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Exacerbation of *Plasmodium chabaudi* malaria in mice by depletion of TCR$\alpha\beta^+$ T cells, but not TCR$\gamma\delta^+$ T cells

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SUMMARY

Although $\gamma\delta$ T cells are found in increased numbers in the spleens of humans and mice infected with malaria, it is not known if these cells are necessary components of an effective immune response. The surface phenotype of spleen cells obtained from mice infected with avirulent *Plasmodium chabaudi adami* or virulent *Plasmodium chabaudi chabaudi* were examined using anti-δ or anti-$\alpha\beta$ T-cell-specific reagents and flow cytometry. Levels of parasitaemia, red blood cell (RBC) counts, and survival times were followed in mice depleted of tumour necrosis factor (TCR)$\gamma\delta^+$ or TCR$\alpha\beta^+$ T cells. Numbers of $\gamma\delta$ T cells increased in the spleens of control antibody-treated infected mice, but not in mice depleted of TCR$\gamma\delta^+$ or TCR$\alpha\beta^+$ T cells. Mice depleted of $\gamma\delta$ T cells had levels of parasitaemia, RBCs, and survival rates similar to control antibody-treated mice. However, mice depleted of TCR$\alpha\beta^+$ T cells had higher levels of parasitaemia, lower RBC counts, and decreased survival rates. These results indicate that TCR$\alpha\beta^+$ but not TCR$\gamma\delta^+$ T cells play an essential role in host defense against *P. chabaudi* infection in mice.

INTRODUCTION

Recent reports show that numbers of $\gamma\delta$ T cells are significantly increased in the spleens of mice[1–3] and peripheral blood and spleens of humans[4–7] infected with malaria. $\gamma\delta$ T cells have been shown to inhibit the growth of *Plasmodium falciparum in vitro*[8] and to contribute to immunity against the liver stages of *P. yoelii* 17X NL in $\alpha\beta$ T-cell-deficient mice.[9] However, $\gamma\delta$ T-cell-deficient but not $\alpha\beta$ T-cell-deficient mice were capable of clearing the erythrocytic stages of *Plasmodium yoelii* 17X NL.[9] The inability of the latter study to show a role for $\gamma\delta$ T cells in the clearance of erythrocytic stages may have been because of the use of *P. yoelii;* infection with this parasite is thought to be controlled primarily by antibody-mediated mechanisms.[10,11] The purpose of the present study was to examine the relative importance of different T-cell subsets in controlling parasitaemia in mice infected with *P. chabaudi.* This murine malaria model is thought to be primarily controlled by cell-mediated immune mechanisms[11] and therefore may be a better model than *P. yoelii* with which to observe effects attributable to $\gamma\delta$ T cells.

MATERIALS AND METHODS

Mice

BALB/cBy female mice between 8 and 10 weeks of age were used. Mice were obtained from the Animal Breeding Facility of the Trudeau Institute. They were free from the common murine viral pathogens according to screening tests routinely performed by the Research Animal Diagnostic Laboratory (University of Missouri, Columbia, MO).

Parasites and infections

Lactate dehydrogenase (LDH) virus-free, *Plasmodium chabaudi adami* and *Plasmodium chabaudi chabaudi* CB were maintained as frozen stock at −150. Mice for each experiment were injected intraperitoneally (i.p.) with 10^6 parasitized RBCs obtained from infected BALB/cBy source mice.

Determination of parasitaemia and anaemia

Blood was obtained from the tails of mice. RBC counts were determined manually by diluting 5 μl of blood in 995 μl of phosphate-buffered saline (PBS) and counting cells placed in a haemacytometer. Thin blood smears were stained with Giemsa stain and levels of parasitaemia determined by counting 200–1000 erythrocytes.

In vivo *cell depletion*

Mice were depleted of $\alpha\beta$ T cells or $\gamma\delta$ T cells by treatment with monoclonal antibodies (mAbs) directed against surface markers of these cells. Rat anti-mouse hybridomas secreting mAb against the CD4 (clone GK1.5, ATCC no. TIB 207) and CD8 (clone 2.43, ATCC no. TIB 210) T-cell determinants and the hamster anti-mouse hybridoma secreting mAb against pan $\alpha\beta$ T cells (clone H57-597, ATCC no. HB 218) were obtained from the American Type Culture Collection (Rockville, MD). The hamster anti-mouse 3A10 hybridoma secreting mAb against

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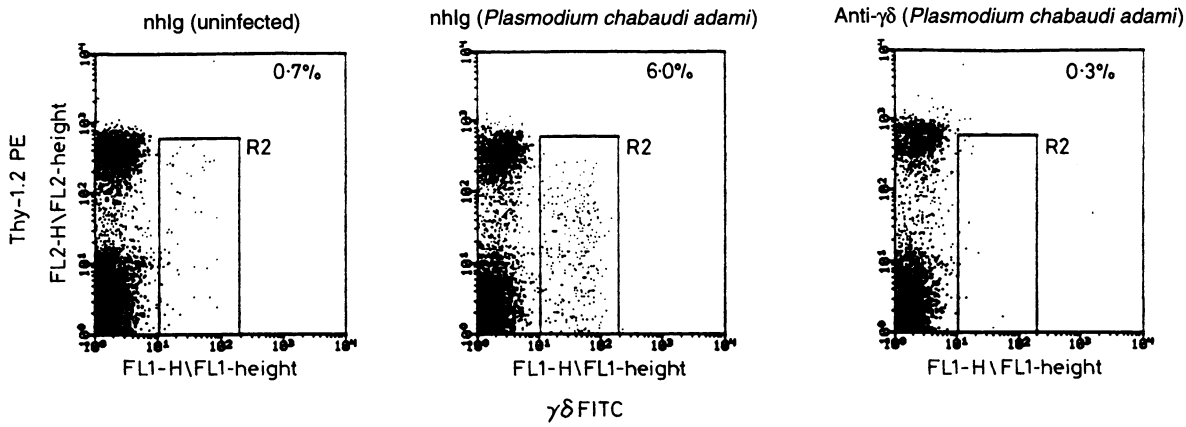

Figure 1. Two-colour flow cytometric analysis of spleen cells obtained from normal mice (panel 1) or mice infected with *Plasmodium chabaudi adami* (panels 2,3) after administration of normal hamster immunoglobulin (panels 1,2) or anti-$\gamma\delta$ mAb (panel 3). Mice were injected i.p. with 200 μg of normal hamster immunoglobulin or anti-$\gamma\delta$ mAb (3A10) mAb 1 day prior to infection and again 3 days following infection. Spleen cells were collected on day 24 of infection and stained with the mAb as described in the Materials and Methods. The percentage of $\gamma\delta$ T cells are shown in the upper right corner of each panel.

pan $\gamma\delta$ T cells[12] was obtained from Dr S. Tonegawa (Massachusetts Institute of Technology, Cambridge, MA). Hybridoma ascites fluids were enriched for IgG by ammonium sulphate precipitation, and mAb were further purified on a diethylaminoethyl (DEAE) column. For *in vivo* depletion of $\alpha\beta$ T cells, mice were injected i.p. with 0·5 mg each of GK1.5 and 2.43 or 200 μg of H57 mAbs 1 day prior to infection and 3 days following infection. For *in vivo* depletion of $\gamma\delta$ T cells, mice were injected i.p. with 200 μg of 3A10 mAb 1 day prior to infection and 3 days following infection. Normal hamster immunoglobulin (nhIg) (Rockland, Gilbertsville, PA) was used as control antibody for 3A10 and H57 mAbs. Rat $IgG2_b$ (LTF6.1, an anti-keyhole limpet haemocyanin mAb developed in our laboratory) was used as isotype-matched control mAb for GK1.5 and 2.43. The extent of T-cell depletion was determined by flow cytometric analysis.

Flow cytometric analysis

$F(ab')_2$ fragments of mAb directed against surface antigens Thy-1 or T-cell receptor (TCR)$\alpha\beta$ (hybridomas 30.H.12 and H57-597; ATCC) were prepared and conjugated to either fluorescein isothiocyanate (FITC) or biotin as described previously.[13] A hamster anti-mouse hybridoma UC7-13D5 producing mAb against TCRcd[14] was obtained from Dr J. Bluestone (The University of Chicago, Chicago, IL). Monoclonal antibodies were purified from ascites fluid by ammonium sulphate precipitation followed by DEAE chromatography. FITC-conjugated $F(ab')_2$ goat anti-hamster IgG was purchased from Jackson ImmunoResearch Lab (West Grove, PA). Single-cell suspensions of spleen cells were obtained by gently pushing spleens through wire mesh and freed of RBCs by lysis. Spleen cells (1×10^6) were first incubated with biotinylated $F(ab')_2$ anti-Thy-1.2 mAb followed by streptavidin-phycoerythrin (PE) (Becton Dickinson, Mountain View, CA). The cells were then either directly stained with FITC-conjugated $F(ab')_2$ anti-TCR$\alpha\beta$ mAb or incubated with whole anti-TCR$\gamma\delta$ mAb (UC7-13D5) followed by FITC-conjugated $F(ab')_2$ goat anti-hamster IgG. It was confirmed that 3A10 or UC7-13D5 mAb were not cross-reactive with $\alpha\beta$ T cells. Data were acquired for 2500–10 000 events per sample on a FACScan cytofluorometer (Becton Dickinson) and analysed using LYSIS II software.

Statistical analysis

A two-sample Student's *t*-test was used to compare numbers of positively stained cells, levels of parasitaemia, or RBC counts. Statistical significance of survival data were analysed using Kruskal–Wallis one-way analysis of variance on ranks followed by Dunn's method of multiple comparison to isolate groups that differ.

RESULTS

FACS analysis of splenic T cells from *P. chabaudi adami*-infected mice

Spleen cells obtained from mAb-treated, *P. chabaudi adami*-infected mice were analysed using two-colour flow cytometry. In contrast to uninfected, nhIg-treated mice, *P. chabaudi adami*-infected mice had significantly increased numbers of splenic $\gamma\delta$ T cells on day 24 of infection (Fig. 1). This result confirms the finding of others[2] that $\gamma\delta$ T cells increase in numbers primarily during resolution of parasitaemia. Treatment of mice with 3A10 mAb 1 day prior to and 3 days following infection with *P. chabaudi adami* resulted in significantly decreased numbers of splenic $\gamma\delta$ T cells detectable by flow cytometry (Fig. 1). The effects of two treatments with 3A10 mAb appeared to last throughout the course of the experiment (Fig. 2a).

Effects of depletion of T-cell subsets on parasitaemia and anaemia

To determine the effects of depletion of T-cell subsets on the course of *P. chabaudi adami* infection, BALB/cBy mice were treated with anti-CD4 plus anti-CD8, anti-TCR$\alpha\beta$, or anti-TCR$\gamma\delta$ mAb as described in the Materials and Methods. Spleens of mice treated with anti-$\gamma\delta$ T-cell mAb (3A10) had barely detectable numbers of $\gamma\delta$ T cells throughout the course

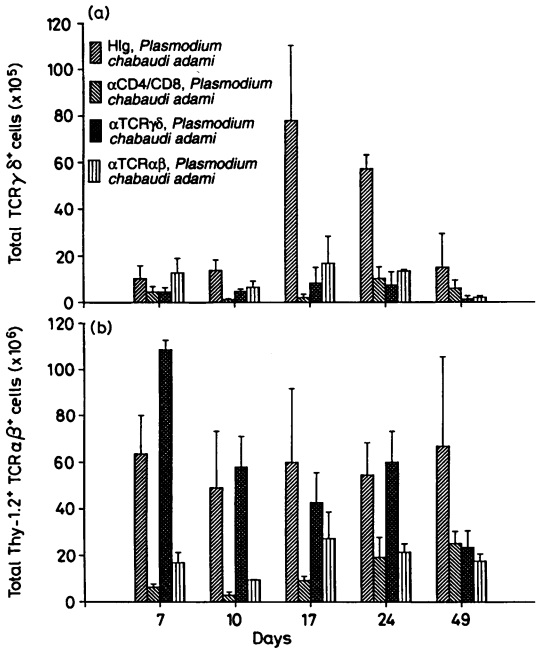

Figure 2. Effects of *in vivo* administration of normal hamster immunoglobulin, anti-CD4/CD8 mAb, anti-TCR$\gamma\delta$ mAb, or anti-TCR$\alpha\beta$ mAb on the total number of (a) TCR$\gamma\delta^+$ or (b) Thy-1.2$^+$ TCR$\alpha\beta^+$ spleen cells obtained from *Plasmodium chabaudi adami*-infected mice on the indicated days. Mice treated as described in the Materials and Methods. Numbers of cells determined by flow cytometry as described in the Materials and Methods. Values are means ± SD for groups of three mice. Mean SD values of total TCR$\gamma\delta^+$ ($\times 10^5$) or TCR$\alpha\beta^+$ ($\times 10^6$) cells in uninfected control mice for the periods tested are 5·4 ± 3·4 and 24·2 ± 7·3, respectively.

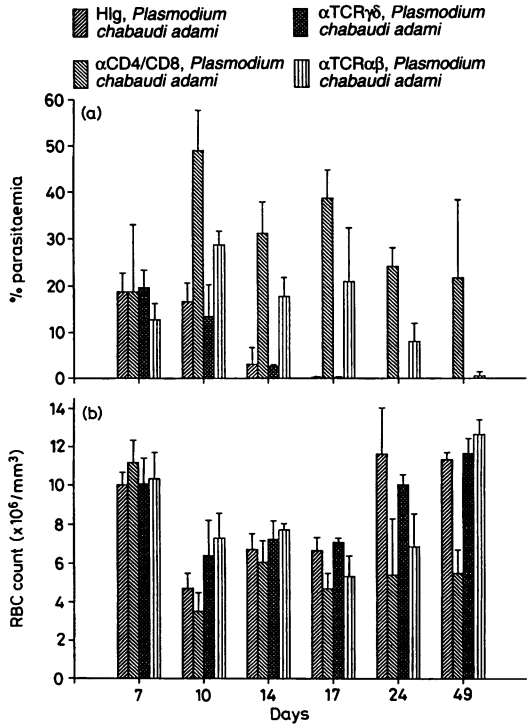

Figure 3. Effects of *in vivo* administration of normal hamster immunoglobulin, anti-CD4/CD8 mAb, anti-TCR$\gamma\delta$ mAb, or anti-TCR$\alpha\beta$ mAb on the (a) percentage of *Plasmodium chabaudi adami*-parasitized erythrocytes or (b) red blood cell count of infected animals. Normal hamster immunoglobulin-treated uninfected mice included as controls. Values are means SD for groups of three mice. Mean ($\times 10^6$/mm^3) ± SD RBC count for uninfected control mice for the period tested was 10·3 ± 0·4.

of infection when compared with nhIg-treated infected mice that had significantly increased numbers of $\gamma\delta$ T cells, especially during the resolution of parasitaemia (Fig. 2a). However, $\gamma\delta$ T-cell-depleted mice also had increased numbers of TCR$\alpha\beta^+$ cells (Fig. 2b) and CD4$^+$ T cells (data not shown) on day 7 of infection. Spleens of mice depleted of TCR$\alpha\beta^+$ cells, by treatment with anti-CD4 plus anti-CD8 or anti-TCR$\alpha\beta$ mAbs, remained relatively depleted of Thy 1.2$^+$ TCR$\alpha\beta^+$ cells through day 49 of infection when compared with nhIg-treated mice (Fig. 2b). Experiments using *P. chabaudi adami*-infected mice treated with LTF6.1 isotype-matched control rat IgG2$_2$ mAb or phosphate-buffered saline had levels of parasitaemia similar to untreated infected mice (data not shown). On days 10, 17 and 24 of infection, mice depleted of TCR$\alpha\beta^+$ T cells also had significantly fewer $\gamma\delta$ T cells than infected mice injected with nhIg ($P < 0{\cdot}05$). Although mice depleted of TCR$\alpha\beta^+$ cells by treatment with H57 mAb had reduced levels of $\gamma\delta$ T cells, these mice had at least as many $\gamma\delta$ T cells throughout the course of infection as mice treated with 3A10 $\gamma\delta$ T-cell-depleting mAb (Fig. 2a). These results suggest that $\alpha\beta$ T cells are required for the expansion of $\gamma\delta$ T cells in malaria-infected mice.[15]

Mice depleted of $\gamma\delta$ T cells following 3A10 treatment had levels of parasitaemia similar to nhIg-treated, infected mice ($P > 0{\cdot}05$); both groups resolved their infections by day 24 of infection (Fig. 3a). In contrast, mice depleted of $\alpha\beta$ T cells had increased levels of parasitaemia that were not resolved by day 49 of infection (Fig. 3a). These two groups differed significantly from the groups treated with nhIg or anti-TCR$\gamma\delta$ antibodies on days 10, 14, 17, 24 and 49 of infection ($P < 0{\cdot}05$). *Plasmodium chabaudi adami*-infected mice depleted of $\gamma\delta$ T cells became no more anaemic than did nhIg-treated infected control mice (Fig. 3b). However, mice depleted of TCR$\alpha\beta$ T cells by treatment with anti-CD4 and anti-CD8 mAb became increasingly more anaemic on days 17, 24 and 49 of infection ($P < 0{\cdot}05$) than did control mice or mice depleted of $\gamma\delta$ T cells (Fig. 3b). Mice treated with anti-TCR$\alpha\beta$ were more anaemic on days 17 and 24 of infection than were mice treated with anti-TCR$\gamma\delta$ antibodies.

Effects of $\gamma\delta$ T-cell depletion on infection with lethal *Plasmodium chabaudi chabaudi* CB

The effects of depleting $\alpha\beta$ T cells or $\gamma\delta$ T cells in BALB/c mice infected with virulent *P. chabaudi chabaudi* CB were also studied. nhIg-treated mice infected with *P. chabaudi chabaudi* had significantly increased numbers of $\gamma\delta$ T cells when compared with nhIg-treated uninfected mice. This was not the case in infected mice treated with 3A10 $\gamma\delta$ T-cell-depleting mAb or in infected mice treated with anti-CD4 and anti-CD8 mAbs to deplete $\alpha\beta$ T cells (Fig. 4a). Treatment of infected mice with nhIg had little effect on the number of Thy-1$^+$ TCR$\alpha\beta^+$ cells produced in response to infection, but injection

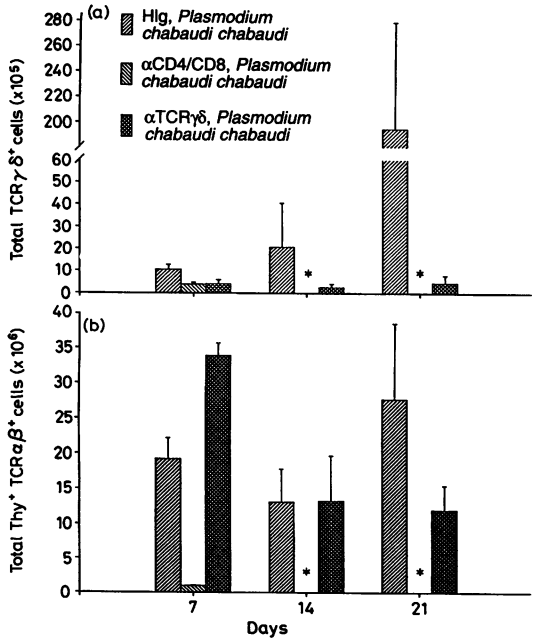

Figure 4. Effects of *in vivo* administration of normal hamster immunoglobulin, anti-CD4/CD8 mAb, or anti-TCR$\gamma\delta$ mAb on the total number of (a) TCR$\gamma\delta^+$ or (b) Thy-1.2$^+$ TCR$\alpha\beta^+$ spleen cells obtained from *Plasmodium chabaudi chabaudi*-infected mice on the indicated days. Mice treated as described in the Materials and Methods. Numbers of cells determined by flow cytometry as described in the Materials and Methods. Values are means ± SD for groups of five mice. Note that * signifies that all mice treated with anti-CD4$^+$ and anti-CD8$^+$ T-cell-depleting mAbs died prior to day 11 of infection. Mean ± SD values of total TCR$\gamma\delta^+$ ($\times 10^5$) or TCR$\alpha\beta^+$ ($\times 10^6$) cells in uninfected control mice for the periods tested are 7·8 ± 0·3 and 16·2 ± 2·8, respectively.

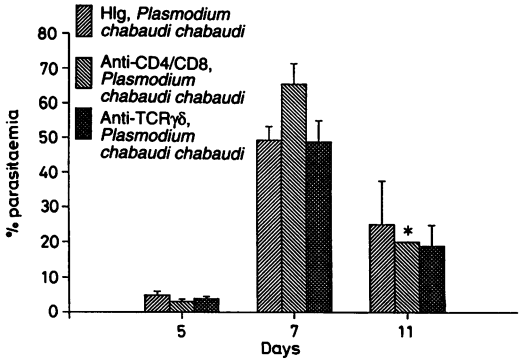

Figure 5. Effects of *in vivo* administration of normal hamster immunoglobulin, anti-CD4/CD8 mAb, or anti-TCR$\gamma\delta$ mAb on the percentage of *Plasmodium chabaudi chabaudi*-parasitized erythrocytes. Values are means ± SD for groups of five mice. Note that * signifies that only one mouse remains in the anti-CD4$^+$ and anti-CD8$^+$ T-cell-depleting mAbs group.

of 3A10 anti-$\gamma\delta$ T-cell-depleting mAb resulted an increased number of TCR$\alpha\beta^+$ cells on day 7 of infection (Fig. 4b). Infected mice depleted of CD4$^+$ and CD8$^+$ T cells had significantly reduced numbers of Thy-1$^+$ TCR$\alpha\beta^+$ cells and all died by day 11 of infection (Fig. 4b). Numbers of parasitized erythrocytes did not differ in infected mice treated with nhIg or anti-$\gamma\delta$ T-cell 3A10 mAb ($P > 0{\cdot}05$), but were significantly increased on day 7 of infection ($P < 0{\cdot}05$ in mice treated with anti-CD4$^+$ and anti-CD8$^+$ T-cell-depleting mAbs (Fig. 5). Moreover, mice treated with anti-CD4$^+$ and anti-CD8$^+$ T-cell-depleting mAbs all died by day 11 of infection; sooner than infected mice treated with nhIg or depleted of $\gamma\delta$ T cells ($P < 0{\cdot}007$). There was no difference in survival between groups of infected mice treated with nhIg or depleted of $\gamma\delta$ T cells (range of 10–34 days).

DISCUSSION

The results of this study show that infection of BALB/cBy mice with ordinarily avirulent *P. chabaudi adami* or virulent *P. chabaudi chabaudi* results in increased numbers of $\gamma\delta$ T cells in the spleen, which confirms the findings of others.[1–3] Depletion of $\gamma\delta$ T cells in mice infected with *P. chabaudi adami* or *P. chabaudi chabaudi* had no significant effects on levels of parasitaemia, anaemia, or survival in either model. However, depletion of TCR$\gamma\delta^+$ cells resulted in a transient increase in TCR$\alpha\beta^+$ cells on day 7 of infection (Fig. 2b and 4b). A role for the regulation of TCR$\alpha\beta^+$ by TCR$\gamma\delta^+$ cells has been previously reported.[16] While the effect of the increased number of TCR$\alpha\beta^+$ cells observed in $\gamma\delta$ T-cell-depleted mice is not known, the observation does not alter the finding that $\gamma\delta$ T-cell-depleted mice respond to *P. chabaudi* infection in a similar way to NHIg-treated mice. In contrast, depletion of TCR$\alpha\beta^+$ T cells resulted in increased levels of parasitaemia and decreased numbers of RBCs in *P. chabaudi adami*-infected mice, and decreased survival times in *P. chabaudi chabaudi*-infected mice. The most straightforward interpretation of these findings is that $\gamma\delta$ T cells, although expanding in the infected mice, are not essential for the resolution of these infections. We think that it is unlikely that $\gamma\delta$ T cells were insufficiently depleted in the treated mice since the numbers of $\gamma\delta$ T cells found in the spleen of $\gamma\delta$ T-cell-depleted mice were at the limits of detection using flow cytometry and far below the numbers in control infected mice. Also, infected mice treated with H57 mAb to deplete TCR$\alpha\beta^+$ T cells had as many or more $\gamma\delta$ T cells on days 17 and 24 of infection than mice treated with 3A10 mAb to deplete $\gamma\delta$ T cells (Fig. 2a); the H57-treated mice were significantly more susceptible to infection than were controls. It is unlikely that remaining $\gamma\delta$ T cells would act to eliminate parasites in one case but not the other unless they act in concert with $\alpha\beta$ T cells.

It is possible that treatment with 3A10 mAb may have resulted in selective depletion of some $\gamma\delta$ T-cell subsets, sparing a subpopulation that is critical to resistance. However, 3A10 mAb has been reported to recognize a determinant on the constant region of δ chains and there seems to be only one C_δ gene segment in the mouse. Thus, 3A10 appears to be a pan-murine anti-$\gamma\delta$ T-cell mAb. We conclude that $\gamma\delta$ T cells do not play an important or necessary role in controlling erythrocytic stages of malaria parasites in *P. c. adami* and *P. c. chabaudi*-infected mice.

Previous reports concerning $\gamma\delta$ T cells and murine malaria show that malaria infection results in increased numbers of $\gamma\delta$ T cells, a finding confirmed in this study. However, increased numbers of $\gamma\delta$ T cells at sites of infection do not represent cause-and-effect evidence that these T cells are involved in

immunity. Certainly relevant to this issue is the temporal kinetics of $\gamma\delta$ T cells in relation to the course of the malaria infection. Data shown in Fig. 2 locate the peak of $\gamma\delta$ T-cell induction in nhIg-treated control mice around day 17 of infection. In contrast, parasitaemia peaked on day 10 (Fig. 3a) and declined progressively thereafter. Similarly, anaemia was most severe on day 10 (Fig. 3b) and recovered progressively from that point onward. Thus, recovery, measured as reduced parasitaemia or increase in RBC count began approximately 1 week before the peak induction of $\gamma\delta$ T cells. This correlative evidence is consistent with the direct evidence from our cell-depletion experiments that suggest that $\gamma\delta$ T cells are not essential mediators of resistance to murine malaria.

Tsuji *et al.*[9] have recently reported studies of *P. yoelii* 17X NL infections of gene knockout mice lacking either TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$ T cells. In these experiments, infected mice deficient in $\alpha\beta$ T cells succumbed to blood-stage infection whereby mice deficient in $\gamma\delta$ T cells resolved their infections with normal kinetics. Langhorne *et al.*[17] have also reported similar findings using gene knockout mice. Although gene knockout mice may possess compensatory resistance mechanisms that could explain the resistance of $\gamma\delta$ T-cell knockout mice to *P. yoelii* infection, the striking similarity of our results to the results of Tsuji *et al.*[9] and Langhorne *et al.*[17] suggests that $\gamma\delta$ T cells are not likely to be important mediators of protective immunity in murine malaria infections.

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