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 Shinhara, 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)

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 (TCR-V) 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–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 granulocyte-colony-stimulating factor (G-CSF), human interleukin-12 (IL-12), human IL-4, and recombinant human interferon-γ (IFN-γ) were purchased from PeproTech (Rocky Hill, NJ). Pyranine (Sigma–Aldrich, St. Louis, MO) was used for flow cytometry analysis.

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)
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 CD40b/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 IgG1 mAb (A85-1), biotin–conjugated anti–mouse CD1d mAb,21 FITC–conjugated anti–mouse CD40 mAb (32/3), FITC–conjugated anti–mouse CD86 mAb (GL1), FITC–conjugated anti–mouse H-2Kd mAb (SF1-1.1), FITC–conjugated anti–mouse IFN-γ mAb (XMG1.2), biotin–conjugated anti–mouse I-A^d 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). Anti-CD11c (N418) MicroBeads was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Culture media
Cells were cultured in Iscove modified Dulbecco medium (Sigma Chemical, St Louis, MO) supplemented with 10% heat-inactivated fetal calf serum, 30% NIH-3T3 culture supernatants, 10 ng/mL GM-CSF, 100 U/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.22,23 Culture medium for the generation and expansion of DC was R1 medium.

Spleen-derived DCs (SDDCs) were generated from BALB/c splenocytes as previously described.24-26 Splenocytes were cultured in R1 medium for 14 days, and CD11c^+ cells were positively selected using anti-CD11c (N418) MicroBeads and magnetic-activated cell-sorting (MACS) column (Miltenyi Biotec) according to the manufacturer’s protocol. The purified cells (> 90% CD11c^+ 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-12p70: BioSource International, Camarillo CA).

Flow cytometry
Cells were stained with FITC–, PE–, or biotin–conjugated mAb and streptavidin-PerCP. Expression of cell-surface markers was analyzed by flow cytometry on EPICS XL (Coulter, Miami, FL).22

Treatment of DCs with Th1/Th2 cytokine and stimulation of NKT cells with α-GalCer–loaded DCs in vitro
SDDCs or BC1 cells suspended in R1 medium were loaded with α-GalCer (100 ng/mL) or vehicle for 24 hours and then cultured with IFN-γ (1 ng/mL) or IL-4 (20 ng/mL) for 3 days in the presence or absence of the α-GalCer. The doses of IFN-γ and IL-4 were determined on the basis of their maximum effects. The IFN-γ– or IL-4–treated DCs were considered IFN/DCs or IL-4/DCs. Each DC group was cocultured with nylon nonadherent splenocytes for 48 hours on a U-bottom 96-well plate at various DC/splenocyte ratios (0.001 to 0.1). In some experiments, B220, I-A^d, or CD8^+ cells in splenocytes were depleted by MACS column. The cells were stained with α-GalCer–loaded CD1d-dimer (CD1d:Ig fusion protein) and PE–conjugated anti–mouse IgG1 mAb followed by staining with FITC–anti–TCRβ. 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^+ TCRβ^+ cells (> 96%). Sorted splenic NKT cells (1 × 10^3 cells/well) were cultured with α-GalCer (100 ng/mL)–pulsed DCs (1 × 10^4 cells/well) from BC1 for 48 hours on a V-bottom 96-well plate.

Stimulation of DCs with α-CD40 and α-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
α-GalCer–loaded IFN/DCs, IL-4/DCs, or iDCs from SDDCs (5 × 10^5) suspended in 50 µL PBS were injected into mouse spleens using a 30-gauge needle. Mice were killed 6 hours (for IL-4 production) or 12 hours (for IFN-γ production) after injection and the blood samples were collected. The levels of IFN-γ and IL-4 in the sera were measured by ELISA.

IL-4 and α-GalCer treatment in vivo
Mice were given IL-4 using the long-lasting IL-4 formulation (IL-4C),27-30 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 × 10^5 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.14

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 (Cr)–release assay.31 The cytotoxicity (%) was calculated as follows: cytotoxicity (%) = ([experimental release − spontaneous release]/(maximum release − spontaneous release)) × 100.

Experimental metastasis assay
RenCa (5 × 10^5) 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.
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.14 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.14

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 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 as iDCs, IFN/DCs, or IL-4/DCs from BC1 cells were cocultured with nylon nonadherent splenocytes (prepared from WT or CD1d—/— mice) for 48 hours at various DC/splenocyte ratios (0.001 to 0.1). Each symbol represents the mean ± SE of triplicate wells. The analysis was repeated twice with similar results. (D) CD40-mediated IL-12 production by DCs. iDCs, IFN/DCs, or IL-4/DCs from BC1 cells were stimulated with anti-CD40 mAb for 48 hours. Each column represents the mean ± SE of 3 independent experiments. (E,F) IL-12 production in DC cultures with nylon nonadherent splenocytes. α-GalCer–loaded iDCs, IFN/DCs, or IL-4/DCs from SDDCs (E) or BC1 cells (F) were cocultured with nylon nonadherent splenocytes for 48 hours at various DC/splenocyte ratios (0.001 to 0.1). Each symbol represents the mean ± SE of 3 independent experiments. Statistical significance was calculated by Student t test (*P < .05; **P < .01; and ***P < .001 vs 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).32 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.22 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.

Figure 1. Cytokine production by splenocytes stimulated with α-GalCer–loaded DCs in vitro. SDDCs (prepared from WT or IL-12—/— mice) or BC1 cells were unstimulated (iDCs) or stimulated with IFN-γ (IFN/DCs) or IL-4 (IL-4/DCs). α-GalCer or vehicle-loaded iDCs, IFN/DCs, or IL-4/DCs were cocultured with nylon nonadherent splenocytes (prepared from WT or CD1d—/— mice) for 48 hours at various DC/splenocyte ratios (0.001 to 0.1). The amount of cytokines in the culture supernatants was measured by ELISA. (A) IFN-γ and IL-4 production in cultures with SDDCs. Each symbol represents the mean ± SE of 3 independent experiments (WT SDDC or IL-12—/— SDDC vs. WT splenocytes). Data are representative of 2 independent experiments (WT SDDC vs. CD1d—/— SDDC vs. WT splenocytes). (B) IFN-γ and IL-4 production in cultures of sorted splenic NKT cells and BC1 cells. Each column represents the mean ± SE of 3 independent experiments. Statistical significance was calculated by Student t test (*P < .05; **P < .01; and ***P < .001 vs iDCs).
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.33 Thus, we analyzed CD40-mediated 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 significantly 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.27,30 As a result, the in vivo half life of IL-4 increases from a few minutes to approximately 24 hours.27 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 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

**Figure 2. Cytokine production by NKT cells stimulated with α-GalCer–loaded DCs in vivo.** α-GalCer or vehicle-loaded iDCs, IFN/DCs or IL-4/DCs of WT or CD1d−/− SDDCs were directly injected into the spleen. Six hours (for IL-4 production) or 12 hours (for IFN-γ production) after injection, blood samples were collected and the levels of IFN-γ (□) and IL-4 (△) in the sera were measured. Each column represents the mean ± SE of 3 independent experiments. Statistical significance was calculated by Student t test (*P < .05 vs iDCs).
derived from BC1 cells and SDDCs (WT or CD1d/−/−) were analyzed on CD1d dimer + TCRβ+ NKT cells or DX5+ TCRβ− NK cells in BALB/c strain (Figure 4D) and on NK1.1+ TCRβ+ NK cells 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 production 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 these 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 administration of α-GalCer alone significantly increased the cytotoxicity 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 cytotoxicity of splenocytes.

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

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

Statistical significance was calculated by Student t-test (*P<.05; **P< .01 vs. cont.).

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 6E-F). This is consistent with previous reports. IL-4C pretreatment further enhanced the antimetastatic 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

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<th>B cell</th>
<th>T cell</th>
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<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>
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<td>IL-4C</td>
<td>53.8 ± 1.8</td>
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<td>8.2 ± 1.2</td>
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<td>Anti-IL-4 mAb</td>
<td>54.9 ± 1.4</td>
<td>29.8 ± 1.1</td>
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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.

Several reports have shown the negative feedback regulation of immune responses by DCs. In human systems, monocyte-derived DCs (DC1) are primarily involved in the development of Th1 responses, whereas plasmacytoid-derived DCs (DC2) support Th2 development. 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 maturational stimuli such as microbial components or CD40 ligation in vitro. In addition, production of IL-12 p70 was significantly increased by DCs derived from IL-4C–pretreated mice, whereas pretreatment of DCs with low-dose IL-4C suppressed IL-12 p70 production compared with untreated DCs. These results demonstrate that negative feedback regulation of Th1/Th2 cytokine balance between DCs and T cells is not seen between DCs and mainstream T cells.

Discussion

Several reports have shown the negative feedback regulation of immune responses by DCs. In human systems, monocyte-derived DCs (DC1) are primarily involved in the development of Th1 responses, whereas plasmacytoid-derived DCs (DC2) support Th2 development. 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 maturational stimuli such as microbial components or CD40 ligation in vitro. In addition, production of IL-12 p70 was significantly increased by DCs derived from IL-4C–pretreated mice, whereas pretreatment of DCs with low-dose IL-4C suppressed IL-12 p70 production compared with untreated DCs. These results demonstrate that negative feedback regulation of Th1/Th2 cytokine balance between DCs and T cells is not seen between DCs and mainstream T cells.
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 same IL-4 treatment, however, decreased the LPS or CpG-induced IL-12 p40 production in vivo.

In the present study, we investigated the regulation (negative feedback) of Th1/Th2 balance from a different point of view; we focused on the interaction between DCs and NKT cells. We found that pretreatment of myeloid DCs 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 involving 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 DCs are thought to be the most potent activators of NKT cells.

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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 < .05, ***P < .01 vs. cont.). (E) IL-4C– or PBS (cont.)–pretreated mice were intravenously injected with 5 × 104 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+ T 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 < .05 vs DCs).
cell activation. The levels of CD86 on IFN/DC 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-1, an inhibitory molecule in NKT activation, 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-1 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. 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. 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 tried treating 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. 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. The antitumor effects of α-GaLCer are thought to require NKT cell–mediated IFN-γ production. 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. NKT cells exhibit direct NK-like nonspecific cytolytic activity against NK-sensitive and NK-resistant tumor cells including P815. 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 Fujii et al 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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