Liver CD4−CD8− NK1.1+ TCRαβ Intermediate Cells Increase During Experimental Malaria Infection and Are Able to Exhibit Inhibitory Activity Against the Parasite Liver Stage In Vitro

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Liver CD4<sup>−</sup>CD8<sup>−</sup> NK1.1<sup>+</sup> TCRαβ Intermediate Cells Increase During Experimental Malaria Infection and Are Able to Exhibit Inhibitory Activity Against the Parasite Liver Stage In Vitro<sup>1</sup>

Sylviane Pied, Jacques Roland, Anne Louise, Danièle Voegtle, Valérie Soulard, Dominique Mazier, and Pierre-André Cazenave<sup>2,3</sup>

Experimental infection of C57BL/6 mice by Plasmodium yoelii sporozoites induced an increase of CD4<sup>−</sup>CD8<sup>−</sup> NK1.1<sup>+</sup> TCRαβ<sub>int</sub> cells and a down-regulation of CD4<sup>+</sup> NK1.1<sup>+</sup> TCRαβ<sub>int</sub> cells in the liver during the acute phase of the infection. These cells showed an activated CD69<sup>+</sup>, CD122<sup>+</sup>, CD44<sup>high</sup>, and CD62L<sup>high</sup> surface phenotype. Analysis of the expressed TCRβ<sub>β</sub> segment repertoire revealed that most of the expanded CD4<sup>−</sup>CD8<sup>−</sup> (double-negative) T cells presented a skewed TCRβ<sub>β</sub> repertoire and preferentially used Vβ2 and Vβ7 rather than Vβ8. To get an insight into the function of expanded NK1.1<sup>+</sup> T cells, experiments were designed in vitro to study their activity against P. yoelii liver stage development. P. yoelii–primed CD3<sup>+</sup> NK1.1<sup>+</sup> intrahepatic lymphocytes inhibited parasite growth within the hepatocyte. The antiplasmodial effector function of the parasite-induced NK1.1<sup>+</sup> liver T cells was almost totally reversed with an anti-CD3 Ab. Moreover, IFN-γ was in part involved in this antiparasite activity. These results suggest that up-regulation of CD4<sup>−</sup>CD8<sup>−</sup> NK1.1<sup>+</sup> αβ T cells and down-regulation of CD4<sup>+</sup> NK1.1<sup>+</sup> TCRαβ<sub>int</sub> cells may contribute to the early immune response induced by the Plasmodium during the prime infection.

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Cellular immunity is regarded as an important mechanism in resistance to infection against liver stages of malaria parasite (1). T cell–mediated immunity induced by the sporozoite, the first invasive form of the malaria parasite, and by the subsequent liver stage is directed against the infected hepatocytes able to process and present malaria Ags to various effector T cells (2, 3). These cell-mediated immune mechanisms have been extensively studied in murine models in which acquired resistance representing 40–50% of the lymphocyte population found in the liver during the infection remains to be elucidated.

T lymphocyte subpopulations of the liver contain, in addition to the conventional single-positive CD4<sup>+</sup> and CD8<sup>+</sup> T cells, a particular set of CD4<sup>−</sup>CD8<sup>−</sup> or CD4<sup>+</sup>CD8<sup>+</sup> (DN) (3) TCRαβ cells representing 40–50% of the lymphocyte population found in the liver (19, 20). These cells exhibit unusual properties because they express the NK1.1 marker (NK1.1<sup>+</sup>), lower levels of TCRs (TCRγ<sub>γ</sub>) than conventional T cells, and a variety of NK cell markers, including CD16, Ly-49A, Ly-49C, and CD122 (β-chain of the IL-2R) (21–23). Liver NK1.1<sup>+</sup> T cells have a restricted usage of the TCRβ<sub>β</sub> gene, mainly Vβ2, Vβ7, and Vβ8.2, and a single V<sub>b</sub> domain V<sub>b</sub>14 (24, 25), suggesting that these cells are selected by nonpolymorphic ligands. It has been reported that NK1.1<sup>+</sup> TCRαβ cells may recognize hydrophobic Ags, particularly lipids and glycolipids involving presentation by CD1 molecules (26–33). It is noteworthy that DN αβ T cells account for a significant proportion of the T cells in other cellular compartments (34, 35), while they are rare in peripheral lymphoid organs and in the blood (23). This unusual liver T cell subset predominantly produces IL-4 (36–39) or IFN-γ (40), and it has been suggested that it may develop independently of the thymus (21, 22, 41).

In the present study, we have examined the kinetics of T cell responses induced in the liver of C57BL/6 mice during a malaria infection initiated by the injection of P. yoelii sporozoites. We found that P. yoelii induces the increase of intrahepatic DN NK1.1<sup>+</sup> αβ T cells during the acute phase of the infection and after remission, whereas CD4<sup>+</sup> NK1.1<sup>+</sup> αβ T cells were down-regulated.

<sup>1</sup>Abbreviations used in this paper: DN, double negative; iHL, intrahepatic lymphocyte; TCRγ<sub>γ</sub>, TCR intermediate.
Materials and Methods

Mice and parasite infection

C57BL/6 mice were purchased from Charles River (St-Aubin les Elbeuf, France). Mice were used at 6–10 wk of age. P. yoelii yoelii 265 BY strain, maintained as described previously (42), was used for these experiments. Sporozoites were obtained from infected salivary glands of Anopheles stephensi mosquitoes, 16 to 21 days after an infectious blood meal. After aseptic dissection, salivary glands were homogenized in a glass grinder and diluted in sterile PBS. Mice were infected by i.v. injection of 4000 sporozoites. Control animals were injected with sterile PBS. Parasitemia was monitored by detecting parasites every day in blood smears after Giemsa staining.

Antibodies

mAbs specific for mouse CD3 ε-chain (145-2C11), CD4 (H129.19), and CD8a (53-6.7) were obtained from Boehringer Mannheim (Meylan, France). Biotin-conjugated anti-TCRβ (HI9T-579), anti-TCRγ8 (GL3), anti-NK-1.1 (PK 136), anti-CD69 (H1.2F3), anti-CD44 (IM-7), anti-CD62L (Mel-14), and R-PE anti-CD122 (TM-β1) were purchased from PharMingen (Clinisciences, Montrouge, France). mAbs to the different TCRV gene families, Vβ2 (B20.6), Vβ3 (KJ25), Vβ4 (KT4), Vβ6 (RR4-7), Vβ7 (TR310), Vβ8.1,2,3 (F23.1), Vβ8.2 (F23.2), Vβ9 (MR10-2), Vβ10 (B21.5), Vβ11 (RR3-15), Vβ12 (MR11-1), Vβ13 (MR12-3), and Vβ14 (14.2), were all biotinylated according to the procedure described by Guesdon et al. (43). R4-6A2, a purified rat IgG1 anti-mouse IFN-γ (hybridoma ATCC HB 170), was a gift of G. Milon (Institut Pasteur, Paris, France).

Cell preparation

Livers were removed from control uninfected mice and mice infected with sporozoites 3, 10, and 30 days after infection. Liver lymphocytes (iHLS) were prepared as described by Watanabe et al. (44). Briefly, the liver was passed through stainless steel mesh and suspended in RPMI medium. After one washing, the cells were resuspended in 30% Percoll containing 100 U/ml heparin and centrifuged at 2600 rpm for 20 min at room temperature. The pellet was resuspended in ACK (ammonium chloride/potassium) buffer to lyse erythrocytes and washed twice in 3% FCS-PBS before counting.

FACS analysis and cell sorting

Staining of iHLS was performed, at 4°C for 30 min, by incubating the cells first with biotinylated mAb described before and subsequently with anti-CD3 FITC, anti-CD4 FITC, or anti-CD8 FITC in the presence of PE-conjugated streptavidin. For three-color analysis, Tri-color-conjugated CD3 FITC, anti-CD4 FITC, or anti-CD8 FITC in the presence of PE-conjugated streptavidin. For three-color analysis, Tri-color-conjugated CD3 FITC, anti-CD4 FITC, or anti-CD8 FITC in the presence of PE-conjugated streptavidin.

Enrichment in CD3int NK1.1+ T cells and CD3high NK1.1+ T cells was done by sorting performed with a FACStar (Becton Dickinson). Mononuclear cells from liver, removed 10 days after sporozoite inoculation, were two color stained with FITC-conjugated anti-CD3 and PE-conjugated anti-NK1.1 (PK 136).

Culture of malaria hepatic stages

C57BL/6 hepatocytes were prepared as described (42) with minor modification. Cells were isolated by collagenase perfusion (Boehringer Mannheim, Mannheim, Germany) of liver fragments and were further purified over a 60% Percoll gradient (Pharmacia Biotech, Uppsala, Sweden). Hepatocyte purity and viability were >95%, as assessed by trypan blue dye exclusion. Cells (8 × 10^6) were cultured in eight-chamber plastic Lab-Tek slides (Nunc, Naperville, IL) in William’s medium (Life Technologies, Edinburgh, Scotland) supplemented with 5% FCS (Life Technologies), 100 μM penicillin-100 μg/ml streptomycin solution (Life Technologies), and incubated at 37°C in 5% CO_2 for 24 h. After removal of medium from the culture chambers, 5 × 10^6 sporozoites were added in 100 μl of fresh supplemented medium. Three hours later, medium was replaced by fresh complete medium and 45 h later, cultures were stopped by ethanol fixation.

In vitro assay of parasite liver stage elimination by liver cells

This was done as follows: 3 h after addition of sporozoites to hepatocyte cultures, the medium was replaced by CD3^high NK1.1+ and CD3^int NK1.1+ T cell preparations (purity >99%) from infected and uninfected control mice were added. Cultures were incubated for 45 h, with a change of 50 μl of medium 24 h after parasite inoculation. Anti-IFN-γ (dilution 1/100)or anti-CD3 (dilution 1/300) was added with or without NK1.1+ T cells to hepatocyte cultures 3 h after sporozoite inoculation, and was maintained constant throughout the experiment by adding Abs in fresh medium during medium change. Schizont numbers were assessed in triplicate cultures by immunofluorescence staining using hyperimmune sera recognizing P. yoelii liver stages. Percent inhibition was calculated by comparing the number of parasites in the experimental cultures with the number in control wells.

Results

Increase of CD4+CD8+ TCRαβ+ cells in the liver of mice infected with P. yoelii

Liver lymphocytes were prepared from naive and P. yoelii-infected C57BL/6 mice. The phenotype of liver T cells was first examined by two-color FACS analysis with anti-TCRαβ, anti-TCRγ8, and anti-CD3 mAbs at different time points after sporozoite inoculation (3, 10, and 30 days). Results obtained from three separate experiments (Table I) showed that the inflammatory response induced in the liver by P. yoelii infection may cause a cellular influx to the site of parasite development or a proliferation of in situ lymphoid cells. Accordingly, the total number of iHLS recovered from the livers of infected mice increased in time when compared with noninfected control mice. The most marked expansion (11.5-fold) was observed at day 10 after sporozoite inoculation, corresponding to the peak of parasitemia. On day 30, more than 1 wk after mice had recovered from the infection, the absolute numbers of all lymphocyte populations were still high compared with control. Both TCR γδ and αβ cell populations expanded, showing a polyclonal proliferation of the iHLS induced by the parasite.

αβ T lymphocytes in the liver have been reported to be a heterogeneous population comprising both conventional cells and an

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Table 1. Phenotype of liver T cells from noninfected C57BL/6 mice and after infection with P. yoelii

<table>
<thead>
<tr>
<th>Days After Infection</th>
<th>Mean Number of Total Cells (×10^6 ± SD)</th>
<th>Percentage of Positive Cells (absolute number × 10^6 ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD3</td>
</tr>
<tr>
<td>Control</td>
<td>0.89 ± 0.1</td>
<td>43.5 ± 5.7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>(0.38 ± 0.005)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>63.6 ± 8.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.50 ± 0.05)</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>46.1 ± 4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.7 ± 0.08)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54.7 ± 4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.7 ± 0.03)</td>
</tr>
</tbody>
</table>

* Data are expressed as the mean number of the results obtained from five mice tested individually in each group.
unusual subset, so-called TCR intermediate (TCR\text{int}), expressing 2-3-fold lower TCR surface density. As αβ T cells constitute the major lymphocyte population stimulated in the liver by the parasite, we have examined the profile of TCR\text{ab} expression during the course of infection. Surprisingly, as shown on Fig. 1A, \textit{P. yoelii} infection did not reduce the proportion of CD3\textsuperscript{+} TCR\textabduce{int} in the liver because the percentages of both TCR\textabduce{int} and TCR\textabduce{high} cells were increased on days 3, 10, and 30 of infection when compared with noninfected control mice. To characterize these TCR\textabduce{int}, three-color cytofluorometric analysis was conducted using anti-CD4 and anti-CD8 mAbs. Gating was done on αβ T cells to display the CD4 and CD8 profiles among these cells. Liver αβ T cells from control mice were largely composed of the CD4 subpopulation of both low and high intensity. After 3 days of infection, \textit{P. yoelii} sporozoites induced a proliferation of CD4\textsuperscript{+} T cells of high intensity and a loss of CD4\textsuperscript{+} T cells of low intensity (data not shown). Then, on days 10 and 30, a decrease in the number of total CD4\textsuperscript{+} TCR\textabduce{int} cells was observed, showing that this cell subpopulation became a minor population in the liver during the acute phase of the infection (Fig. 1B). This decrease in CD4\textsuperscript{+} TCR\textabduce{int} cell subsets was accompanied by an increase in CD8\textsuperscript{+} TCR\textabduce{int} cell subpopulations and the appearance of DN TCR\textabduce{int} cells of high and intermediate intensity that persisted at day 30 (Fig. 1B). However, when the gating was done on total CD3\textsuperscript{+} iHL, an increase in the number of total CD4\textsuperscript{+} cells was observed at days 3 and 10 after infection. This suggests that this increase in total CD4\textsuperscript{+} T cells is due to an expansion of CD4\textsuperscript{+} cells belonging to the γδ lineage (Fig. 1C).

\textit{P. yoelii} infection induces an increase in CD4\textsuperscript{−}CD8\textsuperscript{−}NK1.1\textsuperscript{+} T cells and a down-modulation of CD4\textsuperscript{+}NK1.1\textsuperscript{+} T cells in the liver

Because the liver of C57BL/6 mice contains a major subset of CD4\textsuperscript{−}CD8\textsuperscript{−} αβ T cells expressing the NK1.1 surface marker (19, 20), we have analyzed the kinetics of expression of this T cell subset by estimating the percentage of these cells among the iHLS from the liver of mice infected with \textit{P. yoelii}. As shown in Fig. 2, the percentage of liver CD3\textsuperscript{+} NK1.1\textsuperscript{+} T cells increased significantly during the intrahepatocytic as well as the erythrocytic phase of the parasite development (day 10) and was still high after remission (on day 30 after infection). NK1.1\textsuperscript{+} are a major subset of T cells in the liver, and can be either CD4\textsuperscript{+} or DN (19, 20). As we

![FIGURE 1. A, FACS analysis of expression by liver CD3\textsuperscript{+} T cells isolated from control and infected C57BL/6 mice 3, 10, and 30 days after \textit{P. yoelii} sporozoite inoculation. Black histograms represent the controls and open histogram, cells from infected mice. Gating was done on small lymphocytes. M1 indicate CD3\textsuperscript{+} TCR\textsuperscript{\alpha\beta}\textsuperscript{int} cells and M2, CD3\textsuperscript{+} TCR\textsuperscript{\alpha\beta}\textsuperscript{high} cells. B, CD4, CD8, and DN T cells representation among TCR\textsuperscript{\alpha\beta}-positive cells. Recovered liver cells were triple stained with biotin-conjugated anti-CD4 mAb revealed with Tri-color, FITC-conjugated anti-CD8 mAb, followed by PE-conjugated anti-TCR\textsuperscript{\alpha\beta} mAb. DN T cells among TCR\textsuperscript{\alpha\beta}-positive cells were estimated after labeling with FITC-conjugated anti-CD4 + anti-CD8 mAbs. Numbers of lymphomyeloid liver cells recovered from five individual mice per group were naive, 7.45 \times 10^5 \pm 7.9 \times 10^5; day 3, 14.2 \times 10^5 \pm 7 \times 10^5; day 10, 10.4 \times 10^6 \pm 4.6 \times 10^6; and day 30, 3.2 \times 10^6 \pm 0.9 \times 10^6. C, Representative numbers of CD4\textsuperscript{+}, CD8\textsuperscript{+}, and CD4\textsuperscript{−}CD8\textsuperscript{−} cells among CD3\textsuperscript{+} T cells recovered from liver of mice infected with \textit{P. yoelii} 3, 10, and 30 days after sporozoite inoculation. Data are represented as the mean number of results obtained from five individuals per group. Similar results were obtained in two separate experiments.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.72.2.1465)
FIGURE 2. Effect of *P. yoelii* infection on NK1.1⁺ CD3 T cells in C57BL/6 mice. The data are displayed as a dot plot after gating on small lymphocytes. Absolute numbers of NK1.1⁺ CD3 liver lymphocytes: uninfected (0.17 × 10⁶ ± 6 × 10⁵), day 3 (0.4 × 10⁶ ± 4 × 10⁵), day 10 (1.35 × 10⁶ ± 0.6 × 10⁵), and day 30 (1.38 × 10⁶ ± 0.5 × 10⁵). Representative results from a total of five mice per group.

FIGURE 3. FACS analysis of cell surface phenotype of liver lymphocytes from C57BL/6 mice 3, 10, and 30 days after infection with *P. yoelii* sporozoites and in control mice. Liver lymphocytes were triple stained with biotin-conjugated anti-CD4 + anti-CD8 mAbs revealed with Tri-color, followed by FITC-conjugated anti-CD3 mAb and with either PE-conjugated anti-CD69, anti-CD44, anti-CD62L, or anti-CD122, respectively. Gating was done on CD4⁺ CD8⁺ CD3⁺ cells. Data are displayed as a histogram plot that represents the percentage of positive cells for each phenotypic surface marker.

previously showed that the percentage of CD4⁺ αβ T cells decreased and that of DN αβ T cells increased in *P. yoelii*-infected liver, this suggests that most of the expanded CD3⁺ NK1.1⁺ T cells are of DN TCRαβ phenotype. Accordingly, we can conclude that CD4⁺ NK1.1⁺ T cells were down-regulated.

We further analyzed the surface phenotype of DN αβ T cells, and as observed in Fig. 3, infection by *P. yoelii* up-regulates the expression of activation surface marker CD69 and the IL-2R β-chain (CD122) by CD4⁺ CD8⁺ αβ T cells. Moreover, the relative numbers of CD69⁺ and CD122⁺ cells were increased with time and reached a maximum at day 30 (Fig. 3), indicating a stimulation and an activation by the parasite itself. At day 3 of infection, no difference was observed between infected and control mice in the expression of CD44 and CD62L. DN TCRαβ cells presented a CD44low and CD62Llow phenotype, which became CD44high, CD62Llow at day 10 and CD44high, CD62Lhigh at day 30. This is consistent with the expansion of cells with a memory phenotype, followed by a proliferation and/or a recruitment of both naive and memory cells.

**TCRVβ usage of liver CD3int T cells in *P. yoelii*-infected mice**

As it has been shown that liver NK1.1⁺ TCRαβ lymphocytes use a preferential set of Vβ genes, namely Vβ2, Vβ7, and Vβ8 (45–47), we have analyzed TCRVβ-chain expression by CD3int⁺ T cells expanded in the liver during *P. yoelii* infection. As observed in two separate experiments (Fig. 4), CD3int⁺ iHL from infected mice expressed much less Vβ8 when compared with uninfected C57BL/6 control mice, whereas the frequency of Vβ2⁺ and Vβ7⁺ cells was higher among CD3int⁺ T cells from infected liver. Thus, *P. yoelii* molecules preferentially select CD3⁻ TCR Vβ2⁺ and/or Vβ7⁺ cells rather than CD3int⁺ cells bearing the Vβ8 chains.

**Inhibitory activity of CD3int NK1.1⁺ αβ T cells against *P. yoelii* liver stages**

To characterize the activity of CD3int NK1.1⁺ T cells, the effect of this cell subset on the intrahepatocytic development of the parasite was analyzed in vitro. For this purpose, CD3int⁺ NK1.1⁺ and CD3int⁺ NK1.1⁻ T cells were isolated from livers of C57BL/6 mice either noninfected or 10 days after sporozoite inoculation. Sorting was performed with a FACStar after two-color staining with FITC-conjugated anti-CD3 and PE-conjugated anti-NK1.1. Antiplasmodial activity of these different T cell subpopulations was examined by adding these cells at different ratios to primary cultures of hepatocyte, 3 h after *P. yoelii* sporozoite inoculation (Fig. 5). Both CD3int⁺ NK1.1⁺ and CD3int⁺ NK1.1⁻ T cells, primed in vivo with the parasite and isolated 10 days later, greatly inhibited parasite development when compared with CD3int⁺.
the anti-CD3 Ab. The anti-IFN-γ antibody inhibited the lytic activity of these cells at different ratios, but much less so than did the anti-CD3 Ab. The lytic activity was reversed by an anti-CD3 Ab that had no effect by itself on the lytic activity of liver CD3 Int NK1.1+ T cells. Experiments with CD3 Int NK1.1+ T cells, except at the ratio of 20 T cells to 1 hepatocyte. Experiments were performed to determine the role of CD3 Int NK1.1+ T cells in the expansion of the unconventional CD3 Int NK1.1+ T cell subset. The role of CD3 Int NK1.1+ T cells in the expansion of the unconventional CD3 Int NK1.1+ T cell subset was almost totally reversed by an anti-CD3 Ab that had no effect by itself on parasite growth (Fig. 6). As it was reported that liver CD3 Int NK1.1+ T cells produce high levels of IL-4 (36-39) and IFN-γ (40), we addressed whether IFN-γ was implicated in the inhibitory activity of liver CD3 Int NK1.1+ T cells by adding anti-IFN-γ mAb to the syngeneic infected liver cells. Data showed that anti-IFN-γ Abs, when added to the cultures at the same time as CD3 Int NK1.1+ T cells, were able to partially reverse the inhibition mediated by these cells at different ratios, but much less so than did the anti-CD3 Ab. The anti-IFN-γ alone had no inhibitory activity on parasite development (Fig. 6).

**Discussion**

In this study, we demonstrate the expansion of the unconventional CD3 Int NK1.1+ T cells in the liver of C57BL/6 mice during the acute phase of malaria infection induced by the inoculation of sporozoites of a nonlethal *P. yoelii* strain. This T cell subset exhibited a CD4+CD8- phenotype and presented all of the characteristics previously described for NK T cells, such as expression of CD122 and intermediate level of TCRαβ and CD3 molecules (48). DN NK1.1+ αβ T cells were still present in the liver at high frequency after the mice had recovered from the disease. In parallel to the increase of DN NK1.1+ αβ T cells, a down-regulation of CD4+ NK1.1+ αβ T populations cells and an inversion in the ratio of CD4/CD8 were observed in the liver of C57BL/6 mice during the infection. It is worthy of note that DN NK1.1+ αβ T cells were also found expanded in the liver of mice during infection initiated with erythrocytic stages of the same parasite strain (data not shown). A similar observation was also recently made by the group of T. Abo in C57BL/6 and C57/HeJ mice inoculated with *P. yoelii* yoelii 17X, and *P. yoelii* yoelii 17XNL, another lethal and nonlethal *P. yoelii* strain, respectively (A. Weerasinghe, H. Sekikawa, H. Watanabe, and T. Abo, unpublished data).

In addition, the progressive increase with time of the relative number of DN αβ T cells and the level of expression of the activation surface marker CD69 observed in the liver of infected mice compared with control mice indicated that these cells are directly or indirectly stimulated and activated by plasmodial products providing either from the intrahepatic stage and/or the erythrocytic stage. This increase in DN NK1.1+ αβ T cells and the disappearance of CD4+ NK1.1+ αβ T lymphocyte subpopulations identified in the liver of C57BL/6 mice were also recently observed after infection by several other pathogens, thereby suggesting a regulatory role for this subpopulation of αβ T cells in the early antiparasite-host response by promoting Th1 responses in vivo through down-regulation of IL-4 secretion (49-52). Further analysis also revealed that the DN αβ T cells stimulated by *P. yoelii* at different times after infection have the CD44intCD62Llow and CD44hiCD62Lhi phenotype, suggesting a proliferation and/or a recruitment of both naive and primed DN TCRαβint, as indicated by the level of expression of CD69 and CD122.

NK αβ T cells have been described as using a preferential set of Vβ genes and predominantly Vβ8, which can represent up to 50% of the TCRVβ segments expressed (45-47). To determine the TCRVβ repertoire usage by DN TCRαβint liver cells selected during *P. yoelii* infection, we assessed the percentage of cells using the restricted set of Vβs and found that Vβ2 and Vβ7 were preferentially used rather than Vβ8 gene products. These results also indicated that the DN T cell populations induced by *P. yoelii* molecules were not self-reactive forbidden clones that have been described belonging to the Vβ8- set (41, 45). Moreover, if Vβ2 is preferentially expressed at day 10, both Vβ2 and Vβ7 were used at day 30, suggesting an evolution in the NK T cell response that may depend on the parasite stage specificity of plasmid molecules that stimulate these T cell clones.

Our results, which indicate the expansion of DN NK1.1+ TCRαβint liver cells during *P. yoelii* infection, raise the question of the key role of this T cell subset in the immune response against malaria parasite. Because DN NK1.1+ αβ T cells were present at high frequency only in the liver of the infected mice, and none in the thymus, the spleen, or the blood, we addressed whether a control is exerted by NK T cells upon the development of the liver stage. To obtain more evidence, we analyzed the ability of these cells to inhibit in vitro the intrahepatic development of *P. yoelii*. For technical reasons (low number of cells present in the liver of 3 days postinfection), NK T cells from 10-day infected mice were used in the in vitro assay of intrahepatic parasite elimination. The data indicated that, when added at different ratios to primary cultures of hepatocytes 3 h after sporozoites inoculation, *P. yoelii*-induced NK1.1+ TCRαβ+ effector liver cells were able to decrease the number of mature schizonts. Addition of anti-CD3 mAb

![Graph](image-url)
markedly reduced the antiplasmodial activity of *P. yoelii*-primed NK.1.1+ TCRαβ+ cells, showing that the inhibitory activity exhibited by these cells required Ag presentation by the infected hepatocyte and recognition by the TCRs. This observation of inhibitory activity of NK T cells from 10-day infected mice raised the concern of parasite stage Ag specificity of this T cell subset against the liver stage. We cannot exclude a possible effect of NK T cell clones induced by blood stage parasite molecules on the liver stage. It is interesting to note also that liver NK T cells from blood stage-infected mice can also destroy *P. yoelii* erythrocytic stage in vitro (A. Weerasinghe, H. Sekikawa, H. Watanabe, and T. Abo, unpublished data).

The mechanism by which NK.1.1+ TCRαβ+ iHLs exert their antiparasite effect remains unclear. Nevertheless, consistent with the detection of Fas ligand mRNA in NK.1.1+ T cells described by Arase et al. (53) and the high level of perforin observed in the cytoplasm of activated CD3+ NK.1.1+ cells (54), we can propose that these liver lymphocytes may eliminate parasites within the hepatocyte through a Fas ligand/ Fas- or perforin-mediated mechanism. However, the expression of Fas molecules by infected hepatocytes remains to be demonstrated. On the other hand, cytokines are known to have an important role as mediators and effectors in the host response to plasmodial infection (55). So, the partial but significant reduction by the anti-IFN-γ Ab of the inhibition of *P. yoelii* intrahepatocytic schizogony induced by parasite-primed NK.1.1+ TCRαβ+ liver cells also suggests that IFN-γ may be produced by these iHLs themselves and play a role in their inhibitory activity (34). IFN-γ is known to directly eliminate liver stages in vitro by eliciting NO-dependent mechanisms (16). On the other hand, an increasing number of reports shows that IL-12 is a major factor in the proliferation and activation of NK.1.1+ TCRαβ+ cells (56–58). Because IL-12 was shown to protect 100% of mice or monkeys against infection with *P. yoelii* and *P. cynomolgi* sporozoites, respectively, and that this protection is associated with the high plasma level of IFN-γ (11, 12), we postulate that early production of IL-12 by Kupffer cells induced by parasite component(s) may lead to the subsequent expansion and activation of DN NK.1.1+ αβ T cells that produce IFN-γ (40) and in parallel, to the down-regulation of CD4+ NK.1.1+ αβ T cells that secrete IL-4 (37, 39). This regulation of the NK.1.1+ liver T cell subpopulation during primary infection by malaria parasite would promote a Th1 response, which is associated with a protective response against the parasite liver stage (instead of a Th2-type response).

Several studies have reported that NK.1.1+ TCRαβ+ cells recognize components presented by the CD1 molecules that are abundantly expressed in murine liver (59, 60). Based on these observations, we hypothesize that DN NK.1.1+ TCRαβ+ cells primed by the plasmodial molecules are able to recognize parasite ligands presented in the context of CD1 expressed by the infected hepatocyte. The absence of any detectable expansion of DN NK.1.1+ αβ T cells in *P. yoelii*-infected β2m−/− mice (data not shown), which shows a MHC class I control of the induction of this T cell subset, is in full agreement with this hypothesis, which is the subject of ongoing studies. GPI was defined as a ligand of CD1 molecules that is abundantly expressed in murine liver and intestine. As placental malarial GPI was shown to be recognized by liver NK T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. *Proc. Natl. Acad. Sci. USA* 88:575. 4. Weiss, W. R., S. Mellouk, R. A. Houghten, M. Sedegah, S. Kumar, M. F. Good, J. A. Berzofsky, L. H. Miller, and S. L. Hoffman. 1990. Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. *J. Exp. Med.* 171:765. 5. Renia, L., M. Marussig, D. Grillot, S. Pied, G. Corradin, F. Milgten, G. Del Giudice, and D. Mazier. 1991. In vitro activity of CD4+ and CD8+ lymphocytes from mice immunized with a malaria synthetic peptide. *Proc. Natl. Acad. Sci. USA* 88:796.


