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Superior Protection against Malaria and Melanoma Metastases by a C-glycoside Analogue of the Natural Killer T Cell Ligand α-Galactosylceramide

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Abstract

α-Galactosylceramide (α-GalCer) is a glycolipid that stimulates natural killer T cells to produce both T helper (Th) 1 and Th2 cytokines. This property enables α-GalCer to ameliorate a wide variety of infectious, neoplastic, and autoimmune diseases; however, its effectiveness against any one disease is limited by the opposing activities of the induced Th1 and Th2 cytokines. Here, we report that a synthetic C-glycoside analogue of α-GalCer, α-C-galactosylceramide (α-C-GalCer), acts as natural killer T cell ligand in vivo, and stimulates an enhanced Th1-type response in mice. In two disease models requiring Th1-type responses for control, namely malaria and melanoma metastases, α-C-GalCer exhibited a 1,000-fold more potent antimalaria activity and a 100-fold more potent antimetastatic activity than α-GalCer. Moreover, α-C-GalCer consistently stimulated prolonged production of the Th1 cytokines interferon-γ and interleukin (IL)-12, and decreased production of the Th2 cytokine IL-4 compared with α-GalCer. Finally, α-C-GalCer's enhanced therapeutic activity required the presence of IL-12, which was needed to stimulate natural killer cells for optimal interferon-γ production, but did not affect IL-4. Overall, our results suggest that α-C-GalCer may one day be an excellent therapeutic option for diseases resolved by Th1-type responses.

Key words: NKT cell • NK cell • α-C-galactosylceramide • IFN-γ • IL-12

Introduction

Natural killer T cells (NKT cells) are a unique population of lymphocytes that coexpress markers of NK cells along with a semiinvariant TCR. In mice, the TCR of most NKT cells consists of an invariant Vα chain encoded by the Vα14 and Jα18 gene segments paired with a variable set of Vβ chains encoded mainly by the Vβ8.2, Vβ7, or Vβ2 gene segments. This TCR enables NKT cells to recognize the MHC class I–like molecule CD1d, which is capable of presenting hydrophobic molecules such as lipids and hydrophobic peptides to NKT cells (1, 2).

Thus far, only a few molecules have been shown to activate NKT cells. Of these, α-galactosylceramide (α-GalCer), a glycolipid originally derived from a marine sponge extract, is the best characterized. When NKT cells recognize α-GalCer presented by APCs in the context of CD1d, they respond by rapidly producing large amounts of both IFN-γ and IL-4, and disappearing within 6 h after activation (2–5). In addition, proliferation of and IFN-γ production by NK cells also occurs after NKT cell activation by α-GalCer (2, 4, 6, 7), as do bystander activation of conventional T cells (2, 4, 8) and IL-12 production by APCs (2, 4, 9–11). α-GalCer's ability to stimulate large amounts of both IFN-γ and IL-4 endows it with therapeutic efficacy in various disease models. IFN-γ stimulated by α-GalCer enables the glycolipid to combat a wide variety of infectious diseases, including malaria and hepatitis B, as well as various tumor metastases including melanoma metastases to the lungs and liver. IL-4 stimulated by α-GalCer allows the glycolipid to ameliorate a number of different autoimmune diseases, including autoimmune type 1 diabetes and autoimmune encephalomyelitis (4).

Despite α-GalCer's therapeutic efficacy against a wide variety of disease states, a recent paper indicates that α-Gal-

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Abbreviations used in this paper: α-C-GalCer, α-C-galactosylceramide; α-GalCer, α-galactosylceramide; ICCS, intracellular cytokine staining; NKT cell, natural killer T cell.

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Cer's concomitant stimulation of both IFN-γ and IL-4 limits its ability to maximally combat any one disease due to the antagonistic activities of the two cytokines (12). Recently, a synthetic analogue of α-GalCer possessing a truncated sphingosine chain was shown to stimulate IL-4 production only, and better protect mice from autoimmune encephalomyelitis, showing that small changes in the molecule can stimulate altered, but useful phenotypes (13). In this regard, we synthesized a C-glycoside analogue of α-GalCer (α-C-galactosylceramide [α-C-GalCer]) bearing a single change: the replacement of the glycosidic O by CH_2. This alteration is expected to render the analogue resistant to α-galactosidase catabolism in vivo (14, 15). In addition, the substitution of the glycosidic O, a polar hydrogen bond acceptor, by CH_2, a nonpolar group, might influence the quality of the NKT cell response to the CD1d–glycolipid complex in a manner different than α-GalCer. Given these differences, we were curious to see if our analogue exhibited altered activity in vivo compared with α-GalCer.

Materials and Methods

Glycolipids. α-GalCer [(2S,3S,4R)-1-O-(α-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol] was synthesized by Kirin Brewery. The stock solution was dissolved in a 0.5% polysorbate-20 (Nikko Chemical), 0.9% NaCl solution at a concentration of 200 μg/ml, and diluted in PBS just before injection into mice. α-C-GalCer [(2S,3S,4R)-1-CH_2-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol] was synthesized as described in the Results section. The stock solution was originally dissolved in 100% DMSO at a concentration of 1 mg/ml. Before injection into mice, it was diluted to a concentration of 200 μg/ml in a 0.5% polysorbate-20 (Nikko Chemical), 0.9% NaCl solution, and diluted in PBS just before injection into mice.

Mice. 6–8-wk-old female BALB/c mice were purchased from the National Cancer Institute. 6–8-wk-old female C57BL/6 mice were purchased from the Jackson Laboratory. CD1d-deficient mice were a gift from L. Van Kaer (Vanderbilt University School of Medicine, Nashville, TN). Jα18-deficient mice were a gift from T. Nakayama and M. Taniguchi (Chiba University School of Medicine, Chiba-shi, Chiba, Japan). IFN-γ–deficient mice of BALB/c background were purchased from the Jackson Laboratory. IFN-γ receptor–deficient mice were bred and maintained in our animal facility (New York University School of Medicine). IL-12p40–deficient mice of BALB/c and C57BL/6 backgrounds were purchased from the Jackson Laboratory. All mice were maintained in our animal facility (New York University School of Medicine) under pathogen-free conditions.

Parasites and Their Use for Challenge. Plasmodium yoelii (17XNL strain) was maintained by alternate cyclic passages in *Anopheles stephensi* mosquitoes and Swiss Webster mice. Sporozoites obtained from dissected salivary glands of infected mosquitoes 2 wk after their infective blood meal were used for challenge of the mice. Challenge of mice to determine the development of liver-stage malaria infection was performed by an intravenous injection of 10,000 viable sporozoites into the tail vein. The outcome of the challenge was determined 40–42 h later by measuring the parasite burden in the livers of the mice using a quantitative real-time RT-PCR method (16). Challenge of mice to determine the development of blood-stage malaria infection was performed by an intravenous injection of 75 viable sporozoites into the tail vein. Starting 4 d after challenge, daily peripheral blood smears were obtained from each mouse and examined microscopically for the presence of blood stage parasites until day 17 after challenge. Mice were considered positive for parasitemia if at least one blood-stage parasite was observed during the time of examination.

Determination of Malaria Liver Stage Development. The degree of liver-stage development in challenged mice was determined by quantifying the amount of *P. yoelii*–specific 18S rRNA molecules in the livers of the mice by way of a recently developed real-time RT-PCR technique (16). In brief, a 2-μg sample of total RNA prepared from the livers of challenged mice was reverse transcribed, and an aliquot of the resulting cDNA (133 ng) was used for real-time PCR amplification of *P. yoelii* 18S rRNA sequences. This amplification was performed in a sequence detection system (model GeneAmp™ 5700; PE Applied Biosystems). For this purpose, we used primers 5′-GGGGATTGGTTTTG-ACGTTTTTGCG-3′ (54 nM) and 5′-AAGCATTAAATAAA-GCGAATACATCCTTAT-3′ (60 nM) together with the ds-DNA-specific dye SYBR Green I incorporated into the PCR reaction buffer (PE Biosystems) to detect the PCR product generated. The temperature profile of the reaction was 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

Determination of Melanoma Lung Metastases. The development of melanoma lung metastases in C57BL/6 mice was determined by first challenging mice intravenously with syngeneic B16 melanoma cells suspended in DMEM supplemented with 10% FCS. 2 or 3 wk after challenge the mice were killed, the lungs were removed, and the number of metastatic nodules were counted as described previously (17).

Determination of Serum Cytokine Concentrations. The serum concentrations of IFN-γ and IL-4 were measured 2, 6, 12, 24, 48, and 72 h after treatment with α-GalCer, α-C-GalCer, or nothing by way of a sandwich ELISA (eBioscience). The serum concentrations of IL-12p70 were also measured at 2, 6, 12, 24, 48, and 72 h after treatment by way of a sandwich ELISA (BD Biosciences).

Flow Cytometry and Intracellular Cytokine Staining (ICCS). Freshly isolated liver lymphocytes from C57BL/6 mice were first incubated for 15 min at 4°C with unlabeled anti–mouse FcγIII/II receptor mAb clone 2.4G2 (BD Biosciences) in staining buffer (PBS containing 1% FBS and 0.1% NaN_3). Next, the cells were surface stained with PE-labeled anti-NK1.1 mAb clone PK136 (BD Biosciences) and FITC-labeled anti-CD3ε mAb clone 145-2C11 (BD Biosciences) in staining buffer for 30 min at 4°C. After two washes in staining buffer, the cells were fixed, permeabilized using Cytofix/Cytoperm Plus™ (BD Biosciences), and stained for intracellular IFN-γ using allophycocyanin-labeled anti–IFN-γ mAb clone XMG1.2 (BD Biosciences) or for intracellular IL-4 using allophycocyanin-labeled anti–IL-4 mAb clone 11B11 (BD Biosciences) according to the manufacturer's protocol. Finally, the stained cells were analyzed using a FACSCalibur™ instrument (Becton Dickinson) with CELLQuest™ software (Becton Dickinson).

Results

Synthesis of α-C-GalCer. Our synthesis of α-C-GalCer proceeded by linking a phytosphingosine homologated by one carbon to D-galactose by way of the Ramberg–Backlund protocol (18, 19). The necessary α-anomeric configuration of the C-glycoside was estab-

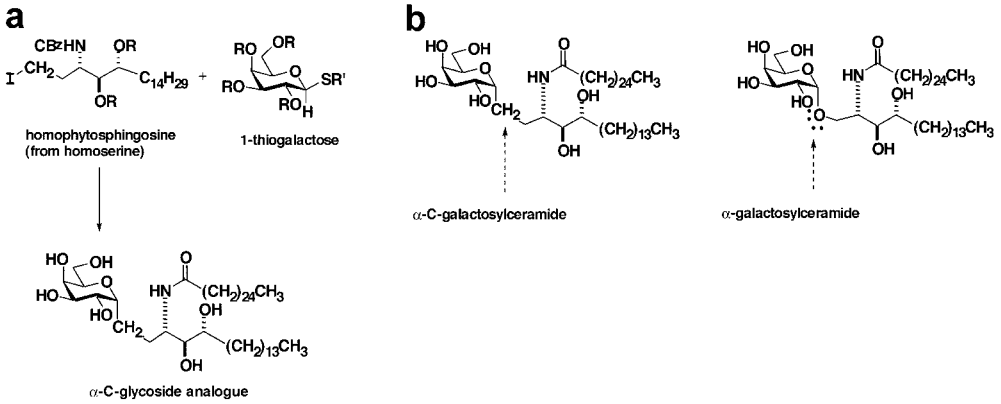

Figure 1. Synthesis and structure of α-C-GalCer. (a) Overview of the pathway used to synthesize α-C-GalCer. The Ramberg–Backlund link-up was performed and the α-configuration was established. Protecting groups were removed and the C-26 fatty amide was installed. (b) Structural comparison of α-C-GalCer and α-GalCer, showing the main differences between the two molecules. Glycoside CH_2 is nonpolar and hydrophobic; it repels hydrogen bond donors. In contrast, glycoside oxygen is polar and the electron pairs can form hydrogen bonds with N–H and O–H donors.

lished using an intramolecular hydride donor methodology. The synthetic route for the homophytosphingosine side chain followed known procedures (20, 21), and the source of chirality for the amine was L-homoserine (as compared with L-serine for the phytosphingosine parent). The necessary hydroxyls were established by a Sharpless dihydroxylation. In the final step, after the α-galacto-C-phytosphingosine assembly was complete, and all protecting groups had been removed, the fatty amide chain was introduced using *p*-nitrophenyl hexadecanoate (22) as the acylating agent to form the final product. Purification was done by flash chromatography on silica gel eluting with $CHCl_3$/MeOH (4:1). Mass spectroscopy and ^1H nuclear magnetic resonance analysis of the fully acylated compound confirmed that it was the desired C-glycoside analogue of α-GalCer, which we have given the trivial name CRONY-101 (Fig. 1).

α-C-GalCer Protects Mice from the Liver Stages of Malaria in a Manner Dependent on CD1d, Vα14⁺ NKT Cells, and IFN-γ. We showed previously that α-GalCer, when administered to mice 2 d before challenge with plasmodial sporozoites, suppressed development of malaria liver stages in a manner dependent on both CD1d-restricted, Vα14⁺ NKT cells and IFN-γ–IFN-γ receptor signaling (23). To see if α-C-GalCer exhibited a similar behavior, we injected WT mice with either α-GalCer or α-C-GalCer 2 d before challenge with live *P. yoelii* sporozoites, and measured the degree of liver stage development using a quantitative real-time RT-PCR assay (16). Mice treated with either α-GalCer or α-C-GalCer showed virtually no liver stage development as compared with untreated control mice, showing that α-C-GalCer has in vivo antimalaria activity similar to that of α-GalCer (Fig. 2 a).

Next, to see if α-C-GalCer's antimalaria activity requires the same determinants as α-GalCer, we injected mice deficient in CD1d, Jα18 (formerly Jα281), IFN-γ, or IFN-γ receptor with the analogue, and measured liver stage development. As with α-GalCer, α-C-GalCer was unable to suppress *P. yoelii* liver-stage development in the absence of these molecules (Fig. 2, b and c). Because both CD1d- and Jα18-deficient mice lack Vα14 NKT cells (24, 25), our results show that α-C-GalCer is a ligand for this cell type in

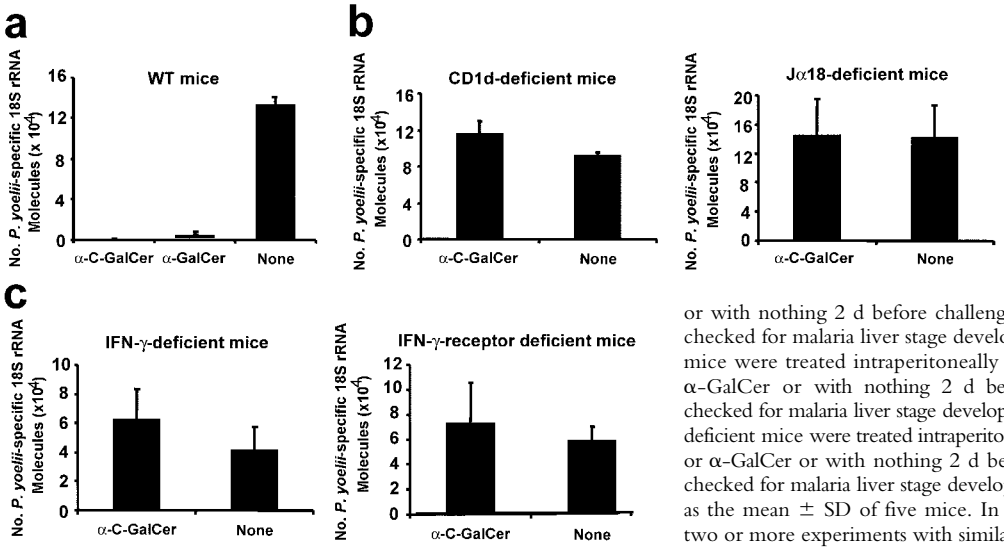

Figure 2. α-C-GalCer protects mice from malaria liver stages in a manner dependent on CD1d, Vα14 NKT cells, and IFN-γ. (a) WT BALB/c mice were treated intraperitoneally with 2 μg of either α-C-GalCer or α-GalCer or with nothing 2 d before challenge with live *P. yoelii* sporozoites, and checked for malaria liver stage development. (b) CD1d- or Jα18-deficient mice were treated intraperitoneally with 2 μg of either α-C-GalCer or α-GalCer or with nothing 2 d before challenge with sporozoites, and checked for malaria liver stage development. (c) IFN-γ– or IFN-γ receptor–deficient mice were treated intraperitoneally with 2 μg of either α-C-GalCer or α-GalCer or with nothing 2 d before challenge with sporozoites, and checked for malaria liver stage development. (a–c) The results are expressed as the mean ± SD of five mice. In all figures, the data represent one of two or more experiments with similar results.

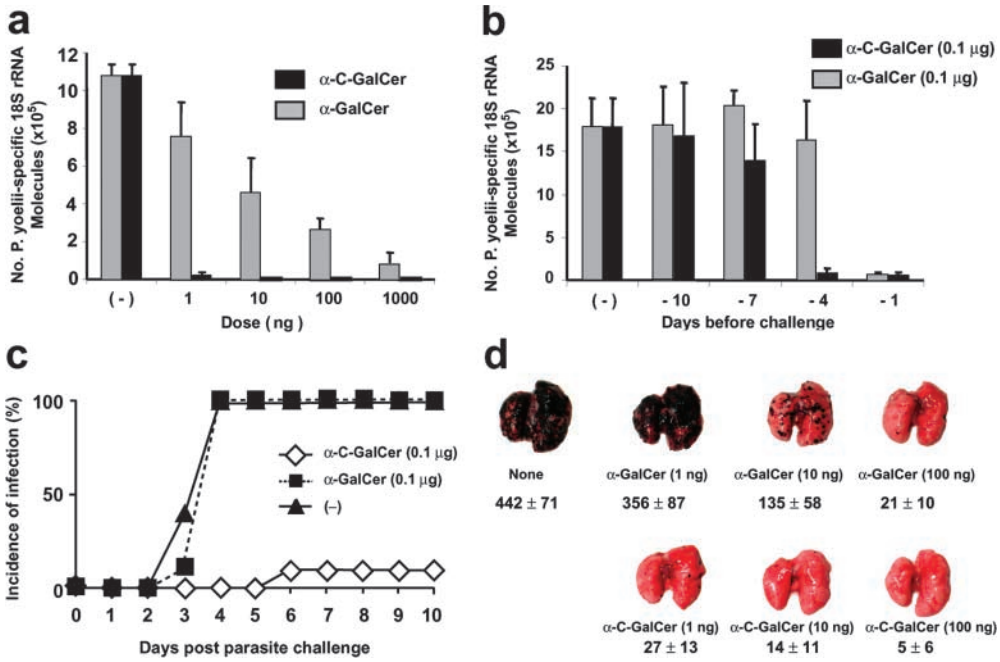

Figure 3. α-C-GalCer better protects mice from malaria liver stages and melanoma metastases than α-GalCer. (a) WT BALB/c mice were treated intraperitoneally with different doses of either α-C-GalCer or α-GalCer or with nothing 3 d before challenge with live *P. yoelii* sporozoites, and checked for malaria liver stage development. (b) WT BALB/c mice were treated intraperitoneally with 100 ng of either α-C-GalCer or α-GalCer or with nothing at various times before challenge with sporozoites, and checked for malaria liver stage development. (c) WT BALB/c mice were injected intraperitoneally with 100 ng of either α-C-GalCer or α-GalCer or with nothing 3 d before challenge with live *P. yoelii* sporozoites. Mice were monitored daily for the presence of blood stage parasites. (d) WT C57BL/6 mice were treated intravenously with different doses of either α-C-GalCer or α-GalCer or with nothing 2 d before intravenous challenge with 5×10^4 syngeneic B16 melanoma cells. 3 wk later, the lungs were checked for tumor metastases. (a, b, and d) The results are expressed as the mean ± SD of five mice. The images shown come from one representative mouse out of five per group.

vivo. Moreover, the requirement of IFN-γ–IFN-γ receptor signaling indicates that α-C-GalCer stimulates a Th1-type response.

α-C-GalCer Exhibits Enhanced Antimalaria Activity Compared with α-GalCer. To see if a difference exists between α-C-GalCer and α-GalCer, we compared both the dose response and kinetic effect of the two glycolipids against the liver stages of malaria. For the dose response, we treated mice with various doses of either glycolipid 3 d before *P. yoelii* sporozoite challenge, and measured liver stage development. We found that α-C-GalCer exhibited a much more potent antimalaria activity, with a dose of 1 ng α-C-GalCer slightly more potent than 1 μg α-GalCer (Fig. 3 a). For the kinetic effect, we treated mice with an identical dose of either glycolipid at different time points before sporozoite challenge, and measured the liver stages. Here, we also observed a striking difference, with α-C-GalCer exhibiting an extended antimalaria effect of up to 3 d longer than α-GalCer (Fig. 3 b).

To confirm the superior antimalaria effect of α-C-GalCer, we assessed the ability of either glycolipid to prevent blood stage malaria infection in mice challenged with live sporozoites. When given 3 d before challenge, α-C-GalCer completely protected 9 out of 10 mice from blood-stage malaria, whereas an identical dose of α-GalCer protected 0 out of 10 mice, the same as untreated controls (Fig. 3 c). Because sporozoite-induced blood-stage infection requires prior successful development of the liver stages, these results corroborate the liver-stage data shown earlier, and clearly show the superior effect of α-C-GalCer in vivo.

α-C-GalCer Exhibits Enhanced Activity against Melanoma Metastases Compared with α-GalCer. Because NKT cell–mediated protection against the liver stages of malaria requires IFN-γ–IFN-γ receptor signaling, the enhanced activity we observed with α-C-GalCer suggested it might be superior in other disease models requiring Th1-type responses for control. One such model involves melanoma metastases to the lungs, in which α-GalCer–mediated inhibition requires an IFN-γ response initiated by NKT cells (26). To see if α-C-GalCer is better able to prevent such metastases than α-GalCer, we treated mice with various doses of either glycolipid 2 d before intravenous challenge with syngeneic melanoma cells, and 2 wk later checked the lungs for the number of metastatic nodules that developed (17). Parallel to our malaria results, α-C-GalCer exhibited a more potent antimetastatic response than α-GalCer, with 1 ng α-C-GalCer equal to 100 ng α-GalCer (Fig. 3 d).

α-C-GalCer Stimulates an Enhanced Th1-type Response in Mice. The superior effect of α-C-GalCer against both malaria liver stages and melanoma metastases suggested it might stimulate an enhanced Th1-type response in vivo compared with α-GalCer. To test this idea, we injected mice with the same dose of either α-C-GalCer or α-GalCer, and at various time points afterwards obtained blood samples for ELISA analyses of IFN-γ, IL-4, and IL-12 concentrations in the sera. For IL-4, both glycolipids stimulated peak concentrations 2 h after treatment; however, α-GalCer stimulated concentrations roughly four times higher than α-C-GalCer (Fig. 4 a). For IFN-γ, both glycolipids stimulated detectable levels starting at 2 h, but α-GalCer's peak occurred 12 h after treatment, returning

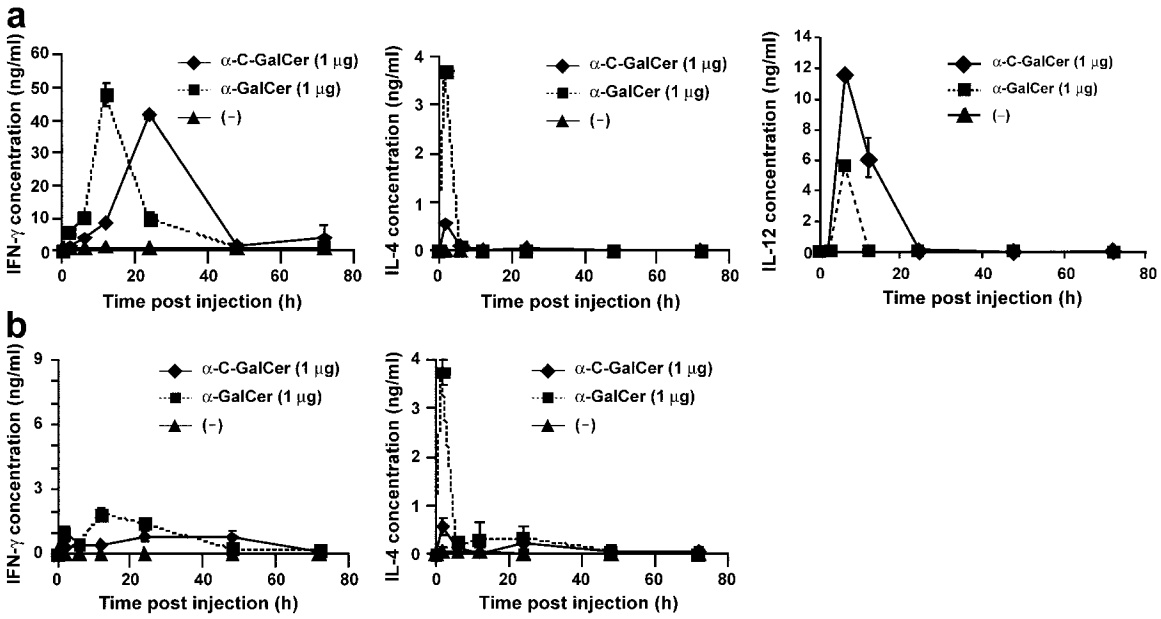

Figure 4. α-C-GalCer stimulates an enhanced Th1-type response in vivo compared with α-GalCer that depends on IL-12. (a) WT C57BL/6 mice were treated intravenously with 1 μg of either α-C-GalCer or α-GalCer or with nothing, and serum samples were obtained at the indicated time points after injection for ELISA analyses of IL-4, IFN-γ, and IL-12 concentrations. (b) IL-12–deficient C57BL/6 mice were treated intravenously with 1 μg of either α-C-GalCer or α-GalCer or with nothing, and serum samples were obtained at the indicated time points after injection for ELISA analyses of IL-4 or IFN-γ. The data are expressed as the mean ± SD of two different dilutions of pooled sera.

to baseline by 24 h. In contrast, α-C-GalCer's peak occurred 24 h after treatment, returning to baseline by 48 h (Fig. 4 a). Finally, for IL-12, both glycolipids stimulated peak concentrations 6 h after treatment; however, α-C-GalCer stimulated concentrations two times higher than α-GalCer. Moreover, α-C-GalCer continued stimulating detectable IL-12 levels 12 h after treatment, whereas by this time point α-GalCer–stimulated IL-12 levels were undetectable (Fig. 4 a). Thus, over time, α-C-GalCer stimulated enhanced levels of the Th1 cytokines IFN-γ and IL-12, and diminished levels of the Th2 cytokine IL-4 as compared with α-GalCer, showing that in vivo it does stimulate an enhanced Th1-type response compared with α-GalCer.

α-C-GalCer's Differential Activity Requires IL-12. Optimal IFN-γ production by NKT cells requires APC-derived IL-12 (2, 4, 9–11). Because α-C-GalCer stimulates much greater IL-12 production than α-GalCer (Fig. 4 a),

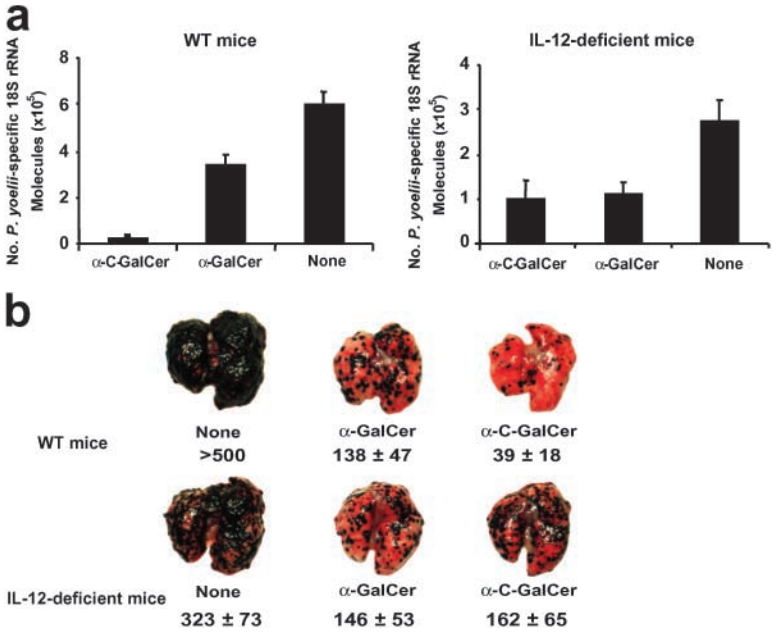

Figure 5. α-C-GalCer's superior therapeutic activity against malaria liver stages and melanoma metastases requires IL-12. (a) WT or IL-12–deficient BALB/c mice were treated intraperitoneally with 10 ng of either α-C-GalCer or α-GalCer or with nothing 3 d before challenge with live *P. yoelii* sporozoites, and checked for malaria liver stage development. (b) WT or IL-12–deficient C57BL/6 mice were treated with 10 ng of either α-C-GalCer or α-GalCer or with nothing 2 d before intravenous challenge with 2×10^5 B16 melanoma cells. 2 wk later, the lungs were checked for tumor metastases. (a and b) The results are expressed as the mean ± SD of five mice. The images shown come from one representative mouse out of five per group.

we wanted to see if IL-12 is necessary for the difference between α-GalCer– and α-C-GalCer–stimulated IFN-γ and IL-4 levels (Fig. 4 a). To address this issue, we treated IL-12–deficient mice with equal doses of either glycolipid, and at various time points afterwards obtained blood samples for ELISA analyses of IFN-γ and IL-4 concentrations in the sera. In the absence of IL-12, IFN-γ levels were greatly reduced in both α-GalCer– and α-C-GalCer–treated mice, and the difference between the two glycolipids was abrogated (Fig. 4 b). In contrast, IL-4 levels stimulated by α-GalCer and α-C-GalCer were unaffected in the absence of IL-12; α-GalCer still stimulated roughly four times more IL-4 than α-C-GalCer. Thus, IL-12 affects α-GalCer– and α-C-GalCer–induced IFN-γ levels, but not IL-4.

Because α-C-GalCer's ability to stimulate prolonged IFN-γ production is abrogated in the absence of IL-12, we wanted to see if its superior therapeutic activity against both malaria liver stages and melanoma metastases to the lung was abrogated as well. To address this issue, we first treated both WT and IL-12–deficient mice with equal doses of either glycolipid 3 d before challenge with sporozoites, and measured malaria liver-stage development. As expected, in WT mice, α-C-GalCer suppressed liver stage development to a much greater degree than α-GalCer; however, in IL-12–deficient mice, there was no difference in antimalaria activity between the two glycolipids (Fig. 5 a). Thus, IL-12 is a key factor driving α-C-GalCer's superior antimalaria effect.

Next, to see if IL-12 is also a key factor driving α-C-GalCer's superior antimetastatic effect, we treated WT and IL-12–deficient mice with equal doses of either α-C-GalCer or α-GalCer 2 d before intravenous challenge with syngeneic melanoma cells; 2 wk later, we checked the lungs for the number of metastatic nodules that developed. As expected, in WT mice, α-C-GalCer better prevented the lungs from melanoma metastases than did α-GalCer; however, in IL-12–deficient mice, there was no difference in the antimetastatic effect between the two glycolipids (Fig. 5 b). Thus, as with malaria liver stages, IL-12 is a key factor driving α-C-GalCer's superior antimetastatic effect.

NK Cells Are the Primary IFN-γ Source after α-GalCer and α-C-GalCer Injection. To determine what cell types are the principle IFN-γ sources after α-GalCer and α-C-GalCer injection, we treated WT mice with equal doses of ei-

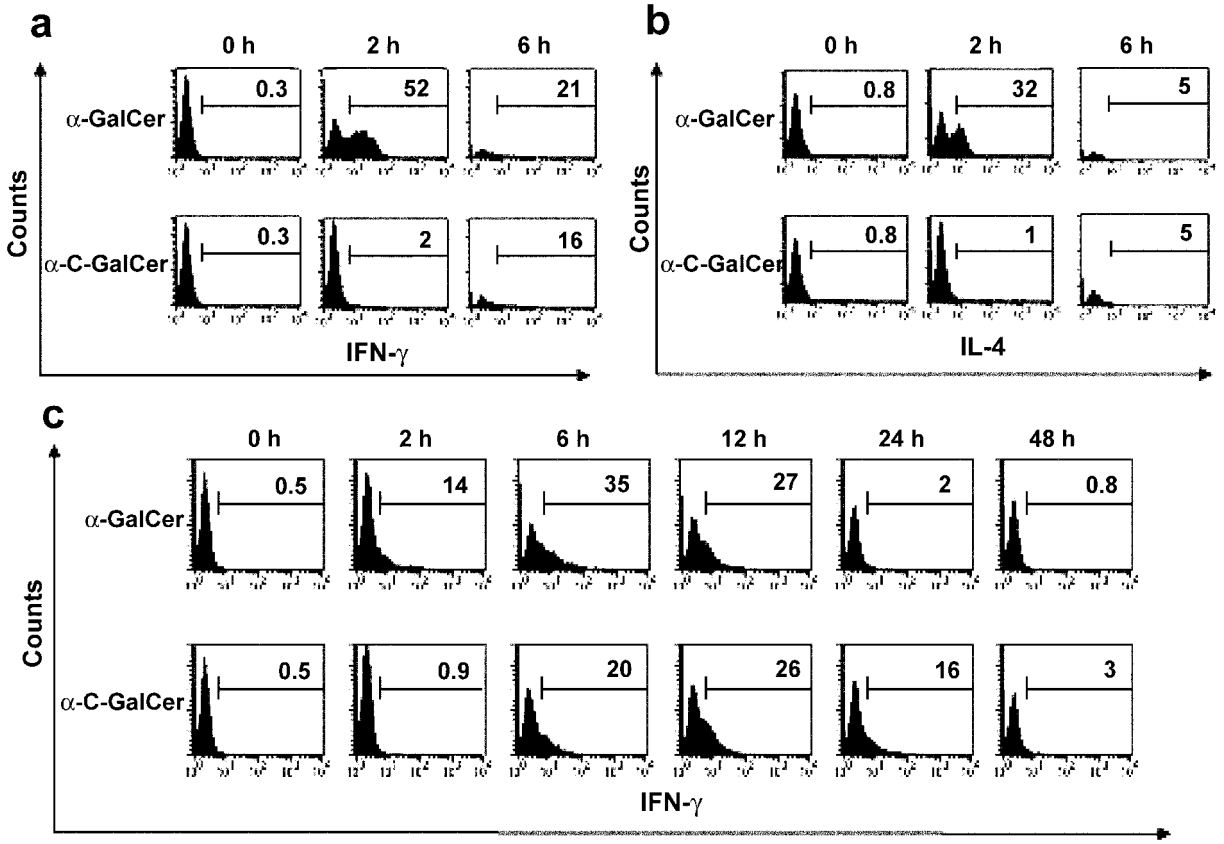

Figure 6. NK cells are the primary IFN-γ source after α-GalCer and α-C-GalCer injection. WT C57BL/6 mice were treated intraperitoneally with 1 μg of either α-GalCer or α-C-GalCer, and at various time points afterwards hepatic lymphocytes were obtained and subjected to ICCS. (a) $NK1.1^+$, $CD3^+$ NKT cells were gated and analyzed for the presence of intracellular IFN-γ. (b) $NK1.1^+$, $CD3^+$ NKT cells were gated and analyzed for the presence of intracellular IL-4. (c) $NK1.1^+$, $CD3^-$ NK cells were gated and analyzed for the presence of intracellular IFN-γ. Numbers indicate the percent of cytokine-positive cells in the gated population. The experiments shown are representative of three independent experiments for each staining protocol.

ther glycolipid, and 2, 6, 12, 24, or 48 h later obtained liver lymphocytes from the mice for ex vivo ICCS analysis of IFN-γ–positive NK ($NK1.1^+$, $CD3^-$), NKT ($NK1.1^+$, $CD3^+$), T ($NK1.1^-$, $CD3^+$), and non–T ($NK1.1^-$, $CD3^-$) cells. At 2 h after injection, a high percentage of NKT cells from α-GalCer–treated mice stained positive for IFN-γ; however, at this same time point, very few NKT cells from mice treated with α-C-GalCer stained positive for the cytokine (Fig. 6 a). A similar result was obtained when we looked at IL-4 production in NKT cells at the 2-h time point; a high percentage of NKT cells from α-GalCer–treated mice stained positive for IL-4, but very few stained positive from the α-C-GalCer–treated mice (Fig. 6 b). Interestingly, at 6 h after injection, the numbers of NKT cells were reduced in both α-GalCer– and α-C-GalCer–treated mice (Fig. 6, a and b), and remained that way up to the 48-h time point (not depicted), indicating that over time NKT cells are not the major IFN-γ source after administration of α-GalCer and α-C-GalCer.

In contrast to NKT cells, NK cells from both α-GalCer– and α-C-GalCer–treated mice stained positive for IFN-γ at time points later than 2 h after injection. In α-GalCer–treated mice, high percentages of NK cells stained positive for IFN-γ at 2, 6, and 12 h after injection, returning to low levels by 24 h. In α-C-GalCer–treated mice, the pattern was different, with high percentages of NK cells staining positive for IFN-γ at 6, 12, and 24 h after injection, returning to low levels by 48 h (Fig. 6 c). Interestingly, these patterns of NK cell IFN-γ production correlate closely with the serum IFN-γ profiles induced by the glycolipids (Fig. 4 a), strongly suggesting that NK cells are the principal IFN-γ sources after α-GalCer and α-C-GalCer administration. Importantly, no IFN-γ staining was observed in $NK1.1^-$, $CD3^+$ T cells and $NK1.1^-$, $CD3^-$ non–T cells at any time point (unpublished data), further attesting to the important role of NK cells in glycolipid-induced IFN-γ production.

Optimal IFN-γ Production by NK Cells after α-GalCer and α-C-GalCer Administration Requires IL-12. To see how IL-12 affects IFN-γ production by NK and NKT cells after α-GalCer and α-C-GalCer injection, we repeated the ICCS kinetics experiment detailed in the preceding paragraphs, this time using IL-12–deficient mice. At 2 h after injection, we observed a smaller percentage of IFN-γ–positive NKT cells in both α-GalCer– and α-C-GalCer–treated IL-12–deficient mice than in WT mice; however, there were still more IFN-γ–positive NKT cells in the α-GalCer–treated IL-12–deficient mice than those treated with α-C-GalCer (Fig. 7 a). At 6 h after injection, just like in WT mice, NKT cell numbers were greatly reduced

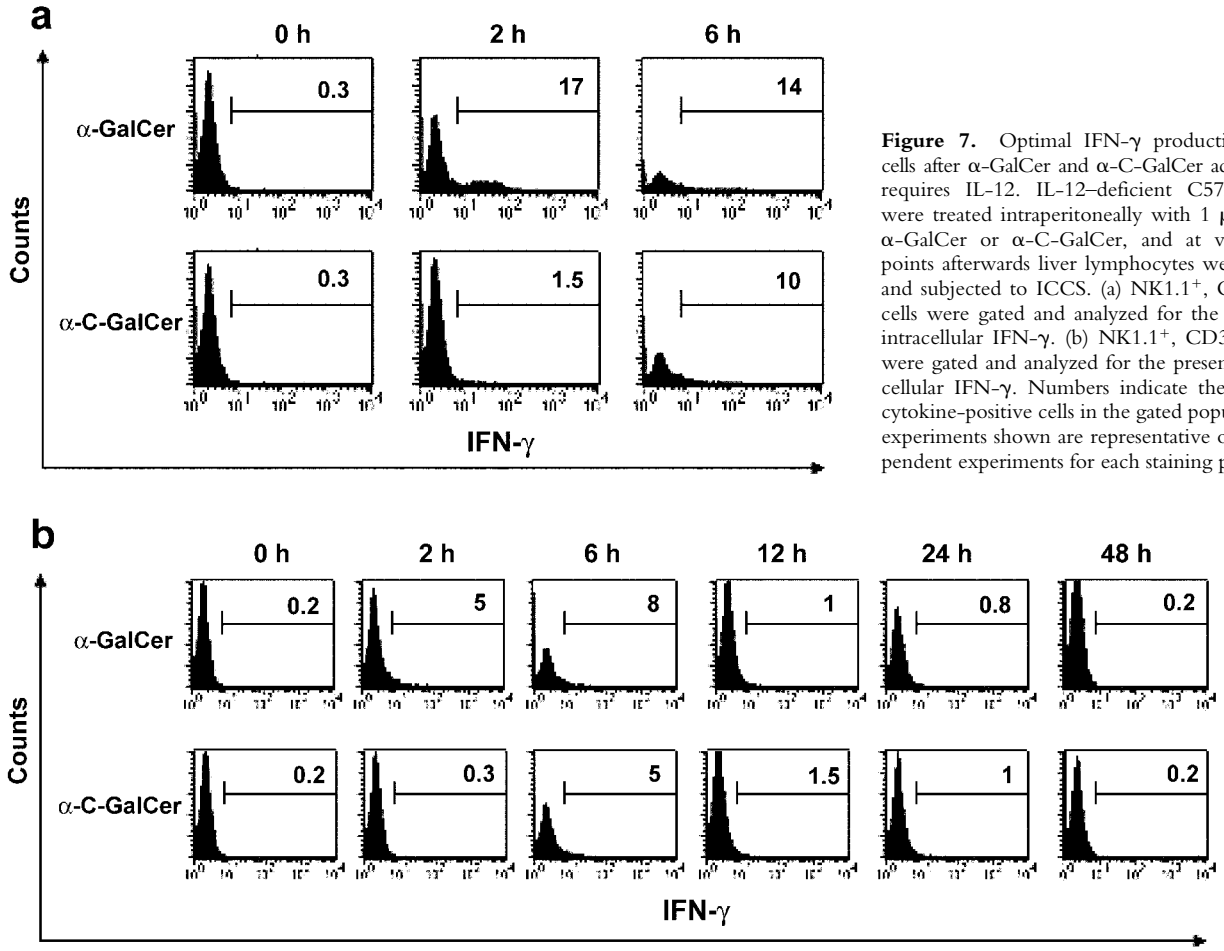

Figure 7. Optimal IFN-γ production by NK cells after α-GalCer and α-C-GalCer administration requires IL-12. IL-12–deficient C57BL/6 mice were treated intraperitoneally with 1 μg of either α-GalCer or α-C-GalCer, and at various time points afterwards liver lymphocytes were obtained and subjected to ICCS. (a) $NK1.1^+$, $CD3^+$ NKT cells were gated and analyzed for the presence of intracellular IFN-γ. (b) $NK1.1^+$, $CD3^-$ NK cells were gated and analyzed for the presence of intracellular IFN-γ. Numbers indicate the percent of cytokine-positive cells in the gated population. The experiments shown are representative of two independent experiments for each staining protocol.

in both α-GalCer– and α-C-GalCer–treated IL-12–deficient mice, and remained so up to the 48-h time point. Thus, IL-12 does appear to facilitate IFN-γ production by NKT cells, but it is not an absolute requirement, and does not appear to seriously affect NKT cell survival after glycolipid injection.

In contrast to NKT cells, NK cell production of IFN-γ was severely affected by the lack of IL-12. In both α-GalCer– and α-C-GalCer–treated IL-12–deficient mice, the numbers of IFN-γ–positive NK cells were markedly reduced at all time points as compared with WT mice (Fig. 7 b). This reduction in NK cell IFN-γ in IL-12–deficient mice closely parallels the reduced serum IFN-γ induced by the glycolipids in these same mice (Fig. 4 b). Thus, IL-12 produced in response to α-GalCer and α-C-GalCer injection appears to act primarily on NK cells, which respond by producing the bulk of the IFN-γ that follows glycolipid administration.

Discussion

In the current work, we synthesized a C-glycoside analogue of the NKT cell ligand α-GalCer, which we refer to as α-C-GalCer. Structurally, α-C-GalCer differs from α-GalCer only in the identity of the chemical group involved in the glycosidic linkage between the galactose and ceramide moieties of the molecule; α-C-GalCer's glycosidic linkage involves a CH_2 group, whereas α-GalCer's glycosidic linkage involves an O atom. Despite this small difference, we found that the biological activities of the two molecules differ greatly.

To assess the biological activity of α-C-GalCer, and compare it with that of α-GalCer, first we used a murine malaria model. In a previous paper, we showed that α-GalCer can protect mice from the liver stages of malaria when administered before live sporozoite challenge, and that this protection requires CD1d presentation of α-GalCer, $V\alpha14^+$ NKT cell activation, and IFN-γ production (23). In the current work, we show that α-C-GalCer can also protect mice from liver-stage malaria when administered before sporozoite challenge, and that this protection also requires CD1d presentation of the glycolipid, $V\alpha14^+$ NKT cell activation, and IFN-γ production. Moreover, we found that α-C-GalCer exhibited a much more potent and longer-lasting anti–liver-stage activity compared with α-GalCer, and that this translated into superior protection against sporozoite-induced blood-stage infection.

Although the malaria model we used to compare the activities of both α-C-GalCer and α-GalCer provided a well-understood, uncomplicated system that yielded clear results showing a striking difference between the two glycolipids, the potential practicality of using either α-C-GalCer or α-GalCer against the liver stages of malaria in humans is severely limited because of the need to administer the glycolipids before sporozoite exposure. At best, the glycolipids might be used for short-term prophylaxis against malaria, or as a possible therapy for the hypnozoite stage of *Plasmodium vivax*. To see if α-C-GalCer has therapeutic efficacy in a more practicable disease system, we used a melanoma lung metastasis model because cancer metastases are one of the disease states it is hoped α-GalCer will one day treat (4). It has been shown in a previous paper that α-GalCer is able to prevent melanoma metastases to the lungs of mice in a manner dependent on IFN-γ (26). Because α-C-GalCer better protects mice from malaria liver stages, and because this effect requires IFN-γ, we expected α-C-GalCer to exhibit a superior antimetastatic effect compared with α-GalCer as well. Indeed, when administered before challenge with melanoma cells (i.e., before the metastatic event), α-C-GalCer exhibited a much more potent prophylactic activity against metastasis than α-GalCer. Thus, in two different disease models requiring IFN-γ for control, α-C-GalCer displayed far superior therapeutic activity than α-GalCer.

α-C-GalCer's superior therapeutic activity is probably due to its ability to stimulate an enhanced Th1-type response relative to that stimulated by α-GalCer. After injection into mice, α-C-GalCer induced prolonged production of the Th1 cytokines IFN-γ and IL-12 and decreased production of the Th2 cytokine IL-4 compared with α-GalCer. It is likely that the increased amount of IFN-γ resulting from its prolonged production after α-C-GalCer injection is the main reason for α-C-GalCer's enhanced potency against malaria liver stages and melanoma metastases, as well as its longer-lasting activity against malaria liver stages. This assertion is supported by the fact that α-C-GalCer's superior activity against both malaria liver stages and melanoma metastases is abrogated in the absence of IL-12, as is its ability to stimulate prolonged IFN-γ production relative to α-GalCer. The importance of IL-12 for optimal stimulation of IFN-γ production by α-C-GalCer and α-GalCer suggests that it is α-C-GalCer's ability to stimulate prolonged IL-12 that allows for its ability to stimulate prolonged IFN-γ as well. The fact that peak IL-12 production precedes peak IFN-γ production is consistent with this conclusion. Interestingly, stimulation of IL-4 production by α-C-GalCer and α-GalCer is unaffected in the absence of IL-12. This result makes sense inasmuch as peak IL-4 production occurs before peak IL-12 production. Because peak IL-4 production precedes peak IL-12 and IFN-γ production, it is possible that α-C-GalCer's ability to stimulate decreased IL-4 early on somehow allows for the later prolonged production of IL-12 and IFN-γ. Further experiments are needed to resolve this issue.

It is generally believed that by 6 h after stimulation with α-GalCer, NKT cells die by apoptosis (2, 4, 5), although one very recent paper suggests that NKT cells may not die after in vivo stimulation with α-GalCer (27). It is also known that downstream of NKT cell activation, NK cells are stimulated to both proliferate and secrete IFN-γ (2, 4, 6, 7). Because most of the IFN-γ produced after α-GalCer treatment appears 12 h after injection, it is likely that most of this IFN-γ is provided by NK cells. Indeed, our WT ICCS results showed that in α-GalCer–treated mice, NKT

cells were a major source of IFN-γ only at the 2-h time point after injection because by 6 h they disappeared. In contrast, NK cells were the principal cell type producing IFN-γ 6 and 12 h after α-GalCer injection, the very time points when the level of α-GalCer-induced serum IFN-γ was highest, and they also provided some of the IFN-γ 2 h after injection. In addition, our ICCS data showed that NK cells were also the primary IFN-γ source in mice treated with α-C-GalCer. NK cells from α-C-GalCer–treated mice produced most of their IFN-γ 6, 12, and 24 h after injection, which overlaps closely with the serum IFN-γ profile induced by α-C-GalCer. Our results also showed that α-C-GalCer stimulated very few IFN-γ–producing NKT cells, although it did induce NKT cell disappearance in a manner comparable to α-GalCer. Thus, NK cells are the major IFN-γ source after administration of α-GalCer and α-C-GalCer.

As discussed before, optimal IFN-γ production after either α-GalCer or α-C-GalCer injection requires the appearance of IL-12. IL-12 is a known stimulator of NK cell proliferation and IFN-γ production (28). In addition, a recent paper has demonstrated a major contribution of NK cells to IFN-γ production after NKT cell stimulation by α-GalCer that is dependent on IL-12 signaling (29). In light of these facts, we examined whether the lack of IL-12 affected the ability of NK and NKT cells to produce IFN-γ in response to α-GalCer and α-C-GalCer administration. Our ICCS experiment using IL-12–deficient mice showed that the lack of IL-12 attenuated the number of IFN-γ–positive NKT cells stimulated by either α-GalCer or α-C-GalCer, but did not affect the survival of these cells. More importantly, the lack of IL-12 severely affected the ability of NK cells to produce IFN-γ after injection of either glycolipid, showing that IL-12 produced in response to α-GalCer and α-C-GalCer acts on NK cells to stimulate downstream IFN-γ. The likely source of this IL-12 is probably DCs. DCs are known to express CD1d on their surface, present α-GalCer to NKT cells, and produce IL-12 in response to this presentation (2, 4, 9–11, 17). Thus, it is likely that DCs are the APCs responsible for presenting α-GalCer and α-C-GalCer to NKT cells in vivo, and that α-C-GalCer somehow stimulates more IL-12 from DCs after interaction with NKT cells. Further research is needed to clarify the role of DCs in α-C-GalCer's in vivo effect, and how exactly α-C-GalCer stimulates enhanced IL-12.

An important question remains as to exactly why α-C-GalCer, which differs structurally from α-GalCer only slightly, exhibits such striking biological differences. In all likelihood, the differences stem from altered binding of α-C-GalCer to CD1d as compared with that of α-GalCer. Such altered binding would likely result in differential recognition of the CD1d–α-C-GalCer complex by the TCR of $V\alpha14^+$ NKT cells and qualitatively different signal transduction events in both NKT cells and DCs. Such differential signal transduction would result in the altered response characterized by low IL-4 and prolonged IL-12 and IFN-γ production. The lone difference between α-C-GalCer and α-GalCer involves the replacement of α-GalCer's glycosidic O, a polar hydrogen bond acceptor, with CH_2, a nonpolar group incapable of participating in hydrogen bonds. This change in hydrogen bonding capacity might cause α-C-GalCer to sit differently in the CD1d binding pocket as compared with α-GalCer, resulting in an altered structure of the CD1d–α-C-GalCer complex and a change in its affinity with the NKT cell's TCR. In addition, the CH_2 group of α-C-GalCer allows for freer rotation about the axis of the glycosidic bond between the galactose and ceramide than α-GalCer's O. This change might also influence the conformation of α-C-GalCer in the CD1d binding pocket, and thereby affect the interaction with the NKT cell's TCR. In effect, α-C-GalCer might be acting in a manner similar to altered peptide ligands, conventional T cell epitopes bearing changes in certain important amino acid residues that lead to an alteration in the TCR recognition of the peptide–MHC complex, and a differential functional response in the responding T cell (30). Our ICCS results showing lower numbers of both IFN-γ–positive and IL-4–positive NKT cells 2 h after administration of α-C-GalCer, as compared with α-GalCer, strongly suggest that the signal conveyed by α-C-GalCer to NKT cells is indeed of a different nature than that conveyed by α-GalCer. It also shows, in agreement with a recent paper using α-GalCer (29), that α-C-GalCer does not polarize NKT cells in either the Th1 or Th2 direction, but instead seems to provide a weaker stimulus for NKT cell cytokine production than α-GalCer. Given the differential activity stimulated by α-C-GalCer, it might be possible to synthesize other analogues of α-GalCer that can stimulate altered NKT cell responses, which might be potentially useful for treatment of certain diseases.

Overall, we have demonstrated that α-C-GalCer is an NKT cell ligand in vivo that stimulates enhanced Th1-type activity compared with α-GalCer. Due to the conserved nature of NKT cell responses in mammals, it is likely that α-C-GalCer, which stimulates $V\alpha14^+$ NKT cells in mice, also stimulates $V\alpha24^+$ NKT cells in humans (31). In this light, if NKT cell–directed therapy ever comes to fruition in humans, α-C-GalCer's Th1-enhancing activity makes it an excellent chemotherapeutic candidate for a number of human diseases, including cancer metastases, allergy, and various infectious diseases such as hepatitis B and C (4). Given the fact that recent clinical trials have shown that α-GalCer appears to have biological activity in cancer patients (32, 33), α-C-GalCer has promise as a therapeutic agent.

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