function with mAb to IFN-γ reduce the efficacy of IL-12 (*16*, *17*). Although $V_\alpha14$ NKT cells are the major source of IFN-γ (*14*), IFN-γ may not be important in the effector phase. This is because high doses of mAbs to IFN-γ do not inhibit $V_\alpha14$ NKT cell–mediated cytolysis (Fig. 3E).

We examined the potential activity of NK and T cells in vitro in the mutant mice; both NK-mediated and T cell–mediated killing functions were potent. The NK activity induced by polyinosinic-polycytidylic acid [poly(I:C)] was as potent in $J_\alpha281^{-/-}$ mice as in wild-type mice (Fig. 4A). Similarly, significant allospecific CTL activity was detected on P815 (H-2^d) and BALB/c (H-2^d) concanavalin A (ConA) blasts, but not on EL-4 (H-2^b), in $J_\alpha281^{-/-}$ mice to the same extent as in wild-type mice (Fig. 4B). Thus, NK and conventional T cells in $J_\alpha281^{-/-}$ mice are functionally active, yet not indispensable for tumor rejection upon IL-12 stimulation.

The primary effect of IL-12 on $V_\alpha14$ NKT cells is also supported by the fact that IL-12 causes an increase in the actual numbers of $V_\alpha14$ NKT cells (about a fourfold increase; 1.5×10^5 to 6×10^5) and in their cell volume (1.3 to 2.5-fold increase) (*21*). It is now clear that a reevaluation of NK and T cell functions in the absence of $V_\alpha14$ NKT cells may alter our understanding of the functions of various subsets of lymphocytes in vivo.

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22. For measurement of the melanoma antigen (GM3 ganglioside), cells or tissues were homogenated. The homogenates of normal or metastatic livers (50 mg) or B16 melanoma were treated with 1 ml of extraction buffer containing 1% NP-40. Varied concentrations of melanoma cell homogenates were used to determine standard curves. Melanoma antigens in the extracts were quantified by radioimmunoassay with ^{125}I-labeled M2590 mAb to melanoma as described [S. Wakabayashi, S. Okamoto, M. Taniguchi, *Jpn. J. Cancer Res.* **75**, 427 (1984); Y. Gunji and M. Taniguchi, *ibid.* **77**, 595 (1986); G. A. Nores, T. Dohi, M. Taniguchi, S. Hakomori, *J. Immunol.* **139**, 3171 (1987)].
23. Cells (1×10^6) were first incubated with mAb to Fc γ receptors II and III (FcγRII/III) (2.4G2) to block nonspecific staining through FcγR, then the cells were incubated with fluorescein isothiocyanate–labeled anti-TCRβ (H57-597) and phycoerythrin–labeled anti-NK1.1 (PK136). Dead cells were excluded by propidium iodide staining, and 10^5 cells were analyzed by EPICS-ELITE (Coulter Electronics, Hialeah, FL) with a logarithmic amplifier as described (*5*).
24. $J_\alpha281^{+/+}$, $J_\alpha281^{-/-}$, and RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$ mice were injected with 2×10^6 B16 or FBL-3 cells in the spleen for liver metastasis, intravenously with 3×10^5 B16 or 2×10^6 LLC cells for pulmonary metastases, or subcutaneously with 2×10^6 B16 cells for subcutaneous tumor growth on day 0. Recombinant murine IL-12 (2400 U/mouse) or phosphate-buffered saline (PBS) was injected on days 3, 5, 7, and 9. On day 14, the mice were killed and either metastatic nodules counted or GM3 melanoma antigens measured by radioimmunoassay in the liver or lung as described (*22*). For subcutaneous tumor growth, injection of IL-12 or PBS was initiated on day 5, and the mice were treated five times per week. The diameters of tumors were measured every day with calipers. The sizes of tumors were expressed as the products of the longest diameter times the shortest diameter (in square millimeters).
25. Target cells were labeled with 100 μCi of sodium chloride (Amersham) per 5×10^6 cells for 1 hour. Effector cells were seeded in 96-well round-bottomed plates at indicated effector/target (E/T) ratios against 1×10^4 target cells. The release of ^{51}Cr from lysed target cells was counted on a γ-counter after a 4-hour incubation at 37°C in 5% CO_2. The percent of specific ^{51}Cr-release was calculated by the following formula: percent of specific lysis = (sample cpm – spontaneous cpm) × 100/(maximum cpm – spontaneous cpm). Spontaneous cpm was calculated from the supernatant of the target cells alone, and the maximum release was obtained by adding 1N HCl to target cells.
26. MAbs to H-2K^b (AF6-885), H-2D^b (KH-95), CD1d (1B1), $V_\beta8$ (MR5-2), NK1.1 (PK136), Ly49C (5E6), IFN-γ (R4-6A2), Fas (Jo2), and Fas ligand (K10) (Pharmingen) were used for blocking experiments.
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CD1d-Restricted and TCR-Mediated Activation of $V_\alpha14$ NKT Cells by Glycosylceramides

Tetsu Kawano, Junqing Cui, Yasuhiko Koezuka, Isao Toura, Yoshikatsu Kaneko, Kazuhiro Motoki, Hitomi Ueno, Ryusuke Nakagawa, Hiroshi Sato, Eisuke Kondo, Haruhiko Koseki, Masaru Taniguchi*

Natural killer T (NKT) lymphocytes express an invariant T cell antigen receptor (TCR) encoded by the $V_\alpha14$ and $J_\alpha281$ gene segments. A glycosylceramide-containing α-anomeric sugar with a longer fatty acyl chain (C_{26}) and sphingosine base (C_{18}) was identified as a ligand for this TCR. Glycosylceramide-mediated proliferative responses of $V_\alpha14$ NKT cells were abrogated by treatment with chloroquine–concanamycin A or by monoclonal antibodies against CD1d/$V_\beta8$, CD40/CD40L, or B7/CTLA-4/CD28, but not by interference with the function of a transporter-associated protein. Thus, this lymphocyte shares distinct recognition systems with either T or NK cells.

An unusual lineage of lymphocytes, $V_\alpha14$ NKT cells, are characterized by their development before thymus formation (*1*), their expression of an invariant TCR encoded by the $V_\alpha14$ and $J_\alpha281$ gene segments (*2*, *3*) mainly associated with $V_\beta8.2$ (*4*), and by the coexpression of the NK1.1 receptor, a marker of NK cells (*5*). The invariant $V_\alpha14$ TCR is essential for the development and function of $V_\alpha14$ NKT cells (*6*–*8*). Contrary to the general rule that the interaction of the TCR with the major histocompatibility complex (MHC) molecules leads to the development of T cells, $V_\alpha14$ NKT cells are selected by CD1d, a nonclassical class Ib molecule (*9*); mutant mice deficient in

CD1d lack $V_\alpha14$ NKT cells (*10*). However, a ligand for invariant $V_\alpha14$ TCR has not yet been identified. Here, we attempt to define a ligand with which to specifically activate $V_\alpha14$ NKT cells and characterize their activation mechanisms.

Because another CD1 molecule, human CD1b, presents glycolipids (*11*, *12*), and because the development and selection of $V_\alpha14$ NKT cells are independent of transporter-associated protein (TAP) essential for peptide presentation on MHC (*13*), we studied glycolipids as candidate $V_\alpha14$ TCR ligands. Synthetic glycolipids (*14*) were used to avoid effects of minor contaminants in biological samples. Moreover, we generated $V_\alpha14$ NKT mice expressing the invariant $V_\alpha14$ and $V_\beta8.2$ transgenes in a recombination activating gene (RAG)–deficient background ($RAG^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$) that only have $V_\alpha14$ NKT cells, but no T, B, or NK cells (*15*). Spleen cells from $V_\alpha14$ NKT mice were cocultured with fractionated dendritic cells (DCs) from NK-only ($RAG^{-/-}$) mice (no T, B, or $V_\alpha14$ NKT cells) pulsed with various glycosylceramides, and their proliferative responses were measured.

$V_\alpha14$ NKT cells incorporated [^{3}H]thymidine ([^{3}H]TdR) after stimulation with α-galactosylceramide (α-GalCer), whereas activation with ceramide itself or β-galactosylceramide (β-GalCer) resulted in no proliferative responses (Fig. 1, A and C). Because α-glucosylceramide (α-GlcCer) as well as α-GalCer stimulated $V_\alpha14$ NKT cells readily, the α-anomeric conformation of sugar moiety is essential. Indeed, in diglycosylated ceramides (Fig. 1, B and C), the α-anomeric configuration of the inner sugar is important. Galα1-6Galα1-1′Cer, Galα1-6Glcα1-1′Cer, Galα1-2Galα1-1′Cer, or Galβ1-3Galα1-1′Cer, whose inner sugar is either α-glucose or α-galactose despite their α- or β-anomer of the outer sugar moiety, could stimulate $V_\alpha14$ NKT cells, whereas Galβ1-4Glcβ1-1′Cer with the β-anomer inner sugar could not.

Because α-GalCer and α-GlcCer, which differ only in the configuration of the 4-hydroxyl group on the carbohydrate, showed no functional differences, the 4-hydroxyl configuration of the sugar seems not to be important. However, α-mannosyl ceramide (α-ManCer), which showed no stimulatory activity, has the 2-hydroxyl group with an axial configuration that differs from that with an equatorial bond on α-GalCer or α-GlcCer, suggesting the importance of the configuration of the 2-hydroxyl group on the sugar moiety, probably for the TCR contact site of this glycolipid (Fig. 1, A and C).

A mutant derivative lacking the 3- and 4-hydroxyl groups on the phytosphingosine of α-GalCer(3,4-deoxy α-GalCer) was not stimulatory, indicating that the 3,4-hydroxyl groups of the phytosphingosine are also important (Fig. 1, A and C). Although the 3-hydroxyl group of the sphingosine plays a crucial role in the sphingolipid-mediated fusion of Semliki Forest virus (*16*), we could not determine whether the 3- and 4-hydroxyl groups were important for the TCR contact sites or for the stabilization of glycolipid conformation.

CD1d is essential for this ligand presentation and recognition by the invariant $V_\alpha14$ TCR, because proliferative responses of $V_\alpha14$ NKT cells were abrogated by monoclonal antibody (mAb) against CD1d, $V_\beta8$, B7, CTLA-4, CD28, CD40, or CD40L but not against H-$2K^b$ or I-A^b (Fig. 2, A and B), indicating that α-GalCer–mediated stimulation of $V_\alpha14$ NKT cells is CD1d-restricted and TCR/costimulatory molecule–dependent. NKT cell hybridomas have been reported to have CD1d autoreactivity (*9*). This discrepancy may be explained by the different TCR expression of hybridomas made by the fusion of thymocytes lacking $V_\alpha14$ TCR expression on the surface (*3*). β_2-Microglobulin $(\beta_2M)^{-/-}$ DCs did not stimulate $V_\alpha14$ NKT cells, whereas $TAP^{-/-}$ DCs could (Fig. 2C), supporting nonpeptide ligand presentation by

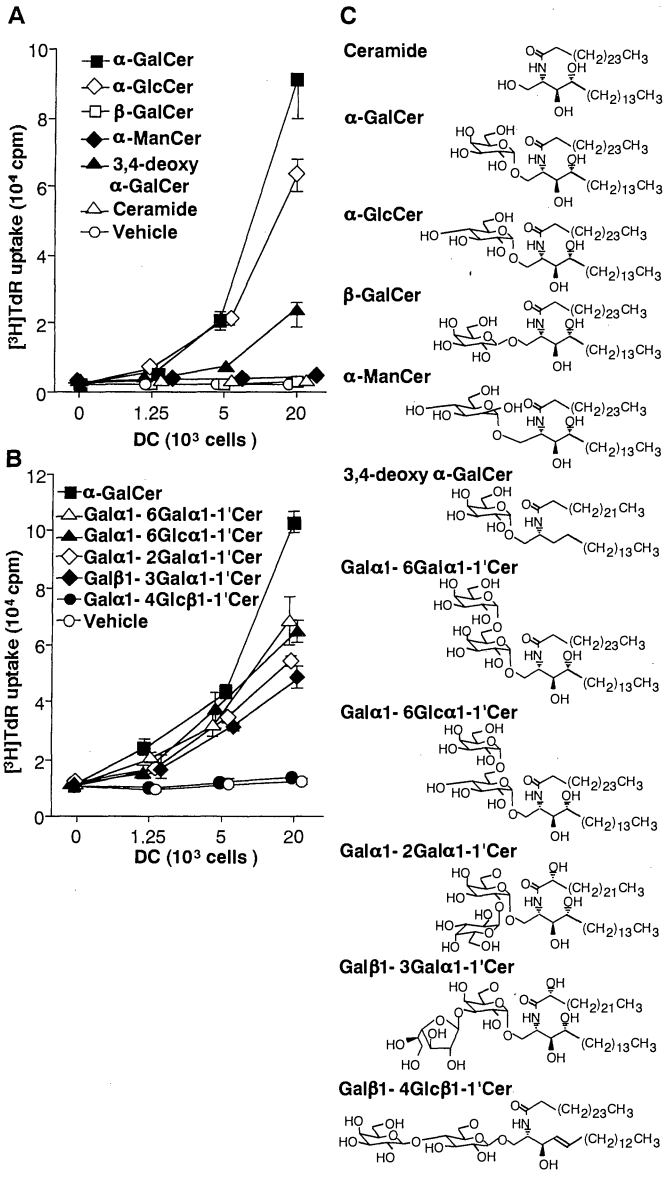

Fig. 1. Proliferative responses of $V_\alpha14$ NKT cells by glycosylceramides. Fractionated DCs prepared from $RAG^{-/-}$ mice as described by Crowley *et al.* (*30*) were pulsed with ceramide [100 ng/ml in 0.1% dimethyl sulfoxide (DMSO)–RPMI], glycosylceramides (100 ng/ml in 0.1% DMSO-RPMI), or control vehicle (0.1% DMSO-RPMI). Spleen cells (2×10^5) from $V_\alpha14$ NKT mice (*15*) were cocultured with pulsed DCs. Three days later, 0.5 μCi of [^{3}H]TdR was added for 12 hours and [^{3}H]TdR uptake was measured. (**A**) Stimulation with monoglycosylated ceramides. (**B**) Stimulation with diglycosylated ceramides. (**C**) Schematic representation of glycosylceramides. The results are expressed as mean counts per minute of three cultures ± SD.

T. Kawano, J. Cui, I. Toura, Y. Kaneko, H. Sato, E. Kondo, H. Koseki, M. Taniguchi, CREST (Core Research for Evolutional Science and Technology) Project, Japan Science and Technology Corporation (JST), and Division of Molecular Immunology, Center for Biomedical Science, School of Medicine, Chiba University, 1-8-1 Inohana, Chuo, Chiba 260, Japan.
Y. Koezuka, K. Motoki, H. Ueno, R. Nakagawa, Pharmaceutical Research Laboratory, Kirin Brewery, 3 Miyahara, Takasaki, Gunma 370-12, Japan.
*To whom correspondence should be addressed. E-mail: taniguti@med.m.chiba-u.ac.jp

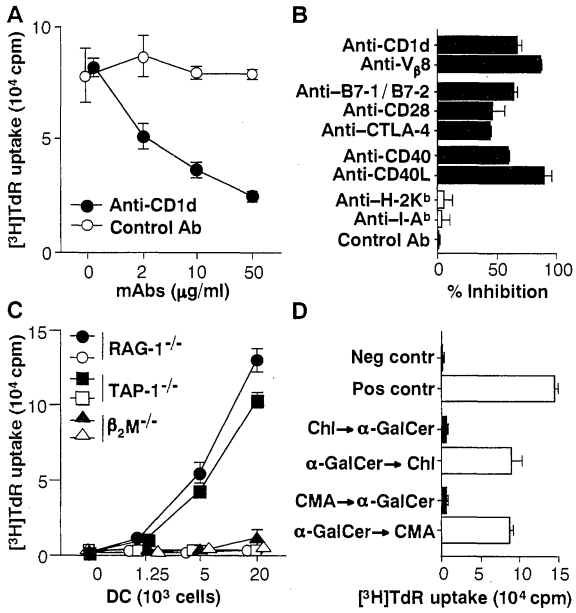

Fig. 2. Mode of recognition and activation of $V_\alpha14$ NKT cells in the induction phase. Cells were prepared as described in Fig. 1 except for the materials indicated below. (**A**) CD1d-dependent recognition. DCs (2×10^4) preincubated with anti-FcγR (50 μg/ml) (2.4G2) were reacted with anti-CD1d (1B1; Pharmingen) or its control antibody (rat immunoglobulin $G_{2b,\kappa}$). (**B**) Blocking of $V_\alpha14$ NKT cell activation. Monoclonal antibodies (50 μg/ml; Pharmingen) against CD1d (1B1), $V_\beta8$ (MR5-2), B7-1(1G10), B7-2 (PO3.1), CD28 (37.51), CTLA-4 (UC10-4F10-11), CD40 (HM40-3), CD40L (MR1), H-2K^b (AF6-88.5), and I-A^b (AF6-120.1), or control mAb, were used. The data were expressed as percent inhibition of the experimental counts per minute over the control counts per minute (81,336 ± 4050 counts per minute). (**C**) Requirement of MHC class I–like molecules but not TAP for stimulation of $V_\alpha14$ NKT cells. α-GalCer–pulsed (closed symbols) or vehicle-pulsed (open symbols) DCs prepared from $\beta_2M^{-/-}$, $TAP^{-/-}$, or $RAG^{-/-}$ mice were used (*30*). (**D**) Effects of Chl or CMA on α-GalCer presentation by DCs. Chl (100 μM; Sigma) or CMA (10 nM; Wako Pure Chemical Industries) was added to the culture of DCs (2×10^4) 1 hour before (Chl or CMA→α-GalCer) or 2 hours after (α-GalCer→ Chl or CMA) the beginning of the 4-hour pulse with α-GalCer. α-GalCer– or vehicle-pulsed DCs without treatment were used as positive (Pos contr) or negative (Neg contr) controls, respectively. The results are expressed as mean counts per minute of three cultures ± SD.

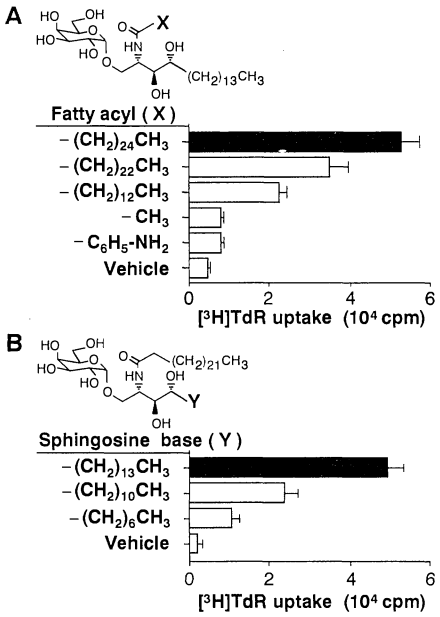

Fig. 3. Effects of lengths of fatty acyl chain and sphingosine base of α-GalCer on activation of Vα14 NKT cells. α-GalCers with different lengths of fatty acyl chains as indicated by X (**A**) and those of sphingosine base as indicated by Y (**B**) were used. The results are expressed as mean counts per minute of three cultures ± SD.

class Ib molecule. On the basis of an analogy with lipoglycan presentation (*12*), it is conceivable that chloroquine (Chl) or concanamycin A (CMA), which prevents acidification or transportation to late endosomes (17), could inhibit α-GalCer presentation by DCs. Treatment of DCs with these drugs before pulse with α-GalCer inhibited proliferation of $V_\alpha14$ NKT cells, whereas treatment after pulse with α-GalCer failed. This finding suggests a requirement for endosomal function in α-GalCer presentation (Fig. 2D).

The structural and functional relation between the lengths of fatty acyl chain and sphingosine base and activity of α-GalCer was examined (Fig. 3, A and B). The most effective lengths of fatty acyl chain and sphingosine base were C_{26} and C_{18}, respectively, whereas the short fatty acyl or short sphingosine base lost their activity, indicating the hydrophobic interaction of α-GalCer with CD1d. The α-GalCer with fatty acyl (C_{26}) and sphingosine base (C_{18}) is estimated to be about 34 Å long, with the fatty acyl chain, the sphingosine base, and the sugar moiety being 28, 17, and 8 Å long, respectively (*18*). Recent studies on the crystal structure of CD1d molecule indicate that the binding groove has two large hydrophobic pockets about 30 Å long and 10 to 15 Å wide (*19*). Therefore, the findings indicate that the α-GalCer with fatty acyl (C_{26}) and sphingosine base (C_{18}) may be suitable for binding to these two pockets of CD1d, possibly through hydrophobic interactions.

To investigate the selectivity of $V_\alpha14$ NKT cell activation with α-GalCer, we cultured spleen cells with α-GalCer from wild-type littermates and $V_\alpha14$ NKT-deficient, $V_\alpha14$ NKT, and NK-only mice whose FACS (fluorescent-activated cell sorting) profiles are shown in Fig. 4A (top). Proliferative responses were observed in $V_\alpha14$ NKT and wild-type mice, but not in the mice without $V_\alpha14$ NKT cells (NK-only mice or $V_\alpha14$ NKT-deficient mice) (Fig. 4A). In addition, $V_\alpha14$ NKT mice produced large amounts of interleukin-4 (IL-4) and interferon γ (Fig. 4B) and also killed the YAC-1 cells upon stimulation with α-GalCer, whereas $V_\alpha14$ NKT-deficient or NK-only mice did not (Fig. 4C). Thus, α-GalCer selectively activates $V_\alpha14$ NKT cells in vivo but not other lymphocytes. Vα14 NKT cells directly kill target tumor cells by an NK-like mechanisms and inhibit tumor growth and metastasis in vivo after activation with α-GalCer (*20*) or IL-12 (*8*), confirming the previous data on the protection of tumor metastasis by the treatment of tumor-bearing mice with α-GalCer (*21*).

Monogalactosylceramide is the smallest size glycosphingolipid, but β-GalCer has been detected mainly in mammals (*22*). Most mammalian normal tissues have ceramides composed of sphingosine with the 4,5-*trans* carbon-carbon double bond in the sphingosine backbone, whereas α-GalCer has phytosphingosine without this carbon double bond (*23*). Although α-GalCer has been isolated from marine sponges and has been hardly detected in normal mammalian tissues (*24*), a form of α-anomeric monoglycolipid or glycolipid with phytosphingosine has been detected in certain bacteria (*25*) and in some conditions of mammalian tissues, such as fetus (*26*), cancer cells (*27*), kidney or intestine (*28*), or in some cultured cells (*29*). An α-glycosylceramide or a natural ligand similar to this glycolipid may thus exist in restricted mammalian tissues or be expressed on cells after activation or during malignancy.

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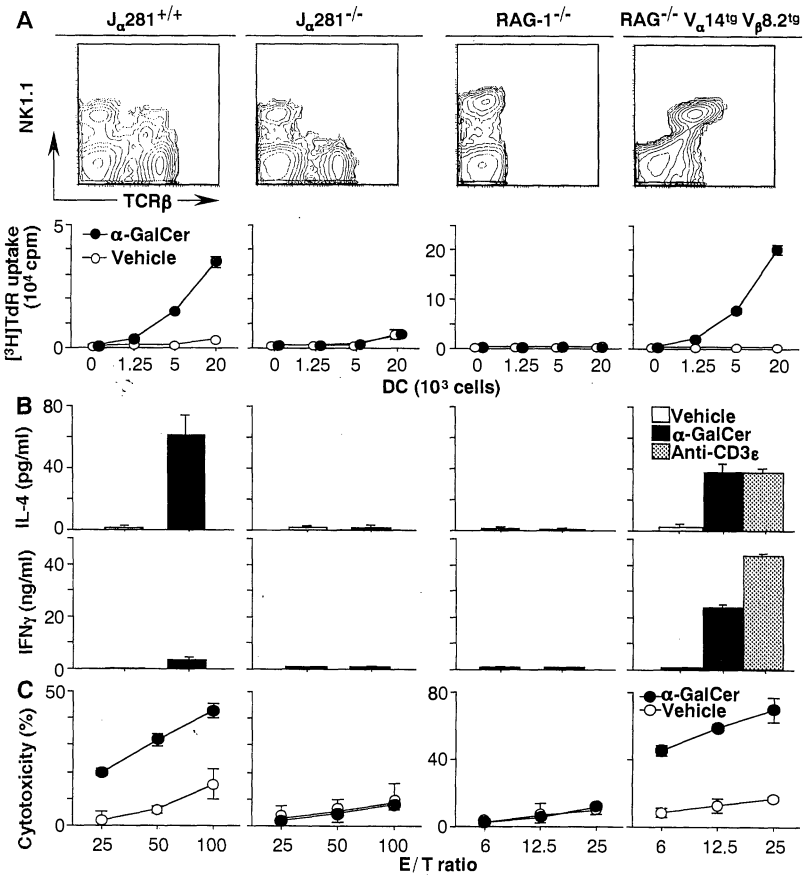

Fig. 4. Selective stimulation of $V_\alpha14$ NKT cells by α-GalCer. (**A**) FACS profiles and in vitro proliferative responses. α-GalCer–pulsed DCs were cocultured with spleen cells from wild-type ($J_\alpha281^{+/+}$) mice, $V_\alpha14$ NKT-deficient ($J_\alpha281^{-/-}$) mice, NK-only (RAG-$1^{-/-}$) mice, or $V_\alpha14$ NKT (RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$) mice as described in Fig. 1. Profiles by FACS analysis (*31*) are also shown. (**B**) Production of IL-4 and IFN-γ by α-GalCer activation. Spleen cells (2×10^5) were cultured at 37°C for 48 hours with α-GalCer (100 ng/ml), immobilized anti-CD3ε (10 μg/ml) (2C11; Pharmingen), or control vehicle. Cytokine production in the supernatants was assayed by an enzyme-linked immunosorbent assay (ELISA) kit (Endogen). (**C**) α-GalCer–mediated cytotoxicity in vitro. Mice were injected intraperitoneally with either α-GalCer (100 μg/kg in 0.025% Polysolvate 20), or vehicle–phosphate-buffered saline (PBS) (0.025% Polysolvate 20 in PBS). Twenty-four hours later, spleen cells were assayed for 4 hours on ^{51}Cr-labeled YAC-1 (H-$2^{k/d}$) cells (1×10^4) as described (*8*). The results are expressed as mean counts per minute of three cultures ± SD.

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14. Synthetic glycolipids used were as follows: α-GalCer, (2*S*, 3*S*, 4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol; ceramide, (2*S*, 3*S*, 4*R*)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol; β-GalCer, (2*S*, 3*S*, 4*R*)-1-*O*-(β-D-galactopyranosyl)- *N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol; α- GlcCer, (2*S*, 3*S*, 4*R*)-1-*O*-(α-D-glucopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol; 3,4-deoxy α-GalCer, [(2*S*)-1-*O*-(α-D-galactopyranosyl)-*N*-tetracosanoyl-2-amino-1-octadecanol]; α-ManCer, (2*S*, 3*S*, 4*R*)-2-amino-*N*-hexacosanoyl-1-*O*-(α-D-mannopyranosyl)-1,3,4-octadecanetriol; Galα1-6Galα1-1′Cer, (2*S*, 3*S*, 4*R*)-2-amino-1-*O*-(α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl)-*N*-hexacosanoyl-1,3,4-octadecanetriol; Galα1-6Glcα1-1′Cer, (2*S*, 3*S*, 4*R*)-2-amino-1-*O*-(α-D-galactopyranosyl-(1-6)-α-D-glucopyranosyl)-*N*-hexacosanoyl-1,3,4-octadecanetriol; Galα1-2Galα1-1′Cer, (2*S*, 3*S*, 4*R*)-2-amino-1-*O*-(α-D-glucopyranosyl-(1-2)-α-D-galactopyranosyl)-*N*-[(*R*)-2-hydroxytetracosanoyl]-1,3,4-octadecanetriol; Galβ1-3Galα1-1′Cer, (2*S*, 3*S*, 4*R*)-2-amino-1-*O*-(β-D-galactofuranosyl-(1-4)-α-D-galactopyranosyl)-*N*-[(*R*)-2-hydroxytetracosanoyl]-1,3,4-octadecanetriol; Galβ1-4Glcβ1-1′Cer, (2*S*, 3*S*, 4*E*)-2-amino-1-*O*-(β-D-galactopyranosyl-(1-4)-β-D-glucopyranosyl)-*N*-hexacosanoyl-4-octadecene-1,3-diol was purchased from Sigma.
15. $V_\alpha14$ NKT (RAG$^{-/-}$ $V_\alpha14^{tg}$ $V_\beta8.2^{tg}$) mice with a C57BL/6 background were obtained by mating RAG$^{-/-}$ $V_\beta8.2^{tg}$ mice and RAG$^{-/-}$ $V_\alpha14^{tg}$ mice generated by mating RAG$^{-/-}$ mice with $V_\beta8.2^{tg}$ or $V_\alpha14^{tg}$ mice, respectively (*6*). All mice were maintained in pathogen-free animal facilities. $V_\alpha14$ NKT mice have only $V_\alpha14$ NKT cells but no other lymphocytes. Lack of NK cells in the mice was also observed in $V_\beta8^{tg}$ mice, suggesting that the transgene of $V_\beta8$ blocks NK cell development (*6*).
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30. M. Crowley, K. Inaba, M. Witmer-Pack, R. M. Steinman, *Cell. Immunol.* **118**, 108 (1989). For preparation of fractionated DCs, spleens were digested with collagenase type III (400 U/ml; Worthington Biochemical). Single-cell suspension was loaded on a dense bovine serum albumin and centrifuged at 1500*g* for 30 min. The low-density fraction was further applied to culture dishes for 1.5 hours. Adherent cells were cultured overnight with ceramide, glycosylceramides, or control medium. The nonadherent cells in the overnight culture were used as pulsed DCs. Pulsed DCs from $\beta_2M^{-/-}$ or TAP$^{-/-}$ mice were x-rayed (30 gray) to eliminate contaminated lymphocytes before assay.
31. Cells (1×10^6) preincubated with antibody to FcγR (anti-FcγR) (2.4G2; Pharmingen) were stained with fluorescein isothiocyanate–conjugated anti-TCRβ (H57-597; Pharmingen) and biotin-conjugated anti-NK1.1 (PK136; Pharmingen) with avidin-conjugated Cy-Chrome (Pharmingen). Dead cells were gated out by propidium iodide staining, and live cells were analyzed by EPICS-XL (Coulter Electronics) with a logarithmic amplifier.

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