Ex vivo expanded human CD4+ regulatory NKT cells suppress expansion of tumor antigen-specific CTLs

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Abstract

NKT cells can produce large amounts of both Th1- and Th2-type cytokines and are an important regulatory cell type. To elucidate their role in acquired immunity, we examined the effect of human Vα24+Vβ11+ NKT cells or CD1d-specific ligand α-galactosylceramide (αGalCer) on the in vitro generation of antigen-specific CTLs from PBMCs using autologous MART-1(26–35) peptide-pulsed dendritic cells as stimulators. Flow cytometry using tetramer for MART-1(26–35) peptide revealed that NKT cells have inhibitory effects on CTL generation. Cytokine analysis using cytometric bead array assay and ELISA showed higher IL-4 and IL-10 secretion in the αGalCer(+) and/or NKT cell(+) culture setting, whereas IL-13 secretion in the culture was not affected by the presence of αGalCer. The CD4+ NKT cell subset seemed to play a major role in this inhibitory effect by secreting large amounts of Th2-type cytokines. Interestingly however, unlike recent reports utilizing mouse models, IL-13 was not a main effector molecule in our human system. Culture with αGalCer in the presence of cytokine-neutralizing antibodies for the Th2 cytokines, IL-4, IL-5 and IL-10, resulted in enhanced CTL generation, suggesting the dominant role of Th2 cytokines over Th1 cytokines. Thus, CD4+ NKT cells can work as immunoregulatory T cells that suppress anti-tumor immune response and, therefore, αGalCer could be used as therapeutic modalities to modulate systemic immune responses, such as autoimmune diseases. Conversely, the use of NKT cells along with anti-Th2 cytokine-neutralizing antibodies or CD4-negative NKT cell subset could enhance the generation of antigen-specific CTLs for adoptive immunotherapy.

Introduction

NKT cells recognize glycolipid antigen in the context of the non-polymorphic, MHC class I-like molecule, CD1d, via their highly limited TCR repertoire (1–3), and can produce large amounts of both pro-inflammatory Th1 cytokines, such as IFN-γ and tumor necrosis factor (TNF)-α, and anti-inflammatory Th2 cytokines, such as IL-4 and IL-10 (4, 5). Recent studies have revealed various roles of NKT cells in innate and adaptive immunity. Development of a wide variety of autoimmune diseases in mice and humans was found to be associated with a reduction of NKT cells, implying an NKT cell immunoregulatory function (6–8). NKT cells prevented graft versus host disease (GVHD) after bone marrow transplantation in an IL-4-dependent manner (9), and transfer of NKT cells from the normal mice to non-obese diabetic mice or activation of NKT cells by the CD1d-specific ligand, α-galactosylceramide (αGalCer), delayed or prevented the onset of diabetes. Thus, NKT cells play a critical role in the inhibition of autoimmune diseases (10–13). Recently, several studies revealed an immunosuppressive function of NKT cells in tumor control (14, 15). Those findings support the idea that NKT cells can function as regulatory T cells to negatively regulate anti-tumor immunity, and some murine experiments have suggested the key role of IL-13 in this suppressive effect mediated by CD4+ NKT cells (15, 16). Conversely, NKT cells activated by αGalCer can induce Th1 differentiation of naive CD4+ T cells, resulting in promotion of anti-tumor immunity (17–19). These findings indicate that NKT cells are an important regulatory cell type that can modulate differentiation of CD4+ T cells toward Th1 or Th2 type. And, since it is difficult to precisely predict the consequences of NKT cell activation, we wanted to investigate...
the relative contributions of T<sub>n</sub>1 and T<sub>n</sub>2 cytokines produced by NKT cells on the activation of human anti-tumor T cell responses.

Dendritic cells (DCs) are another important immunoregulatory cell type that is capable of directing T<sub>n</sub>1 differentiation toward a T<sub>n</sub>1 or T<sub>n</sub>2 type according to the cytokines that co-exist in their microenvironment. For efficient antigen presentation to T cells, DCs need to be matured by maturation signals, such as IFN-γ or CD40 ligand (CD40L) (20, 21). However, DCs also express significant amounts of CD1d molecules, which can present specific glycolipid antigen to NKT cells, resulting in their activation. Since NKT cells are potent producers of key cytokines, such as IFN-γ, and a subset of NKT cells is known to up-regulate CD40L expression when activated (22, 23), the mutual interaction of these two cell types, NKT cell and DC, seems to be critical in modulating immune responses.

We speculated that if NKT cells secreted large amounts of IFN-γ because of their interaction with DCs while co-existing DCs are presenting specific antigens to naive T cells, the establishment of antigen-specific CTLs might be more efficient. Conversely, if the NKT cells secreted predominantly T<sub>n</sub>2-type cytokines, NKT cells could play an inhibitory role in CTL establishment. This study investigated the effect of NKT cells in the establishment of antigen-specific CTLs using a human in vitro cell culture system. We found that NKT cells, especially CD4<sup>+</sup> NKT cells, have inhibitory effects on MART-1- and HER2/neu peptide-specific CTL establishment in our culture system. T<sub>n</sub>2-type cytokines, such as IL-4 and IL-10, played crucial roles in this inhibition, whereas IL-13 was not a main effector. These results suggest differences in cytokine involvement in NKT cell-mediated immunity between mouse and human.

**Methods**

**Reagents**

HLA-A*0201-restricted MART-1(26-35) peptide (EAAAGILTV) was purchased from Research Genetics (Huntsville, AL, USA). Peptide-MHC tetramers for HLA-A*0201-restricted MART-1(26-35) and CMV/pp65(495-503) peptides were supplied by the NIAID Tetramer Facility and NIH AIDS Research and Reference Reagent Program. Specificity of each tetramer was demonstrated by staining of peptide-specific CTLs and lack of staining of irrelevant CTLs (data not shown). Each tetramer was titrated to determine the optimized concentration for use. αGalCer was kindly provided by Kirin Brewery, Tokyo, Japan. Vehicle for αGalCer, polysorbate-20 solvents, was used as a negative control for αGalCer in CTL cultures.

**DC culture**

Peripheral blood monocyte-derived DCs were obtained as previously reported (24). Briefly, PBMCs were isolated from leukapheresis products, obtained from HLA-A*0201-positive healthy donors, by density gradient centrifugation over Ficoll–Hypaque<sup>™</sup> Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden). A total of 10<sup>5</sup> PBMCs were put into 150-cm<sup>2</sup> flask and cultured for 2 h at 37°C. After removing non-adherent cells, attached cells were cultured for 7 days with granulocyte macrophage colony-stimulating factor (800 U ml<sup>−1</sup>; Immunex, Seattle, WA, USA) and IL-4 (25 ng ml<sup>−1</sup>; R&D Systems, Minneapolis, MN, USA) in AIM-V medium (GIBCO, Grand Island, NY, USA) supplemented with 10% human AB serum.

**NKT cell culture**

NKT cells were expanded as previously reported with slight modification (25). Briefly, peripheral blood Vα24/Vβ11+ NKT cells were expanded by culturing PBMCs with autologous DCs in the presence of 100 ng ml<sup>−1</sup> αGalCer and 10 ng ml<sup>−1</sup> IL-7 (R&D Systems) for 10 days, followed by 40 U ml<sup>−1</sup> IL-2 (Chiron, Emeryville, CA, USA). On day 14, Vα24/Vβ11 double-positive cells were sorted on a FACS Vantage (Becton Dickinson, Mountain View, CA, USA), and kept in culture with the addition of IL-7 weekly and IL-2 every 3–4 days. More than 98% of the cultured cells were CD3+Vα24+Vβ11+ by FACS analysis. CD4<sup>+</sup> NKT cell and CD4<sup>−</sup>CD8<sup>−</sup> double-negative (DN) NKT cell subsets were separated using CD4 and CD8 Microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

**DC–NKT cell co-culture**

For cytometric bead array (CBA) assays and IL-13 ELISA, 10<sup>5</sup> NKT cells were put into each well of 96-well U-bottomed plates and various numbers of DCs were added to make a DC : NKT cell titration. For IL-12 ELISA, 10<sup>6</sup> DCs were cultured with various numbers of NKT cells. In half of the wells, αGalCer (100 ng ml<sup>−1</sup>) was added to the medium. After 40 h of incubation at 37°C, culture supernatants were collected and kept frozen at −80°C until the assay was performed. To examine NKT cell effect on DC maturation, 10<sup>6</sup> DCs and 10<sup>5</sup> NKT cells were co-cultured for 40 h in 24-well plates. As a control for NKT cell, T cells were negatively selected from PBMCs using Pan T Cell Isolation Kit (Miltenyi Biotec GmbH) and were co-cultured with DCs. For some experiments, 10<sup>5</sup> CD4<sup>+</sup> or DN NKT cell subsets were used. Harvested cells were immediately stained with antibodies for flow cytometry and the supernatants were collected for CBA assays and ELISAs.

**CTL culture**

AIM-V medium supplemented with 10% human AB serum and 10 ng ml<sup>−1</sup> IL-7 was used for CTL culture. β2-Microglobulin (1 μg ml<sup>−1</sup>) (Sigma, St Louis, MO, USA) was also added to the medium on day 0. PBMCs (1.2 × 10<sup>6</sup> per well) were plated into 24-well plates. PBMCs were cultured alone, with autologous DCs (1.2 × 10<sup>5</sup>) or with autologous DCs and NKT cells (1.2 × 10<sup>5</sup> each) in the presence or absence of MART-1 peptide (10 μg ml<sup>−1</sup>). The same volume of dimethyl sulfoxide (peptide solvent) was added to the antigen-negative wells. αGalCer (100 ng ml<sup>−1</sup>) or equal volume of vehicle was added to the wells. The culture was supplemented with new media and peptide on days 7, 11 and 14. Re-stimulation of CTLs was done on day 14, as per the initial culture condition, by addition of no cells, DCs alone or DC + NKT cell. On day 19, cells were harvested and CTL for MART-1 peptide was determined by MHC tetramer staining. To examine the differential effect of NKT cell subsets on CTL generation, PBMCs (1.2 × 10<sup>5</sup>) were cultured with autologous NKT cell subset (1.2 × 10<sup>5</sup>) and re-stimulated with the same subset on day 14.
In order to reveal the involvement of soluble factors in inhibition of CTL expansion, culture supernatants from DC–NKT cell co-culture (1.2 × 10^6 cells each per well of 24-well plate) were collected after 2 days culture and were kept frozen at −80°C until use. As negative controls, supernatants from DC-alone culture or NKT cell-alone culture were also collected. On day 0, CTL culture was initiated with the medium that consisted of half supernatants and half fresh medium. On days 7, 11 and 14, half of the medium was replaced with supernatants, and on day 14, re-stimulation with DCs or DCs + NKT cells was done.

To examine if NKT cells in PBMCs physiologically inhibit CTL induction, CTL culture was initiated from MART-1 peptide-pulsed DCs and NKT cell-depleted PBMCs using biotinylated anti-Vα24 mAb (ImmuneTech, Marseille, France) and Streptavidin-Microbeads (Miltenyi Biotec GmbH). In some experiments, CD8 T cells were isolated from PBMCs using CD8 T Cell Isolation Kit (Miltenyi Biotec GmbH) and used in the place of PBMCs.

Flow cytometry

Cells were analyzed on a FACSCaliber using CellQuest software (Becton Dickinson). mAbs were as follows: FITC-labeled anti-Vα24 and PE-labeled anti-Vβ11, anti-CD40 and anti-CD83 from ImmuneTech. FITC-labeled anti-CD3, PE-labeled anti-CD25, anti-CD80, anti-CD86, PerCP-labeled anti-CD4, anti-CD8, anti-CD40L, anti-CD28 and anti-CD44 were from BD PharMingen (San Diego, CA, USA). FITC-labeled anti-CD3, PerCP-labeled anti-CD8, anti-CD14 and anti-CD19 and PE-labeled anti-CD4 were from Immunotech (Marseille, France) and Streptavidin-Microbeads (Miltenyi Biotec GmbH). In some experiments, CD8 T cells were isolated from PBMCs using CD8 T Cell Isolation Kit (Miltenyi Biotec GmbH) and used in the place of PBMCs.

MHC-peptide tetramer staining analysis

Peptide-MHC tetramer staining was performed using ~10^6 cells, stained in the dark for 60 min at room temperature with FITC-labeled anti-CD3, PerCP-labeled anti-CD8, a cocktail of APC-labeled anti-CD4, anti-CD14 and anti-CD19 and PE-labeled MART-1 tetramer. As a negative control, PE-labeled CMVpp65 tetramer was also used. Cells were washed twice and analyzed by multiparameter flow cytometry. Approximately 25,000 gated events were collected. Lymphocytes were gated by forward scatter and side scatter gram, and then the CD3+CD4−CD14−CD19− cell population was analyzed for their dual expression of CD8 and TCR recognized by tetramer. In order to calculate the proportion of tetramer-positive cells in CD8+ T cells, the number of CD8+ tetramer+ cells was divided by the total number of CD8+ T cells.

ELISA

IL-12 p70 concentration was measured by ELISA (Mabtech, Nacka, Sweden) according to the manufacturer's instructions. IL-13 concentration was also measured by ELISA (R&D Systems). To examine the antigen specificity of the established CTLs, IFN-γ ELISA was performed. Briefly, cells were harvested from PBMC + DC culture. After intensive washes, cells were counted and plated into U-bottomed 96-well plates (5 × 10^4 or 5 × 10^5 cells per well). A total of 5 × 10^3 peptide-pulsed or unpulsed T2 cells were added into these wells to make 10 : 1 or 100 : 1 effector : target ratio. After 24 h incubation, culture supernatants were collected for IFN-γ ELISA (Endogen, Woburn, MA, USA).

\[ T_{1}/T_{2} \text{ CBA analysis} \]

Culture supernatants were collected at the indicated time points and frozen at −80°C until analysis. The concentrations of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ were measured with the CBA kit (BD PharMingen), according to the manufacturer’s instructions, and analyzed on a FACSCaliber flow cytometer using BD CBA software (BD BioScience; San Diego, CA, USA).

Neutralization of cytokines produced in vitro culture

IL-4, IL-5, IL-10 and IL-13 produced in cultures were neutralized by addition of 5 μg ml⁻¹ anti-IL-4 (IgG1), anti-IL-5 (IgG1), anti-IL-10 (IgG2b) or anti-IL-13 (IgG1) mAbs (R&D Systems). Purified mouse IgG1 and mouse IgG2B (R&D Systems) were used as isotype controls. The same amount of mAbs was added to individual wells on days 7, 11 and 14 when half the medium was exchanged.

Statistical analysis

Differences in CTL frequency were analyzed using Student’s t-test. Differences at P < 0.05 were considered statistically significant.

Results

**DC–NKT cell interaction**

DCs are known to activate NKT cells when the appropriate antigen (αGalCer) is presented on their CD1d molecules. We examined the mutual interaction of these two cell types in the presence or absence of αGalCer by CBA assay (Fig. 1A and B). IL-12 ELISA (Fig. 1C), IL-13 ELISA (Fig. 1D) and flow cytometry assay (Fig. 2). Purified, ex vivo expanded NKT cells and autologous DCs were cultured in 96-well plates, and 2 days later, culture supernatants were harvested and analyzed. In the presence of αGalCer (Fig. 1A), NKT cells secreted a large amount of IFN-γ and also other cytokines, including TNF-α, IL-4, IL-5 and IL-10. Titration of DC : NKT cell showed that even 1 DC to 81 NKT cells could induce significant cytokine secretion by NKT cells, suggesting the important role of the mutual interaction of these two cell types in vivo. IL-12 secretion by DCs was also greatly enhanced by αGalCer (Fig. 1C). In accordance with the results of IL-12 ELISA, IL-13 Elispot assay also showed increasing IL-12-positive DCs when DCs were co-cultured with NKT cells in the presence of αGalCer (data not shown). Significant levels of IL-13 were detected in the DC–NKT cell co-culture regardless of the presence of αGalCer, although it was slightly higher in the culture with αGalCer, particularly as the DC ratio approached 1 : 1 (Fig. 1D). Control cultures of DCs alone or NKT cells alone with or without αGalCer showed no cytokine secretion (data not shown). In addition, expression intensity of some molecules which are known to be markers of DC maturation (CD80, CD83, CD86 and CD40) is moderately enhanced by co-culture with NKT cells but not with conventional T cells (Fig. 2). These results indicate that DC–NKT cell interaction can induce maturation of DCs as well as activation of NKT cells to secrete significant quantities of cytokines, in agreement with the previous observations (26).
Interestingly, even in the absence of αGalCer (Fig. 1B), coculture of these two cell types at high DC : NKT cell ratio resulted in significant increases of several cytokines, including IL-4, IFN-γ and IL-12. Moreover, NKT cells could also similarly enhance expression of maturation markers on DCs (Fig. 2). Thus, these results indicate that DC–NKT cell interaction can induce activation of NKT cell and maturation of DCs even without αGalCer, suggesting a critical role for this DC–NKT cell interaction in vivo.

MART-1-specific CTL establishment
In order to determine the effect of αGalCer or NKT cells on tumor antigen-specific immune responses, we cultured PBMC alone, PBMC + DC and PBMC + DC + NKT cell with and without MART-1 peptide and αGalCer, and enumerated MART-1-specific T cells by tetramer staining. Figure 3(A) shows the expansion of Vα24+Vβ11+ NKT cells in each culture setting. In the absence of αGalCer, NKT cell population was <0.5% of CD3+ lymphocytes in the culture; however, in the presence of αGalCer, it reached >20% in the PBMC or PBMC + DC culture, and ~40% in the PBMC + DC + NKT cell culture after 19 days culture. There were no obvious differences in the frequency of CD8+ T cells between αGalCer(+) and αGalCer(−) culture or between antigen-positive and -negative culture settings (data not shown).

Figure 3(B) shows MHC tetramer staining of the cultured cells. CD3+CD4−CD14−CD19− lymphocytes were gated,
and these cells were analyzed for their dual expression of CD8 and TCRs bound by HLA-A*0201/MART-1 tetramer. We have previously demonstrated that the frequency of MART-1-specific CTLs is <0.1% in unstimulated PBMCs from the donors used in this study (data not shown). CMVpp65 tetramer and PE-labeled control IgG were used in the place of MART-1 tetramer as negative controls. There were no detectable CMVpp65 tetramer(+) cells in any culture setting (data not shown). Table 1 shows the average percentage of CTLs (MART-1 tetramer(+) cells in the CD8+ T cell population) calculated from the data of three experiments. As shown in Fig. 3(B) and Table 1, no MART-1 tetramer(+) cell was observed in antigen(−) settings. The most efficient culture condition for the CTL generation was the PBMC + DC culture in the absence of αGalCer (13.7%) (Fig. 3B). Addition of NKT cells to the culture inhibited CTL establishment significantly as can be seen by the decreased percentage of CD8+/MART-1 tetramer(+) cells compared with PBMC + DC culture in Fig. 3 (αGalCer+/antigen(+) setting: 13.7 versus 2.91%, αGalCer+/antigen(−) setting: 5.04 versus 0.83%). Moreover, addition of NKT cells reversed the positive effect of DCs for the CTL generation because the CD8+/MART-1 tetramer(+) cell population was even smaller in PBMC + DC + NKT cell cultures than PBMC-only cultures (αGalCer−/antigen(+) setting: 4.85 versus 2.91%, αGalCer+/antigen(+) setting: 3.54 versus 0.83%). There was a clear trend that addition of αGalCer decreased the number of CD8+ tetramer(+) cells in each cell setting, although the differences were not statistically significant.

To rule out the possibility of helper CD4+ T cell or CD4+ regulatory T cell involvement in this inhibition of CTL generation, negatively isolated CD8+ T cells were plated with MART-1-pulsed DCs and allogenic NKT cells, and CTL culture was performed (Fig. 3C). In the presence of αGalCer, CD8 T cell (CD4 T-depleted) culture and PBMC culture showed similarly suppressed CTL expansion compared with αGalCer− condition. Thus, depletion of CD4+ T cells did not enhance CTL generation, suggesting that NKT cell, but not CD4+ T cell, is playing a major role in the inhibition of CTL expansion. In the absence of αGalCer, PBMC culture showed higher CTL frequencies than CD8 T cell culture did, suggesting the T(h)1-type helper function of CD4+ T cells might be involved in CTL expansion.

To reveal if NKT cells already existing in PBMCs can work to inhibit CTL generation, we made CTL culture using NKT cell-depleted PBMCs as a starting cell population. In the presence of αGalCer, NKT cell-depleted culture showed significantly better expansion of CTL compared with ordinary PBMCs culture (Fig. 3D). Although the difference was smaller, a similar trend was observed even in αGalCer− culture, suggesting that under more physiologic conditions represented by NKT cells present in the PBMCs, NKT cells can inhibit CTL expansion. This argues that our observations of suppressive NKT cells are not just a result of the use of in vitro expanded NKT cells.

The function of MART-1 peptide-activated cells was analyzed by IFN-γ secretion in response to peptide-pulsed T2 cells as target cells (Fig. 4). In accord with the results of tetramer staining, cells from the αGalCer−/antigen(+) condition showed higher IFN-γ production when cultured with MART-1-pulsed T2 cells compared with cells from αGalCer+/antigen(+) culture. Cells from antigen-negative wells showed no reaction. No IFN-γ secretion was detected when these harvested cells were co-cultured with CMVpp65(495–503) peptide-pulsed T2 cells or peptide-unpulsed T2 cell. Thus, these data indicate that NKT cells, particularly in the presence of αGalCer, suppress antigen-specific CD8+ T cell activation and proliferation in vitro.

Involvement of soluble factors in the inhibition of CTL generation

To examine the involvement of soluble factors in this inhibition of CTL generation, culture supernatants from DC + NKT cell co-culture, DC-alone culture, or NKT cell-alone culture were

Fig. 2. Maturation of DCs by DC–NKT cell co-culture. A total of 1 × 10^6 DCs and 1 × 10^5 autologous NKT cells or T cells were cultured in 24-well plates in the presence or absence of αGalCer. After 40 h incubation, cells were harvested and stained with anti-CD3–FITC, anti-HLA-DR–PerCP, anti-CD14–APC and the following PE-labeled mAbs, anti-CD80, CD83, CD86, CD40. Large cells were gated using scatter grams and CD14 plates in the presence or absence of a line, DCs cultured alone; thin line, DCs cultured with T cells; bold line, DCs cultured with NKT cells. DR+ cells were analyzed for their expression of CD80, CD83, CD86 and CD40. Results are representative of two independent experiments. Dotted generation, suggesting that NKT cell, but not CD4+ T cell, is

Suppression of CTL expansion by NKT cell

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Fig. 3. NKT cell expansion and MART-1-specific CTL generation in each culture condition. (A) A total of $1.2 \times 10^6$ PBMCs from normal donors were plated into each well of 24-well plates. PBMCs were cultured alone, with autologous DCs ($1.2 \times 10^5$ cells) or with autologous DCs and NKT cells ($1.2 \times 10^5$ cells each) in the presence or absence of MART-1 peptide (10 µg ml$^{-1}$) with or without αGalCer (100 ng ml$^{-1}$). Re-stimulation of CTLs was done on day 14, using the same cell condition as the initial culture, by addition of no cells, DCs alone or DCs + NKT cells. On day 19, cells were harvested from the plates and were stained with anti-Vα24-FITC, anti-Vβ11-PE and anti-CD3–PerCP for flow cytometry analysis. Lymphocytes (FSC$^\text{lo}$/SSC$^\text{lo}$ and CD3$^+$) were analyzed for their Vα24/Vβ11 expression. Percentages of double-positive cells are indicated in each dot plot. Representative data from three independent experiments are shown. (B) On day 19, cells were harvested and stained with anti-CD3–FITC, PE-labeled MART-1 tetramer, anti-CD8–PerCP and APC-labeled anti-CD4, CD14, CD19 mAb cocktails. CD3$^+$CD4$^+$/CD14$^+$/CD19$^+$ lymphocytes were analyzed for their CD8 expression and tetramer positivity. The percentages of tetramer-positive cells in CD8$^+$ T cells are indicated in each dot plot. Results are representative of three independent experiments. Ag: MART-1(26–35) peptide. (C) CTL culture was initiated from MART-1 peptide-pulsed DCs, NKT cells and CD8 T cells negatively isolated from PBMCs. On day 19, tetramer staining was performed as described above. Representative data from two independent experiments are shown. *$P < 0.05$. (D) CTL culture was initiated from MART-1 peptide-pulsed DCs and NKT cell-depleted PBMCs with or without αGalCer. On day 19, tetramer staining was performed as described above. Representative data from two independent experiments are shown. *$P < 0.05$.

Table 1. Frequency of MART-1 tetramer-positive CTLs in the CD8+ T cell population

<table>
<thead>
<tr>
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<th>PBMC</th>
<th>PBMC + DC</th>
<th>PBMC + DC + NKT cell</th>
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<tr>
<td>αGalCer−/antigen−</td>
<td>0.01 ± 0.01</td>
<td>0.04 ± 0.03</td>
<td>0.03 ± 0.02</td>
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<tr>
<td>αGalCer+/antigen−</td>
<td>0.01 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.04</td>
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<td>αGalCer−/antigen+</td>
<td>3.65 ± 1.65</td>
<td>9.88 ± 4.42*</td>
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<tr>
<td>αGalCer+/antigen+</td>
<td>1.81 ± 1.57</td>
<td>4.16 ± 1.20**</td>
<td>0.32 ± 0.44**</td>
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Cell culture for CTL generation was done as described in Methods using PBMCs, autologous DCs and autologous NKT cells. On day 19, 5 days after re-stimulation, cells were collected and analyzed by flow cytometry using MART-1-specific tetramer. The averages of three experiments are shown. Antigen, MART-1(26–35) peptide. *$P < 0.05$, **$P < 0.01$. 

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added to the medium. The percentage of MART-1 tetramer+ cells in DC + NKT cell culture was significantly lower compared with those wells to which supernatants from DC-alone culture or NKT cell-alone culture were added (Fig. 5). Although it did not show as strong an inhibition as PBMC + DC + NKT cell culture did, this result indicates that soluble factors are playing important roles in this inhibition.

Cytokine analysis of the culture supernatants by CBA assay and ELISA

Culture supernatants were collected on days 7, 14 and 19 from each culture setting, and cytokine concentrations were measured by CBA assay (for IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ), IL-12 ELISA and IL-13 ELISA (Fig. 6). On day 7, PBMC + DC + NKT cell culture in each αGalCer/antigen setting showed the highest concentration of both T\textsubscript{H}1- and T\textsubscript{H}2-type cytokines with the exception of IL-4 compared with PBMC-only or PBMC + DC cultures. After re-stimulation, however, PBMC + DC culture showed higher secretion of most cytokines, except IL-4, than PBMC + DC + NKT cell in αGalCer(+) setting. PBMC + DC + NKT cell culture showed the highest IL-12 concentration on days 7 and 14, perhaps suggesting the activation of DCs by co-culture with NKT cells. IL-13 concentration was the highest in PBMC + DC + NKT cell culture on days 7 and 19 but dipped on day 14 prior to re-stimulation. Interestingly, the presence of αGalCer did not
significantly affect IL-13 secretion in spite of suppressive effect of αGalCer on CTL generation (Fig. 3B), suggesting that IL-13 was not the key inhibitory factor for CTL generation in this in vitro culture system. There was no significant IL-2 secretion in any culture setting at any time points (data not shown). Comparing αGalCer(+) setting with αGalCer(-) setting, the former showed higher secretion of several cytokines on day 19, although their differences were not obvious on days 7 and 14. In particular, Th2-type cytokine secretion, especially IL-4, was enhanced greatly on day 19, suggesting that αGalCer induced a Th2-biased cytokine milieu in the latter part of this co-culture period. However, cytokine secretion was not decisively biased toward the Th1 or Th2 type by αGalCer at any time during the culture period.

Different function of NKT cell subsets on CTL establishment

To determine which NKT cell subset is responsible for this inhibitory effect on CTL generation, co-culture of PBMCs with NKT cell subsets, either CD4+NKT cells or CD4−CD8−(DN) NKT cells, was performed. CBA assay and ELISA showed different cytokine secretion capabilities between these two NKT cell subsets after co-culture with autologous DCs. CD4+NKT cells secreted large amounts of both Th1-type and Th2-type cytokines (Fig. 7A). On the other hand, DN NKT cells were more Th1 biased since they secreted large amounts of Th1-type cytokines but very small amounts of Th2-type cytokines. However, unlike other Th2-type cytokines, both NKT cell subsets secreted comparable amounts of IL-13 in the presence of αGalCer. Even without αGalCer, the difference of IL-13 secretion between these two subsets was less pronounced compared with the other Th2-type cytokines. Interestingly, CD25 expression on CD4+NKT cell subset was significantly higher in percentage (~60%) compared with that on DN NKT cells (~20%) (Fig. 7B). After a 19-day culture for CTL expansion, tetramer staining revealed that only CD4+NKT cells, but not DN NKT cells, suppressed antigen-specific CTL generation (Fig. 7C). The CD4+NKT cell(+) culture showed only slightly higher IL-13 concentration compared with DN NKT cell(+) culture, and αGalCer increased IL-13 concentration in both settings (Fig. 7D). Therefore, IL-13 is not likely to be the main cytokine inhibiting CTL generation in this in vitro culture setting. These findings suggested to us that the CD4+NKT cell subset, acting as immunoregulatory T cell, might play a major role in the inhibitory effect of NKT cells on CTL generation and that Th2-type cytokines, including IL-4, IL-5 and/or IL-10, might be the key factor.

Critical cytokines inhibiting CTL establishment

In order to examine whether Th2-type cytokines are playing major roles in the inhibitory effect of αGalCer and NKT cells on CTL generation, PBMCs were cultured with DCs and αGalCer in the presence or absence of anti-Th2-type cytokine-neutralizing antibodies. As shown in Fig.8(A), cultured cells with medium containing anti-IL-4, anti-IL-5 or anti-IL-10 mAb showed much higher percentage of MART-1 tetramer(+) cells, showing that these Th2-type cytokines are working as critical factors suppressing CTL establishment. On the other hand, anti-IL-13 mAb could not enhance CTL generation, suggesting again that IL-13 is either not the key inhibitory factor or its effect is not being measured in our in vitro cell culture system (Fig. 8B). Isotype-matched mouse IgG1 or IgG2b were used as negative controls, and did not affect CTL frequency. There were no additive effects of these neutralizing antibodies (data not shown). Importantly, addition of these neutralizing antibodies to the αGalCer(+) media greatly enhanced CTL generation for MART-1 peptide compared with the culture without αGalCer (data not shown). These data suggest that if Th2-type cytokines produced in the culture are adequately neutralized, αGalCer can strongly induce a Th1 cytokine bias, resulting in highly enhanced CTL generation. Neutralization of IL-4 had the greatest effect, with 3.5-fold more CTLs produced in this condition compared with neutralization of IL-5 or IL-10.
Fig. 7. Different functions of NKT cell subsets in CTL establishment. (A) A total of 10^6 DCs and 10^6 cells of NKT subsets were co-cultured in the presence or absence of αGalCer for 40 h in 24-well plates. The supernatants were collected for CBA assays, IL-12 ELISA and IL-13 ELISA. Black bar, αGalCer+ culture, and white bar, αGalCer− culture. Note: y-axis scale is logarithmic and represents picograms per milliliter of the individual cytokines. (B) CD25 expression on NKT cell subsets. Magnetically separated NKT cell subsets were stained with anti-Vα24, anti-CD25, anti-CD4 and anti-CD8 fluorescence-labeled antibodies and analyzed by flow cytometry. Their Vα24/CD25 expressions are shown. (C) PBMCs (1.2 × 10^6) were cultured alone or with the autologous NKT cell subset (1.2 × 10^5 cells each). Re-stimulation of CTLs was done on day 14, using the same cell condition as the initial culture, by addition of no cells, CD4+ NKT cells or DN NKT cells. Tetramer staining was performed as described in the legend of Fig. 3(B). The average percentages of tetramer-positive cells in CD8+ T cells, calculated from hexaplicate wells for each condition, are indicated in each dot plot. Results are representative of two independent experiments. Ag(−), culture without MART-1 peptide, and PB, PBMCs. (D) The culture supernatants on days 7 and 19 were analyzed with IL-13 ELISA.

Suppression of CTL expansion by NKT cell
Similar results have also been obtained using HER2/neu(369–377) peptide (data not shown), demonstrating that this effect is generalizable and not due to properties intrinsic to MART-1-specific CTLs.

**Discussion**

In this study using αGalCer, a specific ligand for the invariant TCR of NKT cells that activates NKT cells, we demonstrated the suppressive effect of the addition of αGalCer and/or NKT cells on CTL establishment in an in vitro human cell culture system. The inhibitory effect of NKT cells was dependent on the CD4+ NKT cell subset and appeared to be induced by secretion of Th2-type cytokines, such as IL-4, IL-5 and IL-10. In addition, neutralization of Th2-type cytokines not only reversed the suppressive effect of αGalCer but also greatly enhanced CTL generation. In spite of the co-existence of Th1-type cytokines in large amounts, IL-4, IL-5 and IL-10 individually could produce this inhibitory effect on CTL generation.

NKT cells have been shown to produce large quantities of both Th1-type and Th2-type cytokines upon stimulation with specific ligand (αGalCer) presented on antigen-presenting cells, like DCs (4, 5). And, DC–NKT cell interaction can induce IL-12 production by DCs (22, 23). In this study, we confirmed high secretion of these cytokines by co-culturing DCs and NKT cells (Fig. 1) and also demonstrated moderately enhanced expression of cell-surface maturation markers on DCs (Fig. 2). Thus, as expected, interaction of these two cell types resulted in DC maturation as well as NKT cell activation, supporting previous observations of Fuji et al. (26). Maturation is important for the antigen-presenting function of DCs (21, 27), and maturation of DCs may occur through Th1-dependent or -independent stimuli. Therefore, we hypothesized that the interaction of DCs and NKT cells could be beneficial in eliciting antigen-specific immune responses. However, contrary to our hypothesis, NKT cells seemed to suppress CTL generation by secretion of Th2-type cytokines in our co-culture system (Fig. 3B). Importantly, we demonstrate that this suppression was the result of the interaction between NKT cells and DCs (Fig. 5) and also that this is not just an artifact of in vitro NKT cell expansion because when NKT cells were depleted from PBMCs, enhanced CTL expansion was observed (Fig. 3D). Depletion of CD4+ T cell did not enhance CTL generation (Fig. 3C), ruling out the possibility that the suppression was dependent upon or the direct result of CD4+ regulatory T cells. Importantly, under more physiologic conditions represented by NKT cells present in the PBMCs, NKT cells can inhibit CTL expansion. This argues that our observations of suppressive NKT cells are not just a result of the use of in vitro expanded NKT cells but apply to NKT cells under more physiologic conditions too.

Recent reports in the literature have described both immunostimulatory and immunosuppressive roles for NKT cells. Consistent with our results, recent reports demonstrated a Th2-like function of NKT cells, showing the correlation of NKT cells with autoimmune disease or their suppressive effect on anti-tumor immunity (9–15). Similarly, NKT cell activation with αGalCer inhibited development of experimental autoimmune encephalomyelitis in mice (28–30). Singh et al. reported that administration of αGalCer with protein antigen (ovalbumin peptide) resulted in the generation of antigen-specific Tc1 cells in mice (31). Moreover, NKT cells might be involved in the induction of immune tolerance (32–34). CD4+Vα14 NKT cells have been demonstrated to play a crucial role in the acceptance of rat islet xenografts in mice, suggesting their function as immunosuppressive regulatory cells (32). Thus, in some circumstances, NKT cells are playing a critical role in promoting a Tc1 response in vivo. In contrast to our data and the other studies discussed above, others have reported a Th1-like function of NKT cells and an enhanced anti-tumor immunity by αGalCer-activated NKT cells (17–19). The enhanced anti-tumor immunity is thought to be mainly due to cytotoxic activity of the NKT cells or the IFN-γ secreted by activated NKT cells (18, 35–37).
cells might also work collaboratively with NKT cells as effector cells (38–40). Nishimura et al. (41) reported that injection of αGalCer into mice resulted in the generation of CTLs as well as the activation of NKT cells. They immunized mice twice with tumor cells at a 2-week interval and later administered αGalCer. However, in our culture system to generate CTLs from human PBMCs, tumor antigen (MART-1 peptide) and αGalCer were added to the culture medium simultaneously. These apparently contradictory findings suggesting both immunostimulatory and immunoregulatory roles for NKT cells are likely the results of difference in the type of antigen, different disease models, different priming methods and, in our case, species-specific differences.

Recent reports demonstrated that the NKT cell subsets, CD4+ and DN NKT cells, exhibited differences in the expression of chemokine receptors, integrins and NKRs, and also in cytokine-producing capacity (42–44). Based on these data, Lee et al. (44) proposed that CD4+ and DN subsets of NKT cells represent distinct lineages with markedly different functional properties. In the present study, we have confirmed the different capacities of these two NKT cell subsets in cytokine production, i.e. CD4+ NKT cells secreted large amounts of both Th1- and Th2-type cytokines, whereas DN NKT cells were more Th1,1 biased (Fig. 7A). We found significantly lower secretion of IL-13 by DN NKT cells compared with CD4+ NKT cells, although it was not completely negative like previous reports (43, 44). In the present study, we sorted NKT cell subset from ex vivo expanded NKT cells and used αGalCer-pulsed DCs to stimulate NKT cell subsets, while previous reports used fresh NKT cells for analyses and phorbol myristate acetate + ionomycin for stimulation. Thus, different conditions might be the cause of this discrepancy. More importantly, we could demonstrate that only the CD4+ NKT cell subset had inhibitory effect on antigen-specific CTL generation (Fig. 7C) and that IL-4 was the major inhibitory cytokine. Interestingly, the majority of CD4+ NKT cells expressed CD25 molecules, while DN NKT cells did not (Fig. 7B). It should be noted that the addition of IL-7 to the in vitro cultures may potentially have increased CD25 expression, but Lee et al. (44) reported a similar finding by analyzing two NKT cell subsets in fresh PBLs using CD1d-αGalCer tetramer. It is intriguing to note that CD4+CD25+ regulatory T cells are reported to have immunoregulatory effect on acquired immunity (45), similar to the inhibitory effect of CD4+ NKT cells that we demonstrated in this study. Thus, CD4+ NKT cell might also be classified as one subset of regulatory T cells. Indeed, quantitative RT-PCR analysis demonstrates FoxP3 expression is markedly higher in the CD4+ NKT cell subset compared with the DN NKT cell subset (our unpublished results), again supporting the contention that these CD4+ NKT cells have immunoregulatory capacity.

Accumulating evidence supports a role for IL-4 secreted by NKT cells in controlling various Th1-mediated pathological conditions such as type 1 diabetes and GVHD (9, 10). IL-4 secreted by NKT cells is also thought to be involved in Th2 differentiation (46), although Cui et al. (47) reported that IL-4 had only a minor effect on Th2 differentiation in their murine model, suggesting that IFN-γ secreted by activated NKT cells was negatively regulating Th2 differentiation. In the present study, probably because of the ex vivo expanded NKT cell population, the αGalCer(+)- culture showed larger secretion of Th2-type cytokine, such as IL-4, IL-5 and IL-10, compared with αGalCer(−) culture, suggesting a relatively more Th2-biased cytokine milieu in αGalCer(+)- culture. Burdin et al. (48) reported that repeated exposure of mice to αGalCer also induced Th2-biased cytokine secretion by splenocytes, supporting this hypothesis. Recently, Terabe et al. (15) reported using murine system that IL-13, an IL-4-related Th2 cytokine, plays a central role in NKT cell-mediated suppression of tumor immunosurveillance. They showed that CD4+ NKT cells were the major source of IL-13 and that elimination of NKT cells or blocking of IL-13 in vitro resulted in prevention of tumor recurrence (15, 16). In our human cell culture system, we could confirm large secretion of IL-13 especially in PBMC + DC or PBMC + DC + NKT cell culture. However, αGalCer(+) and αGalCer(−) culture showed no major differences in IL-13 levels (Fig. 6) in spite of significant differences in the number of NKT cells (Fig. 3A) and yet the resulting expansion of antigen-specific CTLs differed markedly (Fig. 3B). In addition, we found that DN NKT cells had no inhibitory effect on CTL expansion (Fig. 7C), in spite of significant levels of IL-13 being produced (Fig. 7D). Finally, neutralization of IL-13 in CTL culture did not enhance CTL generation (Fig. 8B). We conclude that IL-13 is not a major factor in our human in vitro cultures but must be cautious about this interpretation being generalized beyond this in vitro CTL expansion system because it may simply not recapitulate the in vivo role of IL-13. Indeed, T lymphocytes do not express IL-13Rs (49), so the in vivo effects are mediated through other cells (50, 51) and these may not be present and/or acting in the same capacity in this in vitro setting.

Based on our findings, we speculated that Th2 cytokines, such as IL-4, IL-5 and IL-10, secreted mainly by CD4+ NKT cell subset were having a dominant suppressive effect on CTL generation in spite of the presence of large amounts of Th1 cytokines in these cultures. To examine this hypothesis, we neutralized these Th2 cytokines in our CTL culture system and found that elimination of these cytokines not only reversed the suppressive effect of αGalCer on CTL generation but also greatly enhanced the frequency of CTLs (Fig. 8). The data indicate that these Th2-type cytokines, especially IL-4, are playing a major role in the suppression of CTL generation caused by αGalCer and/or NKT cells, in spite of enormous IFN-γ and TNF-α secretion co-existing in many of the culture settings. This suggests the dominant role of Th2 cytokines over Th1 cytokines in T2,1 differentiation. More importantly, our results indicate that when Th2 cytokines are efficiently blocked with neutralizing antibodies, αGalCer can strongly enhance CTL generation in a Th2,1-biased manner. One possible mediator of the enhanced CTL response following neutralization of Th2 cytokines is CD40L expressed on activated NKT cells. Alternatively, other unknown factors associated with NKT cells might be involved in this pronounced CTL generation. Further study is required to determine the precise mechanism of enhanced CTL generation.

Our results suggest potential avenues for immune-based therapy. αGalCer and/or NKT cells might be useful for the treatment of a variety of diseases that are mediated by pathogenic Th1 cells such as diabetes and GVHD. Conversely, combination of αGalCer with anti-Th2 cytokine-neutralizing
antibodies can be an efficient method to expand tumor antigen-specific CTLs in vitro, which could enhance the generation of cells for adoptive immunotherapy or the expansion of CTL clones. However, further study is needed to investigate the possible clinical application of these methods.

Acknowledgements

The authors thank the Clinical and Regulatory Group and Cell Processing staff of the Program in Molecular Therapeutics for their assistance. This work was supported by NIH/NCI grant number P01 CA78673 and R01 CA095447. Tetramers were provided by the NIAID Tetramer Facility and NIH, AIDS Reference Reagents Program.

Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>APC</td>
<td>alphycoerythrin</td>
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<tr>
<td>CBA</td>
<td>cytometric bead array</td>
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<tr>
<td>CD40</td>
<td>CD40 ligand</td>
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<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DN</td>
<td>double negative</td>
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<tr>
<td>sGalCer</td>
<td>α-galactosylceramide</td>
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<td>GVHD</td>
<td>graft versus host disease</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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References