

# Evaluation of the Function of Human Invariant NKT Cells from Cancer Patients Using $\alpha$ -Galactosylceramide-Loaded Murine Dendritic Cells<sup>1</sup>

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NKT cells play a role in immunological regulation of certain diseases, and their frequency and/or function may be related to disease prognosis. However, it is often difficult to evaluate NKT cell function in patients with malignancies due to reduced numbers of NKT cells as well as the dysfunction of the APCs used as stimulators. We found that NKT cell function could not be evaluated by conventional ELISPOT assays, confirming the impaired function of APCs in chronic myelogenous leukemia (CML)-chronic phase patients. To overcome this problem, we have established a sensitive assay using murine dendritic cells to evaluate the function of small numbers of human NKT cells independent of autologous APCs. We found that imatinib-treated CML-chronic phase patients showing a complete cytogenetic response had NKT cells capable of producing IFN- $\gamma$ , whereas NKT cells from patients who were only partially responsive to imatinib treatment did not produce IFN- $\gamma$ . Functional NKT cells found in imatinib-treated, CML-complete cytogenetic response patients may offer the promise of effective immunotherapy with ex vivo-generated  $\alpha$ -galactosylceramide-pulsed dendritic cells. This new approach should be available for evaluating the functions of NKT cells and APCs in cancer patients. *The Journal of Immunology*, 2006, 177: 3484–3492.

Natural killer T cells constitute a lymphocyte lineage with features of both T cells and NK cells (1). When activated, NKT cells produce IFN- $\gamma$  or IL-4, and also exhibit MHC-independent cytolytic activity against tumor cells. In addition, they can serve a link between innate and adaptive immunity by inducing the full maturation of dendritic cells (DCs),<sup>3</sup> resulting in strong T cell-mediated responses (2, 3).

NKT cells are reduced in number and in their ability to secrete cytokines in patients with malignancies, autoimmune disorders, and viral infections (4). In some patients with advanced cancer, NKT cells are strikingly low in number and are unable to produce IFN- $\gamma$  (5). The number and function of NKT cells were often shown to have prognostic value in certain types of cancer (6–8).

We previously demonstrated that IFN- $\gamma$  production by NKT cells can have antitumor effects in vivo (9). Recent promising reports (10–12) showed NKT cell immunotherapy using  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer)-loaded DCs (DC/Gal) was well-tolerated in clinical trials and effective in mediating antitumor cytotoxic and adjuvant effects. Tracking the frequency and function of NKT cells may contribute to an understanding of disease pathogenesis and provide a tool to measure patient response to  $\alpha$ -GalCer treatment.

We have previously shown that  $\alpha$ -GalCer-pulsed mature DCs activate primary NKT cells in peripheral blood (PB) better than monocytes and immature DCs (13). However, tumors develop mechanisms of immune escape including the secretion of immunosuppressive factors, such as IL-10, TGF- $\beta$ , VEGF, M-CSF or IL-6, which render DCs and macrophages tolerogenic (14, 15). APCs could be responsible for the failure of the immune system to respond to tumors. APCs, especially monocyte-derived DCs, express reduced levels of costimulatory and MHC class II molecules and produce lower levels of inflammatory cytokines in patients with cancer (16–19). Therefore, it is difficult to compare the potency of NKT cells and other effector cells from different patients using autologous APCs when the function of both cell types may be affected by the patient's underlying condition.

To detect NKT cell function independent of human APCs, we focused on a homogenous population of murine DCs. NKT cells recognize glycolipids presented by the nonpolymorphic MHC class I-like molecule CD1d, which is related to  $\beta_2$ -microglobulin-associated transmembrane proteins (1, 4, 9, 20). The strong homology in sequence between human and murine CD1d (60.4% for the  $\alpha_1$  domain, and 62.4% for the  $\alpha_2$  domain) allows human V $\alpha$ 24<sup>+</sup> NKT cells to respond to murine CD1d, and murine V $\alpha$ 14<sup>+</sup> NKT cells to respond to human CD1d (1, 20–23). NKT cells from both species also can be activated by the same glycolipids on APCs, including  $\alpha$ -GalCer, isoglobotrihexosylceramide (24), and

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell;  $\alpha$ -GalCer,  $\alpha$ -galactosylceramide; DC/Gal,  $\alpha$ -GalCer-loaded DC; PB, peripheral blood; CML, chronic myelogenous leukemia; CP, chronic phase; CCR, complete cytogenetic response; PR, partial response; SFC, spot-forming cell; BM, bone marrow; LDA, limiting dilution assay; xeno-DC, xenogeneic DC; auto-DC, autologous DC; allo-DC, allogeneic DC.

$\alpha$ -GalCer-related analogues ( $\alpha$ -C-GalCer) (25), GD3 (26), on CD1d.

We developed this approach to analyze NKT cell function in patients with chronic myelogenous leukemia (CML), a clonal myeloproliferative disorder of hemopoietic progenitors in which the Ph1 chromosome generates a *bcr/abl* chimeric gene that expresses an abnormal fusion protein with altered tyrosine kinase activity (27). Because CML is a stem cell disorder, *bcr-abl*<sup>+</sup> leukemic cell-derived APCs are sometimes reported to be less functional than APCs from healthy donors (28, 29).

Imatinib mesylate (imatinib) is a reversible tyrosine kinase inhibitor that selectively targets the *ABL* portion of the *bcr-abl* fusion protein (30) and is currently used as the first line therapy for chronic phase (CP) CML (31). However, there have been some reports that imatinib has unfavorable effects on cell-mediated immune responses by inhibiting DNA synthesis in primary T cells (32, 33), inducing abnormalities in plasmacytoid DCs (34), suppressing macrophage function through inhibition of the tyrosine kinase receptor, *c-fms* (M-CSF receptor) (35) and blocking the differentiation of myeloid DCs due to a down-regulation of nuclear-localized RelB protein (36, 37). Recently, Cebo et al. (38) demonstrated that imatinib affects cell surface glycosylation of targets and hampers the binding formations of the NK/target immunological synapse, resulting in the subsequent modulation of NK cell-mediated lysis.

In this study, we provide a method to evaluate the function of NKT cells from cancer patients, in this case CML, using murine DCs whose function could be standardized. We show that this approach is able to detect NKT cells in PB, even when their frequencies are low, and we present data suggesting that NKT cell responses are strong in imatinib-treated, CML-CP patients with complete cytogenetic response (CCR) but not in patients with a partial response (PR) to therapy.

## Materials and Methods

### Sample collection and preparation

PBMC were obtained from buffy coats from healthy blood donors (Tokyo Red Cross, Tokyo, Japan) and separated by Ficoll-Hypaque (Amersham Pharmacia Biotech) density centrifugation. PBMC were washed three times with PBS and stored in liquid nitrogen until use. PB samples from 20 CML patients who were in CCR or PR at the cytogenetic level were obtained from National Hospital Organization Kumamoto Medical Center (Kumamoto, Japan) and Kyoto University Hospital (Kyoto, Japan). All of the patients tested were under imatinib treatment when samples were obtained. Written informed consent was obtained from patients according to Declaration of Helsinki. All studies were approved by RIKEN institutional review board.

### Reagents and Abs

$\alpha$ -GalCer was synthesized in the RIKEN Research Center for Allergy and Immunology (Yokohama, Japan). Human rGM-CSF, IL-4, IL-1 $\beta$ , and TNF- $\alpha$  were purchased from R&D Systems. IL-2 was provided by Shionogi, and PGE<sub>2</sub> was obtained from Sigma-Aldrich.  $\alpha$ -GalCer was diluted in 0.05% polysorbate containing PBS (vehicle). The following mAbs were from BD Pharmingen: anti-human mAbs; FITC-conjugated anti-CD3, anti-invariant NKT (6B11), anti-CD4, or PE-conjugated anti-CD8, anti-CD11c, anti-CD14, anti-CD40, anti-CD80, anti-CD83, anti-CD86, anti-invariant NKT (6B11), perforin and allophycocyanin-conjugated anti-CD3, anti-CD11c, anti-CD154, anti-IFN- $\gamma$ , anti-IL-4. Anti-human V $\alpha$ 24-FITC and anti-human V $\beta$ 11-PE mAbs were purchased from Beckman Coulter. Anti-human granzyme B-mAb was purchased from Serotec. Anti-mouse mAbs (BD Pharmingen), PE-conjugated anti-CD11c, biotinylated anti-CD40, CD80, and CD86 were detected using staining with streptavidin-allophycocyanin. CD1d expression was analyzed using mouse anti-human CD1d (NOR3.2) Ab (Abcam) or mouse IgG1 isotype control (BD Pharmingen), followed by secondary Ab, PE-conjugated goat anti-mouse IgG Ab (Jackson ImmunoResearch Laboratories). For flow cytometric analysis, cells were incubated with mAb conjugates for 30 min, washed, and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

### Single-cell ELISPOT assay for quantifying cytokine-producing NKT cells

The detection of IFN- $\gamma$  or IL-4-secreting cells by ELISPOT assay was described previously (13). In brief, 96-well filtration plates (Millipore) were coated with mouse anti-human IFN- $\gamma$  or anti-human IL-4 mAb (both at 10  $\mu$ g/ml; Mabtech). PBMC ( $1 \times 10^6$ /well) were incubated for 16 h either with vehicle or  $\alpha$ -GalCer (100 ng/ml) in RPMI 1640 containing 5% human AB serum or 10% FBS. PMA (50 ng/ml) and ionomycin (1  $\mu$ g/ml) (Sigma-Aldrich) were used for the activation of T cells. After culture, the plates were washed and incubated with biotinylated anti-human IFN- $\gamma$  or anti-IL-4 (both 1  $\mu$ g/ml; Mabtech). IFN- $\gamma$  or IL-4 spot-forming cells (SFCs) were quantified by microscopy. To compare the capacity of different APCs to activate naive NKT cells in culture, in some experiments CD11c/CD14<sup>+</sup> cell-depleted PBMC ( $1 \times 10^6$  cells/well) were cultured with vehicle or  $\alpha$ -GalCer-loaded APCs ( $1 \times 10^3$ ,  $1 \times 10^4$ , or  $1 \times 10^5$  cells/well) in culture medium for 16 h. CD11c/CD14<sup>+</sup> cell-depleted PBMC were prepared by negative selection using PE-conjugated anti-CD11c and CD14 mAbs and anti-PE magnetic beads (Miltenyi Biotec).

### Generation of human and murine DCs

CD14<sup>+</sup> monocytes from PBMC were purified by magnetic beads (Miltenyi Biotec) and cultured in 500 U/ml human IL-4 and 100 ng/ml human GM-CSF for 7 days.  $\alpha$ -GalCer (100 ng/ml) or an equal amount of vehicle was added to the DC cultures on day 5 with the following maturation stimuli; 10 ng/ml IL-1 $\beta$ , 10 ng/ml TNF- $\alpha$ , and 1  $\mu$ g/ml PGE<sub>2</sub> (hereafter referred to as cytokine mixture) for 1 day.

Murine bone marrow (BM)-derived DCs (hereafter referred to as xenogenic (xeno)-DCs) were grown from BM progenitors in RPMI 1640 containing 5% FCS and murine GM-CSF as described previously (39). On day 6,  $\alpha$ -GalCer (100 ng/ml) was added to immature DCs for two more days of culture. To mature the DCs, 100 ng/ml LPS was added on day 7 for 16 h. Mature  $\alpha$ -GalCer-pulsed DCs (DC/Gal) were collected on day 8.

### In vitro generation of V $\alpha$ 24<sup>+</sup> NKT cell lines using mature DCs

To prepare NKT cells,  $\alpha$ -GalCer-pulsed DCs ( $10^4$  cells/well) were added to each well of  $1 \times 10^5$  CD14<sup>+</sup> monocyte-depleted PBMC in the presence of IL-2 (100 U/ml). After 10–14 days, V $\alpha$ 24<sup>+</sup> NKT cells were stained using FITC-labeled anti-V $\alpha$ 24 mAb and selected with anti-FITC magnetic beads. V $\alpha$ 24<sup>+</sup> NKT cells were maintained as cell lines in the presence of 100 U/ml IL-2 in complete medium and restimulated with irradiated autologous PBMC in the presence of  $\alpha$ -GalCer. In some experiments, we used an established and stable NKT cell line, NKT-B1, from a healthy donor.

### Cytokine production by NKT cells

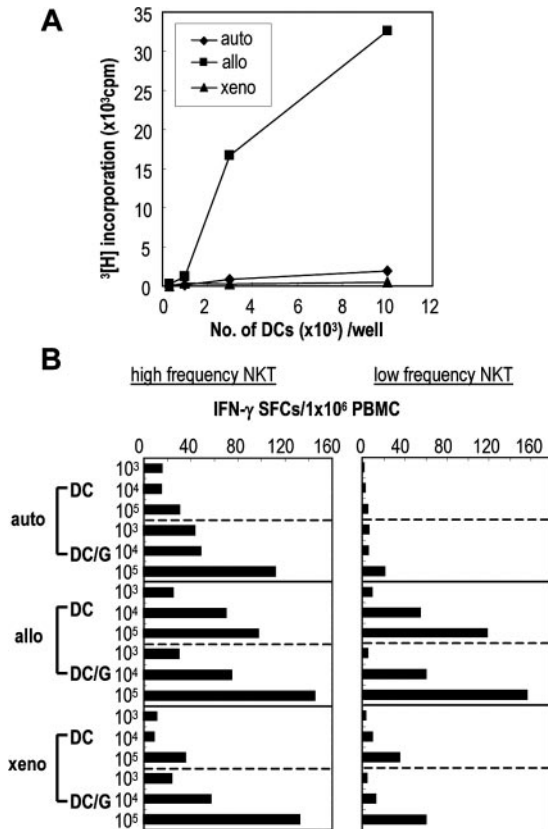
Five thousand NKT cells were cocultured with  $5 \times 10^4$  mature DCs (either autologous or xenogenic), pulsed with vehicle or 100 ng/ml  $\alpha$ -GalCer for 24 h in 96-well plates. The supernatants were assayed by ELISA for cytokines using human IFN- $\gamma$  Opti EIA kit (BD Bioscience Pharmingen).

### Quantitation of NKT cell frequency by multiple microculture and limiting dilution assays (LDAs)

The threshold for quantifying NKT cell frequency was determined by a LDA using NKT cell lines. NKT cells from established cell lines were diluted with CD11c/CD14<sup>+</sup> cell-depleted PBMC from which NKT cells had already been removed by sorting. These NKT cell lines and PBMC from the same donors were used at ratios from 10 to  $10^5$  cells per  $10^7$  PBMC. The graded numbers of NKT cells in PBMC ( $1 \times 10^5$  cells/well) were cocultured with  $\alpha$ -GalCer-pulsed xenogenic DCs (xeno-DCs) or autologous DCs (auto-DCs) ( $1 \times 10^4$  cells/well) in the presence of IL-2 for 10 days. The NKT cell frequencies were determined by FACS.

### Expansion of human NKT cells by coculture with xeno-DC/Gal

To detect low frequencies of NKT cells in blood, PBMC ( $1 \times 10^5$  cells/well) after depletion of CD11c/CD14<sup>+</sup> cells were cocultured with xeno-DC/Gal ( $1 \times 10^4$  cells/well) for 9 days. On day 10, we tested the frequency of expanded NKT cells and determined their intracellular cytokine profile. To perform intracellular cytokine staining, brefeldin A (BD Pharmingen) was added for the last 10 h of culture. These cells were surface stained with CD3-FITC and 6B11-PE, an Ab that recognizes a unique epitope within V $\alpha$ 24 $\alpha$ Q junction of human NKT cells. Fluorescence amplification (FASER-kit-PE; Miltenyi Biotec) was used to enhance the 6B11-PE cell surface staining. Following permeabilization, the cells were stained for intracellular cytokines using IFN- $\gamma$ -APC or IL-4-APC and then analyzed using a FACSCalibur instrument (BD Biosciences).



**FIGURE 1.** Activation of primary NKT cells or T cells from healthy donors by different sources of DCs. *A*, Graded numbers of mature auto, allo-, and xeno-DCs were irradiated and added to  $1 \times 10^5$  monocyte-depleted PBMC in 96-well round-bottom plates for 6 days. T cell proliferation was measured by uptake of [ $^3\text{H}$ ]thymidine ( $1 \mu\text{Ci}/\text{well}$ ) added during the final 16 h of culture. *B*, PBMC were classified by FACS analysis to three groups dependent on the frequency of NKT cells (high NKT cell,  $>0.05\%$ ; low NKT cell,  $>0.01\%$ ,  $<0.05\%$ ; extremely low,  $<0.01\%$ ). Mature murine and human DCs were prepared as described in *Materials and Methods*. In these experiments, different sources of DCs (auto, allo-, or xeno-) pulsed with vehicle (DC) or  $\alpha$ -GalCer ( $100 \text{ ng/ml}$ ) (DC/G) for 2 days were cocultured with  $1 \times 10^6$  PBMC depleted of CD11c/CD14 $^+$  cells at the indicated ratios. The IFN- $\gamma$ -producing activity of NKT cells was tested by ELISPOT after culturing  $1 \times 10^6$  PBMC for 16 h. Data are representative of three independent experiments.

#### Evaluation of the capacity of CML patient-derived CD14 $^+$ monocytes or DCs to present $\alpha$ -GalCer to NKT cells

DC or CD14 $^+$  monocytes, isolated by MACS from healthy volunteers or CML patients, were cultured ( $1 \times 10^4$  cells/well) with an established NKT cell line (NKT-B1) ( $1 \times 10^4$  cells/well) in 96-well round-bottom plates for 48 h in the presence of vehicle or  $\alpha$ -GalCer. IFN- $\gamma$  production from culture supernatants was measured by ELISA.

#### Statistical analysis

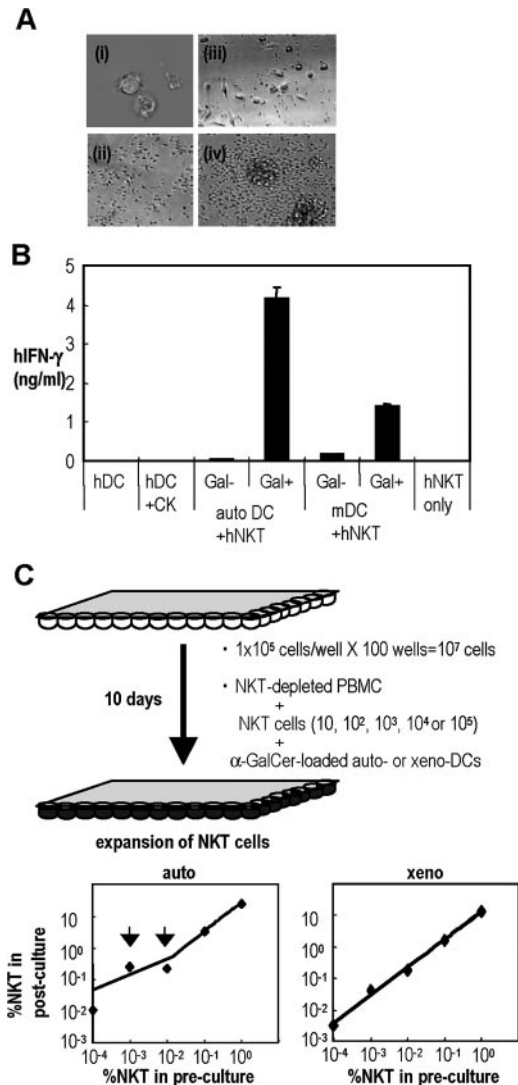
Data were analyzed by the Mann-Whitney  $U$  test. Significance was set at  $p < 0.05$ .

## Results

### The activation of primary T cells or NKT cells from PBMC of healthy donors using different sources of $\alpha$ -GalCer-loaded mature DCs

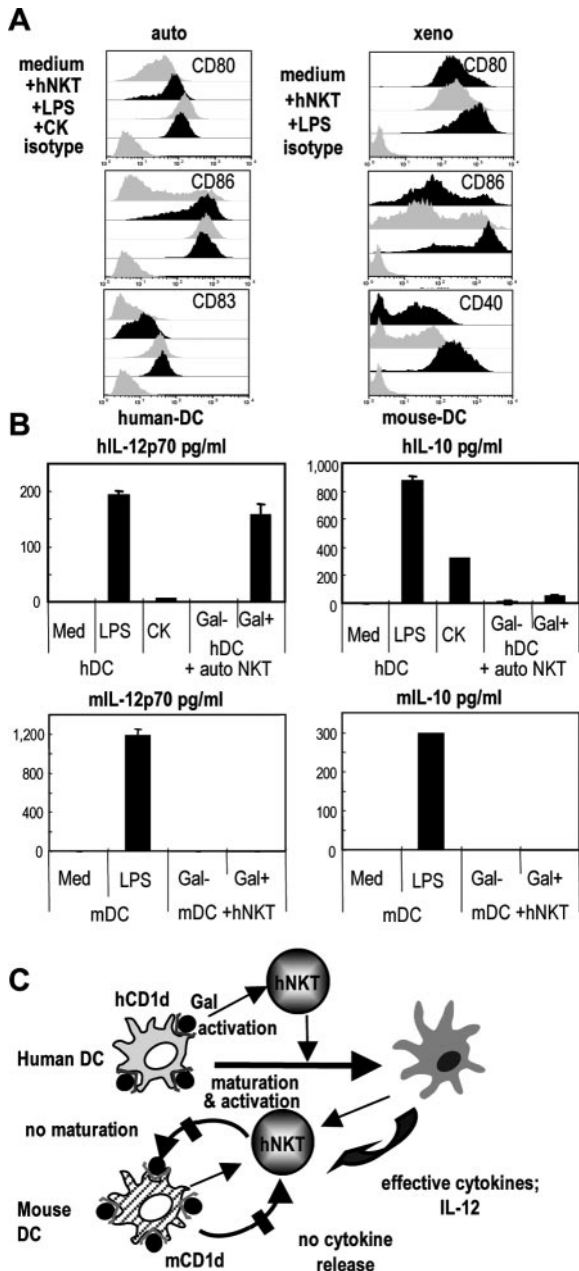
Evaluation of NKT cell function, particularly in disease states, is often difficult due to a low number of NKT cells and/or APC dysfunction. Therefore, we compared the activity of autologous to non-autologous APCs, i.e., allogeneic (allo)-DCs and xeno-DCs.

Because murine DCs do not stimulate human T cells in a MLR (Fig. 1A), we decided to test murine DCs as APCs for human NKT cell responses to glycolipids, as a measure of NKT cell function that is independent of patients' APCs. We determined the number of NKT cells in the PBMC of healthy donors by two-color flow cytometry with CD3-FITC and 6B11-PE mAb, and confirmed their identity by staining with V $\alpha$ 24-FITC and V $\beta$ 11-PE mAb, which

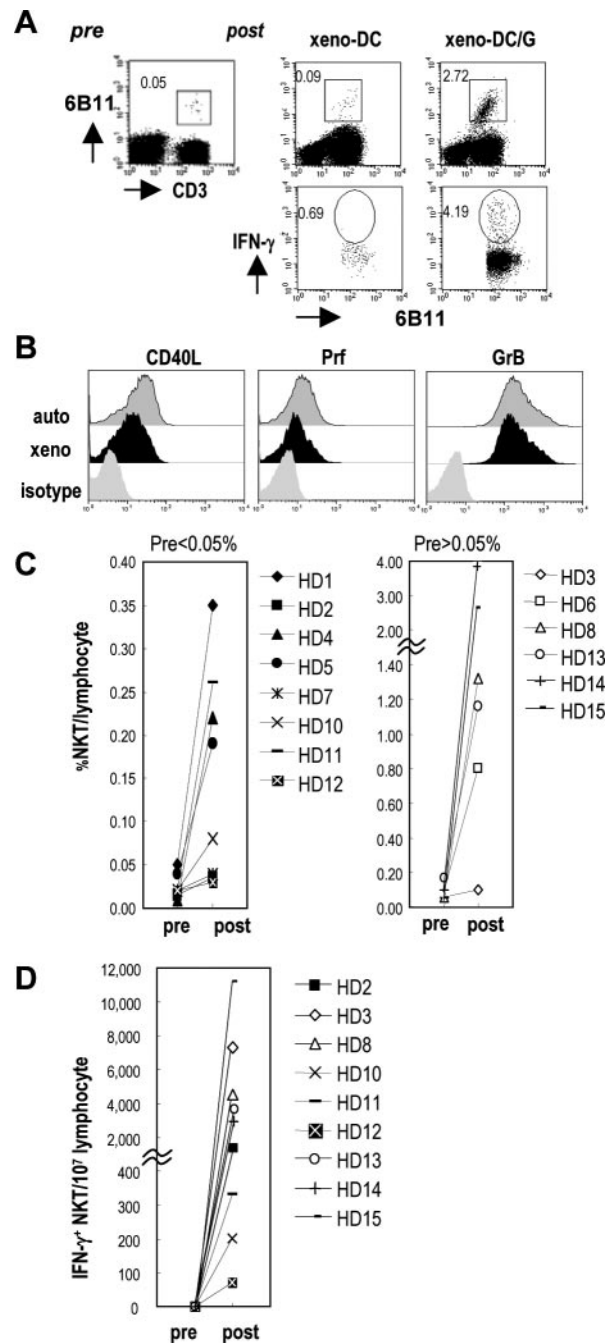


**FIGURE 2.** Quantitation of NKT cell frequency by multiple microculture and LDAs using  $\alpha$ -GalCer-loaded xeno-DC. *A*, Human NKT cell lines were established using auto-DC/Gal as described in *Materials and Methods*. Human NKT cells from an established NKT cell line were cultured with or without murine DC or DC/Gal. Seven days later the cultures were examined by a phase contrast microscopy. *i*, Murine DCs only; *ii*, human NKT cells only; *iii*, human NKT cells with murine DCs; and *iv*, human NKT cells with murine DC/Gal. *B*, Human NKT cells ( $5 \times 10^3$  cells/well) from an established NKT cell line were cultured with or without auto- or xeno-DC or DC/Gal ( $5 \times 10^4$  cells) for 24 h. Culture supernatants were tested for IFN- $\gamma$  production by ELISA. Data are means obtained from four samples in independent experiments. *C*, NKT cells were mixed with NKT cell-depleted PBMC at the graded ratios from  $10$  to  $10^5$  NKT cells/ $10^7$  PBMC/plate in 96-well round plates. The cells were cocultured with auto-DC/Gal or xeno-DC/Gal in the presence of IL-2 ( $100 \text{ U/ml}$ ) for 10 days, and then the percentage of NKT cells was determined by flow cytometry. The arrows indicate instances where there were more NKT cells found in the autologous vs xenogeneic setting. Representative data are from three independent experiments.

are Abs specific for invariant NKT cells. In this assay, the 6B11 mAb was used for detecting NKT cells because it specifically recognizes a unique determinant in the CDR3 region of the invariant (V $\alpha$ 24-J $\alpha$ Q) TCR chain of NKT cells comparable to CD1d tetramer (5). Based on the results, we divided donors into three categories: 1) high-frequency NKT cells (>0.05%); 2) low-frequency NKT cells (0.01–0.05%); and 3) and extremely low frequency NKT cells (<0.01%).



**FIGURE 3.** Human NKT cells can mature human but not murine DCs capable of producing IL-12. At day 5 of culture,  $5 \times 10^4$  human or murine immature DCs were stimulated with 100 ng/ml LPS or cytokine mixture (CK), or cocultured with  $5 \times 10^3$  human NKT cells in the presence of  $\alpha$ -GalCer. A, DCs were harvested after 24 h and analyzed by flow cytometry. Human DCs (auto) or murine DCs (xeno) are shown after gating on CD11c<sup>+</sup> cell fraction. B, The supernatants from the various DC cultures were collected 24 h after the maturation stimulus was added and measured for hIL-12p70, hIL-10, and murine (m)IL-10 by ELISA. Representative data from three independent experiments. C, A model depicting the interaction between human or murine DCs and human NKT cells.



**FIGURE 4.** Functional assays for human NKT cells expanded from PB by xeno-DC/Gal. A, To characterize the function of NKT cells,  $1 \times 10^5$  CD11c/CD14<sup>+</sup> cell-depleted human PBMC from healthy donors were cocultured with xeno-DC/Gal in the presence of IL-2 for 10 days. The frequency of NKT cells was determined by flow cytometry using CD3 and 6B11 mAb. The production of IFN- $\gamma$  from NKT cells was analyzed by intracellular staining assays after culturing 10 h in the presence of brefeldin A. B, NKT cells expanded by auto-DC/Gal or xeno-DC/Gal were analyzed by flow cytometry for levels of perforin, granzyme B, and CD40L expression after gating on the CD3<sup>+</sup>6B11<sup>+</sup> cells. C, The number of expanded NKT cells after 10 days culture was enumerated in low (<0.05%) and high (>0.05%) NKT cell frequency groups. D, All of the expanded NKT cells were tested for IFN- $\gamma$  production. Representative data are from three independent experiments.

As we have shown previously, autologous mature DCs are more useful than immature DCs or monocytes in evaluating the function of NKT cells in blood (7, 13). We evaluated NKT cell function by

ELISPOT, comparing allo-, xeno-, and auto-DCs as stimulators (Fig. 1B). When we compared,  $\alpha$ -GalCer-loaded, mature DCs from these three sources, high numbers of IFN- $\gamma$ -producing spots were detected in all groups (percentage of NKT cells, >0.05%) (Fig. 1). However, when we used allo-DCs for activation, there was a high background due to IFN- $\gamma$  secretion by alloreactive T cells. In contrast, both auto- and xeno-DCs were comparable to each other in their ability to activate NKT cells in a dose-dependent and  $\alpha$ -GalCer-dependent manner.

In PBMC from donors with low NKT cells (0.01–0.05%), some  $\alpha$ -GalCer-responsive IFN- $\gamma$  SFCs were detected when auto-, allo-, and xeno-DCs pulsed with  $\alpha$ -GalCer were used as stimulators (Fig. 1B), but results were inconstant and not statistically significant. When we tested samples from donors with extremely low frequency NKT cells (<0.01%), we could not detect any  $\alpha$ -GalCer-dependent spots in any of the DC groups (data not shown).

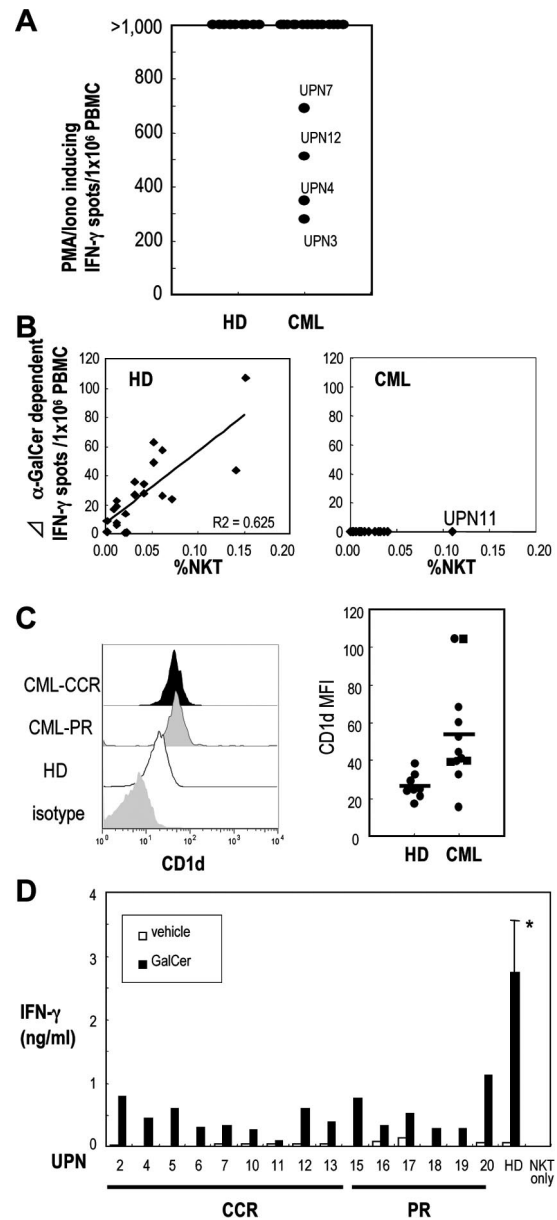
As described above, the frequency of V $\alpha$ 24<sup>+</sup> NKT cells even in most healthy subjects is only ~0.05% in PBMC (13, 40). Additionally, patients with some types of cancer have been shown to have <0.05% NKT cells. Although the approach of using DCs and a 16-h culture doubles the number of SFCs seen with PBMC in a direct ELISPOT assay, it is still difficult to quantify the function of NKT cells in patients with low frequency and extremely low frequency NKT cells. Thus, a 16-h culture cannot increase the number of NKT cells nor can it lower the threshold for detecting functional, IFN- $\gamma$ -secreting NKT cells. A method of expanding existing NKT cells to monitor the low levels of functional NKT cells in the blood of cancer patients was clearly required.

#### Short-term culture of PBMC with $\alpha$ -GalCer-pulsed xeno-DCs leads to NKT cell expansion that correlates with input numbers of NKT cells

When human NKT cells were cocultured with murine DC/Gal, they formed clusters (Fig. 2A), whereas murine DCs alone, human NKT cells alone, and human NKT cells cultured with murine DCs did not form clusters. Both auto- and xeno-DC/Gal were able to activate human NKT cells to produce IFN- $\gamma$  (Fig. 2B), although the levels were lower when murine-DC/Gal were used as stimulator cells.

The threshold for quantifying NKT cell frequency cells was determined by a LDA, in which multiple microcultures of NKT cell-depleted PBMC were mixed with a graded number of NKT cells and expanded with  $\alpha$ -GalCer-loaded auto- or xeno-DCs. When we cultured fewer than  $10^4$  cells/well, we could not reliably detect NKT cells. Therefore,  $10^5$  cells/well was adopted as the standard cell concentration for subsequent assays. Our results also show a close correlation between the frequencies of NKT cells in the preculture and the number of NKT cells expanded by xeno-DC/Gal, even at low NKT cell preculture frequencies (0.0001–1%) (Fig. 2C). The number of NKT cells increased 10-fold after culture, and the auto-DC/Gal expanded the NKT cells in culture more robustly than the xeno-DC/Gal.

To address the mechanism of the difference in expansion of NKT cells between auto-DC/Gal and xeno-DC/Gal, we analyzed the DCs stimulated by NKT cells. After activation, NKT cells can convert immature DCs into mature ones in vitro. In an autologous setting, human DCs matured by activated autologous NKT cells highly up-regulate costimulatory molecules and produce bioactive IL-12 comparable to the levels seen in DCs matured by other stimuli such as LPS (Fig. 3, A and B). Interestingly, NKT-cell-matured DCs produce less IL-10 than DCs matured with LPS (Fig. 3B). In contrast, mouse DCs can present  $\alpha$ -GalCer to human NKT cells, but cannot be matured by activated human NKT cells, and do not produce any cytokines. In the auto-DC/Gal expansion group, we



**FIGURE 5.** Enumeration of IFN- $\gamma$ -producing T or NKT cells from imatinib-treated, CML-CP patients. **A**, To test the function of T cells, PBMC from healthy donors (HD) or CML patients were cultured for 16 h in the presence of PMA (50 ng/ml) and ionomycin (1  $\mu$ g/ml), followed by measurement of IFN- $\gamma$  secretion by an ELISPOT assay. **B**, The frequency of NKT cells (6B11<sup>+</sup>CD3<sup>+</sup>, x-axis) is plotted against the number of IFN- $\gamma$  SFCs obtained after culturing PBMC from HD or CML-CP patients in the presence of  $\alpha$ -GalCer for 16 h (y-axis). **C**, CD1d expression on CD14<sup>+</sup> monocytes from CML patients and healthy donors was compared. The representative data of FACS was shown (left), and mean fluorescence intensity of CD1d expression was enumerated (right). ● and ■ indicate CCR and PR patients in the part of CML, respectively (\*, HD vs CML-PR or CML-CCR;  $p < 0.005$ ). **D**, CD14<sup>+</sup> monocytes were isolated from HD or patients with CML-CP in CCR or PR using CD14-magnetic MACS beads and cultured with NKT-B1 cells for 48 h in the presence of vehicle or  $\alpha$ -GalCer. Culture supernatants were tested for IFN- $\gamma$  production by ELISA (\*, HD vs CML-PR or CML-CCR;  $p < 0.001$ ).

found more expanded NKT cells than would be expected if expansion was entirely ligand-specific (Fig. 2C, arrows). The additional NKT cell expansion is likely due to activation by IL-12 produced by the auto-DC. Therefore, when cultured with xeno-DCs, NKT cells expanded in a manner that closely correlated with

starting numbers of cells, as opposed to auto- and allo-DC expansions.

*Evaluation of IFN- $\gamma$ -producing NKT cells from healthy donors using  $\alpha$ -GalCer-loaded xeno-DCs*

After depletion of CD11c/CD14<sup>+</sup> cells, primary PBMC were cultured with xeno-DC/Gal for 10 days, and then analyzed for IFN- $\gamma$ -producing NKT cells using intracellular cytokine staining (Fig. 4A). Using this approach, we were able to consistently detect an expanded population of NKT cells that produced IFN- $\gamma$ , but not IL-4 (data not shown). In addition, when we cultured CD11c/CD14<sup>+</sup> cell-depleted PBMC with auto-DC/Gal, we detected more IFN- $\gamma$ -producing NKT cells than xeno-DC/Gal (data not shown). This result may be due to the released secondary cytokines, such as IL-12. When we cultured CD11c/CD14<sup>+</sup> cell-depleted PBMC with allo-DC/Gal, we detected less IFN- $\gamma$ -producing NKT cells than xeno-DC/Gal (data not shown). The findings might be explained due to the consumption of cytokines, especially IL-2 by expanded alloreactive T cells, because IL-2 is a key factor for supporting and expanding both T cells and NKT cells. As shown in the previous section, expansion with xeno-DCs more closely correlates with the pre-expansion numbers of functional NKT cells in cultures.

We noted that control murine DCs without  $\alpha$ -GalCer had some background expansion of NKT cells, but did not result in an increase in IFN- $\gamma$ -secreting cells (Fig. 4A), which might be due in part to an endogenous ligand on the murine DC. In addition to secretion of IFN- $\gamma$ , human V $\alpha$ 24<sup>+</sup> NKT cells use granzyme B and perforin to lyse tumor cells (20, 41, 42). When we measured expression of these molecules on NKT cells expanded with auto-DC/Gal or xeno-DC/Gal, we found similar levels of granzyme B and perforin in each group (Fig. 4B). Even PBMC cultures with initially low numbers of NKT cells (<0.05%) produced quantifiable numbers of IFN- $\gamma$ -producing NKT cells after culture, confirming the efficacy of  $\alpha$ -GalCer-loaded CD1d molecules on murine DCs (Fig. 4, C and D). Overall, these findings demonstrate that human

NKT cells can be expanded by murine-DC/Gal in a ligand-specific way and retain their phenotype and function (Fig. 4, C and D).

*Monocytes from imatinib-treated CML patients are impaired in their ability to stimulate NKT cells from healthy donors*

Several studies have documented immune dysfunction in imatinib-treated patients (32, 33), and we have confirmed that T cell responses to PMA/ionomycin are suppressed in a subset of these patients (Fig. 5A).

Compared with the data of healthy volunteers, the frequency of NKT cells in PBMC of CML patients by FACS was relatively low (mean, 0.02%) but still detectable (Table I). Strikingly, IFN- $\gamma$  SFCs were not detected in PB of any CML patients (Fig. 5B), suggesting that T cell response are inhibited in only certain imatinib-treated patients, whereas NKT cell function is reduced in nearly all of them. Depletion of CD14<sup>+</sup> cells abolished IFN- $\gamma$  spots in our ELISPOT assays, indicating that IFN- $\gamma$  production by NKT cells in PB depends on the presence of CD14<sup>+</sup> monocytes. In fact, IFN- $\gamma$ -producing NKT cells were not detected in bulk ELISPOT assays of PBMC from imatinib-treated CML patients. Because monocytes are the most commonly found APCs in this assay, we wanted to evaluate these cells for possible dysfunction.

We analyzed the expression of CD1d on CD14<sup>+</sup> monocytes from CML patients and healthy donors by flow cytometric analysis (Fig. 5C). We found that CD1d expression on monocytes from CML patients, including CCR and PR, was up-regulated more than those of healthy donors.

When we cultured patients' monocytes with NKT-B1 cells, an established NKT cell line from a healthy donor, in the presence of  $\alpha$ -GalCer (100 ng/ml), we found that IFN- $\gamma$  production from activated NKT cells was significantly reduced in imatinib-treated CML patients (Fig. 5D). These results suggest that monocytes from imatinib-treated CML patients have an impaired ability to present  $\alpha$ -GalCer. APC dysfunction in these patients might be related to inhibition of monocyte signaling by imatinib.

Table I. Characteristics of CML-chronic phase patients

	Hematology WBC ( $\times 10^{-9}/L$ )	Hb (g/dl)	Plt ( $\times 10^{-9}/L$ )	PB %CD3 <sup>+</sup> invariant NKT <sup>+</sup>	%CD4	%CD8	%CD3 <sup>-</sup> CD56 <sup>+</sup>	Therapy Imatinib
CR								
UPN1	3.1	10.1	115.0	0.000	49.6	11.0	28.4	— <sup>a</sup>
UPN2	3.0	10.6	117.0	0.026	19.1	18.1	18.0	+
UPN3	4.9	13.6	209.0	0.036	21.6	5.9	20.5	+
UPN4	8.6	14.1	181.0	0.032	34.7	18.2	10.2	+
UPN5	5.6	9.7	237.0	0.009	20.2	12.0	5.0	+
UPN6	2.8	12.6	95.0	0.002	30.2	14.2	19.9	+
UPN7	4.7	13.5	169.0	0.040	15.2	23.8	21.8	+
UPN8	3.1	9.8	98.0	0.030	32.7	10.6	29.3	+
UPN9	2.8	10.8	195.0	0.010	27.2	27.2	9.4	+
UPN10	2.9	10.0	65.0	0.000	27.3	27.3	9.1	+
UPN11	3.0	12.1	113.0	0.110	46.8	30.9	4.7	+ <sup>b</sup>
UPN12	6.0	12.5	204.0	0.020	24.3	19.0	12.2	+
UPN13	5.5	14.7	150.0	0.006	23.5	32.4	33.2	+
UPN14	3.9	9.8	63.0	0.011	28.0	21.2	7.0	+
PR								
UPN15	2.5	10.3	38.0	0.012	48.3	15.7	8.2	+
UPN16	4.2	9.7	107.0	0.002	12.0	15.3	6.7	+
UPN17	8.3	9.0	298.0	0.003	32.5	2.2	5.6	+
UPN18	2.3	10.4	200.0	0.015	42.9	30.8	18.5	+
UPN19	8.4	11.5	1791.0	0.002	13.6	10.0	6.5	+
UPN20	7.7	13.7	124.0	0.010	35.1	15.4	38.5	+

<sup>a</sup> Pre-bone marrow transplantation.

<sup>b</sup> Combination with IFN- $\alpha$ .

*Successful expansion of functional NKT cells from imatinib-treated CCR, but not PR patients in CML-CP*

To detect functional NKT cells in imatinib-treated patients, we expanded their numbers using  $\alpha$ -GalCer-pulsed xeno-DC as described in *Materials and Methods* (Fig. 6, A and B). There was no significant difference in the number of NKT cells found in the PB of CCR and PR patients in preculture (0.024 vs 0.007%, respectively;  $p = 0.25$ ). IFN- $\gamma$ -producing NKT cells were found in all 14 CCR patients after culture (Fig. 6, A and C), suggesting that the NKT cells were normal in function even though they were present in low numbers compared with healthy donors. In contrast, none of the imatinib-treated PR patients generated NKT cells that pro-

duced IFN- $\gamma$  (Fig. 6C). As shown in Table I and Fig. 6, NKT cell function might be a prognostic indicator for likelihood for the remission in CML.

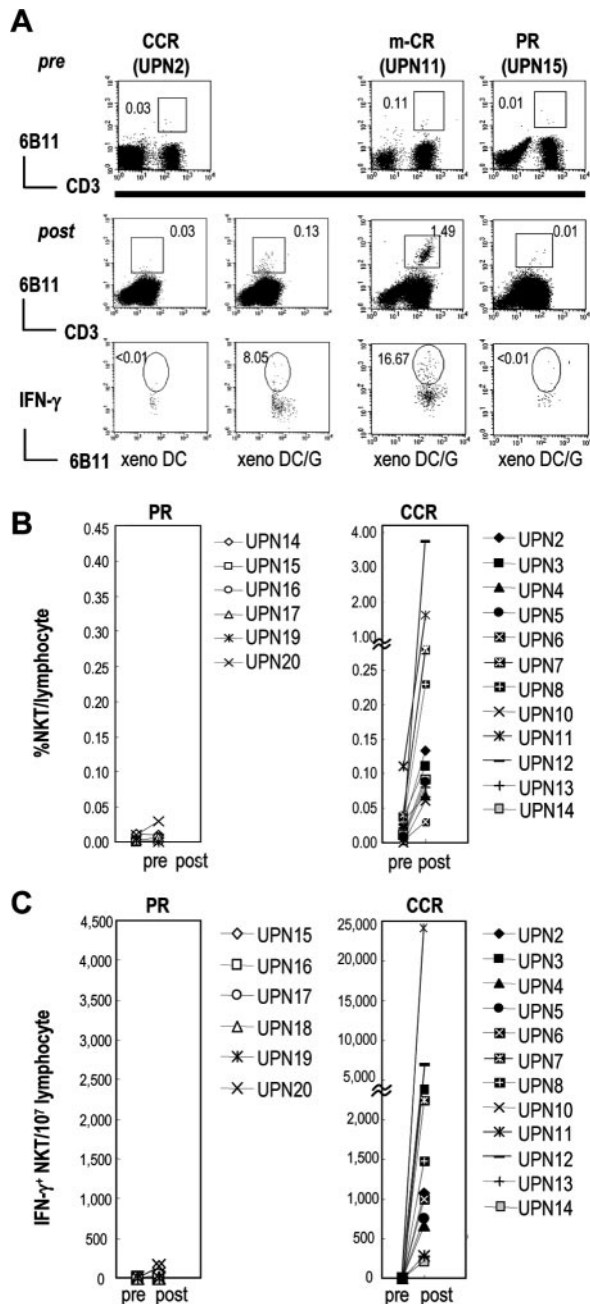
*DCs from imatinib-treated CML patients are able to stimulate NKT cells from healthy donors*

As shown in Figs. 5A and 6, patients with impaired T cell responses to PMA/ionomycin are readily distinguishable from the patients with dysfunctional NKT cells and/or monocytes. Detrimental effects of imatinib on immune responses of T cells and NKT cells thus appear to be different. In marked contrast,  $\alpha$ -GalCer-loaded monocyte-derived DCs from both imatinib-treated patients and healthy donors activated NKT cells equivalently (Fig. 7). This finding indicates that ex vivo-generated auto-DCs can overcome the poor stimulatory effects of monocytes on NKT cells.

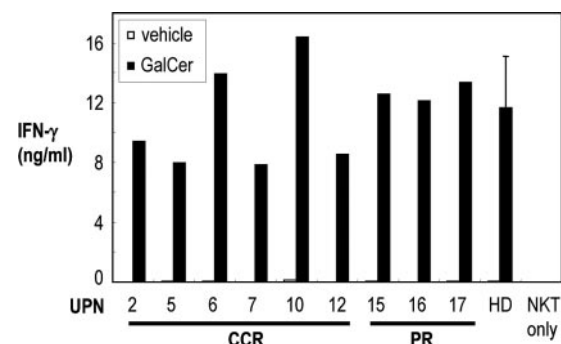
## Discussion

In this study, we developed a new approach in which murine DCs rather than autologous human DCs are loaded with  $\alpha$ -GalCer and used to expand a small population of NKT cells from the PBMC of healthy donors and cancer patients. By using murine DCs to measure NKT cell number and function in patients with CML, one difficult variable is eliminated, namely autologous APC dysfunction secondary to an underlying disease states.

Recent results of three clinical studies demonstrated the tolerability and efficacy of  $\alpha$ -GalCer-pulsed DC therapy to augment NKT cell responses (10–12). In the patients responding to these trials, elevation of inflammatory cytokines in serum as well as proliferation of NKT cells, including IFN- $\gamma$ -producing cells, was demonstrated. Another anticancer therapy involves the vaccination of cancer patients with Ag peptides with the goal of generating an antitumor CTL response against the tumor. However, the frequency of antivaccine T cells in PB is often undetectable or  $<10^{-6}$  of T cells when tested by LDAs (43, 44). The successful vaccination by peptide therapy for inducing adaptive immunity may depend on the frequency and function of CTL precursors in PB. The frequency of NKT cells is normally much lower than that of T cells in PB. However, if we compared the frequency of CTL precursors for a specific tumor Ag in cancer patients, the frequency of NKT cells is no longer low, but instead roughly equivalent. Because the effectiveness of cancer vaccine therapy requires circulating immune cells, evaluating the frequency and function of NKT cells in PB should be useful.



**FIGURE 6.** Analysis of the immunological functions of NKT cells from imatinib-treated CML-CP patients. A, IFN- $\gamma$  secretion was examined by intracellular staining at 10 days after culture using xeno-DC/Gal (m-CR; CR at molecular level). B, Proliferation and C, IFN- $\gamma$  production of NKT cells from imatinib-treated CML-CP (CCR or PR) patients are enumerated.



**FIGURE 7.** Ex vivo-generated monocyte-derived DCs from PB of imatinib-treated CML-CP patients can activate NKT cells. Vehicle- or  $\alpha$ -GalCer-pulsed DCs were generated from CD14<sup>+</sup> cells from CML patients or six healthy donors as described in *Materials and Methods*. The DCs ( $1 \times 10^4$  cells/well) were then cultured with NKT-B1 cells ( $1 \times 10^4$  cells/well) in a 96-well-microtiter plate for 48 h. Culture supernatants were used for the IFN- $\gamma$  ELISA.

Significant reductions in NKT cells have been reported in patients with solid tumors such as melanoma, prostate cancer (5, 45), and lung cancer (11), whereas other studies show that the number and/or function of NKT cells in disease states, such as glioma, are comparable to those found in healthy individuals (46). In addition, there are reports demonstrating a reduction of NKT cells in malignant hematological disorders (47). A possible explanation for these inconsistent findings is an impairment in function of either the APCs or NKT cells or both. As one approach to help eliminate this problem, we tested allo-DCs from healthy donors. However, we could not see any specific NKT cell responses due to strong allogeneic T cell responses (Fig. 1B). Because of the similarity of CD1d molecules between mice and humans, we decided to test the efficacy of xeno-DCs derived from murine BM.

In the current study, we used this new approach to study one of the stem cell diseases, CML, and we found that the frequency of NKT cells in most of the patients with CML-CP was low (Table I). Furthermore, we could not detect IFN- $\gamma$  spots with a direct ELISPOT assay (Fig. 5). Inhibition of tyrosine kinases after imatinib treatment or inhibition of phosphorylation of *c-fms* has been described previously (35). Consistent with these findings, we observed that CD14<sup>+</sup> monocytes of CML patients were less able to activate an NKT cell line established from a healthy donor (Fig. 5). The diminished number and function of NKT cells in CML patients may be due in part to tumor- or therapy-related dysfunction of APC or down-regulation of NKT cell cycle in situ.

When we used  $\alpha$ -GalCer-loaded xeno-DCs to analyze IFN- $\gamma$ -producing NKT cells in patients, we detected functional NKT cells in CCR patients, but not in most of the PR patients. Thus, xeno-DCs can only activate circulating NKT cells from CML patients, but not from patients in PR. These findings suggest normal NKT cell function in most of the CML-CCR patients, but impairment in PR patients. NKT cell reactivity in PR patients might be affected by the persistence of leukemic cells. Low NKT cell numbers found even in CCR patients may indicate APC dysfunction due to imatinib treatment in which circulating CD14<sup>+</sup> cells are unable to activate NKT cells. Contrary to our expectation, CD1d expression on monocytes from CML patients was up-regulated in comparison with monocytes from healthy donors (Fig. 5C). These findings may suggest that the pathway leading to expression of the exogenous CD1d ligand on APCs might be impaired, or that the CD1d expressed on APCs might already be occupied by a natural ligand, making it difficult for macrophages to present  $\alpha$ -GalCer. To further develop this strategy, murine DC cell lines, such as D1 or DC2.4, might be useful instead of BM-derived DC (48, 49).

Imatinib has been shown to be well tolerated and efficient in inducing CCR in most patients (27, 31). However, even patients with CCR must be observed at the molecular level for a long period, because some CCR patients have been reported to have a suboptimal response and a recurrence of their malignancy (50). In fact, imatinib rarely induces molecular remission of CML (51, 52). That may be because imatinib does not kill leukemic stem cells as it does leukemic cells in the periphery (53). Chronic treatment with imatinib may also induce resistance. In addition, imatinib suppresses the activity of many different types of immune cells, making support of these cells by immunotherapy a logical adjunct. Therefore, the development of other effective therapies is necessary to provide a cure for patients with CML-CCR. Two arms of treatment, immunotherapy and imatinib therapy, might be a promising approach to cure. In fact, it was recently reported that a combination of imatinib with peptide immunotherapy or with leukocyte-derived heat shock protein peptide complexes drove CR at the molecular level (54, 55).

In this study, we also demonstrated that DCs derived from CD14<sup>+</sup> monocytes taken from imatinib-treated patients were able to efficiently activate NKT cells in vitro. The findings indicate that DCs from CD14<sup>+</sup> cells would be beneficial in some CML-CP patients with a suboptimal response to imatinib. Moreover, our findings suggest that the combination of imatinib with an immunotherapy using  $\alpha$ -GalCer-pulsed auto-DCs might be an ideal strategy for CML-CP patients with normal NKT cell function.

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## Disclosures

The authors have no financial conflict of interest.

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