The Natural Killer T (NKT) Cell Ligand α-Galactosylceramide Demonstrates Its Immunopotentiating Effect by Inducing Interleukin (IL)-12 Production by Dendritic Cells and IL-12 Receptor Expression on NKT Cells

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Summary

The natural killer T (NKT) cell ligand α-galactosylceramide (α-GalCer) exhibits profound antitumor activities in vivo that resemble interleukin (IL)-12–mediated antitumor activities. Because of these similarities between the activities of α-GalCer and IL-12, we investigated the involvement of IL-12 in the activation of NKT cells by α-GalCer. We first established, using purified subsets of various lymphocyte populations, that α-GalCer selectively activates NKT cells for production of interferon (IFN)-γ. Production of IFN-γ by NKT cells in response to α-GalCer required IL-12 produced by dendritic cells (DCs) and direct contact between NKT cells and DCs through CD40/CD40 ligand interactions. Moreover, α-GalCer strongly induced the expression of IL-12 receptor on NKT cells from wild-type but not CD1−/− or Vα14−/− mice. This effect of α-GalCer required the production of IFN-γ by NKT cells and production of IL-12 by DCs. Finally, we showed that treatment of mice with suboptimal doses of α-GalCer together with suboptimal doses of IL-12 resulted in strongly enhanced natural killing activity and IFN-γ production. Collectively, these findings indicate an important role for DC-produced IL-12 in the activation of NKT cells by α-GalCer and suggest that NKT cells may be able to condition DCs for subsequent immune responses. Our results also suggest a novel approach for immunotherapy of cancer.

Key words: natural killer T cells • dendritic cells • α-galactosylceramide • interleukin 12 • interleukin 12 receptor

Natural killer T (NKT) cells represent a novel lymphoid lineage distinct from mainstream T cells, B cells, and NK cells. NKT cells are characterized by the expression of an invariant TCR encoded by Vα14 and Jα281 gene segments and Vβ8, 7, or 2 gene segments (1, 2). It was demonstrated recently that NKT cells are strongly stimulated by the glycolipid α-galactosylceramide (α-GalCer), a potent inducer of antitumor immunity in mice (3–5). Recognition of α-GalCer by NKT cells appeared to depend on the interaction of the invariant TCR of these cells with α-GalCer presented by the nonclassical MHC molecule CD1d on APCs (6). Stimulation of NKT cells by α-GalCer resulted in the production of large amounts of IFN-γ and some IL-4, and the development of a cytotoxic phenotype (7).

The in vivo antitumor activity of α-GalCer strongly resembles the antitumor activity mediated by the cytokine IL-12 (8, 9). Moreover, both α-GalCer and IL-12 are...
Materials and Methods

Mice: C57BL/6 mice were purchased from Charles River Japan. NKT cell-deficient (Jα281−/−) and CD1d−/− mice were established by specific deletion of the Jα281 and CD1d gene segment, respectively (3, 10). All mice used in this study were 5–8 wk of age and were maintained in specific pathogen-free conditions.

α-GalCer, α-GalCer [(2S,3S,4R)-1-0:α-(4-d-galactopyranosyl)-2-(N-hexanoylamino)-1,3,4-octadecanetriol] used for this study was provided by Dr. Y. Koezuka (Kirin Brewery Co., Ltd., Gunma, Japan (4, 5). The stock solution of α-GalCer (220 μg/ml) was diluted in 0.5% polysorbate 20 (Nikkko Chemical) in 0.9% NaCl solution. This stock solution was further diluted into an appropriate concentration with saline and used for the experiments. A vehicle control solution was prepared from a solution of 0.5% polysorbate 20 in 0.9% NaCl solution. The vehicle control was used in all experiments.

Isolation of Lymphoid Cell Subsets by FACS®. Spleen cells were incubated on nylon wool columns for 45 min, and the nonadherent cells were used for the isolation of NKT cells. N K cells, CD4+ T cells, and CD8+ T cells by cell sorting using a FACS Vantage® instrument (Becton Dickinson). All mAbs used in these experiments (mAbs against NK1.1, CD4, CD8, and TCR-α/β) were purchased from PharMingen. Unless noted otherwise, NK1.1+ TCR-α/β+ cells were used as purified NKT cells. The stained cells were isolated using the FACS Vantage®. The purity of the sorted cells was 98%. The details of the staining and sorting have been described previously (11).

Coculture of DCs and NKT Cells. DCs were prepared according to the method of Steinman et al. (12) with some modifications. In brief, spleen cells were incubated in 10-cm plastic dishes (Falcon; Becton Dickinson) for 2 h, and the nonadherent cells were removed from the culture. The adherent cells were further incubated overnight and the nonadherent cells were harvested. Then, CD11c+B220+CD4+CD8− cells were isolated from the nonadherent populations by cell sorting and used as the source of DCs. Generally, DCs (104) were cocultured with purified NK1.1+ TCR-α/β+ NKT cells (2 x 105) in the presence of 50 ng/ml of α-GalCer in 96-well U-bottomed plates (Costar Corp.). After incubation for 36 h, the culture supernatants were harvested to detect cytokine levels.

Detection of Cytokine Activity. IL-4 or IFN-γ activity in culture supernatants was determined using the Biotrak™ mouse IL-4 or Biotrak™ mouse IFN-γ ELISA system (Nycomed Amersham plc). Serum samples were obtained from C57BL/6 mice 24 h after infection of α-GalCer (200 ng/mouse) and/or IL-12 (200 U/mouse; donated by Genetics Institute, Inc., Cambridge, MA), and cytokine levels were measured using ELISA kits (Nycomed Amersham plc). IL-12 p70 activity in culture supernatants was measured using Interleukin-12 ELISA kits (Genzyme Corp.).

Cytotoxicity Assay. The natural killing activity of spleen cells was determined by 4-h 51Cr-release assays using YAC-1 cells as target. 1 lytic unit (LU) was defined as the number of effector cells required to cause 25% lysis of 2,500 target cells as described previously (13).

Assessment of the Synergistic Effect of α-GalCer and IL-12. In vivo. Wild-type C57BL/6 mice received an injection of α-GalCer (200 ng/mouse i.v.), and 6 h later mice were injected with recombinant IL-12 (200 U/mouse i.p.) or saline. 1 d after treatment with IL-12, IFN-γ production in the serum and NK activity of spleen cells were determined. Control mice were treated with vehicle only.

Quantitative Reverse Transcriptase PCR. RNA was isolated from IL-12−/−mice (11). C57BL/6, CD1d−/−, and NKT cell-deficient mice were injected with α-GalCer (2 μg/ml i.v.) or vehicle. At different time points (0–6 h) after treatment, mice were killed and spleen cells were isolated. TaqMan™ real-time quantitative reverse transcription (RT)-PCR was carried out for the detection of IL-12 mRNA expression by these cells according to published methods (11). In brief, total RNA extracts from the cells were added to the master mixture. To detect the amount of the IL-12 mRNA RT-PCR amplification, target (IL-12 mRNA) and control (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) hybridization probes were mixed with target and control PCR primers, respectively. This mixture was transferred to a set of thermocyclers and transcribed at 42°C for 30 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1.5 min, and analyzed using an ABI PRISM 7700 sequence detector (Applied Biosystems). IL-12 mRNA and IL-12 mRNA expression were estimated from the ratio of fluorescence intensity to GAPDH. IL-12 mRNA expression induced by α-GalCer is indicated in the figures as induction index, calculated as follows: induction index = IL-12 mRNA stimulated sample/IL-12 mRNA unstimulated sample.

TaqMan® probes used for these analyses are as follows: IL-12 mRNA−605T, 5′-CGGATGGCACAACAGATTGGA-3′; IL-12 mRNA−529F, 5′-AGCCACTCTAAAACATATCATGTCGAGG-3′; GAPDH−542T, 5′-CCTGGCCAAAGGTTCATCCATCAGACCTT-3′.

PCR primers used for these analyses are as follows: IL-12β1 mRNA, forward primer (−563F) 5′-AATGTGTGCTGGAGAACGGCGT-3′ and reverse primer (−657R) 5′-GAGTTAACCCTGAGTGTCGCCAGT-3′; IL-12β2 mRNA, forward primer (−529F) 5′-ATTTACGTTTGGTCTGGTCACA-3′ and reverse primer (−602R) 5′-GCCACAGTTCTTTTCTCTC-3′; GAPDH, forward primer (−368F) 5′-CTTCCACCATGGGAAAGGC-3′ and reverse primer (−605R) 5′-GGCATGACTGTTGCTCATG-3′.

Blocking of IL-12R Induction by Anti–IFN-γ mAb. Wild-type C57BL/6 mice were injected with 500 μg i.p. anti–IFN-γ mAb (R-4-6A2; PharMingen) or IL-12 (C15.1 and C15.6, donated by Dr. G. Trinchieri, Wistar Institute of Anatomy and Biology, Philadelphia, PA) at 0 and 1 d before priming with α-GalCer. As a
control, the same amount of rat IgG1 (PharMingen) was injected intraperitoneally into control mice before injection of α-GalCer.

Results

α-GalCer Selectively Activates NK1.1⁺ TCR-αβ⁺ NKT Cells. To provide direct evidence that NKT cells are the only target cells for activation by α-GalCer, various lymphoid subsets were isolated from mouse spleen cell suspensions by flow cytometry and cocultured with DCs in the presence of α-GalCer. After 36 h of culture, the supernatants were harvested and their IL-4 and IFN-γ contents were measured by ELISA. Fig. 1 shows that purified NK1.1⁺ T cells produce higher levels of IL-4 and IFN-γ than unfractionated spleen cells. The IFN-γ produced in these cultures was not derived from classical NK cells, because enrichment of NK1.1⁺ TCR-αβ⁻ NK cells showed no significant cytokine production. In contrast, NK1.1⁺ TCR-αβ⁺ cells, which represent the NKT cell population, revealed markedly high levels of IL-4 and IFN-γ production. Although CD4⁺ T cells produced higher levels of cytokines compared with unfractionated spleen cells, this appeared to be due to the presence of CD4⁺NK1.1⁺ NKT cells, because CD4⁺NK1.1⁻ cells produced neither IL-4 nor IFN-γ in response to α-GalCer. Culture of NK1.1⁺ TCR-αβ⁺ NKT cells alone or with DCs in the absence of α-GalCer caused no significant production of IFN-γ or IL-4, indicating that DCs are essential for the stimulation of cytokine production by NKT cells.

Endogenous IL-12 Production by DCs Is Essential for the Triggering of NKT Cells. Fig. 2 A shows that coculture of DCs and NKT cells in the presence of α-GalCer resulted in high levels of IFN-γ production. However, addition of anti-IL-12 mAb into these cultures caused a marked inhibition of IFN-γ production. Such inhibition was not observed when control anti-CD8 rat IgG mAb was added. Therefore, these results indicated that endogenously produced IL-12 by DCs was essential for the early activation of NKT cells by α-GalCer. The effect of mAbs against CD40 and CD40L on the activation of NKT cells by α-GalCer was also investigated (Fig. 2 B). Both anti-CD40 mAb and anti-CD40L mAb greatly inhibited the production of IFN-γ by NKT cells in response to α-GalCer. These findings suggested that direct contact between DCs and NKT cells through CD40/CD40L interactions is critically important for the triggering of NKT cells.

Figure 1. α-GalCer selectively activates NK1.1⁺ TCR-αβ⁺ NKT cells. Spleen cells from C57BL/6 mice were separated into a variety of lymphoid cell subsets by cell sorting as described in Materials and Methods. Their responsiveness to α-GalCer in the presence of DCs was then determined by measuring IL-4 (■) and IFN-γ (□) levels in the culture supernatants using ELISA. As a control, NK1.1⁺ TCR-αβ⁻ NK cells were cultured alone or with DCs in the absence of α-GalCer. The bars represent mean ± SE of triplicate samples.

Figure 2. Endogenously produced IL-12 and CD40/CD40L interactions during coculture of DCs and NKT cells is essential for NKT cell activation by α-GalCer. Purified NKT cells were cocultured with DCs in the presence of α-GalCer for 36 h. The IFN-γ levels in culture supernatants were then determined by ELISA. (A) The ability of anti-IL-12 mAb to block NKT cell activation by α-GalCer. A anti-CD8 mAb was used as control rat IgG Ab. (B) The ability of anti-CD40 mAb and anti-CD40L mAb to block NKT cell activation by α-GalCer. As a control, rat anti-CD8 IgG mAb was added to the culture. (C) IL-12 production by DCs cultured with α-GalCer and NKT cells. DCs (5 × 10⁵) were activated with 50 ng/ml of α-GalCer for 8 h in the presence or absence of NK1.1⁺ TCR-αβ⁻ NK cells (10⁵) or NK1.1⁺ TCR-αβ⁺ NKT cells (10⁵). The bars represent mean ± SE of triplicate samples.
for the activation of NKT cells by α-GalCer. To study the requirements for IL-12 production by DCs in these cultures, we injected anti–IL-12 mAb or anti–IFN-γ mAb before injection of α-GalCer alone or when cultured with α-GalCer and NKT cells. The effect of α-GalCer on the induction of IL-12 mRNA expression in spleen cells was examined by RT-PCR. As shown in Fig. 3, intravenous injection of α-GalCer into C57BL/6 mice caused the induction of mRNA for both IL-12Rβ1 and IL-12Rβ2 in spleen cells within 4 h. This upregulation of IL-12R was strongly blocked by administration of anti–IL-12 mAb or anti–IFN-γ mAb before injection of α-GalCer (Fig. 4). Moreover, the IL-12R induction by α-GalCer was almost completely abolished in both CD1d−/− and Vα14 NKT cell-deficient mice (Fig. 5, A and B). Thus, these results suggested that CD1d-dependent α-GalCer-induced IFN-γ production by NKT cells may be critically important for the upregulation of IL-12R on NKT cells. To provide direct evidence for this hypothesis, we measured the expression of IL-12R on purified NKT cells that were previously activated in the presence of DCs and α-GalCer, either in vitro or in vivo. Fig. 6 C shows that in vitro activation of spleen cells by DCs plus α-GalCer strongly induced the expression of IL-12R on NKT cells. Similar findings were made when mice were injected in vivo with α-GalCer (Fig. 5 D).

α-GalCer Synergistically Acts with Exogenously Administered IL-12 in the Activation of Natural Killing Activity and IFN-γ Production In Vivo. C57BL/6 mice were injected intravenously with α-GalCer, either in vivo or in vitro. Fig. 7 C shows that in vitro activation of spleen cells by DCs plus α-GalCer strongly induced the expression of IL-12R on NKT cells. Similar findings were made when mice were injected in vivo with α-GalCer (Fig. 5 D).

Discussion
The finding that NKT cells recognize α-GalCer presented by DCs in a CD1d-dependent manner represents a novel recognition mechanism in the immune system (15). NKT cells, which can produce both IFN-γ and IL-4 (16, 17), play an important role in immunoregulation and have been considered to play a central role as innate effector cells involved in both the protection and the onset of immune diseases (18). The NKT cell ligand α-GalCer has a strong immunopotentiating effect in vivo, and this chemical mediates strong antitumor activity (3–5, 9). Therefore, it is important to dissect the mechanism by which α-GalCer activates NKT cells.

The previous finding (3) that NKT-deficient mice did not respond to α-GalCer strongly suggested that NKT cells may be the primary target cells to α-GalCer. However, it...
IL-12 production by DCs appears to be essential for NKT cell activation by α-GalCer, because neutralization of endogenously produced IL-12 by anti–IL-12 mAb caused a strong inhibition of IFN-γ production by NKT cells. The important role of CD40/CD40L for the production of IFN-γ in the cocultures of DCs and NKT cells with α-GalCer is also apparent from these experiments (Fig. 2 B). As demonstrated in Fig. 2 C, DCs produce IL-12 only when they are cultured with α-GalCer in the presence of NKT cells, indicating that direct contact between α-GalCer–bound DCs and NKT cells may be essential for IL-12 production by DCs. This interaction may be required for the production of IFN-γ by IL-12–activated NKT cells, because mAbs directed against CD40/CD40L greatly inhibited IFN-γ production by NKT cells (Fig. 2). These findings indicate that the interaction of NKT cells with DCs may be very similar to the interaction of helper T cells with DCs (22, 26, 27). Since the interactions between DCs and NKT cells occur very quickly after administration of α-GalCer, NKT cells may be able to condition DCs very early in an immune response, and affect subsequent adaptive responses.

In this paper, we also demonstrate that α-GalCer upregulates IL-12R expression in vivo (Fig. 3). IL-12R upregulation is blocked by mAbs against IL-12 or IFN-γ and is absent in CD1d−/− and NKT-deficient mice (Figs. 4 and 5). Moreover, activation of NKT cells in vitro and in vivo results in a strong induction of IL-12R β1 and IL-12R β2 on these cells (Fig. 5, C and D). Therefore, we speculate that the following series of events is induced upon culture of α-GalCer with DCs and NKT cells: (a) α-GalCer first binds to CD1d molecules on DCs; (b) NKT cells recognize α-GalCer–bound DCs via their TCRs and also interact with DCs via CD40/CD40L; (c) during this interaction, DCs produce IL-12; (d) the endogenously produced IL-12 stimulates IFN-γ production by NKT cells; and (e) IFN-γ produced by NKT cells upregulates IL-12R on NKT cells in an autocrine manner. The dramatic synergistic effect of
suboptimal α-GalCer and exogenously administered IL-12 indicates that expression of IL-12R β1 and β2, detected by quantitative RT-PCR, is functionally upregulated in vivo. Moreover, since this synergistic effect of α-GalCer and IL-12 was not demonstrated in NKT-deficient mice, we conclude that in wild-type mice coadministration of α-GalCer and IL-12 leads to upregulation of IL-12R on CD1-dependent NKT cells.

Both α-GalCer and IL-12 have been demonstrated to exhibit potent antitumor activity in vivo. IL-12 has multiple effects on the immune system that are beneficial for the induction of antitumor immunity in vivo (28–30). However, the unexpected severe side effects of IL-12 have made it difficult to use this cytokine in clinical trials (31). We demonstrated that α-GalCer synergistically acts with small doses of IL-12 in vivo to activate NKT cells and to induce IFN-γ production (Fig. 6). These findings suggest that coadministration of α-GalCer with IL-12 could be used as a new approach for tumor immunotherapy.

Recent studies have demonstrated that Th1 immunity regulated by IL-12 and IFN-γ plays a critical role in the induction of protective immunity against tumors and infectious agents (32, 33). Although NKT cells are involved in both Th1 and Th2 immunity through IFN-γ or IL-4 production, the immunomodulating protocol using α-GalCer and IL-12 preferentially induces NKT cells that produce large amounts of IFN-γ (34). These NKT cells may facilitate the development of Th1-dominant cellular immunity essential for the induction of protective immunity against tumors and some infectious agents. Recently, it was demonstrated that α-GalCer can stimulate human NKT cells in a CD1d-dependent manner (35, 36), indicating that our proposed immunotherapy protocol using α-GalCer and IL-12 will be useful for the application to human immune diseases, including cancer.

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