

Inhibition of T Helper Cell Type 2 Cell Differentiation and Immunoglobulin E Response by Ligand-activated V α 14 Natural Killer T Cells

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Summary

Murine V α 14 natural killer T (NKT) cells are thought to play a crucial role in various immune responses, including infectious, allergic, and autoimmune diseases. Because V α 14 NKT cells produce large amounts of both interleukin (IL)-4 and interferon (IFN)- γ upon in vivo stimulation with a specific ligand, α -galactosylceramide (α -GalCer), or after treatment with anti-CD3 antibody, a regulatory role on helper T (Th) cell differentiation has been proposed for these cells. However, the identity of the cytokine produced by V α 14 NKT cells that play a dominant role on the Th cell differentiation still remains controversial. Here, we demonstrate by using V α 14 NKT-deficient mice that V α 14 NKT cells are dispensable for the induction of antigen-specific immunoglobulin (Ig)E responses induced by ovalbumin immunization or *Nippostrongylus brasiliensis* infection. However, upon in vivo activation with α -GalCer, V α 14 NKT cells are found to suppress antigen-specific IgE production. The suppression appeared to be IgE specific, and was not detected in either V α 14 NKT- or IFN- γ -deficient mice. Consistent with these results, we also found that ligand-activated V α 14 NKT cells inhibited Th2 cell differentiation in an in vitro induction culture system. Thus, it is likely that activated V α 14 NKT cells exert a potent inhibitory effect on Th2 cell differentiation and subsequent IgE production by producing a large amount of IFN- γ . In marked contrast, our studies have revealed that IL-4 produced by V α 14 NKT cells has only a minor effect on Th2 cell differentiation.

Key words: interferon γ • interleukin 4 • *Nippostrongylus brasiliensis* • ovalbumin • suppression

V α 14 natural killer T (NKT)¹ cells constitute a novel lymphoid lineage distinct from T, B, or NK cells in the mouse immune system. V α 14 NKT cells are characterized by the coexpression of NK1.1 NK receptor and a single invariant antigen receptor encoded by V α 14 and J α 281 segments (1, 2) in association with a highly skewed set of V β s, mainly V β 8.2 (3–9). The invariant V α 14/V β 8.2 receptor appears not to be expressed on conventional T cells

and its expression is essential for V α 14 NKT cell development (10–12). In fact, deletion of J α 281 gene segment results in the selective loss of V α 14 NKT cell development (NKT-KO mice) (12). Furthermore, transgenic V α 14/V β 8.2 receptor expressed in recombination-activating gene 1-deficient (RAG-1^{-/-}) mice leads to the development of V α 14 NKT cells but not of other lymphoid populations (V α 14 NKT mice) (13). Taken together, these findings strongly indicate that V α 14/V β 8.2 is a unique antigen receptor for V α 14 NKT cells but not for conventional T cells. The NKT cell development was largely inhibited in β 2-microglobulin-deficient mice (14–16) and CD1d-deficient mice (17–19). These results suggested the existence of CD1d-dependent positive selection of V α 14-expressing immature NKT cells during their development. Recently,

¹Abbreviations used in this paper: α -GalCer, α -galactosylceramide; B6, C57BL/6; Nb, *Nippostrongylus brasiliensis*; NKT cells, natural killer T cells; NKT-KO mice, V α 14 NKT-deficient mice; PCA, passive cutaneous anaphylaxis; RAG^{-/-}, recombination-activating gene 1-deficient; RT, reverse transcriptase; V α 14 NKT mice, RAG^{-/-} V α 14Tg V β 8.2Tg mice.

a ligand for the invariant V α 14 NKT cell receptor has been identified as a glycolipid, α -galactosylceramide (α -GalCer, KRN7000), which is presented by CD1d (13). In addition, recent reports also suggest that glycosylphosphatidylinositol-anchored proteins may stimulate NKT cells in a CD1d-dependent manner (20, 21).

Mouse CD4⁺ helper T cells can be divided into two distinct subpopulations on the basis of their cytokine production patterns, and are designated as type 1 (Th1) and type 2 (Th2) cells (22). Th1 cells produce IL-2, IFN- γ , and TNF- β , whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (23–26). The development of Th1 and Th2 cells is central to the diversity of CD4 T cell-dependent immune responses in infectious, allergic, and autoimmune diseases (23, 24). Th1 cells mediate delayed-type hypersensitivity and organ-specific autoimmune diseases, whereas Th2 cells are involved in the development of allergies and the defense against extracellular microorganisms.

Th1 and Th2 cells are thought to be differentiated from a common precursor (23) and the direction of Th cell differentiation into Th1 and Th2 cells is dependent on exogenous cytokines present during the primary antigenic stimulation of naive T cells. It is now well documented that the potent inducer of Th1 is IL-12 (27–30). Th1 cell differentiation is inhibited in mice deficient for p40, a subunit of IL-12 receptor (31), and also in mice deficient for STAT4, a downstream signaling molecule of IL-12 receptor (32, 33). IFN- γ , which is reported to induce IL-12 secretion from various antigen presenting cells (34, 35), stimulates the induction of Th1 cells (36), and in addition inhibits Th2 cell development (37). On the other hand, IL-4 is required for the differentiation of naive T cells into Th2 effector cells (38–41), and no Th2 responses are generated in IL-4-deficient mice (42, 43) or STAT6-deficient mice (44–46).

Unlike from Th1 and Th2 cells with their restricted ability to produce particular cytokines, V α 14 NKT cells produce both IFN- γ and IL-4 after stimulation with either anti-CD3 (47, 48) or α -GalCer (13). Since IL-4 and IFN- γ have an opposite effect on Th2 cell differentiation, extensive studies on the role of NKT cells have been performed. However, this issue remains controversial. Thus, although Yoshimoto et al. reported evidences indicating an important role for NKT cell derived IL-4 in Th2 cell differentiation (49, 50), recent reports by other investigators indicated that CD1-deficient or β 2-microglobulin-deficient mice with few NKT cells produced normal levels of antigen-specific IgE (17, 51–53). In view of this situation, we decided to reexamine the role of V α 14 NKT cells on Th2 differentiation and subsequent IgE antibody responses by using V α 14 NKT cell-deficient mice generated by gene targeting.

We demonstrate in this report that V α 14 NKT cells are not required for IgE responses induced by OVA-immunization or *Nippostrongylus brasiliensis* (Nb) infection. However, antigen-specific IgE production was significantly suppressed when V α 14 NKT cells were activated with α -GalCer. These results indicate that Th2 cell differentiation and subsequent IgE responses can be negatively regu-

lated by IFN- γ produced by ligand-activated V α 14 NKT cells.

Materials and Methods

Mice. V α 14 NKT-deficient (NKT-KO) mice were established by specific deletion of the J α 281 gene segment with homologous recombination and aggregation chimera techniques (12). In these mice, only V α 14 NKT cells are missing and other lymphoid populations, such as T, B, and NK cells remain intact. The V α 14 NKT-KO mice were backcrossed 7 times with C57BL/6 (B6) mice. V α 14 NKT (RAG^{-/-} V α 14Tg V β 8.2Tg) mice with a B6 background were established by mating RAG^{-/-} V β 8.2Tg mice and RAG^{-/-} V α 14Tg mice as previously described (12, 13). In the V α 14 NKT mice, because they lacked gene rearrangement of endogenous TCR- α/β genes, only transgenic TCR- α/β (V α 14Tg and V β 8.2Tg) are expressed, and resulted in preferential development of V α 14 NKT cells with no detectable number of conventional T cells. IFN- γ -deficient mice were provided by Y. Iwakura (Institute of Medical Science, the University of Tokyo, Tokyo, Japan) (54). Pathogen-free B6, (B6 \times BALB/c)F1 mice were purchased from Japan SLC Inc. All mice used in this study were maintained in specific pathogen-free conditions and used at 8–12 wk of age.

Immunofluorescent Staining and Flow Cytometry Analysis. Freshly prepared splenocytes were suspended in PBS supplemented with 2% FCS and 0.1% sodium azide. In general, 10⁶ cells were preincubated with 2.4G2 (PharMingen) to prevent nonspecific binding of mAbs via FcR interactions, and then cells were incubated on ice for 30 min with FITC-conjugated anti-TCR- α/β (H57-597-FITC) and PE-conjugated anti-NK1.1 (PK136-PE) as previously described (12). Both reagents were purchased from PharMingen. Flow cytometry analysis was performed on Epics-Elite (Coulter Electronics).

Treatment with Anti-CD3 mAb and α -GalCer (KRN7000). Wild-type and NKT-KO mice were injected intravenously with 1.5 μ g of anti-CD3 mAb (PharMingen, 145-2C11) in 200 μ l PBS. 90 min after the anti-CD3 treatment, splenocytes were separated and cultured (5 \times 10⁶ cells/ml) in 6-well culture plates (Falcon 3046) for 1 h at 37°C, and then the supernatants were collected and subjected to ELISA for IL-4. For activation of V α 14 NKT cells with α -GalCer, mice were intraperitoneally injected with α -GalCer (100 μ g/kg) or control vehicle as previously described (13). α -GalCer (KRN7000) was provided by Kirin Brewery Co. The α -GalCer stock solution did not contain detectable endotoxins, as determined by Limulus amoebocyte assay (sensitivity limit 0.1 ng/ml) as previously described (55). The stock solution (220 μ g/ml) was diluted in control vehicle, and a mouse received 2 μ g of α -GalCer. Whole spleen cells were prepared 0.5, 1, 2, and 24 h after the injection and washed extensively with ice-cold PBS, and the amounts of IFN- γ were determined by RT-PCR. In some experiments, sera were taken 2 and 24 h after the α -GalCer injection and subjected to ELISA for IFN- γ .

ELISA for Measurement of Cytokine Concentration. IFN- γ (EN2604-50; Endogen) and IL-4 (EN2601-80; Endogen) concentrations in sera or the culture supernatants were measured by ELISA as previously described (13).

Measurement of IFN- γ Transcripts. The amounts of IFN- γ transcript were determined with reverse transcriptase (RT)-PCR. Total cellular RNA from splenocytes was prepared using TRI-ZOL (GIBCO BRL, 15596-018) according to a manufacturer's protocol. 10 μ g of RNA were reverse transcribed in 20 μ l of

mixture by using oligo dT primers, and 1 μ l of reaction mixture was subjected to PCR as previously described (10).

***Nippostrongylus brasiliensis* Infection and OVA Immunization.** Mice were subcutaneously infected with 750 third stage larvae of Nb (56). 3 and 6 wk after infection, the mice were immunized with 10 μ g DNP-conjugated *Nippostrongylus* adult antigen (DNP-Nb) mixed with 2 mg of alum [Al(OH)₃; Wako Chemical. Co.] as an adjuvant.

For OVA immunization, 10 μ g of OVA or DNP-OVA were mixed with 5 mg of alum. The immunized mice were treated intraperitoneally with α -GalCer (100 μ g/kg) or control vehicle on days 1, 5, and 9. 4 wk later, mice were challenged with 10 μ g of DNP-OVA in alum. Serum was collected 2 and 3 wk after primary OVA immunization, and 1 wk after the secondary challenge. IgE production was determined by passive cutaneous anaphylaxis (PCA) and ELISA, and the production of IgG1 and IgG2a was determined by ELISA.

ELISA for Measurement of Antibody Concentration. The total serum IgE level was determined by ELISA as previously described (57). In brief, 96-well plates (Dynatech) were coated with an anti-mouse IgE mAb (6HD5), then plates were blocked with 1% BSA. After application of serum samples and standards (anti-DNP IgE mAb, SPE-7; Seikagaku Kogyo), biotinylated anti-mouse IgE mAb (HMK-12) was added for 30 min, followed by addition of avidin-peroxidase. The plates were washed with PBS containing 0.5% Tween 20. The substrate solution (ABST and H₂O₂) was added, and the reaction was stopped with citrate and read at 450 nm with an ELISA reader (Bio-Rad 550).

For detecting anti-DNP IgG1 or IgG2a antibody, 96-well plates were coated with DNP-BSA. After blocking, standard anti-DNP IgG1 mAb (NKIgG1), standard anti-DNP anti-IgG2a mAb (DO5-1C4), and samples were added. For second antibodies, an affinity-purified rabbit anti-mouse IgG1-peroxidase (Zymed) or a rabbit anti-mouse IgG2a-peroxidase (Zymed) was used, respectively.

Measurement of IgE Antibody Production by PCA Reaction. Anti-DNP-specific and anti-OVA-specific IgE antibody was detected by PCA reaction in rats as previously described (57). Serial dilution of serum was injected intradermally into normal Wistar rats. After a 24-h sensitization period, OVA or DNP-BSA was intravenously injected with Evans Blue. The reaction was examined at 30 min after the challenge. Titers were expressed as the highest dilution eliciting a reaction.

In Vitro Th1/Th2 Cell Induction Culture. Naive CD44^{low}CD4⁺ T cells from (B6 \times BALB/c)F1 or Ly5.1 B6 spleen were prepared as follows: splenocytes were incubated with a mixture of anti-CD8 (53-6.72), anti-NK1.1 (PK136), and anti-CD44 (IM7) mAbs (PharMingen). The treated cells were washed, then incubated on plastic dishes coated with goat anti-mouse IgGs (which cross-react with rat IgG, including 53-6.72, PK136, and IM7). The nonadherent cells were used as naive CD44^{low}CD4⁺ T cell population. Contaminations of CD44^{high} cells, NK1.1⁺ cells, or CD8 T cells were <3% in either marker. The CD44^{low}CD4⁺ T cells (1.5×10^6) were stimulated with immobilized anti-TCR- α/β mAb in the presence of IL-2 (30 U/ml) and graded doses of IL-4 to induce Th1 and Th2 cells in vitro as previously described (58, 59). After stimulation for 2 d, the cells were harvested and cultured for an additional 3 d without anti-TCR stimulation in the presence of IL-2 (30 U/ml). Intracellular staining of IL-4 and IFN- γ was performed as previously described (58, 59). Biotinylated anti-K^d or anti-Ly5.1 mAbs (PharMingen) and allophycocyanin-conjugated avidin (The Jackson Laboratory) were used for identifying responding naive T cells from (B6 \times BALB/c)F1 and

Ly5.1 B6 mice, respectively. V α 14NKT cells from α -GalCer-injected V α 14 NKT mice with a normal Ly5.2 background were added to the induction culture. The cell number of V α 14NKT cells added was adjusted by prestainings of an aliquot of NKT spleen cells with anti-TCR- β and anti-NK1.1 mAbs. Where indicated, anti-IFN- γ mAb (5 μ g/ml; PharMingen) was added in the Th1/Th2 cell differentiation culture.

Results

The goal of this study was to clarify the effector mechanisms of V α 14 NKT cell regulation of Th2 cell differentiation and the subsequent induction of IgE responses. To address this question, we used NKT-KO mice in which the development of V α 14 NKT cells was dramatically inhibited (reference 12, Fig. 1 A), and which produced essentially no primary IL-4 upon anti-CD3 stimulation (Fig. 1 B) or α -GalCer-treatment (data not shown).

No Impairment in the IgE Responses Induced by Nb Infection and OVA Immunization in NKT-KO Mice. NKT-KO mice with B6 background were infected with Nb, and 3 wk later the mice were immunized with DNP-conjugated Nb in alum for the induction of DNP-specific IgE production. To our surprise, the levels of total IgE and DNP-specific IgE detected in wild-type mice were almost identical to those observed in NKT-KO mice (Fig. 2 A). In addition, DNP-specific IgG1 and IgG2a levels in NKT-KO mice were comparable to those observed in the wild-type mice (Fig. 2 A, right, middle and bottom). We also examined the involvement of V α 14 NKT cells in the regulation of IgE response induced by OVA, a conventional protein an-

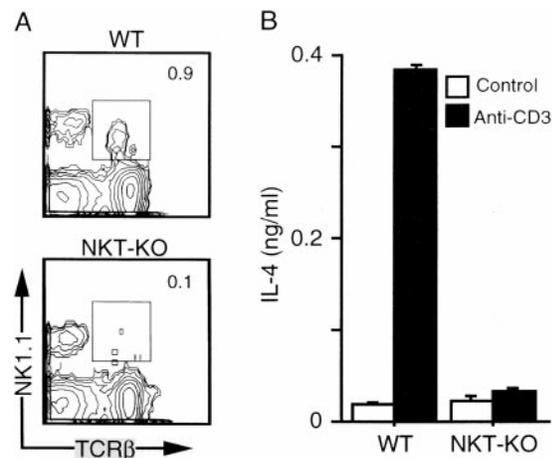


Figure 1. Primary IL-4 production in NKT-deficient mice. (A) TCR- β /NK1.1 staining profiles of spleen cells from wild-type (WT) and NKT-deficient mice (NKT-KO). The percentages of TCR- β ⁺/NK1.1⁺ NKT cells are shown in each panel. (B) IL-4 production of spleen cells from NKT-KO mice after intraperitoneal injection of anti-CD3 mAb. 90 min after the injection of 1.5 μ g of anti-CD3 mAb (black bars) or control hamster Igs (white bars), splenocytes were prepared and cultured in vitro for 60 min (5×10^6 cells/ml). The amount of IL-4 released in culture supernatant was determined by ELISA. Three mice were used in each group.

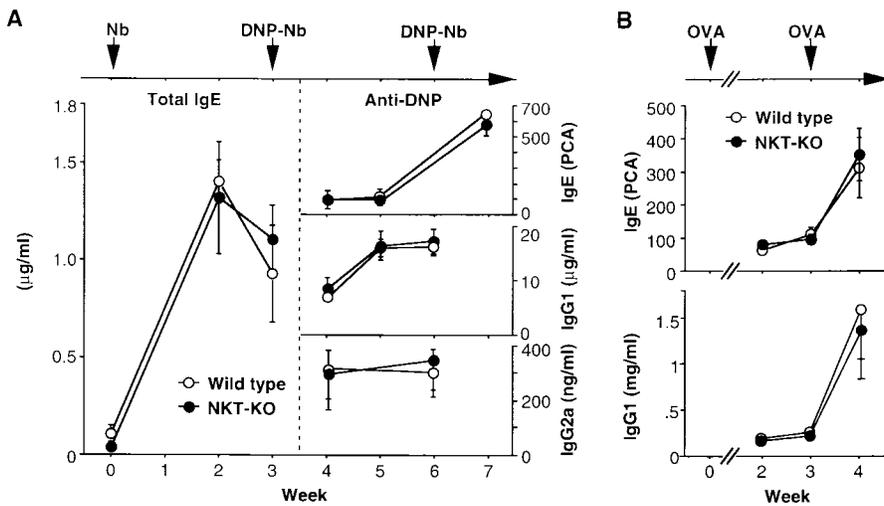


Figure 2. IgE responses induced by Nb infection and OVA immunization in NKT-KO mice. (A) Total serum IgE, antigen-specific IgE, IgG1, and IgG2a production in NKT-KO mice induced by Nb infection and DNP-Nb challenge. NKT-KO mice were infected with Nb (wk 0) and 3 wk later were immunized with DNP-conjugated Nb in alum in order to induce DNP-specific responses. The serum levels of total IgE, anti-DNP-IgG1, and anti-DNP-IgG2a were determined by ELISA. The anti-DNP-specific IgE levels were assessed by PCA reaction. Five mice were used in each group. The mean \pm SD is shown. (B) Antigen-specific IgE and IgG1 production in NKT-KO mice induced by OVA immunization. NKT-KO mice were immunized with OVA in alum (wk 0) and 3 wk later were challenged with OVA. The serum concentrations of anti-OVA-specific IgE and anti-OVA-specific IgG1 were shown. Five mice were used in each group. The mean \pm SD is shown.

tigen. NKT-KO mice were immunized with OVA in alum, and OVA-specific IgE and IgG1 productions were measured. Primary and secondary responses showed no significant differences in the serum antibody levels detected in wild-type and NKT-KO mice (Fig. 2 B). These results indicated that V α 14 NKT cells were not required for antigen-specific IgE responses induced by either Nb infection or OVA immunization.

Effect of α -GalCer Treatment on IgE, IgG1, and IgG2a Responses Induced by OVA Immunization. It is well documented that IgE and IgG1 responses are mediated by antigen-specific Th2 cells, and that IgG2a responses depend on Th1 cells (60). Consequently, we induced a specific activation of V α 14 NKT cells in vivo by using α -GalCer, and the antigen-specific IgE, IgG1, or IgG2a production was assessed. As shown in Fig. 3 A, anti-DNP IgE response induced by DNP-OVA immunization was dramatically reduced in wild-type mice after α -GalCer injection, whereas no inhibition was observed in NKT-KO mice. Some inhibitory effect was also observed in DNP-specific IgG1 response in wild-type mice (Fig. 3 B). In contrast, anti-DNP-IgG2a responses were not reduced, but rather slightly enhanced in wild-type mice (Fig. 3 C). These results suggested that the stimulation of V α 14 NKT cells

with α -GalCer resulted in the suppression of OVA-specific Th2 responses with a subsequent decrease in IgE and IgG1 production while maintaining an intact or enhanced Th1-dependent IgG2a production.

Involvement of IFN- γ in the α -GalCer-induced Suppression of IgE Responses. Since IFN- γ has a potent inhibitory effect on Th2 responses (37), we next assessed serum levels of IFN- γ in B6 mice after in vivo treatment with α -GalCer, which activates V α 14 NKT cells. Spleen cells were prepared 0.5, 1, 2, or 24 h after intravenous administration of α -GalCer, and IFN- γ transcripts were detected by RT-PCR (Fig. 4 A). Serum levels of IFN- γ were also assayed by ELISA (Fig. 4, B and C). IFN- γ transcripts were detected within 2 h after α -GalCer injection in wild-type mice, whereas no transcript was detected in NKT-KO mice. Essentially similar results were obtained by the assessment of serum IFN- γ (Fig. 4, B and C). These results strongly suggested that a large amount of IFN- γ was produced by V α 14NKT cells after α -GalCer treatment in vivo.

Next, we used IFN- γ -deficient mice and examined whether the α -GalCer-induced IgE suppression was detected or not. IFN- γ -deficient mice were immunized with DNP-OVA in alum, and primary IgE and IgG1 responses

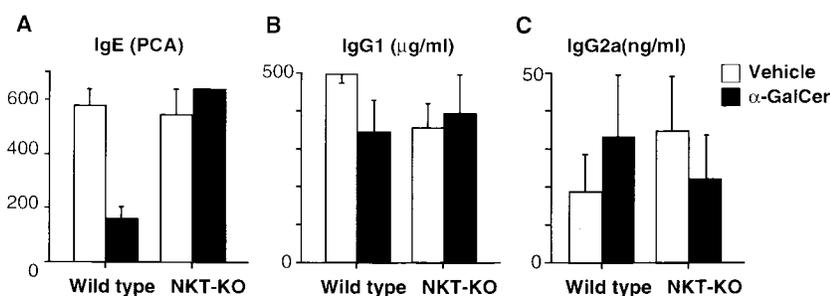


Figure 3. Serum concentration of DNP-specific IgE, IgG1, and IgG2a in NKT-KO mice treated with α -GalCer. NKT-KO mice were immunized with DNP-OVA with alum. 3 wk later, mice were challenged with DNP-OVA in alum. The sera were taken 7 d after the challenge. In addition, mice were treated with α -GalCer (black bars) or vehicle (white bars) on days 1, 3, and 9. Serum concentrations of anti-DNP-IgE, anti-DNP-IgG1, and anti-DNP-IgG2a were determined by ELISA or PCA. Each group consisted of five mice. The mean \pm SD is shown.

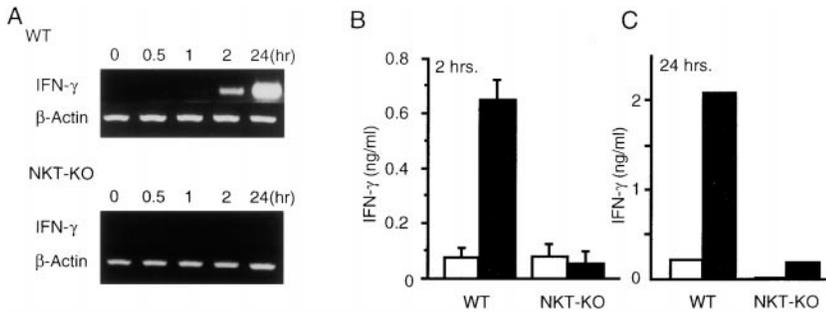


Figure 4. IFN- γ production in NKT-KO mice after α -GalCer treatment. (A) Whole spleen cells were prepared at the indicated times after intraperitoneal injection of α -GalCer, and total RNA was extracted. IFN- γ mRNA in the splenocytes were determined with RT-PCR. Serum concentrations of IFN- γ were assessed 2 (B) and 24 (C) h after the treatment with α -GalCer (black bars) or vehicle (white bars). Each group consisted of three samples.

and secondary IgE responses were assessed (Fig. 5). As we expected, no suppression in the production of IgE was observed in either primary or secondary responses in IFN- γ -deficient mice. In addition, IgG1 response was not impaired. $V\alpha 14$ NKT cells in IFN- γ -deficient mice produced an equivalent level of IL-4 upon stimulation with α -GalCer (data not shown). Thus, it is most likely that the suppressive effect on IgE production is mediated by IFN- γ produced by $V\alpha 14$ NKT cells.

Inhibition of Th2 Cell Differentiation by Activated $V\alpha 14$ NKT Cells Detected in an In Vitro Th1/Th2 Cell Induction Culture System. The results obtained thus far favor the notion that IFN- γ produced by activated $V\alpha 14$ NKT cells inhibits Th2 cell differentiation, and results in suppression of antigen-specific IgE production. Consequently, the role of ligand-activated $V\alpha 14$ NKT cells on Th2 cell differentiation was examined more precisely through the use of an in vitro induction culture system (58, 59). Naive CD4 T cells obtained from (B6 \times BALB/c)F1 mice or Ly5.1 B6 mice were stimulated with immobilized anti-TCR mAb in the presence of IL-4 to allow Th1 and Th2 cell differentiation in vitro. Several doses of $V\alpha 14$ NKT cells from α -GalCer-treated $V\alpha 14$ NKT mice with normal Ly5.2 B6 background were added in the induction culture, and the intracellular production of IFN- γ and IL-4 in K^d-positive T cells or Ly5.1 T cells was assessed as shown in Fig. 6. No detectable alloreactivity of $V\alpha 14$ NKT cells from NKT mice against (B6 \times BALB/c)F1 splenic T cells was detected (data not shown). The numbers of T cells harvested were similar in these different culture conditions (data not shown). In this culture system, an IL-4 dose-dependent increase in the generation of Th2 cells was observed (Fig. 6, A and B, top). However, the addition of activated $V\alpha 14$ NKT cells in the induction culture inhibited IL-4-producing Th2 cell differentiation in a cell-dose-dependent manner (Fig. 6, middle and bottom). In addition, the number of IFN- γ producing Th1 cell differentiation was significantly enhanced in the presence of activated $V\alpha 14$ NKT cells. The addition of nonactivated $V\alpha 14$ NKT cells from vehicle-treated $V\alpha 14$ NKT mice did not have any effect on Th1/Th2 cell differentiation (data not shown). These results clearly indicated that Th2 cell differentiation was inhibited by the addition of preactivated $V\alpha 14$ NKT cells.

Finally, we addressed whether the inhibition of Th2 cell

differentiation induced by ligand-activated $V\alpha 14$ NKT cells was mediated by IFN- γ . Anti-IFN- γ mAb was added to the induction cultures containing responder CD4 T cells and activated $V\alpha 14$ NKT cells. As shown in Fig. 6 C, the inhibition of Th2 cell differentiation induced by $V\alpha 14$ NKT cells was completely rescued by the addition of anti-IFN- γ mAb. Thus, similar to the mechanisms governing IgE suppression in in vivo experimental system (Fig. 5), IFN- γ appeared to be an effector molecule for the inhibition of Th2 cell differentiation induced by activated $V\alpha 14$ NKT cells in vitro.

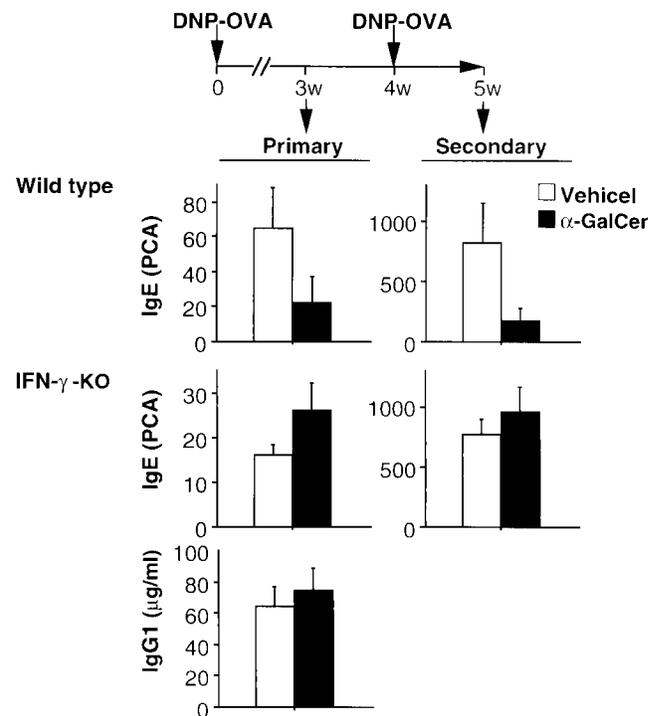


Figure 5. Serum concentration of DNP-specific IgE and IgG1 in IFN- γ -KO mice treated with α -GalCer. Serum levels of anti-DNP-IgE and anti-DNP-IgG1 were determined 3 wk after DNP-OVA immunization. Serum levels of anti-DNP-IgE at 1 wk after secondary challenge with DNP-OVA were also determined. Serum levels of anti-DNP-IgE were determined by PCA. Three mice were used in each group. The mean \pm SD is shown.

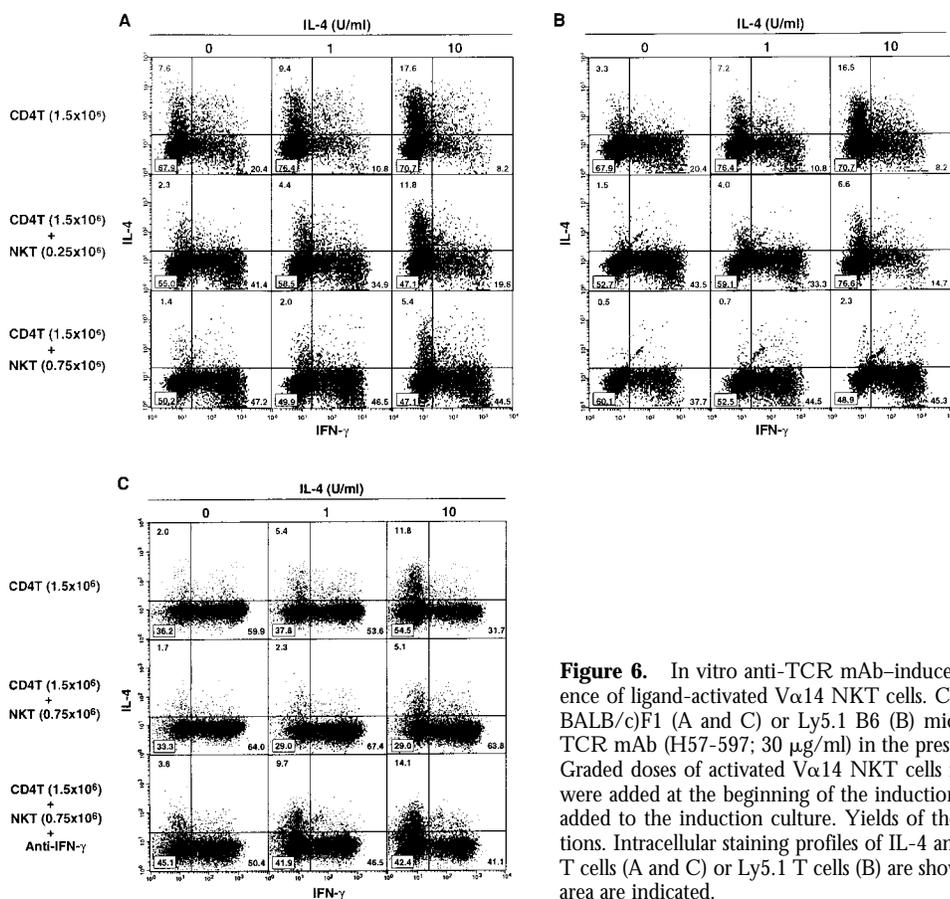


Figure 6. In vitro anti-TCR mAb-induced Th1/Th2 cell differentiation in the presence of ligand-activated V α 14 NKT cells. CD4⁺ naive T cells (1.5×10^6) from (B6 \times BALB/c)F1 (A and C) or Ly5.1 B6 (B) mice were stimulated with immobilized anti-TCR mAb (H57-597; 30 μ g/ml) in the presence of 1 or 10 U/ml of recombinant IL-4. Graded doses of activated V α 14 NKT cells from NKT mice with α -GalCer treatment were added at the beginning of the induction culture. Anti-IFN- γ mAb (5 μ g/ml) was added to the induction culture. Yields of the cells were similar in these culture conditions. Intracellular staining profiles of IL-4 and IFN- γ of electronically gated K^d-bearing T cells (A and C) or Ly5.1 T cells (B) are shown. The percentages of cells present in each area are indicated.

Discussion

In this report, we describe in vivo studies that demonstrate that V α 14 NKT cells are not required for antigen-specific IgE responses induced by Nb infection and OVA immunization (Fig. 2). This conclusion is supported by in vitro studies indicating that the addition of activated V α 14 NKT cells to in vitro Th1/Th2 cell differentiation cultures resulted in the inhibition rather than the induction of Th2 cell differentiation (Fig. 6). These results are in agreement with those reported by others (17, 51–53). Smiley et al. reported that the anti-IgD-induced IgE responses were not impaired in CD1-deficient mice in which NKT cell development is largely inhibited (17). Similarly, β 2-microglobulin-dependent T cells, including NKT cells and conventional CD8 α/β T cells, were reported to be nonessential for Th2 responses induced by immunization with different protein antigens or after infection with certain microorganisms (51–53). However, in contrast, Yoshimoto et al. reported that β 2-microglobulin-dependent T cells were important for anti-IgD-induced IgE production, and that NK1.1⁺ thymocytes restored the defect of anti-IgD-induced IgE production in β 2-microglobulin-deficient mice (49, 50). Regardless of the reasons that may explain the discrepancy with the results obtained with β 2-microglobulin-deficient mice, our data clearly demonstrate that V α 14 NKT

cells are dispensable for IgE responses, at least those induced by Nb infection and OVA immunization. In addition, our results suggest that V α 14 NKT cells are not the major cell source of IL-4 that is required for Th2 cell differentiation. Although several cell types have been proposed as candidates for the source of IL-4 that initiates certain Th2 responses (61–66), the precise mechanisms of how these cells are activated and produce IL-4 leading to Th2 cell differentiation remain to be elucidated.

More interestingly, by using α -GalCer that is a specific stimulating ligand for V α 14 NKT receptor, we found a unique regulatory role of V α 14 NKT cells on Th2 cell differentiation. We observed a selective in vivo suppression of IgE production in mice treated with α -GalCer during OVA priming or Nb infection. A mild but reproducible suppression of the IgG1 response was also observed (Fig. 3). In contrast, IgG2a response was not suppressed and in fact a slight enhancement was detected, suggesting an inhibition in the generation of antigen-specific Th2 cell differentiation. Importantly, V α 14 NKT cells produced large amounts of IFN- γ in the serum when mice were treated with α -GalCer (Fig. 4), and the suppression of IgE was not detected in either NKT-KO or IFN- γ -deficient mice (Figs. 3 and 5). Thus, it is most likely that the selective IgE suppres-

sion observed in α -GalCer-treated mice was due to an impaired Th2 cell differentiation induced by large amounts of IFN- γ secreted from activated V α 14 NKT cells. Consistent with this, the inhibition of Th2 cell differentiation induced by activated V α 14 NKT cells appeared to be an IFN- γ -mediated consequence (Fig. 6 C). A similar suppressive effect on IgE production by IFN- γ was reported in several other experimental systems (67–72). IFN- γ produced by γ/δ T cells suppressed IgE responses in OVA-specific responses (71) and cutaneous contact sensitivity system (72). In addition, since IFN- γ is known to be produced by CD8⁺ α/β -TCR T cells, a possible inhibitory role for these cells in the regulation of IgE responses has been proposed (73).

Recently, Kitamura et al., in collaboration with us, reported a study addressing the role of IL-12 in the production of IFN- γ from α -GalCer-activated NKT cells (74). The majority of IFN- γ production induced by α -GalCer and dendritic cells was found to be inhibited by the addition of anti-IL-12 mAb to the culture, indicating the involvement of IL-12 in the IFN- γ production. IL-12 appeared to be produced by dendritic cells only when they interacted with α -GalCer-activated V α 14 NKT cells. The

IL-12 in turn enhanced the IFN- γ production of the activated V α 14 NKT cells. In addition, transcriptional upregulation of IL-12 receptor was detected after α -GalCer administration (74). Thus, it is conceivable that IL-12 plays a significant role for the IFN- γ -mediated suppressive effect on IgE responses.

Our results suggest that, upon stimulation with certain ligands such as glycosylphosphatidylinositol-anchored protein (21) expressed on parasites or other microorganism, V α 14 NKT cells become IFN- γ -producing cells, leading to the inhibition of Th2 cell differentiation and suppression of IgE responses. In addition, our recent experiments have suggested that a bacteria-derived material, LPS, is able to stimulate V α 14 NKT cells to produce a large amount of IFN- γ (data not shown). Clearly, further analyses are required for addressing the physiological consequences of V α 14 NKT cell-mediated regulation of IgE response. However, it is noteworthy that the successful activation of V α 14 NKT cells and subsequent inhibition of Th2 responses, as described in this report, may open new avenues for research aimed at developing treatment for Th2-dependent diseases, such as systemic autoimmune diseases, chronic graft versus host diseases, and allergic diseases.

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