Brief Definitive Report

Major Histocompatibility Complex Class I Related Molecules Control the Development of CD4+8- and CD4-8- Subsets of Natural Killer 1.1+ T Cell Receptor-α/β+ Cells in the Liver of Mice

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Summary

Normal mouse liver contains prominent subsets of CD4+8- and CD4-8- T cell receptor (TCR)-α/β+ cells with intermediate TCR levels. We show here that these cells express the natural killer (NK)1.1 surface antigen and have a restricted TCRVβ repertoire that is highly skewed to Vβ7 and Vβ8. Surprisingly, both CD4+8- and CD4-8- subsets of NK1.1+TCR-α/β+ cells are absent in the liver of β2-microglobulin deficient mice, which do not express major histocompatibility complex (MHC) class I or "class I-like" molecules. Analysis of reciprocal radiation bone marrow chimeras established with β2-microglobulin deficient and wild-type mice demonstrates that MHC class I expression on radiosensitive (presumably hematopoietic) cells is required for the development of NK1.1+TCR-α/β+ cells in the liver. In the liver of MHC class II deficient mice, the CD4+8- and CD4-8- subsets of NK1.1+TCR-α/β+ cells develop normally. Collectively our data suggest that NK1.1+TCR-α/β+ cells in liver require interaction with a MHC class I-related ligand on hematopoietic cells for their development. This unusual property of liver T cells is shared by a subset of CD4-8-NK1.1+TCR-α/β+ thymocytes, suggesting a common lineage independent of the mainstream of T cell development.

The presence of MHC class I and II molecules on thymic epithelial cells is generally considered to be required for the development of mature CD8+ and CD4+ T cells expressing the TCR-α/β. Recently, mice lacking MHC class I or II expression have been created by homologous recombination techniques and it has been clearly demonstrated that the majority of CD8+ or CD4+ T cells are absent in MHC class I or II deficient mice, respectively (1-6). However, a very minor population of CD4+ T cells is reported to be detectable in the thymus and periphery of MHC class II deficient mice (4-6). Moreover, a small subset of CD4-8- TCR-α/β+ thymocytes, which express the NK1.1 marker (7, 8) and display a highly skewed Vβ repertoire (9, 10), appear to require MHC class I related molecules expressed on hematopoietic cells for their development (11). Hence there is some precedent for the existence of T cell subsets that do not conform to the general rules of MHC-dependent thymic selection.

A T cell subset with unusual properties is abundant in the liver of unmanipulated mice (12-14). These cells, which are mainly CD4+8- CD4-8-, represent 40-50% of T cells in liver and display intermediate levels of TCR-α/β, which are approximately threefold lower than LN T cells (12, 13). As T cells with a similar phenotype appear in old athymic

mice (14), liver is proposed to be an extrathymic T cell differentiation site. In this study, we show that these unusual T cells in the liver of mice express the NK1.1 surface marker and display a TCR repertoire highly skewed towards Vβ7 and Vβ8. Since these cells share many characteristics with thymic CD4-8-TCR-α/β+ cells, we decided to investigate whether or not the development of liver NK1.1+TCR-α/β+ cells is MHC dependent. Our data show that both CD4+8- and CD4-8- subsets of liver NK1.1+ cells are absent in β2-microglobulin (β2m) deficient mice, which lack MHC class I and "class I-like" molecules. A possible relationship between liver NK1.1+ T cells and NK1.1+ thymocytes will be discussed.

Materials and Methods

Mice. C57BL/6 mice were purchased from Harlan Olac LTD (Bicester, UK). β2m deficient (-/-) mice (1, 2) and wild-type (+/-) littermates were bred and maintained in the animal facilities of the Swiss Institute for Experimental Cancer Research (Epalinges, Switzerland). In most experiments, β2m -/- mice used were originally crossed with C57BL/6 mice and the C57BL/6 background was confirmed by staining with an anti-NK1.1 mAb. MHC class II deficient Aα -/- mice (6) and wild-type (+/-) littermates on a C57BL/6 background were kindly provided by Dr. H.

Bluethmann (Hoffmann La Roche, Basel, Switzerland).

Cell Preparation. Mononuclear cells (MNC) from thymus, spleen, LN, and bone marrow were obtained by standard methods. To obtain liver MNC (15), the liver was pressed through stainless steel mesh and suspended in 50 ml of PBS. After being washed once with PBS, the cells (including MNC 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. To obtain intraepithelial lymphocytes (IEL), small intestine dissected free of Peyer's patches was opened longitudinally, washed with PBS, and cut into 5-mm pieces (16). Subsequently, these pieces were incubated in PBS supplemented with 2 mM dithiothreitol and 4 mM NaHCO₃, with stirring for 20 min at 37°C. The supernatant, containing IEL and epithelial cells, was washed with 5% FCS PBS, and fractionated by discontinuous (40 and 80%) Percoll gradient centrifugation for 10 min at 900 g. The interface was harvested and washed with 5% FCS PBS.

Thymocytes expressing low levels of heat stable antigen (HSA), which were used as a source of NK1.1⁺ thymocytes for the ontogeny study, were obtained by treatment of whole thymocytes with IgM mAb B2A2 (anti-HSA) in the presence of rabbit complement (17). Recovered cells (2–3%) were purified by Ficoll-Isopaque gradient centrifugation.

Radiation Bone Marrow Chimeras. To make reciprocal chimeras between β₂m deficient and wild-type mice, recipients were lethally irradiated (950 rad, 117 rad/min, ¹³⁷Cs source) and reconstituted 1 d later with 15–20 × 10⁶ T cell–depleted bone marrow cells. For the following 8 wk, teramycin (1.5 g/liter) was added to the drinking water. Chimeras were killed 10 wk after reconstitution and chimerism was monitored by flow microfluorometry analysis using B8-24-3 (anti-Kᵇ) mAb.

Antibodies and Flow Cytometric Analysis. The following mAb conjugates were used in this study: H57-597-PE (anti-TCRβ; Caltag Laboratories, San Francisco, CA); PK136-biotin (anti-NK1.1; Pharmingen, San Diego, CA); GK1.5-PE and GK1.5-FITC (anti-CD4; Becton Dickinson & Co., Mountain View, CA); 53-6.7-FITC (anti-CD8). Unconjugated rat, mouse, or hamster mAbs against the following TCR Vβ domains were prepared in our laboratory: B20.6.5 (anti-Vβ2); KJ25 (anti-Vβ3); KT4-10 (anti-Vβ4); MR9-4 (anti-Vβ5); 44-22.1 (anti-Vβ6); TR310 (anti-Vβ7); F23.1 (anti-Vβ8.1,8.2,8.3); F23.2 (anti-Vβ8.2); MR10-2 (anti-Vβ9); B21.5 (anti-Vβ10); and RR3-15 (anti-Vβ11). FITC-conjugated goat anti-rat Ig (Caltag Laboratories) or goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Inc., Birmingham, AL) were used with unconjugated mAbs. Rat or mouse Ig was used to block free Ig sites before addition of anti-CD4-PE and anti-NK1.1-biotin. All samples were further stained with streptavidin Tri-color and analyzed by FACScan® and the Lysis II program (Becton Dickinson & Co.).

Results

Liver Is a Major Source of NK1.1⁺TCR-α/β⁺ Cells. 40–50% of total MNC in the liver of 8–10-wk-old C57BL/6 mice are TCR-α/β⁺ (12–14). As shown in Fig. 1, about half of these cells express NK1.1. The NK1.1⁺-TCR-α/β⁺ subset in liver had approximately threefold lower surface TCR-β intensity than NK1.1⁻TCR-α/β⁺ cells in liver, LN, and spleen (Fig. 1). This phenotype clearly corresponds to the so-called "intermediate" T cells in liver which express TCR-β and CD3 at levels higher than
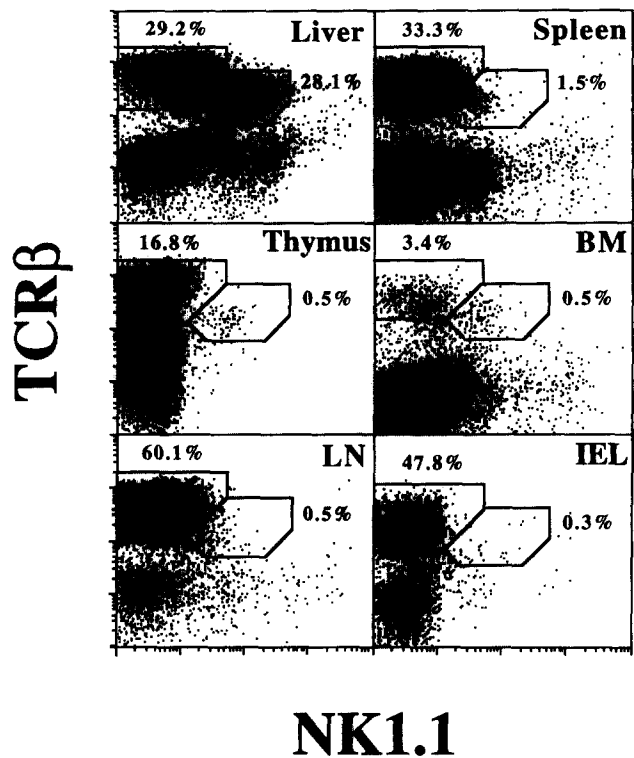

NK1.1

Figure 1. Tissue distribution of NK1.1⁺TCR-α/β⁺ cells. Total MNC separated from indicated organs of 8-wk-old C57BL/6 mice were stained with H57-597-PE (anti-TCRβ, vertical axis) and PK136-Biotin (anti-NK1.1, horizontal axis) plus streptavidin Tri-color. One representative experiment is shown. The numbers correspond to the proportion of NK1.1⁻ or NK1.1⁺TCR-α/β⁺ cells among MNC in each organ.

CD4⁺8⁺ thymocytes but lower than mature T cells (12–14). Since the total number of MNC recovered per liver was 5.0 ± 1.2 × 10⁶, the absolute number of NK1.1⁺TCR-α/β⁺ cells was 10⁶. NK1.1⁺TCR-α/β⁺ cells were rather rare in other organs, i.e., 0.5% in thymus, LN, and bone marrow, 1.5% in spleen, and 0.3% in IEL (Fig. 1). Thus, NK1.1⁺TCR-α/β⁺ cells were most frequently seen in liver. However, 15–20% of HSAˡᵒʷ thymocytes in adult C57BL/6 mice were NK1.1⁺TCR-α/β⁺ (data not shown) as reported elsewhere (18, 19). NK1.1⁺TCR-α/β⁺ cells or "intermediate" TCR cells in thymus and liver appear shortly after birth and increase with age (14, 18, 20). As shown in Fig. 2, neonatal liver contains 10-fold more NK1.1⁺TCR-α/β⁺ cells than thymus; however the absolute number of NK1.1⁺TCR-α/β⁺ cells in these organs is approximately equal in the adult.

We further analyzed the CD4/CD8 phenotype of liver NK1.1⁺TCR-α/β⁺ cells. As shown in Table 1, they were comprised mainly of CD4⁺8⁻ cells (65%) and CD4⁻8⁻ cells (30%). A small subset of CD4⁻8⁺ NK1.1⁺TCR-α/β⁺ cells was also detectable (5%).

TCR-Vβ Usage of NK1.1⁺TCR-α/β⁺ Cells in Liver. A previous study (13) has shown that Vβ8 is overrepresented among TCR intermediate cells in liver. TCR-Vβ expression on the surface of NK1.1⁺TCR-α/β⁺ liver cells and control
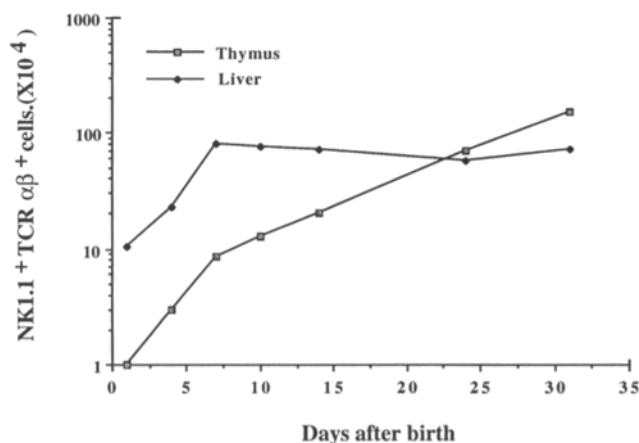
Figure 2. Ontogeny of NK1.1⁺TCR-α/β⁺ cells in the liver and thymus of C57BL/6 mice. At indicated time points, liver MNC and HSAᴸᵒʷ thymocytes (prepared as described in Materials and Methods) were stained with H57-597-PE and PK136-biotin plus streptavidin Tri-color. The absolute number of NK1.1⁺TCR-α/β⁺ cells per organ is shown.

Figure 3. TCR-Vβ repertoire of CD4⁺ LN cells, CD4⁺NK1.1⁺ and CD4⁻8⁻NK1.1⁺ liver T cells. Cells were pooled from three to five C57BL/6 mice aged 8 to 10 wk and stained with a panel of anti-Vβ mAbs. Each bar represents the average of two to three experiments.

LN cells was therefore investigated using a panel of mAbs (Fig. 3). CD4⁺NK1.1⁺ T cells in liver use Vβ8 (69.8 ± 0.9%), Vβ7 (16.0 ± 1.3%), and Vβ2 (8.2 ± 0.3%) at much higher frequency when compared with CD4⁺ LN T cells (21.4 ± 1.5, 1.4 ± 0.2, and 6.1 ± 0.3%, respectively). Among the Vβ8 family, Vβ8.2 (55.0 ± 1.4%) is dominant. Other Vβ such as Vβ4, Vβ6, Vβ10, and Vβ11 were virtually absent in CD4⁺NK1.1⁺ T cells. The Vβ usage of CD4⁻8⁻ NK1.1⁺ T cells was similar to CD4⁺NK1.1⁺T cells, i.e., 67.5 ± 8.5% Vβ8 and 22.0 ± 4.1% Vβ7. It is noteworthy that the intensities of Vβ8, 7, and 2 staining of CD4⁺ NK1.1⁺ T cells were somewhat lower than those of CD4⁺ LN T cells (data not shown). It is interesting to note that both CD4⁻8⁻ and CD4⁺8⁻ subsets of NK1.1⁺TCR-α/β⁺ cells in thymus also overexpress Vβ8, mainly Vβ8.2 (9-11, 18, 21). The similarity of phenotype and Vβ usage suggest that CD4⁺NK1.1⁺ T cells in liver and thymus are more

Table 1. *CD4/CD8 Phenotype of NK1.1⁺TCR-α/β⁺ Cells in the Liver*

	Percent positive among	
Subset	NK1.1⁺TCR-α/β⁺ cells	NK1.1⁻TCR-α/β⁺ cells
	%	
CD4⁺8⁻	65.0, 68.3	65.0, 59.6
CD4⁻8⁺	6.5, 4.9	34.0, 34.5
CD4⁻8⁻	30.1, 29.5	2.0, 2.6

The numbers indicate results from separate experiments. Pools of two to four C57BL/6 mice were used in each experiment, and liver cells were stained with CD4-FITC and/or CD8-FITC, H57-597-PE, and NK1.1-biotin plus avidin Tri-color.
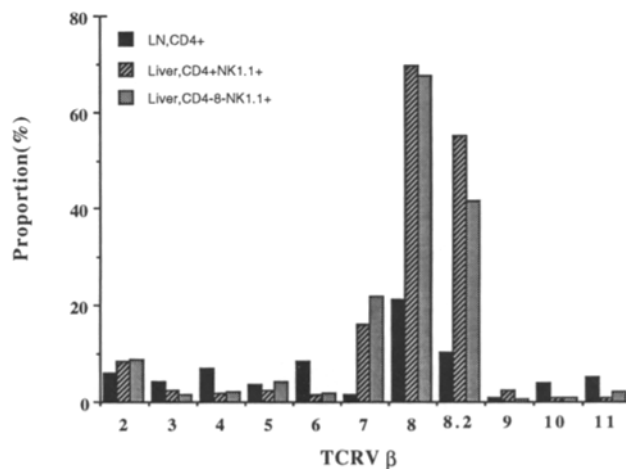
closely related to CD4⁻8⁻NK1.1⁺ T cells than to conventional CD4⁺NK1.1⁻ T cells (18, 21).

Impaired Development of NK1.1⁺TCR-α/β⁺ Cells in the Liver of β₂m Deficient Mice. To investigate the possible role of MHC or MHC-related molecules in the development of NK1.1⁺TCR-α/β⁺ cells in liver, we compared the frequency of these cells in β₂m and Aα deficient mice on a C57BL/6 (NK1.1⁺) background. The former mice (1, 2) failed to express conventional MHC class I molecules (H-2K, H-2D, and H-2L) as well as other β₂m-associated proteins such as Qa, Tla, Hmt, and CD1, whereas the latter (6) lacked conventional MHC class II molecules. Surprisingly, NK1.1⁺TCR-α/β⁺ cells were decreased by >90% in the liver of β₂m −/− mice as compared with their littermate β₂m +/− controls (Fig. 4 and Table 2). This dramatic reduction was apparent in both CD4⁺8⁻ and CD4⁻8⁻ subsets of NK1.1⁺TCR-α/β⁺ cells. In the liver of MHC class II deficient (Aα −/−) mice, the development of NK1.1⁺TCR-α/β⁺ cells was essentially normal for both CD4⁺8⁻ and CD4⁻8⁻ subsets (Fig. 4 and Table 2). Thus, the behavior of NK1.1⁺TCR-α/β⁺ cells in the liver of MHC deficient mice is strikingly similar to that reported recently for CD4⁻8⁻NK1.1⁺thymocytes, which also appear to require MHC class I-related molecules for their development (11).

MHC Class I–related Molecules on Hematopoietic Cells Control the Development of NK1.1⁺TCR-α/β⁺ Cells in Liver. The mechanism underlying the failure of NK1.1⁺TCR-α/β⁺ cells to develop in the liver of β₂m deficient mice was further investigated in radiation bone marrow chimeras. Conventional thymus-derived CD4⁺ or CD8⁺ T cells primarily dependent upon interactions with MHC molecules on radioresistant (presumably epithelial) cells in the thymus of such chimeras in order to be positively selected during development (22). In contrast, the development of the CD4⁺8⁻ NK1.1⁺TCR-α/β⁺ subset in the liver of reciprocal radia-
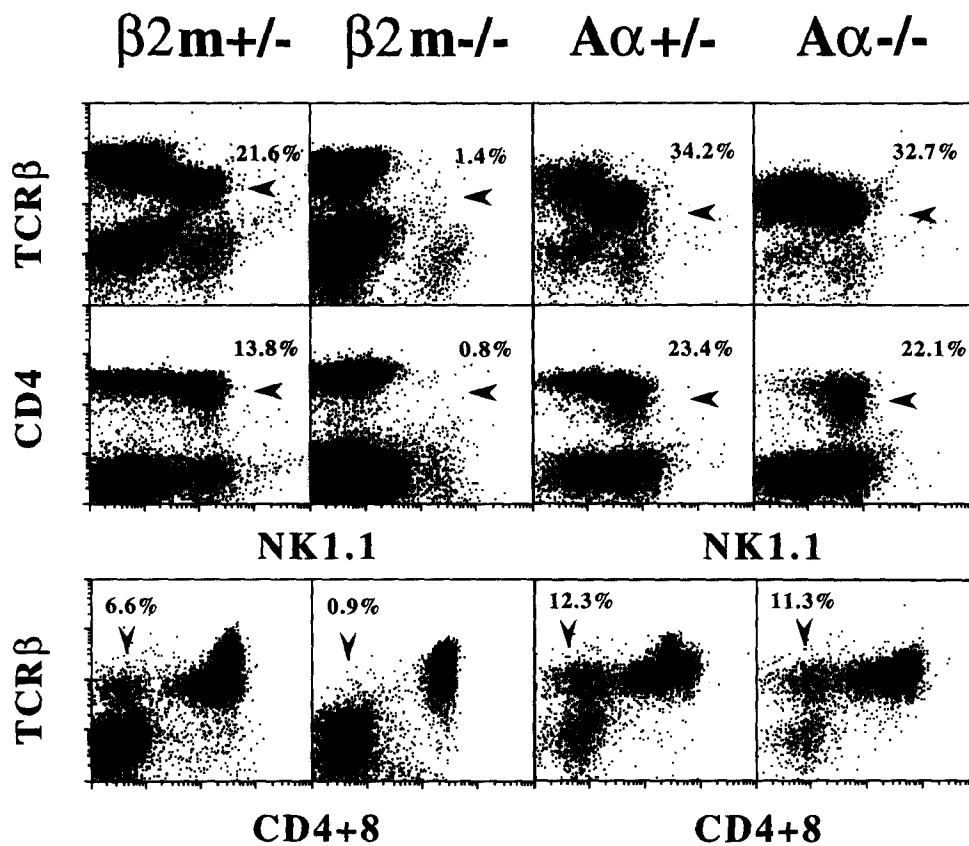
β2m+/-   β2m-/-   Aα+/-   Aα-/-

Figure 4. Analysis of NK1.1+ TCR-α/β+ cells in the liver of MHC deficient mice. For the detection of NK1.1+TCR-α/β+ cells or CD4+NK1.1+ cells, liver MNC were stained with H57-597-PE or CD4-PE and PK136-biotin plus streptavidin Tri-color. For the detection of CD4-8-TCR-α/β+ cells, liver MNC were stained with CD4-FITC, CD8-FITC, and H57-597-PE.

tion bone marrow chimeras established between β2m deficient and wild-type mice correlated strictly with the presence of β2m+ cells of donor (but not host) origin (Table 3). These CD4+8-NK1.1+ cells had a TCR repertoire that was highly skewed towards Vβ8 (Table 3), confirming the phenotype observed for this subset in normal mice (Fig. 3). Collectively, our data indicate that the expression of MHC class I–related molecules on radiosensitive (presumably hematopoietic) cells is required for the development of NK1.1+TCR-α/β+ cells in liver. It is interesting to note that a similar conclusion has recently been reached for

CD4-8-NK1.1+ cells in thymus (11), suggesting that the development of NK1.1+TCR-α/β+ cells in both organs does not follow the conventional rules of MHC-dependent selection on thymic epithelial cells.

Discussion

Our data indicate that a ligand expressed on hematopoietic cells and necessary for the development of NK1.1+-TCR-α/β+ cells in liver is lacking in β2m deficient mice. As discussed earlier, this putative selecting ligand could be

Table 2. *Analysis of NK1.1+TCR-α/β+ Cells in the Liver of β2m or Aα Deficient Mice*

	Liver (Percent NK1.1+TCR-α/β+)				LN (Percent NK1.1-TCR-α/β+)	
Mice	Total	CD4+8-	CD4-8-	CD4-8+	CD4+8-	CD4-8+
	%				%	
β2m+/-	26.8 ± 4.3	17.2 ± 2.9	7.3 ± 0.7	1.0 ± 0.3	37.3 ± 4.4	28.5 ± 2.5
β2m-/-	1.6 ± 0.5	0.9 ± 0.3	1.2 ± 0.2	0.4 ± 0.2	45.7, 51.3	0.4 ± 0.2
Aα+/-	29.5, 34.2	19.8, 23.4	8.8, 12.3	0.7, ND	39.4, 28.2	33.1, 25.6
Aα-/-	35.2, 32.7	23.5, 22.1	13.2, 11.3	ND	1.6, 0.9	72.2, 76.8

Liver MNC from control or mutant mice were analyzed for NK1.1+TCR-α/β+ cells as in Fig. 4. Data are mean ± SD of three to five mice unless otherwise indicated (values for individual mice). LN cells are included for comparison.

Table 3. *Analysis of CD4+NK1.1+ Liver MNC in Reciprocal Radiation Bone Marrow Chimeras Established between β_2m Deficient (−) and Wild-type (+) Mice*

Donor	Host	Percent NK1.1+ in CD4+	Percent Vβ8+ in	
			NK1.1+CD4+,	NK1.1−CD4+
		%		%
+	+	7.4, 8.9	61.2, 63.6	27.7, 29.8
+	−	7.6, 4.4	53.8, 53.4	27.9, 22.1
−	+	1.3, 1.0	ND	22.9, 24.1
−	−	1.1, 0.9	ND	25.1, 27.6

Liver MNC from two individual mice were analyzed in each group. Chimerism was checked by staining with B8-24-3 (anti-K^b) mAb and cells were analyzed after excluding host-derived cells in (+ to −) and (− to +) cases. The proportions of donor-derived cells were 92%, 73% in (+ to −) chimeras and 74%, 76% in (− to +) chimeras. *ND*, not detectable because of the small percentage of NK1.1+CD4+ cells.

either a classical MHC class I molecule or some other β_2m-associated protein such as the product of the Qa, Tla, Hmt, or CD1 locus. Since NK1.1+TCR-α/β+ cells in liver are primarily of the CD4+8− or CD4−8− (but not CD4−8+) phenotype, selection by a classical MHC class I molecule seems rather unlikely. Rather, we would propose that the develop-

ment of NK1.1+TCR-α/β+ cells in liver depends upon interaction of the TCR with a nonclassical class I molecule. Since the latter class of molecules is relatively nonpolymorphic (23) and (at lease in some cases) appears to present a limited number of peptides (24, 25), such an interaction could account for the highly restricted TCR Vβ usage of NK1.1+TCR-α/β+ cells. In the context of this model, the fact that several antigens of microbial origin can be presented by nonclassical class I molecules (26, 27) is of particular interest, since "TCR-α/β intermediate" (presumably NK1.1+) cells in liver have been shown to expand dramatically during bacterial infections (12).

The origin of NK1.1+TCR-α/β+ cells in liver is controversial (28). In this respect, the striking similarities in TCR Vβ phenotype and MHC class I dependence observed for NK1.1+TCR-α/β+ cells in thymus and liver raise obvious questions concerning a possible common lineage independent of the mainstream of T cell development. Indeed, it has been shown that some NK1.1+TCR-α/β+ cells in the spleen of young athymic mice can originate from a grafted neonatal thymus (8). On the other hand NK1.1+TCR-α/β+ cells (or cells with a similar phenotype) clearly develop to some extent in several organs (including spleen and liver) of aged nude mice (14, 20, 29), arguing against an obligatory thymic origin of these cells. Given the apparent requirement for hematopoietic (rather than epithelial) cells for their generation, it remains possible that NK1.1+TCR-α/β+ cells develop independently at multiple sites of hematopoiesis.

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