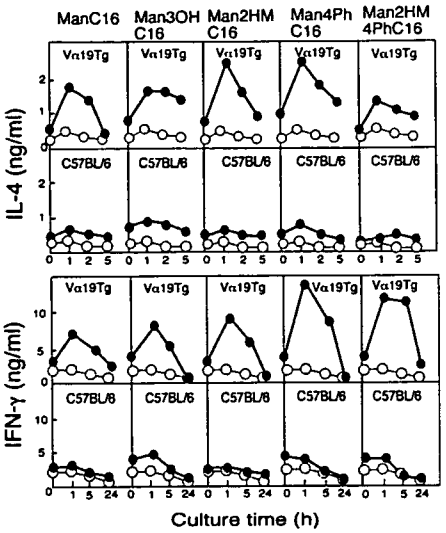

Figure 4. Priming of Vα19 NKT cells *in vivo* with α-ManCer derivatives. Spleen cells from Vα19 Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice previously injected intravenously with glycolipids (20 μg/animal) or vehicle (DMSO) in 200 μL PBS *via* the tail were cultured for the period indicated. Culture supernatants were harvested and tested for production of cytokines at the indicated time points. The thick line with dots and the fine line in each panel represent the cytokine production in the culture with glycolipids and vehicle, respectively. Three independent experiments were performed and essentially the same profiles were obtained.

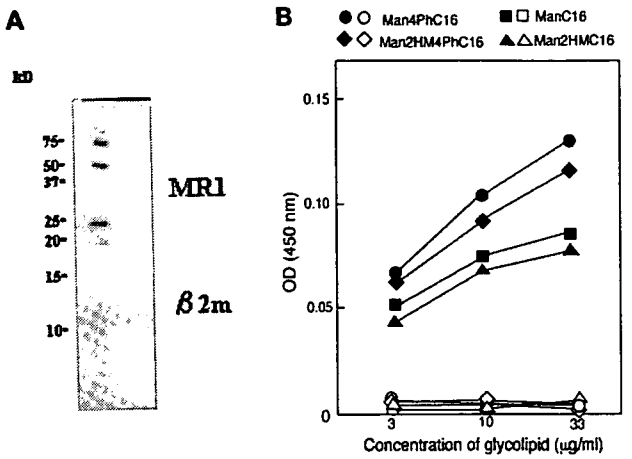

Figure 5. Binding of α-ManCer analogues to immobilized MR1. (A) The MR1/ ß2m proteins expressed in *E. coli* proteins were analyzed by SDS polyacrylamide gel electrophoresis. (B) Binding of α-ManCer analogues to immobilized MR1. MR1/ ß2m proteins (3 μg/mL) were immobilized on a 96-well plate. After washing, α-ManCer analogues were added to the wells and incubated. The glycolipids bound to the MR1 proteins were detected with peroxidase-conjugated Con A. Results are shown as difference in color development (OD 450 nm) from the well added by α-GalCer. One representative experiment of four is shown. Closed symbols represent the binding of peroxidase-conjugated Con A to the well previously coated with the MR1/ ß2m proteins, whereas open symbols represent the binding to the well without coating with the proteins.

MR1-restricted stimulation of Vα19 Tg^+ cells with the α-ManCer derivatives

MHC restriction of the immune responses by Vα19 NKT cells to the α-ManCer derivatives was examined. Affinity of α-ManCer derivatives to immobilized MR1/ß2m proteins were suggested by the binding assay shown in Fig. 5. The binding of α-ManCer derivatives to the plastic well previously coated with MR1/ ß2m proteins was indirectly detected by the subsequent binding of Con A to the glycolipids. The binding of α-ManCer derivatives to MR1 was further supported by the observation that Vα19 Tg^+ cells were activated to some degree when they were cultured in the plastic wells pre-coated with MR1/ ß2m proteins followed by α -ManCer derivatives (Supporting Information online).

Next, MR1-restricted immune responses in culture by Vα19 Tg^+ cells were tested (Fig. 6). Liver MNC from Vα19 Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice were cultured with the α-glycosphingolipid analogues in the presence of anti-MR1 antiserum or pre-immune serum, and the responses were analyzed (Fig. 6C). The immune responses by Vα19 NKT cells to the α-ManCer derivatives were drastically reduced in the presence of anti-MR1 antiserum in culture. On the other hand, the antiserum had no effects on the responses of C57BL/6 liver MNC (where CD1d-restricted Vα14 NKT cells represent about 30% of population) toward α-GalCer (GalC24).

MR1 restriction was further supported by the immune responses of Vα19 Tg^+ cells toward MR1-transfectants. $CD5^+$ cells were prepared from the liver MNC of Vα19 Tg^+ $TCR\alpha^{-/-}$ or $\beta2m^{-/-}$ mice to enrich T-lineage cells, and they were stimulated with the MR1-transfected or non-transfected cells of a B lymphoma line (Raji) [19] previously loaded with α-ManCer derivatives (Fig. 6D). The $CD5^+$ cells from Vα19 Tg^+ $TCR\alpha^{-/-}$ but not $\beta2m^{-/-}$ livers responded to the stimulation with the MR1-transfectants treated with the α-ManCer derivatives.

Taken together, it is strongly suggested by these findings that Vα19 NKT cells respond to α-ManCer derivatives that are presented by non-classical MHC class I-like MR1 molecules.

Discussion

In this study, we demonstrated that TCR engagement of Vα19 NKT cells was induced by the stimulation with the modified α-ManCer that were presented by MR1-transfectants (Fig. 6) or immobilized MR1/ ß2m proteins (Supporting Information). Either Th1- or

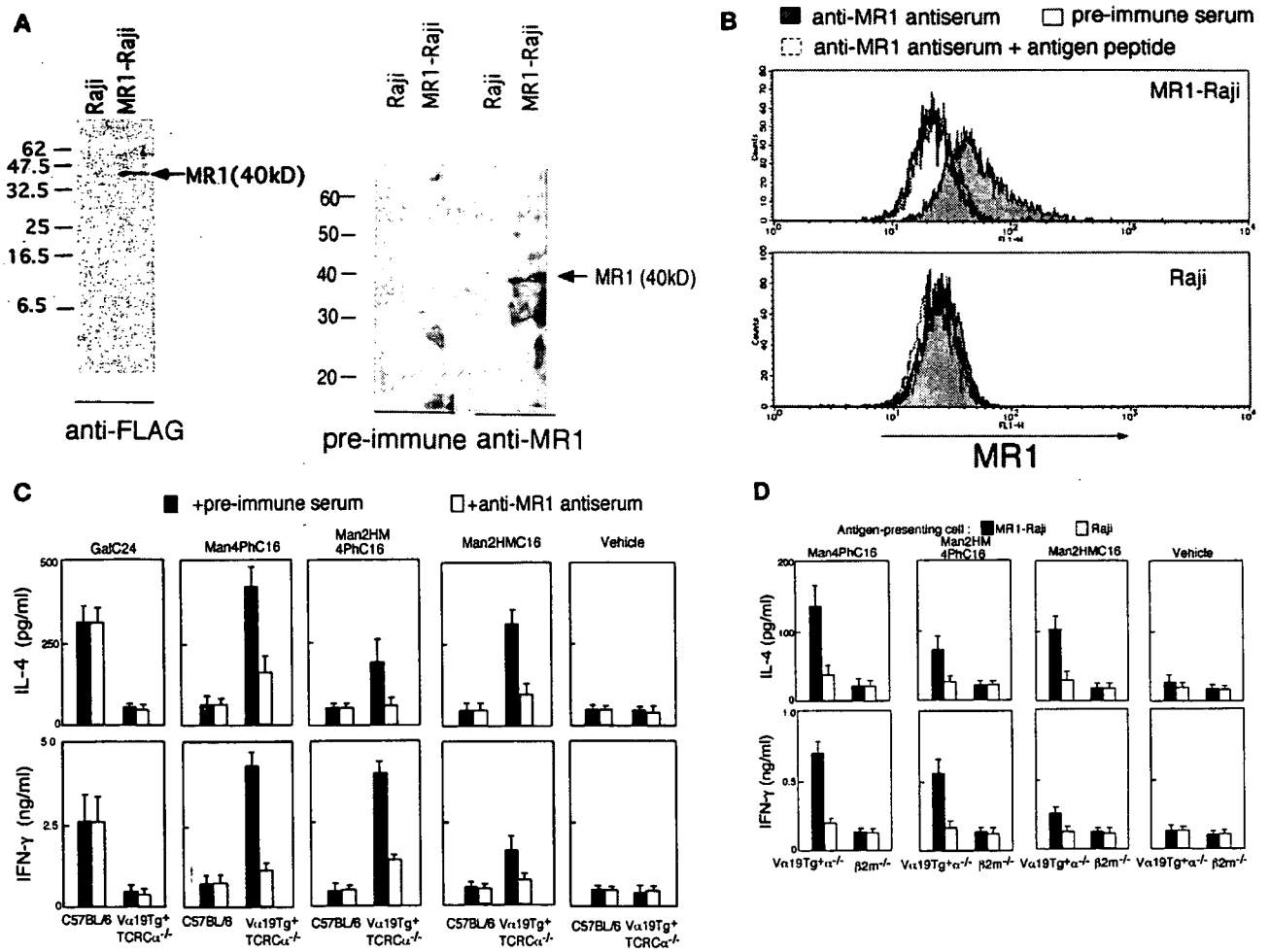

Figure 6. Stimulation of Vα19 NKT cells with α-ManCer derivatives in the context of MR1. (A)Preparation of MR1-transfectants. MR1 cDNA tagged with FLAG was expressed in Raji cells. Cell lysates were analyzed by Western blotting. A 40-kDa band was specifically stained with the partially purified anti-MR1 antiserum for the MR1-Raji cells corresponding to the band stained with anti-FLAG antibody. (B) Expression of MR1 by MR1-transfected cells. MR1- or mock-transfected Raji cells were stained with anti-MR1 antiserum in the presence or absence of the partial MR1 peptide used as an antigen or pre-immune serum peptide and analysed by FACS. (C) Inhibition of the immune responses in the presence of anti-MR1 antiserum. Liver MNC prepared from Vα19 TCR Tg^+ TCR $\alpha^{-/-}$ or C57BL/6 mice were stimulated with α-ManCer derivatives for 2 days in the presence of anti-MR1 antiserum or pre-immune serum. Cytokines were measured by ELISA. The average of triplicate culture in one of the representative three experiments is shown. (D) Stimulation of Vα19 NKT cells with α-ManCer derivatives presented by MR1 transfectants. T-lineage cells ($CD5^+$ cells) were enriched from liver MNC of Vα19 TCR Tg^+ TCR $\alpha^{-/-}$ or $\beta2m^{-/-}$ mice. They were stimulated with MR1-transfected or mock-transfected Raji cells, previously loaded with α-ManCer derivatives. Immune responses were determined on day 2 of culture by measuring the secretion of IL-4 and IFN-γ. The average of triplicate cultures in one of the representative three experiments is shown.

Th2-dominant immune responses of Vα19 NKT cells were brought by the administration of α-ManCer analogues not only *in vitro* but also *in vivo* (Fig. 4); thus the potentials as an immune modulator were found in the glycolipids.

The structural modification of the sphingosine moiety of the α-ManCer analogues could alter the interaction between the antigen-presenting MHC molecule, MR1. It has recently been reported that 2-hydroxymethyl 4-phenyl sphingosine (FTY720) and the related compounds mimic sphingosine-1-phosphate and work as an agonist for sphingosine-1-phosphate receptor [20]. This finding suggests that the modified α-ManCer as well as the natural α-ManCer are capable of being presented by MR1. The altered interaction between the modified α-ManCer and MR1 could presumably influence the spatial location of the α-mannosyl residue to be recognized by the invariant Vα19 TCR and eventually result in the modulation of the immune responses of Vα19 NKT cells. Induction of Th2-

biased immune responses of Vα14 NKT cells with the α-GalCer consisting of a short sphingosine base has been reported [21]. It is proposed in the report that the sporadic stimulation of the invariant Vα14 TCR with the galactose residue of the modified α-GalCer causes insufficient transcription of c-Rel, which is responsible for the expression of Th1 cytokines such as IFN-γ [22]. Provided that this speculation on the immune responses of Vα14 NKT cells is applicable to those of Vα19 NKT cells, introduction of 4-phenyl or 2-hydroxymethyl group into the sphingosine portion of α-ManCer is suggested to stabilize the interaction with MR1 and sustain the stimulation of the invariant Vα19 TCR with the modified α-ManCer, because the modified α-ManCer induced more IFN-γ production in Vα19 NKT cells than α-ManCer without substitution. In fact, the affinity of Man2HM4PhC16 or Man4PhC16 to immobilized MR1 proteins was suggested to be larger than that of ManC16 by the binding assay (Fig. 5). However, the supposed binding affinity of the glycolipids to the MR1 antigen-presenting groove did not necessarily parallel with the activity to induce cytokine production, especially IL-4 production, by Vα19 NKT cells. For example, Man2HMC16 induced Vα19NKT cells to produce more IL-4 than Man2HM4PhC16 or ManC16. The special location of the α-mannosyl residue of the glycolipids, which is influenced by the interaction between the glycolipid and MR1, may also be important to determine the antigenic activity in addition to the stability of the interaction between them.

Antigen nonspecific polyclonal activation of Vα19 cells in culture with immobilized anti-CD3 antibody induced the cytokine production, where the ratio of IL-4/IFN-γ was similar to that when Vα19 cells were stimulated with Man4PhC16 (data not shown). Based on this standard, the cytokine releases of Vα19 NKT cells induced with ManC16, Man3OHC16, Man2HMC16 and Man2HMC24 are assigned to be Th2 biased, whereas those with Man2HM4PhC16 are Th1 biased (Fig. 2D). To our knowledge, the present finding on Man2HM4PhC16 is the first model for the induction of Th1-dominant immunity by administration of glycolipid activators.

Vα19 NKT cells sorted from MNC of Vα19 TCR Tg^+ $TCR\alpha^{-/-}$ livers had potentials to secrete both IL-4 and IFN-γ following TCR engagement. Nevertheless, it is possible that cytokine production found in the cells from Vα19 Tg mice in part arose not only from the Vα19 NKT cells but also from the bystander cells. In fact, intracellular IFN-γ production in $NK1.1^+$ TCR^- and $NK1.1^-$ TCR^+ cells as well as $NK1.1^+$ TCR^+ cells in the Vα19 Tg cells were found following TCR engagement with anti-CD3 antibody (submitted for publication). Similarly, cells in the other lineage such as dendritic cells in the culture may contribute to producing IL-12, for instance, in a secondary manner. However, it should be noted that the specific TCR engagement of Vα19 NKT cells with the modified α-ManCer triggers the following characteristic cytokine production of Vα19 Tg cells *in vitro* and *in vivo* that will contribute to the regulation of Th1/Th2 homeostasis.

Vα19 and Vα14 NKT cells are suggested to share roles in the regulation of the immune system despite being subjected to the controls of independent MHC restriction and antigen specificity. Presumably, these two repertoires are individually involved in certain immune regulatory functions. Recently, localization of Vα19 but not Vα14 invariant TCR α chain-bearing cells in gut lamina propria was reported [13]. In addition, we found that they accumulated in the lesions of patients suffering from multiple sclerosis and autoimmune inflammatory neuropathy [23]. We also found that over-generation of invariant Vα19 TCR^+ cells inhibited the induction of experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis [24]. These findings suggest the possible functional sharing among these subsets. Hence, a series of the α-ManCer derivatives will be important as a prospective immunotherapeutic reagent selectively targeting Vα19 NKT cells for various autoimmune diseases in either Th1 or Th2 excess.

Materials and methods

Synthetic glycolipids

α-ManCer consisting of 3-hydroxy sphingosine (Man3OHC16, Fig. 1) was synthesized as previously described [15]. Other synthetic glycolipids consisting of immunosuppressant FTY720 and its related compounds listed in Fig. 1 were synthesized as described [18].

Mice

C57BL/6 mice and ß2m-deficient ($ß2m^{-/-}$) mice with the C57BL/6 genetic background were obtained from Sankyo Service (Tokyo, Japan) and Jackson Laboratory (Bar Harbor, ME). TCR Cα-deficient mice [25], originally with the 129 genetic background that were backcrossed with C57BL/6 mice for ten times, were a gift of Dr. H. Ishikawa (Keio University).

Vα19-Jα26 invariant TCR Tg mice with the TCR Cα-deficient background were established as described previously (submitted for publication, [15]). In brief, a Vα19-Jα26 invariant TCR gene segment amplified from the genomic DNA of hybridoma NB403 [11] was linked to the TCR α chain promoter, constant, and enhancer regions. The transgene thus produced was introduced into the fertilized eggs of TCR $\alpha^{-/-}$ mice and a Tg line was established. The Tg^+ cells preferentially develop as NKT cells in the Tg mice. The Tg^+, $NK1.1^+$ population accounts for 30% of total MNC in the Tg liver.

All the experiments using mice were reviewed and approved by the experimental animal committee of Mitsubishi Kagaku Institute of Life Sciences.

Bioassay of the synthesized glycolipids in culture

MNC used as responders were prepared from mouse livers by density gradient centrifugation using Percoll (Pharmacia, Uppsala, Sweden) as described previously [26]. Liver MNC from indicated strains of mice (2~4 months of age) were cultured in DMEM supplemented with 10% FCS, 100 U/mL penicillin, 50 μg/mL streptomycin and 5×10^{-5} M 2-mercaptoethanol (10^6 cells/200 μL) in the presence of glycolipids. Glycolipids were dissolved in DMSO and were added to the culture at the final concentration of 2 μg/mL. Each culture included 1/200 v/v of DMSO (used as the vehicle). Concentrations of cytokines in the culture supernatants were determined by ELISA using ELISA kits (PharMingen, San Diego, CA). The error bars indicate the standard deviation. Statistical analysis (Dunnett's multiple comparison post-test) was applied to some results of the immune responses in comparison with the control (the immune responses in culture only with the vehicle).

In some experiments, liver MNC were treated with biotinylated anti-NK1.1 mAb (PK136, PharMingen) or anti-TCR Cß (H57–597, PharMingen) and then with magnetic beads coated with streptavidin (Dynal Biotech, Oslo, Norway) to remove $NK1.1^+$ or $TCR C\beta^+$ cells before culture. The percentage of $NK1.1^+$ or $TCR\alpha\beta^+$ cells after treatments with magnetic beads was less than 3%. In other cases, liver MNC were treated with magnetic beads coated with anti-B220 antibody (Dynal Biotech). After removal of $B220^+$ cells, the remaining cells were then treated with magnetic beads coated with anti-CD5 antibody (Miltenyi Biotech, Gladbach, Germany). The $CD5^+$ cells were enriched by MACS and were used as responders. The percentages of $TCR\alpha\beta^+$ cells in the $CD5^+$ and $CD5^-$ fractions were in the range of 91–96 and 1–4%, respectively.

MR1-transfectants

MR1-transfectants were prepared from cells of a human Burkitt's B lymphoma line, Raji (ATCC) [19]. Mouse MR1 A cDNA [27] was amplified from genomic DNA of C57BL/6 spleen cells using RT-PCR kits (Takara, Tokyo, Japan).The following PCR primers were used for: (5′-MR1), 5′-ATGATGCTCCTGGTTACCTGG-3′; (FLAG-3′-MR1), 5′-CTACTTGTCATCGTCATCCTTGTAGTC(FLAG)-AGAGGGAGAGCTTCCCTCAT-3′.

The PCR product was cloned into pGEM-Teasy (Promega, Madison, WI). The MR1 cDNA was recombined into a eukaryotic expression vector, pCXN [28] (provided by Dr. J. Miyazaki, Osaka University). The expression vector was transfected into Raji cells. The transfectants were selected in the culture medium containing G418 (1 mg/mL) for 1 month. The expression of FLAG (Asp-Tyr-Lys-Asp-Asp-Asp)-MR1 in the transfectants was analyzed by Western blot using the combination of anti-FLAG antibody (Stratagene, La Jolla, CA) and HRP-labeled anti-mouse immunoglobulin (Sigma, St. Lewis, MO) or anti-MR1 antiserum (described below) and HRP-labeled anti-rabbit immunoglobulin (Sigma).

Generation of anti-MR1 antiserum

Rabbits were immunized with a keyhole limpet hemocyanin-conjugated polypeptide corresponding to the α2 domain of mouse MR1 (residue 139–161) [27] with Freund's complete adjuvant (Sigma). Anti-MR1 antiserum was concentrated by affinity chromatography using Sepharose 4B (Pharmacia) conjugated with the antigen peptide.

Cytometric analysis of MR1-transfectant cells

MR1-transfectant cells were pretreated with anti-CD16 mAb (LNK16, Dainippon Pharma, Tokyo, Japan). After immunostaining of the cells with rabbit anti-MR1 antiserum, they were treated with an FITC-labeled second antibody (donkey IgG $F(ab')_2$ fragment against rabbit IgG, Jackson Laboratory). Mouse cells were pretreated with anti-Fc receptor antibody (2.4G2), then stained with anti-TCR Cß antibody (H57–597) or anti-NK1.1 antibody (PK136). The antibodies were obtained from Becton Dickinson (San Jose, CA). The stained cells were analyzed on a flow cytometer (FACScan, Becton Dickinson) equipped with Cell Quest software.

Stimulation of Vα19 Tg^+ cells with MR1-transfectants

MR1- and mock-transfected Raji cells (1×10^5) were incubated in DMEM with glycolipids (5 μg/mL) for 18 h and washed with DMEM twice. After irradiation (3000 rad), they were cocultured with liver MNC (1×10^6) in DMEM (200 μL) for 2 days. Immune responses by the liver MNC in the mixed lymphocyte reactions were monitored by measuring concentrations of cytokines in the culture fluid by ELISA.

Stimulation of Vα19 Tg^+ cells *in vivo*

Stimulation of lymphocytes *in vivo* was performed as reported [29]. Vα19 Tg^+ TCR $\alpha^{-/-}$ or C57BL/6 mice (8-20 weeks of age) were intravenously injected with glycolipids (20 μg/200 μL PBS) instead of anti-CD3 antibody. Spleens were removed from mice 90 min after the injection. MNC were immediately prepared from them by density gradient centrifugation using lymphosepar II (IBL, Gunma, Japan, d = 1.090). They were cultured in DMEM (10^7 cells /mL). Cytokine concentration in the culture fluid was determined by ELISA.

Expression of MR1 proteins

MR1 A and ß2m cDNA were amplified from C57BL/6 spleen cells [27] using RT-PCR kits (Takara, Tokyo, Japan). MR1 cDNA with a sequence for a BirA site [30] was cloned into pRSET expression vector (Invitrogen, Carlsbad, CA). Similarly, ß2m cDNA was cloned into pET28a vector (Novagen, San Diego, CA). The proteins were expressed in BL21(DE3) codon plus RIL host cells (Stratagene, La Jolla, CA). The expressed proteins were dissolved in 6 M guanidine hydrochloride solution. MR1 proteins were labeled with biotin using a labeling kit (Roche, Mannheim, Germany), and purified by avidin gel chromatography (Pierce Biotechnology, Rockford, IL). The proteins were refolded as described [31]. MR1/ ß2m complex yielded 35- and 12-kDa bands in SDS PAGE analysis after staining with Coomassie brilliant blue (more than 95% purity).

Binding assay

MR1 proteins in PBS (3 μg/mL, 100 μL) were incubated in a 96-well plate for overnight at 4°C. The plate was washed with

PBS and blocked with the assay diluent solution for ELISA (Becton Dickinson) for 2 h at room temperature. After washing with PBS, the plate was incubated with the solution of modified α-ManCer or α-GalCer (33, 10 and 3 μg/mL in PBS including 0.1% Tween 20, 50 μL) for 2 h at room temperature. The plate was washed with PBS, then peroxidase-conjugated Con A (Sigma, 0.5 μg/mL in the assay diluent, 50 μL) was added to the plate. It was incubated for 30 min at room temperature. It was washed with PBS including 0.1% Tween 20 seven times, and was added by tetramethylbenzidine liquid substrate (Sigma). The color development was measured by an ELISA reader (Model 550, Bio-Rad, Hercules, CA).

Acknowledgements: The authors thank Dr. O. Kanie for advice. They thank Dr. H. Ishikawa and J. Miyazaki for providing the TCR Cα-deficient mice and the expression vector. They also thank Ms. R. Fujii and Ms. H. Watanabe, for help with experiments, Mr. S. Kamijo and his group for taking care of the mice, and Ms. Y. Murakami for secretarial assistance. This work was supported by a grant from the Ministry of Health, Welfare and Labor, Japan.

References

1 **Bendelac, A., Rivera, M. N., Park, S. H. and Roark, J. H.,** Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* 1997. **15**: 535–562.

2 **Lantz, O. and Bendelac, A.,** An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific $CD4^+$ and $CD4^-8^-$ T cells in mice and humans. *J. Exp. Med.* 1994. **180**: 1097–1106.

3 **Makino, Y., Kanno, R., Ito, T., Higashino, K. and Taniguchi, M.,** Predominant expression of invariant $Vα14^+$ TCR α chain in $NK1.1^+$ T cell populations. *Int. Immunol.* 1995. **7**: 1157–1161.

4 **Bendelac, A., Killeen, N., Littman, D. R. and Schwarz, R. H.,** A subset of $CD4^+$ thymocytes selected by MHC class I molecules. *Science* 1994. **263**: 1774–1778.

5 **Bendelac, A., Lantz, O., Quimby, M. E., Yewdell, J. W., Bennink, J. R. and Brutkiewicz, R. R.,** CD1 recognition by mouse $NK1^+$ T lymphocytes. *Science* 1995. **268**: 863–865.

6 **Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H. *et al.*,** CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science* 1997. **278**: 1626–1629.

7 **Natori, T., Koezuka, Y. and Higa, T.,** Agelasphins, novel α-galactosylceres from the marine sponge *Agelas Mauritianus*. *Tetrahedron Lett.* 1993. **34**: 5591–5592.

8 **Kinjo, Y., Wu, D., Kim, G., Xing, G.-W., Poles, M. A., Ho, D. D., Tsuji, M. *et al.*,** Recognition of bacterial glycosphingolipids by natural killer T cell. *Nature* 2005. **434**: 520–525.

9 **Mattner, J., DeBord, K. L., Ismail, N., Goff, R. D., Cantu III, C., Zhou, D., Saint-Mezard, P. *et al.*,** Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 2005. **434**: 525–529.

10 **Zhou, D., Mattner, J., Cantu III, C., Schrantz, N., Yin, N., Gao, Y., Sagiv, Y. *et al.*,** Lysosomal glycosphingolipid recognition by NKT cells. *Science* 2004. **306**: 1786–1789.

11 **Shimamura, M. and Huang, Y. -Y.,** Presence of a novel subset of NKT cells bearing an invariant Vα19.1-Jα26 TCR α chain. *FEBS Lett.* 2002. **516**: 97–100.

12 **Tilloy, F., Treiner, E., Park, S.-H., Garcia, G., Lemonnier, F., de la Salle, H., Bendelac, A. *et al.*,** An invariant T cell receptor α chain defines a novel TAP-independent major hitocompatibility complex class Ib-restricted α/β T cell subpopulation in mammals. *J. Exp. Med.* 1999. **189**: 1907–1921.

13 **Treiner, E., Duban, L., Bahram, S., Radosavijevic, M., Wanner, V., Tilloy, F., Affaticati, P. *et al.*,** Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* 2003. **422**: 164–169.

14 **Yoshimoto, T., Bendelac, A., Watson, C., Hu-Li, J. and Paul, W. E.,** Role of $NK1.1^+$ T cells in a T_H2 response and in immunoglobulin E production. *Science* 1995. **270**: 1845–1847.

15 **Okamoto, N., Kanie, O., Huang, Y.-Y., Fujii, R., Watanabe, H. and Shimamura, M.,** Synthetic α-mannosyl ceramide as a potent stimulant for a novel NKT cell repertoire bearing the invariant Vα19-Jα26 TCR α chain. *Chemistry & Biology* 2005. **12**: 677–683.

16 **Huang, S., Gilfillan, S., Cella, M., Miley, M. J., Lantz, O., Lybarger, L., Fremont, D. H. and Hansen, T. H.,** Evidence for MR1 antigen presentation to mucosal-associated invariant T cells. *J. Biol. Chem.* 2005. **280**: 21183–21193.

17 **Fujita, T., Hirose, T., Hamamichi, N., Kitao, Y., Sasaki, S., Yoneta, M. and Chiba, K.,** 2-Substituted 2-aminoethanol: minimum essential structure for immunosuppressive activity of ISP-I (myocin). *Bioorg. Med. Chem. Lett.* 1995. **5**: 1857–1860.

18 **Shimamura, M., Okamoto, N., Huang, Y.-Y., Yasuoka, J., Morita, K., Nishiyama, A., Amano, Y. and Mishina, T.,** Induction of promotive rather than suppressive immune responses from a novel NKT cell repertoire Vα19 NKT cell with α-mannosyl ceramide analogues consisting of the immunosuppressant ISP-I as the sphingosine unit. *Eur. J. Med. Chem.* 2006. **41**: 569–576.

19 **Pulvertaft, J. V.,** Cytology of Burkitt's tumor (African lymphoma). *Lancet* 1964. **1**: 238–240.

20 **Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Millingan, J., Thornton, R., Shei, G.-J. *et al.*,** Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* 2002. **296**: 346–349.

21 **Oki, S., Chiba, A., Yamamura, T. and Miyake, S.,** The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NK T cells. *J. Clin. Invest.* 2004. **113**:1631–1640.

22 **Hilliard, B. A., Mason, N, Xu, N., Sun, J., Lamhamedi-Cherradi, S.-E., Liou, C. Hunter, H.-C. and Chen, Y. H.,** Critical roles of c-Rel in autoimmune inflammation and helper T cell differentiation. *J. Clin. Invest.* 2002. **110**: 843–850.

23 **Illés, Z., Shimamura, M., Newcombe, J., Oka, N. and Yamamura, T.,** Accumulation of Vα7.2-Jα33 invariant T cells in human autoimmune inflammatory lesions. *Int. Immunol.* 2004. **16**: 223–230.

24 **Croxford, J. L., Miyake, S., Huang, Y.-Y., Shimamura, M. and Yamamura, T.,** Invariant Vα19i T cells regulate autoimmune inflammation. *Nat. Immunol.* 2006. **7**: 987–994.

25 **Mombaerts, P. A., Clarke, R., Rundnicki, M. A., Iacomini, S., Lafaille, J. J., Wang, L., Ichikawa, Y. *et al.*,** Mutations in T cell receptor genes α and β block thymocytes development at different stages. *Nature* 1992. **360**: 225–231.

26 **Shimamura, M., Ohteki, T., Launois, P., Garcia, A.-M. and MacDonald, H. R.,** Thymus-independent generation of $NK1^+$ T cells *in vitro* from fetal liver precursors. *J. Immunol.* 1997. **158**: 3682–3689.

27 **Hashimoto, K., Hirai, M. and Kurokawa, Y.,** A gene outside the human MHC related to classical HLA I genes. *Science* 1995. **269**: 693–695.

28 **Niwa, H., Yamamura, K. and Miyazaki, J.,** Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991. **108**: 193–199.

29 **Yoshimoto, T. and Paul, W. E.,** $CD4^{POS}$, $NK1.1^{POS}$ T cells promptly produce interleukin 4 in response to *in vivo* challenge with anti-CD3. *J. Exp. Med.* 1994. **179**: 1285–1295.

30 **Schatz, P. J.,** Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. *Biotechnology* 1993. **11**: 1138–1143.

31 **Altman, J. D., Moss, P. A., Goulder, P. J., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I. and Davis, M. M.,** Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996. **274**: 94–96.

the FEBS Journal

Glycolipids with nonreducing end α-mannosyl residues that have the potential to activate invariant Vα19 NKT cells

Michio Shimamura[1], Yi-Ying Huang[1], Naoki Okamoto[1], Yutaka Watanabe[2], Yoshiko Murakami[3], Taroh Kinoshita[3], Yoshio Hirabayashi[4], Chikara Murakata[4], Yukishige Ito[4] and Tomoya Ogawa[4]

1 Developmental Immunology Unit, Mitsubishi Kagaku Institute of Life Sciences, Machida, Japan
2 Faculty of Engineering, Ehime University, Matsuyama, Japan
3 Research Institute for Microbial Diseases, Osaka University, Suita, Japan
4 Riken (The Institute of Physical and Chemical Research), Wakou, Japan

Keywords
glycolipid; immune response; invariant TCR α; MHC class Ib; NKT cell

Correspondence
M. Shimamura, 11 Minamiooya, Machida, Tokyo 194-8511, Japan
Fax: +81 42 724 6317
Tel: +81 42 724 6348
E-mail: michio@libra.ls.m-kagaku.co.jp

(Received 26 January 2007, revised 4 April 2007, accepted 5 April 2007)

doi:10.1111/j.1742-4658.2007.05826.x

We have previously demonstrated that α-mannosyl ceramide and its derivatives promote immune responses of NK1.1$^+$ invariant Vα19-Jα33 T cell receptor (TCR) α$^+$ T cells (Vα19 NKT cells). In this study, attempts were made to determine the structural requirements for natural ligands for Vα19 NKT cells. Naturally occurring and synthetic glycolipids were analyzed for their ability to stimulate the cells prepared from invariant Vα19-Jα33 TCR transgenic mice, in which development of Vα19 NKT cells is facilitated. As a result, α-mannosyl phosphatidylinositols such as 2,6-di-α-mannosyl phosphatidylinositol and α-mannosyl-4α-glucosaminyl-6-phosphatidylinositol (α-Man-GlcNH$_2$-PtdIns) as well as α-mannosyl ceramide derivatives were found to activate the cells from the transgenic mouse liver, gut lamina propria and spleen *in vivo* and *in vitro*. Thus, glycolipids with nonreducing end α-mannosyl residues are suggested to be potent antigens for Vα19 NKT cells. Next, a series of invariant Vα19-Jα33 TCR$^+$ hybridomas, each with variations in the sequence of the Vα-Jα junction and the TCR β chain, were tested for responsiveness toward the α-mannosyl glycolipids. A loose correlation between the primary structure of the TCR and the reactive glycolipids was observed. For instance, hybridomas expressing TCRs consisting of an α chain with a variation in the Vα19-Jα33 junction and a Vβ6$^+$ β chain showed affinity towards α-mannosyl ceramide and α-Man-GlcNH$_2$-PtdIns, whereas those expressing TCRs with an invariant Vα19-Jα33 α chain and a Vβ8$^+$ β chain responded to 2,6-di-α-mannosyl phosphatidylinositol. Thus, it is suggested that Vα19 NKT cells with microheterogeneity in the TCR structure have been generated for defense against various antigens expressing α-mannosyl glycolipids.

Natural killer T (NKT) cells are defined as lymphocytes bearing both the common NK marker NK1.1, a product of a member of the NKR-P1 gene family, and T cell receptor (TCR)–CD3 complex [1]. Cells forming the major component of NKT cells [NK1.1$^+$ Vα14-Jα18 invariant TCR α$^+$ cells (Vα14 NKT cells)]

Abbreviations
α-GalCer, α-galactosyl ceramide; α-ManCer, α-mannosyl-ceramide; α-ManCer(Phe), *N*-[1-(α-mannosyloxymethyl)-3-(4-octylphenyl)propyl] hexadecanamide; α-Man-GlcNH$_2$-PtdIns, α-mannosyl-4α-glucosamine-6-phosphatidylinositol; (α-Man)$_2$-PtdIns, 2,6-di-α-mannosyl phosphatidylinositol; β2m, β$_2$-microglobulin; GPI, glycosylphosphatidylinositol; IFN, interferon; IL, interleukin; LAM, lipoarabinomannan; LPL, lamina propria lymphocyte; MNC, mononuclear cell; NKT, natural killer T; TCR, T cell receptor; Tg, transgene or transgenic; Vα14 NKT cell, NK1.1$^+$ Vα14-Jα18 invariant TCR α$^+$ cell; Vα19 NKT cell, NK1.1$^+$ Vα19-Jα33 invariant TCR α$^+$ cell.

express the invariant TCR α chain (mouse Vα14-Jα18, human Vα24-Jα18) [2,3]. Vα14 NKT cells are responsive to certain glycosphingolipids such as α-galactosyl ceramide (α-GalCer) [4] isolated from marine sponge [5], α-glucuronosyl ceramide and α-galacturonosyl ceramide from α-proteobacteria [6,7], and intracellular lysosomal isoglobotriaosyl ceramide [8] in the context of CD1d [9,10].

Recently, another invariant TCR α chain consisting of Vα19-Jα33 (conventionally Jα26) has been found [11]. We have demonstrated that cells expressing the Vα19-Jα33 invariant TCR α chain are mainly present as NKT cells in mouse livers [12]. These cells [designated as NK1.1$^+$ Vα19-Jα33 invariant TCR α$^+$ cells (Vα19 NKT cells)] represent about 1% of mononuclear cells (MNCs) in the liver, so they are a considerably large population as a lymphocyte clone. Preferential localization of the invariant Vα19-Jα33 TCR$^+$ cells in gut lamina propria has also been reported [13]. Vα19 NKT cells promptly respond to the TCR engagement and produce large amounts of both Th1-promoting and Th2-promoting immunoregulatory cytokines. Thus, they are considered to have important roles in the regulation of the immune system [14,15] (M. Shimamura *et al.*, unpublished results). Recently, participation of Vα19 NKT cells in the regulation of autoimmune diseases has been suggested [16]. Therefore, the search for specific antigens for Vα19 NKT cells is quite important in developing new therapies for various immunoregulatory disorders on the basis of the functional modulation of the repertoire.

The cells bearing the invariant Vα19-Jα33 TCR are positively selected by nonclassic MHC class I molecule MR1 [13]. However, the antigens presented by MR1 have not been identified [17]. We have found that Vα19 NKT cells are specifically stimulated by α-mannosyl ceramide (α-ManCer) [14] and its derivatives with modifications in the sphingosine unit [18] in the context of MR1. In the current study, glycolipids related to α-ManCer were tested for their ability to stimulate Vα19 NKT cells, to determine the structural requirements for natural ligands. In addition, MR1 transfectants were prepared from cell lines deficient in glycolipid biosynthesis, and their ability to stimulate Vα19 NKT cells was compared with that of those prepared from wild-type cell lines in an attempt to determine the endogenous antigens synthesized and presented by the MR1 transfectants. It was suggested that possible candidates were glycolipids independent of the synthetic enzyme for β-glucosylceramide [19] or glycosylphosphatidylinositol (GPI) anchor [20].

Results

Activation of Vα19 NKT cells with α-mannosyl glycolipids

Attempts were made to determine the structural requirements for natural ligands for Vα19 NKT cells. Naturally occurring and synthetic glycolipids were analyzed for their potential to induce immune responses from Vα19 NKT cells. Liver MNCs isolated from invariant Vα19-Jα33 TCR transgenic (Vα19 Tg) mice with the TCR α$^{-/-}$ background [Vα19 Tg$^+$ cells are the sole component of TCR$^+$ cells in them (*c.* 50%)], C57BL/6 mice [Vα14 NKT cells represent the largest proportion in them (*c.* 25%)] and β_2-microglobulin (β2m)$^{-/-}$ mice (negative control) were cultured in the presence of the glycolipids shown in Fig. 1 (1 μg·mL^{-1}). Cytokine secretion into the supernatants and cell proliferation were determined (Fig. 2A). A correlation between the specific antigenicity towards Vα19 Tg$^+$ cells and the structure was suggested in the glycolipids tested here. As well as α-ManCer [14] and its derivatives [18], 2,6-di-α-mannosyl phosphatidylinositol (α-Man)$_2$-PtdIns, a partial structure of bacterial lipoarabinomannan (LAM) [21]) and α-mannosyl 1–4α-glucosamine-1–6-phosphatidylinositol (α-Man-GlcNH$_2$-PtdIns, a partial structure of GPI anchor [22]) were found to be potent stimulators of Vα19 NKT cells. The active glycolipids commonly had α-mannosyl residue(s) at the nonreducing end. In contrast, glycolipids such as porcine blood glycosphingolipids [including β-glucosyl ceramide (β-GlcCer) lactosyl ceramide, globotriaosyl ceramide, and globotetraosyl ceramide]), bovine brain gangliosides (including GM3, GM2, GM1, GD1, and GT1), phospholipids (phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine), yeast glycosyl phosphoinositol ceramide mixture (α-Man-Ino-PO$_4$-Cer, etc. [23]), mycobacterial LAM and its partially degraded derivatives [(α-Man)$_n$-PtdIns, 40 kDa] [24], β-galactosyl phytodiacylglycerol [25] and bivalve α-mannosylated trihexosyl ceramides (α-Man-Man-Glc-Cer, etc.) [26] (see Experimental procedures) did not stimulate Vα19 Tg$^+$ cells up to 10 μg·mL^{-1} (data not shown).

The responses of Vα19 Tg$^+$ cells were dependent on the dose of the glycolipids in culture (Fig. 2B). The cells secreted interleukin (IL)-4 in the early phase, and then interferon (IFN)-γ and IL-17 in the same fashion as they produced cytokines in response to TCR engagement with antibody to CD3 (M. Shimamura *et al.*, unpublished results). Thus, stimulation by glycolipids possibly induces TCR engagement of invariant Vα19 TCR$^+$ cells. However, stimulation by glycolipids was

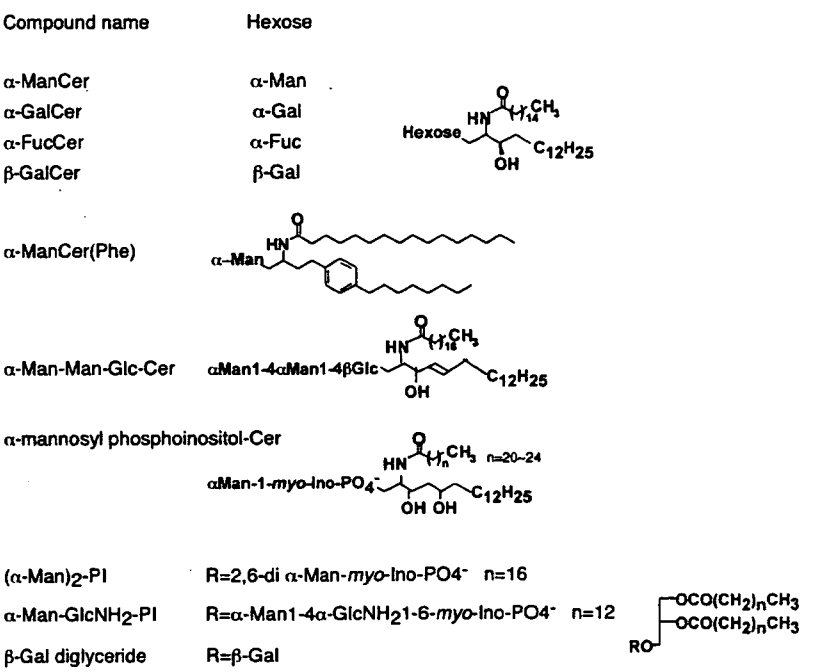

Fig. 1. Diagram of glycolipids characterized in this study.

less intensive than stimulation by antibody to CD3 even at the maximum dose (typically, invariant Vα19 TCR^+ cells produce, upon direct TCR engagement with antibody to CD3, 5–10 times more cytokines than they produce after stimulation with glycolipids).

Next, Vα19 Tg^+ cells before and after depletion of $NK1.1^+$ cells were cultured in the presence of α-mannosyl glycolipids to determine the cell population responding to the glycolipids (Fig. 2C). The immune response to $(α\text{-Man})_2$-PtdIns and α-Man-$GlcNH_2$-PtdIns as well as to *N*-[1-(α-mannosyloxymethyl)-3-(4-octylphenyl)propyl] hexadecanamide [α-ManCer(Phe)] were found entirely in the culture of Vα19 Tg^+ cells before $NK1.1^+$ cell depletion, thus clearly indicating that Vα19 NKT cells are responsible for the reactivity to glycolipids.

The α-mannosyl glycolipids induce immune responses of Vα19 Tg^+ cells of any lymphoid organs examined. The profiles of cytokine production by gut lamina propria lymphocytes (LPLs) in culture are shown in Fig. 3. Like liver MNCs, LPLs isolated from Vα19 Tg^+ TCR $\alpha^{-/-}$ mice but not those from C57BL/6 or $\beta 2m^{-/-}$ mice (data not shown) specifically responded to the α-mannosyl glycolipids. Thus, it is suggested that Vα19 Tg^+ cells with responsiveness towards the α-mannosyl glycolipids are distributed over the lymphoid organs.

The immune responses of Vα19 Tg^+ cells were also observed when they were primed *in vivo* with the α-mannosyl glycolipids. Spleen cells from Vα19Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice injected 90 min previously with the glycolipids were cultured, and cytokines secreted into the supernatants were determined (Fig. 4). Vα19 Tg^+ $TCR\alpha^{-/-}$ splenocytes produced IL-4 and IFN-γ the same as when they were stimulated *in vitro*. On the other hand, C57BL/6 cells displayed less responsiveness to these α-mannosyl glycolipids, presumably due to the lower frequency of Vα19 NKT cells in the spleen. Thus, α-mannosyl glycolipids injected into mice possibly target Vα19 NKT cells, given the lack of immune responses to the glycolipids in the culture of Vα19 Tg cells depleted of $NK1.1^+$ cells. Collectively, a possible application of these glycolipids to immunotherapies is suggested by these observations.

MR1-restricted stimulation of Vα19 Tg^+ cells with the α-mannosyl glycolipids

MHC restriction of the immune responses by Vα19 Tg^+ cells to the α-mannosyl glycolipids was examined (Fig. 5). Liver MNCs were prepared from Vα19 Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice, and they were cocultured with the cells of a B-lymphoma line (Raji) [27] transfected with the cDNA of nonclassic MHC class I molecules (CD1, MR1, Qa2, TL). Vα19 Tg^+ cells were weakly stimulated in coculture with the Raji cells transfected with the cDNA of one of the nonclassic MHC class I molecules, MR1, as suggested previously [14], whereas they were not responsive to stimulation with the transfectants of any other MHC genes (data not

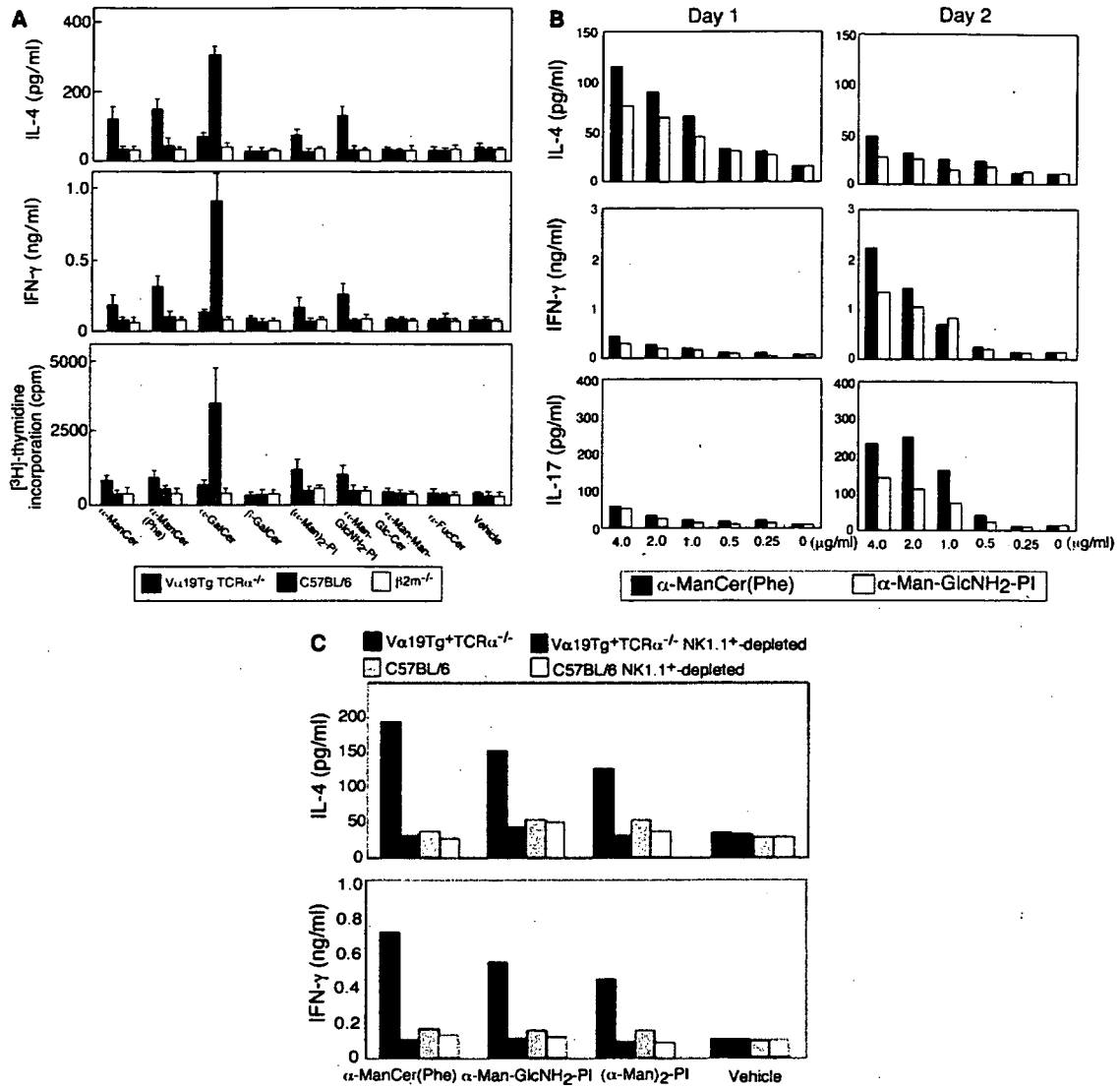

Fig. 2. Activation of invariant Vα19 TCR$^+$ cells with glycolipid antigens in culture. (A) Liver MNCs prepared from Vα19 Tg$^+$ TCRα$^{-/-}$, C57BL/6 and β2m$^{-/-}$ mice were cultured in the presence or absence of glycolipids (1 μg·mL^{-1}). After 2 days, the immune responses were monitored by examining IL-4 and IFN-γ secretion and cell proliferation ([^{3}H]thymidine incorporation for 5 h). As for α-Man-Man-Glc-Cer [27], the results of Man-α1–4-Man-β1–4-Glc-β1–1Cer are shown here, but similar results were obtained when Man-α1–2-Man-β1–4-Glc-β1–1Cer was used. Student's *t*- test was performed to evaluate the statistical significance of the immune responses by Vα19 Tg$^+$ cells towards α-mannosyl glycolipids [α-ManCer, α-ManCer(Phe), Man(Man)-PtdIns, and Man-GlcNH$_2$-PtdIns]. The *P*-values in Student's *t*-test were less than 0.05 when the immune responses to each α-mannosyl glycolipid were compared with the immune responses towards β-GalCer or α-FucCer. (B) Time course and dose-dependent activation of invariant Vα19 TCR$^+$ cells. Liver MNCs prepared from Vα19 Tg$^+$ TCRα$^{-/-}$ mice were cultured in the presence of glycolipids at the indicated dose. After 1 and 2 days, the immune responses were monitored by measuring cytokine secretion. The average of the duplicate cultures in one of the two experiments giving similar results is demonstrated. (C) Determination of the cell population in the Vα19 Tg mice responsive to glycolipid antigens. Liver MNCs were isolated from Vα19 Tg$^+$ TCRα$^{-/-}$ and C57BL/6 mice. Cells were divided into two fractions. One of them was depleted of NK1.1$^+$ cells using magnetic beads. The cells in each fraction were cultured with the glycolipids (2 μg·mL^{-1}) for 3 days. The concentrations of IL-4 on day 1 and IFN-γ on day 3 were determined. One of the representative two experiments giving essentially the same profiles is shown.

shown). Thus, it is likely that invariant Vα19 TCR-bearing cells are restricted by MR1 that is presenting certain endogenous antigens or chaperones. This result is in accord with recent reports that invariant Vα19 TCR$^+$ cells are positively selected by MR1 [13,15].

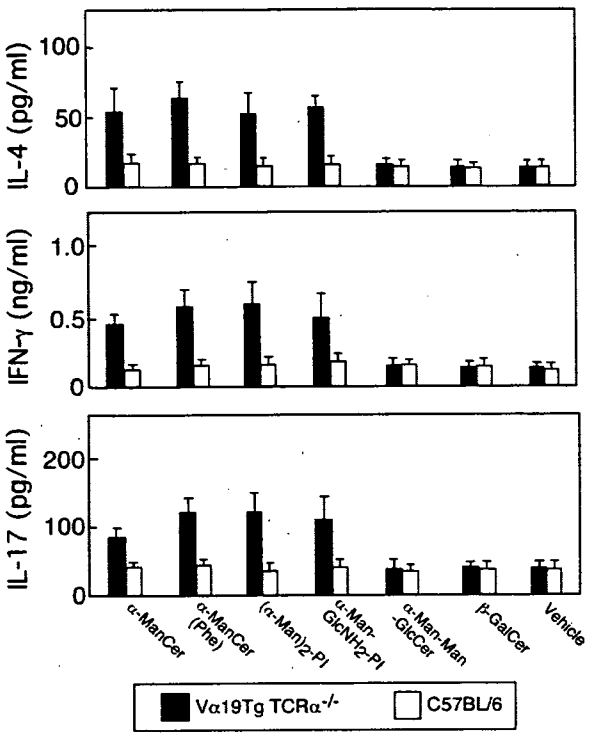

Fig. 3. Stimulation of LPLs isolated from invariant Vα19 TCR Tg mice with glycolipid antigens. LPLs prepared from Vα19 Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice under specific pathogen free conditions were cultured in the presence of glycolipids (2 $\mu g \cdot mL^{-1}$). After 2 days, the immune responses were monitored by analyzing cytokines. The average ± SD of the triplicate cultures is shown. The assays were repeated twice, and similar results were obtained. The *P*-values in Student's *t*-test were less than 0.05 when the immune responses to each α-mannosyl glycolipid were compared with those to β-GalCer. The immune responses of LPLs from Vα19 Tg^+ $TCR\alpha^{-/-}$ or C57BL/6 triggered by the TCR engagement with antibody to CD3 were typically in the range of a 10–20-fold increase compared with the responses by the cells in the control culture.

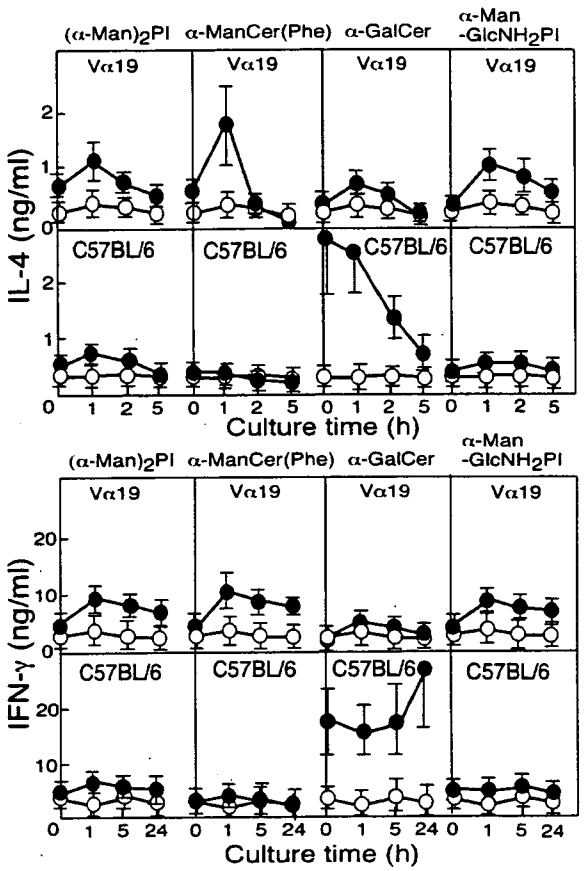

Fig. 4. Stimulation of Vα19 Tg^+ cells *in vivo* following challenge with α-mannosyl glycolipids. Spleen cells from Vα19 Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice injected 90 min previously with glycolipids (20 μg in $NaCl/P_i$ per animal, closed circles) or vehicle (dimethylsulfoxide in $NaCl/P_i$, open circles) via the tail vein were cultured for the periods indicated. Culture supernatants were harvested and tested for production of cytokines. The data points at 0 h represent the concentration of cytokines in the serum 90 min after the glycolipid injection. Representative profiles obtained from one of the three experiments, each using three mice of both strains, are indicated.

The immune responses of Vα19 Tg^+ cells towards MR1 transfectants were enhanced when the transfectants were previously loaded with the α-mannosyl glycolipids as well as α-ManCer [14]. Presumably, putative intracellular ligands were replaced by these glycolipids at the antigen-presenting groove in MR1 molecules. The immune responses were drastically reduced in the presence of anti-MR1 serum but not in the presence of preimmune serum. The immune responses by Vα19 Tg^+ cells upon TCR engagement with antibody to CD3 were about five times as great as those induced in coculture with the glycolipid-loaded MR1 transfectants, and were not reduced in the presence of anti-MR1 serum. Taken together, these findings strongly suggest that invariant Vα19 TCR^+ cells recognize α-mannosyl glycolipids that are presented by MR1.

Correlation between the TCR structure in Vα19 NKT cells and antigen specificity

Two species of glycolipids were observed, after screening, to be potent antigens for Vα19 Tg^+ cells: α-mannosyl sphingolipids and phosphatidylinositols. The responses of Vα19 NKT cell hybridomas were examined to determine the antigen specificity of a single NKT cell clone (Fig. 6). NB116 and NB202, expressing a variant Vα19 TCR α chain coupled with a $V\beta6^+$ β chain,

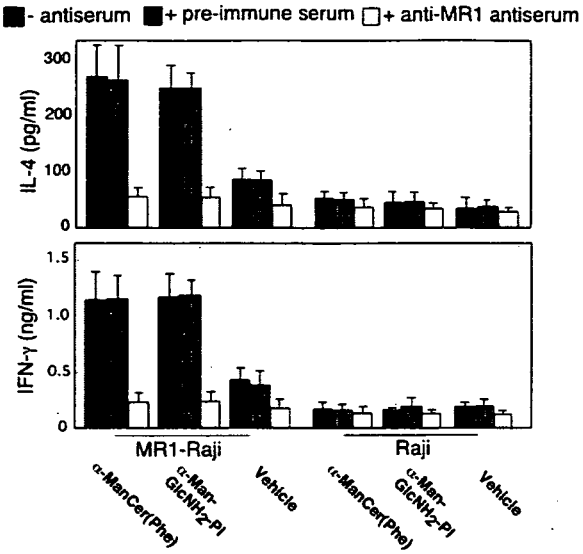

Fig. 5. Stimulation of Vα19 Tg cells with glycolipid antigens in the context of MR1. MR1-transfected or nontransfected Raji cells were incubated with glycolipids (2 $\mu g \cdot mL^{-1}$) for 5 h. They were washed with medium and irradiated (3000 rad), and then cultured with liver MNCs isolated from Vα19 Tg^+ $TCR\alpha^{-/-}$ mice for 3 days in the presence or absence of purified rabbit anti-MR1 serum or preimmune serum (3 $\mu g \cdot mL^{-1}$). The cytokine concentration in the culture fluid was determined by ELISA. The averages of triplicate cultures in one of the representative two results are shown.

showed similar responses towards a series of glycolipids. Hybridomas expressing the invariant Vα19 TCR α chain responded to α-ManCer derivatives and/or $(\alpha\text{-Man})_2$-PtdIns, presumably depending on the CDR3 structure of the β chain. Taken together, these findings suggest a degree of correlation between the structure of the semi-invariant Vα19 TCR αβ and the affinity for glycolipid antigens, although it was not strict.

Possible candidates for the endogenous antigens for invariant Vα19 TCR^+ cells

The MR1 transfectants were able to activate Vα19 Tg^+ cells without them being loaded with any antigens (Fig. 5), although the activation was less efficient than the stimulation with the MR1 transfectants previously loaded with α-mannosyl glycolipids (less than one-third). Thus, it was suggested that certain endogenous antigens were synthesized and loaded over MR1 molecules in a manner independent of Tap (transporter associated with antigen processing) in the transfected cells [17]. To obtain knowledge about the endogenous antigens, the stimulation of Vα19 Tg^+ cells with MR1 transfectants deficient in β-D-GlcCer synthase [19] or GPI anchors (with a defect in PIG-L [20]) was compared with that with wild-type MR1 transfectants. MR1 transfectants deficient in β-D-GlcCer synthase or GPI

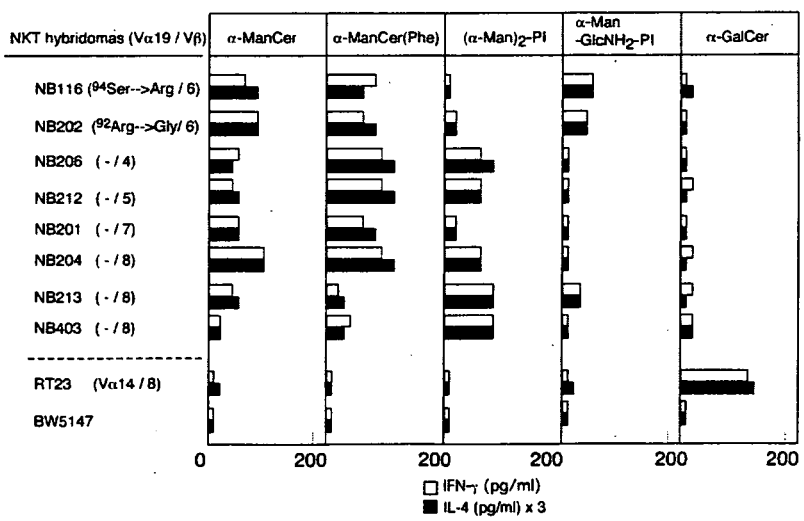

Fig. 6. Stimulation of Vα19 NKT cell hybridomas with α-mannosyl glycolipids. Dendritic cells prepared from C57BL/6 bone marrow were cultured with α-mannosyl glycolipids for 16 h. They were cocultured with Vα19 NKT cell hybridomas for 2 days. Cytokines in the culture fluid were measured by ELISA. The TCR structure of the hybridomas is listed on the left. NB116 and NB202 have an amino acid variation at the CDR3 region of the invariant Vα19-Jα33 α chain, whereas the others have a 'canonical' (germline) sequence. As controls, invariant Vα14 NKT cell hybridoma (RT23 [40]) and TCR-negative parental line BW5147 were examined. A representative profile of five experiments is indicated.

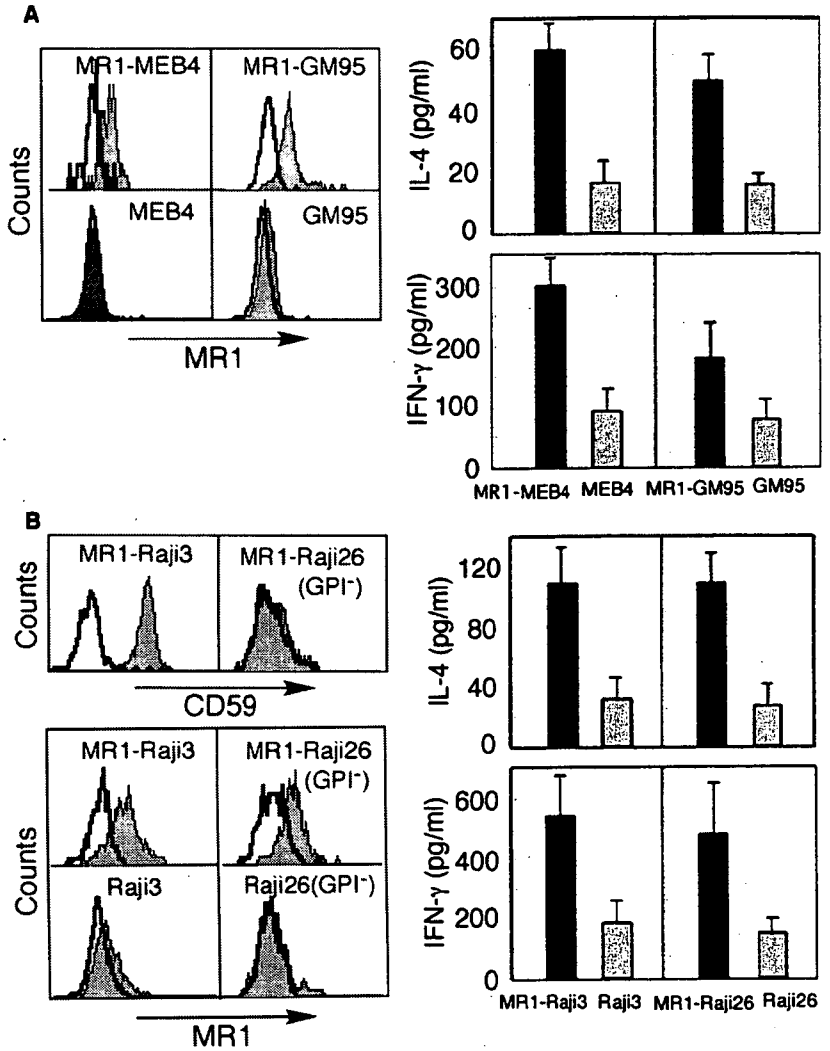

Fig. 7. Stimulation of invariant Vα19 TCR$^+$ cells with mutated MR1 transfectants. (A) Stimulation of Vα19 Tg cells with MR1 transfectants deficient in β-GlcCer synthase [19]. MR1-transfected or nontransfected cells of a melanoma line (MEB-4) and its mutant line deficient in β-GlcCer synthase were immunostained with anti-MR1 serum or preimmune serum, and the expression of MR1 was analyzed by FACS. They were irradiated (3000 rad), and then cultured with liver MNCs from Vα19 Tg$^+$ TCRα$^{-/-}$ mice for 2 days. Production of IL-4 on day 1 and IFN-γ on day 2 of culture was determined by ELISA. (B) Stimulation of Vα19 Tg cells with MR1 transfectants deficient in GPI anchor synthase (PIG-L) [20]. MR1-transfected or nontransfected cells of a B-lymphoma line (Raji3) and its mutant line deficient in GPI anchor (Raji26) were immunostained with anti-CD59 serum, anti-MR1 serum or preimmune serum, and analyzed by FACS. They were irradiated (3000 rad), and then cultured with liver MNCs from Vα19 Tg$^+$ TCRα$^{-/-}$ mice for 2 days. Production of IL-4 on day 1 and IFN-γ on day 2 of culture was determined by ELISA. The averages ± SD of triplicate cultures in one of the representative two results are shown in (A) and (B).

anchors activated the responder cells isolated from Vα19 Tg$^+$ TCRα$^{-/-}$ mice as well as the parental MR1 transfectants (Fig. 7). Thus, it is indirectly suggested that α-mannosyl glycosphingolipids, independent of β-GlcCer synthase or α-mannosyl phosphatidylinositols other than GPI anchors such as (α-Man)$_2$-PtdIns, if there are any in mammalian cells, are frequently presented by MR1 as endogenous antigens.

Discussion

Identifying ligands for the invariant Vα19 TCR is quite important for potential medical applications based on Vα19 NKT cell functions. Specific activators or inhibitors of Vα19 NKT cells may be useful in treating diseases, as specific activators of Vα14 NKT cells such as α-GalCer and its homologs have been shown to be

effective in a number of animal models of disease [28,29]. In the present study, naturally occurring and synthetic glycolipids were comprehensively examined to determine the structural requirements for natural ligands for invariant Vα19 TCR^+ cells.

Certain glycolipids, possessing α-mannosyl residue(s) at the nonreducing end, have been shown to be potent ligands for Vα19 NKT cells when they are presented by MR1. As the truncation of the N-acyl group length from C_{16} to C_8 or C_2 in α-glycosyl ceramides drastically reduced the antigenic activity towards Vα19 NKT cells [14], the lipid portion of antigenic glycolipids possibly binds to the antigen-presenting groove of MR1, similar to CD1, leaving the sugar moiety available for the interaction with the invariant TCR. These active glycolipids are of different glycolipid species, α-ManCer and phosphatidylinositol. Judging from the lack of antigenicity in either of the α-mannosyl inositol phosphoceramides isolated from yeast [23] (data not shown) or bivalve α-mannosylated trihexosyl ceramides (α-Man-Man-Glc-Cer; Fig. 2A) [26] towards Vα19 Tg^+ cells, the α-mannosyl residues must be properly located with respect to the invariant TCR. The result for the bivalve sphingolipids is in contrast to the specific recognition of isoglobotriaosyl ceramide (with a nonreducing end α-galactosyl residue) by Vα14 NKT cells [8].

It is possible that the variations in the primary structure of the semi-invariant Vα19 TCR αβ chains determine the antigenic specificity. In fact, a loose correlation between the structure of semi-invariant Vα19 TCR αβ and the antigen specificity was suggested in Vα19 NKT cell hybridomas (Fig. 6). Invariant Tg Vα19 TCR was cloned from one of the Vα19 NKT cell hybridomas (NB403) with a canonical (germline form) Vα19-Jα33 sequence. The Vα19 Tg^+ cells from the Tg mice expressed TCR β chains with not only Vβ8 but also Vβ6, etc. They were responsive to any of the active α-mannosyl glycolipids. Thus, a possible involvement of the TCR β chain structure was suggested in the determination of antigen specificity, as reported for the specificity of lipid antigens for invariant Vα14 TCR^+ cells [30]. It is not certain whether the responsiveness to the α-mannosyl glycolipids found in the $Vα19^+$ hybridomas represents the whole antigen specificity of invariant Vα19 TCR-bearing cells. A series of semi-invariant Vα19 TCR $αβ^+$ cells against various glycolipids so far characterized and not identified may be generated in the immune system to respond to multiple endogenous and/or exogenous antigens.

It is not clear at present whether the specific antigens for invariant Vα19 TCR^+ cells are of endogenous or exogenous origin. The activation of invariant Vα19 TCR^+ cells by coculture with MR1 transfectants without exogenous antigens suggests the existence of endogenous antigens. The deficiency in β-GlcCer or GPI anchor synthase in MR1 transfectants did not affect the efficiency of stimulation of Vα19 Tg^+ cells (Fig. 7). Thus, the possible endogenous antigens are the glycosphingolipids synthesized by glycosyltransferases other than β-GlcCer synthase or the glycosyl phosphatidylinositols other than the GPI anchors. However, it remains possible that the endogenous antigens are irrelevant to glycolipids [17].

The enhanced responsiveness of Vα19 Tg^+ cells to the MR1 transfectants previously loaded with the α-mannosyl glycolipids suggests that Vα19 NKT cells have the potential to recognize exogenous antigens presented by MR1. Glycosyl phosphatidylinositols such as $(α\text{-Man})_2$-PtdIns and α-Man-$GlcNH_2$-PtdIns may be possible candidates for exogenous antigens. These glycolipids form part of the structure of lipopolysaccharides such as LAM or GPI anchors, which are cell surface components of pathogenic microorganisms. Although mycobacterial LAM or its degraded derivative did not stimulate Vα19 Tg^+ cells in the present study, this is presumably because of the insufficient efficiency of processing and presentation of these compounds with more than 80 mannosyl residues in antigen-presenting cells. On the other hand, the natural occurrence of α-ManCer has not been demonstrated. Thus, glycosphingolipids with an α-mannosyl residue properly located for recognition by Vα19 invariant TCR may be present. Otherwise, α-ManCer may be synthesized by ceramide α-mannosyltransferase, which is so far unknown in mammals or other organisms. The immune responses of Vα19 Tg^+ cells induced by the α-mannosyl glycolipids were less intensive than the immune responses of C57BL/6 cells caused by α-GalCer. However, the immune responses of Vα14 NKT cells induced by the glycolipids found as exogenous antigens for them (α-galactosyl diacylglycerol [31], α-glucuronyl or α-galacuronyl ceramide [7,32], and α-mannosyl phosphatidylinositol [33]) reached a maximum at concentrations of these antigenic glycolipids greater than 10 $μg·mL^{-1}$, and were rather moderate in comparison with those induced by the agonist α-GalCer. Thus, it is not certain whether the putative natural exogenous antigens for Vα19 NKT cells activate them more intensively than the α-mannosyl glycolipids found in this study.

Vα19 and Vα14 invariant TCR^+ cells are possibly involved in the regulation of the immune system despite being subjected to independent MHC controls. We have recently demonstrated that cells bearing Vα7.4-Jα33 (corresponding to mouse Vα19-Jα33) but not Vα24-Jα18

(corresponding to mouse Vα14-Jα18) invariant TCR α chains are frequently present in the lesions of multiple sclerosis patients [34], and that these cells are possibly involved in the suppression of the disease [16]. Thus, these subsets will be possible targets of immunotherapies using glycolipid activators specific to each repertoire.

Experimental procedures

Mice

C57BL/6 mice were purchased from Sankyo Service Co. (Tokyo, Japan). β2m-deficient mice, backcrossed with C57BL/6 mice for six generations, were obtained from Jackson Laboratory (Bar Harbor, ME, USA). TCR Cα-deficient mice, backcrossed with C57BL/6 mice for more than ten generations [35], were provided by H. Ishikawa (Keio University, Japan) and M. Nanno (Yakult Co., Kunitachi, Tokyo, Japan). Experimental animals were treated according to the guidelines of the experimental animal committee of Mitsubishi Kagaku Institute of Life Sciences.

Establishment of Vα19 Tg mice

A Vα19-Jα 33 Tg cloned from Vα19 NKT cell hybridoma (NB403) [12] and combined with the endogenous TCR α promoter and the enhancer was injected into C57BL/6 or TCR Cα-deficient fertilized eggs, and Tg mouse lines were established. Details are described elsewhere [14] (M. Shimamura *et al.*, unpublished results).

Cell preparations

MNCs were prepared from single-cell suspensions of mouse organs by density gradient centrifugation using Lymphosepar II (IBL, Gunma, Japan; $d = 1.090$) for spleen, and Percoll (Pharmacia, Uppsala, Sweden) for liver, as described previously [36]. LPLs were prepared as described by Treiner *et al.* [13].

Flow cytometry

Human lymphoma line Raji cells were pretreated with monoclonal antibody to CD16 (LNK16; Dainippon Pharma Co., Tokyo, Japan). Specific staining was performed with p282 (anti-human CD59; Becton Dickinson, San Jose, CA, USA) or anti-MR1 serum. Fluorescein isothiocyanate-conjugated anti-rabbit IgG [donkey IgG $F(ab')_2$ fragment; Jackson Laboratory] was used as a second antibody for the cells stained with anti-MR1 serum. The stained cells were analyzed on a FACScan flow cytometer equipped with the CELL QUEST software (Becton Dickinson).

In vivo stimulation of Vα19 Tg lymphocytes

Vα19 Tg^+ $TCR\alpha^{-/-}$ and C57BL/6 mice (8 weeks of age) were intravenously injected with glycolipids (20 μg per mouse) in 200 μL of $NaCl/P_i$ including 1 : 200 (v/v) of the vehicle (dimethylsulfoxide). Spleens were removed from mice 90 min after glycolipid injection, and MNCs were immediately prepared from them as described above. They were cultured at a concentration of 5×10^6 mL^{-1} in DMEM (10% fetal bovine serum, 50 $\mu g \cdot mL^{-1}$ streptomycin, 50 $U \cdot mL^{-1}$ penicillin) without further supplements. Cytokines in the supernatants were determined by ELISA.

In vitro stimulation of Vα19 Tg cells with glycolipids

MNCs were isolated from liver or gut lamina propria of mice (8–12 weeks of age) of the indicated strain. Typically, the liver MNCs include 30% NKT cells and 20% T cells, and the gut lamina propria MNCs include 8% NKT cells and 45% T cells in Vα19 Tg^+ TCR $\alpha^{-/-}$ mice. In C57BL/6 mice, the liver MNCs consist of 25% NKT cells and 25% T cells, and the gut lamina propria MNCs consist of 3% NKT cells and 35% T cells. They were cultured (10^6 pwer 200 μL for liver cells, and 5×10^5 per 200 μL for LPLs) for determined periods in DMEM (10% fetal bovine serum) in the presence of glycolipids with 1 : 1000 (v/v) dimethylsulfoxide, and the culture supernatants were analyzed for cytokines by ELISA. Cell proliferation was assessed by measuring the incorporation of [^{3}H]thymidine (0.5 $\mu Ci \cdot mL^{-1}$) for 5 h. In some experiments, the responder cells were cultured on the plate previously coated with antibody to CD3 (2C11; Becton Dickinson; 10 $\mu g \cdot mL^{-1}$ in $NaCl/P_i$). In other experiments, $NK1.1^+$ cells were removed from liver MNCs using magnetic beads (Dynal A. S., Oslo, Norway) before culture. The proportion of $NK1.1^+$ cells in the MNCs prepared from Vα19 Tg^+ TCR $\alpha^{-/-}$ or C57BL/6 livers was reduced from about 40% to less than 2% after the fractionation. The viability of the cells after the treatment was confirmed by the observation that they retained a potential to produce IFN-γ upon TCR engagement with antibody to CD3 that was comparable to that of the same number of the MNCs before NK1.1 depletion.

Glycolipids

α-Glycosylceramides [14], α-ManCer(Phe) [18] $(\alpha\text{-Man})_2$-PtdIns [21], α-Man-$GlcNH_2$-PtdIns [22]and Manα1–4Manα1–4Glcβ1–1Cer [26] were synthesized as previously described. Porcine blood glycolipids, bovine brain gangliosides, β-galactosyl diglyceride isolated from wheat flour [25], phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were obtained from Sigma (St Louis, MO, USA). LAM and its

degraded derivatives [40 kDa $(\alpha\text{-Man})_n$-PtdIns, $n = 80–90$] prepared from *Mycobacterium bovis* Ravenel [24] were provided by M. Sumida (Kagoshima University, Japan).

Stimulation of Vα19 NKT cell hybridomas with glycolipids

Bone marrow cells from C57BL/6 mice were cultured for 5 days in the presence of granulocyte–macrophage colony-stimulating factor (2 $ng{\cdot}mL^{-1}$; Peprotech, London, UK) to prepare dendritic cells. They were incubated with α-mannosyl glycolipids (5 $\mu g{\cdot}mL^{-1}$) with 1 : 1000 (v/v) dimethylsulfoxide for 16 h. After being washed with DMEM, bone marrow cells (1×10^6) were cultured with Vα19 NKT cell hybridomas (1×10^5) [12] in 200 μL of DMEM for 2 days. The concentrations of IL-4 and IFN-γ in the culture supernatants were determined.

Production of MR1 transfectants and anti-MR1 serum

Mouse MR1 A cDNA [37] was amplified from C57BL/6 spleen cells using the following PCR primers: 5′-MR1, 5′-ATGATGCTCCTGGTTACCTGG-3′; Flag-3′-mr1, 5′-CTACTTGTCATCGTCATCCTTGTAGTC(FLAG)AGAGGGAGAGCTTCCCTCAT-3′.

The PCR product was cloned into a eukaryotic expression vector (pCXN) [38]. The vector was transfected into the following cell lines: a human Burkitt's B-lymphoma [Raji [24] (ATCC)], its subline deficient in PIG-L (Raji26 [20]), a C57BL/6 melanoma line [MEB-4 (a subclone of B16)], and its subline deficient in β-GlcCer synthase (GM95, obtained from Riken Bioresource Center, Tsukuba, Japan) [19]. The transfectants were selected in the culture medium containing G418 (1 $mg{\cdot}mL^{-1}$) for 1 month. The expression of FLAG (Asp-Tyr-Lys-Asp-Asp-Asp)-MR1 in the transfectants was analyzed by western blot using antibody to FLAG and horseradish peroxidase-labeled anti-(mouse IgG) (Sigma).

Anti-MR1 serum was prepared by immunization of rabbits with a keyhole limpet hemocyanin-conjugated polypeptide corresponding to the α2 domain of mouse MR1 (residues 139–161, TKQAWEANLHELQYQKNW LEEEC [39]), with Freund's complete adjuvant (Sigma). In western blot analysis, MR1 transfectants gave a 40 kDa band with anti-MR1 serum staining, corresponding to the band with anti-FLAG staining (data not shown).

Stimulation of Vα19 Tg^+ cells with MR1 transfectants

MR1 transfectants or their parental cells (1×10^5 per well in DMEM, 10% fetal bovine serum) were incubated with glycolipids (2 $\mu g{\cdot}mL^{-1}$) with 1 : 1000 (v/v) dimethylsulfoxide used as the vehicle for 5 h, washed twice with DMEM, and irradiated (3000 rad). These cells were cocultured with liver MNCs (1×10^6 per well) from Vα19 Tg or non-Tg mice (8–12 weeks of age) in 200 μL of DMEM (10% fetal bovine serum) for 2–3 days. In some cultures, anti-MR1 serum or preimmune rabbit serum was added to the culture (3 $\mu g{\cdot}mL^{-1}$). Immune responses were monitored by measuring cytokines in the culture fluid.

Acknowledgements

The authors thank Drs R. L. Lester, R. C. Dickson, M. Sumida and J. Miyazaki for providing valuable materials. They thank Mr S. Kamijo and his group members for taking care of the mice. They also thank Ms N. Suzuki and Ms Y. Murakami for technical and secretarial assistance. This work was supported by a grant from the Ministry of Health, Welfare and Labor, Japan.

References

1 Bendelac A, Mivera NR, Park S-H & Roark JH (1997)Mouse CD1-specific NK1 T cells; development, specificity, and function. *Annu Rev Immunol* **15**, 535–562.

2 Lantz O & Bendelac A (1994) An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific $CD4^+$ and $CD4^-8^-$ T cells in mice and humans. *J Exp Med* **180**, 1097–1106.

3 Makino Y, Kanno R, Ito T, Higashino K & Taniguchi M (1995) Predominant expression of invariant $V\alpha14^+$ TCR α chain in $NK1.1^+$ T cell populations. *Int Immunol* **7**, 1157–1161.

4 Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E *et al.* (1997) CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science* **278**, 1626–1629.

5 Natori T, Koezuka Y & Higa T (1993) Agelasphins, novel α-galactosylceramides from the marine sponge *Agelas Mauritianus*. *Tetrahedron Lett* **34**, 5591–5592.

6 Kinjo Y, Wu D, Kim G, Xing G-W, Poles MA, Ho DD, Tsuji M, Kawahara K, Wong C-H & Kronenberg M (2005) Recognition of bacterial glycosphingolipids by natural killer T cell. *Nature (Lond)* **434**, 520–525.

7 Mattner J, DeBord KL, Ismail N, Goff RD, Cantu C III, Zhou D, Saint-Mezard P, Wang V, Gao Y, Yin N *et al.* (2005) Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature (Lond)* **434**, 525–529.

8 Zhou D, Mattner J, Cantu C III, Schrantz N, Yin N, Gao Y, Sagiv Y, Hudspeth K, Wu Y-P, Yamashita T *et al.* (2004) Lysosomal glycosphingolipid recognition by NKT cells. *Science* **306**, 1786–1789.
9 Bendelac A, Killeen N, Littman DR & Schwarz RH (1994) A subset of $CD4^+$ thymocytes selected by MHC class I molecules. *Science* **263**, 1774–1778.
10 Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR & Brutkiewicz RR (1995) CD1 recognition by mouse $NK1^+$ T lymphocytes. *Science* **268**, 863–865.
11 Tilloy F, Treiner E, Park S-H, Garcia G, Lemonnier F, de la Salle H, Bendelac A, Bonneville M & Lantz O (1999) An invariant T cell receptor α chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted α/B T cell subpopulation in mammals. *J Exp Med* **189**, 1907–1921.
12 Shimamura M & Huang Y-Y (2002) Presence of a novel subset of NKT cells bearing an invariant Vα19.1-Jα26 TCR α chain. *FEBS Lett* **516**, 97–100.
13 Treiner E, Duban L, Bahram S, Radosavijevic M, Wanner V, Tilloy F, Affaticati P, Gilfillan S & Lantz O (2003) Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature (Lond)* **422**, 164–169.
14 Okamoto N, Kanie O, Huang Y-Y, Fujii R, Watanabe H & Shimamura M (2005) Synthetic α-mannosyl ceramide as a potent stimulant for a novel NKT cell repertoire bearing the invariant Vα19-Jα26 TCR α chain. *Chem Biol* **12**, 677–683.
15 Kawachi I, Maldonado J, Strader C & Gillfillan S (2006) MR1-restricted Vα19*i* mucosal-associated invariant T cells are innate T cells in the gut lamina propria that provide a rapid and diverse cytokine response. *J Immunol* **176**, 1618–1627.
16 Croxford JL, Miyake S, Huang Y-Y, Shimamura M & Yamamura T (2006) Invariant Vα19*i* T cells regulate autoimmune inflammation. *Nat Immunol* **7**, 987–994.
17 Huang S, Gilfillan S, Cella M, Miley MJ, Lantz O, Lybarger L, Fremont DH & Hansen TH (2005) Evidence for MR1 antigen presentation to mucosal-associated invariant T cells. *J Biol Chem* **280**, 21183–21193.
18 Shimamura M, Okamoto N, Huang Y-Y, Yasuoka J, Morita K, Nishiyama A, Amano Y & Mishina T (2006) Induction of promotive rather than suppressive immune responses from a novel NKT cell repertoire Vα19 NKT cell with α-mannosyl ceramide analogues consisting of the immunosuppressant ISP-I as the sphingosine unit. *Eur J Med Chem* **41**, 569–576.
19 Ichikawa S, Sakiyama NH, Suzuki G, Hidari KI-PJ & Hirabayashi Y (1994) Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyzes the first glycosylation step of glycosphingolipid synthesis. *Proc Natl Acad Sci USA* **93**, 4638–4643.
20 Watanabe R, Ohishi K, Maeda K, Nakamura N & Kinoshita T (1999) Mammalian PIG-L and its yeast homologue Gpi12p are N-acetylglucosaminylphosphatidylinositol de-N-acetylases essential in glycosylphosphatidylinositol biosynthesis. *Biochem J* **339**, 185–192.
21 Watanabe Y, Yamamoto T & Okazaki T (1997) Synthesis of 2,6-di-O-α-D-mannopyranosylphosphatidyl-D-myo-inositol. Utilization of glycosylation and phosphorylation based on phosphite chemistry. *Tetrahedron* **53**, 903–918.
22 Murakata C & Ogawa T (1992) Stereoselective synthesis of glycobiosyl phosphatidylinositol, a part structure of the glycosyl-phosphatidylinositol (GPI) anchor of Trypanosoma brucei. *Carbohydr Res* **234**, 75–91.
23 Dickson RC & Lester RL (1999) Yeast sphingolipids. *Biochim Biophys Acta* **14**(26), 347–357.
24 Sumida M, Hashimoto M, Yasuoka J, Okabe E, Kusumoto S, Takada H, Hayashi T, Tamura T & Kotani S (1996) Immuno-stimulating macromolecular glycolipids from bacterial cell surfaces. *Polymer Preprints* **37**, 129–130.
25 Carter HE, Johnson P & Weber EJ (1965) Glycolipids. *Annu Rev Biochem* **34**, 109–142.
26 Mori T, Ito Y & Ogawa T (1990) Total synthesis of mollu-series glycosyl ceramides. *Carbohydr Res* **195**, 199–224.
27 Pulvertaft JV (1964) Cytology of Burkitt's tumor (African lymphoma). *Lancet* **1**, 238–240.
28 Taniguchi M, Harada M, Kojo S, Nakayama T & Wakao H (2003) The regulatory role of Vα14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* **21**, 483–513.
29 Miyamoto K, Miyake S & Yamamura T (2001) A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature (Lond)* **413**, 531–534.
30 Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, Podrebarac T, Koezuka Y, Porcelli SA, Cardell S, Brenner MB *et al.* (2000) Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* **12**, 211–221.
31 Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR-E-I, Zajonc DM, Ben-Menachem G, Aihge GD, Painer GF *et al.* (2006) Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol* **7**, 978–986.
32 Kinjo Y, Wu D, Kim G, Xing G-W, Poles MA, Ho DD, Tsuji M, Kawahara K, Wong C-H & Kronenberg M (2005) Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature (Lond)* **434**, 520–525.
33 Fischer K, Scotet E, Niemeyer M, Koebernick H, Zerrahn J, Maillet S, Hurwitz R, Kursar M, Bonneville M, Kaufmann SHE *et al.* (2004) Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc Natl Acad Sci USA* **101**, 10685–10690.
34 Ilés Z, Shimamura M, Newcombe J, Oka N & Yamamura T (2004) Accumulation of Vα7.2-Jα33 invariant T

cells in human autoimmune inflammatory lesions. *Int Immunol* **16**, 223–230.
35 Mombaerts P, Clarke AR, Rudnicki MA, Iacomini J, Itohara S, Lafäille JJ, Wang L, Ichikawa Y, Jaenisch RL, Hooper M *et al.* (1992) Mutations in T cell receptor genes α and β block thymocyte development at different stages. *Nature (Lond)* **360**, 225–231.
36 Shimamura M, Ohteki T, Launois P, Garcia A-M & MacDonald HR (1997) Thymus-independent generation of $NK1^+$ T cells in vitro from fetal liver precursors. *J Immunol* **158**, 3682–3689.
37 Yamaguchi H, Hirai M, Kurokawa Y & Hashimoto K (1997) A highly conserved major histocompatibility complex class I-related gene in mammals. *Biochem Biophys Res Commun* **238**, 697–702.
38 Niwa H, Yamamura K & Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199.
39 Hashimoto K, Hirai M & Kurosawa Y (1995) A gene outside the human MHC related to classical HLA class I genes. *Science* **269**, 693–695.
40 Shimamura M, Ohteki T, Beutner U & MacDonald HR (1997) Lack of directed Vα14-Jα281 rearrangements in $NK1^+$ T cells. *Eur J Immunol* **27**, 1576–1579.

nature immunology

Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin

Yoko Hamazaki[1], Harumi Fujita[2], Takashi Kobayashi[3], Yongwon Choi[4], Hamish S Scott[5], Mitsuru Matsumoto[6] & Nagahiro Minato[1,2]

The autoimmune regulator Aire is expressed in a small proportion of medullary thymic epithelial cells (mTECs) and is crucial in the induction of central T cell tolerance. The origin and development of Aire$^+$ mTECs, however, are not well understood. Here we demonstrate that the tight-junction components claudin-3 and claudin-4 (Cld3,4) were 'preferentially' expressed in Aire$^+$ mTECs. In early ontogeny, Cld3,4^{hi} TECs derived from the most apical layer of the stratified thymic anlage first expressed known mTEC markers such as UEA-1 ligand and MTS10. We provide evidence that such Cld3,4^{hi} UEA-1$^+$ TECs represented the initial progenitors specified for Aire$^+$ mTECs, whose development crucially required NF-κB-inducing kinase and the adaptor molecule TRAF6. Our results suggest that Aire$^+$ mTECs represent terminally differentiated cells in a unique lineage arising during thymic organogenesis.

The thymus has a characteristic three-dimensional meshwork structure consisting of two distinct anatomical regions, the cortex and the medulla, each of which contains different types of thymic epithelial cells (TECs)[1–3]. The thymic stroma of each region provides the microenvironment required for distinct aspects of T cell development. Cortical TECs (cTECs) regulate the directional migration and population expansion of T cell progenitors as well as the positive (and in part negative) selection of CD4$^+$CD8$^+$ double-positive thymocytes[4]. Positively selected CD4$^+$ or CD8$^+$ single-positive thymocytes then move toward the medulla[5,6], where medullary TECs (mTECs), in conjunction with dendritic cells, support the late stages of T cell development, including the deletion of self-reactive T cells that escaped negative selection in the cortex[7].

Accumulating evidence indicates that mTECs comprise heterogeneous populations[8]. For example, a small population of mTECs expresses the autoimmune regulator Aire, which is crucial in the induction of T cell tolerance toward tissue-restricted antigens[9,10]. Reports have indicated that both cTECs and mTECs are derived from common progenitors in ontogeny[11–13]. However, it remains to be determined whether the heterogeneity of mTECs reflects distinct mTEC lineages or various stages of differentiation in a single lineage[7,14].

The unique structure of the thymus is organized during embryonic development after the immigration of T cell progenitors[15–17]. Although the thymic anlage initially develops as stratified epithelium from the third pharyngeal pouch endoderm[18], the architecture is reorganized to a meshwork structure of epithelial cells with apparent loss of apico-basal cell polarity[1]. In simple epithelia, the epithelial cell integrity is maintained by junctional complexes at the apical part of cell adhesion sites: tight junctions, adherens junctions and desmosomes[19]. Although adherens junctions and desmosomes are anchoring junctions mechanically attaching cells to their neighbors, tight junctions are defined as occluding junctions. Tight junctions are composed of tetraspanning membrane proteins, claudin proteins and occludin and function as a 'barrier' to separate various microenvironments across the epithelial sheets and as a 'fence' to maintain the epithelial cell polarity[19]. Although the regulation of tight junctions in TECs is probably important, given the transition from a layer to a cluster structure of the thymus, the expression of tight junction components has not been studied extensively.

Here we systematically investigated the expression of the molecular components of tight junctions in the thymus. We found that claudin-3 and claudin-4 (Cld3,4) and occludin were expressed selectively in an mTEC subset expressing Aire and large amounts of major histocompatibility complex (MHC) class II molecules in the thymus of the adult mouse. In early ontogeny, Cld3,4 proteins were expressed selectively in the TECs lining the apical layer of the stratified thymic anlage, and those Cld3,4$^+$ TECs became the initial mTEC progenitors after the disruption of layer structure. We provide evidence that mTECs expressing Aire arise from the Cld3,4$^+$ mTEC progenitors as a unique cell lineage.

[1]Department of Immunology and Cell Biology, Graduate School of Medicine, and [2]Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. [3]Medical Institute of Bioregulation, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan. [4]Department of Pathology and Lab Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. [5]The Walter and Eliza Hall Institute of Medical Research, 3050 Victoria, Australia [6]Institute for Enzyme Research, University of Tokushima, Tokushima 770-8503, Japan. Correspondence should be addressed to N.M. (minato@imm.med.kyoto-u.ac.jp).

Received 29 November 2006; accepted 8 January 2007; published online 4 February 2007; doi:10.1038/ni1438

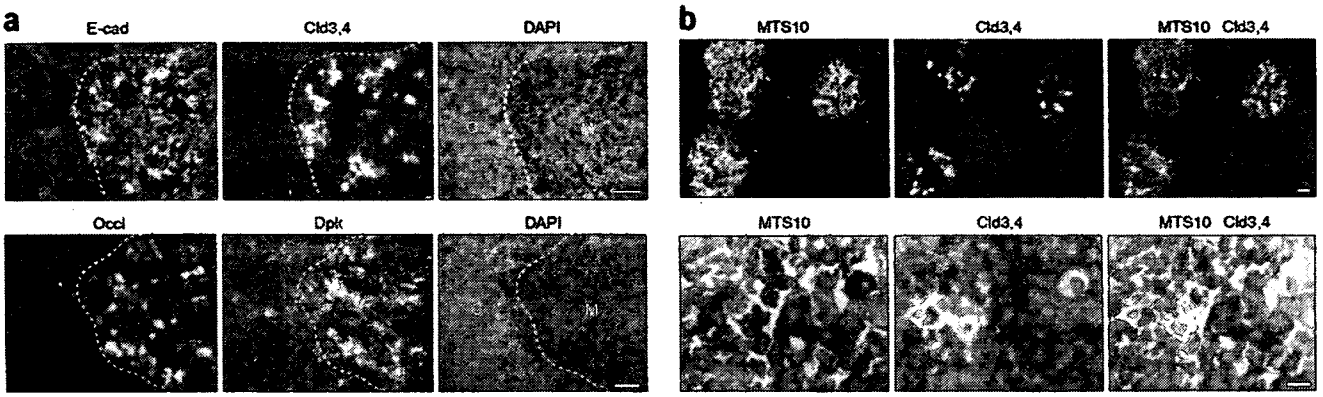

Figure 1 Expression of tight-junction components in a small population of mTECs. (**a**) Immunostaining of adult mouse thymus with antibodies to E-cadherin (E-cad), desmoplakin (Dpk), occludin (Occl) and Cld3,4. DAPI, 4,6-diamidino-2-phenylindole; M, medulla; C, cortex. Broken lines indicate the cortico-medullary borders. (**b**) Two-color immunostaining of adult mouse thymus with anti-Cld3,4 and anti-MTS10. Bottom row, blue indicates DAPI staining. Scale bars, 50 μm (**a**; **b**, top row) or 10 μm (**b**, bottom row). Data are representative of five experiments.

RESULTS

Expression of tight-junction components in Aire$^+$ mTECs

Among the claudin family members, transcripts encoding Cld3,4 were 'preferentially' expressed in mouse thymic stroma cells (data not shown); therefore, we first examined the expression of Cld3,4 and occludin protein, in addition to adherens junction and desmosome components, in the mouse adult thymus. Although E-cadherin and desmoplakin were expressed in all TECs of both cortex and medulla, we detected Cld3,4 and occludin in only a small population of TECs in the medulla (**Fig. 1a**). Double staining with an antibody specific for

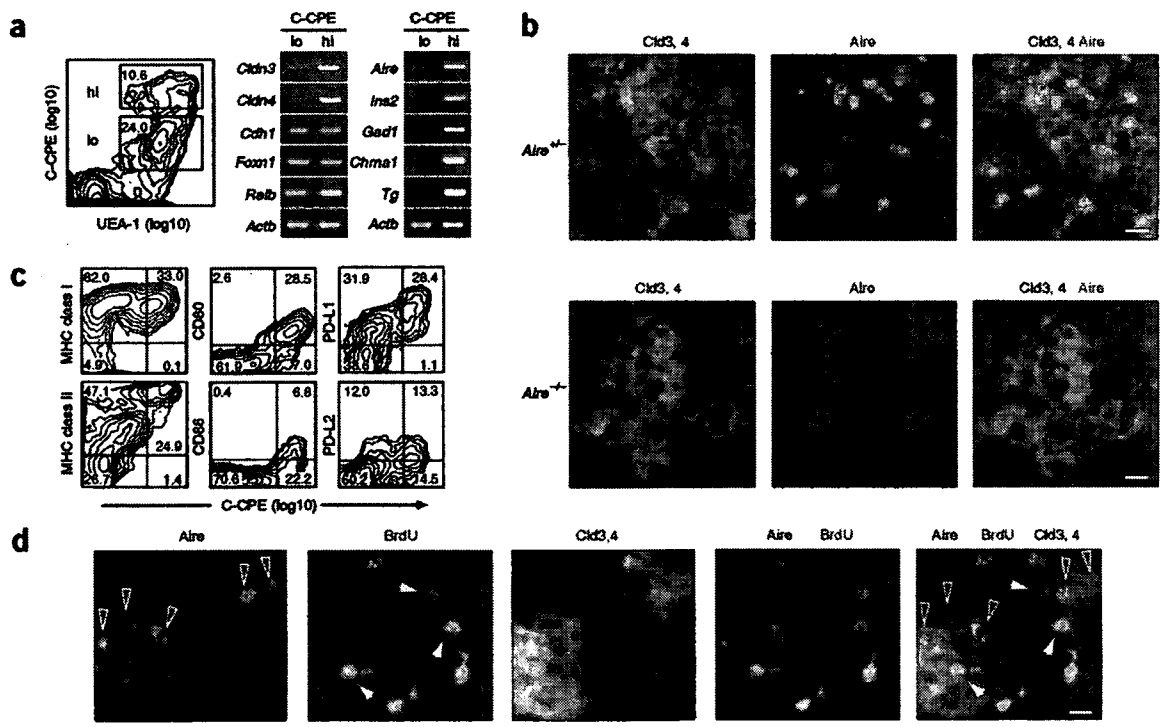

Figure 2 Cld3,4$^+$ TECs express Aire and large amounts of MHC class II and costimulatory molecules. (**a**) Left, flow cytometry of CD45$^-$ stromal cells from adult mouse thymus with biotinylated C-CPE and FITC–UEA-1. Numbers in boxed areas indicate percent C-CPE^{hi} UEA-1$^+$ cells (top) and C-CPE^{lo} UEA-1$^+$ cells (bottom). Right, RT-PCR of gene expression by sorted C-CPE^{hi} UEA-1$^+$ and C-CPE^{lo} UEA-1$^+$ cells (boxed gates at left): *Cldn3*, claudin-3; *Cldn4*, claudin-4; *Cdh1*, E-cadherin; *Foxn1*, forkhead box N1; *Relb*, RelB; *Aire*, Aire; *Ins2*, insulin II; *Gad1*, glutamic acid decarboxylase 1 (GAD67); *Chrna1*, nicotinic cholinergic receptor, α1 subunit; *Tg*, thyroglobulin; *Actb*, β-actin (loading control). (**b**) Two-color immunostaining of thymi from adult *Aire*$^{+/-}$ and *Aire*$^{-/-}$ mice with anti-Cld3,4 and anti-Aire. (**c**) Flow cytometry of CD45$^-$ stromal cells from adult thymus with biotinylated C-CPE and antibodies specific for surface markers (left margins). Numbers in quadrants indicate percent cells in each. (**d**) Three-color immunostaining of thymi from normal adult B6 mice injected intraperitoneally with 1 mg BrdU; 4 h later, thymi were stained with anti-Aire, anti-Cld3,4 and anti-BrdU. Filled arrowheads indicate Cld3,4$^+$Aire$^-$BrdU$^+$ mTECs; open arrowheads indicate Cld3,4$^+$Aire$^+$BrdU$^-$ mTECs. Scale bars (**b**,**d**), 10, μm. Data are representative of five (**a**), two (**b**) and three (**c**,**d**) experiments.

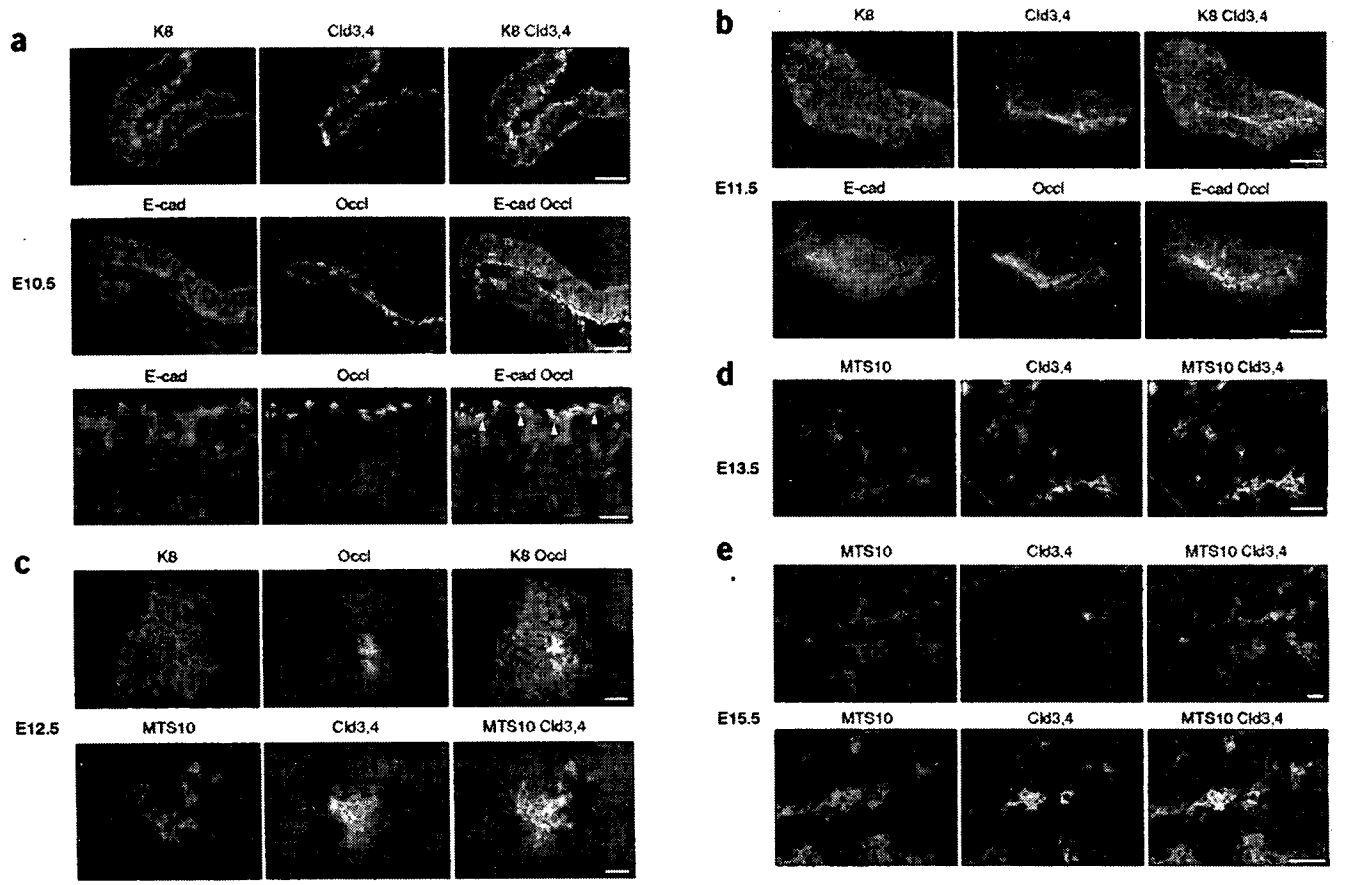

Figure 3 Expression of Cld3,4 marks the origin and development of mTECs during ontogeny. Thymic anlage at various mouse embryonic stages (left margin) was stained with various combinations of antibodies (above images). Blue (**a**, bottom) indicates DAPI staining. Arrowheads (**a**, bottom right image) indicate Cld3,4 at the apical part of cell adhesion sites, characteristic of tight junctions. K8, cytokeratin 8. Scale bars, 40 μm (**a**, top and middle), 10 μm (**a**, bottom), 50 μm (**b**; **c**, top; **d–f**) and 25 μm (**c**, bottom). Data are representative of three experiments.

MTS10, the marker of a chief subset of mTECs[20], showed that the Cld3,4$^+$ TECs had almost no MTS10 expression (**Fig. 1b**). Cld3,4$^+$ TECs were scattered in the medulla as single cells or in small cell clusters, and Cld3,4 proteins on such solitary mTECs were expressed diffusely around the cell surface with no sign of tight-junction formation with the neighboring cells (**Fig. 1b**).

To isolate Cld3,4$^+$ mTECs, we used a biotinylated C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), which binds specifically to the extracellular domains of Cld3 and Cld4 with high affinity[21] (**Supplementary Fig. 1** online). Flow cytometry of adult thymic stromal cells indicated that a small proportion (around 30%) of UEA-1$^+$ mTECs bound large amounts of C-CPE (C-CPE^{hi}), whereas the rest of the UEA-1$^+$ mTECs bound less C-CPE (C-CPE^{lo}; **Fig. 2a**). C-CPE did not bind to non-epithelial cells negative for expression of the epithelial cell–specific adhesion molecule EpCAM in the CD45$^-$ thymic stromal cell fraction (such as CD31$^+$ endothelial cells; **Supplementary Fig. 1**). Sorted C-CPE^{hi} UEA-1$^+$ TECs contained abundant *Cldn3* and *Cldn4* transcripts, whereas C-CPE^{lo} UEA-1$^+$ TECs had marginal expression of *Cldn3* and *Cldn4*; in contrast, the two subsets had similar expression of transcripts for E-cadherin, confirming the feasibility of C-CPE staining (**Fig. 2a**). Sorted C-CPE^{hi} TECs bound Cld3- and Cld4-specific antibodies, whereas C-CPE^{lo} TECs had minimal surface expression of Cld3,4 protein by immunostaining (data not shown). Therefore, we use 'C-CPE^{hi}' and 'C-CPE^{lo}' here synonymously with Cld3,4$^+$ and Cld3,4$^-$, respectively. C-CPE^{hi} TECs had more expression transcripts for the transcription factor RelB than did C-CPE^{lo} TECs, whereas expression of the gene encoding forkhead box N1 was similar in the two subsets. *Aire* and tissue-specific genes encoding insulin, glutamic acid decarboxylase (GAD67), the α1 subunit of the nicotinic cholinergic receptor, and thyroglobulin were expressed almost exclusively in C-CPE^{hi} TECs (**Fig. 2a**). Immunostaining analysis confirmed that most Aire$^+$ cells expressed Cld3,4 (**Fig. 2b**). Careful examination showed the presence of rare Aire$^+$ cells lacking Cld3,4 expression; such cells expressed CD11c and were thus considered to represent thymic dendritic cells (**Supplementary Fig. 2** online). We detected Cld3,4$^+$ mTECs in *Aire*$^{-/-}$ and *Aire*$^{+/-}$ thymi (**Fig. 2b**), indicating that expression of Cld3,4 was independent of Aire. Flow cytometry indicated that most C-CPE^{hi} mTECs had high expression of MHC class II and CD80, with a small portion (around 25%) also expressing CD86 (**Fig. 2c**). In addition, all C-CPE^{hi} mTECs also had high expression of PD-L1, a ligand for the inhibitory receptor PD-1; however, expression of PD-L2, another ligand for PD-1, was marginal. Because MHC class II–high Aire$^+$ mTECs are thought to represent terminally differentiated cells[7], we also examined the proliferation of Cld3,4$^+$ mTECs *in vivo*. At 4 h after injection of 5-bromodeoxyuridine