Stringent Vβ Requirement for the Development of NK1.1+ T Cell Receptor–α/β+ Cells in Mouse Liver
By Toshiaki Ohteki and H. Robson MacDonald

Summary
The liver of C57BL/6 mice contains a major subset of CD4+8- and CD4-8- T cell receptor (TCR)-α/β+ cells expressing the polymorphic natural killer NKI.1 surface marker. Liver NK1.1+ TCR-α/β+ (NK1+ T) cells require interaction with β2-microglobulin-associated, major histocompatibility complex class I-like molecules on hematopoietic cells for their development and have a TCR repertoire that is highly skewed to Vβ8.2, Vβ7, and Vβ2. We show here that congenic C57BL/6.V13− mice, which lack Vβ8-expressing T cells owing to a genomic deletion at the Vβ locus, maintain normal levels of liver NK1+ T cells owing to a dramatic increase in the proportion of cells expressing Vβ7 and Vβ2 (but not other Vβs). Moreover, in C57BL/6 congenic TCR-Vβ3 and -Vβ8.1 transgenic mice (which in theory should not express other Vβ, owing to allelic exclusion at the TCR-β locus), endogenous TCR-Vβ8.2, Vβ7, and Vβ2 (but not other Vβs) are frequently expressed on liver NK1+ T cells but absent on lymph node T cells. Finally, when endogenous Vβ expression is prevented in TCR-Vβ13 and -Vβ8.1 transgenic mice (by introduction of a null allele at the Cβ locus), the development of liver NK1+ T cells is totally abrogated. Collectively, our data indicate that liver NK1+ T cells have a stringent requirement for expression of TCR-Vβ8.2, Vβ7, or Vβ2 for their development.

A mature T cell subset comprising CD4+8− and CD4−8− double-negative (DN) TCR-α/β+ cells expressing the polymorphic NK1.1 marker has been demonstrated to reside in thymus, bone marrow, spleen, and liver of appropriate mouse strains (reviewed in references 1–3). NK1.1+ TCR-α/β+ (NK1+ T) cells have a restricted usage of TCR-Vβ genes (mainly Vβ8.2, Vβ7, and Vβ2) and require β2-microglobulin (β2m)-associated (MHC class I-like) molecules on hematopoietic cells for their development. Other characteristics of NK1+ T cells are well studied, especially in thymus. They have a potential to secrete large amounts of IL-4 and IFN-γ upon primary stimulation in vitro and in vivo, and freshly isolated NK1+ T cells can directly kill CD4+8+ thymocytes via the Fas pathway. IL-7 seems to induce a preferential expansion of NK1+ T cells in normal but not in β2m-deficient mice. DN NK1+ T cells were originally proposed as a possible source of lymph node DN T cells in autoimmune lpr/lpr mice. Although both populations are absent in β2m−/− mice, they seem to belong to different lineages, because lpr DN T cells undergo negative selection mediated by endogenous superantigen, whereas DN NK1+ T cells do not. Most recently it has been reported that thymic NK1+ T cells predominantly use an invariant α chain, Vα14-Jα281 (4), suggesting an interaction with a restricted set of ligands.

Since NK1+ T cells preferentially use Vβ8.2, Vβ7, and Vβ2 gene segments in normal mice, we have investigated whether they formally require these Vβs, by three approaches. First, we studied congenic C57BL/6 (B6) V13− mice, which express the NK1.1 marker and have no Vβ8+ T cells, owing to genomic deletion of the Vβ8 locus. Liver NK1+ T cells in these mice were present at normal levels and expressed either Vβ7 or Vβ2 but not other Vβs. Second, analysis of TCR-Vβ3 and -Vβ8.1 transgenic mice on a B6 background revealed that liver NK1+ T cells selectively expressing endogenous Vβ8.2, Vβ7, and Vβ2 (but not other Vβs) still can arise. Finally, we derived TCR-Vβ3 and -Vβ8.1 transgenic mouse strains unable to express endogenous Vβ chains by backcrossing them to TCR-β−/− mice that have a homozygous deletion encompassing both Cβ genes (5). NK1+ T cells were totally absent in the liver of these mice, whereas conventional T cells developed normally. Collectively, our data demonstrate a stringent TCR-Vβ requirement for the development of liver NK1+ T cells.

Materials and Methods
Mice. B6 mice were purchased from Harlan Olac (Bicester, UK). Congenic B6.V13− mice (a kind gift of Dr. A. Livingstone, Basel Institute for Immunology, Basel, Switzerland) were derived by transferring the Vβ haplotype (which has an extensive deletion at the TCR-β locus, including Vβ5, 8, 9, 11, 12, and 13 gene segments [6]) from C57L (H-2b, Vβ) to B6 mice (H-2b, Vβ). The B6.Vβ mice used were backcrossed for 15 generations.
to B6. TCR-Vβ3 (7) and -Vβ8.1 (8) transgenic mice on a B6 background were kindly provided by Dr. M. Dohlen (Pharmacia Biotech, Lund, Sweden) and Dr. H. Pircher (University Hospital, Zurich, Switzerland). TCR-Vβ3 and -Vβ8.1 transgenic mice lacking endogenous Vβ expression were obtained by backcrossing to TCR-β−/− mice (The Jackson Laboratory, Bar Harbor, ME), which are homozygous for a deletion in the TCR Cβ locus (5). F2 progeny were typed for expression of transgenic and/or endogenous TCR-β chains by staining of PBLs with appropriate anti-Vβ mAbs. All mice were used between 2 and 5 months of age.

Cell Preparation. To obtain liver mononuclear cells (MNCs), the liver was pressed through a stainless steel mesh and suspended in 50 ml of PBS (9). After being washed once with PBS, the cells (including MNCs and hepatocytes) were fractionated by discontinuous (40% and 80%) Percoll gradient centrifugation for 10 min at 900 g. The interface was harvested, washed with 5% FCS PBS, and used for experiments. MNCs from lymph nodes were obtained by a standard method.

Antibodies and Flow Cytometric Analysis. The following mAb conjugates were used in this study: H57-597-PE (anti-TCR-β; Caltag Laboratories, San Francisco, CA); GK1.5-PE (anti-CD4; Becton Dickinson and Co., Mountain View, CA); PK136-biotin (anti-NK1.1; Pharmingen, San Diego, CA). F23.1-FITC (anti-Vβ8.1-8.3), F23.2-FITC (anti-Vβ8.2), and 44-22-FITC (anti-Vβ6) were prepared in our laboratory. Unconjugated KJ25 (anti-Vβ3) was developed with FITC-conjugated goat anti-rat IgG (Caltag Laboratories) or goat anti-mouse IgG and IgM (Tago, Inc., Burlingame, CA). Rat or mouse Ig was used to block free Ig sites before addition of streptavidin Tri-color and analyzed by FACScan using the Lysis II program (Becton Dickinson and Co.). To detect co-expression of β7 (Vβ3) and β8 (Vβ8) on liver CD4+NK1+ T cells of TCR-Vβ3 transgenic mice, four-color flow cytometric analysis was performed. Unconjugated KJ25 (anti-β7) was developed with PE-conjugated goat F(ab')2 anti-mouse IgG (Caltag Laboratories), mouse Ig was used to block, and F23.1-FITC (anti-β8), PK136-biotin, and GK1.5-PE (anti-CD4) plus streptavidin Tri-color. One representative experiment is shown. The numbers correspond to the population of total or CD4+NK1+ T cells in each strain. (B) Vβ expression of liver CD4+NK1+ T cells in B6 and B6.Vβa mice. Lymph node CD4 + T cells of Vβ3 transgenic mice (95.4 ± 0.9%) and Vβ2 (14.0 ± 3.6%) at moderately higher levels than in B6 mice, presumably owing to the absence of several Vβs in this haplotype (6). Thus, liver CD4+NK1+ T cells seem to require TCR-β-Vβ3, Vβ7, or Vβ2 for development, since other Vβs cannot substitute for Vβ8.2 in B6.Vβa mice.

To further confirm the stringency of the Vβ requirement for development of liver NK1+ T cells, we also investigated NK1+ T cells in the liver of TCR-Vβ3 and -Vβ8.1 transgenic mice (Fig. 2 A and Table 2). As expected, most lymph node CD4+ T cells of Vβ3 transgenic mice (95.4 ± 0.9%) expressed transgenic Vβ3 (β3), whereas endogenous Vβs (β8) such as Vβ8, Vβ7, Vβ2, and Vβ6 were very rare. In liver, the proportions of both total NK1+ T cells (7.8 ± 0.9%) and CD4+NK1+ T cells (4.6 ± 0.6%) were reduced.

Results and Discussion

We first compared the proportion of total NK1+ T cells, CD4+NK1+ T cells, and TCR-Vβ usage among CD4+NK1+ T cells in the livers of normal B6 mice and congenic B6.Vβa mice (Fig. 1 and Table 1). As expected from our previous study (9), liver CD4+NK1+ T cells of control B6 mice express Vβ8 (69.3 ± 3.4%), Vβ7 (14.4 ± 1.7%), and Vβ2 (6.5 ± 0.8%). Other Vβs were virtually absent in liver CD4+NK1+ T cells (reference 9; data not shown). In congenic B6.Vβa mice, a normal frequency of liver NK1+ T cells and CD4+NK1+ T cells was observed as compared with that in B6 mice, despite the total absence of Vβ8+ cells (<1%). Instead, B6.Vβa liver CD4+NK1+ T cells use Vβ7 (46.2 ± 5.7%) and Vβ2 (47.1 ± 3.6%) much more frequently than cells from normal mice; however, they do not express Vβ6, Vβ3, Vβ4, and Vβ10, which are not deleted in B6.Vβa mice (data not shown). Lymph node CD4+ T cells in B6.Vβa mice used Vβ7 (3.0 ± 0.7%) and Vβ2 (14.0 ± 3.6%) at moderately higher levels than in B6 mice, presumably owing to the absence of several Vβs in this haplotype (6). Thus, liver CD4+NK1+ T cells seem to require TCR-β-Vβ8.2, Vβ7, or Vβ2 for development, since other Vβs cannot substitute for Vβ8.2 in B6.Vβa mice.

Figure 1. (A) Proportion of total and CD4+NK1+ T cells in liver of B6 and B6.Vβa mice. Lymph node CD4+NK1+ T cells were stained with H57-597-PE (anti-TCR-β) or GK1.5-PE (anti-CD4) and PK136-biotin (anti-NK1.1) plus streptavidin Tri-color. One representative experiment is shown. The numbers correspond to the population of total or CD4+NK1+ T cells in each strain. (B) Vβ expression of liver CD4+NK1+ T cells in B6 and B6.Vβa mice. Lymph node CD4+NK1+ T cells were stained with the indicated FITC-conjugated anti-Vβ mAbs followed by GK1.5-PE and PK136-biotin plus streptavidin Tri-color. Histograms are gated on CD4+NK1+ T cells.
Table 1. **TCR-Vβ Usage among Liver CD4+NK1+ T Cells and Lymph Node CD4+ T Cells of B6 and B6.Vβ Mice**

| Vβs   | Liver CD4+NK1+ | LN CD4+ |
|-------|----------------|---------|
|       | B6            | B6.Vβ   | B6            | B6.Vβ   |
| %     | %             | %        | %             | %        |
| 8     | 69.3 ± 3.4    | 0.6 ± 0.2 | 22.1 ± 2.5    | 0.7 ± 0.2 |
| 7     | 14.4 ± 1.7    | 46.2 ± 5.7 | 1.8 ± 0.3    | 3.0 ± 0.7 |
| 2     | 8.1 ± 0.4     | 47.1 ± 3.6 | 6.5 ± 0.8    | 14.0 ± 0.5 |
| 6     | 1.1 ± 0.3     | 1.8 ± 0.4 | 8.3 ± 0.4    | 11.5 ± 0.5 |

Four B6 or B6.Vβ mice aged 4 mo were analyzed individually. Cells were stained with the indicated anti-Vβ mAbs and gated as described in Fig. 1. Data are expressed as mean ± SD. Proportions of total liver NK1+ cells and CD4+NK1+ cells were 23.1 ± 2.1% and 14.7 ± 2.3% in B6 mice, and 19.9 ± 4.7% and 13.9 ± 3.8% in B6.Vβ mice, respectively.

about threefold as compared with normal age-matched B6 mice (23.1 ± 2.1% and 14.7 ± 2.5%). The majority of liver CD4+NK1+ T cells expressed βT (76.9 ± 2.7%), although the intensity of staining was approximately fivefold lower than that of lymph node CD4+ T cells (Fig. 2 B). Surprisingly, liver CD4+NK1+ T cells from the transgenic mice also expressed endogenous VB8 (72.1 ± 4.3%), VB7 (11.2 ± 2.9%), or VB2 (3.2 ± 0.9%) at similar frequencies as in nontransgenic controls (Fig. 2 B and Table 2). Coexpression of βT (Vβ3) and βE (Vβ8) on a majority of liver CD4+NK1+ T cells was directly confirmed by four-color flow microfluorometry (Fig. 2 C). Other βE, such as Vβ6, were not seen in liver CD4+NK1+ T cells of TCR-β3 transgenic mice.

The results obtained in TCR-β3 transgenic mice were basically confirmed in TCR-β8.1 transgenic mice (Fig. 2 and Table 2). In the latter mice, most lymph node CD4+ T cells expressed βE (96.3 ± 1.8%) but not ββ, whereas liver CD4+ NK1+ T cells expressed βE at levels close to those of normal B6 mice (Vβ8.2, 47.6 ± 2.3%; Vβ7, 19.5 ± 2.6%; Vβ2, 7.5 ± 1.7%). Analysis of βT expression in liver CD4+NK1+ T cells was complicated by the fact that KJ16 mAb, which was used for staining, recognizes both Vβ8.1 (βE) and Vβ8.2 (βE).

The simultaneous expression of βT and βE on a high proportion of liver CD4+NK1+ T cells is unexpected in view of the fact that inhibition of endogenous rearrangement at the TCR-β locus via allelic exclusion is usually efficient in TCR transgenic mice (10). However, there are several reported transgenic models where βE genes are rearranged and expressed, particularly under conditions of low levels of transgene expression (11) and strong negative selection (12). In the case of liver NK1+ T lineage cells, the expression of two β chains seems rather to reflect a strong positive selection for rare cells that have endogenously rearranged β chains with "permissive" VB domains. Indeed, βE expression on liver NK1+ T cells in both Vβ3- and Vβ8.1-transgenic mice was restricted to Vβ8.2, Vβ7, and Vβ2. Moreover, the relative proportion of transgenic NK1+ T cells expressing these endogenous VB domains was virtually identical to what is found in normal liver. Lack of allelic exclusion at the TCR-β locus is not a general property of liver NK1+ T cells, since no cells expressing two VB domains could be detected in normal mouse liver (data not shown).

To formally test whether NK1+ T cells are able to develop in the absence of appropriate VB expression, we crossed TCR-β3 and -Vβ8.1 transgenic mice with TCR-β-/- mice that have a homozygous deletion encompassing both CI3 domains (5). TCR transgenic F1 mice were then backcrossed to TCR-β-/- mice, and the F2 progeny were typed for expression of the TCR transgenes as well as for endogenous VB expression. As shown in Fig. 3 and Table 3, no NK1+ T cells were detectable in the liver of TCR-β3 and -Vβ8.1 transgenic TCR-β-/- mice, whereas (as expected from Fig. 2) liver NK1+ T cells expressing endogenous VB domains could be detected in normal mouse liver.

Table 2. **Predominant Usage of Endogenous VBs among Liver CD4+NK1+ T Cells of TCR-β-Chain Transgenic Mice**

| Vβs | Liver CD4+NK1+ | LN CD4+ |
|-----|----------------|---------|
|     | Vβ3T          | Vβ8.1T  | B6            | Vβ3T   | Vβ8.1T   |
| βT  | 3.81–8.2      | 76.9 ± 2.7 | 75.3 ± 8.3    | 95.4 ± 0.9 | 96.3 ± 1.8 |
| βE  | 8.1–8.3       | 69.8 ± 0.9 | 72.1 ± 4.3    | 21.4 ± 1.5 | 3.1 ± 0.5 |
|     | 8.2           | 55.0 ± 2.4 | ND            | 47.6 ± 2.3 | 10.4 ± 0.3 |
|     | 7             | 18.3 ± 1.3 | 11.2 ± 2.9    | 19.5 ± 2.6 | 1.7 ± 0.2 |
|     | 2             | 8.6 ± 0.4  | 3.2 ± 0.9     | 7.5 ± 1.7 | 6.1 ± 0.3 |
|     | 6             | 1.3 ± 0.1  | 1.0 ± 0.2     | 1.1 ± 0.3 | 8.6 ± 0.3 | 0.4 ± 0.2 | 1.7 ± 0.5 |

Three to four mice in each group were individually analyzed. Liver MNC and lymph node cells were stained with indicated anti-Vβ mAbs and gated as in Fig. 2. Data are expressed as mean ± SD. Proportions of total liver NK1+ cells and CD4+NK1+ cells were 23.1 ± 2.1% and 14.7 ± 2.3% in B6 mice, 7.8 ± 0.9% and 4.6 ± 0.6% in Vβ3 transgenic mice, and 8.1 ± 0.7% and 5.2 ± 0.6% in Vβ8.1 transgenic mice. ND, not done.
dogenous Vβ domains were frequent in TCR transgenic TCR β⁺⁻ littermate controls. In contrast, the development of normal (NK1.1⁻) T cells in liver and lymph nodes of TCR-Vβ3- or -Vβ8.1-transgenic mice was not affected by the presence or absence of endogenous Vβ expression (Fig. 3; data not shown). These data formally establish that liver NK1⁺ T cells fail to develop unless they are able to express permissive Vβ domains.

In conclusion, we show here that the development of NK1⁺ T cells in mouse liver is strictly dependent upon the utilization of a highly restricted subset of Vβ domains, including Vβ8.2, Vβ7, and Vβ2. In contrast, most Vβ-restricted responses of peripheral CD4⁺ or CD8⁺ T cells to conventional protein antigens in vivo are more plastic, since in the absence of a dominant epitope, T cells expressing other Vβ domains specific for previously cryptic or subdominant epitopes appear (13). By analogy with these heterogeneous protein antigen responses, it seems probable that the physiological ligand responsible for the development (and/or expansion) of liver NK1⁺ T cells in vivo is highly monomorphic. In this regard, it has recently been

Table 3. Endogenous Vβ Expression Is Required for NK1⁺ T Cell Development in the Liver of TCR-Vβ3 and -Vβ8.1 Transgenic Mice

| Mouse strain | Endogenous Vβ expression | TCR-β⁺⁺ NK1⁺ | CD4⁺ NK1⁺ | Vβ8.2⁺ in CD4⁺ |
|--------------|--------------------------|--------------|----------|---------------|
| Vβ3⁺⁺        | 11.3 ± 2.2               | 3.5 ± 0.5    | 16.6 ± 2.2 |
| Vβ8.1⁺⁺      | 12.5 ± 1.0               | 3.9 ± 0.7    | 13.6 ± 2.7 |
| Littermate   | 28.7 ± 2.9               | 12.2 ± 2.4   | 30.7 ± 2.2 |

Two to four mice in each group were analyzed individually. Liver MNCs were stained as in Fig. 2. Data are expressed as mean ± SD unless otherwise indicated (individual mice). Proportions of TCR-β⁺NK1⁺ cells and CD4⁺NK1⁺ cells were estimated in B220⁻ cells.
shown that most thymic NK1+ T cells bear a highly conserved TCR-\(\alpha\) chain consisting of V\(\alpha\)14-J\(\alpha\)281 rearrangements with little (or no) junctional diversity (4). Similar V\(\alpha\)14-J\(\alpha\)281 rearrangements, which are believed to occur extrathymically (14), are frequent in other tissues, such as bone marrow or liver (15). Interestingly, DN TCR-\(\alpha/\beta\)+ T cells using a conserved V\(\alpha\)24-J\(\alpha\)Q rearrangement that is highly homologous to the mouse V\(\alpha\)14-J\(\alpha\)281 sequence are expanded in the peripheral blood of many normal individuals (4, 16), suggesting that a related (or identical) ligand in both mouse and humans, The precise nature of the are expanded in the peripheral blood of many normal individuals (4, 16), suggesting that a related (or identical) ligand in both mouse and humans, the ligand recognized by NK1+ T cells remains controversial.

Since NK1+ T cells are present in TAP-1-deficient mice (17) but fail to develop in \(\beta_{2m}\)-deficient mice (1–3), it is likely that the ligand should consist (at least in part) of a TAP-independent \(\beta_{2m}\)-associated molecule such as thymus leukemia antigen (18) or CD1 (19). Indeed, NK1+ T cells and hybridomas have recently been shown to recognize fibroblast stimulator cells infected with a vaccinia virus construct expressing the mouse CD1 gene (20). Moreover, certain constituents of mycobacteria, such as lipoglycans and mycolic acid, can be recognized by some human DN TCR-\(\alpha/\beta\)+ cell lines in association with CD1b (21, 22). Whether mouse NK1+ T cells can also recognize CD1-associated mycobacterial antigens remains, however, to be determined.

We thank Drs. A. Livingstone, M. Dohlsten, and H. Pircher for providing mice; Dr. I.N. Crispe for helpful discussion; and Pierre Zeach and Christian Knabenhans for FACS® analysis.

Address correspondence to H. Robson MacDonald, Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, CH-1066 Epalinges, Switzerland.

Received for publication 13 February 1995 and in revised form 14 November 1995.

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