

Tracking the Response of Natural Killer T Cells to a Glycolipid Antigen Using CD1d Tetramers

By Jennifer L. Matsuda,^{*†} Olga V. Naidenko,^{*} Laurent Gapin,^{*} Toshinori Nakayama,[§] Masaru Taniguchi,[§] Chyung-Ru Wang,^{||} Yasuhiko Koezuka,[¶] and Mitchell Kronenberg^{*†}

From the ^{*}La Jolla Institute for Allergy and Immunology, San Diego, California 92121; the [†]Department of Biology, University of California at San Diego, San Diego, California 92093; [§]CREST (Core Research for Evolutional Science and Technology) and the Department of Molecular Immunology, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan; the ^{||}Department of Pathology, Gwenn Knapp Center For Lupus and Immunology Research, University of Chicago, Chicago, Illinois 60637; and the [¶]Pharmaceutical Research Laboratory, Kirin Brewery Company Limited, Gunma 370-12, Japan

Abstract

A major group of natural killer (NK) T cells express an invariant V α 14⁺ T cell receptor (TCR) specific for the lipoglycan α -galactosylceramide (α -GalCer), which is presented by CD1d. These cells may have an important immune regulatory function, but an understanding of their biology has been hampered by the lack of suitable reagents for tracking them in vivo. Here we show that tetramers of mouse CD1d loaded with α -GalCer are a sensitive and highly specific reagent for identifying V α 14⁺ NK T cells. Using these tetramers, we find that α -GalCer-specific T lymphocytes are more widely distributed than was previously appreciated, with populations of largely NK1.1⁻ but tetramer-binding T cells present in the lymph nodes and the intestine. Injection of α -GalCer leads to the production of both interferon γ and interleukin 4 by nearly all NK T cells in the liver and the majority of the spleen within 2 h. These cells mostly disappear by 5 h, and they do not reappear after 1 wk. Curiously, tetramer-positive thymocytes do not rapidly synthesize cytokines, nor do they undergo decreases in cell number after lipid antigen stimulation, although they express equivalent TCR levels. In summary, the data presented here demonstrate that α -GalCer-specific NK T cells undergo a unique and highly compartmentalized response to antigenic stimulation.

Key words: antigen presentation • lipid antigen • T lymphocyte • natural killer cell • tetramer

Introduction

NK T cells are a specialized T cell subset that share some traits with NK cells, such as the expression of the NK1.1 molecule, as well as characteristics of activated or memory T cells. NK T lymphocytes may be classified into those that are positively selected by CD1d, a nonclassical class I molecule (for review see reference 1), and those that are CD1d independent. Many members of this first population, the CD1d-dependent NK T cells, express a semiinvariant TCR composed of a specific V α 14 rearrangement paired

preferentially with a diverse set of V β 8.2, V β 7, or V β 2 rearrangements.

CD1d-dependent NK T cells are believed to be involved in the regulation of immune responses as a result of their potent ability to secrete cytokines. Evidence supporting a regulatory role for this population has been derived from the study of several animal models of autoimmune diseases (2–6) as well as from studies of human patients with type I diabetes (7) and systemic sclerosis (8). CD1d-dependent NK T cells also might be involved in some circumstances in the prevention of tumor metastases (9) and the response to some infectious agents (10–13).

Currently, the natural ligand(s) for CD1d-restricted NK T cells is unknown, although phosphoinositol-containing compounds have been suggested to be natural ligands for some of these cells (13–15). However, NK T cell reactivity

J.L. Matsuda and O.V. Naidenko contributed equally to this work.

Address correspondence to Mitchell Kronenberg, La Jolla Institute for Allergy and Immunology, 10355 Science Center Dr., San Diego, CA 92121. Phone: 858-678-4540; Fax: 858-678-4595; E-mail: mitch@liai.org

to CD1d is greatly augmented by the glycosphingolipid α -galactosylceramide (α -GalCer)¹ (16), obtained from an extract of the marine sponge *Agelas mauritanus*. This compound was initially identified in a screen for agents that would prevent metastases of tumors to the livers of mice (17). α -GalCer is a specific agent for activating V α 14⁺ CD1d-dependent NK T cells (16, 18). It can be used to manipulate both NK T cell and conventional T cell responses in mice (19, 20) and potentially in humans (21–23). Although analysis for the coexpression of NK1.1 and an α/β TCR is widely used to identify CD1d-dependent NK T cells, there are several reasons why this method is not entirely satisfactory. First, only three common inbred mouse strains (B6, NZB, and SJL) express an allelic form of NK1.1 seen by the available NK1.1 mAb PK136 (1). Second, NK1.1⁺ T cells may downregulate the NK1.1 marker upon activation (24), making it difficult to follow these cells once they have encountered antigen. Lastly, the NK1.1 marker is also found on CD1d-independent T cells (25–27).

The development of MHC multimer technology has provided a breakthrough in the ability to follow T cell populations defined by their antigen specificity (28, 29). The successful application of MHC dimer and tetramer reagents is due to the fact that a multimerized MHC-peptide complex has a significantly higher avidity for the TCR compared with a monomer (30, 31). Tetramers have been used widely to obtain a detailed analysis of the distribution and frequency of conventional CD4⁺ and CD8⁺ antigen-specific T cells during a variety of immune responses. Multimers also have been instrumental in identifying the receptors for nonclassical class I molecules such as HLA-E (32), Qa-1^b (33, 34), and HLA-G (35). There have been no previous reports, however, of the formation of multimeric antigen-presenting molecules loaded with a lipid antigen. Here we describe the formation and use of multimeric CD1d molecules. We demonstrate that α -GalCer-loaded mouse (m)CD1d tetramers are a highly specific reagent for detection of the in vivo response of NK T cells to a model lipid antigen.

Materials and Methods

Reagents. α -GalCer was synthesized by the Pharmaceutical Research Laboratory of Kirin Brewery Co. as described previously (17). A stock solution of α -GalCer was diluted to 220 μ g/ml in 0.5% polysorbate-20 and 0.9% NaCl (hereafter referred to as “vehicle”).

Dimeric CD1d- α -GalCer Complexes. Soluble recombinant mCD1d1 and human (h)CD1d proteins expressed in *Drosophila* SC2 cells have been previously described (36, 37). For CD1d- α -GalCer complex formation, mCD1d or hCD1d proteins were incubated at room temperature for 12–18 h with threefold molar excess of α -GalCer. Dimeric CD1d complexes were formed with 2B9 hamster mAb, which recognizes an epitope near the

COOH terminus of the recombinant mCD1d and hCD1d proteins (Wang, C.-R., and O.V. Naidenko, unpublished data). Dimeric complexes were purified by gel filtration on Superdex 200 column (Amersham Pharmacia Biotech). For flow cytometry analysis, the 2B9 antibody was biotinylated before dimer formation, and streptavidin-tricolor or streptavidin-PE were used for detection.

Tetrameric mCD1d- α -GalCer Complexes. Tetrameric mCD1d complexes were made in a baculovirus expression system as described previously (38). A modified dual promoter baculovirus transfer vector, pBacp10pH (39), was provided by Dr. J. Kappler (Howard Hughes Medical Institute, University of Colorado, Denver, CO). Recombinant mCD1d protein was produced with a BirA tag followed by a 6-histidine tag. The amino acid sequence at the COOH terminus is GSLHHILDAQKMVWNHRHHH-HHH. To generate this construct, the mCD1d1 heavy chain was cloned by PCR using 5' primer CACGTCGACACATGCGG-TACCTACCATGGCTG (Sal I site underlined) and two 3' primers, ACATGCATGCTTAACGATTCCACACCATTCTGTG-CATCCAGAATATGATGCAGGGATCCCAGTAGAGGATGATATCCTG and CATGCATGCTTAGTGATGATGATGATGATGACGATGATTCCACACCATTTC (Sph I sites underlined). Mouse β 2 microglobulin (m β 2m) was cloned using the 5' primer TCCGCTCGAGCAGCATGGCTCGCTCGGT-GACC (Xho I site underlined) and the 3' primer TGGTCCG-GAGATCACATGTCTCGATCCCAGTAG (BspEI site underlined). In the expression construct, the mCD1d heavy chain was expressed under the control of polyhedrin promoter, and m β 2m was expressed under the control of p10 promoter of baculovirus transfer vector (39). The mCD1d/m β 2m expression vector was cotransfected with linearized BaculoGoldTM baculovirus DNA (PharMingen) into High FiveTM (BTI-TN-5B1-4) cells (Invitrogen) using the Lipofectin Reagent[®] (GIBCO BRL) by following the manufacturer's protocol. Recombinant virus was collected 5 d after transfection, amplified, and cloned by serial dilution method. The virus with highest level of mCD1d secretion was used for protein production. Soluble protein was produced by infecting adherent High FiveTM cells at multiplicity of infection of 5–10. mCD1d-containing supernatant was harvested on days 4–5 after the infection, dialyzed against 0.15 M sodium phosphate buffer, pH 7.4, and passed over Ni-agarose (QIAGEN) for one-step affinity purification. Purified mCD1d protein was biotinylated with BirA enzyme (Avidity) following the manufacturer's protocol. α -GalCer-loaded mCD1d was tetramerized by adding neutravidin-PE (Molecular Probes) in 4:1 molar ratio. “Unloaded” mCD1d tetramers were prepared by preincubating biotinylated protein with an equivalent amount of α -GalCer dilution vehicle. For flow cytometry staining, 2.5–5 μ g of tetramerized mCD1d was used.

T Cell Hybridomas. The derivation and characterization of the mCD1d-autoreactive NK T cell hybridomas N38-2C12 (hereafter 2C12), N38-2H4 (hereafter 2H4), DN3A4-1.2 (hereafter 1.2), and DN3A4-1.4 (hereafter 1.4) has been described previously (16, 40). A V β 8.2 CD4⁺ T cell hybridoma, 780D5, was provided by K. Jensen (La Jolla Institute for Allergy and Immunology, La Jolla, CA). mCD1d-restricted V α 14⁻ hybridomas VIII24 (V α 3.2V β 9) and VII68 (V α 4V β 11), a gift of Dr. S. Cardell (Lund University, Lund, Sweden), have been previously described (41).

Competition for Tetramer Binding. The minimal amount of mCD1d- α -GalCer-neutravidin-PE tetramer required for staining of the 1.2 hybridoma was determined by titration of the tetramers. Increasing amounts of competitors were incubated with 1.5×10^4 1.2 hybridoma cells for 15 min before the addition of

¹Abbreviations used in this paper: DN, double-negative; α -GalCer, α -galactosylceramide; IELs, intestinal intraepithelial lymphocytes; LAK, lymphokine-activated killer.

0.75 μg (0.5 μM) of PE-labeled, α -GalCer-loaded mCD1d tetramers. Competitors included α -GalCer-loaded mCD1d and hCD1d monomers and dimers, using proteins derived from *Drosophila melanogaster* SC2 cells, as well as α -GalCer-loaded mCD1d tetramer, made using unlabeled neutravidin. After addition of the labeled tetramers, the cells were incubated for 2 h at 4°C, washed extensively, and analyzed by flow cytometry.

Mice and In Vivo Treatments. C57Bl/6 mice were purchased from The Jackson Laboratory. BALB/c mice and $\beta 2\text{m}^{-/-}$ mice on the C57Bl/6 background were offspring of stock originally obtained from The Jackson Laboratory. $\text{J}\alpha 281^{-/-}$ mice on the C57Bl/6 background have been previously described (42). mCD1d1 $^{-/-}$ mice on the C57Bl/6 background were generated originally by the laboratory of Dr. L. Van Kaer and provided by Dr. K. Andrews (University of California, Los Angeles, CA). All mice were maintained under specific pathogen-free conditions at the vivarium of the La Jolla Institute for Allergy and Immunology and were used between 6 and 15 wk of age. For experiments with primed animals, mice were injected intravenously with 4 μg of α -GalCer or an equivalent volume of vehicle and killed at the times indicated (see Figs. 6 and 7) for analysis.

Cell Preparation. Single-cell suspensions were prepared from the liver, spleen, thymus, bone marrow, lymph nodes, and intestinal intraepithelial lymphocytes (IELs). Liver was perfused with PBS via the portal vein until the liver was opaque and pressed through a 70- μm cell strainer (Becton Dickinson). Total liver cells were then resuspended in a 40% isotonic Percoll solution (Amersham Pharmacia Biotech) underlaid with a 60% isotonic Percoll solution. After centrifugation for 20 min at 900 g , mononuclear cells were isolated at the 40/60% interface. The cells were washed once with RPMI 1640 medium (Life Technologies) supplemented with 5% FBS (HyClone). For the spleen, lymphocytes were isolated by separation of total spleen cells on a lympholyte gradient (Cedarlane Labs.). Bone marrow cells (femur, tibia) were depleted of B cells using magnetic separation. CD19 microbeads (Miltenyi Biotec) were incubated with bone marrow cells, washed, and run over a column as per the manufacturer's protocol. To isolate IELs, the small intestine was opened longitudinally and flushed of fecal content. The intestine was then cut into 0.5-cm pieces, transferred into 250-ml Erlenmeyer flasks, and shaken three times at 200 rpm for 30 min, each time at 37°C, in HBSS without Ca^{2+} or Mg^{2+} and containing 1 mM dithiothreitol (Sigma-Aldrich). The cell suspensions were passed through a 60- μm nylon mesh, and cells were pelleted by centrifugation at 1,200 rpm. The cell pellets were resuspended in 40% Percoll, layered over 70% Percoll, and centrifuged at 900 g for 20 min. Cells from the interface were collected and washed once before analysis.

Flow Cytometry and Intracellular Cytokine Staining. For characterization of the mCD1d- α -GalCer tetramer, cells isolated from various organs were resuspended in PBS staining buffer containing 2% BSA and 0.02% NaN_3 . Cells were incubated for 15 min at 4°C with the blocking 2.4G2 anti-Fc γ R mAb and neutravidin (Molecular Probes) in a twofold excess of the neutravidin-PE contained in the amount of tetramer to be used for the staining. Neutravidin blocking was done to avoid any nonspecific binding of the neutravidin to biotin on the cells. Staining of FITC-, PE-, Cy-Chrome-, and allophycocyanin-conjugated mAbs was done simultaneously with the tetramer in PBS staining buffer at 23°C (room temperature) for 20 min. Cells were washed twice, and staining with biotinylated mAb was performed at 4°C for 20 min. After washing cells, tricolor-conjugated streptavidin was added as a secondary staining reagent for the biotinylated mAb and incubated for 15 min at 4°C, and cells were washed two times before

analysis. mAbs used in this study include FITC-, Cy-Chrome-, or allophycocyanin-labeled anti-TCR- β clone H57-597, biotinylated or PE-labeled anti-NK1.1 clone PK136, Cy-Chrome-labeled anti-CD4 clone RM4-4, allophycocyanin-labeled anti-CD8 α clone 53-6.7, FITC-labeled anti-CD5 clone 53-7.3, FITC-labeled anti-CD44 clone IM7, biotinylated anti-CD69 clone H1.2F3, FITC-labeled anti-V β 2 clone B20.6, FITC-labeled anti-V β 7 clone TR310, FITC-labeled anti-V β 8.1/8.2 clone MR5-2, and FITC-labeled anti-V β 12 clone MR11-1 (PharMingen). For intracellular staining, cells were incubated with blocking 2.4G2 anti-Fc γ R mAb and neutravidin (Molecular Probes) and then surface stained with TCR- β -FITC and either tetramer or anti-NK1.1-PE at 23°C. Cells were permeabilized using Cytofix/Cytoperm PlusTM (PharMingen) and stained using either FITC-labeled anti-IL-4 clone BVD4-1D11 or FITC-labeled anti-IFN- γ clone XMG1.2 (PharMingen) according to the manufacturer's protocol.

Enrichment of CD1d- α -GalCer Tetramer-positive Cells. Thymocytes were enriched for tetramer-positive cells using anti-PE microbeads (Miltenyi Biotec). In brief, cells were prepared as described above, passed through a 30- μm filter, and incubated at 4°C with blocking 2.4G2 anti-Fc γ R mAb and neutravidin for 15 min. Cells were stained at 23°C with the tetramer for 15 min at room temperature, washed, and incubated with the appropriate amount of anti-PE microbeads. Magnetic separation was performed according to the manufacturer's instructions.

Results

mCD1d and hCD1d Multimers Bind Specifically to NK T Cell Hybridomas. Initially we attempted to make CD1d tetramers by refolding the heavy chain and $\beta 2\text{m}$ from bacteria in the presence of α -GalCer. As we did not succeed in this endeavor, we turned to the native CD1d molecules produced in insect tissue culture cells. These molecules had been shown previously to be capable of forming α -GalCer-CD1d complexes in vitro that are antigenic for NK T cells (37). α -GalCer-loaded monomers were formed by incubation of soluble recombinant CD1d molecules with the lipid, while dimers of mCD1d or hCD1d were formed using the anti-CD1d antibody 2B9 after lipid antigen loading. Both dimers bind specifically to the V α 14V β 8 NK T cell hybridomas 1.2 (Fig. 1 A) and 2C12 (data not shown). The level of staining by the hCD1d dimers was equivalent to or greater than that of the mCD1d dimers, consistent with the cross-reactivity of hCD1d for these two cells (22). The mCD1d dimer also stained the V α 14V β 10 hybridoma 1.4. The signal was reduced, however, despite approximately equivalent levels of TCR- β staining of this hybridoma. Interestingly, the hCD1d- α -GalCer dimers did not stain 1.4 at all, in agreement with a previous report of the poor cross-reactivity for hCD1d by this T cell (22). Vehicle-treated or "unloaded" CD1d dimers did not stain any of the hybridomas. α -GalCer-loaded mCD1d tetramers also stained the NK T cell-derived hybridomas (Fig. 1 B). There was a lower background, a more pronounced shift for the positive cells, and more evident reactivity of 1.4, which reacted only weakly with the mCD1d dimers. In most cases there was little reactivity of unloaded mCD1d tetramers, although weak staining of 2C12 was observed.

This last result is in agreement with the ability of 2C12 to respond to plates coated with the insect cell-derived mCD1d, even in the absence of α -GalCer (37). Besides the V α 14V β 8 and V β 10 hybridomas shown in Fig. 1 B, the tetramers also stained the V α 14V β 7 NK T cell hybridoma 2H4 (data not shown), suggesting that NK T cells with the invariant TCR α chain paired with diverse β chains are likely to be tetramer positive. We did not detect any binding of either α -GalCer-loaded or unloaded mCD1d tetramers to two CD1d-restricted but non-V α 14-positive T cell hybridomas, VIII24 and VII68. This is consistent with the inability of α -GalCer to augment the mCD1d reactivity of these T cells (16) and with the inability of these hybridomas to recognize plate-bound mCD1d molecules

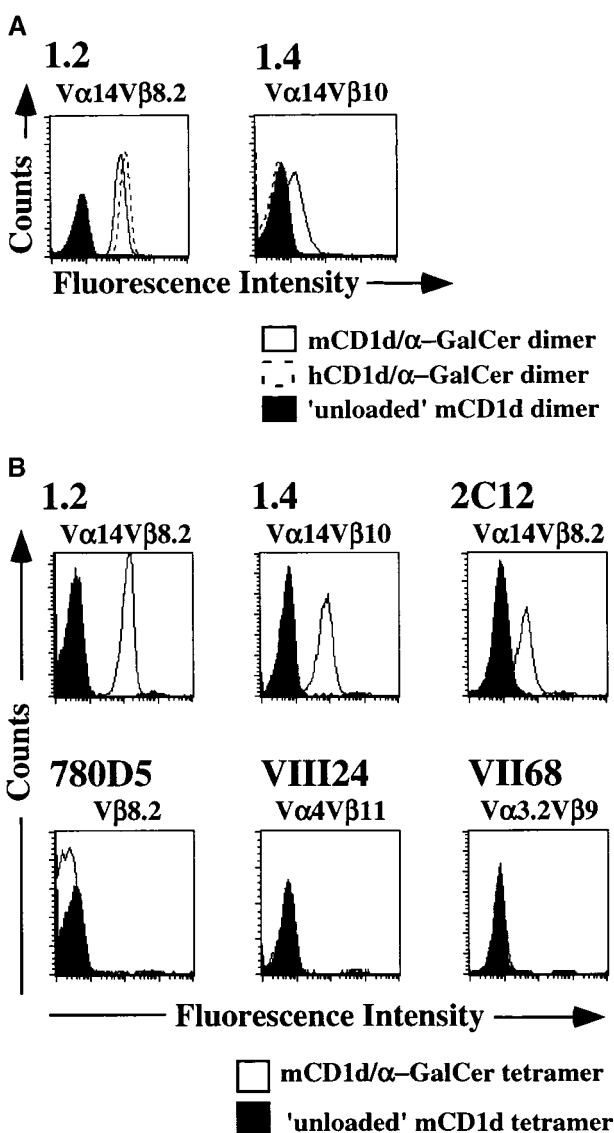


Figure 1. Specific CD1d multimer staining of T cell hybridomas. (A) Comparison of mCD1d and hCD1d dimer staining of the 1.2 and 1.4 NK T cell hybridomas. (B) Comparison of mCD1d tetramer staining of the indicated T cell hybridomas using α -GalCer-loaded and unloaded tetramers. One representative experiment of three is shown.

(Naidenko, O., and M. Kronenberg, data not shown). Additionally, 780D5, a V β 8.2 hybridoma that is not CD1d reactive, failed to bind the mCD1 tetramer, regardless of the presence of α -GalCer.

Competition for Tetramer Binding by Unlabeled CD1d Multimers. We performed competition experiments to confirm the specificity of tetramer staining, as well as to get an estimate of the relative affinities of different CD1d- α -GalCer complexes for a V α 14V β 8 TCR (Fig. 2). mCD1d monomers were the least effective competitors, consistent with the inability of these monomers to stain NK T cells (data not shown). mCD1d dimers were slightly more effective than monomers, with the strongest inhibition achieved with mCD1d tetramers. Therefore, we conclude that effective competition required mCD1d multimerization, and the competitors were active in the low micromolar concentration range. Interestingly, hCD1d was a more effective competitor for binding to 1.2 than the homologous mCD1d molecule. hCD1d monomers were nearly as effective as mCD1d dimers, whereas hCD1d dimers were as effective as mCD1d tetramers. This heteroclitic reactivity of the 1.2 TCR for hCD1d also was evident from IL-2 release assays carried out using this cell (22). Judging from the results of functional assays carried out with other NK T cell hybridomas, this heteroclitic reactivity for hCD1d may not be unique to 1.2, as 2C12 also responded slightly more strongly to hCD1d transfectants (22). Because the mCD1d tetramer gave somewhat better results than the dimer for both staining and competition, the remaining experiments were carried out with the tetrameric reagent.

Tetramer-positive T Cells Express Phenotypic Markers of CD1d-dependent NK T Cells. We also tested the ability of the mCD1d- α -GalCer tetramers (hereafter called "tetramers") to specifically identify NK T lymphocytes in complex mixtures of cells from the thymus and liver. We used multiparameter flow cytometry analysis to evaluate cells bind-

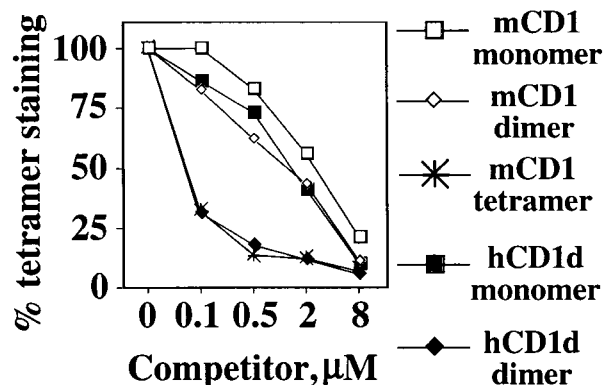


Figure 2. Inhibition of tetramer binding by recombinant CD1d molecules. 1.2 hybridoma cells were incubated with the indicated concentrations of competitor molecules before addition of tetramerized mCD1d. The data are represented as percent of tetramer staining in the absence of a competitor. As a control, even the highest concentration of competing multimerized CD1d molecules did not affect the T cell hybridoma staining with the anti-TCR C β antibody H57-597 (data not shown). This experiment was repeated three times with similar results.

ing to the tetramers for coexpression of activation markers, particular V β regions, and other cell surface proteins typical of NK T cells. Based on the parameters we tested, tetramer-positive cells have the same phenotype as CD1d-restricted NK1.1⁺ T cells, which were defined previously by a combination of NK1.1 and TCR- β staining. They appear to be activated, on account of their expression of high levels of CD44 and CD69 (Fig. 3 A). They also stained positively for CD5, a marker previously reported to be found on NK T cells (1). Additionally, the tetramer-positive cells were compared with conventional T cells from the same organ for their usage of V β gene segments. The

invariant V α 14 expressed by mCD1d-restricted NK T cells associates predominantly with, in the order of decreasing frequency, V β 8.2, V β 7, V β 8.3, V β 2, and V β 8.1 (for review see reference 1). In Fig. 3 B, the bias in the TCR- β repertoire of tetramer-positive cells is apparent. For example, \sim 60% (thymus) or 38% (liver) of the tetramer-positive cells are either V β 8.2 or V β 8.1 positive, as compared with 8–12% positive in the corresponding conventional T cell populations from the same organs. The percentage of V β 7⁺ and V β 2⁺ cells in the tetramer-positive T cell population also reveals this repertoire bias, although more weakly, and for V β 2 only in the thymus. Staining for an irrelevant V β gene segment, V β 12, showed equivalent or decreased expression by NK T cells. These results corroborate those from the hybridomas in showing that tetramer staining is not restricted to a particular V α 14–V β combination.

Tetramer Staining Is Dependent upon CD1d and Ja281 Expression. To further test tetramer specificity, we analyzed cells from inbred mouse strains deficient for genes required for the development of CD1d-dependent NK T cells. These inbred strains included β 2m^{-/-}, CD1d1^{-/-}, and Ja281^{-/-} mice. The semiinvariant TCR of CD1d-restricted NK T cells utilizes a V α 14Ja281 rearrangement, so the Ja281^{-/-} mice also should have decreased tetramer-positive cells. Additionally, we analyzed BALB/c mice with the tetramer, to quantitate α -GalCer-reactive, CD1d-dependent NK T cell numbers in a strain that does not express a form of NK1.1 seen by the antibody PK136. Thymus, liver, and spleen of each of these mice were analyzed for tetramer or NK1.1 staining in conjunction with TCR- β reactivity. Representative tetramer staining of the liver of each of these strains is shown in Fig. 4 A, and the data for all three organs are compiled in Fig. 4 B. Only the C57Bl/6 and BALB/c wild-type strains showed significant populations of tetramer-positive TCR- β ^{int} cells, each \sim 25–30% of the total mononuclear cells in the liver (Fig. 4 B). As expected, β 2m^{-/-}, CD1d1^{-/-}, and Ja281^{-/-} mice failed to stain distinct tetramer-positive populations, staining \leq 1.3% of the total lymphocytes, which is comparable to the staining with the unloaded tetramer. The percentage of positive cells detected with the unloaded tetramer averaged 0.16% in the thymi, 1.5% in the livers, and 0.37% in the spleens of C57Bl/6 mice (data not shown).

The gene-deficient mice retain significant populations of NK1.1⁺ T cells in the liver, however, averaging 6.7% in CD1d1^{-/-}, 3.8% in β 2m^{-/-}, and 7.6% in Ja281^{-/-} mice, reflecting the presence of populations of V α 14⁺ and CD1d-independent NK T cells. As in the liver, the great majority of NK1.1⁺ T cells are also tetramer positive in the thymus. This was less evident in the spleen, where the effect of either CD1d1 or β 2m deficiency on the percentage of NK1.1 T cells was modest, reflecting the presence of a population of CD1d-independent NK T cells in that site. Additionally, whereas the two inbred strains are similar with regard to the distribution of tetramer-binding cells, BALB/c mice showed an approximately twofold increase in tetramer-positive T cells in the thymus compared with the C57Bl/6 strain ($0.7 \pm 0.2\%$ in C57Bl/6 as compared

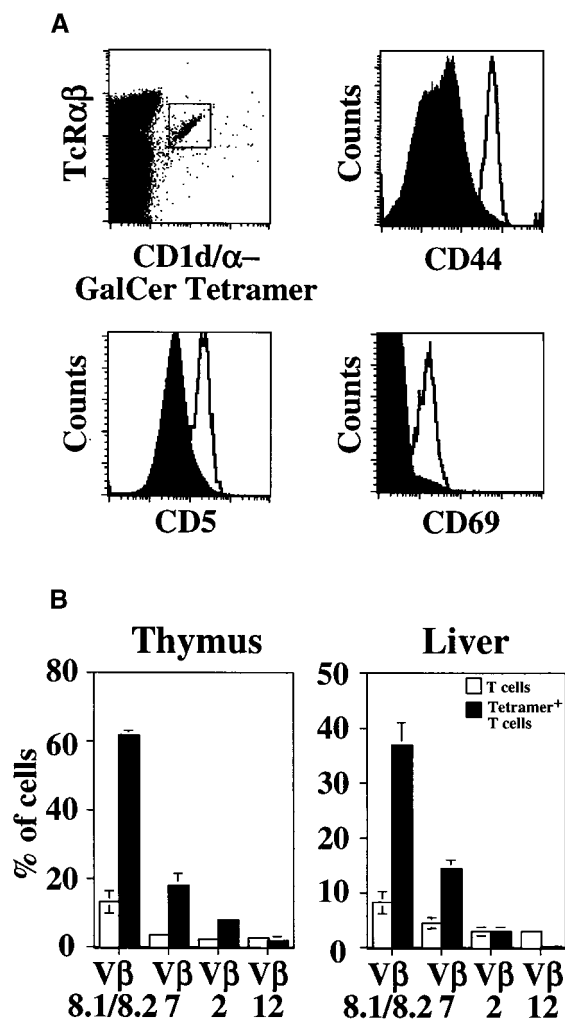


Figure 3. Tetramer-positive cells have the expected phenotypic markers of NK T cells. (A) Phenotypic analysis of tetramer-positive cells in the thymus. Histogram overlays of tetramer-positive TCR- β ^{int} cells (open histogram) and control stainings (shaded histogram) reveal the tetramer-positive population to be CD5⁺CD44^{high}CD69⁺. Conventional T cells served as the control staining for CD44, and for CD5 and CD69, background staining was used due to the presence of conventional T cells staining positive for these markers. (B) Tetramer-positive T cells show a bias in V β gene segment usage as compared with conventional T cells. Conventional T cell controls are the lymphocytes expressing high levels of TCR- β in the thymus (upper left gate in A) and TCR- β ^{high} and NK1.1⁺ T cells in the liver. Each of these figures is derived from two experiments with similar results.

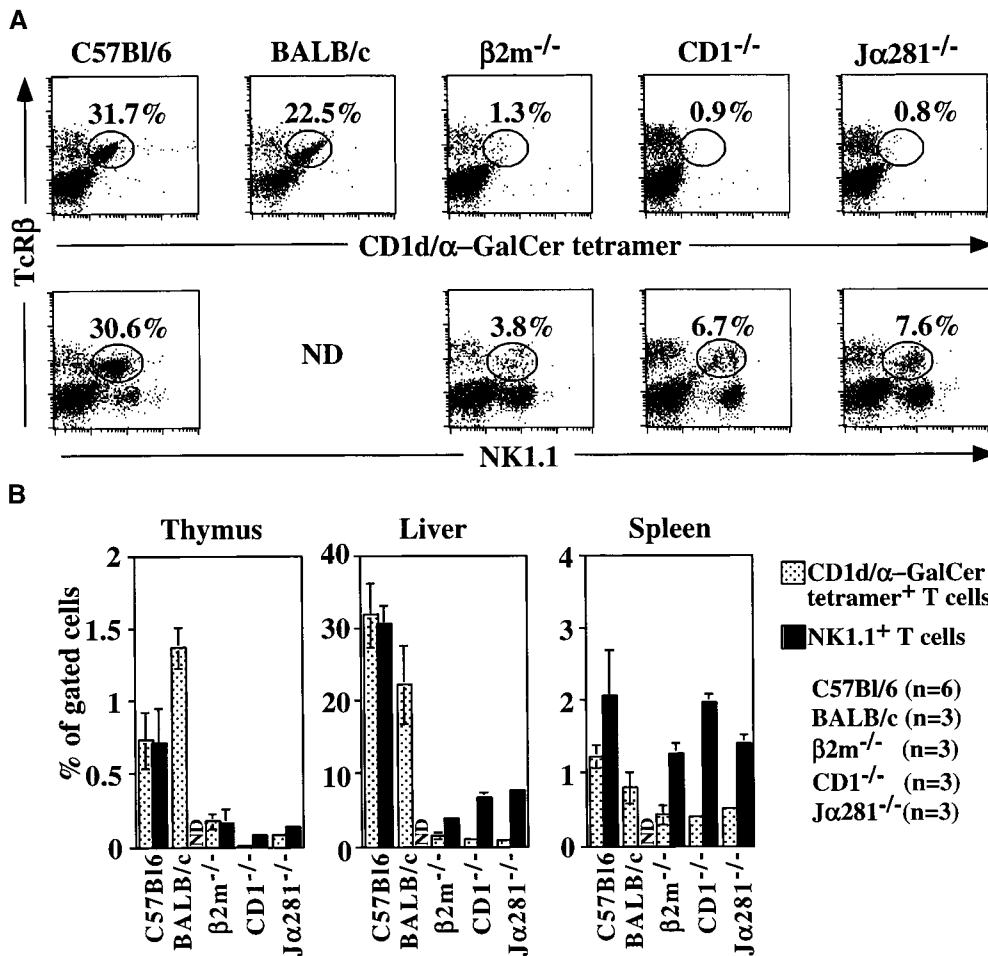


Figure 4. Tetramer binding is dependent upon CD1d and Jα281 expression. (A) Representative analysis for expression of TCR-β and either tetramer (top row) or NK1.1 (bottom row) of liver mononuclear cells from the indicated strains of mice. The numbers indicate the percentage of the liver mononuclear cell preparation that is positive. (B) Comparison of the percentages of tetramer-positive T cells and NK1.1⁺ T cells in the thymus, liver, and spleen for the different strains of mice. The average values for the indicated number of mice of each strain are shown.

with $1.4 \pm 0.1\%$), although this increase was not reflected in the spleen or liver (Fig. 4 B).

Tissue Distribution and Phenotype of Tetramer-reactive Cells. NK T cells are known to be localized in the thymus, liver, spleen, and bone marrow (1). We sought to test whether we could detect CD1d-dependent NK T cells in each of these sites using tetramers and if they are present in any other locations. Fig. 5 A shows representative data from this analysis. α-GalCer-reactive NK T cells could be detected in unfractionated cells from the liver, spleen, and thymus and in B cell-depleted bone marrow. NK T cells also could be detected in peripheral lymph nodes and in the lymphocyte gate of IEL preparations. In each case, the reactive cells had the characteristic TCR-β intermediate phenotype (Fig. 5 A). We also analyzed CD4 and CD8 coreceptor expression by the tetramer-reactive T cells. Without a means to directly detect the specific TCR of NK T cells, investigators have previously used differences in coreceptor expression, along with an analysis of CD1d-deficient mice, to differentiate between three populations of NK T cells (25–27). Two of these populations, CD1d-independent cells and CD1d-dependent but Vα14⁻ cells with diverse TCRs, are more prevalent in the spleen and bone marrow, and they include some CD8⁺ T lymphocytes. By contrast, the third subpopulation, consisting of

NK T cells that are CD1d dependent, α-GalCer reactive, and mostly Vα14⁺, which is most enriched in the thymus and liver, has not been reported to contain CD8⁺ cells. Analysis with tetramers confirmed these earlier findings. α-GalCer-reactive cells are either CD4⁺ or double-negative (DN) in every peripheral organ analyzed (Fig. 5 A). This pattern of coreceptor expression is particularly striking in IELs from the small intestine, because TCR-α/β⁺ IELs are typically >80% CD8⁺. Although there is a small double-positive population evident in the thymus (Fig. 5), this was not consistently reproducible and it is not known if there are double-positive precursors of the major CD4⁺ and DN, tetramer-positive thymocyte populations.

Because NK T cells may lose NK1.1 expression, we analyzed T lymphocytes (TCR-β⁺) for coexpression of NK1.1 and reactivity with the tetramers. The results from this analysis are compiled in Fig. 5, B and C. The great majority of tetramer-positive cells in the thymus, liver, and bone marrow are also NK1.1⁺ (Fig. 5 B). In lymph node and IELs, by contrast, only a minority expresses NK1.1. The potentially α-GalCer-reactive populations in the lymph node and the intestine therefore could have been overlooked, in part due to diminished NK1.1 expression. When the opposite analysis was done by gating on NK1.1⁺ T cells and enumerating the tetramer-positive cells, we found that the vast

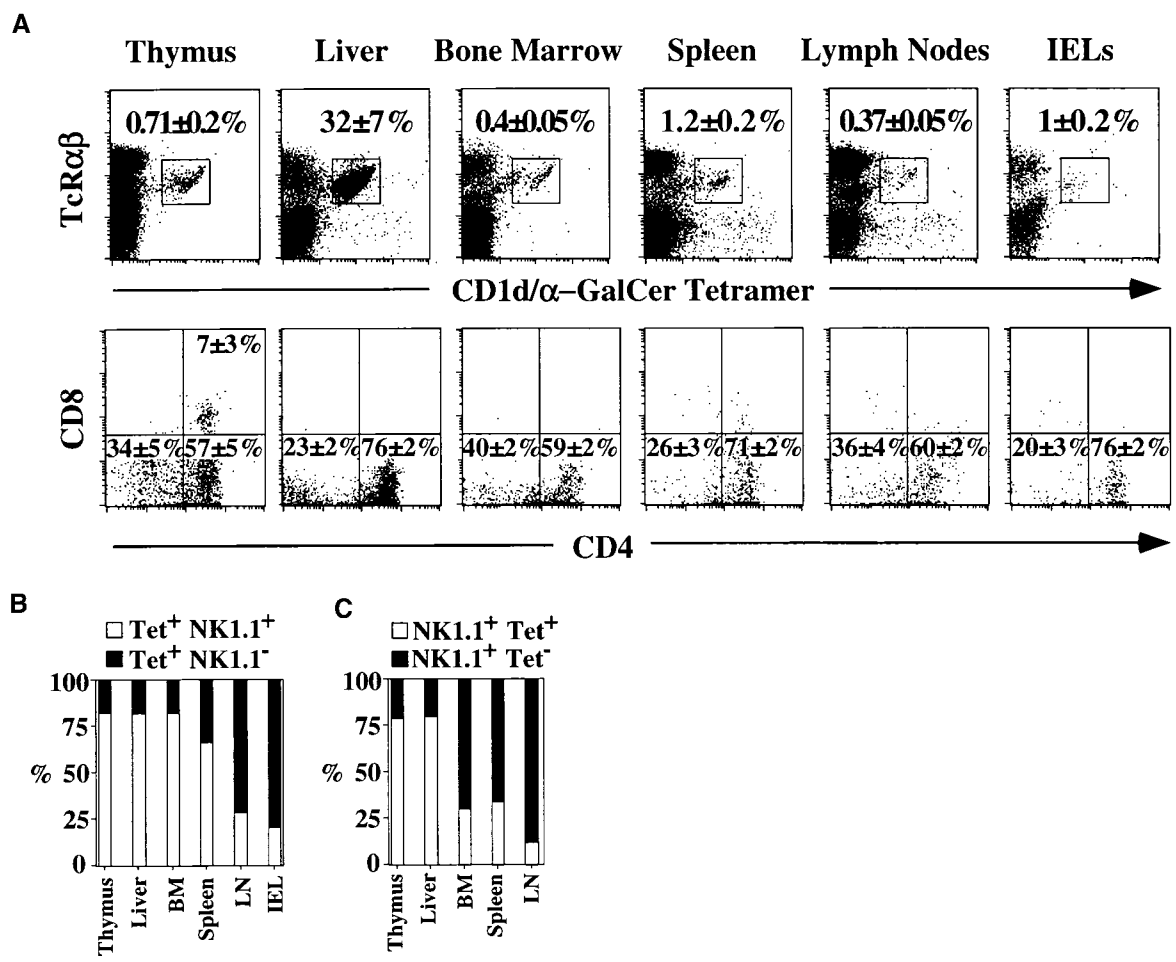


Figure 5. Frequency and phenotype of tetramer-binding cells from various organs of C57Bl/6 mice. (A) Tetramer staining and the CD4/CD8 phenotype of the tetramer-positive cells. Depicted are representative data of cells from the thymus, liver, bone marrow (BM), spleen, peripheral lymph nodes (LN), and IELs. The percentages indicate the proportion of tetramer-positive T cells in the lymphocyte gate. For each organ, the gated tetramer-positive cells were analyzed for their CD4 and CD8 phenotype. (B) Analysis of NK1.1 expression by cells from different organs gated for tetramer binding. (C) Analysis of tetramer reactivity of cells from different organs gated for NK1.1 expression. Percentages and standard deviations were determined based on the analysis of six mice for the liver, spleen, and thymus and for two mice in the lymph node, bone marrow, and IELs.

majority of thymus and liver NK1.1⁺ T cells were tetramer positive, in contrast to those in the bone marrow and spleen (Fig. 5 C). This is reflective of the previously described differences in the distribution of the V α 14⁺ and other NK T cell populations (25–27). The lymph node was similar to the spleen and bone marrow in having a significant population of NK1.1⁺, tetramer-negative T lymphocytes. IELs contain very few NK1.1⁺ T cells, and therefore this analysis for the percentage of tetramer-positive lymphocytes among NK1.1⁺ T cells could not be carried out. Some of the NK1.1⁺ cells that are not also tetramer positive in spleen, bone marrow, and elsewhere could be CD1d-dependent T cells with diverse TCRs (41). We cannot currently identify this CD1d-dependent population with CD1d multimers, however, regardless of whether the cells express NK1.1, because the ligands they require are mostly uncharacterized. Furthermore, as suggested by the analysis of two hybridomas that fit into this category, it is unlikely that the unloaded tetramers can detect the majority of these cells. To

determine if the tetramers might bind to cell types other than T cells, we also analyzed the TCR⁻ cells in various organs. In the thymus, liver, B cell-depleted bone marrow, and IELs, essentially all tetramer-positive cells were TCR- β ⁺ (Fig. 5 A). A population of NK1.1⁻TCR⁻ cells with diverse levels of tetramer binding reproducibly could be observed in the spleen and lymph node (Fig. 5 A); these cells constituted ~2% of the TCR- β ⁻ cells in each of these organs. Staining with the unloaded tetramer revealed the same population, indicating that binding of the tetramer to these cells does not require α -GalCer. The majority of these TCR⁻, tetramer-positive cells are NK1.1⁻ and positive for the B cell marker CD19 (data not shown). Additionally, in the liver, spleen, and lymph nodes, a very minor population (0.26–0.3% of total TCR⁻ cells) was both tetramer and NK1.1 positive (data not shown). Although these data are intriguing, the specificity and significance of the reactivity of unloaded CD1d tetramers with a small subset of B lymphocytes and NK cells remains to be determined.

Potent and Short-lived Cytokine Production by NK T Cells in Response to α -GalCer. The tetramer provides the opportunity to follow an NK T cell population in response to antigen administration. We injected α -GalCer and analyzed the mice at 2 and 5 h for the production of cytokines by tetramer-positive cells. At 0 h, very few of the tetramer-positive cells stain positive for intracellular IFN- γ or IL-4 ($0.8 \pm 0.3\%$ IFN- γ and $2.4 \pm 1.0\%$ IL-4 for the liver). We then analyzed the liver and spleen at 2 h after antigen administration. An average of 22% of the cells in the liver at this time point are tetramer-positive IFN- γ^+ , and 22% are tetramer-positive IL-4 $^+$ (Fig. 6 A). For technical reasons, we were unable to stain for IFN- γ and IL-4 concurrently. It is likely, however, that the majority of NK T cells are secreting both cytokines, because an average of $91 \pm 3\%$ of the tetramer-positive cells in the liver were producing IL-4 and $85 \pm 9\%$ of them were making IFN- γ . NK T cells in the spleen also responded rapidly to α -GalCer, although the average percentage of responding tetramer-positive cells was lower, $55 \pm 5\%$ for IL-4 and $48 \pm 4\%$ for IFN- γ . Strikingly, this large α -GalCer-responding population in

the liver and spleen almost completely disappeared by 5 h. Tetramer-positive IFN- γ^+ cells comprised only 3.7% of the total lymphocyte population in the liver, and tetramer-positive IL-4 $^+$ cells made up 3.4%. The disappearance of cytokine producing tetramer-positive liver cells parallels a similar reduction both in the total tetramer-positive cells (Fig. 6 A) and in the CD4 $^+$ tetramer-positive cells in particular (Fig. 6 B). At 0 and 2 h after antigen, CD4 $^+$ cells comprise $\sim 70\%$ of the tetramer-positive population. By 5 h, however, this CD4 $^+$ population is reduced to 16.6%. This suggests that there may be a selective early loss of the CD4 $^+$ cells or of CD4 expression. There is a reduction in the number of tetramer-positive cells 5 h after antigen in the spleen, although this reduction is less extreme than in the liver (data not shown). Although the total number of binding cells and cytokine-producing tetramer-positive cells decreases rapidly after antigen, there is a concomitant increase in the number of IFN- γ -producing, tetramer-negative cells in the liver. This tetramer-negative and cytokine-producing population was found to be predominantly NK1.1 $^+$, and these cells do not contain IL-4. They com-

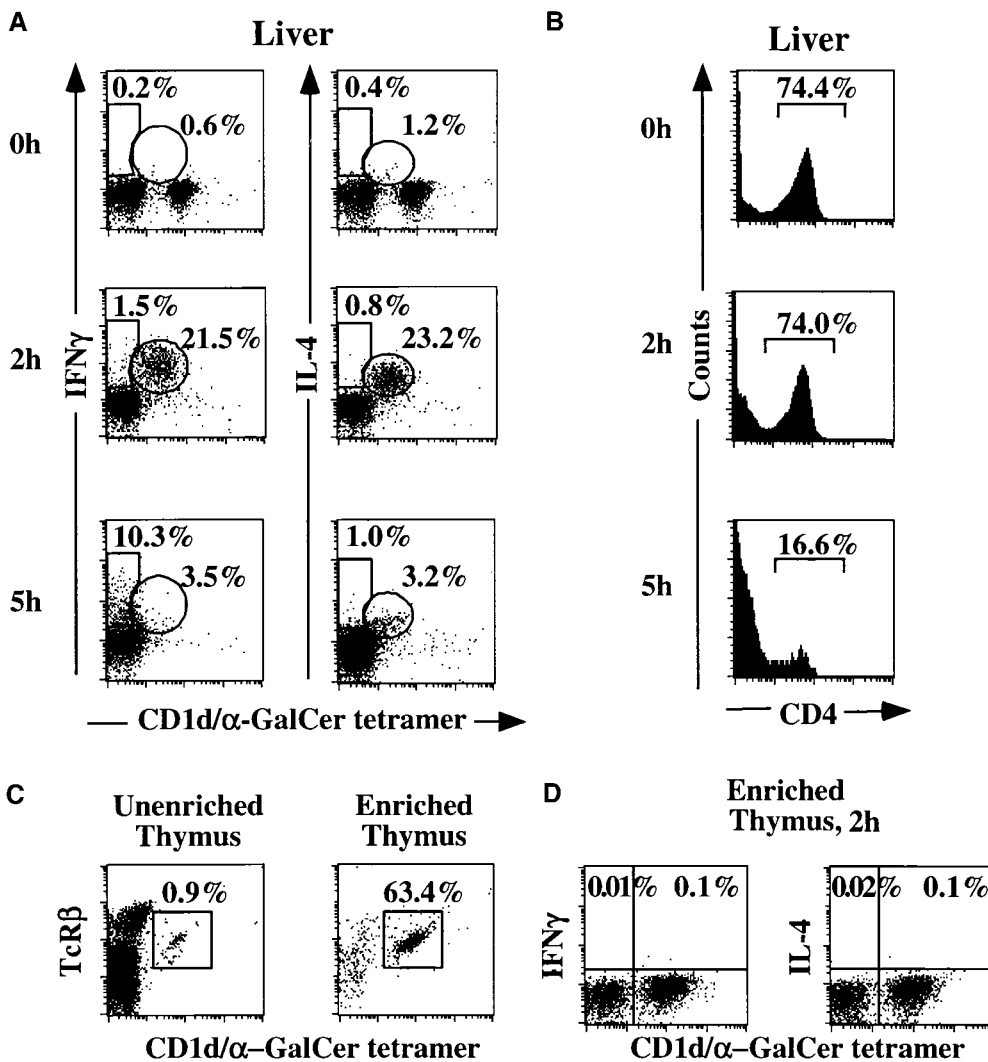


Figure 6. Kinetics and tissue specificity of in vivo response of NK T cells to α -GalCer. (A) Time course of the response in the liver to intravenous injection of α -GalCer. Intracellular cytokine staining was carried out as described in Materials and Methods. (B) CD4 $^+$, tetramer-positive cells in the liver are rapidly and selectively diminished after α -GalCer injection. The percentage of CD4 $^+$ cells among the gated tetramer-positive cells at various time points is shown. (C) Enrichment of tetramer-positive cells in the thymus using magnetic beads. (D) Thymocytes fail to produce IL-4 or IFN- γ 2 h after α -GalCer injection. Three mice were analyzed showing similar results for each time point depicted. Percentages for individual mice are shown in the figure, with averages of several mice in the text.

prised an average of ~2% of the liver mononuclear cells at 2 h and 11% by 5 h. A similar pattern was identified in the spleen (data not shown). These cells express higher levels of NK1.1 (mean fluorescence = 504) than tetramer-positive NK T cells (mean fluorescence = 84), and therefore it is likely that they are NK cells that have been rapidly activated in response to NK T cells. This would be consistent with a recent report (43).

The data presented above suggest that the tetramer-binding cells in the liver are fully responsive to α -GalCer, whereas those in the spleen are only partially responsive. We therefore also analyzed the NK T cells in the thymus. The thymus has a 40–50-fold reduced percentage of tetramer-positive cells compared with mononuclear cells from the liver. We enriched for the tetramer-binding thymocyte population by staining the cells *ex vivo* with tetramer followed by enrichment of the NK T cell using magnetic beads. In this way, the percentage of tetramer-positive thymocytes was increased from <1% to >60% (Fig. 6 C). Although the enriched thymocytes contained ~60% tetramer-positive cells, none of these cells from mice immunized 2 h earlier (Fig. 6 D) or 5 h earlier (data not shown) with α -GalCer contained intracellular IL-4 or IFN- γ . Consistent with our intracellular staining results, there was no reduction in the number of tetramer-positive thymocytes after α -GalCer injection (Fig. 7).

Long-Term Depletion of Tetramer-positive NK T Cells in the Liver in Response to α -GalCer. To assess the reappearance or recovery of the α -GalCer-reactive T cells, C57Bl/6 mice were injected intravenously with 4 μ g of α -GalCer and analyzed 1 wk later for the presence of NK T cells. Representative flow cytometry data are shown in Fig. 7 A. The livers of α -GalCer-primed mice have reduced numbers of tetramer-positive T cells, similar to the numbers observed 5 h after immunization. The majority of the remaining NK1.1⁺ T cells in the liver are tetramer negative, and both the CD4⁺ and DN tetramer-positive cells were decreased (Fig. 7 B). As in the previous cases, the spleen showed less dramatic but similar changes. In a slightly different protocol, cells from mice primed with 11 μ g of α -GalCer at days 0 and 7 were analyzed 6 wk after the second priming. In this case, the percentage of tetramer-positive cells in the liver remained greatly reduced, averaging 2.5% of total monocytes. Collectively, these data indicate that the decrease in tetramer-positive cells is not due to a transient downregulation of the NK T cell TCR. In the thymus, by contrast, tetramer-positive thymocyte numbers are unchanged at 1 wk after lipid antigen priming (Fig. 7 A) and at 6 wk (data not shown), and the proportions of CD4⁺ and DN tetramer-positive thymocytes are unaltered (Fig. 7 B).

Discussion

CD1d-dependent NK T lymphocytes are a distinct T cell subset that may be important for the regulation of the immune response (3–5, 7, 9, 42). Reliable cell surface markers that define this population have been lacking,

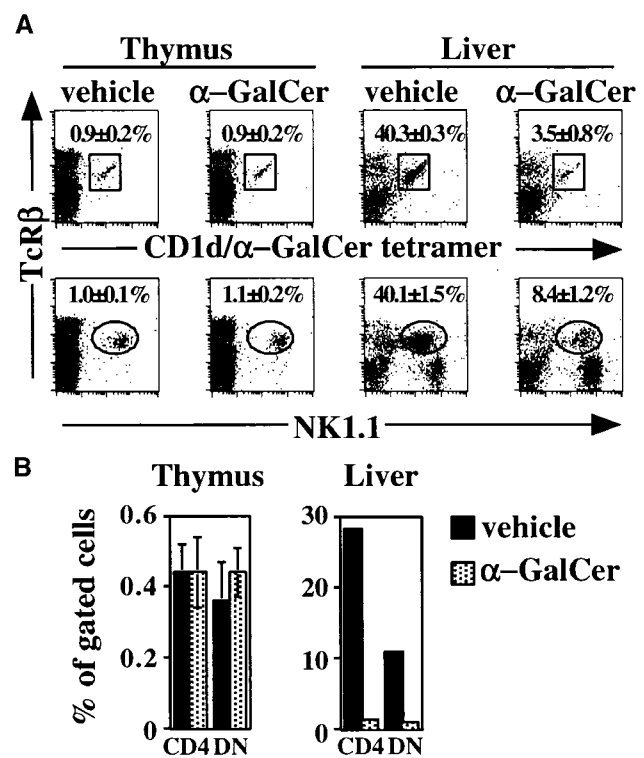


Figure 7. C57Bl/6 mice primed with α -GalCer fail to recover tetramer-positive T cell numbers in the liver 1 wk after priming. (A) Representative flow cytometry analysis of tetramer-positive T cells and NK1.1⁺ T cells in thymi and livers of vehicle- or α -GalCer-primed mice. (B) CD4/CD8 phenotype analysis of tetramer-positive T cells found in the livers and thymi of vehicle- or α -GalCer-primed mice. Three mice were analyzed, each for the vehicle and α -GalCer.

however, and therefore it has been difficult to precisely enumerate these lymphocytes in ontogeny or during immune responses. The combination of NK1.1 and TCR- β expression frequently has been used to define CD1d-dependent NK T cells. This has several important limitations, including the inability of the available mAbs to detect the NK1.1 allele in most mouse strains, the expression of NK1.1 by T cells that are not CD1d reactive (44, 45), and the possible loss of NK1.1 expression by activated NK T cells (24). In this report, we describe the generation and use of several kinds of CD1 multimers. These reagents, particularly α -GalCer-loaded mCD1d tetramers, have allowed us to identify CD1d-reactive NK T cells based solely upon their TCR specificity.

mCD1d dimers and tetramers were both found to be capable of staining α -GalCer-reactive T lymphocytes, although tetramers gave a more reproducible and uniform staining. The reactivity requires multimerization, and consistent with this, mCD1d monomers were relatively ineffective competitors for tetramer binding. α -GalCer loading, which was carried out at neutral pH, is also required in most cases for tetramer binding. These data imply that α -GalCer can effectively replace the endogenous lipid present in CD1d molecules derived from insect cells and that the α -GalCer-CD1d complexes thereby formed are

highly stable. The effective binding at neutral pH agrees with our previous findings on the pH dependence of the lipid-CD1d interaction, which were obtained using surface plasmon resonance to measure the binding of soluble recombinant CD1d to immobilized lipid (37). In that previous study, however, the k_{off} was rapid and the measured half-life of the α -GalCer-CD1d complexes was ~ 7 min (37). It is likely that several factors contribute to the apparently increased stability of α -GalCer binding in the experimental system used in this report. First, rebinding of α -GalCer should be greatly facilitated in the presence of multimerized CD1d molecules, as opposed to mCD1d monomers flowing over a biosensor chip that contains a low density of ligand. Second, as we noted previously (37), because of the chemistry of the α -GalCer immobilization, it is likely that only one of the two hydrophobic chains of the glycosphingolipid was available for CD1d binding in the surface plasmon resonance study, whereas both chains were available in the experiments reported here. The results from a number of control experiments demonstrated the specificity of the CD1d multimers. First, the flow cytometry analysis of T cell hybridomas with the multimers was highly consistent with the functional assays of these cells. Concordant IL-2 release and binding results were obtained for reactivity to mCD1d in the absence of α -GalCer, for the α -GalCer requirement for different CD1d restricted T cells, and for the analysis of cross-reactivity with hCD1d (16, 21, 22). Second, the distribution and phenotype of tetramer-binding cells agrees with previous phenotypic analyses of CD1d-dependent NK T lymphocytes. The reactive cells express activation markers, lack CD8, have preferential expression of V β 8 and V β 7, and are predominantly NK1.1⁺. Additionally, as suggested by previous studies (25–27), the percentage of CD1d-dependent NK T cells in the NK1.1 subset was greater in thymus and liver compared with spleen and bone marrow. Third, tetramer-binding cells were greatly decreased in strains deficient for expression of the CD1d antigen-presenting molecule or the J α 281 component of the invariant TCR α chain, consistent with the inability of these strains to respond to α -GalCer (16, 18).

Staining with the tetramers yielded several unexpected results regarding the phenotype and distribution of the reactive cells. First, although the majority of CD1d-dependent NK T cells are in the thymus, liver, spleen, and bone marrow, populations also were revealed in lymph nodes and IELs. Based on these findings, we conclude that CD1d-dependent and α -GalCer-specific NK T cells are likely to be found in most places where conventional T cells also are located, although the percentage of T cells that fit into this category clearly varies between organs. Second, the great majority of freshly isolated NK cells did not stain with the tetramers, nor did lymphokine-activated killer (LAK) cells generated by culture with IL-2 (Naidenko, O.V., L. Brossay, and M. Kronenberg, unpublished observations). Previously, we had shown that expression of mCD1d by RMA-S cells inhibited LAK activity, although this was only revealed upon repeated culture with IL-2. This result suggested an interaction of CD1 with an inhibi-

tory NK receptor (46). The negative results obtained with the tetramers on LAK cells are not consistent with this hypothesis, but the tetramers might not be able to bind to a putative inhibitory receptor if the RMA-S cells provide a ligand required for this interaction. The altered glycosylation pattern of the insect cell-derived CD1d molecules also might prevent binding. Finally, the affinity might be too low to detect binding, by analogy with the interaction of CD8 with most MHC class I tetramers. It is clear, however, that some NK receptor-class I interactions have a higher affinity (32–34, 47, 48). Further experiments will be required to uncover the basis for this CD1d-mediated inhibition of NK cells.

NK T cell populations exhibit a dynamic response to lipid antigen stimulation. 2 h after antigen injection, nearly all of the tetramer-positive cells in the liver and spleen are making both IFN- γ and IL-4; there are almost no cells polarized to either a Th1 or Th2 cytokine pattern. Rather than expanding in response to antigenic stimulation, however, by 5 h, >80% of the responding NK T cells can no longer be visualized as TCR⁺, CD4⁺, or IL-4-containing cells. What is the fate of the NK T cells that have disappeared from the liver and spleen? We did not find a major increase in the number of tetramer-positive cells in any organ that was analyzed, and therefore we consider it unlikely that the NK T lymphocytes have migrated out to another site after activation. TCR downregulation after antigen activation is a possibility, but this would have to occur concomitantly with a loss of CD4 expression and intracellular IL-4. Furthermore, the population of tetramer-positive cells is greatly reduced even 6 wk after antigen stimulation. Based on these results, we hypothesize that the majority of NK T cells in liver and spleen undergo activation-induced cell death in response to lipid antigen stimulation and that the renewal of this population is surprisingly slow. Consistent with this, Eberl and MacDonald have also provided evidence for a decrease in NK T cell numbers after α -GalCer priming (43). They also have demonstrated that NK T cells rapidly undergo activation-induced cell death after stimulation with either anti-CD3 or IL-12 injection (49). Although they report a greater degree of recovery of the NK T cells based upon NK1.1 and TCR staining at various time points after anti-CD3 injections, their observations with α -GalCer parallel ours. However, we find that the number of tetramer-binding cells does not increase proportionately with the increase in NK1.1⁺ T cells, even 1 wk after antigen administration.

A population of tetramer-negative, NK1.1⁺ cells, which contains intracellular IFN- γ but not IL-4, appears after 5 h. These could be NK cells that have been activated rapidly by NK T cell cytokines, suggesting a close connection between these two lymphocyte populations. This interpretation is consistent with the higher level of NK1.1 expression by these cells, as well as with earlier work showing an α -GalCer-mediated increase in NK cell activity (43, 50–52) and a previous study demonstrating the production of IFN- γ by NK cells after NK T cell activation (53). It therefore is possible that much of the IFN- γ that we (19) and others

(20, 54) have reported in the blood of mice injected with α -GalCer 16 h previously is derived from NK cells that have been activated by NK T cells.

Although CD1d-dependent NK T cells in the liver and spleen respond rapidly and then disappear and apparently die, tetramer-binding cells in the thymus undergo neither response. One possible explanation for these results is that α -GalCer is incapable of reaching the thymus tissue. Consistent with this, thymic NK T cells can respond to α -GalCer when stimulated in suspension culture with the lipid in the absence of exogenous APCs. Alternatively, we speculate that thymocytes may be prevented from responding in vivo by inhibitory receptors or by some other means. Interestingly, it has been shown recently, based upon long-term BrdU labeling, that thymus NK T cells are turning over more slowly than those in the liver (55), consistent with these cells being in a quiescent or negatively regulated state. Further experiments will be required to establish the mechanism for the striking difference in NK T cell responses to lipid antigen in the thymus as compared with the periphery.

The data presented here provide direct insight into the surprising dynamics of antigen-activated NK T cells in vivo. It should be noted that α -GalCer exposure does not lead to the all-encompassing death of NK T cells in the spleen and liver. In fact, the NK T cell responses of α -GalCer-primed mice to a second antigen exposure have been reported to be highly polarized in a Th2 direction (19, 20), or in some cases a Th1 direction (56, 57). The properties that permit the survival and polarized cytokine response of a minority of NK T cells in certain sites remain to be determined. Once natural antigens for NK T cells are identified, it also will be of great interest to determine if they have in vivo effects similar to those of α -GalCer. It is likely that the use of tetramers, which has permitted the definition of these new problems in NK T cell biology, will also contribute to their eventual solution.

We would like to thank Dr. John Kappler for kindly providing the modified dual promoter baculovirus transfer vector pBacp10pH, Drs. Kahlil Andrews and Katsuji Sugie for providing gene knock-out mice, and Kent Jensen for the 780D5 hybridoma. We also thank Dr. Laurent Brossay for helpful suggestions and Drs. Hilde Cheroutre and Ted Prigozy for helpful review of this manuscript.

This project was funded by National Institutes of Health grant RO1 CA52511 (to M. Kronenberg) and a fellowship from the Cancer Research Institute (to L. Gapin). This is manuscript number 370 from the La Jolla Institute for Allergy and Immunology.

Submitted: 4 May 2000

Revised: 14 June 2000

Accepted: 28 June 2000

Note added in proof. After the submission of this manuscript, another report of CD1d tetramers was published (Benlagha, K., A. Weiss, A. Beavis, L. Teyton, and A. Bendelac. 2000. *J. Exp. Med.* 191:1895–1903).

References

1. Bendelac, A., M.N. Rivera, S.H. Park, and J.H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* 15:535–562.
2. Bendelac, A., R.D. Hunziker, and O. Lantz. 1996. Increased interleukin 4 and immunoglobulin E production in transgenic mice overexpressing NK1 T cells. *J. Exp. Med.* 184:1285–1293.
3. Gombert, J.M., A. Herbelin, E. Tancrede-Bohin, M. Dy, C. Carnaud, and J.-F. Bach. 1996. Early quantitative and functional deficiency of NK1⁺-like thymocytes in the NOD mouse. *Eur. J. Immunol.* 26:2989–2998.
4. Zeng, D., D. Lewis, S. Dejbakhsh-Jones, F. Lan, M. Garcia-Ojeda, R. Sibley, and S. Strober. 1999. Bone marrow NK1.1⁻ and NK1.1⁺ T cells reciprocally regulate acute graft versus host disease. *J. Exp. Med.* 189:1073–1081.
5. Hammond, K.J.L., L.D. Poulton, L.J. Palmisano, P.A. Silveira, D.I. Godfrey, and A.G. Baxter. 1998. α/β -T cell receptor (TCR)⁺CD4⁻CD8⁻ (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. *J. Exp. Med.* 187:1047–1056.
6. Falcone, M., B. Yeung, L. Tucker, E. Rodriguez, and N. Sarvetnick. 1999. A defect in interleukin 12-induced activation and interferon gamma secretion of peripheral natural killer T cells in nonobese diabetic mice suggests new pathogenic mechanisms for insulin-dependent diabetes mellitus. *J. Exp. Med.* 190:963–972.
7. Wilson, S.B., S.C. Kent, K.T. Patton, T. Orban, R.A. Jackson, M. Exley, S. Porcelli, D.A. Schatz, M.A. Atkinson, S.P. Balk, et al. 1998. Extreme Th1 bias of invariant V α 24 α Q T cells in type 1 diabetes. *Nature.* 391:177–181.
8. Sumida, T., A. Sakamoto, H. Murata, Y. Makino, H. Takahashi, S. Yoshida, K. Nishioka, I. Iwamoto, and M. Taniguchi. 1995. Selective reduction of T cells bearing invariant V α 24 α Q antigen receptor in patients with systemic sclerosis. *J. Exp. Med.* 182:1163–1168.
9. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, H. Sato, E. Kondo, M. Harada, H. Koseki, T. Nakayama, et al. 1998. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated V α 14 NKT cells. *Proc. Natl. Acad. Sci. USA.* 95:5690–5693.
10. Denkers, E.Y., T. Scharton-Kersten, S. Barbieri, P. Caspar, and A. Sher. 1996. A role for CD4⁺ NK1.1⁺ T lymphocytes as major histocompatibility complex class II independent helper cells in the generation of CD8⁺ effector function against intracellular infection. *J. Exp. Med.* 184:131–139.
11. Flesch, I.E., A. Wandersee, and S.H. Kaufmann. 1997. IL-4 secretion by CD4⁺ NK1⁺ T cells induces monocyte chemoattractant protein-1 in early listeriosis. *J. Immunol.* 159:7–10.
12. Emoto, M., Y. Emoto, I.B. Buchwalow, and S.H. Kaufmann. 1999. Induction of IFN γ -producing CD4⁺ natural killer T cells by *Mycobacterium bovis* bacillus Calmette Guerin. *Eur. J. Immunol.* 29:650–659.
13. Schofield, L., M.J. McConville, D. Hansen, A.S. Campbell, B. Fraser-Reid, M.J. Grusby, and S.D. Tachado. 1999. CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. *Science.* 283:225–229.
14. Joyce, S., A.S. Woods, J.W. Yewdell, J.R. Bennink, A.D. De Silva, A. Boesteanu, S.P. Balk, R.J. Cotter, and R.R. Brutkiewicz. 1998. Natural ligand of mouse CD1d1: cellular glycosylphosphatidylinositol. *Science.* 279:1541–1544.
15. Gumperz, J.E., C. Roy, A. Makowska, D. Lum, M. Sugita, T. Podrebarac, Y. Koezuka, S.A. Porcelli, S. Cardell, M.B. Brenner, et al. 2000. Murine CD1d-restricted T cell recogni-

- tion of cellular lipids. *Immunity*. 12:211–221.
16. Burdin, N., L. Brossay, Y. Koezuka, S.T. Smiley, M.J. Grusby, M. Gui, M. Taniguchi, K. Hayakawa, and M. Kronenberg. 1998. Selective ability of mouse CD1 to present glycolipids: α -galactosylceramide specifically stimulates $V\alpha 14^+$ NK T lymphocytes. *J. Immunol.* 161:3271–3281.
 17. Morita, M., K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi, and H. Fukushima. 1995. Structure-activity relationship of α -galactosylceramides against B16-bearing mice. *J. Med. Chem.* 38:2176–2187.
 18. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of $V\alpha 14$ NKT cells by glycosylceramides. *Science*. 278:1626–1629.
 19. Burdin, N., L. Brossay, and M. Kronenberg. 1999. Immunization with α -galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. *Eur. J. Immunol.* 29: 2014–2025.
 20. Singh, N., S. Hong, D.C. Scherer, I. Serizawa, N. Burdin, M. Kronenberg, Y. Koezuka, and L. Van Kaer. 1999. Cutting edge: activation of NK T cells by CD1d and α -galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J. Immunol.* 163:2373–2377.
 21. Couedel, C., M.A. Peyrat, L. Brossay, Y. Koezuka, S.A. Porcelli, F. Davodeau, and M. Bonneville. 1998. Diverse CD1d-restricted reactivity patterns of human T cells bearing “invariant” AV24BV11 TCR. *Eur. J. Immunol.* 28:4391–4397.
 22. Brossay, L., M. Chioda, N. Burdin, Y. Koezuka, G. Casorati, P. Dellabona, and M. Kronenberg. 1998. CD1d-mediated recognition of an α -galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J. Exp. Med.* 188:1521–1528.
 23. Spada, F.M., Y. Koezuka, and S.A. Porcelli. 1998. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J. Exp. Med.* 188:1529–1534.
 24. Chen, H., and W. Paul. 1998. A population of CD62L^{low} NK1.1⁻CD4⁺ T cells that resembles NK1.1⁺CD4⁺ T cells. *Eur. J. Immunol.* 28:3172–3182.
 25. Eberl, G., R. Lees, S.T. Smiley, M. Taniguchi, M.J. Grusby, and H.R. MacDonald. 1999. Tissue-specific segregation of CD1d-dependent and CD1d-independent NK T cells. *J. Immunol.* 162:6410–6419.
 26. Zeng, D., G. Gazit, S. Dejbakhsh-Jones, S.P. Balk, S. Snapper, M. Taniguchi, and S. Strober. 1999. Heterogeneity of NK1.1⁺ T cells in the bone marrow: divergence from the thymus. *J. Immunol.* 163:5338–5345.
 27. Hammond, K., S. Pelikan, N. Crowe, E. Randle-Barrett, T. Nakayama, M. Taniguchi, M. Smyth, I. van Driel, R. Scollay, A. Baxter, et al. 1999. NKT cells are phenotypically and functionally diverse. *Eur. J. Immunol.* 29:3768–3781.
 28. Altman, J.D., P.A.H. Moss, P.J.R. Goulder, D.H. Barouch, M.G. McHeyzer-Williams, J.I. Bell, A.J. McMichael, and M.M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science*. 274:94–96.
 29. McMichael, A.J., and C.A. O’Callaghan. 1998. A new look at T cells. *J. Exp. Med.* 187:1367–1371.
 30. Cochran, J.R., T.O. Cameron, and L.J. Stern. 2000. The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity*. 12:241–250.
 31. Boniface, J.J., J.D. Rabinowitz, C. Wulfig, J. Hampl, Z. Reich, J.D. Altman, R.M. Kantor, C. Beeson, H.M. McConnell, and M.M. Davis. 1998. Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands. *Immunity*. 9:459–466.
 32. Braud, V.M., D.S. Allan, C.A. O’Callaghan, K. Soderstrom, A. D’Andrea, G.S. Ogg, S. Lazetic, N.T. Young, J.I. Bell, J.H. Phillips, et al. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature*. 391:795–799.
 33. Vance, R.E., J.R. Kraft, J.D. Altman, P.E. Jensen, and D.H. Raulet. 1998. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1^b. *J. Exp. Med.* 188:1841–1848.
 34. Salcedo, M., P. Bousso, H.G. Ljunggren, P. Kourilsky, and J.P. Abastado. 1998. The Qa-1b molecule binds to a large subpopulation of murine NK cells. *Eur. J. Immunol.* 28:4356–4361.
 35. Allan, D.S., M. Colonna, L.L. Lanier, T.D. Churakova, J.S. Abrams, S.A. Ellis, A.J. McMichael, and V.M. Braud. 1999. Tetrameric complexes of human histocompatibility leukocyte antigen (HLA)-G bind to peripheral blood myelomonocytic cells. *J. Exp. Med.* 189:1149–1156.
 36. Castaño, A.R., S. Tangri, J.E. Miller, H.R. Holcombe, M.R. Jackson, W.D. Huse, M. Kronenberg, and P.A. Peterson. 1995. Peptide binding and presentation by mouse CD1. *Science*. 269:223–226.
 37. Naidenko, O.V., J.K. Maher, W.A. Ernst, T. Sakai, R.L. Modlin, and M. Kronenberg. 1999. Binding and antigen presentation of ceramide-containing glycolipids by soluble mouse and human CD1d molecules. *J. Exp. Med.* 190:1069–1080.
 38. Crawford, F., H. Kozono, J. White, P. Marrack, and J. Kappler. 1998. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. *Immunity*. 8:675–682.
 39. Kozono, H., J. White, J. Clements, P. Marrack, and J. Kappler. 1994. Production of soluble MHC class II proteins with covalently bound single peptides. *Nature*. 369:151–154.
 40. Brossay, L., S. Tangri, M. Bix, S. Cardell, R. Locksley, and M. Kronenberg. 1998. Mouse CD1-autoreactive T cells have diverse patterns of reactivity to CD1⁺ targets. *J. Immunol.* 160:3681–3688.
 41. Cardell, S., S. Tangri, S. Chan, M. Kronenberg, C. Benoist, and D. Mathis. 1995. CD1-restricted CD4⁺ T cells in major histocompatibility complex class II-deficient mice. *J. Exp. Med.* 182:993–1004.
 42. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for $V\alpha 14$ NKT cells in IL-12-mediated rejection of tumors. *Science*. 278:1623–1626.
 43. Eberl, G., and H.R. MacDonald. 2000. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. *Eur. J. Immunol.* 30:985–992.
 44. Coles, M., C. McMahon, H. Takizawa, and D. Raulet. 2000. Memory CD8 T lymphocytes express inhibitory MHC-specific Ly49 receptors. *Eur. J. Immunol.* 30:236–244.
 45. Slika, M.K., R.R. Pagarigan, and J.L. Whitton. 2000. NK markers are expressed on a high percentage of virus-specific CD8⁺ and CD4⁺ T cells. *J. Immunol.* 164:2009–2015.
 46. Chang, C.S., L. Brossay, M. Kronenberg, and K.P. Kane. 1999. The murine nonclassical class I major histocompatibility complex-like CD1.1 molecule protects target cells from lymphokine-activated killer cell cytotoxicity. *J. Exp. Med.* 189:

- 483–491.
47. Hanke, T., H. Takizawa, C.W. McMahon, D.H. Busch, E.G. Pamer, J.D. Miller, J.D. Altman, Y. Liu, D. Cado, F.A. Lemonnier, et al. 1999. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity*. 11:67–77.
 48. Michaelsson, J., A. Achour, M. Salcedo, A. Kase-Sjostrom, J. Sundback, R.A. Harris, and K. Karre. 2000. Visualization of inhibitory Ly49 receptor specificity with soluble major histocompatibility complex class I tetramers. *Eur. J. Immunol.* 30: 300–307.
 49. Eberl, G., and H.R. MacDonald. 1998. Rapid death and regeneration of NKT cells in anti-CD3 ϵ - or IL-12-treated mice: a major role for bone marrow in NKT cell homeostasis. *Immunity*. 9:345–353.
 50. Kobayashi, E., K. Motoki, T. Uchida, H. Fukushima, and Y. Koezuka. 1995. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol. Res.* 7:529–534.
 51. Kobayashi, E., K. Motoki, T. Natori, T. Uchida, H. Fukushima, and Y. Koezuka. 1996. Enhancing effects of agelasphin-11 on natural killer cell activities of normal and tumor-bearing mice. *Biol. Pharm. Bull.* 19:350–353.
 52. Nakagawa, R., K. Motoki, H. Ueno, R. Iijima, H. Nakamura, E. Kobayashi, A. Shimosaka, and Y. Koezuka. 1998. Treatment of hepatic metastasis of the colon26 adenocarcinoma with an alpha-galactosylceramide, KRN7000. *Cancer Res.* 58:1202–1207.
 53. Carnaud, C., D. Lee, O. Donnars, S.H. Park, A. Beavis, Y. Koezuka, and A. Bendelac. 1999. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J. Immunol.* 163:4647–4650.
 54. Kitamura, H., A. Ohta, M. Sekimoto, M. Sato, K. Iwakabe, M. Nakui, T. Yahata, H. Meng, T. Koda, S. Nishimura, et al. 2000. α -galactosylceramide induces early B-cell activation through IL-4 production by NKT cells. *Cell. Immunol.* 199: 37–42.
 55. Coles, M.C., and D.H. Raulet. 2000. NK1.1⁺ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4⁺CD8⁺ cells. *J. Immunol.* 164:2412–2418.
 56. Kitamura, H., K. Iwakabe, T. Yahata, S. Nishimura, A. Ohta, Y. Ohmi, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, et al. 1999. The natural killer T (NKT) cell ligand α -galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med.* 189:1121–1128.
 57. Cui, J., N. Watanabe, T. Kawano, M. Yamashita, T. Kamata, C. Shimizu, M. Kimura, E. Shimizu, J. Koike, H. Koseki, et al. 1999. Inhibition of T helper cell type 2 cell differentiation and immunoglobulin E response by ligand-activated V α 14 natural killer T cells. *J. Exp. Med.* 190:783–792.