Negative feedback regulation of T helper type 1 (Th1)/Th2 cytokine balance via dendritic cell and natural killer T cell interactions

Keita Minami, Yoshiki Yanagawa, Kazuya Iwabuchi, Nobuo Shinohara, Toru Harabayashi, Katsuya Nonomura, and Kazunori Onoe

The ability of extracellular stimuli to modulate dendritic cell (DC) activation of natural killer T (NKT) cells was not well understood. We investigated the effects of the T helper type 1 (Th1)/Th2-cytokine environment on DC induction of NKT cell-mediated cytokine production in mice. Pretreatment of myeloid DCs with Th1 or Th2 cytokines, interleukin (IL)–4 or interferon (IFN)–γ, led to the enhanced production of reciprocal cytokines by NKT cells (eg, IL-4 pretreatment led to the enhanced production of Th1 cytokines) in vitro and in vivo. Thus, the recognition of Th1 or Th2 cytokines by DCs acts as a negative feedback loop to maintain Th1/Th2-cytokine balance via NKT cell functions. Using these data, we manipulated cytokine levels and innate cytolytic activity in vivo to increase an antitumor response. This is the first description of a novel regulation system governing Th1/Th2 cytokine balance involving DCs and NKT cells. (Blood. 2005;106:1685-1693)

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Introduction

Natural killer T (NKT) cells are a unique subset of lymphocytes that express some surface markers, as well as functional characteristics, of both T and NK cells. A substantial fraction of NKT cells express an invariant T-cell receptor variable α (TCR-Vα) chain, Vα14Jα18, paired with a restricted set of TCR-Vβ chains. Most NKT cells are restricted to the major histocompatibility complex (MHC) class I b molecule, CD1d, but not classic MHC. CD1d-restricted NKT cells recognize glycolipid antigens such as α-galactosylceramide (α-GalCer) in the context of CD1d on antigen-presenting cells (APCs). Following TCR ligation, NKT cells up-regulate their expression of Fas ligand (FasL). NKT cells are capable of producing large amounts of the T helper type 1 (Th1) cytokine interferon-γ (IFN-γ), and the Th2 cytokine interleukin-4 (IL-4). IFN-γ produced by NKT cells enhances the innate and Th1-dependent immune responses of NK and CD8+ T cells, ultimately leading to the elimination of pathogens and tumor cells. Conversely, NKT cells down-regulate the immune response in several autoimmune diseases in rodents and humans, and this is dependent on IL-4 production. Thus, NKT cells are capable of apparently opposite functions, the IFN-γ-mediated enhancement of host defense and IL-4-mediated immune regulation. To better understand these differing roles of NKT cells, it is essential to understand the mechanism(s) controlling the production of IL-4 and IFN-γ.

Dendritic cells (DCs) are potent professional APCs that are the cells primarily responsible for the initiation and regulation of immune responses against various antigens. Extracellular stimuli such as cytokines, chemical mediators, and pathogen-associated molecular patterns modulate DC activation of T cells and Th1 or Th2 differentiation. DCs can also activate NKT cells via CD1d.

Injection of α-GalCer–loaded DCs to mice induced prolonged IFN-γ production by NKT cells and potently inhibited the metastasis of B16 melanomas compared with mice treated with α-GalCer alone. This datum suggests that DC-presented α-GalCer promotes the development of an effective NKT response. Thus, DCs are capable of stimulating both NKT cells and T cells. However, the influence of extracellular stimuli on DC activation of NKT cells has not yet been investigated. In the present study, we examined the effect of the DC Th1/Th2 cytokine environment on the induction of NKT cell cytokine production in mice. DCs pretreated with Th1 or Th2 cytokines in vitro led to increased NKT cell production of Th2 or Th1 cytokines, respectively. We exploited this observation to manipulate the Th1/Th2 balance in vivo in antitumor models.

Materials and methods

Mice

Six- to 10-week-old female mice were used throughout the study. BALB/c mice (wild-type [WT]) were purchased from Japan SLC (Hamamatsu, Japan) and The Jackson Laboratory (Bar Harbor, ME). CD1d–/– mice, IL-12−/− mice, or ovalbumin (OVA)–specific TCR-transgenic mice (DO11.10 mice) on the BALB/c background were obtained from The Jackson Laboratory. C57BL/6 (B6) mice were purchased from Japan SLC.

Reagents

α-GalCer (Pharmaceutical Research Laboratories, Kirin Brewery, Gunma, Japan) dissolved in 0.5% polysorbate-20 at 220 μg/mL was further diluted with phosphate-buffered saline (PBS) before use. Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF), murine IL-12, murine IFN-γ, and murine IL-4 provided by R&D Systems (Minneapolis, MN) were used.

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Culture media

Cells were cultured in Iscove modified Dulbecco medium (Sigma Chemical, St Louis, MO) supplemented with 10% heat-inactivated fetal calf serum, 30% NIH3T3 culture supernatants, 10 ng/mL GM-CSF, 100 IU/mL penicillin, 100 μg/mL streptomycin, 600 μg/mL L-glutamine, and 50 μM 2-mercaptoethanol (R1 medium).

DC culture

BC1 cells were generated from splenocytes of BALB/c mice as previously described. Culture medium for the generation and expansion of DC was R1 medium. Spleen-derived DCs (SDDCs) were generated from BALB/c splenocytes by culture supernatants of IL-4 and prostaglandin (100 ng/mL) (23). Spleen cells were cultured in R1 medium for 14 days, and CD11c<sup>+</sup> cells were positively selected using anti-CD11c MicroBeads (N418) and a MACS column (Miltenyi Biotec) according to the manufacturer’s protocol. The purified cells (> 90% CD11c<sup>+</sup> cells) were considered SDDCs. SDDCs expressed moderate levels of MHC class I, MHC class II, CD80, and CD86.

Cytokine detection

The cytokine levels in culture supernatants and sera were evaluated using enzyme-linked immunosorbent assay (ELISA) kits (IFN-γ, IL-4, and IL-12p70; Opti EIA, BD Biosciences, San Diego, USA; IL-12p40: Opti EIA, BD Biosciences, San Diego, USA; IL-12p70: BioSource International, Camarillo CA).

Flow cytometry

Cells were stained with FITC-, PE-, or biotin-conjugated mAbs and streptavidin-PerCP. Expression of cell-surface markers was analyzed by flow cytometry on EPICS XL (Coulter, Miami, FL). The cytokine levels in culture supernatants and sera were evaluated using enzyme-linked immunosorbent assay (ELISA) kits (IFN-γ, IL-4, and IL-12p70; Opti EIA, BD Biosciences, San Diego, USA; IL-12p40: Opti EIA, BD Biosciences, San Diego, USA; IL-12p70: BioSource International, Camarillo CA). The intensity of the PE fluorescence was enhanced using FASTER Kit-PE (Miltenyi Biotec) according to the manufacturer’s protocol. The cells were then sorted into CD1d<sup>+</sup> TCR<sup>+</sup> cells (> 96%). Sorted splenic NKT cells (1 × 10<sup>5</sup> cells/well) were cultured with α-GalCer (100 ng/mL)–pulsed DCs (1 × 10<sup>4</sup> cells/well) from BC1 for 48 hours on a V-bottom 96-well plate.

Stimulation of DCs with anti-CD40 mAb

IFN/DCs, IL-4/DCs, or immature (i) DCs derived from BC1 cells were stimulated with an optimal concentration (5 μg/mL) of anti-CD40 mAb, HM40-3.

Stimulation of NKT cells with α-GalCer–loaded DCs in vivo

Mice were given IL-4 using the long-lasting IL-4 formulation (IL-4C), consisting of 4 μg IL-4 mixed with 20 μg 11B11, anti–mouse IL-4 mAb (rat IgG1). IL-4C (4 μg IL-4 with 20 μg 11B11) in 100 μL PBS was injected into mice intravenously. After 3 days, each spleen was separated and a single-cell suspension was prepared. The splenocytes (2.5 × 10<sup>5</sup> cells/well in a 24-well plate) were stimulated with various concentrations of α-GalCer for 48 hours.

In some experiments, 2 μg α-GalCer was injected intravenously into IL-4C–pretreated or untreated mice and the amount of cytokines in the sera was measured.

Intracellular cytokine staining

Two hours after α-GalCer (2 μg/mouse) injection into IL-4C–pretreated or untreated mice, spleens were separated and a single-cell suspension was prepared. The cells were stained using anti-TCRβ mAb, α-GalCer–loaded CD1d-dimer, anti-DX5 mAb, and anti-NK1.1 mAb. The intensity of the PE fluorescence of the CD1d-dimer was enhanced using FASTER Kit-PE (Miltenyi Biotec). The cells were fixed, permeabilized, and stained with FITC–anti–IFN-γ mAb. The fluorescence intensity of the cells was analyzed by flow cytometry.

Cytotoxicity assay

Twenty-four hours after α-GalCer injection (2 μg/mouse) into IL-4C–pretreated or untreated mice, cytotoxicity of the splenocytes against YAC-1 (NK-sensitive leukemia cell line), P815 (mastocytoma cell line), RenCa (BALB/c-derived renal adenocarcinoma cell line), or CT26 (BALB/c-derived colon tumor cell line) was measured by a standard 4-hour chromium 51 (51Cr)–release assay. The cytotoxicity (%) was calculated as follows: cytotoxicity (%) = [(experimental release − spontaneous release)/ (maximum release − spontaneous release)] × 100.

Experimental metastasis assay

RenCa (5 × 10<sup>5</sup>) cells were injected intravenously together with 2 μg α-GalCer or vehicle to IL-4C–pretreated or untreated mice. Three weeks after tumor inoculation, the number of lung metastases was counted with the aid of a dissecting microscope.

IFN-γ, and murine IL-4 were purchased from PeproTech (Rocky Hill, NJ). Purified anti–mouse IL-4 monoclonal antibody (mAb) (11B11), CD1d dimer (CD1d:immunoglobulin [Ig] fusion protein), fluorescein isothiocyanate (FITC)–conjugated anti–mouse TCR β chain mAb (H57-597), phycoerythrin (PE)–conjugated anti–mouse CD49b/Pan-NK cells mAb (DX5), PE-conjugated anti–mouse NK1.1 mAb (PK136), PE-conjugated anti–mouse CD11c mAb (HL3), PE-conjugated anti–mouse CD45R/B220 mAb (RA3-6B2), PE-conjugated anti–mouse IgG<sub>1</sub> mAb (A85-1), biotin-conjugated anti–mouse CD1d mAb, FITC-conjugated anti–mouse CD40 mAb (3/23), FITC-conjugated anti–mouse CD86 mAb (GL1), FITC–conjugated anti–mouse H-2K<sup>d</sup> mAb (SF1-1.1), FITC–conjugated anti–mouse IFN-γ mAb (XMG1.2), biotin-conjugated anti–mouse I-A<sup>d</sup> mAb (AMS-32.1), biotin-conjugated anti–mouse TCRβ mAb (H57-597), and streptavidin–peridinin chlorophyll protein (PerCP) were obtained from BD Biosciences Pharmingen (San Diego, CA). Biotin-conjugated KJ1-26 (a clonotypic mAb for the transgenic TCR of DO11.10 mice) and PE–biotin-conjugated anti–mouse CD11c-dimer (CD1d:Ig fusion protein) and PE–conjugated anti–mouse IgG<sub>1</sub> mAb (100 ng/mL) or vehicle for 24 hours and then stimulated with IFN-γ/DCs or IL-4/DCs. Each DC group was cocultured with nylon fiber– or IL-4–treated DCs were considered SDDCs. SDDCs expressed CD11c<sup>+</sup> cells in splenocytes were positively selected using anti-CD11c MicroBeads (N418) and a MACS column (Miltenyi Biotec) according to the manufacturer’s protocol. The purified cells (> 90% CD11c<sup>+</sup> cells) were considered SDDCs. SDDCs expressed moderate levels of MHC class I, MHC class II, CD80, and CD86.

IFN-γ and α-GalCer treatment in vivo

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CD11c<sup>+</sup> cells prepared from the splenocytes of IL-4C–pretreated or untreated mice were positively selected using anti-CD11c MicroBeads and a MACS column. DC-depleted splenocytes (CD11c<sup>+</sup> splenocytes) were prepared using anti-CD11c MicroBeads and MACS column from the splenocytes of IL-4C–pretreated or untreated mice. Purified DCs and CD11c<sup>+</sup> splenocytes were mixed and cultured with various concentrations of α-GalCer for 48 hours.

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Differentiation of T cells in vitro and analysis of intracellular cytokine

Th polarization of DO11.10 CD4+ T cells was performed as described in our previous study. IFN/DCs, IL-4/DCs, or iDCs from BC1 cells were cocultured with purified CD4+ DO11.10 T cells in the presence of a low- or high-dose OVA323-339 peptide (0.01 or 10 μM). After 5 days, intracellular IFN-γ staining of KJ1-26+ T cells was performed according to our previous study.

Statistical analysis
Student t test was employed to compare the data of 2 different groups.

Results
Stimulation of NKT cells with α-GalCer–loaded DCs in vitro

We examined the ability of DC cytokine treatment to affect NKT cell cytokine production. SDDCs were positive for CD11b and negative for CD8 and B220 (data not shown), a pattern typical of myeloid DCs. We refer to SDDCs stimulated with IL-4 or IFN-γ for 3 days as IL-4/DCs or IFN/DCs, respectively. Unstimulated SDDCs were used as iDCs.

To evaluate the ability of the DC subtypes to stimulate NKT cells, nylon nonadherent splenocytes were cocultured with α-GalCer–loaded DCs for 48 hours (Figure 1A). Significant IFN-γ and IL-4 production was observed in splenocytes cocultured with α-GalCer–loaded iDCs, IL-4/DCs, or IFN/DCs in a dose-dependent manner. No IFN-γ or IL-4 production was detected in the absence of α-GalCer. Interestingly, α-GalCer–loaded IL-4/DCs induced significantly higher IFN-γ production by NKT cells than α-GalCer–loaded iDCs or IFN/DCs (Figure 1A, top left panel). α-GalCer–loaded iDCs and IFN/DCs induced similar levels of IFN-γ production. In contrast, α-GalCer–loaded IFN/DCs induced considerably greater IL-4 production by NKT cells than α-GalCer–loaded iDCs or IL-4/DCs (Figure 1A, top right panel). No differences in IL-4 production were detected between α-GalCer–loaded iDCs and IL-4/DCs. Similar results were obtained using SDDCs derived from IL-12−/− mice (Figure 1A, middle panels). Splenocytes from CD1d−/− mice did not respond to any of the α-GalCer–loaded DC groups (Figure 1A, bottom panels).

We repeated these experiments using a DC line, BC1 cells. As shown in Figure 1B, α-GalCer–loaded IL-4/DCs or IFN/DCs induced substantial levels of IFN-γ or IL-4 production by NKT cells, respectively, compared with other DC groups including α-GalCer–loaded iDCs. These findings are consistent with those obtained with SDDCs.
We then performed the culture of sorted CD1d-dimer+ T cells (NKT cells) with each group of DCs (Figure 1C). α-GalCer–loaded IL-4/DCs induced significantly higher IFN-γ production by sorted NKT cells than α-GalCer–loaded iDCs. In contrast, α-GalCer–loaded IFN/DCs induced a considerably smaller amount of IFN-γ production by the sorted NKT cells than α-GalCer–loaded iDCs.

**IL-12 production by IFN-γ– or IL-4–treated DCs**

IL-12 enhances IFN-γ production by NKT cells. Thus, we analyzed CD40- and IL-12 production by DCs using the BC1 cell line. IFN/DCs produced a considerable amount of IL-12 p40 in the absence of anti-CD40 mAb, whereas neither IL-4/DCs nor iDCs produced IL-12 under these conditions (Figure 1D). After stimulation with anti-CD40 mAb, considerable IL-12 p40 production was observed in all cultures. The production of IL-12 p40 was greatest in the IFN/DC cultures. No detectable level of IL-12 p70 was demonstrated in our culture condition (data not shown). Further, no considerable production of IL-4 and IFN-γ was detected in any cultures analyzed upon stimulation with anti-CD40 mAb (data not shown).

IL-12 p40 production was also analyzed during the interaction between NKT cells and DCs. Nylon nonadherent splenocytes were cocultured with each group of α-GalCer–loaded DCs derived from SDDCs (Figure 1E) or that derived from BC1 cells (Figure 1F). The presence of IL-12 p40 was detected in all combinations of NKT cells and DCs. The amount of IL-12 p40 was directly related to the DC number in all cultures examined, and no differences were seen among these cultures (Figure 1E-F). IL-12 p40 was not detected in the culture with each DC derived from IL-12−/− SDDCs (data not shown). IL-12 p70 production was not detected in any cultures tested (data not shown).

**Stimulation of NKT cells with α-GalCer–loaded DCs in vivo**

To analyze the ability of each DC group to stimulate NKT cells in vivo, we directly injected each type of α-GalCer–loaded SDDCs into mouse spleens and serially examined IL-4 and IFN-γ levels in the serum. After the injection of α-GalCer–loaded DCs, serum levels of IL-4 and IFN-γ were significantly increased, and these levels peaked at 6 and 12 hours, respectively (data not shown). Figure 2 shows the serum levels of IL-4 and IFN-γ at 6 and 12 hours, respectively, after intrasplenic injection of vehicle-loaded or α-GalCer–loaded DCs. All α-GalCer–loaded DCs from WT mice induced high levels of IFN-γ and IL-4 in the serum, whereas vehicle-loaded WT DCs and α-GalCer–loaded DCs from CD1d−/− mice had no appreciable effect on serum cytokine levels. The DCs from CD1d−/− mice were functional, however, when compared with WT DCs in a mixed lymphocyte reaction (data not shown). Thus, considerable levels of IFN-γ and IL-4 are produced by α-GalCer–specific, CD1d-restricted NKT cells in vivo.

Consistent with the results presented in Figure 1, α-GalCer–loaded IL-4/DCs induced a considerably higher amount of IFN-γ than iDCs or IFN/DCs. Additionally, α-GalCer–loaded IFN/DCs induced the production of significantly greater levels of IL-4 than iDCs or IL-4/DCs did.

**Flow cytometric analysis of cell-surface molecules on DCs**

We next compared the expression of different cell-surface markers on iDCs, IL-4/DCs, and IFN/DCs (Figure 3). IFN-γ markedly increased CD1d expression on BC1 cells and SDDCs compared with nontreated iDCs (Figure 3B). In contrast, IL-4 significantly decreased CD1d expression. IFN-γ also increased CD40, CD86, and H-2Kd expressions, while no significant effects were seen on I-Aα expression on BC1 cells and SDDCs. IL-4 did not affect CD40, CD86, H-2Kd, and I-Aα expression on either cell type. CD1d−/− SDDCs behaved similarly to WT cells when treated with IFN-γ or IL-4 (with the exception of changes in CD1d expression).

**The effect of IL-4 administration on α-GalCer–induced cytokine production in vitro and in vivo**

As discussed in Figures 1 and 2, IL-4 modulates DC function leading to enhanced IFN-γ production by NKT cells. It has been reported that a complex of IL-4 with an anti–IL-4 mAb (IL-4C) protects IL-4 from degradation and slowly releases biologically active IL-4 in vivo. As a result, the in vivo half life of IL-4 increases from a few minutes to approximately 24 hours. Using IL-4C, we examined the direct effect of IL-4 on cytokine production by NKT cells in vivo. Splenocytes were prepared from the mice pretreated with IL-4C or anti–IL-4 mAb alone and stimulated with various concentrations of α-GalCer for 48 hours in vitro. Treatment with IL-4C or anti–IL-4 mAb did not affect the cellular composition of the spleen (Table 1). Either treatment showed no significant effects on the cellular composition including the proportion of NK1.1+ T cells in B6 mice (data not shown).

Following α-GalCer treatment, the level of α-GalCer–induced IFN-γ production was significantly higher in splenocytes from IL-4C–treated mice than the control mice (Figure 4A, left panel). In contrast, IL-4 production was decreased in the IL-4C–treated splenocytes (Figure 4A, right panel). Treatment with anti–IL-4 mAb alone showed no significant effects on α-GalCer–induced cytokine production by splenocytes.

We next examined cytokine production in mice stimulated with α-GalCer in vivo (Figure 4B). After α-GalCer injection, serum IFN-γ levels were markedly increased at 3 hours, peaked at 12 hours, and decreased at 24 hours in control mice. Conversely, IL-4 levels increased dramatically by 3 hours but rapidly decreased at 6 hours. Pretreatment with IL-4C markedly enhanced the α-GalCer–induced IFN-γ production at 3 to 12 hours. IL-4 production was not significantly affected at 3 hours. However, in IL-4C–treated mice, IL-4 levels were sustained for 6 hours or more following α-GalCer injection. Pretreatment with anti–IL-4 mAb alone also similarly delayed the decrease in IL-4 levels, but had no significant effect on IFN-γ production (data not shown). α-GalCer failed to induce IFN-γ and IL-4 production in CD1d−/− mice (Figure 4B).

We also analyzed IL-12 p40 and IL-12 p70 production in mice treated with IL-4C or anti–IL-4 mAb. After the injection of α-GalCer, serum IL-12 p40 levels significantly increased at 3
hours, reached a peak at 6 hours, and decreased at 12 hours (data
not shown). In contrast, IL-12 p70 production was detected only at
6 hours after α-GalCer injection. Figure 4C shows the levels of
IL-12 p40 and IL-12 p70 6 hours after α-GalCer stimulation. No
significant differences were observed in IL-12 p40 and p70
production between IL-4C–pretreated and untreated mice. α-GalCer
failed to induce appreciable levels of IL-12 p40 and IL-12 p70
production in CD1d−/− mice.

To directly demonstrate IFN-γ production by NKT cells, we
performed intracellular IFN-γ staining of splenic NKT cells.
Intracellular IFN-γ production by splenic NKT or NK cells was
analyzed on CD1d dimer+ TCRβ+ NKT cells or DX5+ TCRβ− NK
cells in BALB/c strain (Figure 4D) and on NK1.1+ TCRβ+ NKT
or NK1.1+ TCRβ− NK cells in B6 strain (Figure 4E) 2 hours
after α-GalCer injection. Following α-GalCer administration,
marked or slight intracellular IFN-γ production was detected in
NKT cells or NK cells, respectively. The α-GalCer–induced IFN-γ
production by NKT cells was significantly enhanced by IL-4C
pretreatment (Figure 4D-E, bottom panels). In contrast, IL-4C
exerted no significant effects on α-GalCer–induced IFN-γ produc-
tion by NK cells.

The effect of IL-4C treatment in vivo on induction of cytokine
production by NKT cells in vitro

We evaluated the ability of DCs from IL-4C–pretreated mice to
induce NKT cell cytokine production in vitro. DCs prepared from
the splenocytes of IL-4C–pretreated or untreated mice (IL-4C DCs
or control DCs) were mixed with DC-depleted splenocytes derived
from IL-4C–pretreated or untreated mice (IL-4C SP or control SP).
The mixed cells were then stimulated with various concentrations
of α-GalCer. Control SP and IL-4C SP produced considerable
levels of both IFN-γ and IL-4 after 2 days of culture in the presence
of either control DCs or IL-4C DCs, while only slightly producing
cytokines in the absence of DCs (Figure 5). In the presence of
100 ng/mL α-GalCer, IFN-γ production by control SP with IL-4C
DCs was significantly higher than that by control SP with control
DCs (Figure 5A, P < .05). Similarly, the level of IFN-γ produced
by IL-4C SP with IL-4C DCs was high compared with that by
IL-4C SP with control DCs. Notably, IFN-γ production by IL-4C
SP with control DCs was considerably higher than that by control
SP with control DCs in the presence of 100 ng/mL α-GalCer
(P < .05). IL-4C treatment showed no significant effects on IL-4
production in these cultures (Figure 5B). No cytokine production
was detected in cultures of either control DCs or IL-4C DCs alone.

The effect of IL-4C pretreatment on α-GalCer–induced
cytotoxicity against various tumor cells

We examined the effect of IL-4C pretreatment on α-GalCer–
induced cytotoxicity. Consistent with previous studies,6,34,35 admin-
istration of α-GalCer alone significantly increased the cytoxicity
of splenocytes against all tumor cell targets, YAC-1, P815, RenCa,
or CT26 (Figure 6A-D). It should be noted that α-GalCer–induced
cytotoxicity was markedly augmented in mice pretreated with
IL-4C compared with control mice. IL-4C pretreatment alone did
not affect the innate cytoxicity of splenocytes.

The effect of IL-4C pretreatment on α-GalCer–induced
antimetastatic effects against RenCa cells

We next evaluated the effect of IL-4C pretreatment on α-GalCer–
induced antimetastatic effects in a mouse lung metastasis model.
Treatment with α-GalCer alone markedly inhibited metastasis

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**Figure 3. Expression of surface molecules on DCs treated with IFN-γ or IL-4.**

Expressions of CD1d, CD40, CD86, H-2Kd, and I-A^d on DCs derived from BC1 cells and SDDCs (WT or CD1d−/−) were analyzed by flow

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**Table:**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Control</th>
<th>IL-4DC</th>
<th>IL-4C-DC</th>
<th>IFN-DC</th>
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<td>CD1d</td>
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<tr>
<td>I-A^d</td>
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Statistical significance was calculated by Student's t-test (*P < .05, **P < .01 vs iDCs).
by Student SE of 3 independent experiments. Statistical significance was calculated of IFN-CD1d-dimer or isotype-matched Ig. Each symbol or column represents the mean MFI (cont.) or IL-4C. The intracellular IFN-3 days after administration of IL-4C or PBS (cont.). The amount of IFN-

**Effect of IL-4 and IFN-γ on capability of DCs for Th1/Th2 differentiation**

We also evaluated the ability of IL-4/DC and IFN/DCs to induce Th1/Th2 development in OVA-specific T cells (DO11.10) (Figure 4). This is consistent with previous reports.34,35 IL-4C pretreatment further enhanced the antimitastatic activity of α-GalCer (Figure 6E). Significantly reduced numbers of metastases were observed in α-GalCer–treated mice that had been pretreated with IL-4C compared with mice treated with α-GalCer alone. IL-4C pretreatment alone did not affect the number of lung metastases.

**Table 1. Cell populations in spleens of mice treated with IL-4C or anti–IL-4 mAb in vivo**

<table>
<thead>
<tr>
<th>Cell populations in spleens, %</th>
<th>B cell</th>
<th>T cell</th>
<th>DC</th>
<th>NK cell</th>
<th>DX5+ T cell</th>
<th>CD1d-dimer+ T cell</th>
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<td>Control</td>
<td>53.7 ± 1.7</td>
<td>32.5 ± 1.6</td>
<td>2.6 ± 0.4</td>
<td>6.4 ± 1.2</td>
<td>0.45 ± 0.02</td>
<td>0.13 ± 0.03</td>
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<tr>
<td>IL-4C</td>
<td>53.8 ± 1.8</td>
<td>33.4 ± 2.6</td>
<td>3.5 ± 0.5</td>
<td>8.2 ± 1.2</td>
<td>0.36 ± 0.03</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Anti-IL-4 mAb</td>
<td>54.9 ± 1.4</td>
<td>29.8 ± 1.1</td>
<td>3.2 ± 0.4</td>
<td>8.1 ± 1.4</td>
<td>0.33 ± 0.05</td>
<td>0.15 ± 0.01</td>
</tr>
</tbody>
</table>

BALB/c mice were given IL-4C, anti–IL-4 mAb, or PBS as a control. Three days after the injection, cell populations in the spleen were analyzed by flow cytometry. Each datum represents the mean of 3 independent experiments.

(Figure 6E-F). In agreement with our previous study,14 DCs with a high- or low-dose antigen predominantly promoted Th1 or Th2 development, respectively. The ability of IL-4/DCs to induce Th1/Th2 development was almost the same as that of untreated DCs in the presence of low-dose antigen, whereas Th1 development by IL-4/DCs with high-dose antigen was suppressed compared with that by untreated DCs. In contrast, the ability of IFN/DCs to induce Th1/Th2 development was almost the same as that of untreated DCs in the presence of high-dose antigen, whereas Th1 development induced by IFN/DCs with low-dose antigen was significantly greater than that by untreated DCs. Thus, the Th1 or Th2 cytokine environment of DCs promoted Th1 or Th2 development of DO11.10 T cells, respectively, depending on the antigen dose used. These results demonstrate that negative feedback regulation of Th1/Th2 cytokine balance between DCs and NKT cells is not seen between DCs and mainstream T cells.

**Discussion**

Several reports have shown the negative feedback regulation of immune responses by DCs,36,37 In human systems, monocyte-derived DCs (DC1) are primarily involved in the development of Th1 responses, whereas plasmacytoid-derived DCs (DC2) support Th2 development.36 The Th2 cytokine IL-4 enhances DC1 maturation and kills DC2 precursors, while the Th1 cytokine IFN-γ rescues the IL-4–induced killing of the DC2 precursors. Thus, cytokine production by mature Th1 or Th2 cells governs a negative feedback loop that selectively inhibits prolonged Th2 or Th1 responses, respectively, by regulating the survival of the DC subset involved in the generation of a polarized immune response.

In the murine system, however, Hochrein et al reported that IL-4 enhanced the production of IL-12 p70 by splenic DCs stimulated with IFN-γ plus maturation stimuli such as microbial components or CD40 ligation in vitro. In addition, production of
IL-12 p70, a major Th1-inducing cytokine, induced by lipopolysaccharide (LPS) or cytidine phosphate guanosine (CpG) stimulation in vivo was enhanced by simultaneous treatment with IL-4. The negative feedback regulation of Th1/Th2 cytokine balance involving DCs and NKT cells was also observed in in vivo experiments where DCs pretreated with IL-4 or IFN-γ resulted in enhanced NKT cell IFN-γ production following stimulation with α-GalCer ex vivo. Although α-GalCer–induced IFN-γ production by splenocytes from IL-4C-treated mice was higher than that by control splenocytes in the presence of control DCs at a concentration of α-GalCer (100 ng/mL), we consider that NKT cells in IL-4C–treated mice had been influenced by IL-4–conditioned DCs in vivo, which resulted in the enhanced production of IFN-γ upon encountering α-GalCer in vitro.

Thus, the Th1/Th2 cytokine environment provides a negative feedback loop by regulating the ability of DCs to preferentially induce contrasting cytokine production by NKT cells. The negative feedback regulation via DCs and NKT cells likely suppresses substantial deviations from Th1/Th2 balance. Thus far, no such regulation was demonstrated between DCs and mainstream T cells. This is the first description of a novel regulation system governing Th1/Th2 cytokine balance involving DCs and NKT cells.

Concerning mechanisms underlying the negative feedback regulation, IL-12 enhances IFN-γ production by NKT cells. However, we found that IL-12 productions were at similar levels among the NKT cell cultures with various DCs. In addition, the negative feedback regulation of Th1/Th2 cytokine balance via DCs and NKT cells was demonstrated in the absence of IL-12. Thus, IL-12 appears to be dispensable for this regulation system.

DCs are thought to be the most potent activators of NKT cells via CD1d compared with other APCs. However, the regulation of CD1d expression on DCs is not fully understood. LPS and IFN-α increased CD1d expression on bone marrow–derived DCs in parallel with MHC class II, CD80, CD86, and CD40. In the present study, we found that IFN-γ also increased CD1d expression on DCs, but IL-4 significantly decreased CD1d expression. It is generally considered that CD86 expressed on APCs promotes NKT

Figure 6. The effect of IL-4C pretreatment on α-GalCer–induced cytotoxicity and prevention of lung metastasis. α-GalCer (2 μg) or vehicle was intravenously injected into IL-4C–pretreated or untreated (cont.) mice. After 24 hours, cytotoxicity of the spleen cells against (A) YAC-1, (B) P815, (C) RenCa, or (D) CT26 was measured by a 4-hour 51Cr-release assay. Each symbol represents the mean ± SE of 3 independent experiments. Statistical significance was calculated by Student t test (***P < .001 vs cont.). (E) IL-4C– or PBS (cont.)–pretreated mice were intravenously injected with 5 × 106 RenCa cells with or without 2 μg α-GalCer. After 3 weeks, the number of lung metastases was counted. Each column represents the mean ± SE of 5 individuals. Statistical significance was calculated by Student t test (**P < .01). (F) Representative views of lung metastasis in nontreated (cont.), IL-4C–treated, α-GalCer–treated, and IL-4C plus α-GalCer–treated mice.

Figure 7. The effect of IL-4 or IFN-γ on preference of DCs to induce Th differentiation. DO11.10 CD4+ T cells were cocultured with iDCs, IL-4/DCs, or IFN/DCs from BC1 cells in the presence of a high- or low-dose OVA323-339 peptide for 5 days. The cells were stained by KJ1-26 mAb and monitored for intracellular staining of IFN-γ and IL-4. (A) Dot plots gated by KJ1-26+ cells. Results are representative of 3 independent experiments. (B) Proportions of IL-4+ IFN-γ+ (Th2, left side) or IFN-γ IL-4– (Th1, right side) cells in the KJ1-26+ population. Each column represents the mean ± SE of 3 independent experiments. Statistical significance was calculated by Student t test (***P < .001 vs iDCs).
cell activation. The levels of CD86 on IFN/DCs were significantly higher than that on iDCs and IL-4/DCs. No differences were noted in the expression level of CD86 between IL-4/DCs and iDCs. On the other hand, our preliminary experiments showed that the expression of Qa-1b, an inhibitory molecule in NKT activation, 39 was reduced on IL-4/DCs, but increased on IFN/DCs (data not shown). It seems to us that the balance of TCR-mediated signal, costimulatory signal, and inhibitory signal via CD1d, CD86, and Qa-1b on DCs is important to induce selective promotion of cytokine production by NKT cells with each DC group. Thus, the mechanism underlying the negative feedback regulation may be not simple.

It has been reported that IL-18 promotes productions of both IL-4 and IFN-γ by NKT cells, while IL-7 selectively enhances IL-4 production. 40,41 In the present study, α-GalCer–loaded IFN/DCs selectively enhanced IL-4 production by NKT cells, raising a possibility that IL-7 is involved in the enhancement of IL-4 production by NKT cells stimulated with IFN/DC. Thus, we are currently trying to clarify the mechanism underlying negative feedback regulation of Th1/Th2 cytokine balance via DCs and NKT cells by focusing our analysis on DC-produced cytokines including IL-7 and IL-18 and costimulatory, inhibitory, and adhesion molecules on DCs.

In the present study, we used IL-4C to prolong the half-life of IL-4 in vivo. IL-4C protects IL-4 from degradation and slowly releases biologically active IL-4 in vivo. 27,30 In the presence of IL-4C, α-GalCer primarily induced IFN-γ production by NKT cells in vivo. Since a method to achieve the prolonged release system of biologically active IFN-γ in vivo has not been established, we treated mice with IFN-γ alone to examine the effect of a Th1 cytokine environment on the subsequent NKT cell–mediated cytokine productions in vivo. However, treatment with IFN-γ alone did not significantly affect cytokine production (data not shown). It has been reported that IFN-γ very rapidly disappears from the serum after injection into mice. 43 Similarly, we found that pretreatment with IL-4 alone only slightly enhanced NKT cell IFN-γ production (data not shown). Consequently, we cannot fully address the ability of a dominant Th1 environment to modulate NKT cell cytokine production until the advent of a better technology to effect sustained IFN-γ levels in the serum.

α-GalCer has been found to be antitumorogenic in a variety of different tumor cell models. 44 The antitumor effects of α-GalCer are thought to require NKT cell–mediated IFN-γ production. 21,45-47 α-GalCer–induced IFN-γ production enhances innate and Th1-dependent immune responses such as NK and CD8+ T-cell–mediated tumor killing. Consequently, we exploited our newly found understanding of Th1/Th2 feedback regulation to enhance the antitumor effects of α-GalCer. IL-4C administration significantly enhanced α-GalCer–induced cytotoxicity against various tumor cell targets including NK-resistant P815 cells, and this treatment also inhibited tumor metastases. 21,34,35 NKT cells exhibit direct NK-like nonspecific cytolytic activity against NK-sensitive 45 and NK-resistant tumor cells including P815. 49 Although the killing of P815 cells was low compared with other targets (ie, YAC-1), this demonstrates that IL-4C pretreatment enhances the direct cytolytic activity of NKT cells induced by α-GalCer, in addition to IFN-γ–augmented NK cytotoxicity.

Finally, to further evaluate the effects of IL-4C on antitumor activity in vivo, we examined the effect of IL-4C treatment on α-GalCer–mediated metastasis suppression. Interestingly, the metastasis of RenCa cells was nearly completely inhibited by α-GalCer treatment in IL-4C–pretreated mice but not in mice treated with α-GalCer alone. These findings are consistent with a report of Fuji et al 21 demonstrating that enhanced IFN-γ production by NKT cells resulted in decreased metastases in mice.

We described a novel immune regulation system involving DC interactions with NKT cells. Using the knowledge gained from our feedback model, we demonstrated that the augmentation of NKT cell–mediated IFN-γ production and antitumor immunity was actually possible in vivo. Thus, our present findings will hopefully lead to the development of clinical applications exploiting this new regulation system for a potent antitumor immune therapy.

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References


Negative feedback regulation of T helper type 1 (Th1)/Th2 cytokine balance via dendritic cell and natural killer T cell interactions

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