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Tissue-Specific Segregation of CD1d-Dependent and CD1d-Independent NK T Cells[1]

Gérard Eberl,* Rosemary Lees,* Stephen T. Smiley,† Masaru Taniguchi,‡ Michael J. Grusby,†§ and H. Robson MacDonald[2]*

NKT cells, defined as T cells expressing the NK cell marker NK1.1, are involved in tumor rejection and regulation of autoimmunity via the production of cytokines. We show in this study that two types of NKT cells can be defined on the basis of their reactivity to the monomorphic MHC class I-like molecule CD1d. One type of NKT cell is positively selected by CD1d and expresses a biased TCR repertoire together with a phenotype found on activated T cells. A second type of NKT cell, in contrast, develops in the absence of CD1d, and expresses a diverse TCR repertoire and a phenotype found on naive T cells and NK cells. Importantly, the two types of NKT cells segregate in distinct tissues. Whereas thymus and liver contain primarily CD1d-dependent NKT cells, spleen and bone marrow are enriched in CD1d-independent NKT cells. Collectively, our data suggest that recognition of tissue-specific ligands by the TCR controls localization and activation of NKT cells. ***The Journal of Immunology*****, 1999, 162: 6410–6419.**

Natural killer T (NKT)[3] cells express markers common to the NK cell lineage, such as NK1.1, IL-2Rβ (CD122), members of the Ly49 killer-inhibitory receptor family (1–3), and the recently described DX5 Ag (4). The TCR repertoire of the majority of NKT cells consists of an invariant Vα14-Jα281 chain (5) paired preferentially with a polyclonal Vβ8.2 (and to a lesser extent polyclonal Vβ7 and Vβ2) chain (6–8). Most NKT cells also express high levels of CD69 (very early activation marker) and CD44 (Pgp-1), and low levels of CD62L (L-selectin) (2, 7), a phenotype reminiscent of memory T cells (9). NKT cells are found mainly in thymus, liver, spleen, and bone marrow at a relatively constant number of 0.5–1.5 million cells per organ (2, 8, 10–12). Interestingly, the CD4/CD8 coreceptor phenotype of NKT cells varies in these organs. In particular, most thymus and liver NKT cells are $CD4^+$ or $CD4^-$ $CD8^-$ (DN) (2, 8), whereas spleen and bone marrow are enriched in DN and $CD8^+$NKT cells (12, 13).

Most NKT cells are positively selected by the monomorphic MHC class I-like and β_2-microglobulin (β_2m)-dependent molecule CD1d, since mice deficient in β_2m or CD1d have a marked reduction in the number of NKT cells (8, 14–17). Moreover, a panel of V$\alpha14^+$ T cell hybridomas (derived from splenocytes or thymocytes) secretes IL-2 when cultured with $CD1d^+$ splenocytes and thymocytes or CD1d-transfected cell lines (18, 19). Interestingly, individual V$\alpha14^+$ T cell hybridomas differ in their reactivity to CD1d-expressing cells, suggesting that NKT cells may recognize diverse CD1d-restricted epitopes. CD1d has a profound hydrophobic groove that may accommodate hydrophobic peptides and lipid moieties (20) such as phosphatidylinositol derivatives (21) and glycosylceramides, and the latter have been shown to induce CD1d-restricted proliferation of splenic V$\alpha14^+$NKT cells (22, 23). Finally, a significant number of NKT cells are found in CD1d-deficient mice, suggesting that some NKT cells recognize ligands distinct from CD1d.

Both a thymic and an extrathymic origin of NKT cells have been suggested. NKT cells can be generated in fetal thymic organ cultures (14), and neonatal thymus grafts implanted in congenitally athymic (nude) mice give rise to donor-derived NKT cells in the recipient organs (13), suggesting that NKT cells develop in the thymus. On the other hand, low numbers of NKT cells are also detected in bone marrow (12), spleen (24), and liver (25) of nude mice, and reconstitution of nude or adult-thymectomized irradiated mice with syngeneic bone marrow cells gives rise to NKT cells (24), in particular to $CD8^+$NKT cells (26), in the recipient organs, suggesting that at least some NKT cells develop extrathymically from bone marrow.

In this study, we have determined the phenotype and the requirement for thymus, CD1d, and the invariant Vα14-Jα281 chain for the development of each individual (DN, $CD4^+$, or $CD8^+$) NKT cell subset in various tissues. We find that most NKT cells in thymus and liver are thymus and CD1d dependent, and express a biased TCR repertoire associated with an activated T cell phenotype. In contrast, a high proportion of NKT cells in spleen and bone marrow is thymus and/or CD1d independent, and expresses a diverse TCR repertoire associated with a phenotype found on naive T cells and NK cells. Thus, our results demonstrate a tissue-specific segregation of two types of NKT cells. Moreover, they suggest that TCR specificity, restricted by CD1d and other unknown ligands, influences the localization and activation of NKT cells.

Materials and Methods

Mice

C57BL/6 females were purchased from Harlan/Netherlands (Zeist, The Netherlands), and C57BL/6-*nu/nu* (nude) mice were purchased from BRL Biological Research Laboratories (Fullensdorf, Switzerland). $CD1^{-/-}$

*Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, Epalinges, Switzerland; †Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115; ‡CREST (Core Research for Evolutional Science and Technology) and Department of Molecular Immunology, Chiba University Graduate School of Medicine, Chiba, Japan; and §Department of Medicine, Harvard Medical School, Boston, MA 02115

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[2] Address correspondence and reprint requests to Dr. H. R. MacDonald, Ludwig Institute for Cancer Research, Ch. des Boveresses 155, 1066 Epalinges, Switzerland. E-mail address: HughRobson.MacDonald@isrec.unil.ch

[3] Abbreviations used in this paper: NKT cells, T cells expressing the NK cell marker NK1.1; β_2m, β_2-microglobulin; DN, double negative; HSA, heat-stable Ag.

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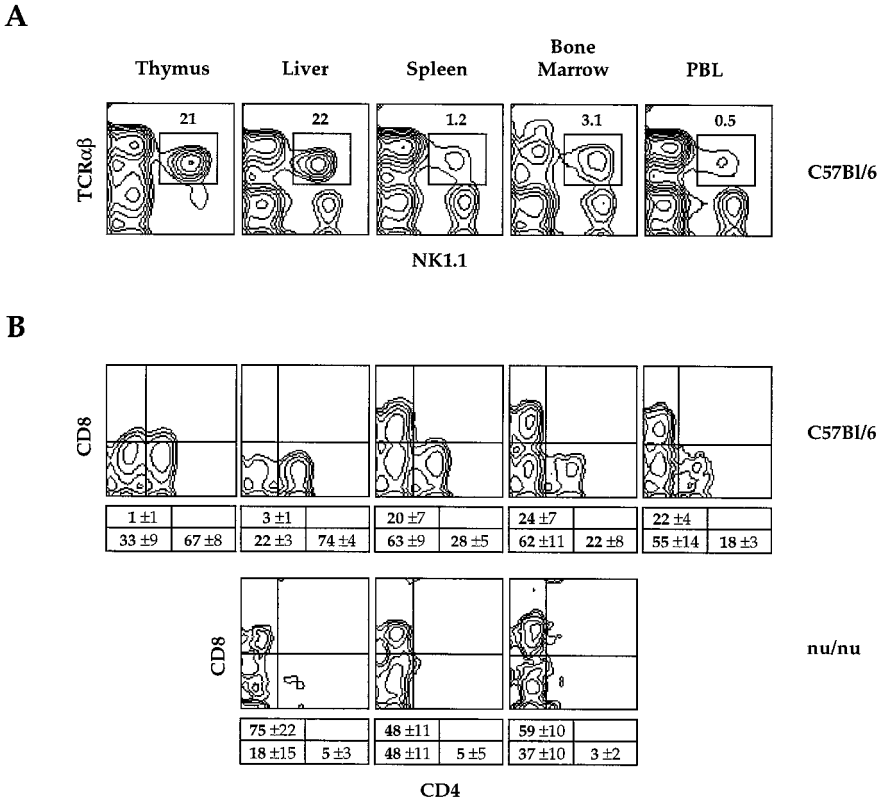

FIGURE 1. CD4 and CD8 expression by NKT cells. HSA^- thymocytes, mononuclear liver cells, B cell-depleted spleen and bone marrow cells, and PBL isolated from C57BL/6 or nude mice were quadruple stained with mAbs against TCRβ, NK1.1, CD4, and CD8. *A*, TCRβ versus NK1.1 staining in the different organs of C57BL/6 mice. Numbers are percentage of NKT cells (see Table I). *B*, NKT cells from C57BL/6 or nude mice were gated by the region depicted in *A*, and analyzed for CD4 and CD8 expression. Numbers are percentage of $CD8^+$, DN, and $CD4^+$ cells among NKT cells and are the mean ± SD of at least five (C57BL/6) and three (nude) independent experiments.

(BALB/c × 129Sv)F_2 mice and Jα281$^{-/-}$ 129 mice were backcrossed to C57BL/6 mice for three generations. All mice were used at 8–12 wk of age, except nude mice, which were used at 6 mo of age.

Cell preparation

Single cell suspensions were prepared from the liver, spleen, thymus, and bone marrow. Total liver cells were resuspended in a 40% isotonic Percoll solution (Pharmacia, Uppsala, Sweden) and underlaid with an 80% isotonic Percoll solution. Centrifugation for 20 min at 1000 × *g* isolated the mononuclear cells at the 40–80% interface. Cells were washed twice with PBS containing 2% FCS. Spleen cells and bone marrow (femur, tibia) cells were resuspended in DMEM medium supplemented with 5% FCS and 1% HEPES (FH-med) and loaded onto 10-ml nylon wool columns that had been preincubated overnight at 37°C with FH-med. The columns were incubated for 45 min at 37°C, and cells depleted of B cells and monocytes were harvested by washing the columns with 20 ml of FH-med. Thymocytes were resuspended in PBS containing 2% FCS together with a 1/10 dilution of J11d or B2A2 (anti-HSA) hybridoma culture supernatants. After an incubation of 45 min at 4°C, the cells were washed and incubated for another 45 min at 37°C with an appropriate dilution of rabbit complement. The live mature (HSA^-) thymocytes were isolated and washed twice.

Flow cytometry

Cells were preincubated with 2.4G2 culture supernatant to block Fcγ receptors, then washed and incubated with the indicated mAb conjugates for 40 min in a total volume of 100 μl of PBS containing 2% FCS. Cells were washed and, if required, incubated with streptavidin conjugates for 20 min. After a further wash, cells were resuspended in PBS containing 2% FCS and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) for three-color stainings or on a FACScalibur flow cytometer (Becton Dickinson) for four-color stainings.

Antibodies

The following mAbs were purchased from PharMingen (San Diego, CA): FITC-, Cy-Chrome-, APC-, or biotin-conjugated anti-TCRβ (H57-597); PE- or biotin-conjugated anti-NK1.1 (PK136); Cy-Chrome-conjugated anti-CD4 (H129.19) and anti-CD8α (53-6.7); FITC-conjugated anti-TCR Vα2 (B20.1); anti-TCR Vα3.2 (RR3-16); anti-TCR Vα8 (B21.14); anti-TCR Vα11 (RR8-1); DX5 and anti-Ly49A (A1); and PE-conjugated anti-CD69 (H1.2F3). The PE-conjugated anti-CD62L (Mel-14) was purchased from Caltag (San Francisco, CA), and APC-conjugated streptavidin at Molecular Probes Europe (Leiden, The Netherlands). FITC-conjugated anti-CD4 (GK1.5) and anti-TCR Vβ8.2 (F23.2) were prepared at the Ludwig Institute (Epalinges, Switzerland).

Results

Tissue distribution of DN, $CD4^+$, and $CD8^+$ NKT cells

Fig. 1*A* and Table I report the proportion of $NK1.1^+TCR\alpha\beta^+$ cells (NKT cells) in thymus, liver, spleen, bone marrow, and PBL of wild-type C57BL/6 mice. Even though NKT cells are highly enriched in mature (HSA^-) thymocytes and in liver mononuclear

Table I. *Tissue distribution of NKT cells in C57BL/6 wild-type and mutant mice*[a]

Strain	Thymus	Liver	Spleen	Bone Marrow	PBL
C57BL/6	21 ± 8	22 ± 7	1.2 ± 0.5	3.1 ± 0.9	0.5 ± 0.1
nu/nu		0.5 ± 0.4	0.5 ± 0.3	0.2 ± 0.1	n.d.
$CD1d^{-/-}$	1.8 ± 0.8	3.4 ± 1.9	0.5 ± 0.1	1.4 ± 0.3	n.d.
Jα281$^{-/-}$	2.0 ± 0.6	2.8 ± 0.8	0.5 ± 0.1	0.8 ± 0.1	n.d.

[a] The percentage of $NK1.1^+$ TCR-β^+ cells was determined in HSA^- thymocytes, total mononuclear cells in liver and PBL, and B cell-depleted mononuclear cells in spleen and bone marrow (see *Materials and Methods*). Data are expressed as the mean ± SD of three to five individual mice per group. All mutant mice were backcrossed to C57BL/6 for at least three generations.

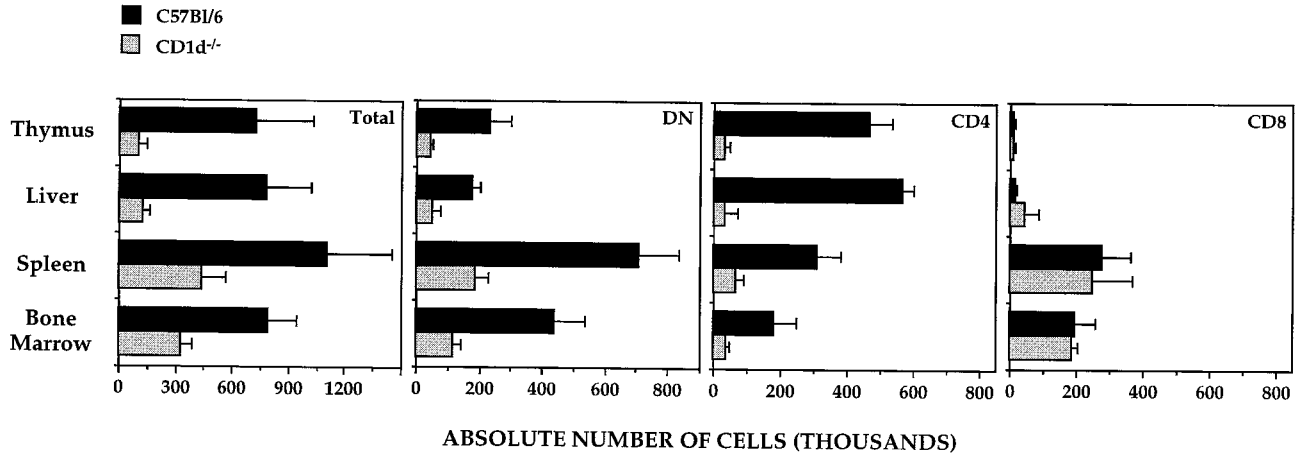

FIGURE 2. CD1d dependency of NKT cells. Cells isolated from C57BL/6 or CD1d-deficient mice were quadruple stained with mAbs against TCRβ, NK1.1, CD4, and CD8. Absolute numbers of cells per organ were calculated for each NKT cell subset on the basis of total numbers of mononuclear cells in liver (4×10^6) and total nucleated cells in thymus (100×10^6), spleen (100×10^6), and bone marrow (300×10^6) (53). For graphical convenience, only one-half of the total number of bone marrow cells are reported. Numbers are the mean ± SD of at least four independent experiments.

cells as compared with spleen and bone marrow mononuclear cells, the absolute number of NKT cells is similar in all four organs (Fig. 2) (2). In contrast, only a small proportion of NKT cells is found in PBL. As reported in part previously, the distribution of DN, $CD4^+$, and $CD8^+$ NKT cells varies considerably between organs (Fig. 1*B*) (2, 8, 12, 13). In thymus and liver, most NKT cells are $CD4^+$ (2/3) or DN (1/3), and only a negligible fraction is $CD8^+$. In contrast, spleen, bone marrow, and PBL are enriched in DN and $CD8^+$ NKT cells, representing ~60% (DN) and ~20% ($CD8^+$) of the total NKT cell population. Hence, even though liver and spleen are both abundantly perfused with blood, NKT cells in PBL are similar, in terms of CD4 and CD8 expression, to spleen rather than to liver NKT cells.

Thymus dependency of NKT cells

Previous studies have established that the number of NKT cells in liver, spleen, and bone marrow of congenitally athymic (nude) mice or neonatally thymectomized mice is markedly reduced as compared with wild-type controls (12, 13, 25, 27). Table I confirms that most NKT cells are dependent on the thymus for development. Interestingly, however, the residual NKT cell population in nude mice contains almost no $CD4^+$NKT cells, but a majority of $CD8^+$NKT cells and a significant proportion of DN NKT cells (Fig. 1*B*). Hence, the development of essentially all $CD4^+$NKT cells and a majority of DN NKT cells is thymus dependent, whereas a large fraction of $CD8^+$NKT cells is thymus independent.

CD1d dependency of NKT cells

CD1d is an MHC class I-like molecule that is dependent on β_2m for its cell surface expression (28). In β_2m- or CD1d-deficient mice, the number of NKT cells in thymus, liver, and spleen is markedly reduced (Table I) (8, 14–17). In particular, $CD4^+$NKT cells are more affected by the absence of β_2m or CD1d as compared with total NKT cells (15) or with DN NKT cells (8). In this study, we directly compared the CD1d dependency of each individual NKT cell subset in thymus, liver, spleen, and bone marrow. Fig. 2 shows that the development of most $CD4^+$NKT cells is indeed dependent on CD1d, whereas $CD8^+$NKT cells are CD1d independent, irrespective of the organ considered. The majority of DN NKT cells is also CD1d dependent, although to a lesser extent than $CD4^+$NKT cells.

Together, these results show that thymus and liver are enriched in CD1d-selected NKT cells that express CD4. In addition, most liver NKT cells are dependent upon the thymus for their development. In contrast, spleen and bone marrow are enriched in CD1d-independent NKT cells that express CD8 and can develop in the absence of thymus. Interestingly, spleen and bone marrow are also enriched in DN NKT cells, in particular in CD1d-independent DN NKT cells.

TCR repertoire of NKT cells

The data presented above support the hypothesis that thymus and liver select for different NKT cell subsets than spleen and bone marrow. To further test this possibility, we characterized the TCR repertoire expressed by NKT cells in these different organs. It is known that approximately one-half of NKT cells in thymus, liver, spleen, and bone marrow express a TCR Vβ8 chain (7, 8, 13), shown in thymus and liver to consist mainly of Vβ8.2. In addition, most thymus and liver NKT cells express an invariant TCR Vα14-Jα281 chain, whereas a lower proportion of spleen and bone marrow NKT cells expresses Vα14-Jα281, as estimated by two independent semiquantitative PCR methods (29). We determined directly the expression of Vβ8.2 and indirectly the expression of Vα14 (at a single cell level) by each individual NKT cell subset. In particular, we estimated the bias in the Vα repertoire by determining the expression of a pool of Vα-chains (Vα2, Vα3.2, Vα8, and Vα11) (Vα pool) (3) to which mAbs are available. Fig. 3*A* shows that the Vβ repertoire of liver NKT cells is indeed strongly biased toward Vβ8.2 as compared with conventional liver T cells. Moreover, the expression of the Vα pool is greatly reduced in liver NKT cells as compared with T cells, indicating a strong bias (presumably toward Vα14) in the Vα repertoire of liver NKT cells. Fig. 3*B* shows that in thymus and liver, ~50% of $CD4^+$ or DN NKT cells express Vβ8.2 and a strongly biased Vα repertoire. In contrast, in spleen and bone marrow, $CD4^+$NKT cells still express a strongly biased Vβ repertoire (40–50% Vβ8.2^+), but the bias in the Vα repertoire is significantly reduced. Moreover, spleen and bone marrow DN NKT cells express a Vβ repertoire that is biased to intermediate levels (20–30% Vβ8.2^+) and an unbiased Vα repertoire. Finally, in all organs considered, $CD8^+$NKT cells express

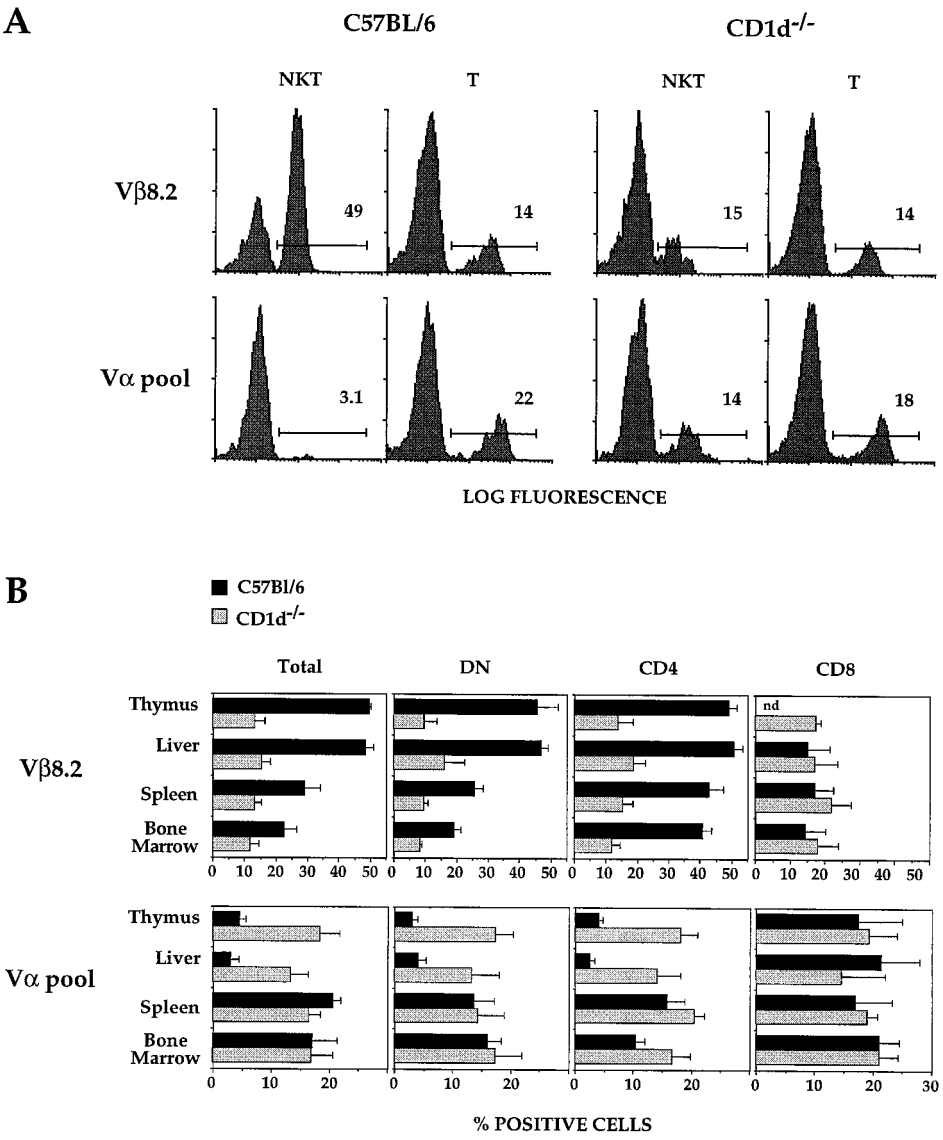

FIGURE 3. The TCR repertoire of NKT cells. *A*, Liver mononuclear cells isolated from C57BL/6 or CD1d-deficient mice were triple stained with mAbs against TCRβ, NK1.1, and Vβ8.2 or a pool of mAbs against Vα2, Vα3.2, Vα8, and Vα11 (Vα pool). Numbers are percentage of Vβ8.2$^+$ or Vα pool$^+$ cells among NKT cells (gated as in Fig. 1*A*) or conventional T cells (gated as NK1.1$^-$TCRβ^+ cells). *B*, Cells isolated from C57BL/6 or CD1d-deficient mice were quadruple stained with mAbs against TCRβ, NK1.1, CD4 and/or CD8, and Vβ8.2 or Vα pool. All cells analyzed were gated on TCRβ^+NK1.1$^+$ phenotype. The experiment reported in *A* is representative of at least four independent experiments, and the data presented in *B* are the mean ± SD of at least three independent experiments.

a TCR repertoire that is unbiased for both the TCR Vα- and Vβ-chains (Fig. 3*B*), and is similar to the TCR repertoire of conventional T cells in terms of expression of individual Vα- and Vβ-chains (data not shown). Hence, thymus and liver strongly select for NKT cells (CD4$^+$ and DN) that express a TCR repertoire biased for both the TCR Vα- and Vβ-chains. In contrast, spleen and bone marrow are enriched in CD4$^+$NKT cells that express a highly biased Vβ repertoire together with a weakly biased Vα repertoire, DN NKT cells that express a weakly biased Vβ repertoire together with an unbiased Vα repertoire, and CD8$^+$NKT cells that express a diverse TCR repertoire. Unexpectedly, these data reveal the existence, in wild-type spleen and bone marrow, of NKT cells that express a Vβ8.2 chain in the apparent absence of the invariant Vα14 chain.

CD1d selects for NKT cells expressing a biased TCR repertoire

We tested whether NKT cells in thymus, liver, spleen, and bone marrow of CD1d-deficient mice still expressed a biased TCR repertoire. Fig. 3*A* shows that in CD1d-deficient mice, similar proportions of liver NKT cells and conventional T cells express Vβ8.2 and the Vα pool. Moreover, in all organs considered, the TCR repertoire of each individual NKT cell subset is unbiased for both the Vα- and the Vβ-chains in the absence of CD1d (Fig. 3*B*). Thus, the biased TCR repertoire of DN and CD4$^+$ NKT cells is strictly dependent on CD1d, independently of whether the repertoire bias includes both Vα- and Vβ-chains or only the Vβ-chain.

NKT cells can express a biased TCR Vβ repertoire in the absence of the invariant Vα14-Jα281 chain

The invariant TCRα-chain expressed by NKT cells is encoded by the Vα14 gene segment rearranged to the Jα281 gene segment without V-J junctional diversity (5, 30, 31). This invariant Vα14 chain is expressed by most thymus and liver NKT cells and is selected by CD1d (14, 17, 32). In Jα281-deficient mice (33), the number of NKT cells is reduced to a similar extent as in CD1d-deficient mice (Table I). Fig. 4*A* shows the TCR repertoire expressed by the individual subsets of NKT cells in Jα281-deficient mice. Interestingly, CD4$^+$ and DN NKT cells expressing a Vβ repertoire biased to intermediate levels (toward Vβ8.2) are found in thymus and liver, but not in spleen and bone marrow. In contrast, no NKT cell subset expresses a biased Vα repertoire in any of the organs considered. These data demonstrate formally that NKT cells expressing a biased TCR Vβ repertoire can develop in the absence of the invariant Vα14-Jα281 chain and, moreover, in the absence of any apparent bias in the Vα repertoire. Importantly, in wild-type mice, NKT cells expressing a biased Vβ repertoire in

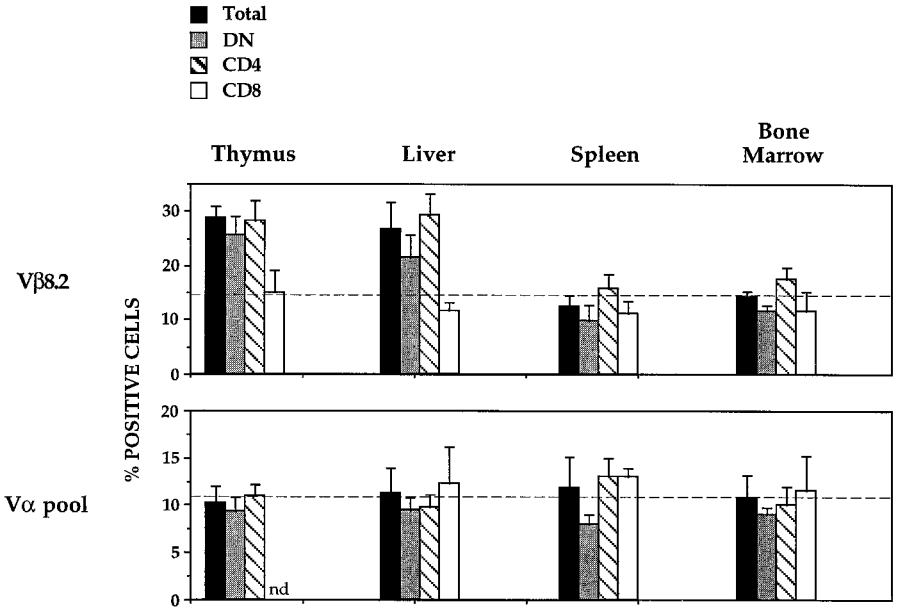

FIGURE 4. The TCR repertoire of NKT cells in Jα281-deficient mice. Cells isolated from Jα281-deficient mice were quadruple stained with mAbs against TCRβ, NK1.1, CD4 and/or CD8, and Vβ8.2 or a pool of mAbs against Vα2 and Vα8 (Vα3.2 and Vα11 are not expressed in these mice; data not shown). All cells analyzed were gated on TCRβ^+NK1.1$^+$ phenotype. Among NKT cells, percentage of (mean ± SD) DN, CD4$^+$, or CD8$^+$ cells were respectively 55 ± 7, 34 ± 4, 8 ± 4 (thymus), 36 ± 4, 46 ± 4, 18 ± 7 (liver), 52 ± 5, 15 ± 3, 32 ± 7 (spleen), and 61 ± 2, 11 ± 3, 27 ± 3 (bone marrow). The dotted lines report, for comparison, the percentage of Vβ8.2$^+$NKT cells and Vα2$^+$ or Vα8$^+$ NKT cells in CD1d-deficient mice (which do not contain NKT cells expressing a biased TCR repertoire; Fig. 3). The data are the mean ± SD of at least four independent experiments.

the absence of a biased Vα repertoire are found only in spleen and bone marrow, whereas in Jα281-deficient mice (lacking NKT cells expressing a TCR repertoire biased for both the TCR Vα- and Vβ-chains), these cells are present exclusively in thymus and liver. Collectively, these data suggest that thymus and liver strongly select for NKT cells expressing the most biased (and CD1d-dependent) TCR repertoire available.

Expression of T cell activation markers by NKT cells

Thymus NKT cells express high levels of CD44 and CD69 and low levels of CD62L (L-selectin) (2, 7), a phenotype found on activated or memory T cells in the periphery. We confirmed that NKT cells express high levels of CD44 in all organs considered, namely thymus, liver, spleen, and bone marrow (data not shown). We further investigated the expression of CD62L and of the very early activation marker CD69 in each individual NKT cell subset. Fig. 5 shows that a very low proportion (~2%) of thymus and liver NKT cells expresses CD62L, whereas 50–60% of spleen and bone marrow NKT cells are CD62L$^+$. An opposite expression pattern was seen with CD69, since ~70% of thymus and ~85% of liver NKT cells express CD69, whereas only ~20% of spleen and ~40% of bone marrow NKT cells are CD69$^+$. In general, CD4$^+$NKT cells contain a lower proportion of CD62L$^+$ and a higher proportion of CD69$^+$ cells as compared with total or DN NKT cells, whereas CD8$^+$NKT cells contain a higher proportion of CD62L$^+$ cells. Taken together, these results show that most NKT cells in thymus and liver display a phenotype found on activated T cells. In contrast, a high proportion of spleen and bone marrow NKT cells displays a phenotype found on naive T cells (with the exception of CD44). Moreover, as compared with total or DN NKT cells, a higher proportion of CD4$^+$NKT cells (mostly CD1d dependent and expressing a biased TCR repertoire) displays an activated phenotype, whereas a higher proportion of CD8$^+$NKT cells (mostly CD1d independent and expressing a diverse TCR repertoire) displays a naive phenotype.

Expression of NK cell markers by NKT cells

NKT cells, by definition, express the NK cell marker NK1.1. In addition, several reports show that NKT cells in thymus, liver, and spleen express the NK cell marker CD122 (IL-2Rβ) (34, 35). Indeed, we found that all subsets of NKT cells in all tissues examined express CD122 (data not shown). More recently, Moore et al. have reported the expression of a novel NK cell marker, DX5, on a minor subset of TCR$^+$ thymocytes (4). Fig. 6*A* shows that NKT cells express DX5, but interestingly, the expression level varies considerably between organs and NKT cell subsets. In thymus and liver, NKT cells express intermediate to low levels of DX5, whereas a majority of NKT cells in spleen and bone marrow express high levels of DX5. CD4$^+$NKT cells express in general lower levels of DX5 as compared with total or DN NKT cells, whereas CD8$^+$NKT cells express higher levels of DX5.

Liver and thymus NKT cells have been reported to also express members of the Ly49 killer-inhibitory receptor gene family (including Ly49A, C, and G2) (2, 3). Fig. 6*B* shows that the proportion of NKT cells expressing Ly49A is lower in thymus and liver as compared with spleen and bone marrow. Moreover, the proportion of cells expressing Ly49A is lowest among CD4$^+$NKT cells and highest among CD8$^+$NKT cells. In summary, all NKT cells express the NK cell markers NK1.1 and CD122, but variable proportions of the individual NKT cell subsets express the NK cell markers DX5 and Ly49A. In addition, individual NKT cell subsets display a similar expression pattern for both NK cell markers, DX5 and Ly49A. Expression of these NK cell markers is highest among CD1d-independent NKT cells, and correlates with the expression of a diverse TCR repertoire and a naive phenotype.

CD1d-dependent NKT cells are activated and express low levels of NK cell markers

We directly assessed, at a single cell level, whether the biased TCR repertoire of CD1d-dependent NKT cells correlates with activation markers and low levels of NK cell markers such as DX5 or Ly49A. This analysis was performed on CD4$^+$NKT cells using four-color flow cytometry, but not on DN NKT cells, which would require five-color flow cytometry. CD8$^+$NKT cells were not analyzed, since they express in general a diverse TCR repertoire and high levels of NK cell markers. Fig. 7*A* shows that, in all organs considered, activated (CD62L$^-$) CD4$^+$NKT cells express a TCR repertoire that is highly biased toward Vβ8.2, whereas, remarkably, naive (CD62L$^+$) CD4$^+$NKT cells express a diverse TCR repertoire. Moreover, activated CD4$^+$NKT cells express low to intermediate DX5 levels, whereas naive CD4$^+$NKT cells express high DX5 levels (Fig. 7*B*). In summary, a clear dichotomy exists in the

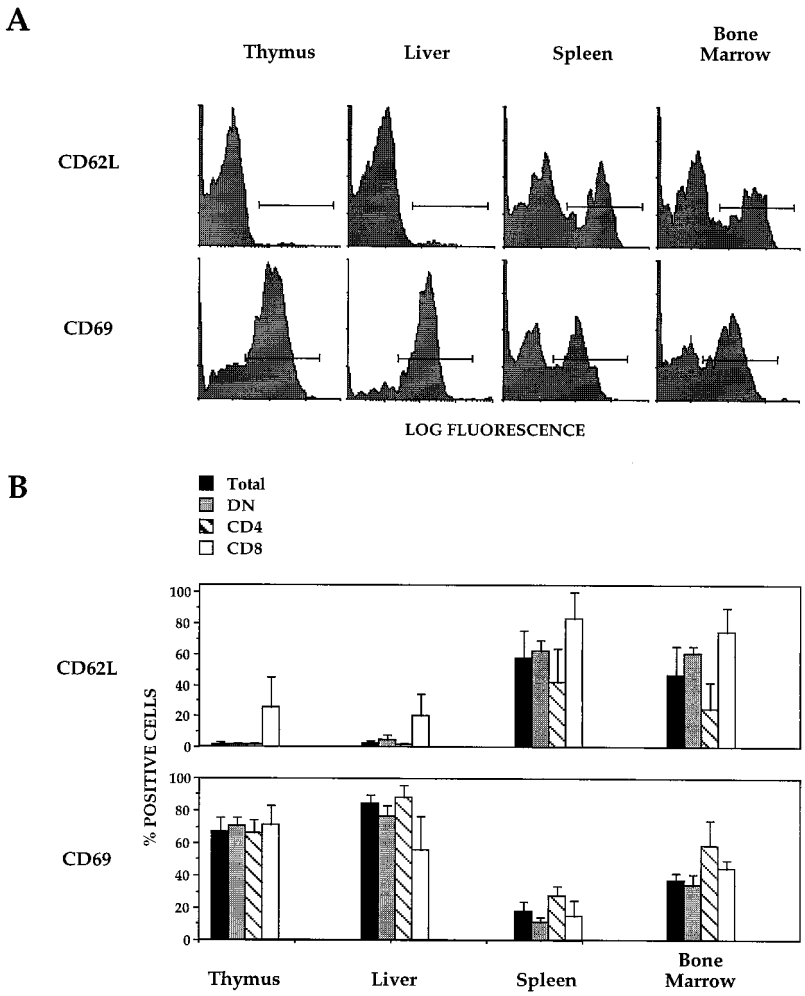

FIGURE 5. Expression of T cell activation markers by NKT cells. Cells isolated from C57BL/6 mice were *A*, triple stained with mAbs against TCRβ, NK1.1, and CD62L or CD69; and *B*, quadruple stained with mAbs against TCRβ, NK1.1, CD4 and/or CD8, and CD62L or CD69. All cells analyzed were gated on TCRβ^+NK1.1$^+$ phenotype. In *A*, bars indicate the cells considered as positive. The experiment reported in *A* is representative of at least four independent experiments, and the data presented in *B* are the mean ± SD of at least four independent experiments.

tissue distribution, specificity, and phenotype of NKT cells (Table II). One type of NKT cell segregates preferentially to thymus and liver, and depends on CD1d for development. CD1d-dependent NKT cells express a biased TCR repertoire, an activated phenotype, and low levels of NK cell markers. A second type of NKT cell, enriched in spleen and bone marrow, develops in the absence of CD1d, and expresses a diverse TCR repertoire, a naive phenotype, and high levels of NK cell markers.

Discussion

A large fraction (15–30%) of mature (HSA$^-$) thymocytes and liver mononuclear cells express the TCR$\alpha\beta$ as well as the NK cell marker NK1.1 and are termed NKT cells (1, 2). Most (80–90%) thymus and liver NKT cells express an invariant Vα14-Jα281 chain paired preferentially to a polyclonal Vβ8.2, Vβ7, or Vβ2 chain (5–8) and are positively selected by the monomorphic MHC class I-like molecule CD1d (14–17). Moreover, a T cell subset similar to mouse NKT cells exists in human CD3$^+$CD56$^+$PBL. These cells are CD1d restricted (36), and express NKRP1A, a human homologue of NK1.1 (37), and an invariant Vα24-JαQ chain, which has high sequence homology with the mouse Vα14-Jα281 chain (5). Moreover, this Vα24-JαQ chain is paired preferentially to a restricted set of Vβ-chains, including Vβ11 and Vβ13, which have sequence homology with the mouse Vβ8 and Vβ7 chains (5). These CD1d-restricted NKT cells in mice and humans apparently carry out a conserved immunologic function that remains to be clearly established (38, 39).

In contrast to thymus and liver, substantial numbers of NKT cells in spleen and bone marrow do not express the invariant Vα14-Jα281 chain (29). Moreover, five independent reports show that NKT cells still develop in thymus, liver, and spleen of CD1d-deficient mice and represent 10–20% of the number of NKT cells in wild-type tissues (8, 14–17). Thus, some NKT cells exist that do not express the invariant Vα14-Jα281 chain and are not positively selected by CD1d. In this study, we have analyzed in detail the phenotype, TCR$\alpha\beta$ repertoire, activation status, and CD1d dependency of NKT cells in various tissues. Our data demonstrate the existence of two major NKT cell subsets that are strongly selected by the tissue environment they encounter.

One NKT cell subset (Table II) can be defined as expressing a biased TCR$\alpha\beta$ repertoire that is positively selected by CD1d. Importantly, CD1d is able to select NKT cells that express only a partially (Vβ8.2) biased TCR repertoire, in the apparent absence of bias toward Vα14. CD1d-dependent NKT cells consist of CD4$^+$ and DN cells, have a phenotype found on activated T cells (CD62L$^-$CD69$^+$), and express low levels of the NK cell markers Ly49A and DX5. Similarly, human CD1d-dependent Vα24$^+$NKT cells have been reported to consist mainly of CD4$^+$ and DN cells and to express low levels of killer-inhibitory receptors of both the C-type lectin family (CD94) and the Ig superfamily (37). A second NKT cell subset (Table II) can be defined as expressing a CD1d-independent and diverse TCR$\alpha\beta$ repertoire (i.e., a TCR$\alpha\beta$ repertoire comparable with that expressed by conventional T cells). CD1d-independent NKT cells consist mainly of CD8$^+$ and DN

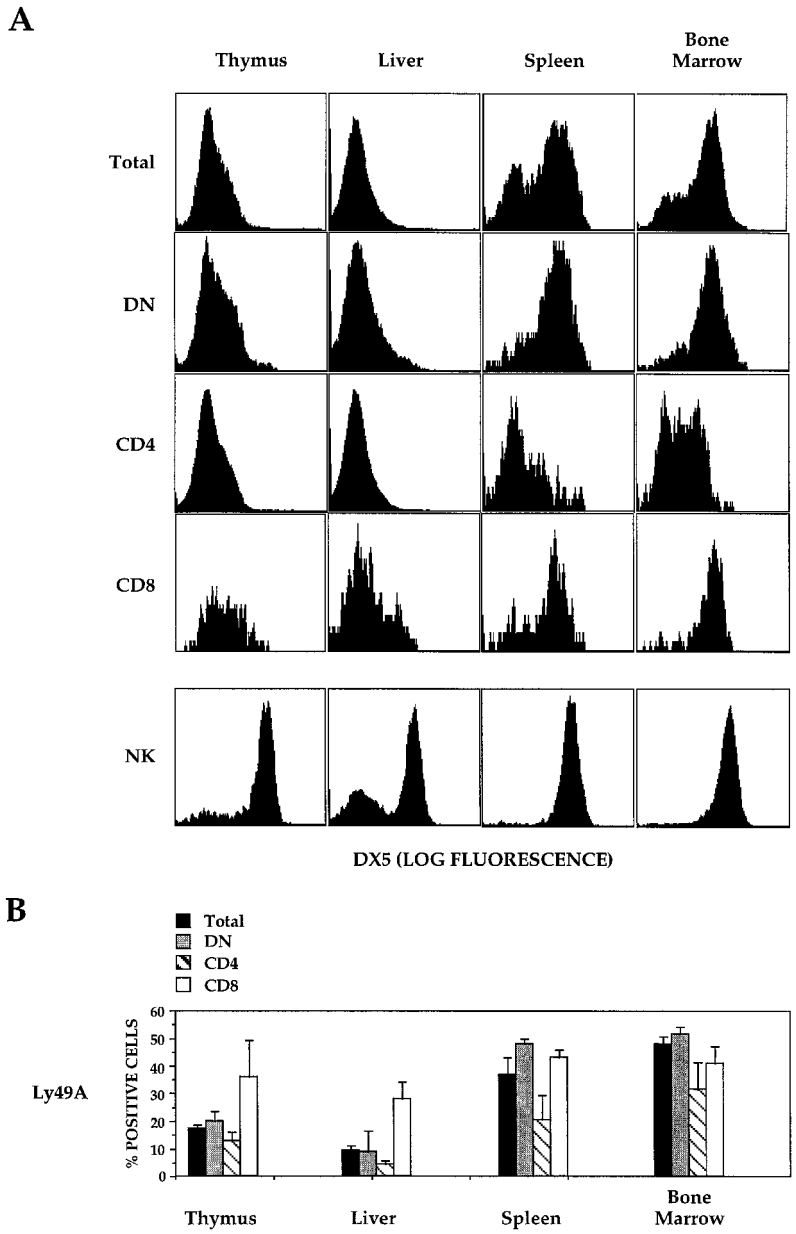

FIGURE 6. Expression of NK cell markers by NKT cells. *A*, Cells isolated from C57BL/6 mice were quadruple stained with mAbs against TCRβ, NK1.1, CD4 and/or CD8, and DX5. All cells analyzed were gated on TCRβ^+NK1.1$^+$ phenotype, except for the *lower panels* showing the expression of DX5 by TCRβ^-NK1.1$^+$ cells, which are mostly NK cells. *B*, Cells isolated from C57BL/6 mice were quadruple stained with mAbs against TCRβ, NK1.1, CD4 and/or CD8, and Ly49A. The experiment reported in *A* is representative of at least four independent experiments, and the data presented in *B* are the mean ± SD of at least three independent experiments.

cells, have a phenotype found on naive T cells (CD62L$^+$CD69$^-$), and express high levels of the NK cell markers Ly49A and DX5. The absence of CD1d-dependent CD8$^+$NKT cells is consistent with earlier results showing that Vα14-Jα281$^+$NKT cells do not develop in CD8$\alpha\beta$ transgenic mice (14), suggesting that the presence of CD8 causes negative selection of precursors of CD1d-dependent NKT cells.

NKT cells are found mainly in thymus, liver, spleen, and bone marrow, whereas they are rare in lymph nodes and gut (2, 8, 10–12). Thymus and liver contain mostly NKT cells that belong to the CD1d-dependent subset (Table II). In contrast, spleen and bone marrow contain both CD1d-dependent and CD1d-independent NKT cells. Therefore, two types of tissues can be defined that contain either mostly CD1d-dependent NKT cells or high proportions of CD1d-independent NKT cells. This finding suggests that the two types of tissues express different ligands and thereby select different NKT cell subsets on the basis of TCR specificity or, alternatively, that distinct tissue-specific homing receptors are expressed by CD1d-dependent and -independent NKT cells.

Several recent reports indicate that CD1d-dependent NKT cells may recognize a variety of (tissue-) specific ligands. Brossay et al. (19) and Park et al. (18) have shown that a panel of Vα14-Jα281$^+$ T cell hybridomas was stimulated by different CD1d$^+$ tissues and CD1d-transfected cell lines to produce IL-2. However, the amount of IL-2 produced varied both with the T cell hybridoma and the stimulators used in the assay, independently of the level of CD1d. Moreover, a particular set of synthetic CD1d-restricted ligands, α-glucosyl- and α-galactosylceramides, has been found by Kawano et al. to induce proliferation of splenic Vα14-Jα281$^+$NKT cells, when loaded on dendritic cells (22). Although Joyce et al. have proposed that cellular glycosylphosphatidylinositol is a natural ligand of CD1d (21), Burdin et al. have recently reported that phophatidylinositol and phosphatidylinositol dimannoside do not activate NKT cells (23). In the case of CD1d-independent NKT cells, molecules responsible for their positive selection and activation remain to be defined. The number of CD8$^+$NKT cells, the major population of CD1d-independent NKT cells, is nevertheless greatly diminished in

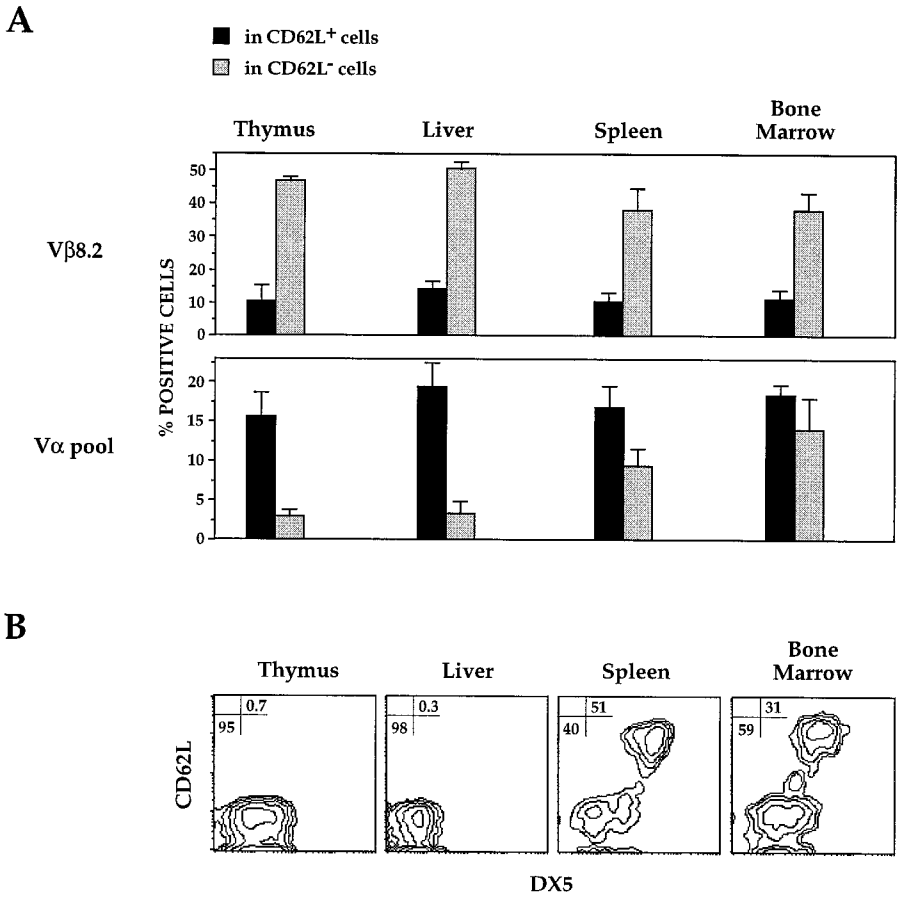

FIGURE 7. TCR repertoire and NK cell markers in activated $CD4^+$NKT cells. Cells isolated from C57BL/6 mice were quadruple stained with *A*, mAbs against CD4, NK1.1, CD62L, and Vβ8.2 or a pool of mAbs against Vα2, Vα3.2, Vα8, and Vα11 (Vα pool), and *B*, mAbs against CD4, NK1.1, CD62L, and DX5. All cells analyzed were gated on $CD4^+NK1.1^+$ phenotype. Numbers (*B*) are percentage of $CD4^+$NKT cells in the $DX5^{low}CD62L^-$ (*lower left*) or the $DX5^{high}CD62L^+$ (*upper right*) compartment. It should be emphasized that essentially all $CD4^+NK1.1^+$ cells express TCR$\alpha\beta$ in all organs considered. The data in *A* are the mean ± SD of five independent experiments, and the experiment depicted in *B* is representative of four independent experiments.

β_2m- and in TAP-1-deficient mice (data not shown), indicating that MHC class I or class I-like molecules are involved in the selection of $CD8^+$NKT cells. In contrast, a comparable reduction of DN NKT cells is found in CD1d- and β_2m-deficient mice (data not shown), indicating that putative ligands of CD1d-independent DN NKT cells do not belong to the MHC class I or class I-like family of molecules.

In addition to different ligand specificities, CD1d-dependent and CD1d-independent NKT cells also express different patterns of activation markers. Indeed, CD1d-dependent NKT cells may encounter activating ligands preferentially in thymus and liver and, consequently, acquire a phenotype found on activated T cells ($CD62L^-$, $CD69^+$). In contrast, CD1d-independent NKT cells may not encounter activating ligands under normal circumstances and express a phenotype found on naive T cells ($CD62L^+$, $CD69^-$). However, all NKT cell subsets express the T cell activation marker CD44. In this context, Walker et al. have shown that conventional $CD8^+$ T cells specific for an HLA-CW3-derived peptide are mostly $CD62L^-$ and $CD44^+$ at the peak of the response, whereas some long-term immune $CD8^+$ T cells are $CD62L^+$, but still express CD44 (40). Accordingly, expression of CD44 on NKT cells may indicate that all NKT cells have undergone activation, but since CD1d-dependent and -independent NKT cells are $CD62L^-$ and $CD62L^+$, respectively, only CD1d-dependent NKT cells may undergo chronic activation in thymus and liver.

The expression of different levels of NK cell markers by CD1d-dependent and CD1d-independent NKT cells (i.e., $Ly49A^-DX5^{low}$ versus $Ly49A^+DX5^{high}$) may indicate that the two types of NKT cells undergo different selection events during development. As proposed earlier by Bendelac et al., NK cell markers such as Ly49A may be directly involved in the selection process of NKT cells (2). Since Ly49A is a member of the killer-inhibitory receptor family (41), it may deliver a negative signal to NKT cells when it binds the cognate MHC class I ligand, dampen the effect of TCR signaling, and allow positive selection of NKT cells only when

Table II. *Dichotomy in tissue distribution, CD1d dependency, and phenotype of NKT cells*

NKT Cells	Preferential Tissue Distribution	CD1d Dependency	TCR Repertoire	CD4/CD8 Expression	Activation Phenotype	NK Cell Markers[a]
Type I	Thymus Liver	Dependent	Biased[b]	$CD4^+$, DN	Activated[c]	Low
Type II	Spleen Bone marrow	Independent	Diverse	$CD8^+$, DN	Naive[c]	High

[a] Ly49A and DX5.
[b] Preferential expression of Vα14 and Vβ8.2.
[c] Activated NKT cells are $CD62L^-$ and $CD69^+$, whereas naive NKT cells are $CD62L^+$ and $CD69^-$.

their TCR has sufficient affinity for its ligand. In agreement with this hypothesis, CD1d-dependent NKT cells constitutively expressing Ly49A (as a transgene) fail to fully develop in mice expressing a ligand of Ly49A (H-2D^d) (3), showing that Ly49A interferes to some degree with positive selection of NKT cells by CD1d. Similar to CD1d-dependent mouse NKT cells, human CD1d-dependent Vα24$^+$NKT cells also express low levels of killer-inhibitory receptors (37).

A major issue raised by this study is whether CD1d-dependent and CD1d-independent NKT cells develop along distinct cell lineages, and whether they share a common tissue of origin. Kikly et al. have reported that transfer of syngeneic bone marrow cells into congenitally athymic (nude) mice gives rise to CD3$^+$NK1.1$^+$ cells of donor origin in the recipient spleen (24). Sato et al. have extended these findings by showing that transfer of syngeneic bone marrow cells into adult thymectomized and irradiated mice gives rise to CD8$^+$NKT cells in recipient liver and spleen (26). Hence, CD8$^+$NKT cells (i.e., CD1d-independent NKT cells) can apparently be generated from bone marrow in the absence of thymus. Indeed (as shown in this study), the majority of residual NKT cells present in various tissues of athymic mice are CD8$^+$. On the other hand, the strong reduction in the number of NKT cells in nude mice (12, 13) or neonatally thymectomized mice (27) as compared with wild-type mice mostly affects CD4$^+$NKT cells (i.e., CD1d-dependent NKT cells), and to a lesser extent DN NKT cells. Hence, most CD1d-dependent NKT cells presumably develop in the thymus, and a significant proportion of CD1d-independent NKT cells develops extrathymically (presumably from the bone marrow). In addition, since the absolute number of CD1d-independent NKT cells (in particular DN NKT cells) is decreased in nude and neonatally thymectomized mice as compared with wild-type mice, it is probable that a fraction of CD1d-independent NKT cells develops in the thymus and then emigrates to spleen and bone marrow. It may also be proposed that the thymus is indirectly involved in the extrathymic generation of CD1d-independent NKT cells. Tsukahara et al. have reported that injection of the thymic hormone thymosin α or transfer of CD3^{high}CD122$^-$ cells (presumably thymus-derived conventional T cells) into nude mice favors the expansion of host CD3^{int}CD122$^+$ (a phenotype that includes NKT cells), but not of CD3^{high}CD122$^-$ cells (42), suggesting that thymus-derived humoral and cellular components may promote extrathymic development of some NKT cells.

Of particular interest is our finding that in wild-type mice, CD1d-dependent NKT cells in thymus and liver express both a biased TCR Vβ and Vα repertoire, whereas in spleen and bone marrow, CD1d-dependent NKT cells express a TCR Vβ repertoire biased to high or intermediate levels and a TCR Vα repertoire that is only slightly biased. In mice deficient for the TCR Jα281 gene segment (used by most thymus and liver NKT cells in wild-type mice), some NKT cells also express a biased TCR Vβ repertoire in the absence of an apparent bias in the Vα repertoire, similar to CD1d-dependent spleen and bone marrow NKT cells in wild-type mice. However, in Jα281-deficient mice, these cells are found in thymus and liver, but not in spleen and bone marrow. These results show that in the absence of CD1d-dependent NKT cells expressing a fully biased TCR repertoire (for both Vβ- and Vα-chains), NKT cells expressing a partially (i.e., Vβ-) biased TCR repertoire segregate in thymus and liver. It seems reasonable to propose that a TCR repertoire biased for both Vβ- and Vα-chains is the optimal repertoire selected by CD1d in wild-type mice, whereas a partially biased TCR repertoire is a suboptimal repertoire in wild-type mice, but an optimal repertoire in Jα281-deficient mice. Therefore, thymus and liver may in all cases select for NKT cells expressing TCRs with the highest affinities for CD1d. A strong selection of CD1d-dependent NKT cells by the liver is further suggested by our earlier observations made in TCR Vβ3 and Vβ8.1 transgenic mice. Indeed, NKT cells in liver (6), but not in bone marrow (data not shown), express both the transgenic Vβ-chain and an endogenously rearranged Vβ8.2 chain (which is presumably selected by CD1d). Finally, we have found that blood NKT cells are enriched in CD8$^+$ and DN NKT cells, confirming that recirculating NKT cells consist mainly of CD1d-independent NKT cells, whereas CD1d-dependent NKT cells segregate in specific tissues such as thymus and liver.

It may be proposed that the tissue-specific segregation of CD1d-dependent and -independent NKT cells results from the expression of distinct homing receptors. Examples of such homing receptors include L-selectin (CD62L), which mediates adhesion of lymphocytes to high endothelial venules in secondary lymphoid organs (43), and β_7 integrins involved in homing to mucosal tissues (44, 45). Nevertheless, our finding that NKT cells expressing a partially (Vβ-) biased TCR repertoire are found in spleen and bone marrow in wild-type mice, but segregate in thymus and liver in Jα281-deficient mice (i.e., in the absence of NKT cells expressing a fully biased TCR repertoire), suggests that TCR specificity rather than homing receptors determines tissue-specific segregation of NKT cells.

The existence of a subset of CD1d-independent NKT cells may be relevant to studies of CD1d- and β_2m-deficient mice, which are frequently considered to be functionally deficient in NKT cells (15–17, 46–49). In this respect, it will be important to establish whether CD1d-independent NKT cells are capable of secreting IL-4 and other cytokines. This situation is further complicated by the fact that an IL-4-secreting CD1d-independent subset of NKT cells expressing a TCR$\gamma\delta$ has also been described (50).

In conclusion, two types of NKT cells can be defined on the basis of their reactivity to CD1d. Whereas CD1d-independent NKT cells resemble naive T cells and recirculate, CD1d-dependent NKT cells are activated and segregate mainly in thymus and liver. CD1d-dependent NKT cells express a highly biased TCR repertoire that is conserved across species, and hence, these cells may have a unique and tissue-specific immune function. The potential importance of this function is underscored by our recent finding that homeostasis of liver NKT cells is reached rapidly (within 2 to 3 days) upon depletion by anti-CD3ϵ mAb or IL-12 treatment (51), suggesting that NKT cells are constantly required in the liver. Known functions of CD1d-dependent NKT cells include IL-12-mediated rejection of liver metastases (25, 33, 52) and control of autoimmunity (2). It may therefore be proposed that CD1d-dependent NKT cells segregate in the liver to rapidly detect and control systemic diseases. In contrast, the physiologic role of CD1d-independent NKT cells remains to be established.

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