Murine $\gamma\delta$ T Lymphocytes Elicited during *Plasmodium yoelii* Infection Respond to *Plasmodium* Heat Shock Proteins

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$\gamma\delta$ T cells accumulate during *Plasmodium* infections in both murine and human malarias. The biological role of these cells and the antigens that they recognize are not clearly understood, although recent findings indicate that $\gamma\delta$ T cells in general influence both innate and antigen-specific adaptive host responses. We examined the accumulation of $\gamma\delta$ T cells elicited during infection with virulent and avirulent *Plasmodium yoelii* parasites in relatively susceptible and resistant strains of mice. Our results indicated that in nonlethal malaria infections, $\gamma\delta$ T cells comprise a larger proportion of splenic T cells than in lethal infections and that only a live infection is capable of inducing an increase in the percentage of $\gamma\delta$ T cells in vivo. Furthermore, we demonstrate that $\gamma\delta$ T cells elicited during a *P. yoelii* infection respond by proliferation in vitro to *P. falciparum* heat shock proteins (HSPs) of 60 and 70 kDa, suggesting a possible immunological involvement of parasite HSPs in this arm of the cellular immune response during malarial infection in mice.

Malaria represents a worldwide health concern and poses a challenge in vaccine development. To develop an effective vaccine, we must first understand the immunological mechanisms involved in the protection against infection. Great advances in the understanding of the humoral and cellular effector arms of the immune response during malaria infection have occurred within the last several years. Many unanswered questions remain, however, such as the role of $\gamma\delta$ T cells in infection.

In malaria infections, $\gamma\delta$ T cells accumulate in the peripheral blood and spleens of individuals infected with *Plasmodium falciparum* and *P. vivax* (22, 23, 28, 32, 34, 38). These $\gamma\delta$ T cells are stimulated most strongly by the schizont/merozoite stage of the parasite and are able to inhibit the replication of *P. falciparum* in vitro (2, 12, 15, 16, 23). During *P. chabaudi* infections in mice, the splenic $\gamma\delta$ T-cell population expanded more than 10-fold; $\gamma\delta$ T-cell-depleted mice, however, were unable to control parasitemia during the same time frame as wild-type mice (41–43). More recently, Tsuji et al. have shown by passive transfer experiments that a $\gamma\delta$ T-cell clone was able to inhibit the development of *P. yoelii* during the liver stage of infection in mice (40). $\gamma\delta$ T cells are therefore believed to play a beneficial role in disease outcome during malaria infection.

We have examined the biological role of $\gamma\delta$ T cells in a rodent malaria system by using *P. yoelii* strains that cause lethal (17XL) and nonlethal (17XNL) malaria in genetically susceptible and relatively resistant strains of mice. Our results indicate that (i) $\gamma\delta$ T cells increase as a percentage of total T cells in the spleens of the infected mice, (ii) $\gamma\delta$ T cells comprise a smaller percentage of T cells in more susceptible mouse strains and in infections with more virulent forms of the parasite, (iii) a live infection is required to elicit a $\gamma\delta$ T-cell accumulation, and (iv) $\gamma\delta$ T cells elicited during infection with the malaria parasite proliferate in vitro in the presence of *P. falciparum* heat shock proteins (HSPs) of 60 and 70 kDa.

MATERIALS AND METHODS

Mice. The mice [female BALB/c (*H-2^d*) and C57BL/6 (*H-2^b*)] used in these studies were 6 to 10 weeks old and obtained from the National Cancer Institute, Frederick. Three to five mice were used for any given time point of observation in these studies.

Parasites, infections, and immunizations. *P. yoelii* 17XNL and 17XL were maintained by serial passage. Parasites were injected by the intraperitoneal (i.p.) route at 10^6 parasitized erythrocytes (RBCs) per mouse unless otherwise specified. Parasitemia was determined by microscopic examination of Giemsa-stained thin blood smears every 2 days postinfection until resolution. Results are expressed as a mean of results obtained with three to five mice, and experiments were repeated at least three times with representative data shown. For the immunization experiments, blood was collected in heparin from infected mice. Leukocytes were removed by passage of blood through a glass bead column followed by a cellulose column (1). The parasites were then inactivated in a gamma irradiator (7.2×10^4 rads), and irradiated RBCs were injected i.p. at 10^{10} infected RBCs.

Flow cytometry. Spleens were removed from the mice and teased gently in RPMI 1640 to obtain a single-cell suspension. The cells were then treated with ammonium chloride lysing solution to remove RBCs. Washed splenocytes (10^6) were first incubated at 4°C for 15 min in phosphate-buffered saline containing 1% bovine serum albumin, 0.1% sodium azide (pH 7.2), hamster immunoglobulin G ($10 \mu\text{g}/10^6$ cells), and rat immunoglobulin G ($10 \mu\text{g}/10^6$ cells) isotype controls (Pharmingen). The cells were then incubated for 30 min at 4°C with the appropriate monoclonal antibodies (MAbs) (0.5 μg). The cells were washed in phosphate-buffered saline, fixed with 1% formaldehyde, and analyzed on a Becton Dickinson FACSscan apparatus. The following fluorescein isothiocyanate-conjugated MAbs were used for staining: anti-CD3 ϵ (Pharmingen 145-2C11), anti- $\alpha\beta$ T-cell receptor (TCR) (Pharmingen H57-597), and anti-CD8 α (Gibco-BRL 53-6.7). The following phycoerythrin-conjugated MAbs were used: anti- $\gamma\delta$ TCR (Pharmingen GL3), anti-CD3 ϵ (Gibco-BRL 29B), and anti-CD4 (Pharmingen GK1.5).

Cytotoxic-cell depletion. Spleens were removed, teased in RPMI 1640, and treated with an ammonium chloride lysing solution to remove RBCs. Washed cells were resuspended at 10^8 cells/ml in RPMI 1640 supplemented with 25 mM HEPES and 0.3% bovine serum albumin. The appropriate antibodies (see below) were added, and the cells were treated for 30 min on ice. The cells were pelleted, 100 μl of Low-Tox-M complement (Cedarlane) was added to the RPMI 1640, and the mixture was incubated for 30 min at 37°C . The cells were washed, and the procedure was repeated. Fluorescence-activated cell sorter (FACS) analysis was used to determine the effectiveness of the depletion. Cytotoxic-cell elimination removed more than 95% of the appropriate splenocyte populations. The antibody cocktail of anti-CD4 (GK 1.5 culture supernatant; 0.5 ml), anti-CD8 (2.43 ascites; 20 μl), anti-major histocompatibility complex class II (MHC II) *I-A^d* (MK-D6 ascites; 20 μl), and anti- $\alpha\beta$ TCR (H57 culture supernatant; 0.5 ml) was used to obtain a $\gamma\delta$ -T-cell-enriched population. $\alpha\beta$ TCR-enriched populations were obtained after treatment with a cocktail of anti-MHC II (MK-D6 ascites; 20 μl) and anti- $\gamma\delta$ TCR (GL-3 culture supernatant; 0.5 ml).

Lymphocyte proliferation. After cytotoxic-cell depletion, cells were resuspended in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U of penicillin

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FIG. 1. Parasitemia in BALB/c and C57BL/6 mice. Mice were infected i.p. with *P. yoelii* 17XNL (10^6 parasitized RBCs). Parasitemia was measured every other day by microscopic examination of a blood smear stained with Giemsa reagent. The values shown are mean and standard deviation.

per ml, and 100 μ g of streptomycin sulfate per ml. Each cell population (unfractionated, $\gamma\delta$ enriched, and $\alpha\beta$ enriched) was plated at 2×10^5 cells/well in a flat-bottom 96-well plate in the presence of 3×10^5 irradiated antigen-presenting cells (3,000 rads), with triplicate wells used for each antigen. The following antigens were used in the proliferation experiments: medium alone (control), *P. yoelii* 17XNL extract (10^6 /ml), *P. falciparum* HSP 60 (10 μ g/ml) and HSP 70B (10 μ g/ml), and concanavalin A (2.5 μ g/ml). The cells were cultured for 5 days at 37°C under 5% CO_2 and then pulsed with 1 μ Ci of [^3H]thymidine for 18 h. The cells were harvested with a 96-well plate harvester, and radioactivity was measured in a Packard radioactivity counter. Results are expressed as stimulation index (S.I.), defined as average counts per minute in antigen wells/average counts per minute in negative control wells.

$\gamma\delta$ TCR analysis. Mice were sacrificed on day 10 postinfection, and the spleens were removed and prepared as described above. RNA was isolated with Tri Reagent (Molecular Research Center, Inc.) as specified by the manufacturer. First-strand cDNA was synthesized from total RNA as follows. Briefly, 5 μ g of total RNA was mixed with 500 ng of oligo(dT) $_{15}$ and 1 μ l of a 10 mM deoxynucleoside triphosphate (Pharmacia Biotech) mix and incubated for 50 min at 37°C in the presence of 1 $\times$ first-strand buffer (Gibco-BRL), dithiothreitol, and SuperScript II (Gibco-BRL). PCR was performed with a 1:10 dilution of the cDNA template (5 μ l) in a 50- μ l reaction mixture containing 5 μ l of 10 $\times$ PCR buffer (Sigma), 400 ng of the appropriate primer, 100 μ M deoxynucleoside triphosphate mix (Perkin-Elmer), and 1 U of *Taq* DNA Polymerase (Sigma). The PCR cycles consisted of an initial denaturation at 94°C for 5 min followed by 30

cycles of 94°C for 45 s, annealing at 45°C for 1 min, and extension at 72°C for 2 min, with a final extension of 10 min. The PCR-amplified products were analyzed on a 1% agarose gel. The specific primers used have been described previously (39) and were designed to amplify any of the rearranged transcripts containing V γ 1.1, V γ 1.2, V γ 2, J γ 1, J γ 2, or J γ 4 and V δ 4, V δ 5, V δ 6, and J δ 1.

Antigens. *P. falciparum* HSP 60 and HSP 70B recombinant proteins containing the His $_6$ tag were expressed in *Escherichia coli* and purified over a Ni $^{2+}$ column (11, 26), as was the *P. falciparum* 27-kDa protein (35). The *P. yoelii* antigen extract was prepared from BALB/c mice infected with *P. yoelii* (17XNL). Platelets and leukocytes were removed by passing the blood through a glass bead column followed by a cellulose column (1). The cells were then briefly sonicated, sterilized by γ -irradiation, and stored at -70°C .

RESULTS

Parasitemia and splenic T-cell differences in inbred strains of mice. BALB/c and C57BL/6 mice were infected with 10^6 *P. yoelii* 17XNL-infected RBCs and monitored over the course of infection for parasitemia. As seen in Fig. 1, BALB/c mice are relatively more resistant to *P. yoelii* than are C57BL/6 mice as measured by the percent parasitemia and the duration of infection. In both mouse strains, initial parasitemia increased at relatively the same rate; however, on day 10, when BALB/c mice reached a peak parasitemia of 10 to 15% which resolved by day 13, the parasitemia in C57BL/6 mice continued to rise until it was higher than 50% on day 17 and resolved around day 25.

We next measured changes in the $\gamma\delta$ T-cell population in the spleen (Fig. 2) and lymph nodes (data not shown) of both strains of mice. An increase in the percentage of $\gamma\delta$ T cells was seen only in the spleens of the infected mice. There were significant differences in the percentage of $\gamma\delta$ T cells in the spleens of BALB/c (Fig. 2A) and C57BL/6 (Fig. 2B) mice. In BALB/c mice, $\gamma\delta$ T cells accumulated to a level of 20% of total splenic T cells on day 10 before gradually declining to 7.4% by day 24, after the resolution of parasitemia. In contrast, $\gamma\delta$ T-cell percentages continued to increase gradually in C57BL/6 mice. On day 10, both strains had roughly the same percent parasitemia (12%), although the $\gamma\delta$ T-cell population in C57BL/6 mice comprised only 9% of the total splenic T cells, in contrast to 20% in the BALB/c. These results demonstrated that the more resistant BALB/c mice displayed a higher and faster percent increase in $\gamma\delta$ T cells than did the more susceptible C57BL/6 mice.

We also examined changes in the CD4 $^+$ and CD8 $^+$ splenic T-cell populations. CD8 $^+$ cells in both groups of mice showed

FIG. 2. Parasitemia (same data as in Fig. 1) and the percentage of $\gamma\delta$ T cells in the spleens of BALB/c (A) and C57BL/6 (B) mice infected with *P. yoelii* 17XNL (10^6 parasitized RBCs). Spleens were removed on the appropriate day postinfection. The splenocyte cell suspension was stained with anti-CD3 and anti- $\gamma\delta$ TCR for flow cytometry. The values shown are mean and standard deviation.

FIG. 3. Parasitemia (same data as in Fig. 1) and the percentage of CD4⁺ and CD8⁺ cells in the spleens of BALB/c (A) and C57BL/6 (B) mice infected i.p. with *P. yoelii* 17XNL (10^6 parasitized RBCs). On the appropriate day spleens were removed and the cell suspension was stained with anti- $\alpha\beta$ and anti-CD4 or anti- $\alpha\beta$ and anti-CD8 for flow cytometry. The values are expressed as mean and standard deviation.

a similar profile, with an initial population comprising 30 to 35% of the total T cells in the spleen and decreasing to 15% at peak parasitemia in BALB/c mice and later in C57BL/6 mice. In more resistant (BALB/c) mice, the CD4⁺ population remained constant at 65% of the total T-cell number throughout the infection. In contrast, in C57BL/6 mice, the CD4⁺ population showed a gradual reduction from an initial 58% to 33% on day 20 (Fig. 3). Therefore, the two major differences observed in the splenic T cells of the more susceptible and less susceptible mice during *P. yoelii* infection were (i) a lower percentage of $\gamma\delta$ T cells in susceptible mice (C57BL/6) and (ii) a decrease in the percentage of CD4⁺ T cells in the susceptible mice (C57BL/6).

A secondary infection is associated with a lower percentage of $\gamma\delta$ T cells. To evaluate splenic T-cell populations during a challenge infection, BALB/c mice were infected with 10^6 *P. yoelii* 17XNL-infected RBCs and rechallenged with the same dose 3 weeks later when the mice were completely free of any parasitemia. Rechallenged mice were generally resistant to infection since they developed very low parasitemia if any. Eight days after the secondary infection, the mice were sacrificed and the spleens were removed for analysis of $\gamma\delta$, CD4⁺, CD8⁺, and $\alpha\beta$ T cells by flow cytometry. As shown in Fig. 4, the percentage of $\gamma\delta$ T cells did not increase upon rechallenge of the mice, although an elevation in both CD4⁺ and CD8⁺ T-cell numbers was observed.

Comparison of phenotypic differences in the splenic T-cells of mice infected with virulent and avirulent strains of *P. yoelii*. To gain further insight into whether $\gamma\delta$ T cells play a role in controlling malaria infection, we investigated differences in the splenocyte populations in BALB/c mice infected with virulent and avirulent strains of the parasite. Mice were infected with either 10^6 *P. yoelii* 17XL-, 10^4 *P. yoelii* 17XL-, or 10^6 *P. yoelii* 17XNL-infected RBCs, and parasitemia and splenocyte T-cell populations were measured. As shown in Table 1, parasitemia increased much faster in the lethal infections than in the non-lethal infections and the mice infected with lethal parasites were not able to resolve the infection. Examination of splenic T-cell populations showed no difference in the percentage of CD4⁺ or CD8⁺ cells (data not shown). However, significant differences were observed when the percentages of $\gamma\delta$ T cells were measured. The $\gamma\delta$ T-cell percentage did not change in mice infected with the lethal parasites at 10^4 and 10^6 parasit-

ized RBCs, while during the same infection period with the nonlethal parasite the percentage of $\gamma\delta$ T cells increased three-fold. A possible interpretation of these observations is that accumulation of $\gamma\delta$ T cells plays a protective role in less severe malaria caused by *P. yoelii* 17XNL and that immune system changes during fulminating infection by *P. yoelii* 17XL do not allow any significant expansion of the percentage of $\gamma\delta$ T cells. This observation further suggests a potentially protective role for $\gamma\delta$ T cells in less severe malaria infections.

A productive infection is required to elicit $\gamma\delta$ T cells. To investigate if a productive infection is required to elicit $\gamma\delta$ T-cell expansion, BALB/c mice were infected with live *P. yoelii*-infected RBCs (10^6) or immunized with 10^{10} irradiation-inactivated parasites. The mice were sacrificed on day 8, and $\gamma\delta$ T-cell percentages were measured by flow cytometry. Results with the infected group reconfirmed our previous finding in which $\gamma\delta$ T cells accounted for roughly 18% of splenic T cells. On the other hand, no change in the percentage of splenic $\gamma\delta$ T cells was seen when the mice were inoculated with radiation-

FIG. 4. Changes in splenic T-cell populations during primary and challenge infections. BALB/c mice were infected i.p. with *P. yoelii* 17XNL (10^6 parasitized RBCs) and rechallenged with the same dose 3 weeks later. Eight days after the secondary infection, the spleen cells were stained with the appropriate combination of antibodies for flow cytometry. Results are expressed as mean and standard deviation.

FIG. 5. Percentage of $\gamma\delta$ T cells in the spleens of control, infected, and immunized mice. Mice were infected with 10^6 parasitized RBCs, and the $\gamma\delta$ T-cell population was measured by staining splenocytes with anti-CD3 and anti- $\gamma\delta$ TCR on day 8. Immunized mice were given 10^{10} irradiated parasitized RBCs i.p., and $\gamma\delta$ T-cell percentages were measured on day 8. Control mice were uninfected. Results are expressed as mean and standard deviation.

inactivated parasites (Fig. 5), demonstrating that only a productive infection is capable of eliciting an increase in the percentage of $\gamma\delta$ T cells.

$\gamma\delta$ T cells respond to malarial HSPs. It has been well documented that $\gamma\delta$ T cells respond to *Mycobacterium tuberculosis* HSPs (5, 14, 19, 33, 38). Therefore, we tested the ability of $\gamma\delta$ T cells elicited in a malaria infection to respond to *Plasmodium* HSPs. BALB/c and C57BL/6 mice were infected with 10^6 parasitized RBCs, and the spleens were tested in lymphoproliferation assays at three different time points. Splenocytes were separated into three groups: unfractionated, $\gamma\delta$ enriched, and $\alpha\beta$ enriched. The $\gamma\delta$ -enriched and the $\alpha\beta$ -enriched T cells were obtained by treatment with a cocktail of antibodies followed by complement treatment (described in Materials and Methods) and were found to be greater than 90% pure as determined by flow cytometry (data not shown). Figure 6 shows the results obtained with BALB/c mice. The total T-cell population did not respond strongly on days 9 and day 12 postinfection, during which time a productive infection was

TABLE 1. Changes in parasitemia and percent $\gamma\delta$ T cells during infection with nonlethal and lethal strains of *P. yoelii*

<i>P. yoelii</i> strain and dose	Day	% Parasitemia	% $\gamma\delta$ T cells
17XNL (10^6)	0	0	2.8
	3	0.1	3.3
	6	1.8	8.2
17XL (10^4)	0	0	2.3
	3	0	1.8
	5	3.7	1.7
	7	61	2
17XL (10^6)	0	0	2.1
	3	1.8	2.1
	5	74.6	2.4

^a BALB/c mice were infected with 10^6 *P. yoelii* 17XNL-parasitized RBCs or 10^6 or 10^4 *P. yoelii* 17XL-parasitized RBCs. Parasitemia was measured by microscopy, and $\gamma\delta$ T cells were monitored by the staining of splenocytes with anti-CD3 and anti- $\gamma\delta$ TCR MABs.

occurring. On day 20, however (8 days after resolution of the infection), there was a strong proliferative response by total T cells to the *P. yoelii* lysate (SI = 10.5), as well as to *Plasmodium* recombinant HSPs of 60 kDa (SI = 14.6) and 70 kDa (SI = 15.2). Figure 6B shows the results of the proliferation of the $\gamma\delta$ T-cell-enriched population. On day 9 there was little proliferation with all three antigen preparations, but there was a significant proliferative response to *P. falciparum* HSP 60 (SI = 6.5 on day 12 and 6 on day 20) and HSP 70B (SI = 4.5 on day 12 and 9.4 on day 20) later in infection. These $\gamma\delta$ T cells did not proliferate when stimulated in the presence of the *P. yoelii* extract. Likewise, the $\alpha\beta$ -enriched T-cell population showed a proliferative response to HSP 60 (SI = 5.4 on day 12 and 8.0 on day 20) and HSP 70B (SI = 7.7 on day 20). None of these cell populations responded by proliferation (SI < 2.0) in the presence of an unrelated parasite antigen, Pfg27 (35), used as a negative control (data not shown). These results demonstrate that the $\gamma\delta$ T cells elicited during infection in BALB/c mice with *P. yoelii* respond by proliferation in the presence of HSP 60 and HSP 70B of *P. falciparum*. Similar proliferation studies with C57BL/6 mice were inconclusive (data not shown), and

FIG. 6. Proliferative responses to *P. yoelii* extracts and *P. falciparum* HSP 60 and HSP 70. BALB/c mice were sacrificed on day 9, 12, or 20 after infection with *P. yoelii* 17XNL. Spleens were removed and treated with a cocktail of antibodies. (A) Total cells (no antibody treatment); (B) $\gamma\delta$ T-cell enriched (anti-*I-A^d*, anti- $\alpha\beta$, anti-CD4, anti-CD8); (C) $\alpha\beta$ T-cell enriched (anti-*I-A^d*, anti- $\gamma\delta$). The cells were plated at 2×10^5 /well of a 96-well plate in the presence of *P. yoelii* extract (10^6 parasitized RBCs/ml), *P. falciparum* HSP 60 (10 μ g/ml), or *P. falciparum* HSP 70 (10 μ g/ml) with irradiated APCs (3×10^5 /well). On day 5, the cells were pulsed with 1 μ Ci of [3 H]thymidine, and 18 h later they were harvested. Radioactive counts in cells incubated in medium alone were 53 (day 9), 87 (day 12), and 103 (day 20) in group A; 115 (day 9), 88 (day 12), and 76 (day 20) in group B; and 108 (day 9), 140 (day 12), and 127 (day 20) in group C. Results are expressed as mean SI and standard deviation.

TABLE 2. RT-PCR analysis of $\gamma\delta$ TCR^a

TCR chain	Chain expression with:		
	J1	J2	J4
Gamma			
V1.1	—	—	—
V1.2	—	+	+
V2	—	—	—
Delta			
V4	+		
V5	+		
V6	—		

^a +, PCR amplification (various combinations of V and J primers)-positive results; —, negative results.

these were consistent with the experience of other investigators with C57BL/6 mice (20).

$\gamma\delta$ TCR analysis. Finally we examined $\gamma\delta$ TCR usage in mice that had been infected with *P. yoelii* 17XNL-infected RBCs 10 days prior to RT-PCR analysis. The results (Table 2) show that the γ chain, consisting of V1.2 with the J2 and J4 segments, and the δ chain, consisting of V4 and V5 with the J1 segment, were predominantly expressed in the splenocytes of infected mice.

DISCUSSION

$\gamma\delta$ T cells accumulate in a wide variety of infections, providing both potentially beneficial and pathological roles (6, 8, 17, 18, 21, 23–25, 29, 32, 34, 38, 42). The biological role of these cells in immunity is unclear; however, recent studies indicate that $\gamma\delta$ T cells influence both early innate and antigen-specific adaptive host responses. Since the discovery of $\gamma\delta$ T cells in 1986 (7), significant advances in our understanding of the immunobiological role of these cells have been made, although this progress has occurred much more slowly than our understanding of the $\alpha\beta$ T-cell population. The biological role of $\gamma\delta$ T cells seems to vary with the organism causing infection, the anatomical location of the $\gamma\delta$ T cell, and the unconventional ways in which these cells recognize antigen (see references 8, 11a, 18, 36, and 38 for reviews).

The studies described in this paper were undertaken to enhance our understanding of the role of the $\gamma\delta$ T cell in a malaria infection. Our strategy was to analyze $\gamma\delta$ T cells and their antigen specificity during infections with avirulent and virulent strains of *P. yoelii* by using less susceptible (BALB/c) and more susceptible (C57BL/6) strains of mice. The two inbred strains of mice (BALB/c and C57BL/6) display marked differences in the course of infection, which was extended by 12 days and was associated with four- to fivefold higher levels of parasitemia in the more susceptible C57BL/6 mice than in BALB/c mice. Initial experiments examined the spleen and lymph nodes for changes in $\gamma\delta$ T-cell populations. In subsequent studies, we focused on the spleen as the target organ based on our observation that it displayed marked differences in the percentage of $\gamma\delta$ T cells. Moreover, previous studies have demonstrated a critical role for spleen in the control of rodent malarial (45). As shown in Fig. 2, there were significant differences in the accumulation of $\gamma\delta$ T cells in the two mouse strains, with a much slower accumulation in C57BL/6 (relatively more susceptible) mice.

The increase in the percentage of $\gamma\delta$ T cells in both strains of mice coincided with the peak percent parasitemia and declined thereafter. The simplest interpretation of these results

may be that in BALB/c mice, $\gamma\delta$ T cells could play a role in controlling acute parasite infection (percent parasitemia and duration), resulting in a less virulent (resolving) infection. Further support for such an interpretation was provided by the lack of any such increase during infection with highly lethal parasites, even when a 2-log-lower inoculum was used in an attempt to alter the time course of rising parasitemia. Previous studies have also reported up to a 10-fold expansion of splenic $\gamma\delta$ T cells during *P. chabaudi* infections in mice (41). Antibody depletion of $\gamma\delta$ T cells in those studies resulted in exacerbated *P. chabaudi* malaria, suggesting a beneficial role for these T cells. Colocalization of $\gamma\delta$ T cells and parasite infection in the red pulp of the spleen might indicate a possible location for destruction of the parasite (4). More recently, Tsuji et al. have shown a partially protective role for a $\gamma\delta$ T-cell clone against the hepatic stage of malaria infection (40). However, this $\gamma\delta$ T-cell clone had no effect against the erythrocytic stage of infection. In a different rodent malaria model (*P. berghei*), $\gamma\delta$ T cells were shown to participate in the pathogenesis of malaria leading to cerebral malaria (44). Thus, it appears that $\gamma\delta$ T cells might provide a beneficial function in the early phase of infection with less virulent parasites. Other cell-mediated and humoral immune mechanisms might be more effective in chronic phase of infection. Nonetheless, these studies do support the notion that $\gamma\delta$ T cells might provide a first line of defense or early innate immune mechanism during the host response to infectious agents.

We also examined CD4⁺ and CD8⁺ $\alpha\beta$ T-cell changes during infection in these mice. In both strains of mice we observed a decrease in the percentage of CD8⁺ T cells which coincided with peak parasitemia. This could be due to a direct impact of the rising $\gamma\delta$ T-cell percentage on CD8⁺ cells or could indicate that CD8⁺ cells are not essential as the primary mediators of the blood stage malaria infection with nonlethal *P. yoelii*. Several studies have suggested that CD4⁺ T cells play an important role in the elimination of malaria infections in mice (13, 42). In our studies, the number of CD4⁺ cells remained relatively constant in the BALB/c mouse infection but decreased in the C57BL/6 mouse infection from roughly 60% to 35% of total cells at peak parasitemia. We do not know if the drop in the CD4⁺ T-cell population in the more susceptible C57BL/6 mice is directly accountable for our findings of a lower accumulation of $\gamma\delta$ T cells. van der Heyde et al. (42) have suggested that CD4⁺ T cells modulate $\gamma\delta$ T-cell responses. Thus, a combination of decreasing numbers of CD4⁺ T cells and lower accumulation of $\gamma\delta$ T cells in C57BL/6 mice might provide one possible immunological mechanism resulting in a more susceptible phenotype, i.e., extended duration of the course of infection and much higher peak parasitemia than in the less susceptible BALB/c mice.

Further support for a beneficial role for $\gamma\delta$ T cells was provided by studies in which we compared $\gamma\delta$ T cells during infection with lethal and nonlethal parasites. We do not know what triggers the activation of $\gamma\delta$ T cells during infection with avirulent *P. yoelii*. It appears that $\gamma\delta$ T cells or their soluble mediators participate in resolving infection caused by nonlethal parasites. Our studies also suggest that these cells participate primarily early in infections in naive mice. Once CD4⁺ and CD8⁺ T cells have been primed during primary infections, they might provide additional mechanisms involved in suppressing parasitemia during subsequent infections. Further studies are needed to evaluate the role of the $\gamma\delta$ T cell during infections with virulent and avirulent strains of malaria.

Finally, we investigated whether *Plasmodium* HSPs are recognized as antigens by the $\gamma\delta$ T cells elicited during malaria infections. $\gamma\delta$ T cells elicited by various infectious organisms

recognize a peptide epitope in *M. tuberculosis* HSP 65 (5, 6, 14, 18, 19, 33) including a $\gamma\delta$ T-cell clone with protective activity against the liver stage of infection of *P. yoelii* (40). More recent studies have demonstrated that $\gamma\delta$ T cells also recognize epitopes in nonpeptidic phosphorylated antigens (3, 9, 30). The studies described here demonstrate that $\gamma\delta$ T cells elicited during malaria infection proliferate in response to *P. falciparum* HSPs of 60 and 70 kDa. There was very little proliferative response to any of the antigens tested in total splenocytes, as well as those enriched in $\gamma\delta$ T cells and in $\alpha\beta$ T cells at the earliest time point, and this was not a result of high background proliferation, since cells incubated in medium alone had comparable counts. Moreover, the cells in all groups tested at all three time points responded vigorously in the presence of concanavalin A (data not shown). Similar suppressed lymphoproliferative responses early during a malaria infection are well documented (10, 37).

Plasmodium HSPs have approximately 60% homology to mouse and other eukaryotic HSPs, and therefore it is conceivable that these $\gamma\delta$ T cells can also recognize self HSPs and that this could account for the elevated $\gamma\delta$ T-cell levels that remain in the spleen long after the infection has been resolved. Since $\gamma\delta$ T cells elicited during infection with *P. yoelii* respond to epitopes in HSP 70 and HSP 60, we also investigated if immunization with purified HSP 70 will elicit specific $\gamma\delta$ T cells. However, the results of these studies were inconclusive, since the incomplete Freund's adjuvant used during immunization has been shown earlier to elicit $\gamma\delta$ T cells, as was also found in our own studies (results not shown). In *Toxoplasma gondii* infections, $\gamma\delta$ T cells play an essential role in the control of the infection, and HSP 65 expression on macrophages was essential for this apparently $\gamma\delta$ T-cell-dependent protection. In these studies, virulent forms of the parasite were less able to induce HSP 65 expression on macrophages (31). We do not know if similar mechanisms might participate during malarial infection.

In the present study, we also examined the preferential usage of V and J segments of the TCR of the $\gamma\delta$ T cells in the spleens elicited during the infection. Surprisingly, not only did we find $\gamma\delta$ T cells that are located primarily in the spleen but we also found VJ gene rearrangements primarily associated with the epidermal areas of the mice. This might suggest that these cells are eventually recruited to the spleen at the time of infection and could account for differences seen in *Plasmodium* infections in which antibody-mediated $\gamma\delta$ T-cell knockout mice show differences in infection compared to gene-disrupted T-cell knockout mice (27, 43).

Overall, the interaction between $\gamma\delta$ T cells and malaria appears to be complex, with variations not only among strains of mice used but also among strains of parasites. Additionally, interplay of other T cells ($CD4^+$, $CD8^+$) may greatly affect the course of acute versus chronic infection as well as accumulation of $\gamma\delta$ T cells. Since $\gamma\delta$ T cells do respond to HSPs of the parasite, perhaps one strategy would be to obtain *Plasmodium* HSP-specific $\gamma\delta$ T-cell clones and directly evaluate their function in vivo and in vitro during infection with the malaria parasite.

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