

The Mechanism and Significance of Deletion of Parasite-specific CD4⁺ T Cells in Malaria Infection

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Abstract

It is thought that both helper and effector functions of CD4⁺ T cells contribute to protective immunity to blood stage malaria infection. However, malaria infection does not induce long-term immunity and its mechanisms are not defined. In this study, we show that protective parasite-specific CD4⁺ T cells were depleted after infection with both lethal and nonlethal species of rodent *Plasmodium*. It is further shown that the depletion is confined to parasite-specific T cells because (a) ovalbumin (OVA)-specific CD4⁺ T cells are not depleted after either malaria infection or direct OVA antigen challenge, and (b) the depletion of parasite-specific T cells during infection does not kill bystander OVA-specific T cells. A significant consequence of the depletion of malaria parasite-specific CD4⁺ T cells is impaired immunity, demonstrated in mice that were less able to control parasitemia after depletion of transferred parasite-specific T cells. Using tumor necrosis factor (TNF)-RI knockout- and Fas-deficient mice, we demonstrate that the depletion of parasite-specific CD4⁺ T cells is not via TNF or Fas pathways. However, in vivo administration of anti-interferon (IFN)- γ antibody blocks depletion, suggesting that IFN- γ is involved in the process. Taken together, these data suggest that long-term immunity to malaria infection may be affected by an IFN- γ -mediated depletion of parasite-specific CD4⁺ T cells during infection. This study provides further insight into the nature of immunity to malaria and may have a significant impact on approaches taken to develop a malaria vaccine.

Key words: *Plasmodium* • apoptosis • cell-mediated immunity • immune evasion • IFN- γ

Introduction

CD4⁺ T cells play a critical role in protective immunity to blood stage malaria parasite infection. This has been demonstrated by the selective depletion of CD4⁺ T cells in vivo (1–3) and by the adoptive transfer of CD4⁺ T cells to immunocompromised mice (3–7). Recent studies of two of the leading malaria vaccine candidate antigens, the 19-kD carboxyl-terminal segment of merozoite surface protein 1 (MSP1₁₉)* and apical membrane antigen 1, have further con-

solidated previous findings. It has been shown that immunity to *Plasmodium yoelii* YM after immunization with MSP1₁₉, relies upon MSP1₁₉-specific T cells that help B cells continue synthesis of antibody after challenge (8, 9). Effector CD4⁺ T cells, on the other hand, acting independently of antibody, have been shown to contribute to protective immunity to *Plasmodium chabaudi* infection after apical membrane antigen 1 immunization (10). As in mice, CD4⁺ T cells are thought to have a major role in regulating the human immune response to *Plasmodium falciparum* infection. Human CD4⁺ T cells have been shown to inhibit parasite growth in vitro (11). T cell-dependent production of antiparasite antibodies occurs after T cell stimulation with antigen in T cell–B cell cooperative systems in vitro (12, 13). In vitro stimulation of CD4⁺ T cells with malaria antigens may also result in proliferation and/or IFN secretion, neither of which is correlated with levels of serum an-

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*Abbreviations used in this paper: B cell KO mice, C57BL/6J- μ m chain knockout mice; CFSE, 5- and 6-carboxyfluorescein diacetate succinimidyl ester; EMEM, Eagle's minimal essential medium; MSP1₁₉, merozoite surface protein 1; pRBC, parasitized red blood cell; TUNEL, TdT-mediated dUTP nick-end labeling.

tibody against corresponding antigens (14–16). In vitro stimulation may also induce IL-4 secretion that in individual donors is correlated with neither lymphocyte proliferation nor IFN- γ release, but rather with concentrations of the relevant serum antibody (17, 18). These results suggest that the human response is controlled by distinct CD4⁺ T cell subsets that correspond in part to the TH1- and TH2-like cells found in *P. chabaudi*-infected mice (19). These data suggest that both helper and effector functions of CD4⁺ T cells contribute to immunity to malaria infection in both mice and people.

Despite the critical roles of CD4⁺ T cells in controlling malaria infection, immunity to malaria is slow to develop, typically taking more than 5 yr for individuals in endemic areas (20). The reasons for this are not well defined. It could result from poor immunogenicity of malaria antigens (21), antigenic variation and polymorphism (22–24), or from host immunosuppression resulting from malaria infection (25). It is well known that antigen-driven deletion or anergy of immunological responses is a major regulatory strategy to control potentially harmful responses (26). However, these mechanisms might be used by infectious organisms to enable their survival (27, 28). Indeed, previous work from our group has shown that *P. berghei*-specific CD4⁺ T cells that had been adoptively transferred into nude mice were depleted after challenge with homologous parasites (29). As such, further investigation of the mechanism and significance of the depletion of parasite-specific T cells during infection would provide insight into the nature of immunity to malaria infection and would have a significant impact on future malaria vaccine development strategies.

In this study, we show that protective parasite-specific CD4⁺ T cells are depleted by both lethal and nonlethal species of rodent malaria parasites. We further show that depletion only affects parasite-specific CD4⁺ T cells and that IFN- γ is involved in the process. We also demonstrate that protective immunity to malaria infection is significantly affected by such depletion of parasite-specific CD4⁺ T cells.

Materials and Methods

Mice and Parasites

Female inbred BALB/c (H-2^d), BALB/c nu/nu, and C57BL/6J (Thy1.2, H-2^b) mice were purchased from Animal Resources Centre. C57BL/6J- μ m chain knockout (B cell KO) mice (30) were obtained from The Centenary Institute for Cancer Medicine and Cell Biology (Sydney, Australia) and bred in our animal facility. C57BL/6J Thy1.1 mice, C3H (H-2^k) mice, and C3H Lpr (Fas-deficient) mice were obtained from The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). TNF-RI knockout (H-2^b) mice were supplied by Australian National University (Canberra, Australia). All mice ranged in age from 6 to 8 wk when experiments were initiated.

Plasmodium berghei ANKA, *Plasmodium vinckei*, *P. chabaudi* adami, *P. yoelii* 17XNL, and *P. yoelii* YM Parasites

The parasites were maintained by passage through donor mice. Parasitemia was monitored by examining DiffQuick-stained (Lab

Aids Pty. Ltd.) blood smears under an oil immersion lens. To prepare antigen for in vitro stimulation and immunization, blood was collected from infected mice, washed twice with Eagle's minimal essential medium (EMEM; Trace Biosciences), resuspended to 10⁸ cells/ml, and stored at -20°C until used. Parasitized red blood cell (pRBC) lysate was prepared by freeze-thawing pRBCs (6 × 10⁸ per ml) three times followed by sonication.

Generation of Parasite- and OVA-specific CD4⁺ T Cell Lines

T cell lines were generated from the lymph node cells of mice immunized with crude parasite antigen or OVA protein (Sigma-Aldrich). The mice were briefly immunized in the hind footpads with 3 × 10⁷ pRBCs or 100 μ g of OVA protein emulsified in CFA (H37Ra; Difco Laboratories). 7–9 d after immunization, draining inguinal and popliteal lymph node cells were removed and suspended at 2 × 10⁶ cells/ml in culture medium consisting of EMEM supplemented with 10% FCS, 100 U/ml benzyl penicillin (CSL Ltd.), and 5 × 10⁻⁵ M of 2-mercaptoethanol (Sigma-Aldrich). Cells were dispensed in 24-well cluster plates (Corning Glass Works) containing 10⁶ pRBC/ml or 100 μ g/ml of OVA protein. After 4 d, viable cells were isolated on Ficoll-Paque (Amersham Pharmacia Biotech), washed, and dispensed in 24-well plates containing irradiated (2,500 rad) syngeneic spleen cells (APCs) at 2 × 10⁶ per ml in the absence of antigen for 7 to 10 d (rest phase). For expansion, the rested cells were restimulated with antigen in the presence of APCs (2 × 10⁶ cells/well).

The specificity of T cells was assessed by T cell proliferation assays. After 7–10 d of rest, viable T cells were isolated by Ficoll-Paque gradient centrifugation, washed, and resuspended at 10⁶ cells/ml in proliferation medium. 100 microliters of this suspension was added to 96-well flat-bottom plates containing 10⁶ APCs and appropriate antigens. The cells were cultured for 4 d. [³H]thymidine (0.5 μ Ci in 25 μ l of EMEM; Dupont) was added to each well for the final 12–18 h of culture. Cells were harvested onto filter mats (Wallac Oy) and the incorporated radioactivity was determined in a liquid scintillation counter. Results are expressed as mean counts per minute \pm 1 SEM.

Characterizing T Cell Lines

Phenotypic Marker. The phenotypes of established T cell lines were analyzed by FACS[®] analysis (Becton Dickinson). 10⁶ rested T cells were incubated at 4°C for 30 min with 50 μ l of undiluted hybridoma supernatants. After two washes, cells were incubated for an additional 30 min at 4°C with 50 μ l of FITC-labeled goat anti-rat IgG or goat anti-hamster IgG (Caltag), washed, and fixed in 1% paraformaldehyde in PBS. Fluorescence was analyzed using a FACSCalibur[®] flow cytometer with the use of CellQuest[™] software (Becton Dickinson). For each sample, 10,000 events were counted and the percentage of positive cells was determined after correction for nonspecific fluorescence. The following rat IgG mAbs were used for staining: anti-CD3 (KT3), provided by T. Mandel (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia); anti-CD8 (53-6.72; American Type Culture Collection [ATCC], catalog number TIB 105); anti-B220 (ATCC catalog number TIB 146), purchased from ATCC; and anti-CD4 (GK1.5). Hamster IgG mAbs included anti-TCR $\alpha\beta$ (H57-597) provided by T. Mandel and anti-TCR $\gamma\delta$ (GL3-1A) provided by K. Shortman (The Walter and Eliza Hall Institute of Medical Research).

Cytokine Assays. Cell-free culture supernatants were collected after 24, 48, and 72 h of incubation for measurement of IL-4 and IFN- γ activity. IL-4 was measured by ELISA according to the method described by Doyle et al. (31). In brief, PVC plates

(ICN Biomedicals) were coated with protein-G-purified rat anti-mouse IL-4 mAb BVD4-1D11 at a concentration of 5 µg/ml. After coating and between each incubation step, plates were washed three times with PBS-Tween 20. Plates were blocked for 1 h at 37°C with 1% BSA in PBS-Tween 20 before samples of supernatants in PBS-Tween 20 were added to individual wells. After a 1-h incubation at 37°C, 100 µl of a biotinylated BVD6-24G2 anti-IL-4 mAb was added for 1 h at 37°C, followed by the addition of 100 µl/well streptavidin-horseradish peroxidase conjugate (Vector Laboratories). Color was developed with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) at 0.55 µg/ml in 0.1 M of citrate (pH 4.4) with 0.1% H₂O₂ (Sigma-Aldrich). Finally, the plates were read at 415 nm. Titers in bulk culture supernatants were determined from the linear portion of parallel dose-response curves by comparison with titrations of baculovirus-derived murine IL-4 and expressed in arbitrary units (in which 1 U/ml is defined as the concentration stimulating half-maximal proliferation of the IL-4-responsive cell [CT.4S]). Supernatants from limiting dilution cultures were assayed at a single concentration of 50%. Positive supernatants were defined as those that exceeded the value for control wells by at least 3 SD without responders. For measuring IFN-γ, ELISA kit (BD PharMingen) was used according to manufacturer's instruction protocol. rIFN-γ (Sigma-Aldrich) was used as a standard control.

Functional Study of the Role of Generated T Cell Lines. *P. yoelii*- or OVA-specific CD4 T cells were harvested after 7–10 d of rest in bulk culture and enriched over a Ficoll-Paque gradient (400 g for 20 min). 10⁶ viable T cells were resuspended in 200 µl of PBS and injected intravenously via the lateral tail vein into B cell KO mice. The mice were then infected with 10⁵ *P. yoelii* parasites. Parasite densities were monitored by microscopic examination of tail blood films during the course of infection.

In Vivo Monitoring of T Cells

Parasite- (from different species) or OVA-specific T cell lines were stained with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) as described by Lyons and Parish (32). T cells were briefly resuspended at 10⁷ cells/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. After incubation, cells were washed twice with EMEM and resuspended in PBS. 10⁷ labeled cells were adoptively administered to mice (three to five mice per group), and some of these mice were challenged 24 h later with 10⁵ live parasites. Mice were killed at various days after challenge and peripheral blood, spleen, inguinal lymph nodes, liver, lungs, and bone marrow from two femurs were collected. Single cell suspensions were prepared by Ficoll-Paque centrifugation for peripheral blood lymphocytes by flushing through bone marrow and pressing through stainless meshes for cells from spleen, lymph nodes, liver, and lungs. Cells were washed and resuspended in 0.1% BSA/0.1% NaN₃/PBS and analyzed using a FACSCalibur® flow cytometer. For analysis, 200,000–500,000 cells were counted. In another approach to study the fate of transferred cells, 1.5 × 10⁷ T cells generated in Thy1.2 C57BL/6J wild-type mice were adoptively transferred to Thy1.1 C57BL/6J mice. The transferred T cells were then analyzed using PE-labeled anti-Thy1.2 mAb (BD PharMingen) as described above.

Apoptosis Assays

Annexin V Staining. Early stage apoptosis was detected using the Annexin-V-Fluos staining kit (Boehringer). 10⁶ cells were briefly washed once with PBS, resuspended in 100 µl of staining

solution, and incubated for 10–15 min at room temperature. Apoptotic cells were then analyzed by using a FACSCalibur® flow cytometer. Propidium iodide was used to exclude necrotic cells. To identify the source of apoptotic cells, a double staining method was applied. GK1.5, M1/70, and 8c5 mAb (Caltag) were used for staining CD4 T cells, macrophage, and granulocytes, respectively.

TdT-mediated dUTP Nick-end Labeling (TUNEL) Method. Late stage apoptosis was detected using the in situ cell death detection kit (Boehringer). 10⁶ cells were briefly washed once with PBS and then resuspended in 100 µl of 4% freshly prepared paraformaldehyde solution and fixed for 1 h at room temperature. After being washed twice, the fixed cells were permeabilized in 0.1% Triton X-100 solution for 2 min on ice at 4°C. The cells were then washed twice in PBS and resuspended in 50 µl of TUNEL reaction mixture for 60 min at 37°C in a humidified atmosphere in the dark. Finally, apoptotic cells were analyzed using a FACSCalibur® flow cytometer.

Determination of Immunity to Parasite Infection after T Cell Depletion

P. berghei-specific CD4 T cells were harvested and transferred into BALB/c nu/nu mice as described above. The mice were then infected with 10⁵ homologous parasites and treated with pyrimethamine (1 mg daily) on days 3, 4, 5, and 14 after infection. 2 wk later, mice were challenged again with 10⁵ *P. berghei* pRBC. Parasite densities were monitored by microscopic examination of tail blood films during the course of infection.

Antibody Assay

P. berghei-specific antibodies in sera were measured using ELISA. PVC plates (ICN Biomedicals) were coated overnight at 4°C with 0.2 µg/ml of crude pRBC antigen. Plates were washed three times after coating and between each incubation step with PBS-Tween 20. Plates were blocked for 1 h at 37°C with 1% BSA in PBS-Tween 20 before samples of serum diluted in PBS-Tween 20 were added to individual wells. After a 1-h incubation at 37°C, 100 µl of a 1:3,000 dilution of peroxidase-conjugated sheep anti-mouse IgG (The Binding Site) was added for 1 h at 37°C, followed by the addition of 100 µl/well of 2,2'-azino-di-[ethyl-benzthiozolin sulfonate] (Sigma-Aldrich). After 30 min, the absorbance of each well was read at 405 nm. Serially diluted sera were used to determine antibody titers.

Statistical Analysis

Comparisons among experimental groups by Student's *t* test were performed using a statistical analysis program of Sigma Plot for Windows version 4.0. Significance was set at *P* < 0.05.

Results

Generation of Parasite-specific T Cell Lines. Mice were immunized with pRBC lysates or OVA protein. 7–9 d after immunization, single cell suspensions from draining inguinal and popliteal lymph node cells were cultured with antigen as described in Materials and Methods. After four cycles of stimulation and rest, the cell lines were characterized by FACS® analysis and bioassay for cytokine production. T cell lines generated to all the species of *Plasmodium* in this report have a similar phenotype and cytokine production profile. The data in Fig. 1 are for *P. yoelii* 17 XNL. The established lines were composed of 99.8% CD4⁺ T

cells with 99.6% TCR $\alpha\beta$ expression (Fig. 1 A). Their specificity was confirmed by proliferation assays (Fig. 1 A). These T cell lines produced IFN- γ without detectable IL-4 as measured by bioassay (Fig. 1 B). To assess function of these generated T cell lines, they were administered to B cell KO mice. After homologous parasite challenge, the B cell KO mice that had received the T cells had significantly lower parasitemia than the control mice that received OVA-specific T cells over the course of infection (Fig. 1 C; $P < 0.01$).

Both Lethal and Nonlethal Strains of Plasmodium Cause Depletion of Parasite-specific CD4⁺ T Cells. To monitor parasite-specific T cells in vivo, they were either labeled with CFSE and adoptively transferred to syngeneic mice, or we relied on an allelic marker to track cells. Initially, *P. yoelii* 17 XNL-specific T cells generated from BALB/c mice were labeled with CFSE and were monitored by FACS[®] analysis after transfer to syngeneic mice. Division was demonstrated by the sequential halving of the intensity of the fluorescent signal in the cells (32). After transfer, CFSE T

cells in the spleen displayed a single peak in normal BALB/c mice by day 6 (Fig. 2 A). Although spleen sizes increased and some parasite-specific T cells divided after infection with *P. yoelii* 17 XNL, labeled cells were undetectable by day 6 (Fig. 2 A). This was first evident by day 4 after challenge, and by day 6 there were less than 0.01% CFSE T cells detectable (Fig. 2 A). This decrease was not reflected in an increased number of CFSE-labeled cells in other tissues. Cells were monitored in blood, lung, liver, bone marrow, and lymph nodes. Infection by day 6 resulted in an apparent decrease of parasite-specific cells in all locations (Fig. 2 B; $P < 0.05$).

To further assess the fate of T cells after infection and to compare the responses to lethal and nonlethal infections, CFSE-labeled T cell lines specific for both nonlethal (*P. chabaudi*) and lethal species (*P. vinckei* and *P. yoelii* YM) were adoptively transferred to BALB/c nude mice. As shown in Fig. 3 A, the absolute numbers of CFSE-labeled T cells in the spleens were significantly decreased in infected mice compared with uninfected mice by day 6 after

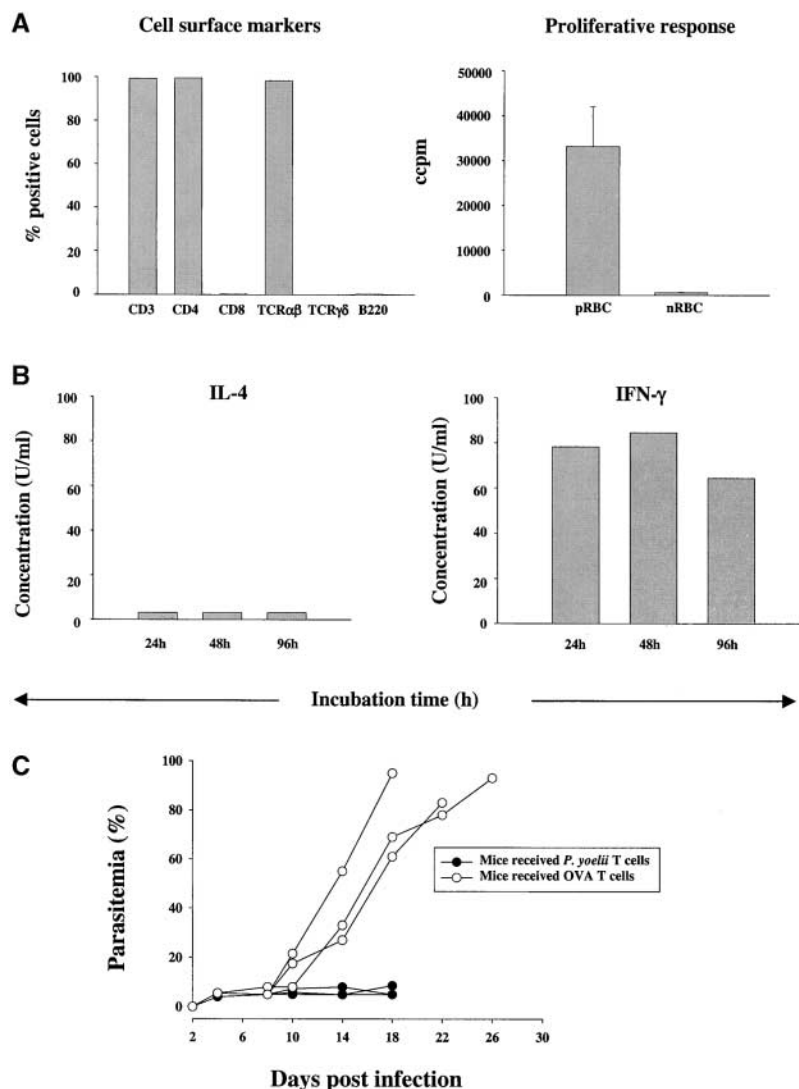


Figure 1. (A) Phenotype and specificity of generated T cell lines. After four cycles of stimulation of *P. yoelii* 17 XNL-specific T cell line, cell surface markers were measured by FACS[®] analysis. The specificity was determined by proliferation assay for T cell lines after four cycles of stimulation. (B) Cytokine production. IL-4 and IFN- γ production by *P. yoelii* 17 XNL-specific T cell line were measured by ELISA. Data show U/ml for IL-4 and IFN- γ production. (C) Protective role of transferred parasite-specific T cells. C57BL/6J B cell KO mice were administered *P. yoelii* 17 XNL-specific T cells (●) or OVA-specific T cells (○) followed by challenge with *P. yoelii* 17 XNL parasites 24 h later. Parasitemia was monitored every second day. Significantly lower peak parasitemias (less than 5% parasitemia over the course of infection) were observed in the mice that received *P. yoelii* 17 XNL-specific T cells compared with the mice that received OVA-specific T cells ($P < 0.01$). The figure represents one of four independent experiments with similar findings.

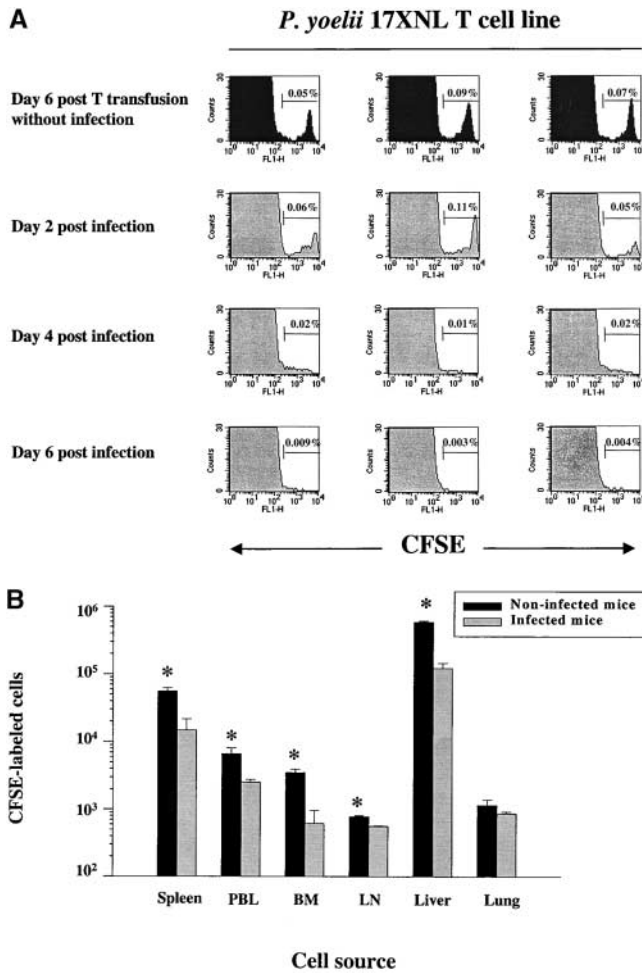


Figure 2. (A) Depletion of parasite-specific CD4⁺ T cells by malaria infection. BALB/c mice (three to four mice per group) were administered labeled *P. yoelii* 17 XNL-specific T cells, followed by challenge with homologous parasites. Control mice received labeled T cells without infection. Splens from control mice were collected by day 6 after cell transfusion (upper panel). Splens from infected mice were collected by day 2 (second panel), day 4 (third panel), or day 6 (bottom panel) after cell transfusion and malaria infection. Percentage of positive CFSE-labeled cells in spleens is indicated in FACS[®] profiles. Each FACS[®] profile represents one of two independent experiments with similar findings. The numbers of donor CFSE cells was significantly lower on day 6 in infected mice ($P < 0.01$) and there were less than 0.01% CFSE T cells detectable. (B) Depletion of parasite-specific T cells in other tissues. Day 6 after cell transfusion, the absolute numbers of CFSE-labeled *P. yoelii* 17 XNL-specific T cells were measured in both infected and noninfected mice in all locations including per spleen, per ml of blood (PBL), per two femurs of bone marrow (BM), per two inguinal and two popliteal lymph nodes (LN), per liver, and per two lungs. Data represent mean cell numbers \pm 1 SEM. *, noninfected mice had significantly higher numbers of CFSE T cells ($P < 0.05$).

transfusion ($2.52 \times 10^5 \pm 0.91$ vs. $0.63 \times 10^5 \pm 0.19$, $P < 0.01$, for *P. Chabaudi*-specific T cells; $4.19 \times 10^5 \pm 1.47$ vs. $1.47 \times 10^5 \pm 0.37$, $P < 0.01$, for *P. vinckei*-specific T cells; and $4.0 \times 10^5 \pm 0.60$ vs. $1.90 \times 10^5 \pm 0.30$, $P < 0.01$, for *P. yoelii* YM-specific T cells). To confirm the deletion, the numbers of CD4⁺ T cells were also assessed. It was found that CD4⁺ T cells in the spleen were not de-

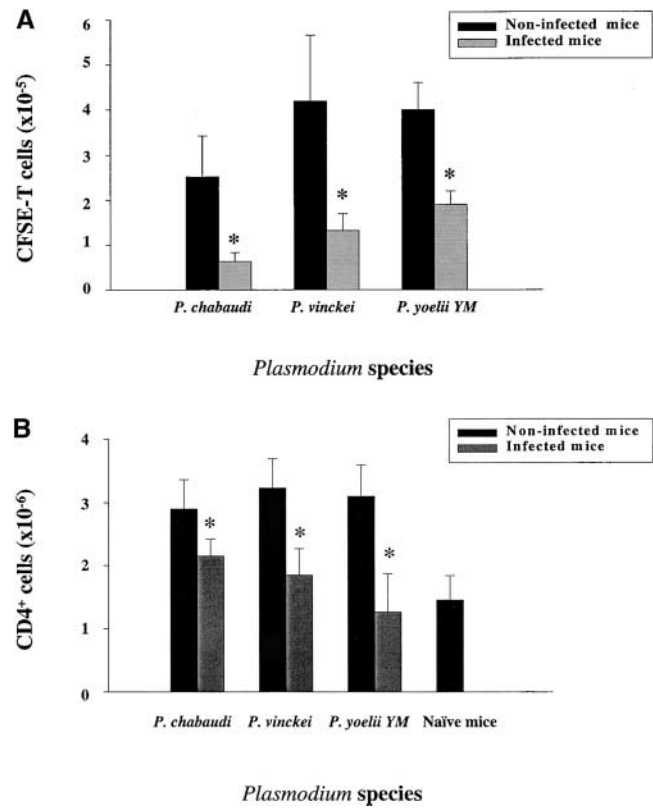


Figure 3. Depletion of parasite-specific CD4⁺ T cells by both lethal and nonlethal species of malaria parasites. (A) BALB/c nude mice (three to four mice per group) were administered labeled *P. chabaudi*-specific, *P. vinckei*-specific, or *P. yoelii* YM-specific T cells. Some mice were then challenged with homologous parasites. Splens were collected at day 6 after challenge. The absolute numbers of CFSE-labeled T cells in the spleens were counted. Data represent mean cell numbers \pm 1 SEM. *, significantly lower numbers of CFSE-labeled T cells were observed in infected mice by day 6 after transfusion in all tested parasite species ($P < 0.01$). (B) The absolute numbers of CD4⁺ T cells (by day 6 after challenge) in the spleens of both infected and noninfected mice are shown. Background levels of CD4⁺ T cells from naive nude mice are also shown. Data represent mean cell numbers \pm 1 SEM. *, significantly lower numbers of CD4⁺ T cells in infected nude mice were observed in all tested parasite-specific T cell lines ($P < 0.01$).

tected at levels above uninfected nude mice for all tested parasite-specific T cell lines (Fig. 3 B).

To formally exclude the possibility that parasite-specific T cells had undergone division in vivo, to the extent that the CFSE label was reduced beyond detection, an allelic marker was used to track transferred T cells. We demonstrated that transferred C57BL/6J Thy1.2 T cells specific to *P. yoelii* 17XNL parasites were deleted in the spleen (Fig. 4) and other tissues (unpublished data) in infected C57BL/6J Thy1.1 mice, but survived in noninfected control mice.

Depletion Only Affects Parasite-specific CD4⁺ T Cells. To determine whether depletion was limited to parasite-specific CD4⁺ T cells, OVA-specific CD4⁺ T cell lines were adoptively transferred to nude mice. Although spleen sizes increased after infection with *P. berghei*, there was a slight reduction in the percentage of OVA-specific T cells

P. yoelii 17 XNL T cell line

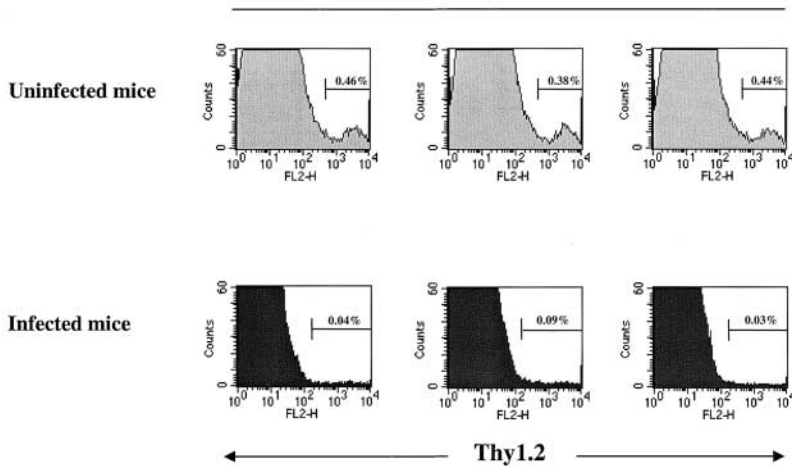


Figure 4. Deletion determined by using allele type markers. Thy1.1 C57BL/6J mice (three to four mice per group) were administered Thy1.2 *P. yoelii* 17 XNL-specific T cells followed by challenge with homologous parasites. Control mice received T cells without infection. Splens from control mice were collected by day 6 after cell transfusion (top). Splens from infected mice were collected by day 6 (bottom) after cell transfusion and malaria infection. Percentage of positive Thy1.2 cells in splens is indicated in FACS[®] profiles. Each FACS[®] profile represents one individual mouse. The number of donor cells was significantly lower in infected mice on day 6 using Thy1.2 to mark the cells ($P < 0.01$).

compared with uninfected mice (Fig. 5 A). However, when corrected for spleen size, there was no diminution of OVA-specific T cells in the splens of infected mice ($1.77 \times 10^5 \pm 0.24$ vs. $1.74 \times 10^5 \pm 0.53$).

We then asked whether depletion was triggered by antigen per se. Thus, nude mice that had been administered OVA-specific T cells were directly challenged with varying doses of OVA antigen ranging from 1 to 1,000 μg . OVA

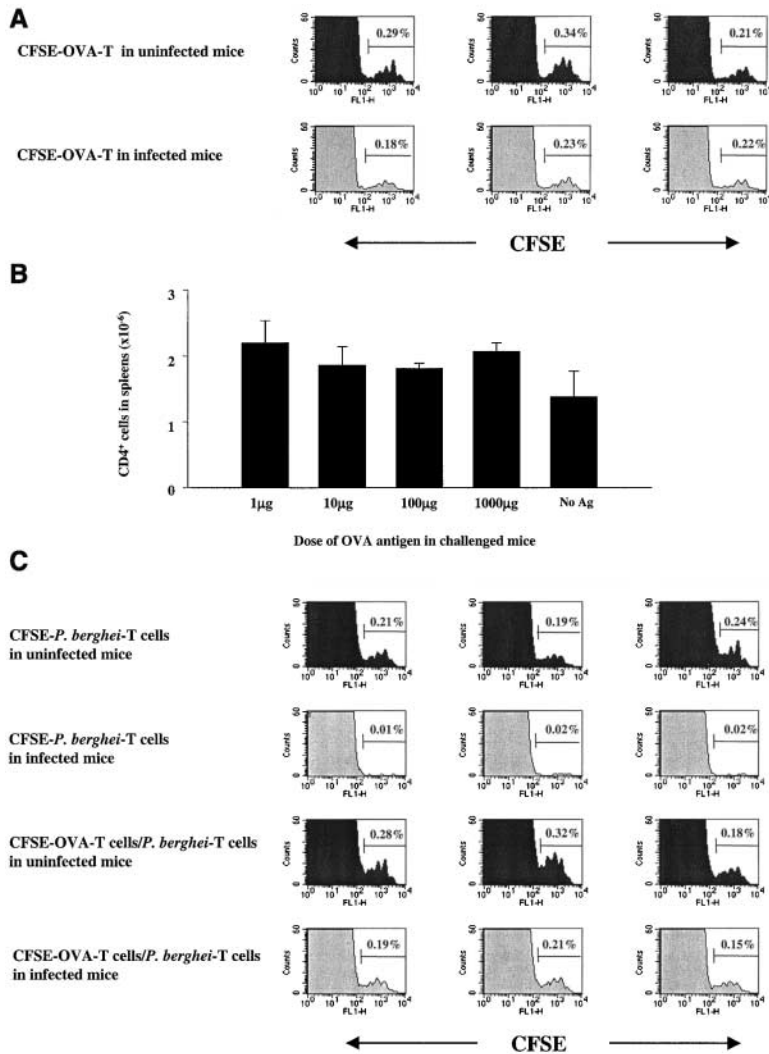


Figure 5. Effect of infection on OVA-specific T cells in nude mice. (A) Nude mice were administered OVA-specific CD4⁺ T cells and followed by challenge with *P. berghei* parasites (bottom) or no challenge (top). Splens were collected on day 6 after cell transfusion. Labeled cells in splens were subsequently detected by FACS[®] analysis. Percentage of positive CFSE-labeled cells in splens is shown in FACS[®] profiles. The figure represents one of five independent experiments with similar findings. Each FACS[®] profile represents one individual mouse. There was no significant diminution of OVA-specific T cells in the splens of infected mice ($P > 0.05$). (B) Mice (three mice per group) that had been administered OVA-specific T cells were either directly challenged, or not challenged as controls, with different doses of OVA antigen as indicated in the figure. The data given are for the absolute numbers of splenic CD4⁺ cells in mice challenged by different doses of OVA. Data represents mean cell numbers \pm 1 SEM. (C) Mice (three mice per group) were administered CFSE-labeled *P. berghei*-specific T cells (upper and second panel) or both CFSE-labeled OVA-specific T cells and nonlabeled *P. berghei*-specific T cells (third and bottom panel). Some mice were infected by *P. berghei* parasites as indicated in the figure. Splens were collected on day 6 after cell transfusion. Percentage of positive CFSE-labeled cells in splens is shown in FACS[®] profiles. The figure represents one of two experiments with similar findings. Each FACS[®] profile represents one individual mouse. Deletion of malaria-specific T cells did not result in the depletion of bystander CFSE OVA T cells ($P > 0.05$).

challenge resulted in an increased number of splenic CD4⁺ T cells for all doses of antigen (Fig. 5 B).

To determine whether depletion of parasite-specific T cells affects bystander T cells, mice were administered both CFSE-labeled OVA-specific and nonlabeled *P. berghei*-specific T cells. As shown in Fig. 5 C, malaria infection resulted in the depletion of CFSE-labeled *P. berghei*-specific T cells in the control group and some diminution in the percentage of CFSE-labeled OVA-specific T cells in the mice that had received both CFSE-labeled OVA-specific and nonlabeled *P. berghei*-specific T cells. When an absolute number of CFSE OVA T cells were enumerated, there was an increase in the number of CFSE OVA T cells in the spleens of infected mice versus noninfected mice ($3.5 \times 10^5 \pm 0.25$ vs. $2.96 \times 10^5 \pm 0.18$) although the difference was not statistically significant ($P > 0.05$).

Acquired Immunity to Malaria Infection Is Significantly Reduced after the Depletion of Parasite-specific CD4⁺ T Cells. To determine whether immunity to malaria infection is affected by the depletion of parasite-specific CD4⁺ T cells, *P. berghei*- or OVA-specific T cells were adoptively transferred to nude mice. Some mice were then given a pulse infection with homologous parasites followed by treatment with pyrimethamine 3, 4, 5, and 14 d after infection with the intention of deliberately depleting parasite-specific T cells, but enabling the mice to survive infection. Successful pyrimethamine treatment was confirmed by checking blood films and transferring blood to naive reporter mice. 2 wk after treatment, the mice were rechallenged. Mice that had received parasite-specific T cells and been previously infected and treated with pyrimethamine had significantly higher levels of parasitemia than mice that received parasite-specific CD4⁺ T cells without previous infection ($18.0\% \pm 2.1$ vs. $3.1\% \pm 0.7$ on day 8; $25.0\% \pm 2.1$ vs. $7.2\% \pm 1.7$ on day 10; and $26.6\% \pm 1.0$ vs. $10.7\% \pm 2.5$ on day 12; $P < 0.01$), although they still had significantly lower levels of parasitemia than nude mice that received no T cells or OVA-specific T cells (Fig. 6 A; $18.0\% \pm 2.1$ vs. $29.2\% \pm 3.1$ or $26.2\% \pm 2.8$ on day 8; $25.0\% \pm 2.1$ vs. $35.5\% \pm 6.1$ or $37.7\% \pm 3.4$ on day 10; and $26.6\% \pm 1.0$ vs. $35.6\% \pm 3.7$ or $44.3\% \pm 3.6$ on day 12; $P < 0.01$). Although recipients of parasite-specific T cells that were initially infected were subsequently less immune, recipients alone developed a low titer antibody response on day 8 after challenge (Fig. 6 B). Thus, these data suggest that cellular immunity to malaria infection was impaired as a result of their initial exposure to parasites.

Mice Infected with Malaria Parasites Have Significantly Higher Levels of Apoptosis. To investigate the mechanism of depletion of parasite-specific T cells, we measured apoptosis during infection. Using an early stage apoptosis Annexin V detection kit, apoptosis was detectable in splenic cells as early as 24 h after infection (Fig. 7 A). Using dual staining, we found that from the various cell populations studied, CD4⁺ T cells alone demonstrated increased apoptosis after infection (Fig. 7 B). Using the TUNEL method, it was found that by day 6 after challenge, infected mice had significantly higher levels of apoptosis in the spleen compared

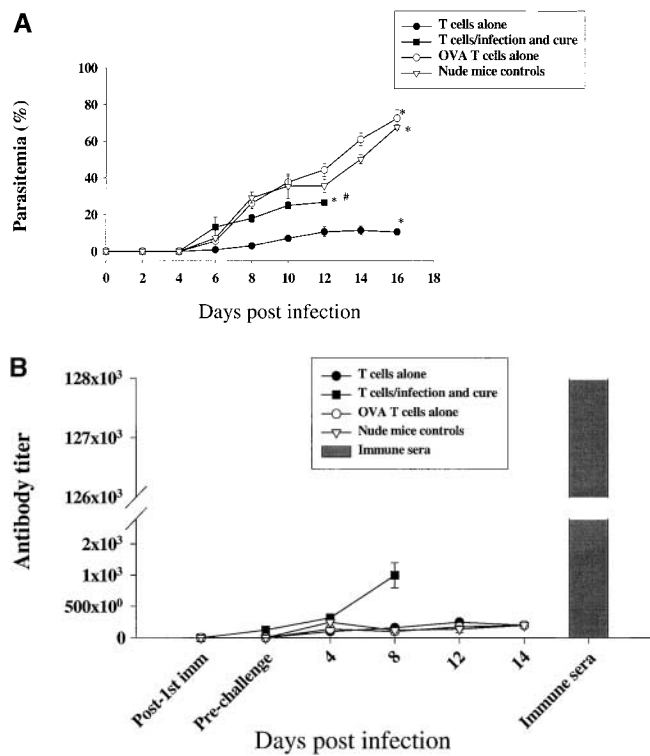


Figure 6. (A) Parasitemia in nude mice after infection-induced depletion of parasite-specific T cells. *P. berghei*-specific T cells were adoptively transferred to nude mice (five mice per group). One group of mice (■) were then infected with homologous parasites followed by treatment with pyrimethamine on days 3, 4, 5, and 14. Another group of mice only received *P. berghei*-specific T cells without infection and treatment (●). Control mice received no T cells (▽) or OVA-specific T cells (○). All groups of mice were challenged with *P. berghei* parasites at the same time as those mice (■) that had received pyrimethamine treatment (14 d after the last dose of pyrimethamine). Parasitemia was monitored every second day. Data represent mean parasitemia \pm 1 SEM. #, mice that had received parasite-specific T cells and had been previously infected and treated with pyrimethamine had significantly higher levels of parasitemia than mice that received parasite-specific CD4⁺ T cells without previous infection ($P < 0.01$), but had lower levels of parasitemia than nude mice that received no T cells or OVA-specific T cells ($P < 0.01$); *, death of mice. (B) Antibody levels. Sera from different groups of mice as indicated above were collected before and after challenge, and anti-*P. berghei* antibody titers were measured by ELISA. Data represents mean titer \pm 1 SEM.

with uninfected mice (Fig. 7 C; $21.48 \pm 3.10\%$ vs. $7.25 \pm 0.32\%$, $P < 0.01$).

Depletion of Parasite-specific CD4⁺ Cells after Infection Is Not via TNF or Fas Pathway. To identify the pathway of apoptosis in malaria infection, *P. berghei*-specific T cell lines were generated in TNF-RI knock-out- and Fas-deficient (C3H Lpr) mice. Similarly to wild-type mice, the established T cell lines generated from these mice consisted of 99% CD4⁺ cells with 99% TCR $\alpha\beta$ expression, and cells secreted IL-2 and IFN- γ (unpublished data). Because the mice lack either TNF-RI or Fas, they will not receive signals from TNF or Fas ligand, respectively. Accordingly, if apoptosis is mediated via the TNF or Fas pathways, the T cell lines generated from these mice will not be depleted. Using the same approach as described above, CFSE-labeled

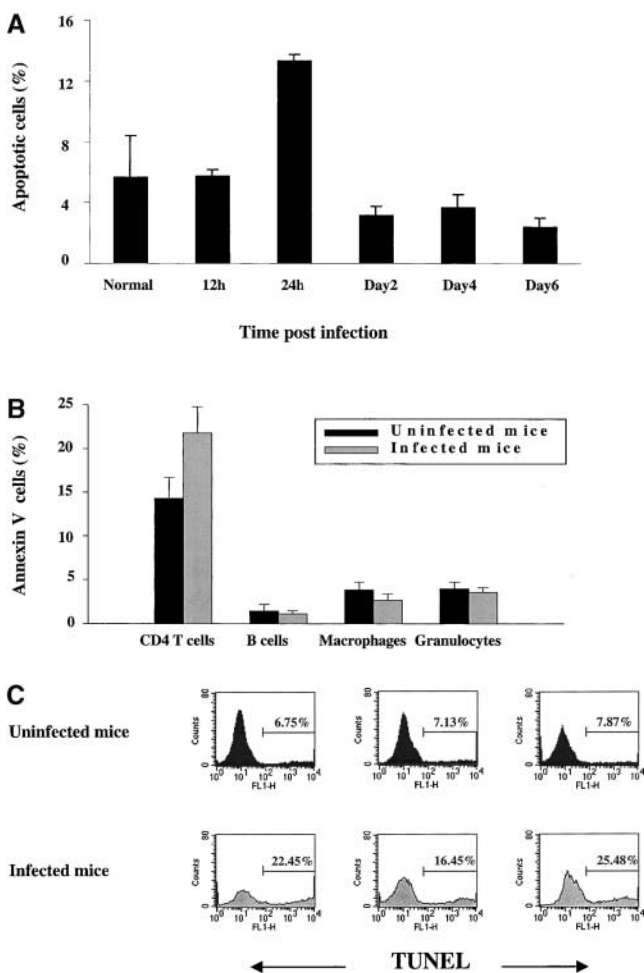


Figure 7. Apoptosis in mice infected with malaria parasites. Mice (three to four per group) were infected with *P. berghei* parasites. (A) At various days after challenge, spleens were removed and apoptosis was determined by an early stage apoptosis detection kit Annexin V. (B) Apoptosis of different types of cells were measured by a double staining method. (C) TUNEL method was used to detect late stage apoptosis in spleens by day 6 after *P. berghei* parasite infection. Percentage of apoptotic cells in infected mice and noninfected mice is indicated in individual FACS[®] profiles. The figures represent one of many independent experiments (including ones that use different strains of parasite infections) with similar findings. All data represent mean percentage \pm 1 SEM. Significantly higher levels of apoptosis in the spleen were observed in infected mice ($P < 0.01$).

parasite-specific T cells generated from these mice were adoptively transferred to wild-type mice. After challenge, the transferred T cells were undetectable by day 6 (Fig. 8), suggesting that the depletion of parasite-specific CD4⁺ T cells during infection is not mediated by either TNF or Fas.

IFN- γ Contributes to the Depletion of Parasite-specific CD4⁺ T Cells in Malaria Infection. To determine whether IFN- γ plays a role in the apoptosis in malaria infection, CFSE-labeled *P. berghei*-specific T cells were adoptively transferred to nude mice. The mice were then administered 1 mg daily, 1, 2, and 3 d after challenge, of either anti-IFN- γ antibody XMG-6 (33) or an isotype-matched control antibody (GL-113). Similarly to previous experiments, T cells were de-

pleted in the mice that were infected and also received control antibody (Fig. 9 A). In contrast, T cells underwent multiple divisions in the infected mice that had simultaneously received anti-IFN- γ antibody (Fig. 9 A). When the absolute numbers of CFSE-labeled cells were counted in the spleens, mice that had received anti-IFN- γ antibody had significantly higher levels of T cells compared with mice that were not infected or were infected and received isotype control antibody (Fig. 9 B; $22.7 \times 10^4 \pm 3.81$ vs. $10.9 \times 10^4 \pm 3.52$ or $5.7 \times 10^4 \pm 1.04$, $P < 0.01$). These data suggest that IFN- γ is necessary for the depletion of parasite-specific CD4⁺ T cells during malaria infection.

Discussion

This study demonstrates that apoptotic deletion of parasite-specific CD4⁺ T cells is a common occurrence after infection with different species of rodent malaria parasites and can lead to impaired T cell-mediated immunity. Deletion requires more than antigenic stimulation alone. IFN- γ is involved in this process, but engagement of Fas or TNF-RI is not required.

The central role of CD4⁺ T cells in protective immunity to blood stage malaria infection has been shown in both murine and human studies (34, 35). Despite the potential for CD4⁺ T cells to contribute to protective immunity, natural immunity to malaria is slow to develop (20). The mechanisms for this remain to be defined and are the subject of intense investigation. To address this question, we first generated parasite-specific T cell lines by immunizing mice with pRBC lysates. These T cell lines were TH1-like (Fig. 1, A and B). T cells were adoptively transferred to B cell KO mice and provided partial protection against challenge with homologous parasites (Fig. 1 C). Because B cell KO mice cannot produce antibody, the control of parasitemia must be dependent on the transferred cells and independent of antibody. Thus, these data indicate that the established parasite-specific T cells have a protective role (36). Using established TH1-like T cell lines, we studied the fate of malaria parasite-specific T cell lines in vivo. Consistent with our previous report (29), the current study showed that transferred T cells were depleted in mice after infection. We have extended these observations by showing that depletion started as early as day 4 after challenge and was completed by day 6 (Fig. 2 A). Furthermore, we demonstrated that lethal and nonlethal strain parasite-specific T cells undergo a similar depletion process in vivo after infection (Fig. 3, A and B, and Fig. 5 C). This indicates that depletion of transferred parasite-specific T cells is a common phenomenon in malaria and may be a strategy used by malaria parasites to eliminate T cells and evade host defenses that can act to limit the infection, as shown in other systems (37, 38).

Significantly, in this study, we showed that the depletion during malaria is confined to parasite-specific CD4⁺ T cells. The evidence comes from three separate sets of experiments. First, OVA-specific CD4⁺ T cell lines were not

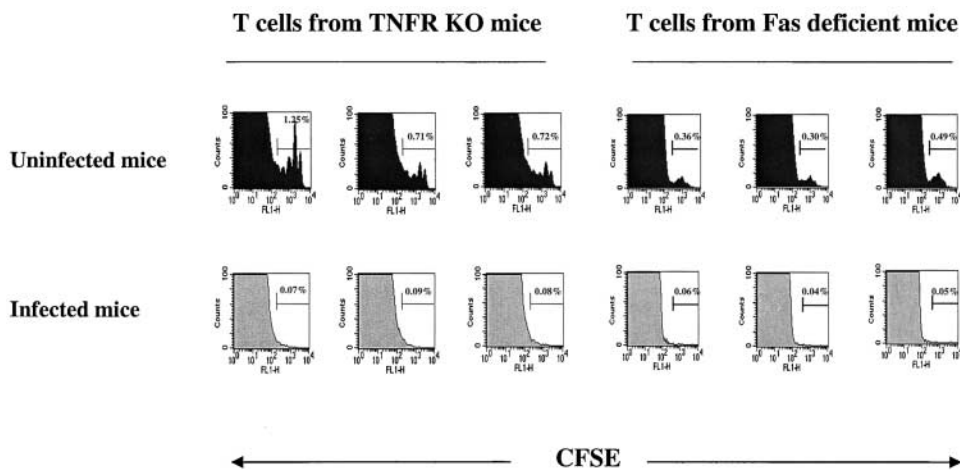


Figure 8. Depletion of parasite-specific CD4⁺ T cells generated from TNF-RI knockout- or Fas-deficient mice. *P. berghei*-specific T cell lines generated from TNF-RI KO- and Fas-deficient (C3H Lpr) mice were adoptively transferred to wild-type mice (three mice per group). Some mice were then challenged by homologous parasites. Spleens were collected by day 6 after T cell transfusion. The labeled cells in spleens were subsequently detected by FACS[®] analysis. Percentage of positive CFSE-labeled cells in spleens is shown in FACS[®] profiles. Figures represent one of two independent experiments with similar findings. Each FACS[®] profile represents one individual mouse.

depleted by malaria infection (Fig. 5 A). Secondly, instead of depletion, OVA-specific T cells were activated and divided in response to direct challenge with a range of doses of OVA antigen (Fig. 5 B). This suggests that at least in the short term, only parasite-specific T cells are depleted once they are engaged by their specific parasite antigen. Finally, using a dual transfusion system, we showed that depletion of parasite-specific T cells did not kill bystander OVA-specific T cells (Fig. 5 C). This strongly suggests that depletion is programmed within the cell after TCR engagement by appropriate parasite antigens. The fact that dead antigen, OVA, does not lead to the depletion of OVA-specific T cells, and that malaria infection did not cause the bystander killing of OVA-specific CD4⁺ T cells (Fig. 5), strongly suggest that other elements of infection, apart from T cell

stimulation or cytokine release alone, are required for depletion apoptosis of parasite-specific T cells.

T cell fate in vivo has been under intensive investigation in many different systems (39–41). During immune responses, large numbers of activated effector T cells are generated. The majority of these cells are then eliminated by apoptosis (42, 43). A balance of survival and apoptosis of immune cells during infection is extremely important as it may affect the generation of protective memory T cells and may eliminate potentially harmful activated T cells. On the other hand, apoptosis may well be induced by infectious organisms to enable their survival. In this study, our data suggest that apoptosis is a key factor involved in the depletion of malaria parasite-specific CD4⁺ T cells. This conclusion comes from our observation that infected mice had

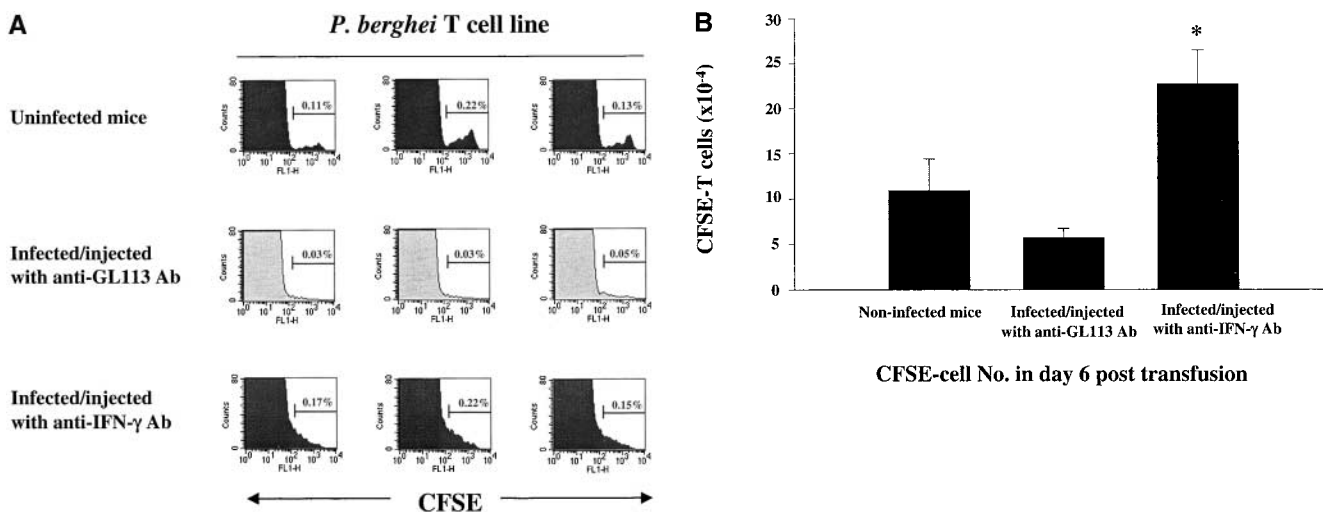


Figure 9. The role of IFN- γ in the apoptosis of malaria parasite infection. (A) CFSE-labeled *P. berghei*-specific T cells were adoptively transferred into nude mice (three mice per group). One group of mice were injected with anti-IFN- γ antibody (1 mg daily, on days 1, 2, and 3 after challenge) and challenged with homologous parasites along with other control groups of mice as indicated in the figure. Spleens were collected by day 6 after T cell transfusion. Labeled cells in spleens were then detected by FACS[®] analysis. Percentage of positive CFSE-labeled cells in spleens is shown in FACS[®] profiles. Each FACS[®] profile represents one individual mouse. (B) Absolute number of CFSE-labeled cells were counted in the spleens in different groups of mice. *, mice that had received anti-IFN- γ antibody had significantly higher levels of T cells compared with mice that were not infected or were infected and received isotype control antibody ($P < 0.01$).

significantly higher levels of early and later stage apoptotic cells in the spleen compared with uninfected mice (Fig. 7, A and C). Using a dual staining method, we also demonstrated that from the range of cells studied, CD4⁺ T cells alone were apoptotic (Fig. 7 B). This has been further confirmed in our recent study that showed high levels of apoptotic cells after infection in CFSE-labeled helper T cells specific for the vaccine candidate homologue, MSP1₁₉ (44).

Apoptosis is strictly regulated through the interaction of proapoptotic and antiapoptotic molecules. Three major mechanisms have been found to be responsible for the activation of the apoptotic machinery. These include the withdrawal of survival signals like growth factors, the intervention of conflicting signals during the cell cycle, or the recognition of a specific molecule at the cell surface (45). Of these three mechanisms, receptor-mediated signaling is the best defined. Death receptors belonging to the TNF receptor super family are transmembrane proteins localized to the cytoplasmic membrane. Well-characterized death receptors are TNF-RI and CD95 (Fas) which bind the cytokine TNF α and CD95 ligand (CD95L), respectively (46–48). To attempt to find the pathway of apoptosis in malaria infection, parasite-specific T cell lines were generated from TNF-RI knockout- and Fas-deficient mice. Our data (Fig. 8) show that after challenge the parasite-specific T cells generated from these knockout mice were depleted, as were the T cells generated from wild-type control mice, which suggests an alternative pathway is involved in depletion.

The central role of cytokines contributing to protective immunity and pathogenesis in malaria infection has been demonstrated in numerous studies (49, 50). In particular, IFN- γ responses have been shown to be associated with resistance to reinfection with *P. falciparum* in young African children (51). On the other hand, IFN- γ has been implicated in the pathogenesis of experimental cerebral malaria (52, 53). An early study also postulated IFN- γ as a key factor responsible for the cell death of effector CD4⁺ T cells and as a possible mechanism for self-tolerance (54). Indeed, more recent studies have suggested that IFN- γ is responsible for eliminating responding CD4⁺ T cells in several other systems by inducing apoptosis of activated CD4⁺ T cells (55–57). To determine whether IFN- γ plays a role in apoptosis in malaria infection, CFSE-labeled *P. berghei*-specific T cells were administered simultaneously with anti-IFN- γ antibody into nude mice. Nude mice that received anti-IFN- γ antibody had significantly higher levels of CFSE-labeled parasite-specific T cells compared with control mice (Fig. 9), indicating that IFN- γ , apart from its role in killing parasites, contributes to the depletion of parasite-specific CD4⁺ T cells. Indeed, IFN- γ has been shown to induce apoptosis of mammalian cells in a manner independent of Fas and TNFR (58). Whether IFN- γ alone or combined with other molecules such as nitric oxide (59) regulates apoptosis in malaria infection merits further investigation.

Although this study shows that impaired immunity occurs after the depletion of protective parasite-specific

CD4⁺ T cells during malaria infection, it is well known that normal mice can be immunized by infection followed by drug cure. In such mice it is likely that induced antibody plays the critical role in protection. We observed (Fig. 6 B) that infection did not prevent an antibody response in mice that had received parasite-specific T cells. However, cell-mediated immunity can control parasite growth (10). A major challenge is to identify target antigens for cell-mediated immunity and understand the regulation of it after infection. Thus, this study, by defining a possible apoptotic pathway, contributes to a better understanding of parasite immunity that may lead to successful vaccine development.

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