of new ring forms was blocked in miceC60d (Fig. 1E, 1H). In contrast, the efficiency of erythrocyte invasion by PcAJ was similar in mice200d and naïve controls. Because mice200d still retained the ability to inhibit erythrocyte invasion by PcAS, we concluded that the clearance of chronic parasitemia is associated with the loss of immunological effector mechanisms that are particularly required for protection against heterologous PcAJ parasites.

The partial loss in protective immunity to heterologous PcAJ parasites may result from a reduction in CD4^+ T_E/T_EM cell populations

The inactivation of the innate and/or acquired immune systems may account for the decline in protective immunity to heterologous PcAJ parasites following clearance of chronic parasitemia. Humoral immunity does not appear to be involved in the partial loss of protective immunity, because parasite-specific IgG1 and IgG2a serum titers were maintained or even increased on day 200 p.i. compared with day 60 p.i. (Fig. 2A). Therefore, we evaluated subsets of CD4^+ T cells from the spleens of these mice based on a recent study that defined CD4^+ T_E cells, CD4^+ T_EM cells, and CD4^+ T_CEM cells (26), as described in Supplemental Fig. 1. We found a significant increase in the CD4^+ T_E/T_EM cell numbers per spleen in miceC60d, whereas these subsets had similar numbers per spleen in mice200d and the age-matched controls (AMCs); only T_CEM cells were maintained at comparable numbers in miceC60d and mice200d (Fig. 2B). Indeed, on day 60 p.i., most CD4^+ T EM cells had an effector memory phenotype in which CD62L was expressed at low levels. The reduction of the CD4^+ T_E/T_EM cell populations observed after parasite elimination was accompanied by a loss of the ability of CD4^+ T cells to proliferate and produce IFN-γ in response to iRBCs and to proliferate in response to MSP-1 (Fig. 2C, 2D). The need for IFN-γ to maintain the protection against heterologous parasites in chronic mice was investigated by treating miceC60d with anti–IFN-γ mAbs. A partial loss of the ability to control PcAJ challenge was observed in IFN-γ–depleted miceC60d when compared with miceC60d who were treated with the isotype control (Fig. 2E). This effect was accompanied by a reduction in body temperature (Fig. 2F).

IFN-γ–induced priming of the innate immune system subsides after the elimination of residual parasitemia

The impaired CD4^+ T cell response to iRBCs may not fully explain the partial loss of protective immunity to heterologous challenge observed after the elimination of chronic parasitemia. This is suggested by the fact that iRBC-stimulated CD4^+ T cells took a few days to secrete IFN-γ, but chronic and cured mice differed in their ability to promptly inhibit the invasion of new erythrocytes (Fig. 1H). Therefore, we postulated that the clearance of residual parasitemia reduces the extent of IFN-γ–induced priming, a potent mechanism by which the optimization of the innate immune system is achieved (17). Because the molecular pathways underlying IFN-γ–induced priming involve the activation of TLRs and other IFN-inducible genes (18), we compared the expression of ifnar and ifnr 2, ifnr 3, il6, and β-actin mRNAs in splenic DCs. This population is located in the red pulp of the spleen, where it is in direct contact with iRBCs, and also in the white pulp, contributing to both parasite clearance and T cell activation (27, 28). We observed a significant increase in the expression of these mRNAs in DCs from miceC60d, with the exception of ifnar mRNA, whereas there was no difference between
mice200d and the AMCs (Fig. 3A). Because the high expression of TLR mRNAs during acute PcAS malaria has been associated with augmented IFN-γ production following stimulation with TLR agonists (19), we then analyzed the spleen cell response to TLR4 and TLR9 agonists (LPS and CpG, respectively). In fact, spleen cells from mice60d produced increased levels of IFN-γ in response to LPS and CpG, whereas there was no significant response for mice200d and the AMCs (Fig. 3B).

MyD88-mediated signaling is required for CD4+ T cell responses and the IFN-γ–induced priming of splenic DCs in chronic mice

Our previous results correlate the persistence of strain-transcending immunity to blood-stage P. chabaudi malaria with the maintenance of increased CD4+ Te/TEM populations and TLR hyperresponsiveness. Thus, it is reasonable to speculate that TLR signaling contributes to generate and/or maintain CD4+ Te/TEM cells and consequently to increase the IFN-γ–induced priming of splenic DCs. In agreement with this hypothesis, mice lacking the TLR signaling MyD88 adaptor had higher chronic parasitemia than C57BL/6 mice (Fig. 4A). Despite the fact that similar numbers of CD4+ Tc cells were found in the spleens of C57BL/6 and MyD88KO mice60d, there was a reduction in the CD4+ TEM cell population in the latter group (Fig. 4B). Accordingly, the CD4+ T cell responses to parasites were impaired in MyD88KO mice60d, as shown by the diminished in vitro proliferation and IFN-γ production following iRBC or MSP-119 stimulation (Fig. 4C, 4D). The absence of the MyD88 adaptor also led to reduced mRNA expression of the IFN-inducible genes $stat3$, $irf7$, and $ifnar$ in splenic DCs (Fig. 4E), indicating that IFN-γ–induced priming also was impaired. Thus, intact TLR signaling machinery appears to be crucial for maintaining CD4+ T cell responses, which ultimately leads to IFN-γ–induced priming and optimizes the control of chronic parasitemia.

In vivo priming with IFN-γ restores the protective immunity to heterologous PcAJ parasites in cured mice

Our previous results suggest that the elimination of chronic parasitemia leads to a reduction in the IFN-γ–induced priming of the innate immune system and consequently to defective control of secondary infection, particularly with heterologous PcAJ parasites. To investigate whether in vivo priming with IFN-γ can restore the immune system activation status observed in the presence of residual parasitemia, starting on day 192 p.i., C57BL/6 mice were treated every 2 d with four suboptimal (priming) i.v. doses of rIFN-γ (Fig. 5A), as established previously (25). To confirm that this treatment primes the innate immune system, spleen cells from IFN-γ–primed mice200d produced significantly higher levels of IFN-γ in response to LPS and CpG compared with unprimed mice200d (Fig. 5B). Accordingly, IFN-γ–primed mice200d...
showed an improved capacity to control a secondary infection with *P. chabaudi* (Fig. 6A) and *P. yoelii* (Fig. 5C). Moreover, in mice challenged with *P. yoelii*, IFN-γ treatment allowed 100% survival and attenuated the clinical manifestations of the disease (i.e., a reduction in body temperature and blood hemoglobin concentration) (Fig. 5D–F). Other parameters, such as body weight and hepatic damage, were similar to the AMCs in both groups of challenged mice (data not shown). The IFN-γ–primed AMCs presented with an in-
crease in spontaneous and LPS-stimulated IFN-γ production (Fig. 6B), but these mice were not protected against primary infection with PcAS (Fig. 6C). These data indicate that IFN-γ–induced priming restores strain-transcending immunity in mice that have been previously exposed to parasites.

In vivo priming with IFN-γ restores the CD4+ T cell response to parasites in cured mice

The relevance of TLR signaling in the development of acquired immunity to P. chabaudi is suggested by our data showing that enhanced parasitemias in chronic MyD88KO mice are associated with low numbers of TEM cells and reduced iRBC-driven CD4+ T cell proliferation and IFN-γ production. Because our previous results show that low doses of IFN-γ restore both TLR hyper-responsiveness and strain-transcending immunity, we sought to investigate whether this treatment also improves CD4+ T cell responses in mice200d. Our data show a decrease in CD62L expression in splenic CD4+ TEM cells (CD3+CD4+CD44hiIL-7Rα+CD62Llo) and TEM cells (CD3+CD4+CD44hiIL-7Rα+CD62Llo) per spleen on day 60 p.i., as determined by flow cytometry. (C) Percentages of proliferating (CFSElow) CD4+ T cells from the spleens of mice200d stimulated with iRBCs (1 T cell/iRBCs) or MSP-119 (10 µg/ml), as determined by flow cytometry. (D) IFN-γ concentrations measured by ELISA in the supernatants from the cell cultures described in (C). (E) Fold increase over control (0 d) samples of stat3, irf7, and ifnar mRNAs, as evaluated by qPCR and analyzed by the 2−ΔΔ threshold cycle algorithm. Data show the mean ± SD (n = 4–6) of one representative experiment out of three. *p < 0.05, significant differences were analyzed between the C57BL/6 and MyD88KO mice (A–E). ND, Not detected.

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**FIGURE 4.** Parasitemias, CD4+ T cell subsets, and CD4+ T cell responses to iRBCs and MSP-119 and IFN-inducible gene expression in C57BL/6 and MyD88KO mice primarily infected with PcAS. C57BL/6 and MyD88KO mice infected with 1 × 10^6 iRBCs were analyzed in comparison with AMCs. (A) Parasitemia curves. (B) Numbers of CD4+ TCM (CD3+CD4+CD44hiIL-7Rα+CD62LhiCD27hi), TEM (CD3+CD4+CD44hiIL-7Rα+CD62Llo) and TE (CD3+CD4+CD44hiIL-7Rα+CD62Llo) cells per spleen were determined by flow cytometry. (C) Percentages of proliferating (CFSElow) CD4+ T cells from the spleens of mice200d stimulated with iRBCs (1 T cell/iRBCs) or MSP-119 (10 µg/ml), as determined by flow cytometry. (D) IFN-γ concentrations measured by ELISA in the supernatants from the cell cultures described in (C). (E) Fold increase over control (0 d) samples of stat3, irf7, and ifnar mRNAs, as evaluated by qPCR and analyzed by the 2−ΔΔ threshold cycle algorithm. Data show the mean ± SD (n = 4–6) of one representative experiment out of three. *p < 0.05, significant differences were analyzed between the C57BL/6 and MyD88KO mice (A–E). ND, Not detected.
immunity to *P. chabaudi* acts almost immediately after parasite challenge, inhibiting the first round of erythrocyte transmigration (15). Furthermore, our results show that the immunological effector mechanism that ensures strain-transcending immunity to *Pc* AS malaria (15) is not primed with IFN-γ and challenged with heterologous *Pc* AJ parasites. Starting on day 192 p.i., C57BL/6 mice were treated every 2 d with four i.v. doses (15 ng/kg; 10,000 U/mouse) of rIFN-γ and then challenged i.v. with $1 \times 10^8$ *Pc* AJ iRBCs. IFN-γ-primed mice were compared with unprimed mice and AMCs. (A) Experimental design. (B) IFN-γ concentrations measured by ELISA in spleen cell supernatants of unchallenged mice stimulated with LPS (10 μg/ml) or CpG ODN 1826 (10 μg/ml) for 72 h. (C) Parasitemia curves. (D) Survival curves. (E) Mouse temperatures. (F) Hemoglobin concentrations. Data show the mean ± SD (n = 4–6) of one representative experiment out of three. *p < 0.05, significant differences were analyzed between IFN-γ-primed and unprimed mice.

FIGURE 6. Parasitemias and CD4+ T cell responses in cured C57BL/6 mice and AMCs primed with IFN-γ and challenged with homologous *Pc* AS parasites. Starting on day 192 p.i., C57BL/6 mice were treated every 2 d with four i.v. doses (15 ng/kg; 10,000 U/mouse) of rIFN-γ. Noninfected AMCs were treated using the same protocol. IFN-γ-primed mice were compared with unprimed mice. (A) Parasitemia curves for mice challenged i.v. with $1 \times 10^8$ *Pc* AS iRBCs. (B) LPS-stimulated IFN-γ production in noninfected AMCs. (C) Parasitemia curves for AMCs challenged i.p. with $1 \times 10^6$ *Pc* AS iRBCs. Data show the mean ± SD (n = 4–6) of one representative experiment out of three. *p < 0.05, significant differences were analyzed between IFN-γ-primed and unprimed mice (A–C).
invasion prior to de novo T cell activation. Theoretically, CD4+ TM cells can activate the innate immune response via IFN-γ, or independently of this cytokine, as recently reported for influenza A virus infection (29). Our data corroborate the idea that persistence of the parasite maintains a pool of CD4+ TE/TEM cells that is able to produce low levels of IFN-γ (26). It also shows that the continuous priming of the innate immune system by IFN-γ, a process that is evidenced in chronic mice by the increased expression of IFN-inducible genes in splenic DCs and TLR hyperresponsiveness in splenocytes, allows the control of the heterologous challenge and avoids the development of the clinical manifestations of the disease and consequent animal death. These results may explain why clinical and sterile immunity against malaria are likely to be excluding events.

The IFN-γ–induced enhancement of TLR expression and function was previously shown during acute human and mouse malaria and was considered to be a booster for antimalarial responses when innate immunity plays a major role (19). This study reports a huge IFN-γ response to TLR agonists by splenocytes 1 wk after PcAS infection that subsides within 2 wk; the levels of IFN-γ produced in the fourth week p.i. are comparable with those described in this study in chronic mice. These findings recapitulate the notion that the spleen CD4+ T cell response to PcAS malaria develops in two phases concomitantly with acute and chronic parasitemias, in which the early phase is intense and short lasting, rapidly providing large amounts of proinflammatory cytokines, and the late phase is characterized by small peaks of IFN-γ production (30).

Our data indicate that the continuity of IFN-γ–induced priming of the innate immune system during the late infection is required to control heterologous parasites, against which humoral immunity is not optimized because of MSP-1 gene polymorphisms (9, 10). It is important to note that, in our study, in vivo priming with low doses of IFN-γ protects against parasite challenge in only previously infected mice but not in naive mice, suggesting that cooperation with other immunological effector mechanisms, such as Ab against conserved epitopes in parasite Ags (5), is required to ensure strain-transcending immunity to P. chabaudi malaria.
The immunological effector mechanisms enhanced by IFN-γ-induced priming are vast, as indicated by the diversity of the IFN-inducible genes that are highly expressed following macrophage treatment with low doses of IFN-γ (17, 18). Our results that correlate the expression levels of trl2, trl4, trl9, md2, and cd36 mRNAs in splenic DCs from chronic and cured mice with the ability to control heterologous challenge implicate these molecules in the maintenance of strain-transcending immunity to malaria. Considering the existence of several TLR ligands in Plasmodium, such as the GPI anchor, which is recognized by the TLR2/TLR1 complex with the contribution of TLR4 (31, 32), and parasite DNA, which is recognized by TLR9 (33), the long-lasting TLR hyperresponsiveness in chronic mice maintains the capacity of macrophages, DCs, and other TLR-bearing cells to recognize iRBCs and merozoites. In fact, it has been shown that DC activation not only requires direct cell-to-cell contact and internalization of iRBCs by DCs but also involves TLR4, TLR9, MyD88, and signaling via NF-κB (27). Moreover, TLR3 and TLR9 promote bacterial uptake by murine and human macrophages through the induction of a phagocytic gene program, which also is induced by TLR4 and TLR5 (34, 35). The class B scavenger receptor CD36, another molecule that shows enhanced mRNA expression in splenic DCs from chronic mice but not from cured mice, mediates the opsonin-independent phagocytosis of iRBCs by monocytes and DCs (36). The binding of iRBCs to these cells is thought to occur through the recognition of externalized phosphatidylserine or P. falciparum–encoded erythrocyte membrane protein 1 (37).

In contrast, the high expression of stat3 mRNA in splenic DCs from chronic mice indicates that IFN-γ-induced priming of the innate immune system is a fine-tuned process that is regulated during the late infection by feedback inhibitory loops, such as those mediated by IL-10, STAT3, and suppressor of cytokine signaling 1 (18). It has been known for quite a long time that exposure to TLR agonists leads to the development of tolerance through a blockade of the corresponding signal transduction pathways (38–40). Tolerance almost certainly occurs in patients from holoendemic areas of malaria that do not exhibit the signs and symptoms of the disease despite the presence of blood parasites (41, 42). Likewise, body temperature and weight are normalized after the control of acute parasitemia in P. falciparum-infected mice (5). In agreement with the concept that stimulatory and regulatory molecular pathways coexist during P. falciparum malaria, CD4+ TCM cells coexpressing IFN-γ and IL-10 have a major role in both protection and the control of the clinical manifestations of the disease (43).

Another important feature of IFN-γ–induced priming observed in this study is its effect on the CD4+ TM cell subsets of cured mice, leading to a shift from CD4+ TCM cells to CD4+ TEM cells and restoring the proliferative and IFN-γ responses to iRBCs and TLR agonists. The need for cognate Ag recognition for the maintenance and survival of CD4+ TM cells has been discussed recently for malaria (14) as well as for other diseases (44, 45). It is possible that the persistence of parasite Ags maintains parasite-specific CD4+ TCM cells in cured mice; IFN-γ–induced priming shifts this population to CD4+ TEM cells that promptly respond to iRBCs and TLR agonists. Alternatively, the IFN-γ–induced priming may rescue cross-reactive CD4+ TEM cells that are driven by Ags from other microbes, as proposed in a model for development of severe falciparum malaria in nonimmune adults (46). Despite that both mechanisms can contribute to restore the CD4+ TEM cell responses following the IFN-γ treatment, the CD4+ T cell proliferation and IFN-γ production in response to iRBCs are considerably improved in cured mice but not in AMCs. This finding supports the idea that parasite-specific CD4+ TM cells are main participants in the proliferative and IFN-γ responses that are restored by treating cured mice with IFN-γ. A further indication of the parasite-specific nature of these CD4+ T cells is that IFN-γ treatment also increases the proliferative response to MSP-1_19 in cured mice. The exact mechanism by which IFN-γ maintains CD4+ TEM cells in an effector–memory state is still not well understood. We hypothesize that the reestablishment of the CD4+ TM cell response to iRBCs is, at least in part, an indirect effect of the IFN-γ–induced TLR hyperresponsiveness of DCs. This possibility is supported by our data from MyD88KO mice indicating that TLR signaling also contributes to maintaining the CD4+ TEM cell responses during chronic infection, which ultimately leads to IFN-γ–induced priming and helps to control the reminiscent parasitemia. This increased chronic parasitemia in MyD88KO mice did not cause animal death in our study, as reported previously (26, 47).

Altogether, our study shows that sustained IFN-γ–induced priming of the innate immune system is required to maintain host surveillance and consequently strain-transcending immunity to blood-stage P. chabaudi malaria. It also suggests that the loss of protection in spontaneously cured mice results from the interruption of a positive cycle in which continuous exposure to parasites maintains the pool of CD4+ Tg/Tg* cells that are able to produce priming doses of IFN-γ and IFN-γ–induced priming of the innate immune system contributes to sustain the CD4+ Tg/Tg* cell responses to the parasites. These results may help to explain why the immunity against Plasmodium is rapidly lost when the parasites are eliminated from the hosts, providing a molecular basis for strain-transcending immunity in human malaria. The knowledge that IFN-γ–induced priming of the innate immune system contributes to maintain strain-transcending immunity may have important implications in developing and boosting immunization strategies against malaria.

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Disclosures
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