Hyporesponsiveness to Natural Killer T-Cell Ligand α-Galactosylceramide in Cancer-Bearing State Mediated by CD11b+ Gr-1+ Cells Producing Nitric Oxide

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Abstract

CD1d-restricted natural killer T (NKT) cells are a potential therapeutic target for cancer, for which several clinical trials have already been reported. NKT cells are specifically activated by a synthetic glycolipid, α-galactosylceramide (α-GalCer). However, it is known that, in human cancer patients, NKT cells express a degree of hyporesponsiveness to α-GalCer. In this study, we have examined the mechanism by which hyporesponsiveness to α-GalCer can be induced. In cancer-bearing mice, α-GalCer-induced NKT cell expansion, cytokine production, cytotoxicity, and antimetastatic effect in vivo were all significantly impaired. In fact, α-GalCer could eliminate metastatic disease in naïve animals but failed to protect cancer-bearing mice. CD11b+ Gr-1+ cells were particularly increased in cancer-bearing mice and were necessary and sufficient for the suppression of the α-GalCer response in a nitric oxide–mediated fashion. Administration of a retinoic acid to cancer-bearing mice reduced the population of CD11b+ Gr-1+ cells and effectively restored α-GalCer-induced protection. These results show a novel feature of NKT cell function in cancer. Furthermore, our data suggest a new strategy to enhance NKT cell-mediated anticancer immune responses by suppressing CD11b+ Gr-1+ cell functions. (Cancer Res 2006; 66(23): 11441-6)

Introduction

CD1d-restricted natural killer T (NKT) cells are a lymphoid lineage characterized by expression of unique invariant T-cell receptor (TCR) encoded by Vα14-Jo281 gene segments in mice and Vα24-Jo18 in humans (1). NKT cells recognize α-galactosylceramide (α-GalCer) and its analogues: glycolipids that can be presented by CD1d (2, 3). It has been shown that α-GalCer selectively stimulates NKT cells to produce large amount of both T helper (Th) 1 and Th2 cytokines and that α-GalCer-activated NKT cells exhibit cytolytic activity and exert antitumor effects (2, 4). It has been shown that α-GalCer presented by CD1d (2, 3). It has been shown that α-GalCer-activated NKT cells to produce large amount of both Th1 and Th2 cytokines and that α-GalCer-activated NKT cells exhibit cytolytic activity and exert antitumor effects (2, 4).

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Materials and Methods

Cells, reagents, monoclonal antibodies, and fluorescence-activated cell sorting. Three cell lines were used: B16 melanoma (American Type Culture Collection [ATCC], Manassas, VA), 3LL Lewis lung cancer (ATCC), and YAC-1 lymphoma cell line (Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan). α-GalCer was provided by Kirin Brewery Co. Ltd. (Gunma, Japan). Phycoerythrin (PE)-labeled α-GalCer/CD1d tetramer was prepared as described previously. The following monoclonal antibodies (mAb) obtained from BD PharMingen (San Diego, CA) were used in this study: anti-TCRβ chain (H57-597), anti-NK1.1 (PK136), anti-CD11b (M1/70), and anti-Gr-1 (RB6-8C5). Anti-transforming growth factor-β (TGF-β) mAb (1D11) was purchased from R&D System (Minneapolis, MN). The bioactivity of the mAb was confirmed using a TGF-β ELISA kit (R&D System) by replacing the capture antibody of the kit by this mAb (data not shown). Anti-FITC or PE magnetic beads were purchased from Miltenyi Biotec and used for magnetic-activated cell sorting (MACS). Fluorescence-activated cell sorting (FACS) analysis was done with FACS Calibur using the CellQuest program.

Mice. Male C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan). To establish tumors in mice, 3 × 106 cancer cells were injected s.c. in the mid-dorsal region. Tumors were allowed to grow for 3 to
5 weeks without treatment. For metastasis experiments, mice were i.v. injected with 2 × 10^6 cancer cells via tail vein. Fifteen days later, the mice were sacrificed, and number of metastatic nodules was counted. For in vivo stimulation with α-GalCer, mice were i.p. injected with α-GalCer (2 μg/animal) or vehicle.

**Cell expansion and cell-mediated cytotoxicity.** Spleen cells (1 × 10^6 per well) obtained from naive or cancer-bearing mice were cultivated in 24-well culture plate with α-GalCer (100 ng/ml) in the presence of recombinant interleukin (IL)-2 (200 units/ml; PeproTech) in 1 ml RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were harvested at indicated time points and assessed by FACS analysis. For cytotoxic assay, vehicle or α-GalCer was injected to naive and cancer-bearing mice. Twenty-four hours later, spleen cells were obtained from those mice and assessed their cytotoxicity against YAC-1 or B16 cells as targets by standard 4-hour Na^251Cr-release assay.

**Cytokine measurement.** For in vitro assay, mice were i.p. injected with 2 μg α-GalCer, and sera were collected from these mice at indicated time points. For in vitro assay, mouse splenocytes (4 × 10^6 cells per well in 48-well plate) were stimulated with α-GalCer (100 ng/ml) for various durations, and culture supernatants were collected. Concentrations of IFN-γ or IL-4 in these samples were measured using ELISA kits.

**Isolation and evaluation of CD11b^+ Gr-1^+ cells.** The population of CD11b^+ Gr-1^+ cells in the spleen was evaluated by FACS. CD11b^+ or Gr-1^+ cells were isolated with MACS (purity, >93%). The isolated CD11b^+ or Gr-1^+ cells (3 × 10^4 per well) with or without pretreatment with 10 mmol/L inducible NO synthase (iNOS) inhibitor, Nω-monomethyl-L-arginine (L-NMMA; Calbiochem), for 24 hours, were added to culture of splenocytes (1 × 10^5 cells per well) in the presence of α-GalCer (100 ng/ml) in 96-well round-bottomed plate. In some experiments, anti-TGF-β mAb (20 μg/ml) was added to the final culture. In another experiment, splenocytes were pretreated with L-NMMA for 24 hours and then stimulated with α-GalCer (100 ng/ml). Culture supernatants were harvested at indicated time points and assessed for their cytokine concentration.

**In vivo treatment with ATRA.** Naive or B16-bearing mice were i.p. injected with PBS or ATRA (10 mg/kg Sigma-Aldrich) for consecutive 5 days, and at day 6 their splenocytes were obtained. The number of CD11b^+ Gr-1^+ cells in the splenocytes and their cytokine production on α-GalCer stimulation were examined as described above. For metastasis experiments, mice were i.v. injected with 2 × 10^6 B16 cells via tail vein at day 6, and the number of metastatic nodules in the lungs was counted at day 21.

**Statistical analysis.** Data are expressed as mean ± SD, and differences among groups were analyzed by the Mann-Whitney U test or Student's t test with StatView software.

**Results**

**Impaired α-GalCer-induced cell expansion and cytotoxic production in cancer-bearing mice.** We first obtained splenocytes from either naive or B16-bearing mice and stimulated them with α-GalCer. It has been reported that this treatment dramatically expanded NK1.1^+ TCRβ^+ population (12). Before culture (day 0), populations of NK1.1^+ TCRβ^+ (NK cells), NK1.1^+ TCRβ^+ cells, CD1d tetramer^+ TCRβ^+ cells (NKT cells), NK1.1^+ TCRβ^+ (conventional T cells), and NK1.1^+ TCRβ^+ cells between naive and B16-bearing mice were not significantly different (Fig. 1A). In the culture of splenocytes from naive mice, the NK1.1^+ TCRβ^+ population expanded well by day 7 (Fig. 1B). In contrast, this expansion of NK1.1^+ TCRβ^+ cells in B16-bearing or 3LL Lewis lung cancer–bearing mice was significantly lowered (Fig. 1B). Thus, the α-GalCer-induced cell expansion of NK1.1^+ TCRβ^+ population is impaired in cancer-bearing mice.

Next, we examined α-GalCer-induced cytokine production in cancer-bearing mice. On α-GalCer injection, the levels of both IFN-γ and IL-4 in the sera of B16-bearing mice were significantly lower than those in naive mice (Fig. 1C, top). When splenocytes from cancer-bearing mice were stimulated with α-GalCer in vitro, reduced level of both IFN-γ and IL-4 production in the supernatants was observed compared with those from naive mice (Fig. 1C, bottom). These results indicate that the cytokine response to α-GalCer is suppressed in cancer-bearing mice.

**Decreased cytotoxic and antimetastatic activity induced by α-GalCer in cancer-bearing mice.** Next, we examined whether the α-GalCer-induced cytotoxic activity in the spleens differs between naive and cancer-bearing mice. Spleen cells obtained from naive mice that had been injected with α-GalCer showed significant cytotoxicity against both YAC-1 and B16 cells (Fig. 2A). However, when B16-bearing mice were injected with α-GalCer, the cytotoxicity induced in the spleens was significantly reduced to both targets. These results indicate that α-GalCer-induced cytotoxicity in the spleen is impaired in the cancer-bearing state. This α-GalCer-induced cytotoxicity is mainly mediated by NK cells activated by NKT cells (13). Judged by the data in Fig. 1A, the population of NK cells in cancer-bearing mice was reduced than that in naive mice (the mean reduction rate was ~60%), although not significantly different. However, the reduction rate of α-GalCer-induced cytotoxicity was apparently ~60% especially for B16 target. Therefore, it is unlikely that the impaired cytotoxicity in the cancer-bearing mice was only due to the reduction of NK cell population.

We next evaluated the antimetastatic effect of α-GalCer in cancer-bearing status. In naive mice that had been i.v. injected with 3LL cells, treatment with α-GalCer effectively inhibited the formation of lung metastasis (Fig. 2B, left). In contrast, in cancer (3LL)-bearing mice, α-GalCer did not efficiently prevent the lung metastasis (Fig. 2B, left), indicating that antimetastatic effect of α-GalCer is impaired in cancer-bearing status. When B16 cells were i.v. injected into B16-bearing mice, similar results were obtained (Fig. 2B, right), suggesting that the immunocompromised status of cancer-bearing mice is not tumor cell specific.

**Effect of CD11b^+ Gr-1^+ cells on α-GalCer-induced immune responses.** Several reports have indicated that CD11b^+ Gr-1^+ cells accumulate in tumor-bearing hosts (both in mice and humans) and contribute to an immunosuppressive state (9, 10). Therefore, we focused on the role of CD11b^+ Gr-1^+ cells in the hypersensitivity to α-GalCer in cancer-bearing mice by using B16 tumor model. The proportion and absolute number of CD11b^+ Gr-1^+ cells were increased in B16-bearing mice (Fig. 3A and B). CD11b^+ and Gr-1^+ cells were separately isolated from naive and cancer-bearing mice and then added to freshly isolated naive splenocytes cultured with α-GalCer. Seventy-two hours later, IL-4 concentration in the supernatants was determined. As production of IL-4 is more specific for NKT cells than IFN-γ (14), CD11b^+ cells derived from naive mice did not significantly change the level of IL-4 production, whereas those from cancer-bearing mice reduced IL-4 production. For Gr-1^+ cell addition, similar results were obtained (Fig. 3C, right). Thus, the CD11b^+ Gr-1^+ population seemed to contribute to the hyporesponsiveness of NKT cells to α-GalCer in cancer-bearing mice. To examine a contribution of TGF-β in this suppression, we added a blocking anti-TGF-β mAb in some cultures. However, it did not cancel the suppression (Fig. 3C, white column).

**NO-mediated suppression by CD11b^+ Gr-1^+ cells.** Because some studies reported that CD11b^+ Gr-1^+ cells induced by tumors suppress T cell responses by their NO production (15), we then tested a possible role of NO in the α-GalCer hyporesponsiveness. We pretreated splenocytes with iNOS inhibitor (1-NNMA) and stimulated them with α-GalCer. There was no significant difference in IL-4 production between 1-NNMA-treated and 1-NNMA-unpretreated splenocytes.
splenocytes obtained from naive mice (Fig. 4A). However, the IL-4 production by splenocytes from cancer-bearing mice was almost completely restored by the pretreatment with L-NMMA (Fig. 4B).

To determine whether the NO-mediated hyporesponsiveness to \( \alpha \text{-GalCer} \) was due to the increased CD11b+ Gr-1+ cells, we pretreated isolated Gr-1+ cells with 1-NMMA and added them into the culture of \( \alpha \text{-GalCer} \)-stimulated naive splenocytes. This pretreatment of Gr-1+ cells derived from cancer-bearing mice significantly

![Figure 1. \( \alpha \text{-GalCer} \)-mediated cell expansion and cytokine production. A, proportion of NK1.1+ TCRh+ , NK1.1+ TCRd+, CD1d tetramer+ TCRh+ , NK1.1+ TCRd+, and NK1.1+ TCRh+ cells in the spleens of naive and cancer-bearing mice. Columns, mean of three mice per group; bars, SD. No significant differences were determined. B, expansion of NK1.1+ TCRh+ cells by \( \alpha \text{-GalCer} \). Splenocytes obtained from naive or cancer-bearing mice were cultured with \( \alpha \text{-GalCer} \) (100 ng/mL) in the presence of recombinant IL-2 in 24-well plates for the indicated duration. Fraction of NK1.1+ TCRh+ cells in the cultured cells were estimated by FACS. Absolute number of NK1.1+ TCRh+ cells. Points, mean of triplicate cultures; bars, SD. * \( P = 0.0062 \); ** \( P = 0.0071 \) versus naive. C, \( \alpha \text{-GalCer} \)-induced cytokine production. Top, naive and cancer-bearing mice were i.p. injected with \( \alpha \text{-GalCer} \) (2 \( \mu \)g), and serum samples were obtained at the indicated time points; bottom, splenocytes obtained from naive or B16-bearing mice were stimulated with \( \alpha \text{-GalCer} \) (100 ng/mL) for the indicated duration in vitro, and culture supernatants were collected. Concentrations of IFN-\( \gamma \) and IL-4 were measured by ELISA. Points, mean of five mice (top) or triplicate wells (bottom); bars, SD. Every experiment was repeated more than twice with similar results.

![Figure 2. \( \alpha \text{-GalCer} \)-mediated cytotoxicity and antimetastatic effect. A, naive and B16-bearing mice were i.p. injected with vehicle or \( \alpha \text{-GalCer} \) (2 \( \mu \)g/animal). Twenty-four hours later, splenocytes were isolated from naive (N) or cancer-bearing (C) mice and used as effector cells in cytotoxic assay. NK-sensitive YAC-1 (left) and B16 (right) cells were used as target cells. Columns, mean of triplicate wells; bars, SD. Representative data from three independent experiments. * \( P = 0.0012 \); ** \( P = 0.0085 \); *** \( P = 0.0014 \); **** \( P = 0.0021 \). B, antimetastatic effect of \( \alpha \text{-GalCer} \) in vivo. Naive and cancer (3LL or B16)-bearing mice were i.v. injected with 2 \( \times 10^7 \) corresponding cancer cells (left, 3LL; right, B16) via tail vein. \( \alpha \text{-GalCer} \) (2 \( \mu \)g/animal) or vehicle was i.p. administrated with 4-day interval. These mice were sacrificed at day 15, and metastatic nodules in the lungs were counted. Columns, mean of five mice in each group; bars, SD. N.D., not detected.
up-regulated the IL-4 production, whereas that of naive Gr-1+ cells did not (Fig. 4B). Therefore, CD11b+ Gr-1+ cells from cancer-bearing mice induce the hyporesponsiveness to α-GalCer in a NO-mediated fashion.

**Treatment of cancer-bearing mice with ATRA restores α-GalCer reactivity in vivo.** Finally, we injected ATRA, which has been shown to differentiate CD11b+ Gr-1+ cells to mature dendritic cells, macrophages, and granulocytes (16), into naive and cancer (B16)-bearing mice for 5 consecutive days. At day 6, the population of CD11b+ Gr-1+ cells and their suppressive activity on cytokine production were examined. The number of CD11b+ Gr-1+ cells in spleens of cancer-bearing mice was significantly reduced by the ATRA treatment, whereas that in naive mice was not significantly changed (Fig. 5A). Accordingly, this treatment restored the α-GalCer-induced IL-4 production from cancer-bearing mice (Fig. 5B), indicating that the ATRA treatment could reverse defective NKT cell response to α-GalCer in cancer-bearing mice. In fact, when the cancer-bearing mice were treated with ATRA, the α-GalCer-induced antimitastic activity was significantly restored, whereas ATRA treatment alone did not (Fig. 5C). These results indicate that suppression of CD11b+ Gr-1+ cells substantially reversed the hyporesponsiveness to α-GalCer in the cancer-bearing state.

**Discussion**

Our previous studies have indicated hyporesponsiveness of Vα24 NKT cells obtained from human cancer patients to α-GalCer stimulation (7, 8). In this study, we have found a similar hyporesponsiveness to α-GalCer in cancer-bearing mice. More importantly, we found that an increased population with markers of CD11b and Gr-1 in cancer-bearing mice is, at least in part, responsible for the hyporesponsiveness to α-GalCer through their NO production. This is consistent with our previous result that the T-cell-depleted fraction in peripheral blood mononuclear cells (containing myeloid cell fraction) is responsible for the hyporesponsiveness of Vα24 NKT cells of cancer patients (7). Because a similar increase of

![Figure 3](image-url) Increase of CD11b+ Gr-1+ cells in cancer-bearing mice and their suppressive activities on α-GalCer-induced cytokine production. A, splenocytes obtained from naive and B16-bearing mice were stained with FITC-conjugated anti-Gr-1 and PE-conjugated anti-CD11b mAb. Representative flow cytometric profiles from naive (left) and B16-bearing (right) mice. Inserted numbers, percentage of each fraction. B, absolute numbers of CD11b+ Gr-1−, CD11b− Gr-1−, and CD11b− Gr-1+ cells in the spleen. Columns, mean of five mice in each group; bars, SD. *, P = 0.032; **, P = 0.012. C, IL-4 production by α-GalCer-stimulated splenocytes cultured with or without CD11b+ (left) or Gr-1+ (right) cells derived from naive or B16-bearing mice. In some wells, blocking anti-TGF-β mAb (20 ng/mL) was added. The culture duration was 72 hours. The experiments were repeated twice with similar results. ***, P = 0.0086. ****, P = 0.0073.

![Figure 4](image-url) Restoration of α-GalCer reactivity by NO blocking. A, splenocytes obtained from naive or B16-bearing mice were pretreated with or without iNOS inhibitor (L-NMMA, 10 mmol/L) for 24 hours. After washing, those cells were stimulated with α-GalCer (100 ng/mL) for 72 hours in vitro. IL-4 concentration in the supernatants was measured. *, P = 0.012. B, freshly isolated splenocytes of naive mice were cultured with α-GalCer in the presence of L-NMMA-pretreated or L-NMMA-untreated Gr-1+ cells derived from naive or B16-bearing mice. **, P = 0.041. Representative results from three independent experiments.
Figure 5. Restoration of α-GalCer reactivity by treatment with ATRA. A, naive and B16-bearing mice received PBS or ATRA (10 mg/kg) once daily for 5 days. Numbers of CD11b+ Gr-1+ cells in the spleens at day 6 were estimated. Columns, mean of five mice; bars, SD. *, \( P = 0.05 \). B, IL-4 production of α-GalCer-stimulated (100 ng/mL) splenocytes from naive mice or PBS- or ATRA-treated B16-bearing mice. The experiments were repeated twice with similar results. **, \( P = 0.0047 \). C, restoration of antitumor activity by ATRA treatment. B16-bearing mice were treated with PBS or ATRA. Then, \( 2 \times 10^5 \) B16 cells were i.v. injected via tail vein. The mice were treated with or without α-GalCer, and lung metastasis was evaluated as described in Fig. 2B. Columns, mean of five mice in each group; bars, SD. ***, \( P = 0.043 \).

immature myeloid cells in cancer patients has been reported (9), our present findings seem to have relevance with the clinical situation. Whereas other reports have indicated NKT cell dysfunction in several diseases and animal models (17, 18), only a few reports have indicated such dysfunction in cancer. Previous murine studies dealing with cancer and NKT cells mostly used naive (healthy) mice at the timing of α-GalCer stimulation. Thus, the present study has significance for cancer immunotherapy.

Terabe et al. have reported that CD11b+ Gr-1+ cells, which are stimulated by IL-13 produced from non-Vα14-Jα281 CD1d-reactive T cells, induce suppression of tumor immunosurveillance of 15-12RM tumor through their TGF-β production (19, 20). Therefore, we have tested the possibility that TGF-β produced by CD11b+ Gr-1+ cells is involved in the hyporesponsiveness to α-GalCer in cancer-bearing mice by using a blocking anti-TGF-β mAb (Fig. 3C). However, this treatment did not restore the cytokine production by NKT cells, suggesting a little contribution of TGF-β in this hyporesponsiveness. Instead, we have identified the NO-mediated mechanism in this study (Figs. 4 and 5). Furthermore, another mechanism may also be involved. A previous report has shown that the immunosuppressive effect of the immature myeloid cells on T cells requires direct cell-cell contact (21). Therefore, it is conceivable that the immature myeloid cells may directly contact and affect NKT cells when presenting α-GalCer on their CD1d.

ATRA is one of the derivatives of vitamin A and is able to induce differentiation of immature myeloid cells to mature ones (22). This is consistent with previous observation that CD11b+ Gr-1+ immature myeloid cells accumulate in vitamin A-deficient mice (22). We here found that in vivo administration of ATRA restored cytokine production and antitumor capacity in cancer-bearing mice with α-GalCer stimulation (Fig. 5). Therefore, when considering a cancer immunotherapy using α-GalCer, it could be beneficial to combine it with another therapy, such as retinoids or other vitamin A derivatives, which could reduce the size of the immature myeloid cell population known to be increased in cancer patients (9).

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