Gradual Decline in Malaria-Specific Memory T Cell Responses Leads to Failure to Maintain Long-Term Protective Immunity to *Plasmodium chabaudi* AS Despite Persistence of B Cell Memory and Circulating Antibody

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Gradual Decline in Malaria-Specific Memory T Cell Responses Leads to Failure to Maintain Long-Term Protective Immunity to Plasmodium chabaudi AS Despite Persistence of B Cell Memory and Circulating Antibody

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The mechanisms responsible for the generation and maintenance of immunological memory to Plasmodium are poorly understood and the reasons why protective immunity in humans is so difficult to achieve and rapidly lost remain a matter for debate. A possible explanation for the difficulty in building up an efficient immune response against this parasite is the massive T cell apoptosis resulting from exposure to high-dose parasite Ag. To determine the immunological mechanisms required for long-term protection against P. chabaudi malaria and the consequences of high and low acute phase parasite loads for acquisition of protective immunity, we performed a detailed analysis of T and B cell compartments over a period of 200 days following untreated and drug-treated infections in female C57BL/6 mice. By comparing several immunological parameters with the capacity to control a secondary parasite challenge, we concluded that loss of full protective immunity is not determined by acute phase parasite load nor by serum levels of specific IgG2a and IgG1 Abs, but appears to be a consequence of the progressive decline in memory T cell response to parasites, which occurs similarly in untreated and drug-treated mice with time after infection. Furthermore, by analyzing adoptive transfer experiments, we confirmed the major role of CD4+ T cells for guaranteeing long-term full protection against P. chabaudi malaria.

Malaria, the infectious disease caused by Plasmodium spp., still remains the most devastating illness in tropical countries, mainly in Sub-Saharan Africa. The disease is also important in Asia and Latin America, resulting in 350–500 million clinical cases and more than 1 million deaths every year, mostly affecting children under 5 years of age (1). The asexual blood stage of the parasite is responsible for the pathology and morbidity caused by the infection and, consequently, has been the focus of many studies concerning protective immunity. Among murine parasites adopted to analyze the immune response against malaria, the infection with Plasmodium chabaudi has been shown to be of great value, being considered a suitable model for the human disease caused by Plasmodium falciparum (2).

During the acute infection by blood-stage P. chabaudi, there is prominent polyclonal lymphocyte activation, with intense proliferation of T and B spleen cells (3–5). This initial immune response is characterized by production of type 1 effector molecules, such as IFN-γ, TNF-α, and IgG2a (3, 6–8). As the disease progresses, a large fraction of these activated lymphocytes is eliminated by apoptosis (9, 10), and the subsequent development of a specific T and B cell response results in parasite clearance and protection against reinfection. CD4 αβ T cells are thought to be essential for the establishment of protective immunity to P. chabaudi (11–14), where this may also hold true for humans, since these cells are critically required for helping B cells to produce specific IgG Abs. Despite the intense initial immune response to the parasite, it is believed that naturally acquired human immunity to malaria is in some way defective or short-lived, in that multiple infections are necessary to generate clinical protection, while chronic infection with low parasitemia is common in the population living in endemic areas, and immunity is lost (or becomes less effective) when a previously immune person moves away from an endemic area (15). However, there is only limited data on the mechanisms underlying the development of T and B cell memory to malaria and therefore the real reason why infected individuals have difficulty in generating and maintaining a protective response has yet to be fully elucidated (16, 17). One of the hypotheses created to explain this difficulty is related to the intense apoptosis observed during the acute infection (18–21).

There is thus great interest in evaluating the immunological mechanisms involved in the development of long-term protective immunity to malaria and the consequences of high and low acute phase parasite loads in this process, an issue that has also been considered of major relevance for establishing the immunization protocols in vaccine preclinical trials (22). In view of this, we have performed a detailed analysis of the T and B cell compartments over an extensive period of time after untreated and drug-treated P. chabaudi malaria. This study covered the chronic phase and the period after disease remission, which occurs between 2.5 and 3 mo after primary infection (23). Our data indicated that the progressive...
decline in the memory T cell response to parasites and not in the serum levels of specific IgG Abs, which occurs in untreated and drug-treated mice with time after infection, is responsible for the loss of full protective immunity to *P. chabaudi* malaria. The major role of CD4+ T cells for ensuring long-term full protection against *P. chabaudi* malaria was also confirmed by adoptive transfer experiments.

**Materials and Methods**

**Mice, parasites, infection, and chloroquine treatment**

Six- to 8-wk-old C57BL/6, CD28−/− (C57BL/6) (24) (originally from The Jackson Laboratory) female mice were bred under specific pathogen-free conditions at the Isogenic Mice Facility (Instituto de Ciências Biomédicas/Universidade de São Paulo, São Paulo, Brazil). *P. chabaudi* AS was maintained as previously described (14). To obtain untreated and drug-treated groups, mice were inoculated i.p. with 10⁶ parasitized RBC (PRBC). Drug-treated mice previously described (14). To obtain untreated and drug-treated groups, mice (10⁶) were incubated in labeling buffer (10 mM HEPES-NaOH (pH 7.4), Pharmingen). To analyze the percentage of apoptotic cells, splenocytes were stained with PE (BD Pharmingen) was performed. To detect surface IgG, cells were stained with PE- or Cy-streptavidin conjugate. Stained cells were analyzed by flow cytometry.

**Splenocyte purification**

For staining with purified mAbs to CD4 (H129.19 or GK1.5), CD8 (53-6.7), CD11b (M1/82), and CD11c (MEL-14) from BD Pharmingen. For staining with purified Abs, a further incubation with polyclonal anti-rat Ig conjugated to PE (BD Pharmingen) was performed. To detect surface IgG, cells were incubated with goat anti-mouse IgG-biotinylated Abs (Southern Biotechnology Associates) followed by FITC- or Cy-streptavidin conjugate (BD Pharmingen). To analyze the percentage of apoptotic cells, splenocytes (10⁶) were incubated in labeling buffer (10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, and 5 mM CaCl₂) with FITC-conjugated annexin V (BD Pharmingen) and Cy- or PE-labeled mAbs to CD4 and CD8. Stained cells were analyzed by flow cytometry using a FACS Calibur with CellQuest software (BD Biosciences).

**Parasite-specific ELISA**

Anti-*P. chabaudi* Abs were quantified by ELISA as previously described (25). In brief, 96-well flat-bottom microtiter plates (Costar) were coated overnight (4°C) with a total of 5 μM for 20 min at 37°C. Cells (10⁶) were then cultured in 96-well plates (Costar) with PRBC (3 × 10⁶) or medium alone, for 72 h at 37°C in a 5% CO₂ atmosphere. In purified T cell cultures, APCs were added at the proportion of 1/1. After incubation, cells were stained with PE- or Cy-labeled mAb to CD4 and analyzed by flow cytometry.

**CFSE proliferation assay**

The proliferative T cell response to parasite was measured as previously described (28). Briefly, 6 × 10⁶ 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. Cells (10⁶) were then cultured in 96-well plates (Costar) with PRBC (3 × 10⁶) or medium alone, for 72 h at 37°C in a 5% CO₂ atmosphere. In purified T cell cultures, APCs were added at the proportion of 1/1. After incubation, cells were stained with PE- or Cy-labeled mAb to CD4 and analyzed by flow cytometry.

**Statistical analysis**

Statistical analysis was generally performed with ANOVA and Tukey’s multiple comparison test. The GraphPad Prism 4 software was used and differences between the three groups were considered significant when the *p* < 0.05 (5%).

**Results**

Experimental design and parasitism in groups of mice subjected to untreated and drug-treated *P. chabaudi malaria*

To evaluate T and B cell memory in experimental conditions of low and high acute phase parasite loads, C57BL/6 mice were infected i.p. with 10⁶ PRBC where half of the animals were treated with two subsequent noncurative doses of chloroquine whenever parasitemia reached 1% of erythrocytes. The different parasitemia levels of untreated and drug-treated mice is shown in Fig. 1. At days 20, 60, 120, and 200 p.i., three to five mice from each group were challenged with 10⁵ PRBC and sacrificed 15 days later for experimental analysis (Fig. 1B). Noninfected mice challenged with 10⁵ PRBC were used as controls. Age-matched unchallenged mice from all groups were also evaluated.
T and B cell populations in the spleen at different times after untreated and drug-treated *P. chabaudi* malaria

To evaluate the effects of low and high parasitemias on T cell activation and apoptosis during the early infection, the surface expression of CD69 and externalized phosphatidylserine was determined at day 7 p.i. in CD4\(^+\) and CD8\(^+\) cells from the spleen of C57BL/6 mice. As shown in Fig. 2A, the expression of CD69 and annexin V binding sites was significantly reduced in CD4\(^+\) cells from drug-treated mice compared with untreated mice \((p < 0.01)\). Similar results were obtained for the CD8\(^+\) cell population with percentages of CD69\(^+\) and annexin V\(^+\) cells of 7.4 \pm 1.1 and 10.3 \pm 1.4 in control mice, 37.6 \pm 3.2 and 20.1 \pm 1.6 in untreated mice, and 13.1 \pm 1.6 and 11.5 \pm 1.9 in drug-treated mice, respectively (data not shown; \(p < 0.01\), for untreated mice compared with control or drug-treated mice). Corroborating the notion that T cells from mice suffering from acute malaria are strongly activated and undergo apoptosis, the numbers of CD4\(^+\) cells per spleen at day 20 p.i. were more than two times higher in drug-treated mice compared with untreated mice (Fig. 2B). At day 60 p.i., however, CD4\(^+\) cell numbers in untreated mice showed a substantial increase. At day 200 p.i., both untreated and drug-treated mice showed similar cell numbers.

**FIGURE 1.** Experimental design and parasitemia in mice with untreated and drug-treated *P. chabaudi* malaria. C57BL/6 mice were infected with 10\(^6\) PRBC. Half of the animals were treated with two subsequent noncurative doses of chloroquine whenever parasitemia reached 1% of erythrocytes. A, Parasitemia curves of untreated and drug-treated mice based on a representative experiment of three performed. Data represented as means \pm SD \((n = 4 – 6)\). Arrows show the days of chloroquine treatment. B, Experimental protocol. At days 20, 60, 120, and 200 p.i., untreated and drug-treated mice, as well as noninfected (control) mice, were reinfected with 10\(^8\) PRBC and sacrificed 15 days later for experimental analysis. Age-matched unchallenged mice were also sacrificed on the same days.

**FIGURE 2.** T and B cell populations in the spleen at different times after untreated and drug-treated *P. chabaudi* malaria. A, Histograms of the expression of CD69 and annexin V binding sites in CD4\(^+\) cells from untreated and drug-treated mice at day 7 of infection. Noninfected mice were used as controls. Numbers in histograms represent means \pm SD \((n = 3 – 5)\) of the percentages of positive cells in gated CD4\(^+\) cells. B, Numbers of CD4\(^+\) and CD45R(B220)\(^+\) cells per spleen in untreated and drug-treated mice at days 20, 60, 120, and 200 p.i., comparing with noninfected controls. Data represent means \pm SD \((n = 3 – 5)\). C, Percentages of CD4\(^+\)CD62L\(^{low}\) and CD45R\(^+\)IgG\(^+\) cells in untreated and drug-treated mice at days 20, 60, 120, and 200 p.i., comparing with noninfected controls. Each point corresponds to a single mouse \((n = 3 – 5)\). Horizontal lines represent mean values of the percentages of CD62L\(^{low}\) and IgG\(^+\) cells in gated CD4\(^+\) and CD45R\(^+\) cells, respectively. *, \(p < 0.05\); **, \(p < 0.01\); and ***, \(p < 0.001\). Data are representative of two or three separate experiments.
increase and differences between the two infected groups disappeared. The CD8\(^+\) cell population showed a comparable behavior, with cell numbers per spleen at days 20 and 60 p.i. of 10.0 ± 0.1 and 15.3 ± 2.2 × 10\(^6\) in control mice, 13.0 ± 2.1 and 34.4 ± 4.5 × 10\(^6\) in untreated mice, and 33.0 ± 4.6 and 32.4 ± 0.1 × 10\(^6\) in drug-treated mice, respectively (data not shown; \(p < 0.01\), for 20-day infected drug-treated mice compared with control or 20-day infected untreated mice). In contrast to T cells, a similar increase in B (CD45R\(^B\)\(^-\)) cell numbers per spleen was observed for both groups of mice at days 20 and 60 of infection. At later times, the numbers of CD4\(^+\) and CD45R\(^B\)\(^-\) cells, as well as those of CD8\(^+\) cells (data not shown), approached control values and differences within groups were not statistically significant.

Analysis of CD4\(^+\)CD62L\(^\text{low}\) and CD45R\(^B\)\(^-\)IgG\(^+\) cells, which represent experienced/memory cell subsets, reinforced the idea that differences in spleen lymphocyte populations of untreated and drug-treated mice were limited to the early T cell response (Fig. 2C). The elevated incidence of CD4\(^+\)CD62L\(^\text{low}\) cells in untreated mice at day 20 p.i. might be a result of their higher level of activation. These data also confirmed our previous findings suggesting that major alterations in T and B cell populations occurred at days 20 and 60 p.i., inasmuch as CD4\(^+\)CD62L\(^\text{low}\) and CD45R\(^B\)\(^-\)IgG\(^+\) cell percentages were similar to controls at days 120 and 200 of infection.

**Protective immunity against a secondary parasite challenge at different times after untreated and drug-treated P. chabaudi malaria**

To evaluate the protective immunity in untreated and drug-treated mice, half of the animals were challenged at days 20, 60, 120, and 200 p.i. where parasitemias were determined in subsequent days. At day 200 p.i., a few mice from each group was also challenged with parasites from a late isolate, which was obtained during the ascending parasitemia of mice inoculated with blood samples of 25-day infected drug-treated mice. For groups challenged from days 20 to 120 p.i., untreated and drug-treated mice were very efficient in controlling parasite growth, and parasitemias remained under 0.3% of erythrocytes (Fig. 3). However, at day 200 p.i., there was a significant decrease in the protective immunity of untreated and drug-treated mice with parasitemias ranging from 1 to 5% of erythrocytes between days 1 and 5 after secondary infection. A similar phenomenon was observed when mice were challenged at day 200 p.i. with the late parasite isolate. However, in this case, parasitemias were slightly higher compared with those of mice reinfected with conventional parasites, a phenomenon that could be explained by the switch to a novel phenotype according to parasite Ags expressed on the erythrocyte membrane (29). In summary, our results have shown that the capacity to control parasite growth declines with a similar kinetic in untreated and drug-treated mice.

**Parasite recognition by specific IgG2a and IgG1 Abs obtained at different times after untreated and drug-treated P. chabaudi malaria**

To determine to what extent the loss of full protective immunity observed at day 200 p.i. in untreated and drug-treated mice is determined by a decline in humoral immunity, we compared the levels of specific IgG2a and IgG1 Abs in the serum at days 20, 60, 120, and 200 after infection. Fig. 4A shows that specific IgG2a serum levels increased until day 120 p.i. in untreated and drug-treated groups, but were significantly higher at all time points in mice subjected to the lower parasitemias. Moreover, whereas for drug-treated mice, the specific IgG2a titers remained high at day 200 p.i. (\(p > 0.05\), compared with day 120 p.i.), a substantial decrease was observed in untreated mice (\(p < 0.05\), compared with day 120 p.i.). The IgG1 serum levels were very low during the whole period for both groups of infected mice (see data from day 0 in Fig. 6B).

To confirm that untreated mice had a lower capacity to sustain the humoral response, a flow cytometry assay was developed to compare the ability of sera from mice at day 200 p.i. to recognize intraerythrocytic parasite Ags. In this assay, schizont-infected erythrocytes were fixed, permeabilized, stained with YOYO-1 (a dye for nucleic acid), and then incubated with serum samples (diluted from 1/100 to 1/1600) followed by an anti-mouse IgG2a developing Ab. As shown in dot blots from Fig. 4B, at a 1/100 dilution, sera from 200 day-infected untreated and drug-treated mice recognized fixed YOYO-1\(^+\) PRBC with a similar intensity. However, after serum titration, higher reactivity was observed in serum samples from drug-treated mice, being the mean fluorescence intensity of IgG2a Abs bound to YOYO-1\(^+\) fixed PRBC comparable when serum samples from untreated and drug-treated mice were diluted to 1/100 and 1/1600, respectively. Furthermore, the possibility that serum IgG2a from drug-treated mice was able to recognize a larger range of parasite Ags expressed on the erythrocyte surface was supported by data showing that the percentages of nonpermeabilized PRBC labeled by these Abs (diluted 1/100) were two times higher compared with those of untreated mice (\(p < 0.01\); Fig. 4C).
FIGURE 4. Parasite recognition by specific IgG2a Abs obtained at different times after untreated and drug-treated P. chabaudi malaria. A, Specific IgG2a Abs in sera from untreated and drug-treated mice at days 20, 60, 120, and 200 after infection. Noninfected mice were used as controls. Each point corresponds to a single mouse (n = 3–7). Horizontal lines represent mean of 1/1iter obtained from OD values at 650 nm. B, Recognition of intraerythrocytic parasite Ags by specific IgG2a Abs from sera of untreated and drug-treated mice at day 200 p.i., comparing with noninfected controls. Dot plots show infected (YOYO-1−) erythrocytes from fixed blood being recognized by IgG2a Abs from representative serum of each group (diluted 1/100). NRBC was used as control. Histograms show the expression of bound IgG2a in gated YOYO-1− erythrocytes from fixed PRBC (PRBC) incubated with diluted serum samples (from 1/100 to 1/1600) from representative mice of each group. The means ± SD (n = 3–5) of the mean florescence intensity for bound IgG2a of diluted sera are shown in the lower part of this graph. C, Recognition of parasite Ags on the erythrocyte membrane by specific IgG2a Abs from sera (diluted 1/100) of untreated and drug-treated mice at day 200 p.i., comparing with sera from noninfected controls. Dot plots show nonpermeabilized PRBC being recognized by IgG2a Abs. NRBC was used as control. A: *, p < 0.05; **, p < 0.01; and ***, p < 0.001 (comparison between indicated groups). B and C: *, p < 0.05; **, p < 0.01; and ***, p < 0.001 (untreated groups compared with drug-treated groups). Comparisons of untreated and drug-treated groups with control groups are not shown for the sake of simplicity (p < 0.01, for all time points). Data are representative of three separate experiments.

**T cell response to infected erythrocytes at different times after untreated and drug-treated P. chabaudi malaria**

As the loss of full protective immunity at day 200 p.i. could not be explained by the specific IgG serum levels, we then compared the T cell response to PRBC in untreated and drug-treated mice at days 20, 60, 120, and 200 after infection. Unexpectedly, for both infected groups, the proliferative response of CD4+ cells, which was considerably high at day 20 p.i., decreased progressively with time such that at day 200 p.i. it was indistinguishable from that of control mice (Fig. 5A). This phenomenon was also observed for CD8+ cells with percentages of CFSElow cells at days 20 and 200 p.i. of 6.6 ± 1.2 and 10.3 ± 0.6 in control mice, 22.8 ± 2.3 and 12.9 ± 1.3 in untreated mice, and 26.9 ± 1.3 and 14.0 ± 1.1 in drug-treated mice, respectively (data not shown; p < 0.01, for 20-day infected mice compared with control or 200-day infected mice). Differences between untreated and drug-treated mice were only statistically significant for CD4+ cells analyzed at day 60 p.i., with higher values being recorded for mice subjected to a controlled infection. However, it should be considered that at day 20 p.i. the T cell numbers per spleen were more than 2-fold higher in drug-treated mice than in untreated mice, which would lead to significantly higher numbers of PRBC-respecting T cells per spleen compared with untreated mice. Regarding the cytokine profile of PRBC-stimulated spleen cells, TNF-α and IFN-γ reached maximum levels in both infected groups at days 20 and 120 p.i., respectively, decreasing thereafter (Fig. 5B). IL-5 was only detected at day 20 p.i., with the same occurring for IL-4 which showed concentrations of around 30 pg/ml for untreated and drug-treated mice (data not shown). Statistically significant differences between the infected groups were restricted to TNF-α production at day 60 p.i., with a higher amount being detected in cell supernatants from drug-treated mice.

One possible explanation for the reduction in PRBC-stimulated T cell proliferation with time after infection could be a gradual decrease in the APC functions. To clarify this issue, T cells from day 20 p.i. were cultured along with APCs from day 200 p.i., and vice versa, in the presence of PRBC. As shown in Fig. 5C, for both untreated and drug-treated mice, CD4+ cells from day 20 p.i. proliferated vigorously when stimulated by APCs from day 20 or 200 p.i. (p < 0.001, compared with cultures of T20 cells without APCs), while CD4+ cells from day 200 p.i. showed a negligible response independently of the APCs (p < 0.001, compared with T20 plus APC200/200 cell cultures). CD8+ cells showed a similar behavior with percentages of CFSElow cells ranging from 87.9 to 95.5 in T20 plus APC200/200 cell cultures and from 5.1 to 9.2 in T200 plus APC200/200 cell cultures (data not shown; p < 0.001, for T20 plus APC200/200 cell cultures compared with T200 plus APC200/200 cell cultures). In any case, the proliferative T cell response in the presence of NRBC was insignificant (data not shown).

**Immune response to parasite challenge at different times after untreated and drug-treated P. chabaudi malaria**

To understand how untreated and drug-treated mice manage to control parasite challenge at different times after the primary infection, we also analyzed several immunological parameters 15
days after reinfection. The percentage of CD5RB^{high} cells within CD4^{+}CD62L^{low} cells is an interesting parameter because it reflects the reactivation of experienced/memory CD4^{+} cells. Data in Fig. 6A show that the CD4^{+}CD62L^{low}CD45RB^{high} cell percentage and spleen weight had parallel kinetics. Thus, huge spleen and CD4^{+}CD62L^{low}CD45RB^{high} cell responses were observed 15 days after the secondary infection in 20-day infected drug-treated mice, whereas these parameters were already increased before challenge in 20-day infected untreated mice and no significant change occurred thereafter. At day 60 p.i., however, parasite challenge induced just slight increases in the spleen weights and CD4^{+}CD62L^{low}CD45RB^{high} cell percentages of both infected groups. Moreover, at later times p.i., no response to challenge was observed in untreated and drug-treated mice.

A different scenario was observed for the B cell response, in which increases in CD45R^{+}IgG^{+} cell percentages occurred across all groups of challenged mice, with differences between untreated and drug-treated mice being detected only in 20-day infected mice. Accordingly, the levels of specific IgG2a Abs were significantly increased in sera from untreated and drug-treated mice from days 5 to 15 after reinfection, with higher amounts being attained in animals challenged at days 120 and 200 p.i. (Fig. 6B). In most time points after secondary challenge, higher IgG2a levels were detected in drug-treated mice compared with untreated mice. The specific IgG1 response was only detected in mice reinfeeted at days 120 and 200 p.i., with differences between untreated and drug-treated groups occurring in animals challenged at day 200 p.i. where untreated mice showed a lower response. The fact that Ab responses show better boosting if the secondary challenge occurs more than 60 days after the primary infection has important implications for the dosing schedule of vaccines designed to induce humoral immunity.

**Requirement of memory CD4^{+} T cells for specific Ab production and protection against P. chabaudi challenge**

The preceding results have shown that the protective immunity and T cell response to parasites decline with time, but the ability to produce specific IgG2a and IgG1 Abs is maintained or even improved at later periods after primary infection. To determine whether memory CD4^{+} T cells are required for humoral response and protection against parasite challenge, adoptive transfer experiments were performed using CD28^{-/-} mice as recipients. The rational behind using this experimental model was based on previous results showing that CD28^{-/-} mice infected with *P. chabaudi* control the acute phase parasitemia, but fail to generate memory T cells and to produce parasite-specific IgG Abs (28). Due to the incapacity to develop acquired immunity, CD28^{-/-} mice have higher chronic phase parasitemia, which remains oscillatory for >3 mo. The fact that the main deficiency in these mice is associated with memory cells make this model suitable to determine the mechanisms involved in long-term protective immunity to *P. chabaudi*. In these experiments, CD28^{-/-} mice were injected...
i.p. with total or CD4− spleen cells containing 20 × 10⁶ B cells obtained from untreated C57BL/6 mice at day 200 of infection. Adoptively transferred CD28−/− mice were infected i.p. with 10⁶ PRBC on the same day of transference.

Concerning specific serum Abs, the transfer of total and CD4− cells from untreated mice to CD28−/− mice resulted in IgG2a and IgG1 production, which was detected for the first time at day 14 p.i. (Fig. 7A). At this point, the two groups of adoptively transferred mice had similar amounts of IgG2a and IgG1 in the sera, but at later periods after transfer, significantly lower levels were observed in mice receiving CD4− cells. Furthermore, when these mice were cured with chloroquine and then challenged with parasites, a higher IgG2a response occurred in mice reconstituted with total cells. Low IgG levels were also detected at days 28 and 35 p.i. in CD28−/− mice that received total cells from noninfected (control) mice, whereas nontransferred mice and mice receiving CD4− (control) cells were negative for both IgG isotypes throughout the whole experiment.

For the primary infection, a delay of 1 day in the first parasitemia peak was observed in CD28−/− mice reconstituted with total and CD4− cells from noninfected (control) mice, whereas a 2-day delay occurred in animals reconstituted with one of the two cell populations from untreated mice (Fig. 7B). In addition, CD28−/− mice receiving total cells from untreated mice showed a significant lower first parasitemia peak compared with those mice that received CD4− (untreated) cells (p < 0.01). Although all of the animals were able to control the first parasitemia peak, none of them succeeded in eliminating the parasites up to day 35 p.i. when they were cured by...
chloroquine treatment. Following reinfection, recrudescent parasitemia peaks were again observed in nontransferred CD28−/− mice, while the animals reconstituted with total cells from untreated and control mice showed full control of parasite growth. This finding is of particular interest because these mice had very different serum levels of specific IgG2a and IgG1 Abs. In contrast, CD28−/− mice receiving CD4+ cells showed a detectable parasitemia peak that was significantly lower when the cells came from untreated mice (p < 0.05).

Taken together, these results indicate that CD4+ cells obtained at day 200 p.i. helped to maintain the serum levels of specific IgG2a and IgG1 Abs and to generate a secondary B cell response against the parasite. Moreover, they suggest that these CD4+ cells contributed toward the protective immunity against *P. chabaudi* through a mechanism that is, at least in part, independent of the humoral response.

Comparison of B cells from 200-day infected untreated and drug-treated mice according to the capacity to produce specific IgG2a and IgG1 Abs and to mediate protection against *P. chabaudi* challenge

Given that untreated and drug-treated mice had different serum levels of specific Abs, subsequent experiments were performed to compare CD45R+B spleen cells from 200-day infected untreated and drug-treated C57BL/6 mice in relation to the ability of transferring the capacity to CD28−/− mice of producing specific IgG2a and IgG1 Abs and of controlling the parasite challenge. To confirm the involvement of CD4+ T cells in the humoral response of untreated and drug-treated mice, CD28−/− mice were also reconstituted with total cells from each group. Fig. 8A shows that IgG2a was initially detected at days 14 and 21 p.i. in CD28−/− mice reconstituted with total cells from untreated C57BL/6 mice at day 200 after infection. CD28−/− mice receiving cells from noninfected C57BL/6 mice were used as controls. From days 35–45 p.i., mice were treated with chloroquine (open arrows) and then reinected (closed arrow). Each point corresponds to a single mouse (n = 3).

Horizontal lines represent mean of 1/titer obtained from OD values at 650 nm. A, Parasitemias in the same groups of mice shown in A. Data represent means ± SD (n = 3). A: *, p < 0.05; **, p < 0.01; and ***, p < 0.001 (comparison between indicated groups). B, Statistics are not shown for the sake of simplicity. Data are representative of two separate experiments.
reconstituted either with total or CD45R<sup>+</sup> cells from control mice in comparison to nontransferred CD28<sup>−/−</sup> mice.

As shown in Fig. 8B, analysis of the first parasitemia peak showed a comparable scenario for CD28<sup>−/−</sup> mice reconstituted with cells from untreated and drug-treated mice, with a 1-day delay being evident in mice receiving CD45R<sup>+</sup> cells, a phenomenon also observed for animals receiving control cells, and a 2-day delay along with a significant lower peak in mice receiving total cells (p < 0.05, compared with all other transferred groups). In addition, after chloroquine treatment and reinfection, the performance was again similar for CD28<sup>−/−</sup> mice reconstituted with cells from untreated and drug-treated mice, in that mice receiving CD45R<sup>+</sup> cells showed a small but evident parasitemia peak and infected erythrocytes were rarely detected in mice receiving total cells. Concerning control groups, it was remarkable that, similar to the experiment shown in Fig. 7, two prominent parasitemia peaks were observed after parasite challenge in nontransferred CD28<sup>−/−</sup> mice, yet only one prominent peak was detected in mice reconstituted with CD45R<sup>+</sup> cells from noninfected mice while very low parasitemias were found in animals reconstituted with total cells from noninfected mice.

In conjunction, these results have shown that B cells from 200-day infected drug-treated mice have an improved capacity to produce specific IgG2a and IgG1 Abs compared with B cells from 200-day infected untreated mice, but this better response does not result in higher protection. These data also corroborate the notion...
that T cells from day 200 p.i. contribute toward protecting mice against *P. chabaudi* challenge, with a similar efficiency in untreated and drug-treated mice, and through an effector mechanism not exclusively associated with their ability to help B cells to produce specific Abs.

**Discussion**

Previous studies have shown that limited exposure to *P. chabaudi* malaria induces prominent cell-mediated immunity associated with T cell protection from apoptosis. In the present study, T and B cell compartments were analyzed over a period of 200 days in C57BL/6 mice subjected to untreated or drug-treated *P. chabaudi* infection. According to this study, T cell apoptosis controls the increase in spleen CD4+ and CD8+ cell populations upon the acute infection but does not impair the generation of memory T cells with specificity for the parasite. We have also demonstrated that B cells from mice subjected to controlled *P. chabaudi* malaria have a long-lasting improvement in their capacity to produce specific IgG2a and IgG1 Abs. Even so, the loss of full protective immunity to *P. chabaudi* malaria is not influenced by the acute phase parasite load nor by the serum levels of specific IgG Abs, but appears to be a consequence of the progressive decline in the memory T cell response to parasites that occurs similarly in untreated and drug-treated mice with time after infection. Furthermore, in the adoptive transfer experiments, we confirmed the major role of CD4+ T cells for guaranteeing long-term full protection against *P. chabaudi* malaria.

The present study has confirmed the notion that high-dose parasite Ag during the acute *P. chabaudi* infection induces T cell apoptosis associated with a delay in the expansion of splenic T cell populations (30). However, according to our study, this process does not impair the generation of memory CD4+ and CD8+ T cells with specificity for the parasite. This conclusion is based on data showing that, at day 20 p.i., the T cell proliferation and cytokine secretion in response to infected erythrocytes are intense and comparable in both untreated and drug-treated mice. Thus, even if a fraction of specific T cells is eliminated by apoptosis during the acute infection, as previously described in mice receiving T cell lines recognizing *Plasmodium berghei*, *Plasmodium yoelli*, and *P. chabaudi* parasites (19–21), a proportion of the cells remain alive and may contribute toward generating the pool of memory CD4+ and CD8+ T cells. Moreover, considering the in vitro and in vivo T cell responses to the parasite, the memory T cell pool attains a similar size in untreated and drug-treated mice at later periods after the acute disease. The cytokine profile is also indistinguishable in the two infected groups with production of comparable amounts of IFN-γ, TNF-α, IL-5, and IL-4 at most analyzed time points. Furthermore, the adoptive transfer of spleen cells from untreated and drug-treated groups to infected CD28−/− mice results in a small but significant reduction in the first parasitemia peak, which reaches similar values and is mediated by CD4+ T cells regardless of their origin.

Another relevant contribution of this work is to demonstrate that the specific B cell response is compromised over an extended period of time in mice subjected to high-dose parasite Ags at early *P. chabaudi* infection. This conclusion is supported by the analysis of serum IgG2a and IgG1 recognizing parasite extracts and infected erythrocytes and also by experiments with adoptively transferred B cells. The data showing higher percentages of nonpermeabilized PRBC being recognized by the sera of 200-day infected drug-treated mice, in relation to Abs generated in untreated mice, indicate that IgG2a produced under these conditions has specificity for a greater range of parasite Ags expressed on the membrane of infected erythrocytes, which could include molecules created through antigenic variation (29). In contrast, the concept that a lower acute phase parasite load determines a general improvement in the humoral response is corroborated by data at day 200 p.i. showing 16 times more serum IgG2a recognizing permeabilized PRBC in drug-treated mice. Furthermore, the adoptive transfer experiments suggest that mice subjected to a controlled *P. chabaudi* infection contain significantly higher numbers of specific B cells per spleen on day 200 p.i. than untreated mice. Although these results seem to conflict with those reported by Pombo et al. (31) showing the absence of specific Abs after a controlled *P. falciparum* infection, it is worth noting that, in this case, human volunteers were treated with a curative drug schedule immediately after parasite inoculation, leading to ultralow doses of Ag that may have been insufficient to induce the humoral response. In agreement with our results, an improvement of the specific-IgG2a secondary response to *P. chabaudi* has been previously observed in CB6F1 mice treated with chloroquine when parasitemias reached 1–10% of erythrocytes (32).

The persistent lower levels of specific IgG2a Abs found in the serum of untreated mice do not appear to be a consequence of an indiscriminate B cell apoptosis since, in contrast to T cells, the acute phase parasite load does not interfere with the increase in B cell numbers per spleen or in the percentages of experienced (sIgM− sIgD−) B cells. However, a delayed increase in the splenic CD4+ T cell population, as observed in 20-day infected untreated mice, could compromise B cell clonal expansion and affinity maturation. Beside this possibility, the higher amounts of immune complexes generated during the untreated infection could inhibit acute phase Ab production with long-term consequences for humoral response, a mechanism known to be mediated by FcγRIIb receptors expressed in B cells (33). Alternatively, the competition for appropriate survival niches among plasma cells originated during polyclonal lymphocyte activation and through the affinity maturation process, a phenomenon that might be more intense in untreated mice, could damp down the production of specific IgG Abs (34). Another feasible explanation is that the untreated infection reinforces the activity of regulatory T cells, a cell population that increases during acute *P. chabaudi* malaria (C. A. Zago and M. R. D’Império Lima, unpublished observation) and which is known to control the humoral response (35).

Another important contribution of this study is the evidence that the T cell response to infected erythrocytes progressively declines over time after *P. chabaudi* infection. This has been observed in vitro by analyzing PRBC-stimulated CD4+ and CD8+ T cell proliferation and in vivo by evaluating the expression of CD45RB in CD4+CD62Llow T cells stimulated by secondary parasite challenge. Moreover, the parallel behavior of spleen weight and CD4+CD62LlowCD45RBhi/hth T cells raises the hypothesis that the spleen response is governed by T cells or, alternatively, that both processes are regulated by common mechanisms, such as those coordinated by APCs. The possibility that dendritic cells from *P. chabaudi*-infected mice had gradually adopted a regulatory pattern can be inferred from data showing that *P. yoelli*-infected PRBC induce a shift in the cytokine profile of LPS-stimulated dendritic cells from IL-12 to IL-10 (36). However, our experiments with T:APC cocultures demonstrate that T cell proliferative unresponsiveness at day 200 p.i. is not due to down-regulation of APC functions, leaving as putative explanations for this phenomenon a progressive reduction in the frequency of specific CD4+ and CD8+ T cells or a gradual increase in their regulation. Although the massive T cell apoptosis during the untreated acute infection does not impair the generation of memory T cells, it is possible that continuous Ag-induced CD4+ and CD8+ T cell death in consequence of chronic parasitemia results in the gradual decrease of the
T cell response observed in untreated and drug-treated mice. It should be noted, however, that CD4⁺ T cells from day 200 p.i. still retain the capacity to help B cells to produce specific IgG2a and IgG1 Abs, as revealed by the adoptive transfer experiments.

Finally, our results indicate that the progressive decline in the memory T cell response to parasites with time after infection is the main cause of the loss of full protective immunity to \textit{P. chabaudi} malaria. This conclusion is supported by the following considerations. First, the efficiency in controlling parasite growth is reduced in untreated and drug-treated mice at day 200 p.i. when the T cell proliferative response and production of inflammatory cytokines reach minimal levels. Second, T cells are always required to achieve complete control of parasite challenge in adoptive transfer experiments. Third, in many situations, serum levels of specific IgG Abs do not correlate with the capacity to restrain the parasite growth. This is clearly demonstrated in CD28⁺ CD4⁺ mice receiving total spleen cells from noninfected C57BL/6 mice. Although these cells provide full protection against a secondary infection, the specific-IgG serum levels are significantly lower than those found in CD28⁻ CD4⁺ mice receiving CD4⁺ or CD45R⁺ spleen cells from untreated or drug-treated mice in which protection to challenge is incomplete. Another example of this lack of correlation is the observation that untreated and drug-treated mice differ in terms of specific IgG2a and IgG1 Abs, including those recognizing the PRBC surface, but have a very similar behavior concerning the ability to eliminate the challenge with an early or a late parasite isolate. A discrepancy between protection and humoral response was also reported in the study by Pombo et al. (31) in which human volunteers were capable of achieving a sterile immunity against \textit{P. falciparum} malaria apparently by a cell-mediated immune response in the absence of detectable levels of specific Abs. In contrast, mice with very recent or current low-level chronic infections with \textit{P. chabaudi} maintain a better protective immune response to challenge than drug-cured animals, even though the parasite persistence does not substantially affect either longevity or avidity of the Ab responses (23).

Taking these results together, we conclude that the loss of full protective immunity to \textit{P. chabaudi} malaria is not determined by the acute phase parasite load nor by the serum levels of specific IgG Abs, but is largely due to the incapacity of infected mice to maintain the pool of memory T cells with potential to mediate an effector response against the parasite. The requirement of the Th1-immune response to achieve protection during the chronic phase of \textit{P. chabaudi} infection has been previously described (26). Although these authors attributed the inefficiency of mice lacking IL-12 to control parasitemia to the predominance of IgG1 over IgG2a, this study analyzed only the first month of infection when the immune system is activated due to parasite persistence. In contrast, our data suggest that CD4⁺ T cells are essential to ensure full protection against a secondary challenge and their lack compromises parasite control even in the presence of specific IgG2a and IgG1 Abs. It is also important to note that the decrease of protective immunity in untreated and drug-treated mice was only observed at day 200 p.i., when the parasite had already been eliminated as suggested by studies in which blood samples were transferred to immune-deficient mice (23). Thus, it is likely that several months after disease remission, when the activation of the immune system had already returned to its basal level, the production of proinflammatory cytokines would be necessary to ensure full protective immunity, which might be achieved even in the presence of low levels of specific Abs. If this were the case, the goal of maintaining a large pool of effector-memory T cells in \textit{Plasmodium}-immunized individuals should be pursued to allow sterile immunity and blockade of parasite transmission.

We believe that this analysis will help further understanding as to why the protective immunity to human malaria is so rapidly lost, knowledge that can help improve our strategies for vaccine development.

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Disclosures

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References


