Phenotypic and Functional Differences Between NKT Cells Colonizing Splanchnic and Peripheral Lymph Nodes

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NKT cells are considered unconventional T cells. First, they are restricted by a nonclassical MHC class I molecule, CD1d, which presents glycolipids; second, their TCR repertoire is very limited. After stimulation by their TCR, NKT cells rapidly release large amounts of cytokines, such as IL-4 and IFN-γ. Little is known about NKT cells present in lymph nodes. In the present report we show that NKT cells are differently distributed in various lymph nodes and are, for instance, abundant in pancreatic and mesenteric lymph nodes of C57BL/6 mice and nonobese diabetic mice. The high frequency of NKT cells in splanchnic lymph nodes is not simply a consequence of inflammatory signals, as draining lymph nodes still contain low frequencies of NKT cells after IFA or CFA injections. NKT cells from splanchnic lymph node harbors a Vβ repertoire similar to that of splenic and liver NKT cells, in contrast to peripheral NKT cells that are not biased toward Vβ8 segments. Analysis of cytokine production by NKT cells from splanchic lymph nodes reveals that they produce at least as much IL-4 as IFN-γ, in contrast to NKT cells from other organs (spleen, liver, and peripheral lymph nodes), which produce much more IFN-γ than IL-4. These specific features of NKT cells from splanchic lymph nodes might explain their protective action against the development of pathogenic Th1 cells in type 1 diabetes. *The Journal of Immunology*, 2002, 168: 3251–3258.

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### References

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3. Abbreviations used in this paper: α-GalCer, α-galactosylceramide; NOD, nonobese diabetic; SA, streptavidin; RT, reverse transcription.

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**Phenotypic and Functional Differences Between NKT Cells Colonizing Splanchnic and Peripheral Lymph Nodes**

Véronique Laloux,* Lucie Beaudoin,* Catherine Ronet,† and Agnès Lehuen2*

NKT cells are unconventional T lymphocytes restricted by a nonclassical MHC class I molecule, CD1d associated to β₂-microglobulin (1, 2). In contrast to polymorphic MHC molecules, which present antigenic peptides, monomorphic CD1d molecules bind and present glycolipids to NKT cells (3, 4). The TCR repertoire of NKT cells shows relatively limited heterogeneity. Almost all NKT cells use an invariant germline-encoded TCR α-chain (Vα14-Jα281) preferentially associated with Vβ8, Vβ7, and Vβ2 chains (5). They express markers common to the NK cell lineage such as NK1.1, CD122 (IL-2R), monomorphic CD1d molecules bind and present glycolipids to NKT cells (3, 4). The TCR repertoire of NKT cells shows relatively limited heterogeneity. Almost all NKT cells use an invariant germline-encoded TCR α-chain (Vα14-Jα281) preferentially associated with Vβ8, Vβ7, and Vβ2 chains (5). They express markers common to the NK cell lineage such as NK1.1, CD122 (IL-2R), CD44 and CD161 (6, 7). The diversity of these networks explains why NKT cells have been implicated in several biological systems, including protection against various infections by bacteria (8) and parasites (9, 10), tumors and metastases (11, 12), and development of various autoimmune diseases, such as type I diabetes (13–16) and multiple sclerosis (17).

NKT cells are present in most lymphoid organs where conventional αβ T cells are found, although the ratio of NKT to αβ T cells varies widely from one organ to another (2, 18). NKT cells are proportionally more abundant in liver (30–50%), bone marrow (20–30%), and thymus (10–20%) than in spleen (3%) and blood (4%). In contrast, it has been consistently reported that NKT cells are rare in peripheral lymph nodes (0.5%) (18–21). The importance of NKT cells in the regulation of immune responses led us to reexamine the presence of these cells in various lymph nodes. Indeed, these secondary lymphoid organs are crucial for the development of efficient local immune responses. Our previous analysis revealed that NKT cells in Vα14-Jα281 transgenic and control nonobese diabetic (NOD) mice are differentially distributed in various lymph nodes, as mesenteric and pancreatic lymph nodes contain a proportion of NKT cells (relative to total organ cells) similar to that found in the spleen. In contrast, in peripheral lymph nodes (popliteal, inguinal, and brachial), NKT cells are 10 times less frequent (22).

In the present study we first sought to determine whether high frequencies of NKT cells in splanchnic lymph nodes were also observed in nonautoimmune animal strains such as C57BL/6 mice, and if their presence in large numbers is a consequence of stimulation/inflammation of lymph nodes. As several studies have described some heterogeneity among NKT cells according to their localization (19, 20, 23–25), we characterized the TCR repertoire and phenotype of NKT cells present in splanchic lymph nodes. The functional capacities of NKT cells from splanchic lymph nodes were compared with those of NKT cells from several other organs after in vivo and in vitro stimulation. Their steady state cytokine production was determined ex vivo by means of quantitative PCR, and the role of APC in NKT cell activation was analyzed.
Materials and Methods

**Mice**

The Va14-Jo281 transgenic NOD line 86 was produced by microinjection of NOD eggs. Va14-Jo281 transgenic mice on NOD, Ca21/2-NOD, and congenic NK1.1 NOD backgrounds have been described in detail previously (13, 22, 26). The Va14-Jo281 C57BL/6 transgenic line was obtained after 10 backcrosses of the Va14-Jo281 NOD line 86 on C57BL/6 mice. They were further backcrossed on Ca21/2 C57BL/6 mice. All mice used in this study were raised and housed in strictly controlled specific pathogen-free conditions.

**Flow cytometry**

Cell suspensions were prepared from spleen, liver, and mesenteric and pancreatic lymph nodes of individual mice. Other lymph nodes were pooled from three mice of identical age. Hepatocytes and red cells were removed by Percoll gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). Cells were stained at 4°C in PBS containing 1% BSA and 0.1% azide after blocking FcYR by incubation with 2.4G2 mAb. Staining was performed with FITC-conjugated anti-TCRαβ mAb (H57) or PerCP-conjugated anti-CD3ε mAb (145-2C11; BD PharMingen, San Diego, CA), PE-conjugated or biotinylated anti-NK1.1 mAb (PK156; BD PharMingen), biotinylated anti-TCRβVβ2 mAb (B2O.6; BD PharMingen), biotinylated anti-TCRβVβ mAb (TR310; BD PharMingen), biotinylated anti-TCRβVβ8 mAb (F23.1; BD PharMingen), biotinylated anti-CD69 mAb (H1.2F3; BD PharMingen), FITC-conjugated anti-CD44 mAb, PE-conjugated anti-CD122 mAb (IL-2Rβ chain; BD PharMingen), PE-conjugated anti-isotype control (hamster IgG; BD PharMingen), and allophycocyanin-conjugated streptavidin (SA; Caltag Laboratories, South San Francisco, CA). Stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software.

**CFA and IFA injections**

Thirteen-week-old mice were injected s.c. in the hind footpads with saline alone or emulsified with IFA or CFA (Difco, Detroit, MI). Two days, 14 days, and 5 mo after the injections, the mice were killed, draining popliteal lymph nodes and pancreatic lymph nodes were isolated, and cell suspensions were prepared and stained to detect NKT cells.

**NKT cell purification**

For immunoscope analysis, cell suspensions were prepared from spleen, pancreatic lymph nodes, and liver of individual Va14-Jo281 C57BL/6 and NOD transgenic mice. Red cells in spleens were lysed with NH4Cl, and B lymphocytes were removed by panning on anti-IgM-coated plates. Cell suspensions were incubated with 2.4G2 mAb and stained with FITC-conjugated anti-TCRαβ mAb (H57) and PE-conjugated anti-NK1.1 mAb (PK136; BD PharMingen). TCRαβNK1.1+ splenocytes, pancreatic lymph nodes, and liver cells were sorted with a Beckman Coulter sorter (Hialeah, FL).

For functional studies, NKT cell purification was performed as follows. After removal of red cells and B cells, splenocytes were incubated with 2.4G2 mAb and anti-mouse CD5 microbeads (Ly-1; Miltenyi Biotec, Auburn, CA). CD5-positive cells were magnetically purified with an LS separation column using VarioMACS (Miltenyi Biotec). Mesenteric or pancreatic lymph node cells preincubated with 2.4G2 mAb and CD5+ spleen cells were stained with FITC-conjugated anti-CD5 mAb (Ly-1, 53-7.3; BD PharMingen) and biotinylated anti-NK1.1 mAb plus SA-allophycocyanin for further electronic purification of CD5+ NK1.1+ cells with a FACSVantage sorter (BD Biosciences). Using both protocols, purity after cell sorting was between 96 and 98%.

**Messenger RNA quantification, PCR procedure, and immunoscope analysis**

RNA from TCRαβNK1.1+ cells was isolated for reverse transcription (RT) into cDNA. Quantification of PCR products was conducted as previously described (27), using a competitive PCR strategy based on size-altered CD3ε cDNA that yields the number of CD3ε copies contained in the sample. A volume of cDNA solution containing 104 copies of cDNA CD3ε was PCR-amplified using each of the 24 Vα-specific primers and a fluorescence-labeled Cβ-specific primer (94°C for 30 s; 60°C for 30 s; 72°C for 30 s) for 31 cycles (remaining within the exponential phase of amplification). Products resulting from this PCR were analyzed on an automated sequencer. The size and intensity of each band were recorded and analyzed using Immunoscope software (Applied Biosystems, Foster City, CA).

**In vivo activation by α-GalCer and intracytoplasmic staining**

Mice were injected either with 4 μg α-GalCer (2 µg i.v. and 2 µg i.p.) or with vehicle alone (saline containing 0.5% polysorbate-20). The mice were killed after 2 h, as kinetic studies showed that this time corresponds to the peak of CD69 up-regulation on NKT cells in spleen and pancreatic lymph nodes. Cell suspensions were prepared from spleen and pancreatic lymph nodes. After incubation with 2.4G2 mAb, surface staining was performed with FITC-conjugated anti-TCRαβ mAb and biotinylated anti-NK1.1 mAb plus SA-PerCP (BD Biosciences). Cells were then fixed in the dark for 20 min at room temperature with 2% paraformaldehyde. Permeabilization was

![FIGURE 1](http://www.jimmunol.org/)

NKT cells are present at high frequencies in mesenteric and pancreatic lymph nodes. Cells from an 11-wk-old Va14-Jo281 Ca21/2 C57BL/6 mouse and a littermate control and from a 13-wk-old Va14-Jo281 Ca21/2 NOD mouse and a littermate control were dually stained with anti-TCRαβ and anti-NK1.1 mAbs. The numbers represent the percentages of αβNK1.1+ cells among total organ cells.
performed with PBS containing 0.5% saponin, 1% BSA, and 0.1% azide. Intracytoplasmic staining was performed with PE-conjugated anti-mouse IFN-γ mAb (XMG1.2; BD PharMingen) and allophycocyanin-conjugated anti-mouse IL-4 mAb (11B11; BD PharMingen) diluted in permeabilization buffer and left in the dark for 30 min at room temperature. Cells were washed in permeabilization buffer and resuspended in normal FACS buffer for flow cytometry on a FACSCalibur.

In vitro activation by PMA plus ionomycin

Cell suspensions were prepared from spleen, liver, and mesenteric, pancreatic, and peripheral lymph nodes of 10- to 11-wk-old mice. Cells were stimulated in vitro at 1 × 10^6 cells/ml by 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 10 mg/ml brefeldin A in RPMI 1640/10% FCS for 4 h at 37°C. Nonstimulated cells were left in medium plus brefeldin A in the same conditions as stimulated cells. The cells were harvested, then surface-stained for TCR and NK1.1, followed by fixation, permeabilization, and intracytoplasmic staining for IL-4 and IFN-γ as described above. Stained cells were analyzed on a FACSCalibur flow cytometer.

**FIGURE 2.** Comparison of NKT cell numbers in various lymph nodes from C57BL/6 and NOD mice. Cells from C57BL/6 (A) or NOD (B) Vα14-Jα281 Cα-Tg transgenic mice (□) and littermate controls (■) were dually stained with anti-TCRαβ and anti-NK1.1 mAbs. The numbers represent the percentages of NKT cells among total organ cells. The means ± SD were determined for 10 individual mice, except for inguinal lymph nodes (three independent experiments with two mice pooled).

**FIGURE 3.** The NKT cell frequency in peripheral lymph nodes is not modified by adjuvant injections. C57BL/6 (A) or NOD (B) Vα14-Jα281 Cα-Tg transgenic mice (open symbols) and littermate controls (filled symbols) were injected s.c. with PBS alone, IFA, or CFA, and lymph node cells were prepared for NKT cell staining 2 and 14 days later (squares and circles, respectively). Nontreated (NT) mice were also analyzed (triangles). Each point represents an individual mouse. The data shown correspond to one of three independent experiments that produced similar results. In total, six mice for each group have been analyzed.

**FIGURE 4.** NKT cells from splanchnic lymph nodes, but not from peripheral lymph nodes, exhibit a similar Vβ repertoire to that of NKT cells from spleen and liver. A, Cells from spleen, mesenteric (Mes), pancreatic (Panc), or peripheral (Peri) lymph nodes and liver of 10- to 15-wk-old C57BL/6 or NOD Vα14-Jα281 Cα-Tg transgenic mice were triply stained with anti-TCRαβ, anti-NK1.1, and anti-Vβ2, anti-Vβ7, or anti-Vβ8 mAbs. The numbers represent the percentages of cells positive for each Vβ among NK1.1+ cells. B, αβ+ NK1.1+ cells from liver, spleen, and pancreatic lymph nodes of Vα14-Jα281 Cα-Tg C57BL/6 mice were purified by cell sorting, and their Vβ repertoires were determined by semiquantitative RT-PCR immunoscope analysis. Values represent the mean ± SD for three individual mice.
CD5ε⁺NK1.1⁺ (0.15 × 10⁵) splenocytes and pancreatic lymph node cells were lysed for RNA extraction and RT into cDNA. Cytokine mRNA expression was analyzed with a kinetic ELISA-PCR method as previously described (22, 28). Because the main source of variability in PCR RNA quantification is not the RT or PCR steps but, rather, the amount of starting material and RNA quality, all samples were tested in duplicate.

**Dendritic cell enrichment and in vitro activation of NKT cells by α-GalCer**

Dendritic cells were enriched from spleens or mesenteric lymph nodes of 10- to 13-wk-old C57BL/6 mice. Tissues were minced and digested for 30 min at 37°C with 1 mg/ml collagenase D (stock solution at 100 mg/ml; Roche, Mannheim, Germany). Pellets were resuspended in 2 ml RPMI 1640 containing 0.01 M HEPES buffer, 1 mM sodium pyruvate, and 1× nonessential amino acids without serum, and centrifuged at 1000 × g for 30 min. Low density cells were harvested from the interphase and washed; FcγRs were blocked with 2.4G2 mAb. CD11c⁺ cells were magnetically enriched using VarioMACS with anti-mouse CD11c microbeads (N418; Miltenyi Biotec) and MS separation columns. For NKT cells purification, Vα14-Jo281 transgenic C57BL/6 mice from 6 to 16 wk of age were killed, and splenocytes and mesenteric lymph node cells were prepared. The purification of NKT cells was performed as described above (purity, >96%). For in vitro stimulation, 0.3 × 10⁵ CD5ε⁺NK1.1⁺ splenocytes or mesenteric lymph node cells were cultured at 37°C in complete medium (RPMI 1640 containing 10% FCS, glutamine, 2-ME, and penicillin-streptomycin) with 5 × 10⁴ irradiated (3000 rad) splenocytes or mesenteric lymph node cells containing 50–65% CD11c⁺ cells, with or without 100 ng/ml α-GalCer (a gift from Kirin Brewery, Guma, Japan). Culture supernatants were harvested after 48 h to measure IL-4 and IFN-γ production. IL-4 and IFN-γ were measured with ELISA methods as previously described (13), using mAbs 11B11 plus R46A2 (a gift from DNAX Research Institute, Palo Alto, CA) respectively. Recombinant mouse IL-4 and IFN-γ were purchased from R&D Systems (Abingdon, U.K.).

**Inhibition of in vitro α-GalCer stimulation with blocking anti-CD1d mAb**

After removal of red cells, splenocytes and mesenteric lymph node cells from Vα14-Jo281 Cαε⁻⁻ C57BL/6 transgenic mice were stimulated during 48 h in vitro (6 × 10⁵ total cells/well) with α-GalCer (100 ng/ml) in the presence or the absence of blocking anti-CD1d mAb (20H2, 1–7%) produced by NKT cells from pancreatic lymph nodes have a less-activated phenotype than splenic NKT cells. Splenocytes and pancreatic lymph node cells from C57BL/6 and NOD Vα14-Jo281 Cαε⁻⁻ C57BL/6 mice from 6 to 16 wk of age were killed, and splenocytes and pancreatic lymph node cells were prepared. The percentages of total cytokine-producing cells and IL-4:IFN-γ ratios are indicated for each panel. Similar results were obtained in four experiments with each strain and also with Vα14-Jo281 Cαε⁻⁻ mice. αβ⁻NK1.1⁺ cells were also analyzed; only 3–7% produced cytokines, and for each organ their cytokine profiles were similar to those of αβ⁻NK1.1⁺ cells (data not shown).

**FIGURE 5.** NKT cells from pancreatic lymph nodes have a less-activated phenotype than splenic NKT cells. Splenocytes and pancreatic lymph node cells from C57BL/6 and NOD Vα14-Jo281 Cαε⁻⁻ C57BL/6 mice were triply stained with anti-TCRαβ, anti-NK1.1, and anti-CD69, anti-CD122 or anti-CD44 mAbs. NKT (αβ⁺NK1.1⁺) cells from spleen (thin line) and pancreatic lymph node (bold) NKT cells. These results are representative of two experiments.

**FIGURE 6.** IL-4 and IFN-γ produced by NKT cells from pancreatic lymph nodes and spleen after in vivo stimulation. Vα14-Jo281 Cαε⁻⁻ C57BL/6 (A) or NOD (B) mice were injected i.v. and i.p. with α-GalCer or vehicle, and splenocytes and lymph node cells were analyzed 2 h later by surface and intracytoplasmic staining. The dot plots correspond to αβ⁺NK1.1⁺ gated cells. The percentages of single IL-4⁺ or IFN-γ⁺ and dual IL-4⁺IFN-γ⁺ producers are indicated in each quadrant. Percentages of total cytokine-producing cells and IL-4:IFN-γ ratios are also indicated for each panel. Similar results were obtained in four experiments with each strain and also with Vα14-Jo281 Cαε⁻⁻ mice. αβ⁻NK1.1⁺ cells were also analyzed; only 3–7% produced cytokines, and for each organ their cytokine profiles were similar to those of αβ⁻NK1.1⁺ cells (data not shown).
Results

The high frequency of NKT cells in mesenteric and pancreatic lymph nodes is not a consequence of inflammation

Although it is commonly accepted that NKT cells are rare in peripheral lymph nodes, our previous study of V\(\alpha\)14-Jα281 transgenic and control NOD mice revealed that NKT cells were abundant in mesenteric and pancreatic lymph nodes. To determine whether this high frequency of NKT cells in splanchic lymph nodes was peculiar to NOD mice (which develop diabetes as a consequence of pancreatic inflammation), we backcrossed the V\(\alpha\)14-Jα281 transgene onto C57BL/6 mice. In such mice, NKT cells were also more abundant in mesenteric and pancreatic lymph nodes (5–7% in the lymphocyte gate) than in peripheral lymph nodes such as popliteal, inguinal, and brachial nodes (~1% of NKT cells; Figs. 1 and 2). Transgenic and nontransgenic C57BL/6 and NOD mice were analyzed at 4 wk to 6 mo of age; NKT cell frequencies in splanchic and peripheral lymph nodes did not vary with age (data not shown). We then examined whether the higher frequency of NKT cells in these splanchic lymph nodes was due to chronic activation by food Ags. To generate a source of inflammation, we backcrossed the V\(\alpha\)14-Jα281 transgene onto C57BL/6 mice. In such mice, NKT cells were abundant in mesenteric and pancreatic lymph nodes. To determine whether this high frequency of NKT cells in splanchic lymph nodes was due to immune response to a local Ag present in these lymph nodes, if this were the case, one would expect to detect a NKT cell repertoire different from that of splenic NKT cells. Immunofluorescence staining showed that NKT cells from spleen, mesenteric and pancreatic lymph nodes, and liver expressed a similar repertoire of V\(\beta\) segments (Fig. 4A). An exhaustive survey of all V\(\beta\) used by NKT cells from pancreatic lymph nodes of V\(\alpha\)14-Jα281 transgenic C57BL/6 mice (Fig. 4B) and NOD mice (data not shown) was determined by semi-quantitative PCR-based analysis and compared with that of splenic and liver NKT cells from the same mice. The V\(\beta\) repertoire of NKT cells from these three organs were very similar, with frequent usage of V\(\beta\)1, -2, -7, -8.1, -8.2, -8.3, -9, and -12.

The expression of surface molecules usually expressed on NKT cells was then analyzed on NKT cells from splanchic lymph nodes after immunofluorescence staining. All surface molecules analyzed were less strongly expressed on NKT cells from splanchic lymph nodes than on NKT cells from spleen. For example, in V\(\alpha\)14-Jα281 NOD mice, CD69 was detected on 68% of NKT cells from spleen, but only on 31% of NKT cells from pancreatic lymph nodes (Fig. 5). CD122 and CD44 were slightly less expressed on NKT cells from pancreatic lymph nodes than on splenic NKT cells. Analysis of NKT cells from mesenteric lymph nodes gave results similar to those obtained with NKT cells from pancreatic lymph nodes (data not shown). These data suggested that NKT cells from splanchic lymph nodes were less activated than splenic NKT cells. NK cell lineage markers, such as three different Ly49 molecules (Ly49 C1, G2, and A), were also expressed on fewer

**FIGURE 7.** IL-4 and IFN-\(\gamma\) produced by NKT cells from spleen, splanchic and peripheral lymph nodes, and liver after in vitro stimulation by PMA and ionomycin. Cells from V\(\alpha\)14-Jα281 C57BL/6 (A) or NOD (B) mice were stimulated in vitro by PMA plus ionomycin for 4 h at 37°C. The cells were harvested and stained with anti-TCR\(\alpha\)β and anti-NK1.1 mAbs then permeabilized and incubated with anti-IL-4 and anti-IFN-\(\gamma\) mAbs. The dot plots correspond to \(\alpha\)\(\beta\) NKT1.1* gated cells. The percentages of single IL-4* or IFN-\(\gamma\)* and dual IL-4*IFN-\(\gamma\)* producers are indicated in each quadrant. The percentages of total cytokine-producing cells (CK* cells) and IL-4:IFN-\(\gamma\) ratios are also indicated in each panel. Similar results were obtained in two experiments with each strain and also with V\(\alpha\)14-Jα281 Cα1* mice. Three mice were pooled for each experiment.

Repertoire and phenotype of NKT cells in splanchic lymph nodes

The large number of NKT cells in splanchic lymph nodes could reflect specific antigenic stimulation by a local Ag present in these lymph nodes. If this were the case, one would expect to detect a NKT cell repertoire different from that of splenic NKT cells. Immunofluorescence staining showed that NKT cells from spleen, mesenteric and pancreatic lymph nodes, and liver expressed a similar repertoire toward V\(\beta\)2, -7, and -8 segments. In contrast, NKT cells from peripheral lymph nodes harbored a distinct V\(\beta\) repertoire with no preferential usage of these three V\(\beta\) segments (Fig. 4A). An exhaustive survey of all V\(\beta\) used by NKT cells from pancreatic lymph nodes of V\(\alpha\)14-Jα281 transgenic C57BL/6 mice (Fig. 4B) and NOD mice (data not shown) was determined by semi-quantitative PCR-based analysis and compared with that of splenic and liver NKT cells from the same mice. The V\(\beta\) repertoires of NKT cells from these three organs were very similar, with frequent usage of V\(\beta\)1, -2, -7, -8.1, -8.2, -8.3, -9, and -12.
NKT cells from splanchnic lymph nodes than from spleen (data not shown).

Functional characterization of NKT cells from pancreatic and mesenteric lymph nodes

To compare the functional capacities of NKT cells from pancreatic lymph nodes and spleen, cytokine production was determined shortly after in vivo stimulation with α-GalCer, a specific ligand of CD1d-restricted T cells. Cytokine contents were analyzed by intracytoplasmic immunofluorescence staining 2 h after α-GalCer injection. As shown in Fig. 6A, 21% of splenic NKT cells from Vα14-Jα281 C57BL/6 mice produced both IL-4 and IFN-γ, while 13 and 7% of NKT cells produced only IFN-γ or IL-4, respectively. In comparison, fewer NKT cells from pancreatic lymph nodes produced cytokines (21 vs 41% of splenic NKT cells).Interestingly, more NKT cells from pancreatic lymph nodes produced IL-4 than IFN-γ, whereas more splenic NKT cells produced IFN-γ than IL-4. Experiments with NKT cells from Vα14-Jα281 NOD mice (Fig. 6B) gave results very similar to those obtained with NKT cells from Vα14-Jα281 C57BL/6 mice. These data showed that after in vivo stimulation NKT cells from pancreatic lymph nodes preferentially produced IL-4, whereas splenic NKT cells preferentially produced IFN-γ. To compare cytokine production by NKT cells from five different organs (spleen; mesenteric, pancreatic, and peripheral lymph nodes; and liver), NKT cells were stimulated in vitro for 4 h with PMA plus ionomycin (Fig. 7). This assay confirmed that NKT cells from pancreatic lymph nodes produced less IFN-γ than NKT cells from spleen. Moreover, it revealed that NKT cells from mesenteric lymph nodes were functionally similar to NKT cells from pancreatic lymph nodes. In contrast, NKT cells from peripheral lymph nodes produced even more IFN-γ than NKT cells from spleen. Similar results were obtained in both C57BL/6 and NOD mice. With the same protocol, IL-10 production by NKT cells from these five organs was below the detection limit (data not shown).

To determine the constitutive level of cytokine messengers in vivo with no exogenous stimulation, NKT cells from spleen and pancreatic lymph nodes were electronically sorted and lysed to extract mRNA. Cytokine mRNA levels were measured by quantitative kinetic RT-PCR. As shown in Fig. 8, for the same amount of TCR β-chain cDNA, NKT cells from pancreatic lymph nodes contained 4–10 times less IFN-γ mRNA than splenic NKT cells and 1.5 times more IL-4 mRNA. All these results showed that NKT cells from splanchnic lymph nodes were functionally different from NKT cells from other organs.

We then examined whether the origin of APC influenced the cytokines produced by NKT cells. NKT cells from mesenteric lymph nodes or spleen were stimulated in vitro by α-GalCer in the presence of APC from mesenteric lymph nodes or spleen. Because anti-TCRαβ or anti-CD3 mAb might have activated the cells, and conventional NK cells are CD5-negative, NKT cells were electronically sorted on the basis of CD5+ NK1.1+. Sorted NKT cells were stimulated by α-GalCer, and IL-4 and IFN-γ release in supernatants was measured 48 h later. As shown in Table I, when NKT cells from both organs were stimulated in the presence of APC from mesenteric lymph nodes, they produced less IFN-γ than in the presence of spleen APC. This showed that the origin of APC could influence the pattern of cytokine production by NKT cells. Surprisingly, NKT cells from mesenteric lymph nodes were more responsive after 48 h than NKT cells from spleen (for cytokine production, see Table I; for proliferation, data not shown). This difference could be due to the increased activation-induced cell death of splenic NKT cells during the 48 h of culture. Indeed, it has been shown that NKT cells die shortly after activation in vivo and in vitro, and this death is Fas mediated (20, 29, 30). Fas expression is higher in splenic NKT cells (35%) than in NKT cells from splanchnic lymph nodes (24%), as shown in Fig. 9A. To confirm

![Image: Figure 8. IL-4 and IFN-γ mRNA levels of NKT cells from pancreatic lymph nodes and spleen. CD5+ NK1.1+ cells from Vα14-Jα281 C57BL/6 or NOD mice were sorted and, without stimulation, lysed for RNA extraction. IL-4 and IFN-γ mRNAs were quantified by kinetic PCR. Each curve corresponds to the mean of two or three separate experiments. The TCR β-chain was also analyzed to normalize the different samples. The negative PCR control (dashes) corresponds to PCR without cDNA. The bars connect the curve obtained with NKT cells from spleen () to the curve obtained with NKT cells from pancreatic lymph nodes (). The length of the bars corresponds to the difference in the number of PCR cycles required to yield the same amount of specific cDNA. Similar results were obtained with Vα14-Jα281 Cα2+ mice.]

Table I. Cytokine released by NKT cells during in vitro stimulation with α-GalCer for 48 h

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<th>Origins of NKT Cells</th>
<th>Origins of APC</th>
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<th>IFN-γ (ng/ml)</th>
<th>Ratio IL-4/IFN-γ</th>
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<tr>
<td>Mesenteric LN</td>
<td>Mesenteric LN</td>
<td>–</td>
<td>ND</td>
<td>0.01</td>
<td>ND</td>
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* Similar results have been obtained in two independent experiments. These results have been generated with Vα14-Jα281 transgenic Cα2+ mice. LN, Lymph node; ND, not detectable.
We characterized NKT cells, which are abundant in mesenteric and pancreatic lymph nodes. NKT cells have usually been found at low frequencies in peripheral lymph nodes relative to other lymphoid organs, and have therefore rarely been analyzed. Analysis of various lymph nodes revealed that, in fact, NKT cells (TCRβ⁺NK1.1⁺) were present in mesenteric and pancreatic lymph nodes at a frequency similar to that found in spleen. This high proportion was not due to the age of the mice, as it was observed in mice as young as 4 wk and in those older than 6 mo. The frequency of NKT cells in these splanchnic lymph nodes was identical in females and males (data not shown). Interestingly, the larger number of NKT cells in splanchic lymph nodes was probably not a consequence of chronic activation by food Ags. Indeed, inflammation of peripheral lymph nodes after CFA or IFA injection did not modify the frequency of NKT cells even though these draining lymph nodes were considerably enlarged, containing up to 50 × 10⁶ cells. Pancreatic lymph nodes were analyzed separately from mesenteric lymph nodes, as they play an important role in autoimmune diabetes and as NKT cells can protect NOD mice against diabetes. The frequency and characteristics of NKT cells were similar in mesenteric and pancreatic lymph nodes. This is not completely unexpected, as pancreatic lymph nodes are closely associated with mesenteric lymph nodes (31). To understand the preferential localization of NKT cells in splanchic lymph nodes relative to peripheral lymph nodes, we analyzed the expression of αβ⁺ integrin on NKT cells from these organs. αβ⁺ binds to the endothelial mucosal addressin MadCAM-1 and is involved in the homing of conventional αβ T lymphocytes to mucosal tissues, including mesenteric lymph nodes (32, 33). However, the percentage of NKT cells expressing αβ⁺ was similar in all lymph nodes (24% in mesenteric, 25% in pancreatic, and 26% in peripheral lymph nodes; data not shown). Another possible explanation for the high frequency of NKT cells in splanchic lymph nodes could be the local presence of a specific Ag. However, the observation that the Vβ repertoire of NKT cells from pancreatic lymph nodes was very similar to that of splenic NKT cells and liver NKT cells argues against this hypothesis. It is still not clear why so many NKT cells are present in splanchic lymph nodes; extensive analysis of cytokines, chemokines, and chemokine receptors expressed by NKT cells might be necessary to elucidate this particular localization.

Our phenotype analyses and functional studies suggested that NKT cells from pancreatic and mesenteric lymph nodes were less activated than splenic NKT cells. Fewer NKT cells from splanchic lymph nodes expressed activation molecules such as CD69, CD122, and CD44 and produced cytokines such as IL-4 and IFN-γ after stimulation (2 h in vivo by α-GalCer, and 4 h in vitro by PMA plus ionomycin). Previous studies have shown that NKT cells from various organs can be more or less activated (19, 23). Indeed, NKT cells from liver are more activated than those from spleen. One cannot exclude that among Vα2, -7, and -8 T cells are not restricted by CD1d. However, it is important to note that splanchic NKT cells harbored a more biased Vβ repertoire characteristic of CD1d-restricted cells (77% of NKT cells expressed Vβ2, -7, and -8) than splenic NKT cells (66% expressed Vβ2, -7, and -8). Therefore, the lower frequency of splanchic NKT cells responsive to α-GalCer compared with splenic NKT cells is probably not due to a higher dilution of CD1d-restricted NKT cells by NKT cells not restricted by CD1d. More importantly, our study showed that the ratio of IL-4/IFN-γ production was higher with NKT cells from splanchic lymph nodes than with NKT cells from spleen, liver, and peripheral lymph nodes. This

The specificity of α-GalCer stimulation for CD1d-restricted T cells, we added blocking anti-CD1d mAb (20H2) to the culture. As shown in Fig. 9B, the addition of 20H2 mAb totally inhibited proliferation and cytokine production with α-GalCer in a dose-dependent manner, whereas anti-CD3 mAb stimulation was not affected (data not shown).
was observed after in vivo stimulation by α-GalCer and after in vitro stimulation by PMA plus ionomycin and also when NKT cells were analyzed ex vivo without exogenous stimulation. This difference between NKT cells from splanchic lymph nodes and NKT cells from other organs is probably not due to the strength of signaling through the TCR, as it was observed after stimulation by PMA plus ionomycin, which bypasses TCR engagement. Moreover, the IL-4/IFN-γ ratios were similar in vitro with α-GalCer concentrations from 0.2–100 ng/ml (data not shown). The fact that splenic NKT cells produced less cytokines in vitro than NKT cells from splanchic lymph nodes could be due to the increased activation-induced cell death of splenic NKT cells during the 48 h of culture, as Fas expression is higher in splenic NKT cells than in NKT cells from splanchic lymph nodes. The lower ratio of IL-4/IFN-γ production observed with the 48-h stimulation compared with short (few hours) stimulations could be due to IL-4 degradation and/or its consumption by NKT cells (34). This difference in the cytokine profiles of NKT cells from various organs might reflect the environment of NKT cells. Indeed, when highly purified NKT cells from splanchic lymph nodes or spleen were stimulated for 48 h by α-GalCer in the presence of splanchic lymph node APC, they produced less IFN-γ than in the presence of splenic APC. In vitro studies have also suggested that the nature of the APC influences the NKT cell cytokine production profile (35). It would be interesting to further analyze APC from various organs and the effects of soluble factors usually associated with mucosal environments, such as TGFB.

In conclusion, this study shows that NKT cells are frequent in splanchic lymph nodes and that NKT cells from these organs secrete little IFN-γ. The presence of pro-Th1 NKT cells in pancreatic lymph nodes, where autoreactive anti-islet T cells are primed (36), could explain the protective role of NKT cells in V14-Jα281 NOD mice.

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References