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Immunology

Deletion of *Plasmodium berghei*-specific CD4$^+$ T cells adoptively transferred into recipient mice after challenge with homologous parasite

(tolerance/malaria immunity)

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ABSTRACT **The immune response to malaria parasites includes T cell responses that reduce parasites by effector T cell responses and by providing help for antibody responses. Some parasites are more sensitive to antibody and others are more sensitive to cell-mediated immunity. We demonstrate that cultured CD4$^+$ T cells that produce interferon γ and interleukin 2, but not interleukin 4, in response to stimulation with the rodent parasite *Plasmodium berghei* can reduce but not eliminate parasites *in vivo* after adoptive transfer. Although cells can persist *in vivo* for up to 9 months in uninfected mice, infection results in elimination of up to 99% of specific T cells in different tissues, as judged by tracking T cells labeled with the fluorescent dye 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester. T cells specific for ovalbumin are unaffected. *In vivo* activation and division of transferred T cells *per se* are not responsible for deletion because T cells positive for 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester divide up to six times within 7 days in uninfected mice and are not deleted. Understanding the factors responsible for parasite-mediated specific deletion of T cells would enhance our knowledge of parasite immunity.**

Immunity to malaria is slow to develop, typically taking more than 5 years for individuals in endemic areas (1). Immunity to blood stages of malaria is dependent upon antibody (2) and T cells (3). The role of CD4$^+$ T cells has been demonstrated by selective depletion *in vivo* (4–6), by adoptive transfer of T cells to immunodeficient mice (4, 7–10), and by the ability of human T cells to inhibit parasite growth *in vitro* (11). CD4$^+$ cells of both T helper cell types Th1 and Th2 have been shown to be effective (9). However, not all rodent malaria parasites are susceptible to T cell-mediated immunity (12) and the reasons for this are unknown. Factors affecting the activation, function, and lifespan of parasite-specific T cells would be expected to have a significant impact on parasite survival.

Many factors have been nominated including the poor immunogenicity of antigens (13), antigenic polymorphism (14–16), immunosuppression as a result of malaria infection (17), and the need for continuous malaria exposure to maintain immunological memory (18). Antigen-driven deletion or anergy of immunological responses is a major regulatory strategy to control potentially harmful responses (19) and may be used by infectious organisms to their advantage, as shown by the exhaustive deletion of lymphocytic choriomeningitis virus-specific CD8$^+$ T cells (20). Although the ability of malaria parasites to persist as a result of deletion of parasite-specific CD4$^+$ T cells has not been investigated, infection is associated with specific nonresponsiveness of peripheral blood mononuclear cells (21, 22). To test whether malaria infection can delete parasite-specific T cells, we studied a parasite known for its lack of sensitivity to T cells and used an adoptive transfer system in which tagged T cells can be followed *in vivo* after infection.

MATERIALS AND METHODS

Mice. Normal and *nu/nu* (nude) female BALB/c mice, 6–8 weeks old, were used.

Parasites and Antigens. *Plasmodium berghei* ANKA was used in all experiments. It was maintained by passage of infected erythrocytes from mouse to mouse. Parasitemia was monitored by examining Diff-Quik-stained blood smears under an oil immersion lens. For preparing antigen for *in vitro* stimulation and immunity, blood was collected from infected mice, washed twice with MEM, resuspended to 1×10^8 cells per ml, and stored at −20°C until used. *P. berghei*-parasitized red blood cell (pRBC) lysate was prepared by freeze-thawing 6×10^8 pRBCs per ml three times followed by sonication.

Generation of T Cell Lines. BALB/c mice were immunized by footpad injection of 100 μl (50 μl per foot) of pRBC lysate emulsified in complete Freund's Adjuvant (Difco). Eight days later, popliteal and inguinal lymph nodes were removed and teased apart in medium. Cells were washed and cultured at 10^6 cells per ml in culture medium (MEM supplemented with 10% heat-inactivated fetal calf serum and 50 μM 2-mercaptoethanol) in the presence of 1×10^6 pRBCs per ml in 24-well tissue culture plates (Costar). After 4 days of stimulation, viable cells were separated by Ficoll/Paque (Pharmacia) centrifugation and rested at 5×10^5 cells per ml in medium containing 10^6 irradiated normal spleen cells per ml (2,500 rad; 1 rad = 0.01 Gy). After 10–14 days, cells were stimulated with *P. berghei*-pRBCs in the presence of fresh irradiated normal spleen cells. Multiple stimulation and resting cycles were undertaken.

An ovalbumin (Ova)-specific line was generated by immunization of mice with 100 μg of Ova (Sigma) in complete Freund's adjuvant and maintained *in vitro* as above but with Ova at 200 μg/ml in the stimulation phase.

Adoptive Transfer Experiments. BALB/c *nu/nu* mice were administered T cells intravenously. After 4 h, the mice were challenged i.p. with 10^6 live *P. berghei*-pRBCs. Controls included mice that received T cells alone, mice that did not receive T cells but that were challenged with parasites, and a group of naive mice.

Abbreviations: Ova, ovalbumin; CFSE, 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester; IFN-γ, interferon γ; IL, interleukin; pRBC, parasitized red blood cell; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; TUNEL, terminal deoxynucleotydyltransferase (TdT)-mediated dUTP-biotin nick-end labeling.

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Lymphoproliferation Assay. The proliferation of spleen, lung, liver, and lymph node cells at the concentration of 2×10^6 cells per ml or *P. berghei*- or Ova-specific T cells at the concentration of 5×10^5 cells per ml in the presence of 1×10^6 irradiated normal spleen cells per ml was tested in a volume of 200 μl of MEM supplemented with 2% heat-inactivated normal mouse serum and 50 μM 2-mercaptoethanol in 96-well flat bottom plates (Costar). Cells were stimulated with *P. berghei*-pRBCs (10^4–10^6 cells per ml), normal RBCs (10^4–10^6 cells per ml), Ova (25–400 μg/ml), purified protein derivative of *Mycobacterium bovis* (20 μg/ml), or Con A (10 μg/ml). Cultures were incubated for 3 days at 37°C in 5% CO_2/95% air, and then pulse-labeled with 0.25 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; 1 Ci = 37 GBq; DuPont) for an additional 18 h. Cells were harvested onto fiberglass filters, and radioactivity was measured with a β scintillation counter (Betaplate, Pharmacia).

Bioassays for Interferon γ (IFN-γ), Interleukin (IL) 2, and IL-4. Cell-free supernatants were collected after 24–48 h of incubation. IFN-γ activity was determined by measurement of inhibition of WEHI-279 cell proliferation (23). IL-2 and IL-4 activities were assayed by using the growth-dependent CTLL-2 and CT.4S cell lines, respectively (24, 25). Briefly, supernatants at different dilutions were plated in triplicate in 96-well flat bottom plates (Costar) in the presence of washed WEHI-279 (5×10^3 cells), CTLL-2 (8×10^3 cells), or CT.4S (8×10^3 cells). The cultures were incubated for 72 h for WEHI-279 cultures and for 24 h for CTLL-2 and CT.4S. All the cultures were pulse-labeled for an additional 18 h before harvesting onto filters and measuring radioactivity. Data presented are mostly for 1:2 dilutions of supernatants, although data from the other dilutions gave the same conclusions. Titers of IFN-γ are presented. The specificity of the IFN-γ response was demonstrated by adding an anti-murine IFN-γ mAb R46A2 (26).

Flow Cytometric Analysis. To characterize the surface phenotype of the cell lines, cells were incubated with rat mAbs specific for CD3 (KT3), CD4 (GK1.5), and CD8 (TIB105) or hamster mAbs specific for T cell receptors $\alpha\beta$ (H57–597) and $\gamma\delta$ (GL3–1A) at 4°C for 30 min. After two washes with 0.1% BSA/0.1% NaN_3/PBS, cells were incubated with a 1:100 dilution of fluorescein isothiocyanate (FITC)-labeled $F(ab')_2$ fragments of goat anti-rat IgG or FITC-labeled $F(ab')_2$ fragments of goat anti-hamster IgG (Caltag, South San Francisco, CA) for 30 min at 4°C. To identify the presence of the cell line in the spleen, a single-cell suspension was stained with anti-CD3 mAb and FITC-labeled $F(ab')_2$ fragments of goat anti-rat IgG and then with phycoerythrin-labeled rat mAb specific for CD4 (Caltag). The percentage of positive cells was measured by a fluorescence-activated cell sorter (FACS). Propidium iodide solution was used to exclude dead cells.

***In Vivo* Study of 5-(and -6)-Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE)-Labeled Cells.** Staining of *P. berghei*- or Ova-specific T cell lines with CFSE (Molecular Probes) was performed as described by Lyons and Parish (27). Briefly, T cells were resuspended at 1×10^7 cells per ml in PBS. A 5 mM stock solution of CFSE in dimethyl sulfoxide was added to a final concentration of 10 μM and cells were incubated for 30 min at 37°C. Cells were washed twice with ice-cold 10% fetal calf serum/MEM and once with MEM and resuspended in MEM. Cells were administered to nude mice, and some of these mice were challenged 4 h later with 10^6 live parasites. Mice were sacrificed 7 days after the challenge and peripheral blood, spleen, inguinal lymph nodes, liver, lung, and bone marrow from two femurs were collected. Single-cell suspensions were prepared by Ficoll/Paque centrifugation for peripheral blood lymphocytes and by pressing through stainless meshes for cells from spleen, liver, and lung. Cells were washed and resuspended in 0.1% BSA/0.1% NaN_3/PBS and analyzed by using a FACScan.

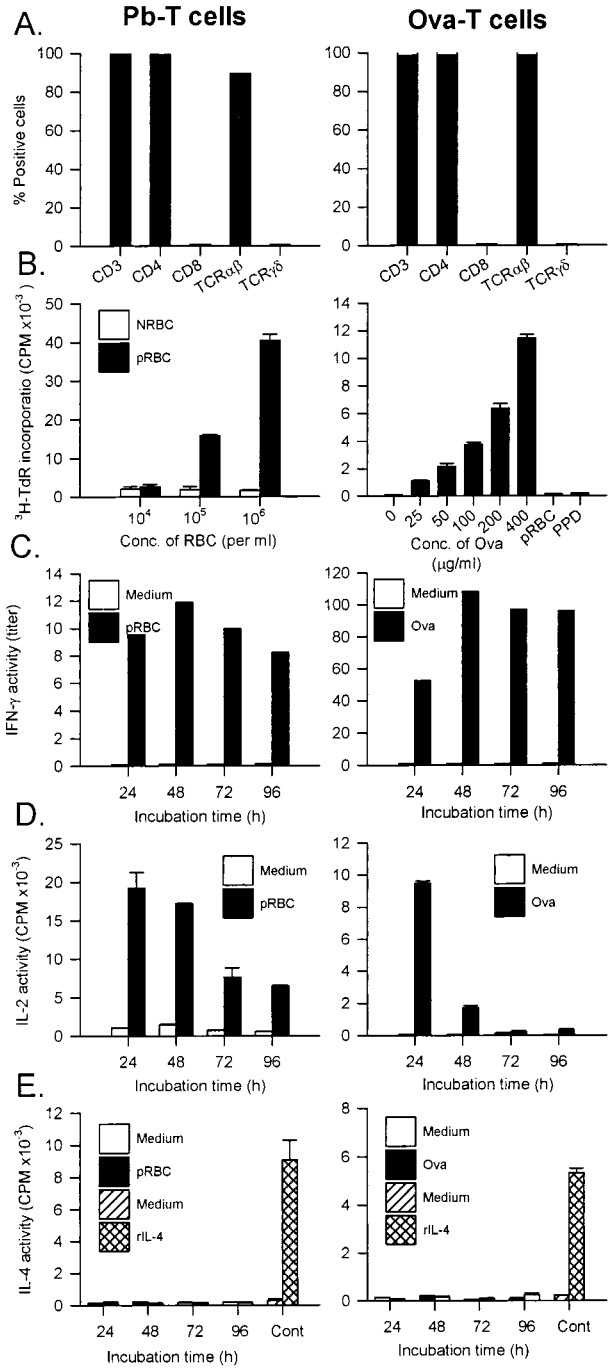

FIG. 1. Characteristics of *P. berghei*- or Ova-specific T cell lines, showing cell surface phenotype (*A*), proliferative response (*B*), IFN-γ (*C*), IL-2 (*D*), and IL-4 production (*E*). Data show mean ± 1 SEM for cell proliferation and IL-2 and IL-4 production. The activity of IFN-γ is shown as a titer, as determined by calculating the dilution that gave 50% inhibition of WEHI-279 cell proliferation. The concentration of antigen used to stimulate cytokine production was 10^6 pRBCs per ml or Ova at 200 μg/ml. The concentration of IL-4 used in the positive control was 400 units/ml, and the sensitivity of the cells in this assay was 3.125 units/ml.

Apoptosis Assay. A rapid flow cytometric method for simultaneous two-color staining of membrane determinants combined with terminal deoxynucleotydyltransferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) *in situ* was used (28). Briefly, 1×10^6 cells were incubated with phycoerythrin-conjugated anti-CD4 mAb on ice for 30 min. Cells were washed twice with FACS buffer and fixed with 1%

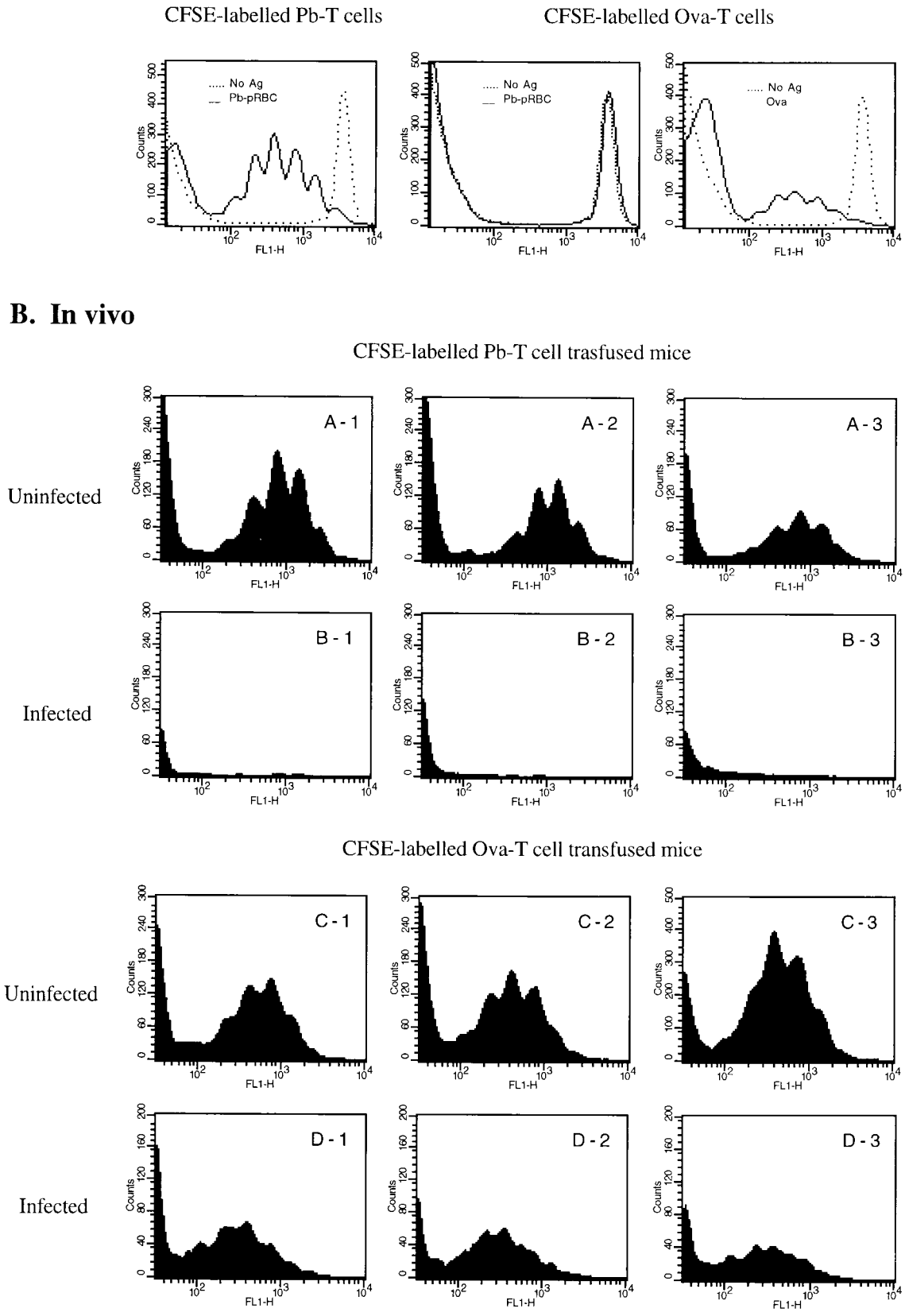

FIG. 2. FACS profiles of CFSE-labeled *P. berghei*- or Ova-specific T cells after *in vitro* culture and *in vivo* adoptive transfer. The cells after rest for 7 days were labeled with CFSE and cultured with or without *P. berghei*-pRBCs or Ova *in vitro* (*A*) or were adoptively transferred into BALB/c nude mice and some of the mice were then infected with 1×10^6 live *P. berghei*-pRBCs (*B*). Cells were harvested after 5 days of *in vitro* culture or from the spleen of mice 7 days after challenge, and FACS analysis was done. (*B*) Each panel represents an individual mouse (A-1, A-2, A-3, B-1, B-2, B-3, etc.).

paraformaldehyde/PBS at room temperature for 30 min. Cells were washed twice with FACS buffer and incubated with 100 μl of 0.1% Triton X-100/0.1% sodium citrate on ice for 2 min. Cells were washed with FACS buffer and then with TdT buffer and incubated in 50 μl of TdT buffer containing 4 μM biotinylated-dUTP (Boerhinger Mannheim), 5 units of TdT (Boerhinger Mannheim), and 60 nM dATP (Boerhinger Mannheim) at 37°C for 1 h in a moist chamber. The reaction was stopped by adding 5 μl of 0.2 M EDTA. Cells were washed twice with FACS buffer and incubated with 50 μl of a 1:100 dilution of FITC-avidin (Sigma) on ice for 30 min. Cells were washed twice and analyzed by using a FACScan.

RESULTS

T cell lines were generated after immunization of BALB/c mice with *P. berghei*-pRBC lysate and Ova. The lines expressed $\alpha\beta$ T cell receptors and CD4 and, in response to specific stimulation, proliferated and secreted both IFN-γ and IL-2 without detectable IL-4 (Fig. 1). After stimulation, cells underwent one to six divisions over a 5-day period, as shown by sequential halving of the fluorescent signal from cells labeled with CFSE (27) (Fig. 2*A*). After *in vitro* "resting," unlabeled cells were washed then administered to naive BALB/c nude mice via i.v. injection (5×10^6 cells per mouse). The mice that received the *P. berghei*-specific T cells experienced lower parasite loads after infection than control mice that either did not receive T cells or that received Ova-specific T cells (10% parasitemia vs. 50% parasitemia at day 8 postinfection). The mice nevertheless died with anemia and weight loss at day 10 after infection. Increasing the dose of transferred *P. berghei*-specific T cells (up to 2×10^7 cells per mouse) did not save the mice (data not shown). The transfer of T cells into mice that were not challenged was not associated with any adverse effects.

Cells for adoptive transfer were then tagged with CFSE. After transfer into nude mice and in the absence of infection, both *P. berghei*-specific and Ova-specific T cells recovered from spleen had divided between one and six times over a 7-day period (Fig. 2*B*), in contrast to the lack of division of unstimulated cells *in vitro* (Fig. 2*A*). Division is demonstrated by the sequential halving of the intensity of the fluorescent signal in the cells. After infection, although spleen sizes increased, no *P. berghei*-specific T cells were detectable (Fig. 2*B*). There was a slight reduction in the percentage of Ova-specific T cells (Fig. 2*B*) compared with uninfected mice. The absence of parasite-specific cells in the spleen after infection was mirrored in the lack of a proliferative and cytokine response *in vitro* of spleen cells harvested from infected mice that had adoptively received parasite-specific T cells. Complete unresponsiveness to parasite or Con A was evident by day 7 after infection with respect to IL-2 production and lymphoproliferation and by day 14 after infection with respect to parasite-induced IFN-γ production (Fig. 3). We can exclude the possibility that all *P. berghei*-specific T cells had undergone extensive division *in vivo* to the extent that the CFSE label was reduced beyond detection because FACS analysis based on CD4 did not detect any cells above background levels for infected mice compared with 1.5×10^6 cells in the spleens of uninfected nude mice that had received 10^7 cells. Spleen cells from uninfected mice that received adoptively transferred cells could respond for at least 9 months after transfer (data not shown). Spleen cells from naive nude mice did not respond to either parasite or Con A stimulation (Fig. 3). Although spleen size increased during infection, the parasite-specific proliferative response, when corrected for spleen size (Δcpm per spleen), was reduced by >99% at days 10 and 14 after infection whereas the proliferative response of spleen cells from mice given Ova-specific T cells was not significantly decreased over the 14 days after infection (Fig. 4).

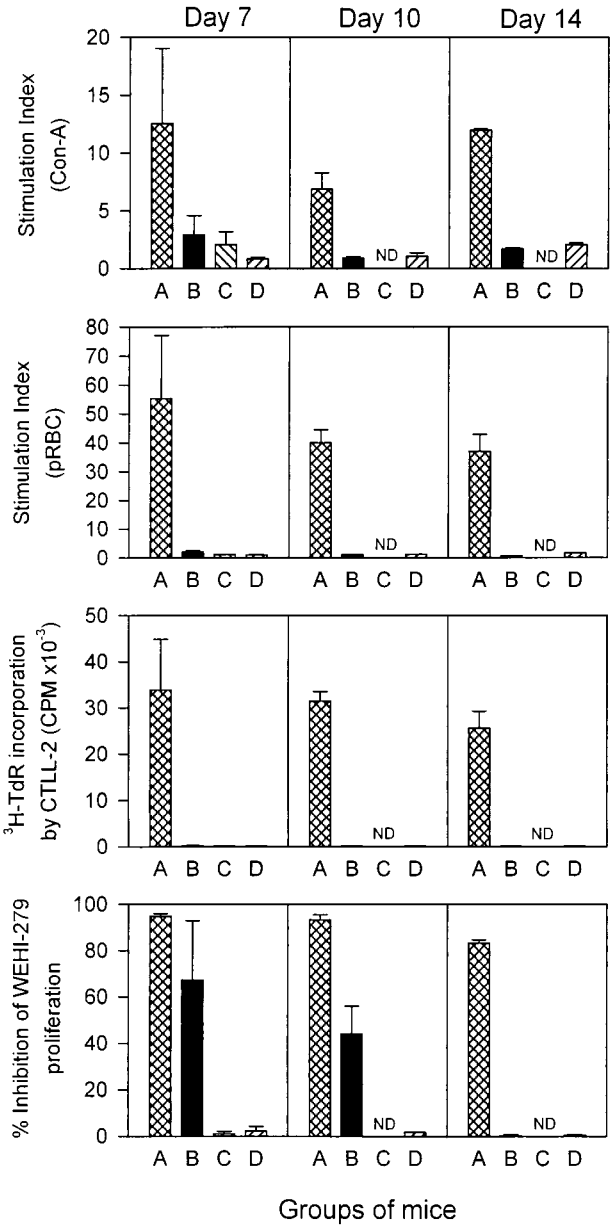

FIG. 3. *In vitro* proliferation and production of cytokines by spleen cells from BALB/c nude mice; some of the mice had received *P. berghei*-specific T cells and/or were challenged with live pRBCs at day 0. Spleen cells were taken at days 7, 10, and 14 from mice that had received the following treatments. Bars: A, received 10^7 specific T cells but were not infected; B, received 10^7 T cells and were infected with 10^6 pRBCs; C, were infected but not given specific T cells; D, were neither infected nor given specific T cells. Cells were investigated for lymphoproliferation in response to Con A or pRBC lysate and for cytokine production in response to pRBC lysate. The data show the mean $\pm$ 1 SEM.

Disappearance of cells from the spleen was not reflected in an increased number of specific cells in other tissues. CFSE-labeled cells were monitored in blood, lung, liver, bone marrow, and lymph node, as well as spleen (Fig. 5). Infection resulted in a decrease in *P. berghei*-specific cells in all locations, except liver and bone marrow, which harbored small numbers of cells not affected by infection. Ova-specific T cells (in all tissues examined) were not affected by parasite infection. This figure also shows that, when corrected for spleen size, there was no diminution of Ova-specific T cells in the spleens of infected mice compared with a 95% reduction in the absolute number of parasite-specific T cells in the spleens of infected

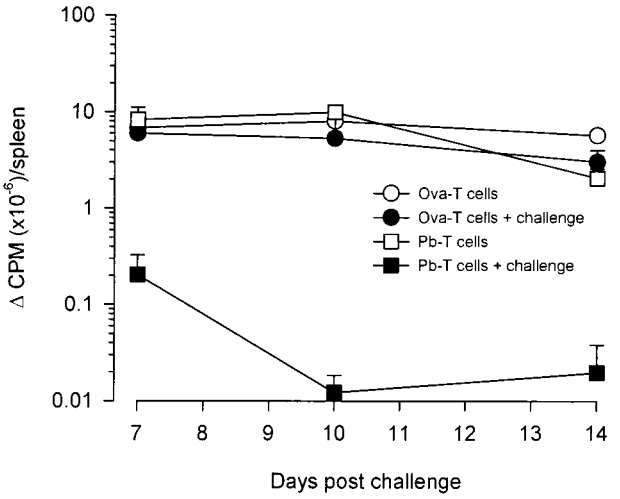

FIG. 4. *In vitro* proliferation by spleen cells from BALB/c nude mice that had received Ova-specific T cells (○, ●) or *P. berghei*-specific T cells (□, ■) and then were (●, ■) or were not (○, □) infected with 10^6 live *P. berghei*-pRBCs at day 0. *In vitro* stimulation is with homologous antigen (*P. berghei* pRBCs or Ova). The data show the mean ± 1 SEM.

mice. There was no proliferative response to parasite antigen from cells recovered from the other tissue sites (data not shown). Immunohistochemical examination of brain tissue did not reveal the presence of T cells.

Infection of normal mice with *P. berghei* resulted in a peak of apoptotic $CD4^+$ T cells in the spleen, 4 days after infection as defined by the TUNEL assay (Fig. 6).

Parasite infection did not affect the function of antigen-presenting cells. Nonadherent spleen cells from infected recipient mice when mixed with adherent cells from uninfected recipients failed to produce IL-2 or IFN-γ in response to parasite *in vitro*, whereas nonadherent cells from uninfected recipients when mixed with adherent cells from infected (or uninfected) recipients responded normally (data not shown).

DISCUSSION

Data from many systems demonstrate that T cells can control parasite growth through antibody-independent and -dependent mechanisms. We demonstrate that $CD4^+$ T cells specific for the lethal parasite *P. berghei* can partially control parasite growth *in vivo* but cannot eliminate parasites, unlike similar T cell populations that can completely protect mice against the nonlethal parasites *P. chabaudi* (6, 7, 9) and *P. yoelii* (29). Although mice that received specific T cells died at a lower parasitemia than mice that did not receive T cells, suggesting T cell-mediated immunopathology, it is likely that if the T cells were able to control parasite growth much more effectively, then the mice would survive. Approximately 10^7 T cells specific for the nonlethal parasite *P. yoelii* 17X when adoptively transferred, for example, can keep peak parasitemia less than 3% before parasites are completely cleared (29). Our data, however, show that such a scenario is not possible with *P. berghei* because parasite-specific T cells are eliminated *in vivo* within 7 days of infection. Deletion is likely to involve apoptosis because significantly greater numbers of apoptotic $CD4^+$ T cells were observed in infected as opposed to uninfected normal mice (Fig. 6).

After transfer, both parasite-specific and Ova-specific T cells undergo up to six rounds of division within 7 days, in the absence of infection. Malaria parasites are known to express antigens that cross-react immunologically with other organisms (30, 31), providing a possible explanation for the *in vivo* response of the parasite-specific T cells. It is more difficult,

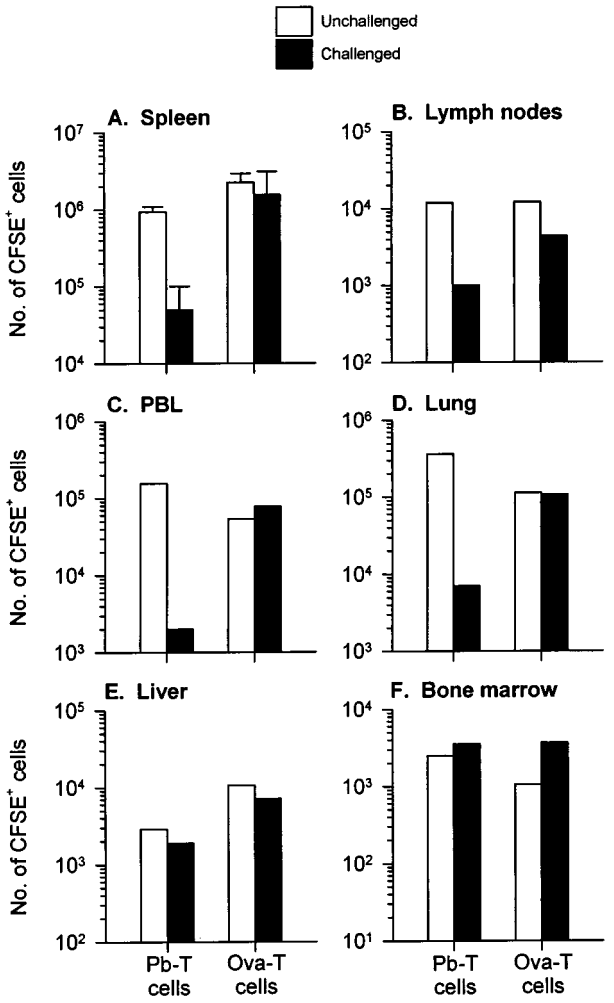

FIG. 5. Recovery of cells from various organs of either infected or noninfected BALB/c nude mice that had received 10^7 CFSE-labeled *P. berghei*- or Ova-specific T cells 7 days earlier. Three mice in each group were studied; cells from individual spleens and pooled cells from other tissues were assessed. Data are the number of $CFSE^+$ cells per spleen (*A*), per two inguinal lymph nodes (*B*), per ml of blood (*C*), per the whole lung (*D*), per the whole liver (*E*), and per two femurs (*F*).

however, to explain the *in vivo* response of the Ova-specific T cells. However, homeostatic regulation of $CD4^+$ T cell pool size *in vivo* is well described (32), if not well understood.

The *in vivo* response (in the absence of antigen) is very similar to the *in vitro* response (in the presence of antigen). It appears unlikely therefore that exhaustive cell activation *per se* is responsible for the deletion after infection, because division *in vivo*, driven by nonparasite factors and comparable to the amount of division seen *in vitro* in response to parasite antigen at optimal concentration, does not result in deletion. It is possible, however, that antigen is more efficiently presented *in vivo* and as a result apoptotic signals are engaged.

It is of interest to compare our data with the results of a study of the nonlethal rodent malaria *P. chabaudi chabaudi* (33). After infection of normal mice with this parasite, parasite-reactive T cells could not be detected in peripheral blood but were detected in the spleen. We have also found that highly responsive T cells specific for *P. chabaudi adami* can be recovered from the spleens of T cell-transfused nude mice after infection and recovery (ref. 34 and F. Amante and M.F.G., unpublished results). T cells are known to play a critical role in resistance to *P. chabaudi* (7, 12). It is likely that a major reason for the survival of mice infected with *P. chabaudi* is that specific T cells are not deleted nor anergized.

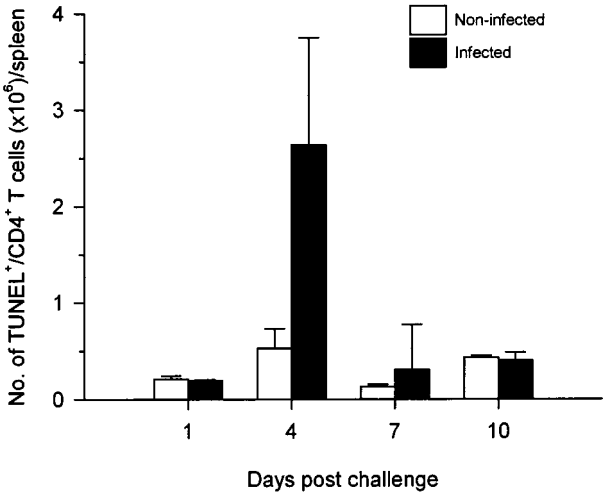

FIG. 6. Apoptosis in *P. berghei*-infected BALB/c mice. BALB/c mice were (■) or were not (□) infected with 10^6 *P. berghei*-pRBCs at day 0. Their spleens were then collected 1, 4, 7, and 10 days after infection and analyzed by using TUNEL technique. Data are the mean ± 1 SEM.

In contrast, we have also found complete malaria parasite-specific T cell anergy from the spleens of infected nude mice that received T cells specific for the lethal murine malaria *P. yoelii* YM, a parasite known not to be controlled well by cell-mediated immunity (data not shown). Spleen cells from unifected transfused mice responded strongly. The situation in human *P. falciparum* infections is less clear; however, acute infection is associated with peripheral blood T cell nonresponsiveness (21, 22) and apoptosis of human mononuclear cells in response to malaria infection has been observed (35). This could contribute to the virulence of falciparum malaria and to the slow rate of acquisition of immunity.

Different apoptotic pathways have been identified (36), but the molecular mechanism of deletion/apoptosis in malaria infected mice is not defined. Understanding the molecular regulation of this response and the responsible parasite factors should aid our understanding of immunity to malaria.

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