The Journal of Immunology

Dissociation of NKT Stimulation, Cytokine Induction, and NK Activation In Vivo by the Use of Distinct TCR-Binding Ceramides

John R. Ortaldo, Howard A. Young, Robin T. Winkler-Pickett, Earl W. Bere, Jr., William J. Murphy and Robert H. Wiltrout

J Immunol 2004; 172:943-953; ;
doi: 10.4049/jimmunol.172.2.943
http://www.jimmunol.org/content/172/2/943

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Dissociation of NKT Stimulation, Cytokine Induction, and NK Activation In Vivo by the Use of Distinct TCR-Binding Ceramides[1]

John R. Ortaldo,[2]* Howard A. Young,* Robin T. Winkler-Pickett,* Earl W. Bere, Jr.,* William J. Murphy,† and Robert H. Wiltrout*

NKT and NK cells are important immune regulatory cells. The only efficient means to selectively stimulate NKT cells in vivo is α-galactosylceramide (αGalCer). However, αGalCer effectively stimulates and then diminishes the number of detectable NKT cells. It also exhibits a potent, indirect ability to activate NK cells. We have now discovered another ceramide compound, β-galactosylceramide (βGalCer) (C12), that efficiently diminishes the number of detectable mouse NKT cells in vivo without inducing significant cytokine expression or activation of NK cells. Binding studies using CD1d tetramers loaded with βGalCer (C12) demonstrated significant but lower intensity binding to NKT cells when compared with αGalCer, but both ceramides were equally efficient in reducing the number of NKT cells. However, βGalCer (C12), in contrast to αGalCer, failed to increase NK cell size, number, and cytolytic activity. Also in contrast to αGalCer, βGalCer (C12) is a poor inducer of IFN-γ, TNF-α, GM-CSF, and IL-4 gene expression. These qualitative differences in NKT perturbation/NK activation have important implications for delineating the unique in vivo roles of NKT vs NK cells. Thus, αGalCer (which triggers NKT cells and activates NK cells) efficiently increases the resistance to allogeneic bone marrow transplantation while βGalCer (C12) (which triggers NKT cells but does not activate NK cells) fails to enhance bone marrow graft rejection. Our results show βGalCer (C12) can effectively discriminate between NKT- and NK-mediated responses in vivo. These results indicate the use of different TCR-binding ceramides can provide a unique approach for understanding the intricate immunoregulatory contributions of these two cell types. ***The Journal of Immunology,*** **2004, 172: 943–953.**

Natural killer T cells represent a small lymphocyte subpopulation that has important immunoregulatory functions (1). NKT cells are characterized by their coexpression of the NK receptor NKR-P1A (CD161) and a TCR. However, the NKT subpopulation exhibits considerable heterogeneity in both phenotypic characteristics and functions (1, 2). Specifically, Kronenberg and Gapin (1) have recently categorized NKT cells into several distinct subsets based on their TCR repertoire, expression of Ag-presenting coreceptor molecules, and their anatomical compartmentalization in the host. Type I NKT cells exhibit a rearrangement of the Vα14-Jα18 variable region of the TCR and are either $CD4^+$ or $CD4^-$, $CD8^-$. The responsiveness of these NKT cells is CD1d restricted, and these cells are primarily located in the thymus, liver, spleen, and bone marrow (1). The reactivity of this subset appears restricted to α-galactosylceramide (αGalCer)[3] (1, 3) and represents a major component of the overall NKT cell population. This NKT subset coexpresses Ly49 receptors that are also found on NK cells (2). A second category of NKT cells is CD1d autoreactive and coexpresses a more distinctive TCR expression pattern (Vα3.2-Jα9/Vα8, Vα8) than type I (1). Although these NKT cells are CD1d autoreactive, they are not truly polyclonal. Like type I NKT cells, they are $CD4^+$ or $CD4^-$, $CD8^-$ and express predominately NK1.1. However they differ from type I NKT cells in both their location in vivo (liver) and the fact that they are nonreactive with αGalCer (1). A third subset of NKT cells is not CD1d dependent, has a diverse TCR expression, and can be either $CD4^+$, $CD8^+$ or $CD4^-$, $CD8^-$. The Ag reactivity of this third subset is not known, and their in vivo distribution is similar to that of type I NKT cells. A fourth subset of NKT cells is defined by their expression of α_2-integrin (CD49B), which is recognized by the NK-reactive Ab, DX5. This NKT population has little overlap with the type I subset and may have a very specialized role in the immune response (1).

Mouse studies have demonstrated that NKT cells can regulate autoimmune responses (4–11) and can reverse some types of immune dysregulation (12–16). A better understanding of the characteristics and functions of NKT cells may provide new insights into treatment of autoimmune diseases, as well as novel strategies to increase reactivity against cancer and AIDS. In this context, many studies of NKT cells have centered on the functions of CD1-restricted, Vα14-Jα18 or Vα3, 2-Jα9/Vα8, Vβ8-expressing NKT cell subsets that represent a majority of spleen-, liver-, and bone

*Laboratory of Experimental Immunology, National Cancer Institute-Center for Cancer Research, Frederick, MD 21702; and †Department Microbiology, University of Nevada Medical School, Reno, NV 89557

Received for publication August 5, 2003. Accepted for publication November 4, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This project has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. N01-CO-12400. Animal care was provided in accordance with the procedures outlined in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publications No. 86-23, 1985). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the U.S. Government.

[2] Address correspondence and reprint requests to Dr. John Ortaldo, National Cancer Institute, Center for Cancer Research, Laboratory of Experimental Immunology, Building 560, Room 31-93, Frederick, MD 21702-1201. E-mail address: ortaldo@mail.ncifcrf.gov

[3] Abbreviations used in this paper: αGalCer, α-galactosylceramide; βGalCer, β-galactosylceramide; mCD1d, murine CD1d; FasL, Fas ligand.

0022-1767/04/$02.00

marrow-associated NKT cells (1, 3). The determination of NKT-mediated biological activities has been largely dependent on the ability of αGalCer to selectively target and perturb these cells and thereby relate their apparent presence or absence to many facets of immune regulation. In this regard, administration of αGalCer in vivo has been reported to rapidly deplete NKT cells through a TCR-dependent apoptotic process (17). However, during this process αGalCer triggers production of IFN-γ from NKT cells and also activates NK cells that are often found in the spleen and liver. This secondary activation of NK cells can have confusing implications for delineating the role of NKT cells in various disease states and immune responses, because the activation of colocated NK cells could account for changes in biological activities that accompany loss of NKT cell detection. Thus, it is quite difficult to differentiate the baseline contribution of NKT cells to various immune responses from NKT-dependent or -independent amplification of other leukocyte responses.

In this study, we have shown that βGalCer (C12) can be efficiently presented on CD1d and rapidly reduces the number of detectable NKT cells in vivo without inducing production of IFN-γ, activation of NK-mediated lysis, or NK-dependent rejection of bone marrow grafts.

Materials and Methods

Mice

Mice were obtained from the Animal Production Area, National Cancer Institute-Frederick and used between 6 and 12 wk of age.

Ceramide reagents

Various reagents including D-glucosyl-β1-1′ ceramide (C8), D-galactosyl-β1-1′ ceramide (C8), D-glucosyl-β1-1′ ceramide (C12), and D-galactosyl-β1-1′ ceramide (C12) were purchased from AvantiLipids (Alabaster, AL). αGalCer (KRN7000) was graciously provided by Kirin Brewery (Tokyo, Japan). The ceramide reagents were first dissolved in DMSO, then diluted in PBS containing 0.5% Tween 20. Control diluent or PBS was used as a control for all studies.

NK cell isolation

NK cells were isolated from the livers or spleens of C57BL/6 (B6) mice and grown for 7–10 days in 1000 IU/ml rIL-2 (Hoffman-LaRoche, Nutley, NJ) as previously described (2). Liver-associated NK cells were isolated from IL-2-treated mice as previously described (2). Liver-associated mononuclear cells (35–70% $CD3^-$, $NK1.1^+$) were used immediately after isolation or after IL-2 expansion.

Proteose peptone method

As previously described (18), mice were injected i.p. with 1 ml of 3% proteose peptone (BD Biosciences, Sparks, MD). After 4 days, the peritoneal exudate cells were removed from the abdominal cavity using a needle and syringe. These cells were washed with PBS and used in the assay.

Flow cytometry reagents and Abs

NK1.1-PE (or allophycocyanin), DX-5-PE and CD3-PerCP (BD PharMingen, San Jose, CA), as well as CD69-FITC, were used for flow cytometric analysis as previously described (2). Intracellular detection of cytoplasmic IFN-γ was performed using kits purchased from BD PharMingen and used as per the manufacturer's instructions. Annexin V staining was used to evaluate early events in apoptosis (BD PharMingen).

Flow cytometry analysis and lymphocyte sorting

Cells were stained as previously described (2) and analyzed on a FACSort or LSR System flow cytometer (BD Biosciences, San Jose, CA). Cells were directly stained using FITC-, PE-, PerCP-, and allophycocyanin-labeled primary Abs. Sorting experiments were performed on a MoFlo cell sorter (DAKO/Cytomation, Ft. Collins, CO) to isolate NK and NKT cells using NK1.1-PE and CD3biotin-SA.Tricolor (Caltag Laboratories, Burlingame, CA) as the defining immunological markers.

Cytokine measurement

IFN-γ and chemokine proteins were measured in culture supernatants or mouse serum using ELISA kits (R&D Systems, Minneapolis, MN) as previously described (2). Cell stimulations were performed at concentrations of $1–5 \times 10^6$ cells/ml, and variation within assays was always <5 pg/ml. In some experiments, B cell lines (A20 and A20/CD1d generously provided by M. Kronenberg, La Jolla Institute, San Diego, CA) were pretreated with various reagents for 30 min at 37°C, washed and mixed with sorted populations of NK or NKT cells (>98% pure), and supernatants were collected for analysis after 24 h of culture.

RNase protection assay

Multiprobe RNase protection assays were performed using the mck-1 or mck-5 probe sets (BD PharMingen). Total cellular RNA was extracted using TRIzol (Life Technologies, Gaithersburg, MD), and 1–5 μg of total mRNA was hybridized with a [^{33}P]UTP-labeled RNA probe (1×10^6 cpm/sample) prepared according to the manufacturers directions (BD PharMingen) using the BD PharMingen RiboQuant in vitro transcription kit. Following hybridization, the samples were treated with RNase A and T1 according to the procedure provided by BD PharMingen. The RNase was inactivated and precipitated using a solution containing 200 μl of Ambion (Austin, TX) RNase inactivation reagent, 50 μl of ethanol, 5 μg of yeast tRNA, and 1 μl of Ambion GycoBlue coprecipitate per RNA sample. The samples were mixed well, incubated at −70°C for 15 min, and centrifuged at 14,000 rpm for 15 min at room temperature. The pellets were then suspended in 3 μl of BD PharMingen sample buffer and subjected to PAGE as recommended by the manufacturer (BD PharMingen).

Cytotoxicity assay

NK cytotoxicity was evaluated against the prototype NK target Yac-1 in a standard 4-h ^{51}Cr release assay as previously described (2).

Bone marrow transplantation

The efficiency of reconstitution after bone marrow transplantation was evaluated using a new label-tracking procedure (19) that uses CFSE (Molecular Probes, Eugene, OR)-labeled bone marrow cells that can be evaluated in vivo after 1–4 days. Briefly, 10×10^6 CFSE-labeled bone marrow cells (20) were injected into irradiated recipient mice (C57BL/6, 900 rad; BALB/c, 800 rad). The spleens were then evaluated by flow cytometry for the total number of labeled cells in treated or untreated autologous vs allogeneic transplant groups. Mice were evaluated individually, and the mean and SD for each group was calculated.

Tetrameric GalCer-CD1d complexes

Tetramers of wild-type CD1d molecules were produced as described by Matsuda et al. (21). CD1d molecules were biotinylated and prepared by the National Institutes of Health tetramer facility. The production and biotinylation of murine CD1d (mCD1d) protocol was as previously reported (21). mCD1d was coexpressed with β_2-microglobulin in insect cells (High 5) using a baculovirus expression system. The monomer was purified from media by concentration on a tangible filter device followed by standard nickle-agarose separation. The correct fractions were combined and exchanged twice in biotinylation buffer. The biotinylation reaction was conducted overnight at 25°C. Biotinylated mCD1d was separated from biotin and ATP on a S300 gel filtration column. Biotinylated CD1d molecules were then incubated overnight at room temperature with a 3-fold molar excess of α or βGalCer (solubilized in 0.5% Tween 20, 0.9% sodium chloride, hereafter called vehicle) or with an equal amount of vehicle alone. CD1d monomers were then tetramerized using a 1:4 molar ratio of allophycocyanin-conjugated streptavidin (BD PharMingen).

Tetramer staining

All staining and washes were performed in a buffer consisting of 2% FCS, 0.075% sodium bicarbonate, and 0.1% sodium azide in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO), as previous described (21). Cells were stained for 3 h at room temperature, then stained with subset Abs, washed two times, and fixed in 1% paraformaldehyde (Sigma-Aldrich) in PBS. The intensity of fluorescence on hybridoma cells was determined by flow cytometry analysis using a FACSort flow cytometer (BD Biosciences).

Results

Dose-dependent NKT depletion and NK activation by αGalCer

To ultimately compare and contrast the immune-stimulating effects of different ceramide compounds, a series of in vivo studies was performed to define the direct effects of αGalCer on NKT cells, as well as subsequent indirect effects on NK cells (Fig. 1). A rapid activation of NK cells was noted as defined by increased NK-mediated lysis (Fig. 1*A*), increased size of NK cells (Fig. 1*B*), increased expression of the CD69 activation Ag (Fig. 1*C*), increased total number (Fig. 1*D*), and percentage (Fig. 1*F*) of NK cells. As expected (1), administration of several doses of αGalCer resulted in a rapid decrease in the number (Fig. 1*E*) and percentage (Fig. 1*G*) of detectable NKT cells. The highest doses of αGalCer caused a 2- to 10-fold increase in total liver cellularity (Fig. 1*H*) and a rapid and potent increase in the amounts of IFN-γ in the serum that persisted for up to 48 h (Fig. 1*I*). Interestingly, a wide range of doses of αGalCer resulted in potent and sustained (> 7 days) activation of NK cells (lytic function, Fig. 1*A*) and increase in NK cell numbers and frequency (Fig. 1, *D* and *F*). Although these effects were dose dependent, doses as low as 0.1 μg/mouse resulted in significantly increased activities. These findings clearly show that in vivo administration of αGalCer results in a rapid decrease in a major NKT subset from the liver that coincides with a rapid and persistent activation of NK cells as defined by multiple parameters.

Because NK activation closely follows the stimulation of NKT cells, it is important to demonstrate whether these events are directly related. In a subsequent study in SCID mice that lack NKT cells, this unique leukocyte subset was shown to be critical for the initiation of NK cell activation by αGalCer (Fig. 2). Administration of αGalCer to SCID mice failed to increase NK cell size or cytolytic activity, whereas expected increases in both NK cell size and lytic functions were seen in control C57BL/6 mice. Other parameters of NK cell activation as shown in Fig. 1 were also not observed in SCID mice (data not shown). These results strongly implicate activation of NKT cells as a prerequisite for subsequent NK cell activation after administration of αGalCer.

αGalCer-induced NKT cell depletion and NK activation is not dependent on TH_1 cytokines, perforin, or Fas ligand (FasL)

Because the decrease in NKT cell detection induced by αGalCer is accompanied by a rapid burst of IFN-γ production and NK activation, we speculated that TH_1 cytokines could be critical regulatory factors in these effects. Furthermore, because NKT cells rapidly become undetectable after αGalCer administration, we speculated that the Fas or

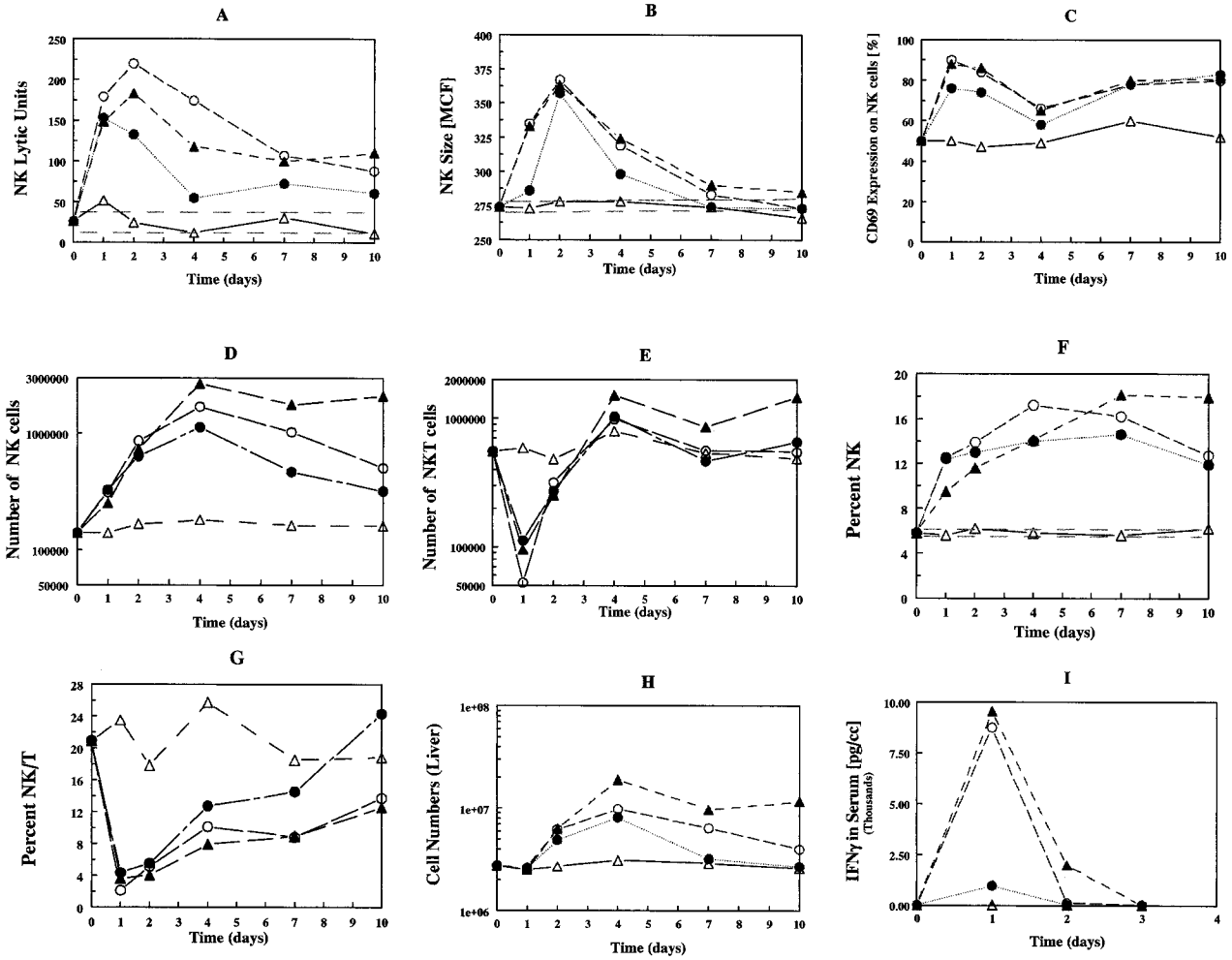

FIGURE 1. Mice were given varying doses 10 (○), 1 (▲), or 0.1 (●) of αGalCer or diluents (△), and their liver lymphocytes were evaluated for cell functions at various times (days). All points represent pooled cells from three to five mice per group. *A*, NK activity measured by ^{51}Cr release from YAC-1 expressed in lytic units. *B*, NK cells based on flow cytometry forward scatter measurements. *C* measures CD69 expression on NK cells ($NK1.1^+$, $CD3^-$). *D*, Liver NK cell numbers. *E*, NKT cells numbers. *F*, The relative percentage NK cells in liver. *G*, The relative percentage NKT ($NK1.1^+$, $CD3^+$) cells in liver. *H*, Total leukocyte number in liver. *I*, Serum IFN-γ levels in serum. All experiments represent a pool of three to five mice.

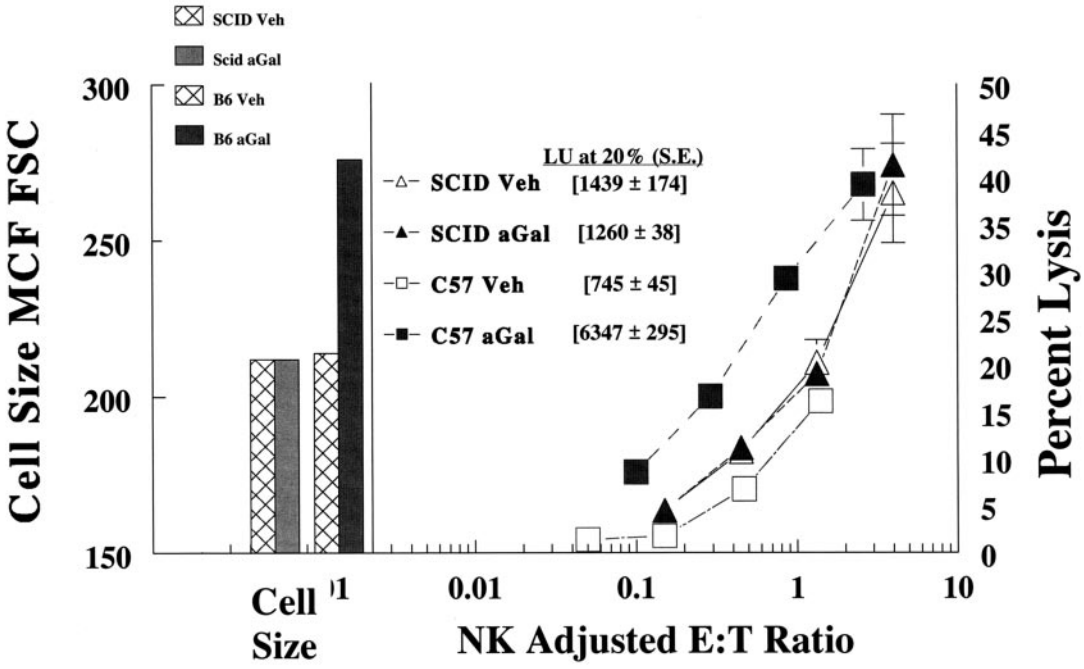

FIGURE 2. C57BL/6 or C57BL/6 SCID mice were analyzed for the ability of 1 μg/ml αGalCer to increase NK cell size (*left*) or NK activity (*right*). Cytotoxicity (and SD) results are shown in line chart with lytic unit values indicated in the legend.

perforin pathways might contribute to this effect. Table I evaluates a series of αGalCer treatments in selected gene disruption mice. However, the ability of αGalCer to reduce the number of detectable NKT cells and activate NK cells were largely retained in mice with dysregulated TH_1 cytokine, TNF superfamily, and perforin genes. Some mutant or knockout mice, such as FasL (gld) and CD40L (−/−), had low baseline percentages of NKT cells, but an additional loss of >50% of these cells was observed after αGalCer treatment. Similarly,

Table I. *Summary of αGalCer effects on liver subsets and function*[a]

		Cells Mouse ($\times 10^6$)	% NK	% NKT	% T	NK Size (MCF)	NK Lysis	No. of NK ($\times 10^5$)	No. of NKT ($\times 10^5$)	No. of T ($\times 10^6$)
Expt. 1										
NT	B6	3.60	6.5	16.5	34.6	292	38.5	2.3	6.0	1.2
αGal 24	B6	4.40	10.6	1.9	33.2	349	743.0	4.7	0.8	1.5
NT	GLD (FasL)	15.40	2.3	3.0	54.0	325	60.3	3.5	4.5	8.3
αGal 24	GLD (FasL)	9.00	4.4	1.3	62.1	355	329.0	3.9	1.2	5.6
NT	PFP	2.90	7.2	13.2	36.0	289	<1	2.1	3.8	1.0
αGal 24	PFP	3.40	9.6	2.1	44.0	337	<1	3.3	0.7	1.5
Expt. 2										
NT	B6	3.00	6.9	28.6	29.6	272	36.0	2.1	8.6	0.9
αGal 24	B6	4.10	11.9	1.3	41.2	319	340.0	4.9	0.5	1.7
NT	TNF	3.50	7.7	26.8	29.1	275	82.0	2.7	9.4	1.0
αGal 24	TNF	8.20	4.6	0.9	46.0	325	473.0	3.8	0.7	3.8
NT	MIP	1.90	11.1	13.0	26.0	274	24.1	2.1	2.5	0.5
αGal 24	MIP	3.10	4.6	0.4	32.8	338	662.2	1.4	0.1	1.0
Expt. 3										
NT	B6	2.70	5.2	23.1	31.0	276	25.9	1.4	6.2	0.8
αGal 24	B6	2.70	9.5	4.5	40.3	316	714.8	2.6	1.2	1.1
NT	GLD (FasL)	2.30	3.8	7.1	44.5	280	29.1	0.9	1.6	1.0
αGal 24	GLD (FasL)	9.00	4.5	3.2	65.1	320	99.2	4.1	2.9	5.9
Expt. 4										
NT	B6	3.45	8.0	23.0	32.1	214	55.0	2.8	7.9	1.1
αGal	B6	2.40	15.8	4.0	37.2	266	830.4	3.8	1.0	0.9
NT	GKO	1.67	5.6	20.0	29.3	207	11.9	0.9	3.3	0.5
αGal	GKO	3.70	14.1	6.5	44.0	268	249.8	5.2	2.4	1.6
NT	IL-12p40	2.80	6.9	13.0	25.6	222	16.8	1.9	3.6	0.7
αGal	IL-12p40	4.00	7.5	3.8	27.9	280	245.0	3.0	1.5	1.1
NT	CD40L	1.80	5.0	7.3	31.5	211	53.7	0.9	1.3	0.6
αGal	CD40L	3.66	6.3	1.7	45.5	272	662.8	2.3	0.6	1.7

[a] Data from liver at 24 h after administration of 1 μg of αGalCer.

Table II. *Comparison of ceramides in their ability to deplete NKT cells and activate NK cells*[a]

Treatment	Dose (μg/mouse)	% NK	% NKT	NK Size	NK CD69	NK Lysis Units
NT		5.2	25.1	237	59	107
αGal	0.1	10.1	**8.9**	**281**	**80**	**1715**
βGal (C8)	10	5.8	24.5	246	57	133
βGal (C8)	1	7.3	23.4	233	44	129
βGal (C12)	10	9.6	**8.2**	245	45	**417**
βGal (C12)	1	11.2	**11.7**	235	41	198
βGluc (C8)	10	4.3	19.2	244	61	48
βGluc (C8)	1	5.3	25.0	242	62	22
βGluc (C12)	10	5.1	21.8	242	58	43
βGluc (C12)	1	4.1	23.3	244	62	44

[a] Bolded values indicate significant increases ($p < 0.05$), as compared to the negative control, induced by treatments. Similar results were obtained for all treatments in at least two additional experiments.

NK bystander activation as assessed by NK lysis, increased size, and up-regulation of CD69 was observed in all knockout mice (as expected perforin (PFP) mice lacked NK-mediated lysis). Interestingly, dysregulation of the *FasL* gene in gld mice impaired the ability of αGalCer to substantially increase both NK cell number and function, suggesting a possible role for the Fas signaling pathway in the NK activation effect. It should be noted that 24 h is a relatively early time point for evaluation of these cellular increases, which are generally maximal at 3–5 days after αGalCer administration, so additional studies will be needed to see if the FasL-dependent effect is due to a qualitative change in the response or to an altered kinetics of response. Perhaps of most interest was the observation that IFN-γ knockout mice demonstrate efficient NK bystander activation, showing that IFN-γ was not required for most of the bystander effects, and suggesting that the activation of NK cells may not be directly linked to the burst of IFN-γ that occurs coincident with NKT cell stimulation.

Dissociation between NKT depletion and NK augmentation by βGalCer (C12)

The results described above re-emphasize the coordinate induction of two quite different αGalCer-induced regulatory events in key innate immune parameters, a reduction in the number of detectable NKT cells and the activation of NK cells. This data set demonstrates the potency of αGalCer for stimulating innate immune functions, but also illustrates the difficulties in using αGalCer to discriminate the roles of these two cell types in various biological effects in vivo.

Therefore, we speculated that perhaps other ceramide compounds might retain the ability to bind to CD1d on B cells and monocytes, but differ qualitatively from αGalCer in their ability to directly deliver signals to NKT cells and indirectly activate NK cells. Therefore, studies were performed to evaluate if ceramides could be identified that would specifically stimulate NKT cells without causing downstream activation events (e.g., NK activation and IFN-γ production). The data presented in Table II show that βGalCer (C12) potently reduces detectable NKT cells, while other ceramide compounds including βGalCer (C8), β-GlucCer (C12), and β-GlucCer (C8) demonstrated no biological activity when compared with the diluent control. As shown in both Table II and Fig. 3, administration of βGalCer (C12) resulted in a similar reduction in detectable NKT cells as seen for αGalCer (Fig. 3*D*), but without the broad bystander NK activation obtained after administration of αGalCer, as illustrated by a lack of increased percentage, lysis, and CD69 expression (Fig. 3, *A–C*, respectively). High doses of βGalCer (C12) were used to ensure that maximal depletion and/or potential activation would be detected. Overall, it is clear that βGalCer (C12) can very effectively reduce the number of detectable NKT cells, and this effect can be mediated in the absence of the NKT-dependent bystander NK activation that occurs after administration of αGalCer.

βGalCer (C12) reduces the number of detectable NKT cells in the absence of TH_1 and TH_2 cytokine induction

Because previous studies (1, 3, 22, 23), as well as our own results (Table I, Fig. 2), have demonstrated that αGalCer activation and subsequent reduction in detectable NKT cells results in strong IFN-γ production, we evaluated the abilities of αGalCer vs βGalCer (C12) to induce cytokine production in vivo in C57BL/6 mice. Cytokine gene expression for IL-4, IL-13, or IFN-γ was evaluated in both liver and spleen 1, 2, or 4 h after administration of αGalCer and βGalCer (C12). As shown in Fig. 4, only αGalCer induced IL-4, IL-13 (data not shown), or IFN-γ. Interestingly, the ability of αGalCer to increase gene expression at 2 h and/or 4 h was demonstrated in both the liver, which contains large numbers of NKT cells (15–30% of all lymphocytes), as well as in the spleen, which generally contains only 1–3% NKT cells. Thus, although both αGalCer and βGalCer (C12) reduce NKT cell detection, only αGalCer induces cytokine gene expression in spleen and liver. These results were extended by further studies where the ability of αGalCer and βGalCer (C12) to induce cytokine proteins in the serum was studied. The results shown in Fig. 5 demonstrate that αGalCer potently increases serum levels of IFN-γ and GM-CSF (Fig. 5*A*) and IL-4 and TNF-α (Fig. 5*B*) at 1, 2, or 4 h after treatment, whereas βGalCer (C12) did not induce appreciable amounts of these cytokines. The rapid induction of cytokine genes and proteins by αGalCer suggests that these effects must be closely linked to the binding and activation of αGalCer to the TCR of NKT cells. However, the failure of βGalCer (C12) to induce cytokine gene expression while effectively reducing the number of detectable NKT cells contrasts sharply with the effects of αGalCer. This apparent dichotomy of activities for these two ceramides suggests on one hand a similar pathway for binding to and stimulating NKT cells, but in contrast a downstream divergence in signaling for cytokine gene expression. To investigate these questions, studies were performed to compare the abilities of αGalCer vs βGalCer (C12) to induce annexin V binding to NKT cells as an early measure of apoptosis induction. The results shown in Fig. 5*C* demonstrate that both αGalCer and βGalCer (C12) rapidly reduce the number of NKT cells (by ~50% within 1.5 h) also induce a rapid increase in annexin V binding on NKT but not NK cells (Fig. 5*D*). The simultaneous induction of both annexin V expression and loss of NKT detection for both αGalCer and βGalCer (C12) suggests a similar molecular mechanism for these effects by both agents. Examination of NK cell numbers over this time period demonstrated

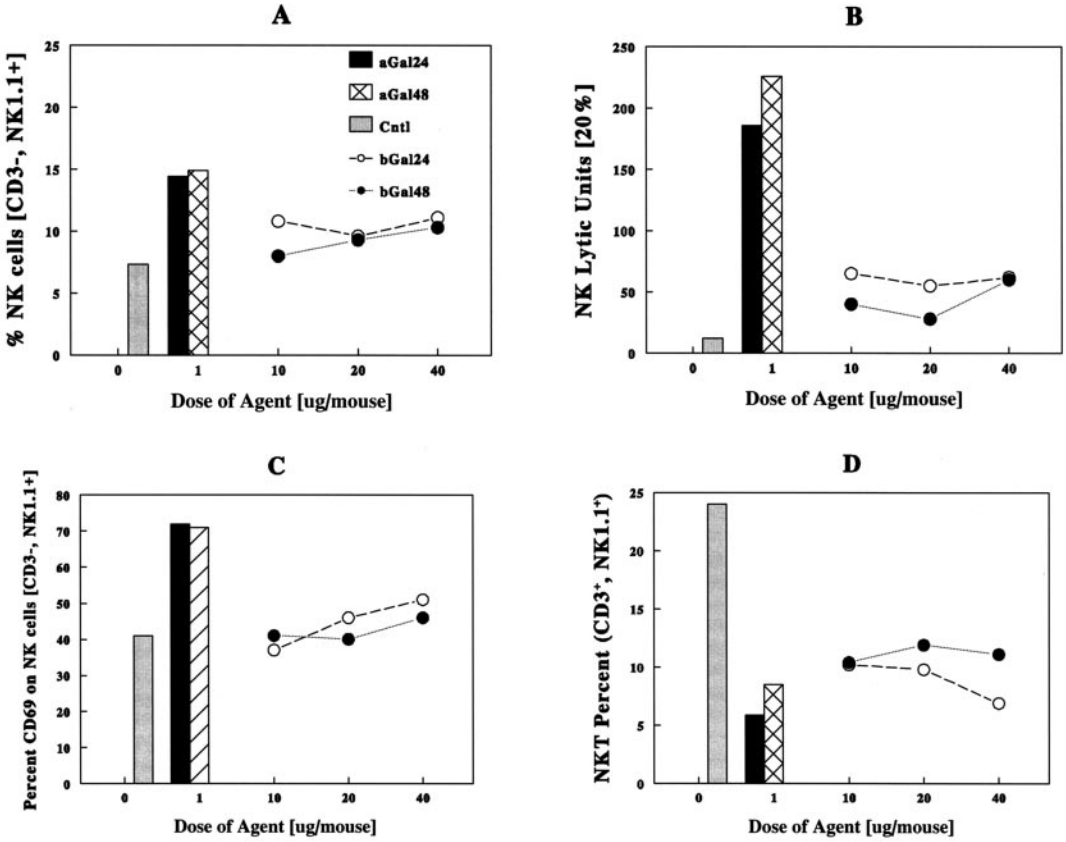

FIGURE 3. NK cell percentage (*A*), NK cytolytic activity in lytic units at 20% (*B*), CD69 expression on NK cells (*C*), and percentage of NKT cells (*D*) are evaluated compared with control (control-diluent; ▨), αGalCer at 24 h ■ or 48 h ▨, 1 μg/mouse, or βGalCer (C12) at 24 h (○) or 48 h (●) at the indicated doses. Data representation of at least three experiments.

no increase in annexin V expression for either αGalCer or βGalCer (C12). Thus, βGalCer (C12) can be used to dissociate NK-activating events from the process of NKT apoptosis and disappearance, suggesting that the quality of the signals induced by βGalCer (C12) and αGalCer binding to the TCR differ.

CD1d-dependent binding of αGalCer and βGalCer (C12) to NKT cells

Because the αGalCer resulted in such a rapid and pronounced increase of NK cell activation and cytokine induction whereas βGalCer (C12) failed to induce this potent stimulation, we evaluated the relative efficiency of binding of both ceramides to NKT cells. The results of a typical binding study are shown in Fig. 6. CD1d tetramers were either loaded with αGalCer or βGalCer (C12) and their binding to NKT-enriched liver lymphocytes was studied. Fig. 6*A* reveals the strong binding of αGalCer-loaded CD1d to NKT cells (Fig. 6*A*, *lower left*), and this was not observed with either NK cells or NK1.1$^-$ T cells. NKT cells evaluated with CD1d tetramers without either αGalCer or βGalCer loading failed to demonstrate any significant binding (data not shown). Fig. 6*B* shows binding with βGalCer (C12)-loaded tetramers where a similar percentage of NKT cells bound the CD1d, albeit at lower intensity, while both NK and NK1.1$^-$ T cells failed to demonstrate this binding (Fig. 6*A* and data not shown). When NKT subsets were evaluated, both CD4 and CD8

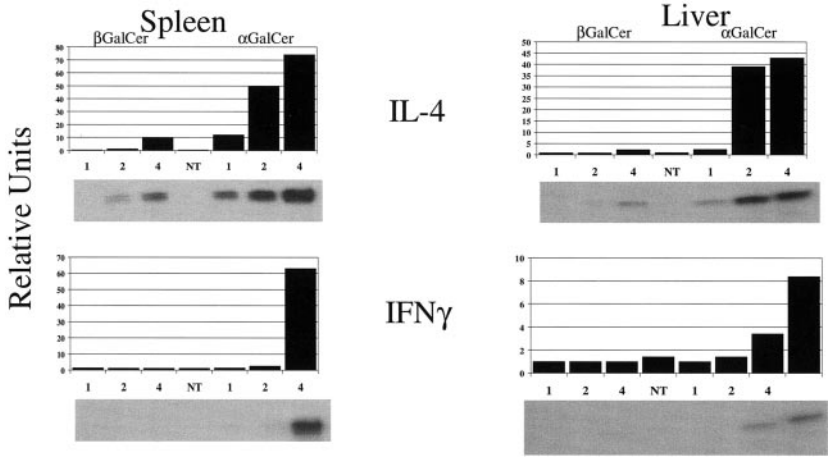

FIGURE 4. Mice (C57BL/6) were injected with αGalCer or βGalCer (C12), and spleen or liver cells were collected at indicated times. Cells were evaluated for mRNA expression using multiprobe RPA analysis. Data is presented as relative changes based on densitometry of expression levels in untreated mice. All values have been corrected based on control RPA (L32) expression (not shown).

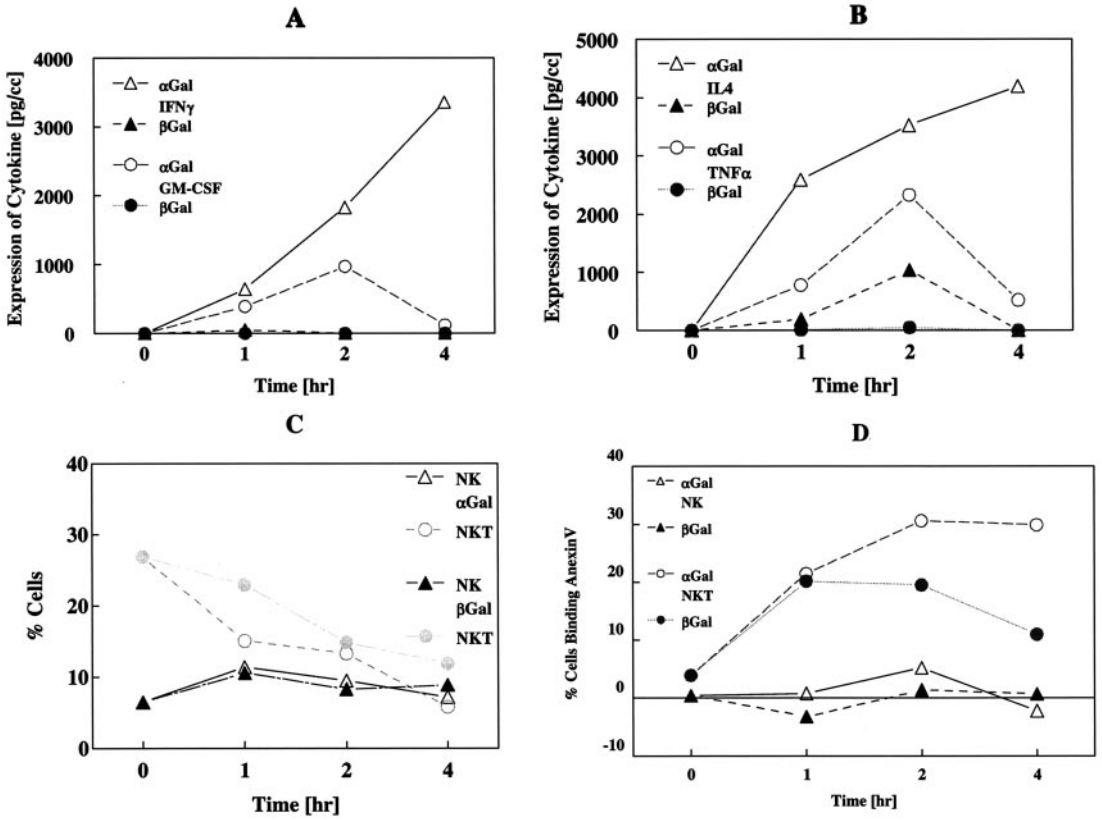

FIGURE 5. Levels of serum cytokines were evaluated by quantitative ELISA and shown in *A* (IFN-γ (△, ▲) and GM-CSF (○, ●)) and *B* (IL-4 (△, ▲) and TNF-α △, ▲)) at various times after treatment with 1 μg of αGalCer (open symbols) or 10 μg of βGalCer (filled symbols) (C12). *C*, Percentage in vivo for the NKT (○, ●) or NK (△, ▲) populations after treatment αGalCer (open symbols) or βGalCer (C12) (filled symbols). *D*, The in vivo expression of annexin V on NKT (○, ●) or NK (△, ▲) cells after treatment with αGalCer (open symbols) or βGalCer (filled symbols) (C12). Data representative of at least three experiments.

subsets bound αGalCer with a strong intensity (Fig. 6*B*, *left*), while βGalCer (C12) binding to $CD4^+$ NKT cells was also similar to its binding to $CD8^+$ cells (Fig. 6*B*, *right*). Overall, these results demonstrate strong binding of αGalCer on both CD4 and CD8 NKT cells (Fig. 6*B*, *left*) with weaker or but quantitatively similar percentages for βGalCer (Fig. 6*B*, *right*). The data shows that ~9% and ~10% of $CD4^+$ and $CD8^+$ cells, respectively, bind αGalCer- and βGalCer-loaded tetramers in fresh liver leukocyte populations. When NKT subsets were evaluated, both CD4 and CD8 subsets bound αGalCer with a strong intensity (Fig. 6, *C* and *D*), while βGalCer (C12) binding to $CD4^+$ NKT cells was significantly stronger than it was to $CD8^+$ cells (see Fig. 9*D*). Binding of unloaded tetramers was used as a control in Fig. 9, *C* and *D*. Similar experiments were performed with NKT cells that had been flow cytometrically sorted and cultured for 3–4 days in IL-2 (data not shown). These results demonstrated a similar intensity difference in binding of αGalCer and βGalCer (C12) to highly purified NKT cells and a lack of binding of αGalCer to either NK or T cells.

To more directly investigate the relative abilities of αGalCer or βGalCer (C12) to directly interact with the TCR of NKT cells in C57BL/6 mice, we used an in vitro assay system in which control A20 cells or A20 cells transfected with CD1d were used to present αGalCer or βGalCer (C12) to isolated NKT vs NK cells (negative control). Cultures containing these enriched subsets of NK or NKT cells and A20 cells were stimulated with αGalCer or βGalCer (C12) for 24 h, and supernatants were evaluated for cytokine release (Fig. 7). IFN-γ, GM-CSF, and IL-4 production was strongly induced from NKT cells by αGalCer, but not βGalCer (C12), only when CD1d-transfected A20 cells were used. Only low levels of cytokine production were observed when transfected A20 cells were treated with βGalCer (C12) or when NK cells were stimulated with either αGalCer or βGalCer (C12). These results clearly show that presentation of αGalCer by CD1d is required for cytokine induction, while βGalCer (C12) is either not efficiently presented to NKT cells by CD1d or the quality of the signal is insufficient to induce cytokine production.

Additional studies were also done in BALB/c mice to evaluate the abilities of αGalCer and βGalCer to activate NKT cells with ceramide presented by autologous peritoneal monocytes. Fig. 8 shows a representative experiment where liver lymphocytes (32% NKT) were mixed 1:1 with protease-peptone-induced peritoneal exudate cells after loading for 1 h at 37°C with varying doses of αGalCer and βGalCer (C12). Similar to the results with the CD1d-transfected cell line, αGalCer rapidly induced activation and production of TNF-α, IFN-γ, IL-4, and IL-13, while βGalCer induced only low levels of TNF-α and no IFN-γ, IL-4, and IL-13. Both αGalCer and βGalCer (C12) were able to induce IL-5 from liver lymphocytes. Overall, these results show that the biological effects of βGalCer (C12) differ dramatically from those of αGalCer in the context of CD1d and macrophage-mediated presentation to NK cells.

βGalCer (C12) depletes NKT cells, but fails to induce rejection of allogeneic bone marrow

The accumulated results above demonstrate that βGalCer (C12) is efficiently recognized by NKT cells but does not effectively activate

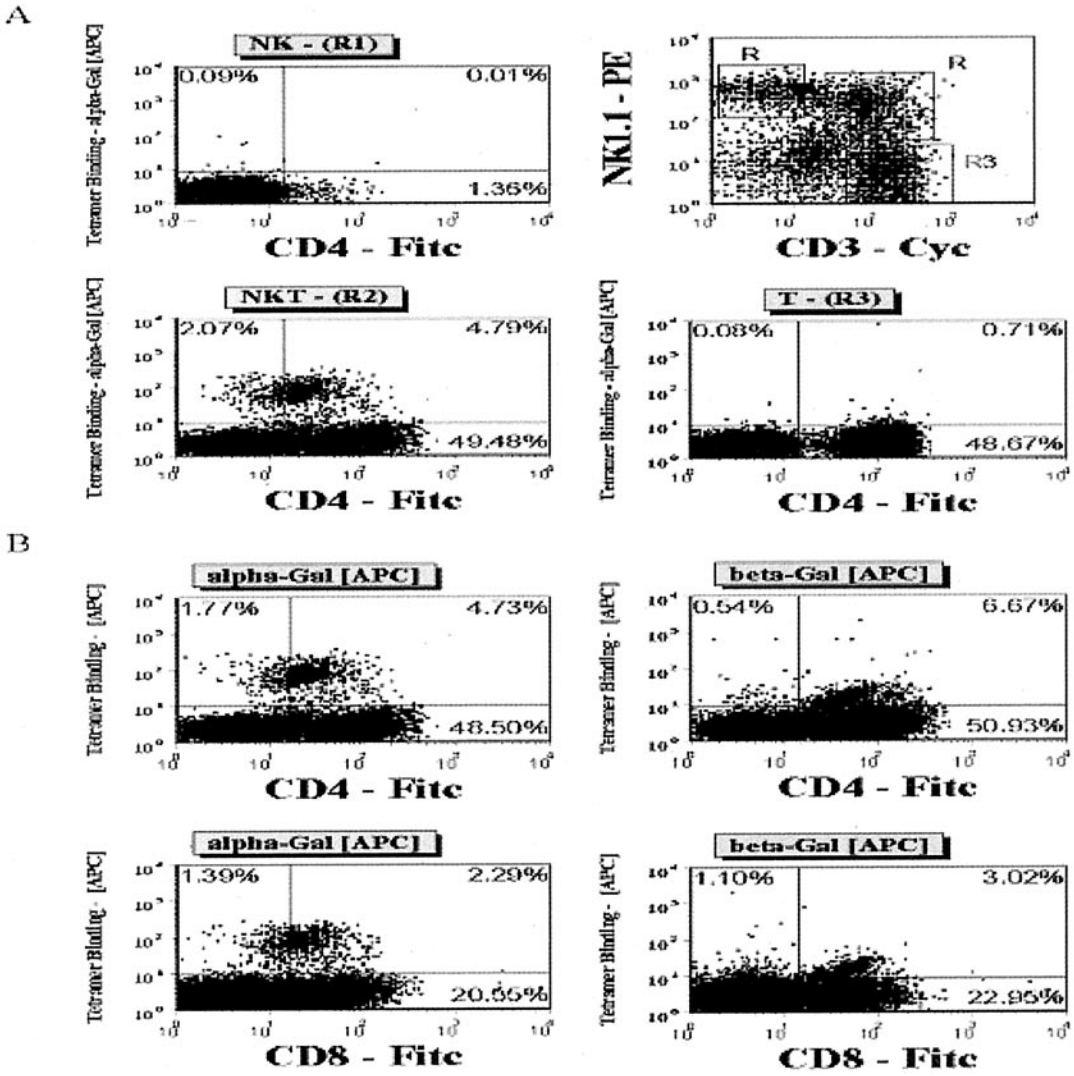

FIGURE 6. Liver-derived lymphocytes were isolated and fractionated on Percoll gradients, and CD1d tetramer binding was evaluated using four-color flow cytometry. *A* shows the expression of αGalCer-loaded tetramers on liver subsets as gated in the *upper right panel* using NK1.1-PE and CD3-cychrome. Binding of ~9% of liver $CD4^+$ NKT cells (*lower left*), which constituted 6.9% of total NKT cells, was observed. In contrast, no binding to NK cells (*upper left*) nor NK1-negative T cells (*lower right*) was observed. *B* compares the binding of αGalCer- and βGalCer-loaded tetramers to NKT cells that were gated similarly to *A* (cells were stained with NK1.1-PE, CD3-cychrome, and CD1d-tetramers loaded with ceramide and with either CD4-FITC or CD8-FITC).

NK cells. This conclusion implies that βGalCer (C12) should not effectively induce cytokines or NK-dependent biological effects in vivo at doses that effectively reduce the number of detectable NKT cells.

Because one of the most sensitive measures of NK activity in vivo is their ability to reject bone marrow, allogeneic transfer of C57BL/6 [H-2^b] marrow into BALB/c [H-2^d] mice was used as a model to confirm that βGalCer (C12) was unable to induce NK-mediated functions in vivo. BALB/c mice are known to exhibit only relatively weak bone marrow rejection, and thus this model can easily detect even a small amount of NK cell activation. The transfer of C57BL/6 bone marrow cells into allogeneic BALB/c mice was >50% rejected by day 3 (Fig. 9*A*). As expected, this event was shown to be NK mediated because prior depletion of NK cells using anti-asGM1 rabbit serum in the BALB/c recipient mice (which lack NK1.1 expression) reduces the degree of allogeneic graft rejection. The treatment of mice with αGalCer, which results in NK activation, further enhanced the rejection of the donor cells to ~90%. In contrast, treatment with βGalCer (C12) did not significantly enhance basal rejection. To determine whether clear dose-dependent differences could be established for these two agents, additional studies were performed. Results from these studies clearly demonstrated that αGalCer doses from 0.1 to 0.01 μg/mouse retained NK activating abilities (Fig. 9*B*), while βGalCer (C12) doses from 1 to 10 μg/mouse failed to induce any in vitro NK activation. The data shown in Fig. 9*C* confirm that both ceramides had the expected ability to reduce the detection of NKT cells at 24 h. Previous studies suggested that even doses of βGalCer (C12) ≥10 μg resulted in NKT depletion with minimal effects on NK cells. Figure 9*D* shows two experiments where several doses of αGalCer and βGalCer (C12) were evaluated for enhancement of marrow rejection. Whereas αGalCer can potently activate marrow rejection at a dose as low as 0.1 μg/mouse, βGalCer (C12) at a dose of 10 μg, which is effective at reducing the number of NKT cells, had no enhancing effect on bone marrow graft rejection. Interestingly, a very high dose of βGalCer (20 μg), which also had no appreciable NK enhancing effects (Fig. 3), did have some ability to enhance rejection of bone marrow cells suggesting

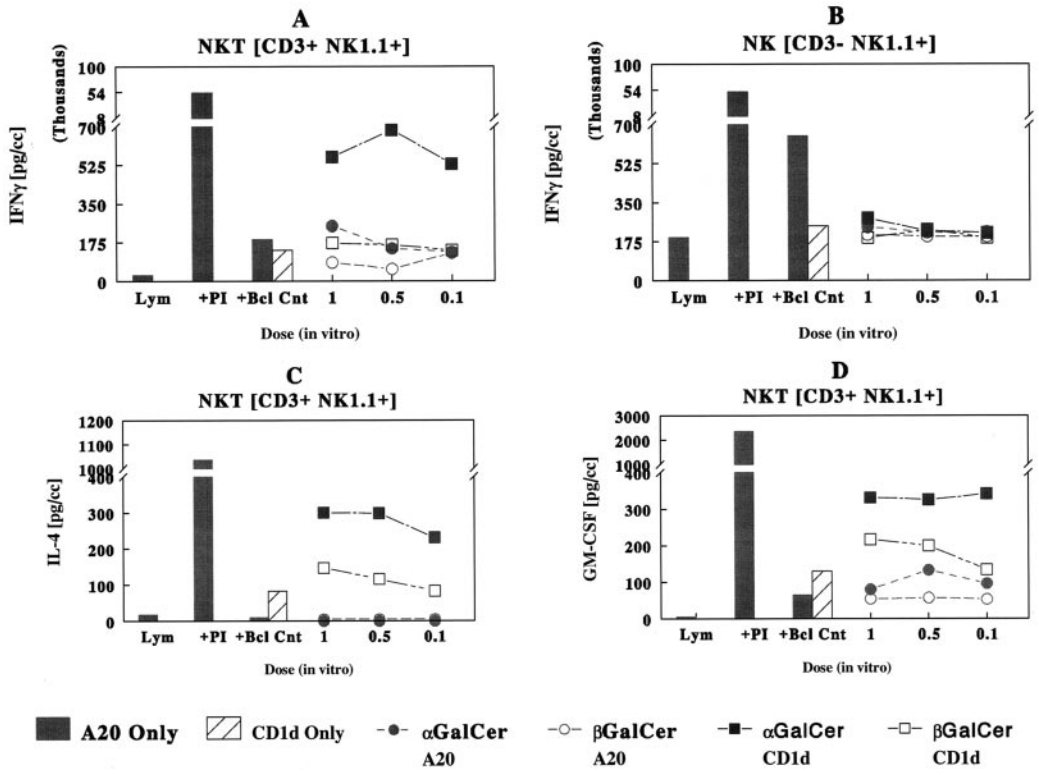

FIGURE 7. Highly purified NK (*B*) or NKT (*A*, *C*, and *D*) cells from C57BL/6 mice were evaluated for in vitro production of cytokines after treatment with A20 or A20/CD1d with various doses of either αGalCer or βGalCer. *A* and *B*, IFN-γ production in NKT and NK cells, respectively. *C*, IL-4; *D*, GM-CSF. Data representative of at least three experiments. SE of cytokine assays is <5%.

that at very high doses βGalCer (C12) can either induce enough augmentation of NK activity (see Table II) to mediate some effects in vivo or induce some biological effects through a non-NK-mediated mechanism. However, it is clear that in all studies a dose of 10 μg of βGalCer can be used in vivo to effectively reduce detectable NKT cells without appreciable NK-activating effects as assessed by NK number, activation stage, cytolytic activity, or very sensitive NK-dependent functions in vitro.

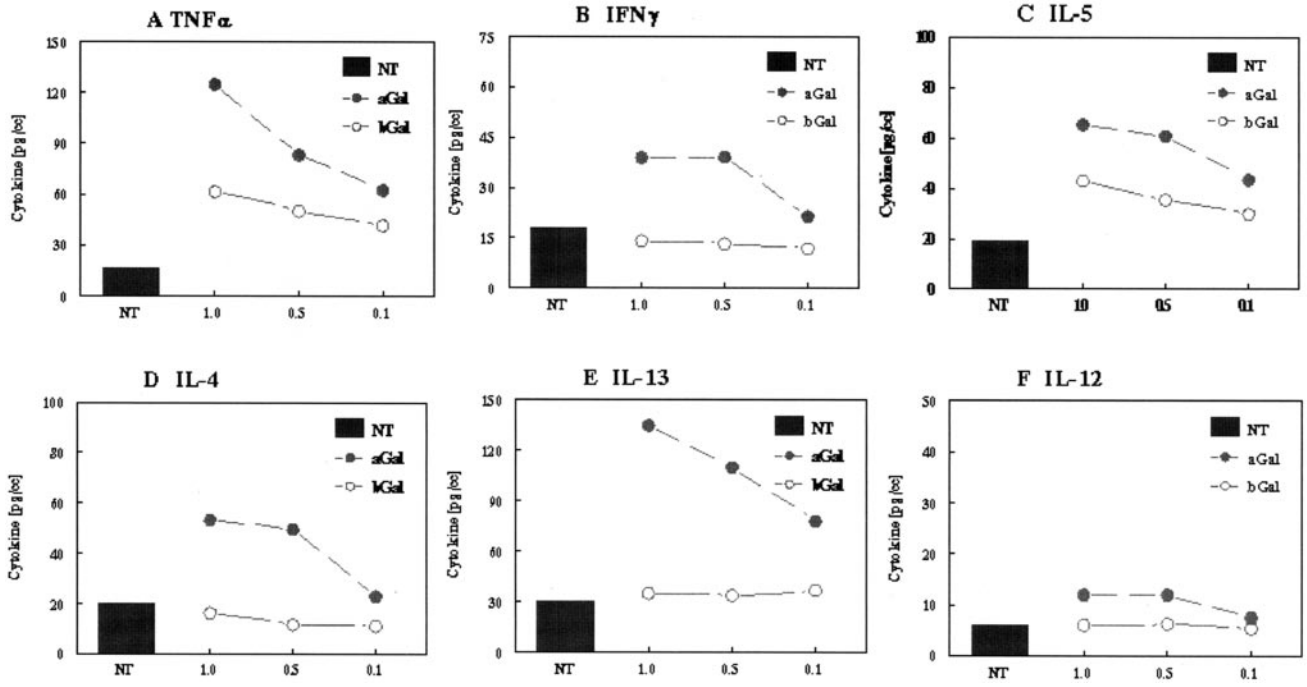

FIGURE 8. Purified liver lymphocyte cells were evaluated for in vitro production of cytokines after treatment of peritoneal macrophages with various doses (1.0, 0.5, and 0.1 μg/ml) of either αGalCer or βGalCer (C12). *A* (TNF-α), *B* (IFN-γ), *C* (IL-5), *D* (IL-4), *E* (IL-13), and *F*, (IL-12) show production of different cytokines after 6 h (or 24 h; data not shown) of coculture.

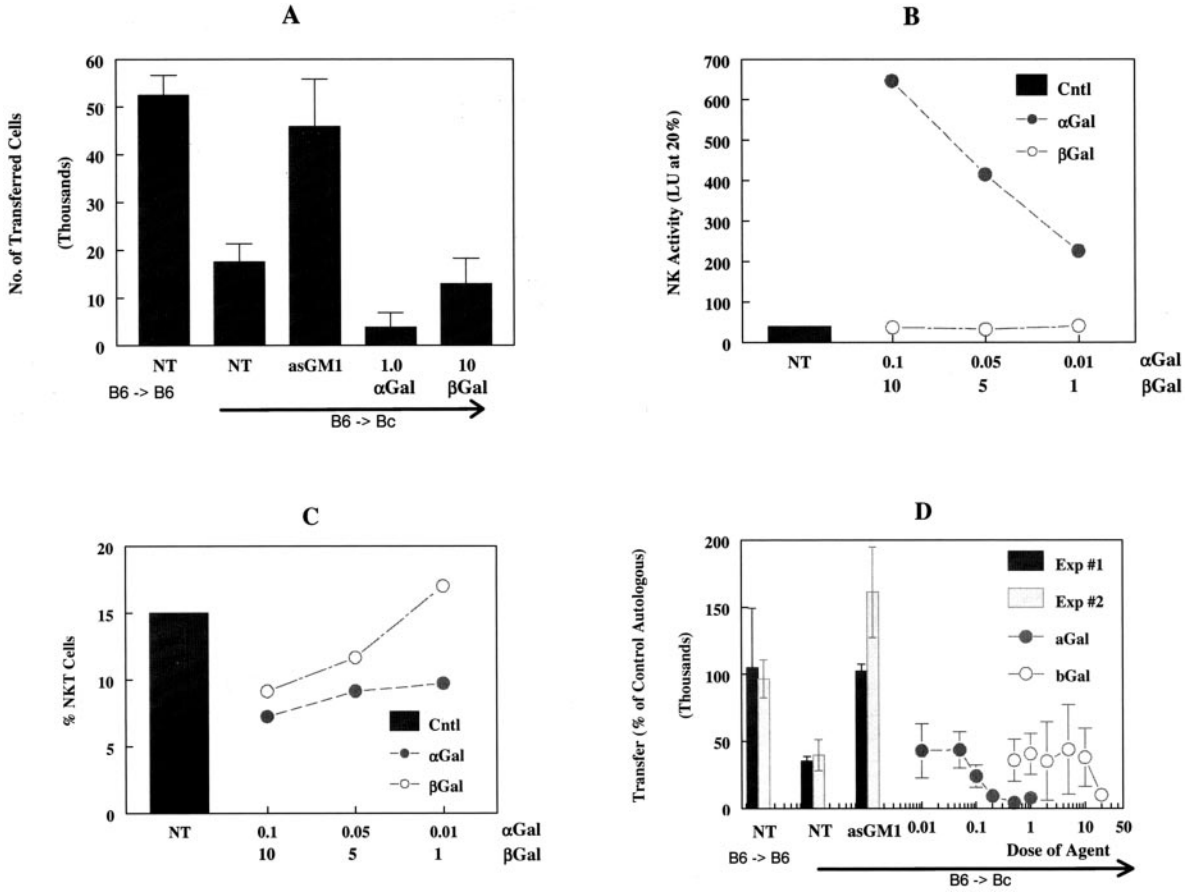

FIGURE 9. In vivo rejection of allogeneic bone marrow cells was examined in a model where C57BL/6 mice were used as bone marrow donors and BALB/c mice were used as recipients. *A*, The effect of pretreatment of mice with asGM1 (day −2) or αGalCer or βGalCer (day −3) before administration of 10×10^6 autologous or allogeneic bone marrow cells. Recipient mice were irradiated with either 800 rad (BALB/c) or 900 rad (C57BL/6). The in vitro effects are depicted in *B* (NK activity) and *C* (percent NKT cells) for mice treated with varying doses of αGalCer or βGalCer. *D*, Several experiments that examine the dose-dependent effects of αGalCer (○) and βGalCer (●) on marrow transfer. Solid and shaded bars represent control marrow transfer in autologous (B6-B6) or allogeneic (B6-BALB/c) models and with asGM1 treatment. Data representative of at least three experiments.

Overall, these results confirm that βGalCer (C12) does directly bind to NKT cells, but that differences in the intensity and subset affinity of this binding may contribute to its dramatically different in vitro and in vivo biological effects.

Discussion

NKT and NK cells are two critically important components of the innate immune system. These leukocyte subsets have distinct and complementary functions for therapeutic responses against microbes and tumors, and for some inappropriate responses to autoantigens (1, 3). To date, it has been difficult to clearly delineate the individual contributions of these subsets in these various disease states and to identify the precise mechanism by which they mediate diverse biological effects in vivo. In the current study, we have developed a new approach to dissociate direct effects on the NKT compartment from NK activation. This approach shows that both αGalCer and another somewhat different ceramide, βGalCer (C12), can efficiently increase apoptosis and decrease detection in vivo of a major subset of NKT cells that are reactive with ceramide-specific TCR components. However, unlike αGalCer, which also potently activates bystander NK cells and induces a storm of cytokines (IL-4, IFN-γ, GM-CSF, and TNF-α), doses of βGalCer (C12) that directly impact NKT cells fail to induce NK activation or cytokine production. Thus, βGalCer is a novel tool for efficiently perturbing functional NKT cells in vivo without additional, indirect host immune activation. The mechanistic basis for reduced NKT detection by αGalCer is not completely understood, although this effect does require interaction with the TCR (1, 3), which in turn results in some process that induces apoptosis in at least some cells (5, 17, 24, 25). It is also possible that at least some stimulated NKT cells might endocytose their NK1.1 and TCR as part of a process that results in apoptosis or that simply renders the cells nondetectable. This conclusion is consistent with recent report (26, 27) that endocytosis of NK1.1 on NKT cells follows finding of αGalCer. The studies by van Kaer (26) also indicated a rapid loss of detection and subsequent return of NKT cells after a 5-μg dose in vivo, a result that differs somewhat from results in our in vivo treatment regimen, where a more gradual return to "the norm" after 6–7 days (Fig. 1) was seen. Our studies have shown that both αGalCer and βGalCer (C12) induced a loss of detectable NKT cells and an increase in NKT cell apoptosis in the liver within 4–6 h. In contrast, in vitro stimulation studies showed that CD1d presentation of βGalCer does not result in the stimulation of NKT cells for direct or indirect production of cytokines, suggesting 1) qualitative differences in the signals induced in NKT cells by αGalCer vs βGalCer (C12) and 2) qualitative differences in the processes of apoptosis and cytokine production after stimulation of NKT cells through the TCR. In addition, CD1d tetramer binding was observed with both αGalCer and βGalCer, but a much weaker binding and different NKT subset affinity was seen with the βGalCer-loaded tetramers. Interestingly, weak binding of Ag to the TCR during thymic development is well established as an anergy/

death signal. Similar processes may be involved with βGalCer (C12) binding to peripheral NKT cells that trigger their loss of detection without potent downstream gene activation. Thus, the use of αGalCer vs βGalCer (C12) may provide a novel approach for studying the biological functions of NKT and NK populations, and perhaps NKT subsets, in vivo.

Although NKT cells represent a diverse population of lymphocytes, a great deal of information has been accumulated relating specifically to the immunological functions of the Vα14 and Vα24 NKT cell subsets. These cells have been identified as potent cytokine producers, and their appropriately controlled manipulation could be critical for initiating and sustaining immune responses against cancer or infectious diseases, or for modifying immunopathological responses. In this context, Hammond and Godfrey (7) recently summarized the implications of NKT cell function in diabetes, autoimmunity, sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and Sjogren's syndrome. The contributions of NKT cells to various types of beneficial and deleterious immune responses may be through production of cytokines and/or modification of the function of other cells such as NK cells. This NKT-NK interaction represents important cross-talk between various components of the innate immune system, and subtle disruptions of this cross-talk may have important qualitative or quantitative implications for the generation and persistence of adaptive immune responses. For example, nonobese diabetic mice have a deficiency in NKT and NK cell numbers and function and develop diabetes (4, 6, 8, 9, 11, 12, 15). The administration of αGalCer protects nonobese diabetic mice from development of diabetes, but the mechanism for this effect is unclear, because presentation of αGalCer in the context of CD1d directly activates NKT cells, and also indirectly stimulates NK cells. In the experimental autoimmune encephalomyelitis and multiple sclerosis models, SJL mice have a deficiency in NKT cells, but a recent report (28) has demonstrated that both 129/J and SJL mice also have NK signaling deficiencies in their activating Ly49 receptors that result in reduced cytokine production. Recent studies have shown that treatment of mice with αGalCer protects mice from experimental autoimmune encephalomyelitis (13, 14, 16). As shown in the present study, both αGalCer and βGalCer (C12) can reduce the number of detectable NKT cells, and thus any in vivo differences in their biological effects could be due to factors or bystander activation of NK cells that are stimulated only by αGalCer. A practical demonstration of the differential capabilities of these uniquely acting ceramides is illustrated in our studies by their disparate effects on bone marrow graft rejection.

The results of our studies with βGalCer (C12) suggest that this agent provides a unique approach for delineating key roles of NKT cells in settings where their presence is specifically required for some therapeutic or disease-causing effect. Conversely, in settings where NK cells play a unique role, the induction of beneficial effects by αGalCer (which activates NK cells) and the failure to induce such effects with βGalCer (which does not activate NK cells) would provide new insights into the roles of this cell subset in innate and adaptive immune responses.

Acknowledgments

We thank Anna Mason for her technical assistance, Susan Charbonneau for secretarial support, Mehrnoosh Abshari for flow cytometric sorting, and Tim Back, John Wine, and Erin Lincoln for their support in animal care and experimentation.

References

1. Kronenberg, M., and L. Gapin. 2002. The unconventional lifestyle of NKT cells. *Nat. Rev. Immunol. 2:557.*
2. Ortaldo, J. R., E. W. Bere, D. Hodge, and H. A. Young. 2001. Activating Ly-49 NK receptors: Central role in cytokine and chemokine production. *J. Immunol. 166:4994.*
3. 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.*
4. Baxter, A. G., S. J. Kinder, K. J. Hammond, R. Scollay, and D. I. Godfrey. 1997. Association between $\alpha\beta TCR^{+}CD4^{-}CD8^{-}$ T-cell deficiency and IDDM in NOD/Lt mice. *Diabetes 46:572.*
5. Eberl, G., and H. R. MacDonald. 1998. Rapid death and regeneration of NKT cells in anti-CD3ε- or IL-12-treated mice: a major role for bone marrow in NKT cell homeostasis. *Immunity 9:345.*
6. Falcone, M., B. Yeung, L. Tucker, E. Rodriguez, and N. Sarvetnick. 1999. A defect in interleukin 12-induced activation and interferon γ 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.*
7. Hammond, K. J., and D. I. Godfrey. 2002. NKT cells: potential targets for autoimmune disease therapy? *Tissue Antigens 59:353.*
8. Hong, S., M. T. Wilson, I. Serizawa, L. Wu, N. Singh, O. V. Naidenko, T. Miura, T. Haba, D. C. Scherer, J. Wei, et al. 2001. The natural killer T-cell ligand α-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat. Med. 7:1052.*
9. Naumov, Y. N., K. S. Bahjat, R. Gausling, R. Abraham, M. A. Exley, Y. Koezuka, S. B. Balk, J. L. Strominger, M. Clare-Salzer, and S. B. Wilson. 2001. Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets. *Proc. Natl. Acad. Sci. USA 98:13838.*
10. Oya, H., T. Kawamura, T. Shimizu, M. Bannai, H. Kawamura, M. Minagawa, H. Watanabe, K. Hatakeyama, and T. Abo. 2000. The differential effect of stress on natural killer T (NKT) and NK cell function. *Clin. Exp. Immunol. 121:384.*
11. Sharif, S., G. A. Arreaza, P. Zucker, Q. S. Mi, J. Sondhi, O. V. Naidenko, M. Kronenberg, Y. Koezuka, T. L. Delovitch, J. M. Gombert, et al. 2001. Activation of natural killer T cells by α-galactosylceramide treatment prevents the onset and recurrence of autoimmune type 1 diabetes. *Nat. Med. 7:1057.*
12. Hammond, K. J., 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.*
13. Jahng, A. W., I. Maricic, B. Pedersen, N. Burdin, O. Naidenko, M. Kronenberg, Y. Koezuka, and V. Kumar. 2001. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J. Exp. Med. 194:1789.*
14. Miyamoto, K., S. Miyake, and T. Yamamura. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature 413:531.*
15. Poulton, L. D., M. J. Smyth, C. G. Hawke, P. Silveira, D. Shepherd, O. V. Naidenko, D. I. Godfrey, and A. G. Baxter. 2001. Cytometric and functional analyses of NK and NKT cell deficiencies in NOD mice. *Int. Immunol. 13:887.*
16. Singh, A. K., M. T. Wilson, S. Hong, D. Olivares-Villagomez, C. Du, A. K. Stanic, S. Joyce, S. Sriram, Y. Koezuka, and L. Van Kaer. 2001. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J. Exp. Med. 194:1801.*
17. Leite-de-Moraes, M. C., A. Herbelin, C. Gouarin, Y. Koezuka, E. Schneider, and M. Dy. 2000. Fas/Fas ligand interactions promote activation-induced cell death of NK T lymphocytes. *J. Immunol. 165:4367.*
18. Fortier, A. H., and L. A. Falk. 1995. Isolation of murine macrophages. In *Current Protocols in Immunology.* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds. Wiley, New York, p. 14.1.1.
19. Oberg, L., S. Johansson, J. Michaëlsson, P. Hoglund, and K. Kärre. 2002. A novel in vivo assay for NK cell mediated elimination of spleen and bone marrow cells. In *19th International Natural Killer Cell Workshop, October 5–8.* San Juan, Puerto Rico.
20. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods 171:131.*
21. Matsuda, J. L., O. V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C. R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med. 192:741.*
22. Smyth, M. J., N. Y. Crowe, D. G. Pellicci, K. Kyparissoudis, J. M. Kelly, K. Takeda, H. Yagita, and D. I. Godfrey. 2002. Sequential production of interferon-γ by $NK1.1^{+}$ T cells and natural killer cells is essential for the antimetastatic effect of α-galactosylceramide. *Blood 99:1259.*
23. Smyth, M. J., E. Cretney, K. Takeda, R. H. Wiltrout, L. M. Sedger, N. Kayagaki, H. Yagita, and K. Okumura. 2001. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon γ-dependent natural killer cell protection from tumor metastasis. *J. Exp. Med. 193:661.*
24. Osman, Y., T. Kawamura, T. Naito, K. Takeda, L. Van Kaer, K. Okumura, and T. Abo. 2000. Activation of hepatic NKT cells and subsequent liver injury following administration of α-galactosylceramide. *Eur. J. Immunol. 30:1919.*
25. Hayakawa, Y., K. Takeda, H. Yagita, S. Kakuta, Y. Iwakura, L. Van Kaer, I. Saiki, and K. Okumura. 2001. Critical contribution of IFN-γ and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of α-galactosylceramide. *Eur. J. Immunol. 31:1720.*
26. Wilson, M. T., C. Johansson, D. Olivares-Villagomez, A. K. Singh, A. K. Stanic, C. R. Wang, S. Joyce, M. J. Wick, and L. Van Kaer. 2003. The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion. *Proc. Natl. Acad. Sci. USA 100:10913.*
27. Crowe, N. Y., A. P. Uldrich, K. Kyparissoudis, K. J. L. Hammond, Y. Hayakawa, S. Sidobre, R. Keating, M. Kronenberg, and M. J. Smyth. 2003. Glycolipid antigen drives rapid expansion and sustained cytokine production by NK T cells. *J. Immunol. 171:4020.*
28. McVicar, D. W., R. Winkler-Pickett, L. S. Taylor, A. Makrigiannis, M. Bennett, S. K. Anderson, and J. R. Ortaldo. 2002. Aberrant DAP12 signaling in the 129 strain of mice: implications for the analysis of gene-targeted mice. *J. Immunol. 169:1721.*