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NKT Cell Stimulation with Glycolipid Antigen In Vivo: Costimulation-Dependent Expansion, Bim-Dependent Contraction, and Hyporesponsiveness to Further Antigenic Challenge

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Activation of NKT cells using the glycolipid α-galactosylceramide (α-GalCer) has availed many investigations into their immunoregulatory and therapeutic potential. However, it remains unclear how they respond to stimulation in vivo, which costimulatory pathways are important, and what factors (e.g., Ag availability and activation-induced cell death) limit their response. We have explored these questions in the context of an in vivo model of NKT cell dynamics spanning activation, population expansion, and subsequent contraction. Neither the B7/CD28 nor the CD40/CD40L costimulatory pathway was necessary for cytokine production by activated NKT cells, either early (2 h) or late (3 days) after initial stimulation, but both pathways were necessary for normal proliferative expansion of NKT cells in vivo. The proapoptotic Bcl-2 family member Bim was necessary for normal contraction of the NKT cell population between days 3–9 after stimulation, suggesting that the pool size is regulated by apoptotic death, similar to that of conventional T cells. Ag availability was not the limiting factor for NKT cell expansion in vivo, and a second α-GalCer injection induced a very blunted response, whereby cytokine production was reduced and further expansion did not occur. This appeared to be a form of anergy that was intrinsic to NKT cells and was not associated with inhibitory NK receptor signaling. Furthermore, NKT cells from mice prechallenged with α-GalCer in vivo showed little cytokine production and reduced proliferation in vitro. In summary, this study significantly enhances our understanding of how NKT cells respond to primary and secondary antigenic challenge in vivo.

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The best-defined and most commonly used agonist ligand for NKT cells is α-galactosylceramide (α-GalCer), a glycolipid originally isolated from a marine sponge (4, 5). α-GalCer has very potent and specific effects on NKT cells, including the induction of rapid cytokine production and proliferation and, as such, can affect a large number of experimental diseases in an NKT cell-dependent fashion, including autoimmunity and cancer (2–4). Based on these studies, the therapeutic potential of α-GalCer is being studied in clinical trials with cancer patients (6, 7).

Within 3–12 h of stimulation with α-GalCer in vivo, TCRα+ NK1.1+ NKT cells are no longer detectable in peripheral lymphoid organs, reappearing 24–48 h later. Their (apparent) disappearance was originally attributed to activation-induced cell death, although this also occurred in mice lacking the proapoptotic Bcl-2 family member Bim (8), which controls death of conventional T cells and contraction of the T cell pool after Ag-induced expansion (9, 10). At least three separate studies have shown that this disappearance is mostly due to transient TCR and NK1.1 down-regulation (8, 11, 12). By day 3, NKT cells expand to up to 10 times their normal number in vivo, with ongoing production of IFN-γ, before contracting to normal levels between 6 and 9 days later. The factors that regulate the cytokine production, expansion, and subsequent contraction of the NKT cell population are unknown, but several studies have suggested roles for CD28 and...
CD40 pathways in this process (13–16). This remains controversial, with at least one paper showing that the initial burst of cytokines from NKT cells in response to α-GalCer is independent of these costimulatory factors (17). The mechanisms underlying the contraction phase that occurs between days 3 and 9 are unknown, but Ag depletion and/or programmed Ag-induced cell death are two likely possibilities.

Therapeutic use of α-GalCer in mouse models typically involves two or more treatments with this reagent, although the effects of secondary treatments on NKT cell expansion and NKT cell-derived cytokine production in vivo are unclear. At least two studies have examined cytokine production by NKT cells after α-GalCer rechallenge; however, these provided conflicting results (17, 18). One study found that despite a bias toward increased IL-4 production with no detectable IFN-γ in serum, NKT cells were producing both IFN-γ and IL-4 after rechallenge in vivo (17), whereas the other found that NKT cells were anergic upon re-stimulation, based on in vitro rechallenge after primary stimulation with soluble α-GalCer in vivo (18). When chronic α-GalCer stimulation was used (weekly injections for 8 wk), this depleted peripheral NKT cells, which were gradually replaced in a thymus-dependent manner; however, the new NKT cells were severely hypo-responsive to further stimulation due to increased expression of inhibitory receptors that appeared to be imprinted during their intrathymic development (19). A more recent study (20) showed that up-regulation of the inhibitory receptor CD94/NKG2A on peripheral NKT cells, and Qa1+ on APCs, occurred even after only a single injection of α-GalCer in vivo and provided data showing that this interaction was responsible for impaired responsiveness to a second challenge with α-GalCer in vivo.

Stimulation of NKT cells is known to induce bystander cytokine production and proliferation of other cells, including NK, T, B, and dendritic cells (DC) (14, 21–25). NK cells appear to be the main source of serum IFN-γ after α-GalCer stimulation in vivo (26), and IFN-γ production by NK cells downstream of NKT cell activation is essential for α-GalCer-mediated tumor rejection (27). For this reason, studies that have examined the NKT cell response via cytokine levels or proliferation in unfractionated cell cultures, or serum cytokine levels cannot clearly distinguish between direct and indirect consequences of NKT cell stimulation. The aim of this study was to understand the factors that regulate the NKT cell response to α-GalCer in vivo, using CD1d/α-GalCer tetramers to specifically examine NKT cells by flow cytometry and directly assess the responses of these cells to primary and secondary challenges and the importance of the CD28 and CD40 costimulatory pathways in NKT cell activation, early and late stage cytokine production, and expansion. We also examined whether NKT cell activation and expansion are limited by Ag availability, whether contraction of the NKT cell pool to prestimulation levels is Bim dependent, as it is for conventional T cells, and whether the expansion or cytokine response from previously challenged NKT cells is different from that of naive NKT cells upon re-stimulation.

Materials and Methods

**Mice**

Inbred C57BL/6J (B6), B6.TAP-1-deficient (TAP-1−/−), B6.CD40L-deficient (CD40L−/−) (28), B6.CTLA-4tg transgenic (CTLA-4tg16) (29), and B6.Bim-deficient (Bim−/−) mice (30) were obtained from Department of Microbiology and Immunology Animal House, University of Melbourne, or the Walter and Eliza Hall Institute Central Animal House. Gene-targeted mice were generated on the 129b background and backcrossed ≥10 generations to B6 before use. CTLA-4tg16 mice were generated on the B6l background and backcrossed eight times to B6. All mice were between 6 and 9 wk of age and were housed under specific pathogen-free conditions.

**Culture medium**

Culture medium contained RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS (JRH), 100 U/ml penicillin (Invitrogen Life Technologies), 100 µg/ml streptomycin (Invitrogen Life Technologies), 2 mM Glutamax (Invitrogen Life Technologies), 1 mM sodium pyruvate (Invitrogen Life Technologies), 50 µM 2-5ME (Sigma-Aldrich), 0.1 mM nonessential amino acids (Invitrogen Life Technologies), and 15 mM HEPES buffer (Invitrogen Life Technologies).

**Abb and flow cytometry**

Thymus as well as spleen cells and liver lymphocyte isolation were performed as previously described (31). Cells were counted using an automated cell counter (Z Series Dual; Coulter Electronics) or a hemocytometer. To avoid nonspecific binding of Abs to FcγRs, hybridoma supernatant containing anti-mouse CD16/32 mAb (2.4G2; grown in-house) was included in cell labeling experiments. Cells were stained with FITC-conjugated NK1.1 (PK136), CD4 (RM4-5), Ly49C/I (5E6), NKG2A/C/E (2D05), or TCR-β (H57-597); PE-conjugated CD4 (RM4-5) or NKG2D (CX5; BioScience); PerCP-conjugated CD4 (RM4-5), allopolycoycyanin-conjugated TCR-β (H57-597) or Ly49G2 (4D11); biotin-conjugated CD28 (37.51), CD94 (18d3), Fas ligand/CD178 (MFL3), Ly49A (A1), NK1.1 (PK136), or NKG2A (all from BD Pharmingen unless otherwise specified), or PE-Cy5–, PE-Cy7–, or PE-Cy5–/7–conjugated mouse α-GalCer-loaded CD1d tetramers (produced in-house as previously described (32), using recombinant baculovirus encoding histamine-tagged mouse CD1d and mouse β2-microglobulin, provided by Dr. M. Kronenberg’s laboratory, La Jolla Institute for Allergy and Immunology, San Diego, CA). Biotinylated Abs were detected using streptavidin-Alexa Fluor 488 (Molecular Probes) or streptavidin conjugated to PerCP, PE-Cy5, or PE-Cy7 (BD Pharmingen). Stained cells were analyzed using a FACS Calibur, LSRII, or FACSAria flow cytometer (BD Biosciences), and data were processed with either CellQuest (BD Biosciences) or FlowJo (Tree Star) software. For B cell depletion, single-cell suspensions were stained with anti-B220 (RA3.6B2; grown in-house), washed, and incubated with sheep anti-rat IgG-conjugated immunomagnetic beads (Dynal Biotech). Bead-labeled cells were magnetically removed according to the supplier’s instructions, and the remaining cells were surface labeled for flow cytometric sorting. Multicolor sorting was performed using a FACS Aria, with purities consistently >95%. Where necessary, digital data were presented using biexponential scaling to resolve on-axis events.

**Intracellular cytokine staining**

After isolation from the liver or spleen, lymphocytes were cultured for 2 h in 2.0 µM monensin (GolgiStop; BD Biosciences) or 10 µg/ml brefeldin A without in vitro stimulation. After cell surface Ab labeling, lymphocytes were fixed once before fixing in 0.5% paraformaldehyde (BDH Chemicals) in the dark for 30 min at room temperature. Cells were then washed twice in FACS buffer before incubation with PE-conjugated Abs to IFN-γ (XM1G2.1) or IL-4 (11B11) or with an IgG1 isotype control (R3-34). Intracellular staining was performed in FACS buffer containing 0.05% saponin (Sigma-Aldrich) for 1 h at room temperature in the dark. In some experiments, the BD Biosciences intracellular staining kit with GolgiStop was used according to the instructions.

**In vivo treatment with α-GalCer**

α-GalCer was provided by the Pharmaceutical Research Division of Kirin Brewery and was prepared in saline supplemented with 0.5% (w/v) polysorbate-20. Control vehicle saline was also supplemented with 0.5% (w/v) polysorbate-20. Mice were injected i.p. with 2 µg of α-GalCer or control vehicle.

**In vitro α-GalCer-induced NKT cell proliferation**

After isolation, 4 × 107 spleen lymphocytes from naive or α-GalCer-pre-treated mice (2 µg/mouse injected i.p. 2 mo, 1 mo, or 2 wk previously) were CFSE-labeled in 1 ml of PBS with 16 µl of CFSE at 0.1 mM in 2 ml of PBS with 0.1% BSA for 10 min at 37°C with gentle mixing. Cells were washed twice with 5 ml of culture medium containing 20% FCS, resuspended in fresh culture medium, and cultured in the presence of α-GalCer (ranging from 1 to 500 ng/ml in different experiments) with or without IL-2 (50 U/ml). At the end of the first day, cells were washed by spinning through an underlay of FCS and were resuspended in fresh culture medium without α-GalCer, but with IL-2 in appropriate wells. This was designed to allow TCR re-expression in the absence of continued Ag exposure over the next
2.5 days of culture, thus permitting identification of NKT cells by CD1d tetramer staining. After the total 3.5-day culture, cells were harvested, counted, and analyzed by flow cytometry.

Results

**CD28 and CD40 signaling pathways are essential for expansion of the NKT cell pool, but not for cytokine production**

The influence of CD28 and CD40 signaling on NKT cell stimulation is controversial, with one study (13) showing that both factors are necessary for NKT cell activation, whereas another (17) showed that NKT cell activation proceeds normally in the absence of these factors. These studies were performed before the realization that NKT cells do not die after α-GalCer stimulation in vivo; but, instead, transiently down-regulate their TCR and NK1.1 markers, undergo significant expansion, and display sustained cytokine production for up to 3 days after treatment (8, 11, 12). It was therefore important to revisit these questions and investigate the involvement of CD40 and CD28 signaling pathways in early (2 h) and late (3–6 days) phases of α-GalCer-induced NKT cell activation in vivo. CD40L−/− and CTLA-4IgTg mice (that secrete soluble CTLA-4-Ig and effectively inhibit the B7/CD28 signaling pathway in vivo (29, 33)) were injected i.p. with α-GalCer and examined between 0 and 6 days after treatment for NKT cell-derived cytokine production and expansion. NKT cells from CD40L−/− and CTLA-4IgTg mice were indistinguishable from those from wild-type (WT) mice in their ability to produce IFN-γ and IL-4, labeled at both 2 h and 3 days after treatment (Fig. 1, A and B). This demonstrates that these signaling pathways are not critical for either the early or late phase of NKT cell-derived cytokine production. In contrast, both groups of mice, particularly CTLA-4IgTg mice, displayed reduced numbers and frequency of NKT cells in both the liver and spleen compared with WT mice (Fig. 1C), demonstrating an important role for CD28 and CD40 signaling in NKT cell expansion. CTLA-4IgTg mice also had lower total liver cell counts compared with WT and CD40L−/− mice (Fig. 1C), suggesting that a CD28-dependent process is necessary for intrahepatic recruitment or proliferation of bystander cells; however, this effect was not observed in the spleen. The transient down-regulation of NK1.1 on activated NKT cells that has been previously reported (8, 11) was observed in each group of mice (Fig. 1C). Taken together, these data suggest that some aspects of NKT cell activation, such as cytokine production, can occur independently of the CD28/CD40 pathway, but both costimulatory factors are required for optimal NKT cell expansion after stimulation.

**Contraction of the NKT cell pool requires the proapoptotic Bel-2 family member Bim**

After α-GalCer-induced expansion (8, 11, 12), NKT cell numbers return to pretreatment levels within 6–9 days after stimulation (8, 12). Upon immunization or challenge with a pathogen, conventional Ag-specific T cells also undergo a period of expansion before contracting to prestimulation levels. The death of conventional T cells during shut-down of an immune response is known to be under the control of the BH3-only protein, Bim (9, 10), a proapoptotic factor important in the mitochondrial pathway of apoptosis (30). To investigate whether contraction of the NKT cell pool was the result of a similar cell death pathway, Bim-deficient mice were injected with α-GalCer and examined for expansion and contraction of the NKT cell pool. NKT cells expanded similarly in WT and Bim−/− mice, but contraction of the NKT cell pool was significantly delayed, in both spleen and liver, until at least 9 days after treatment in Bim−/− mice (Fig. 2).

α-GalCer rechallenge induces NKT cell-derived cytokines but not NKT cell expansion

To assess the ability of Ag-experienced NKT cells to respond to further TCR-mediated stimuli, α-GalCer-challenged mice were re-injected with α-GalCer on day 3, at the peak of NKT cell expansion when they are still producing IFN-γ, but no detectable IL-4, or on day 12, when NKT cell numbers have contracted and cytokine production has ceased. Rechallenge on either day 3 or 12 induced a second, albeit reduced, burst of cytokine production within 2 h (Fig. 3, A and B), and the response at 12 days was similar (Fig. 3B). Rechallenge on day 3 appeared to transiently sustain NKT cell numbers in the spleen, but rechallenge on day 12, after the NKT cell pool had contracted to normal size, failed to induce any additional expansion of the NKT cell pool in either spleen or liver (Fig. 3C), demonstrating that Ag-experienced NKT cells are refractory to secondary α-GalCer-induced expansion. In contrast to primary stimulation, rechallenge on either day 3 or 12 also caused less TCR down-regulation (data not shown). Collectively, these data demonstrate that Ag-experienced NKT cells and/or their progeny are less responsive to antigenic stimulation with α-GalCer than naive NKT cells in vivo despite expressing apparently normal TCR levels. This is the first study to directly examine NKT cell numbers after antigenic rechallenge in vivo.

**Reduced NKT cell responsiveness is not associated with up-regulation of NK cell inhibitory receptors**

Chronic α-GalCer stimulation (2 μg i.p. weekly for 8 wk), followed by 8-wk rest without additional stimulation, results in depletion and thymus-dependent repopulation of the NKT cell pool by NKT cells with increased expression of inhibitory receptors (19). Indeed, this appeared to be the main reason underlying the nonresponsiveness (anergy) of chronically challenged NKT cells, because TAP-1−/− APCs (which lack the MHC class I molecules that are ligands for these inhibitory receptors, but retain CD1d expression) were able to stimulate a full response from the anergized NKT cells. A recent report also suggested that NK2GΔA up-regulation occurred after even a single injection of α-GalCer, and that ligation of this inhibitory receptor was responsible for hyporesponsiveness to a second α-GalCer challenge (20). Therefore, we examined a range of stimulatory and inhibitory NK receptors as well as other relevant receptors on splenic and thymic NKT cells at various times after a single injection of α-GalCer in vivo. Surprisingly, we found little/no evidence of inhibitory receptor up-regulation at any time point tested (including days 3, 6, 14 (Fig. 4), and 18 (data not shown)). Moreover, NK2GΔA expression was undetectable on spleen NKT cells (supported by staining with three different Abs: NK2GΔA, NK2GΔA/C/E, and CD94) at each of these time points, and likewise, the stimulatory receptor NK2GΔD was also down-regulated on these NKT cells. Expression of other receptors, including Ly49D, Ly49F, CD152 (CTLA-4), and CD154 (CD40L) by NKT cells was unaffected by α-GalCer challenge, and none of these markers showed altered expression on conventional (CD1d tetramer-negative) αβ TCR+ T cells after α-GalCer stimulation (data not shown). To test the involvement of inhibitory NK receptors in NKT cell anergy, splenic NKT cells were purified from naive or 7-day α-GalCer-pretreated WT mice and cocultured in vitro with WT or TAP-1−/−-derived DCs for 12 h with or without 200 ng/ml α-GalCer. Because TAP-1−/− mice lack the ligands for Ly49 receptors and NK2GΔA/C/E (MHC class I (34) and the Qa1β-associated nonameric peptide Qdm (35, 36), respectively), but not CD1d, they serve as an ideal tool for
investigating the involvement of these receptors in NKT cell anergy. Cultures of pretreated splenic NKT cells and WT DCs produced lower levels of IFN-γ compared with naive NKT cells, and this effect was not reversed by the use of TAP-1/−/− DCs (Fig. 5).

Reduced NKT cell proliferation after in vitro restimulation following in vivo challenge

Unfractionated splenocytes were isolated from naive or α-GalCer-pretreated mice (2 wk, 1 mo, or 2 mo previously), labeled with...
FIGURE 2. Contraction of the NKT cell pool is Bim dependent. Mice (WT and Bim^{−/−}) were injected i.p. with 2 μg of α-GalCer and examined between 3 and 9 days later. Liver- and spleen-derived lymphocytes were isolated and stained with CD1d/α-GalCer tetramer, anti-αβTCR, and NK1.1 mAbs and analyzed for the percentage of CD1d/α-GalCer tetramer-binding αβTCR⁺ NKT cells, total lymphocyte number, and total NKT cell number in both spleen (top row) and liver (bottom row). Data are derived from two independent experiments with a total of: WT: control (PBS injected), five mice; 3 days, five mice; 6 days, five mice; 9 days, five mice; and Bim^{−/−}: control (PBS injected), five mice; 3 days, five mice; 6 days, six mice; 9 days, five mice. Control (PBS-injected) mice were harvested at each time point to control for unstimulated NKT cell status. Error bars represent the SEM.

FIGURE 3. The NKT cell response to α-GalCer rechallenge in vivo. Mice (WT) were injected i.p. with 2 μg of α-GalCer and re injected on day 3 or 12 after primary challenge. Liver- and spleen-derived lymphocytes were isolated and cultured for 2 h in brefeldin A; surface-labeled with CD1d/α-GalCer tetramer or anti-αβTCR, CD4, or NK1.1 mAbs; and then fixed and permeabilized for intracellular staining with mAbs to IL-4 or IFN-γ. CD1d/α-GalCer tetramer αβTCR⁺ NKT cells were gated and analyzed for their expression of IFN-γ or IL-4, as shown in A with representative data from liver NKT cells after a 3- or 12-day rechallenge experiment. Accumulated cytokine data from day 3 and 12 rechallenge experiments are shown in B. Total NKT cell numbers at each time point are shown in C. Data were derived from three to five mice per group. Control (C; PBS-injected) mice were harvested at each time point to control for unstimulated NKT cell status. Error bars represent the SEM. Arrows indicate the time of α-GalCer injection.
CFSE, and cultured for 3–4 days to test their proliferative response to H9251-GalCer-mediated stimulation in vitro. The data in Fig. 6 show a comparison of NKT cells from naive and 2-wk pretreated mice. NKT cells from naive mice, cultured in the presence of H9251-GalCer, proliferated more extensively (as indicated by CFSE dilution) than NKT cells from mice prechallenged in vivo. A previous study suggested that IL-2 in combination with H9251-GalCer in vitro could enhance the number of IFN-γ-producing splenocytes from mice prechallenged in vivo (18); however, it was not clear from this study exactly how this affected the specific NKT cell response. For example, did this reflect an increase in NKT cell number, NKT cell-derived IFN-γ production, or IFN-γ production from bystander cells? Using the CD1d/H9251-GalCer tetramer to specifically identify NKT cells, we found that IL-2 overcame the impaired proliferative response of NKT cells prechallenged in vivo, resulting in a similar level of CFSE dilution to that seen for naive NKT cells (Fig. 6).

Reduced NKT cell cytokine production after α-GalCer rechallenge in vitro

Lymphocytes were harvested from livers of naive or α-GalCer-pretreated mice (2 wk, 1 mo, and 2 mo previously) and stimulated in vitro in the presence of α-GalCer with or without IL-2, IL-12 plus IL-18, PMA/ionomycin, or anti-CD3 with or without IL-2 to examine cytokine production by intracellular cytokine staining. Data from a 1-mo prechallenge experiment are shown in Fig. 7 and were similar to those observed from the 2 wk and 2 mo prechallenge groups (data not shown). As expected, naive NKT cells produced both IFN-γ and IL-4 in response to α-GalCer stimulation, whereas the response of NKT cells from α-GalCer-prechallenged mice was barely detectable. Although IL-2 enhanced the proliferative response of prechallenged NKT cells (Fig. 6), it had no apparent effect on the cytokine response by these NKT cells in the presence of α-GalCer. In contrast, simultaneous stimulation with IL-12 plus IL-18 in the absence of α-GalCer, a combination that drives potent IFN-γ production by NKT cells (Fig. 7) (37) triggered a partial, but still reduced, IFN-γ response from these in vivo prechallenged NKT cells. PMA/ionomycin stimulation drove strong production of IFN-γ and IL-4 regardless of whether the NKT cells were from naive or prechallenged mice. This in vitro hyporesponsiveness was unlikely to be the result of impaired APC function in prechallenged mice, because similar hyporesponsiveness was observed when plate-bound anti-CD3 was used to stimulate the NKT cells (Fig. 7). This supports our data, shown in Fig. 5, demonstrating that anergy is intrinsic to the NKT cells from α-GalCer-prechallenged mice and cannot be overcome when DC from naive mice are used as stimulators.

Discussion

Until recently, NKT cells were thought to respond very rapidly to Ag-mediated stimulation in vivo, releasing a burst of cytokines and dying within hours due to activation-induced cell death (38). We and others recently published a revised model of NKT cell dynamics after activation in vivo, which postulates that these cells do not die rapidly upon stimulation, but, instead, proliferate within 3 days to reach levels up to 10 times their steady-state numbers, before contracting to prestimulation levels by 6–9 days (8, 11, 12).

**FIGURE 4.** Modulation of cell surface receptors on NKT cells after α-GalCer stimulation in vivo. Mice (WT) were injected i.p. with 2 μg of α-GalCer on day 0 and examined 3, 6, or 14 days later. Spleen- and thymus-derived lymphocytes were isolated and stained with CD1d/α-GalCer tetramer plus anti-αβTCR (*first column*), and NKT cells were electronically gated, as shown, and examined for expression of NK1.1, NK22A/C/E, CD94, Ly49A/C/G2/I, Fas ligand (CD178), and CD28 (□), overlaid onto relevant isotype controls (□). Controls (no injection), three mice; day 3, two mice; day 6, three mice; day 14, three mice.
showed that they were required for the characteristic rapid and extensive expansion of the NKT cell pool in vivo. Because CD80 (CD28 ligand) up-regulation on APCs follows CD40 ligation, it is reassuring that both CD40L−/− mice and CTLA-4IgTg groups of mice displayed similar phenotypes with impaired NKT cell expansion after α-GalCer stimulation in each. The results from these experiments are consistent with the idea that NKT cells themselves can be activated without requiring costimulation via CD28, whereas the production of downstream factors (e.g., IL-12) from bystander cells (e.g., DC) may be necessary for a fully developed NKT cell response (14, 40). We cannot exclude the possibility, however, that other NKT cell intrinsic factors not measured in this study may depend on signaling via the CD40/CD28 pathways, and these may be directly involved in the NKT cell proliferative response, or that some residual B7 function might exist in these mice despite the excess CTLA-4Ig protein present in the serum. It should be added that although CD28 signaling may not be necessary for the normal α-GalCer-induced, NKT cell-derived cytokine response in vivo, it does not necessarily mean that NKT cells are unresponsive to CD28. Indeed, CD28 signaling appears to override MHC class I-mediated inhibition of constitutive NKT cell cytokine production (in the absence of exogenously added Ag) in vitro (16). Moreover, CD28 costimulation was found to enhance NKT cell-derived cytokine production in response to in vitro treatment with anti-CD3 Abs (41). Regardless of which stage of the NKT cell response CD28 and CD40 pathways influence, these factors are likely to play an important role in many α-GalCer-induced responses where disease resolution depends upon increased NKT cell number and/or downstream activation and cytokine production by effector cells (13).

An interesting problem was whether Ag depletion was a key factor that limited NKT cell expansion and/or whether this was responsible for the subsequent contraction of the NKT cell pool to prestimulation levels. The observation that reinjection of α-GalCer 3 and 12 days after primary challenge failed to trigger a second wave of expansion strongly suggested that the Ag availability was not a key limiting factor, but, rather, that the expansion/contraction dynamics of the NKT cell pool were a programmed response. Our results also show that the contraction phase of NKT cells involves a Bim-dependent apoptotic process, which aligned this stage of the NKT cell response with that of conventional T cells that also undergo Bim-dependent apoptosis during shut-down of an immune response (9, 10); however, the partial and delayed decline in NKT cell number in Bim−/− mice suggests that other factors may also contribute to this stage. It is noteworthy that this contrasts with the early stage of NKT cell activation, when they transiently disappear within a few hours of antigenic challenge in vivo, which we previously demonstrated was independent of Bim (8).

The finding that NKT cells are hyporesponsive to rechallenge with α-GalCer is partly consistent with results from a previous study that suggested that NKT cells become anergic after stimulation with free α-GalCer in vivo (18). It must be pointed out, however, that the approach and conclusions of this previous study were significantly different from ours, in that it examined the ability of α-GalCer-pulsed DC to induce an NKT-dependent response in vitro or in vivo and concluded that when NKT cells were isolated from mice previously challenged with free α-GalCer in vivo, they did not respond to α-GalCer-pulsed DC in vitro. However, the assays used in that study failed to demonstrate a clear response to free α-GalCer regardless of whether the cells were isolated from naive or free α-GalCer-treated mice and were therefore unable to compare the specific NKT cell responses to primary and secondary challenges with free α-GalCer, which to date is the most...
common form of treatment in therapeutic models. It is also important to note that in our study, although cytokine production from rechallenged NKT cells was reduced, it was still clearly detectable in vivo and is therefore likely to have significant biological consequences. This is important when considering the regimen for α-GalCer therapy that usually involves more than one injection in vivo. Previous studies have illustrated that α-GalCer recall responses are biased toward IL-4 production in serum and in vitro (17, 42), and that multiple α-GalCer treatment regimens can be used to treat Th1 disorders such as type 1 diabetes (43, 44) and experimental autoimmune encephalomyelitis (45, 46). Our data reveal that, consistent with an earlier report (17), NKT cells themselves remain unpolarized after secondary α-GalCer challenge, suggesting that the systemic loss of IFN-γ after multiple α-GalCer treatment regimens might be primarily due to a downstream effect on other IFN-γ-producing cells, such as NK cells, as originally suggested by Matsuda et al. (17). This is also consistent with an earlier report that NK cells act as the main source of serum IFN-γ (26), highlighting the importance of analyzing cytokine production at a single-cell level, and the requirement for a greater understanding of the interactive biology among NKT, NK, and DCs. Why NKT cells failed to expand in response to a secondary challenge with α-GalCer in vivo, but still produced moderate amounts of cytokines, remains unclear, but this might be determined by the nature of the APC, with DCs providing optimal stimulation (18, 47), although this may vary in a tissue-dependent manner, because

![FIGURE 6](http://www.jimmunol.org/)

Previously challenged NKT cells show reduced in vitro proliferation after rechallenge with α-GalCer. Spleen-derived lymphocytes were labeled with CFSE, cultured for 1 day with α-GalCer (500 ng/ml) or without 50 U/ml IL-2, and washed, and the culture was continued for an additional 2.5 days without α-GalCer (to allow TCR re-expression), but with IL-2 replenished in appropriate cultures. After culture, NKT cells were stained with CD1d/α-GalCer tetramer and anti-αβTCR mAb and examined for evidence of cell division, as indicated by dilution of CFSE labeling. The percentage of cells having undergone one or more divisions is shown as well as the geometric mean fluorescence intensity (MFI) for the entire NKT cell population. These data represent results from one of four similar experiments conducted between 2 wk and 2 mo after primary in vivo α-GalCer stimulation.

![FIGURE 7](http://www.jimmunol.org/)

Impaired in vitro cytokine production by NKT cells from mice previously challenged with α-GalCer. Mice were injected with 2 μg of α-GalCer i.p. and killed 1 mo later. Liver-derived lymphocytes were isolated and cultured for 6 h with no further stimulation, 200 ng/ml α-GalCer with or without 50 U/ml IL-2, 100 ng/ml each of IL-12 and IL-18, 10 ng/ml PMA, and 3 μM ionomycin, or 10 μg/ml plate-bound anti-CD3 with or without 50 U/ml IL-2. GolgiStop was added for the final 5 h of culture before cells were surface-stained with the CD1d/α-GalCer tetramer and anti-CD4, then fixed and permeabilized for intracellular staining with anti-IFN-γ, anti-IL-4, or rat IgG1 isotype control intracellular mAb. CD1d/α-GalCer tetramer+/CD4+ cells were selected by electronic gating and analyzed for their expression of IFN-γ, IL-4, or for non-specific staining by the isotype control. Data are representative of four mice per group, and this experiment is representative of four experiments conducted between 2 wk and 2 mo after primary in vivo α-GalCer treatment.
Kupffer cells appear to be more important for liver NKT cell activation (47). An additional factor may be the affinity of the TCR-Ag interaction on primary challenge, because the lower affinity α-GalCer analog known as OCH, appears to permit a more vigorous secondary response to α-GalCer in vivo (20). However, we were unable to verify part of this study that suggested the mechanism for hyporesponsiveness by NKT cells involved an interaction between up-regulated NKG2A on NKT cells and Qa1b on APCs. In contrast, we found NKG2A and its coreceptor, CD94, were down-regulated to undetectable levels on splenic NKT cells for at least 18 days after challenge with free α-GalCer in vivo. We favor the hypothesis that NKT cell hyporesponsiveness to α-GalCer rechallenge is intrinsic to the NKT cells themselves, because plate-bound anti-CD3 stimulation was unable to enhance cytokine production from prechallenged NKT cells ex vivo, and DCs isolated from naive WT or TAP-1−/− mice (which lack the Qdmin peptide that associates with Qa1b and is required for NKG2A binding (35, 36)) were unable to overcome this anergy. Although we do not see up-regulation of inhibitory receptors on NKT cells after a single stimulation with α-GalCer in vivo, our results are not at odds with an earlier study from our group (19). That study showed chronic α-GalCer stimulation over 8 wk in vivo that gradually depleted peripheral NKT cells, followed by thymus-dependent NKT cell repopulation over the next 8 wk. In that study the repopulating NKT cells in the periphery and thymus expressed higher levels of inhibitory receptors, apparently as a result of altered thymic education. This is obviously very different from the approach used in the current study, where NKT cells were not depleted and repopulated by the thymus over a long period, and thymic NKT cells did not alter their inhibitory receptor profile. Taken together, it appears that multiple mechanisms may exist leading to NKT cell unresponsiveness, the physiological significance of which may be to protect against NKT cell-mediated damage resulting from sustained NKT cell activation, which would otherwise generate high levels of systemic cytokine production and activation of a broad range of bystander cells.

In summary, we have demonstrated that injection of free α- GalCer, the most commonly used approach for experimental NKT cell activation/therapy in vivo, leads to a potent primary NKT cell response that depends on both CD40 and CD28 pathways for maximal NKT cell expansion, but not NKT cell cytokine production. The subsequent contraction phase involved Bim-dependent apoptotic cell death, and surviving NKT cells were shown to be hyporesponsive to further activation by free α-GalCer injection, exhibiting reduced cytokine production and expansion potential in vivo. It remains to be determined whether the primary and secondary responses to recently identified self and other foreign Cd1d-restricted Ags, including iGb3 (48, 49) and bacteria-derived glycolipids (50–52), are regulated in a similar manner.

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Disclosures
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