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 naive 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.

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-Jο281 gene segments in mice and Vα24-Jο18 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 amounts 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). Therefore, manipulation of immune system with α-GalCer has a potential to become an effective tool in cancer immunotherapy. In fact, several clinical trials against cancer using α-GalCer have already been reported (5, 6). Considering its clinical applications, it seems important to examine the α-GalCer-induced immune responses in cancer-bearing hosts. Using clinical samples obtained from cancer patients, we have reported that responses of Vα24 NKT cells against α-GalCer to proliferate or produce cytokines were impaired (7, 8). These observations prompted us to investigate α-GalCer-induced immune responses in cancer-bearing mice and examine corresponding mechanisms. We report here that α-GalCer-induced cell expansion, cytokine production, and antimetastatic activity in cancer-bearing mice were impaired as seen in human samples. It has been reported that myeloid cells, which are characterized as CD11b⁺ Gr-1⁺ cells, were specifically increased in tumor-bearing patients and animals (9, 10). Because CD11b⁺ Gr-1⁺ cells are a heterogeneous population of myeloid cells that comprises immature macrophages, granulocytes, and dendritic cells, these cells have been called “immature myeloid cells” (9, 10). CD11b⁺ Gr-1⁺ cells were increased in the tumor-bearing mice we examined, and they repressed the α-GalCer reactivity in a nitric oxide (NO)-mediated fashion. Finally, we could restore the α-GalCer-induced cytokine production and antimetastatic capacity in cancer-bearing mice with administration of all-trans-retinoic acid (ATRA) that decreased the CD11b⁺ Gr-1⁺ cell population. These findings may be significant for establishing optimal anticancer immunotherapy in which α-GalCer treatment is involved.

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 (11). The following monoclonal antibodies (mAb) obtained from BD PharMingen (San Diego, CA) were used in this study: anti-TCRα 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 FACSCalibur using the CellQuest program.

Mice. Male C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan). To establish tumors in mice, 3 × 10⁵ cancer cells were injected s.c. in the mid-dorsal region. Tumors were allowed to grow for 3 to

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5 weeks without treatment. For metastasis experiments, mice were i.v. injected with $2 \times 10^5$ 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 ($1 \times 10^6$ 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 51Cr-release assay.

**Cytokine measurement.** For in vivo 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 $\times$ 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 were cultured with StatView software.

**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 cytokine 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$^+$ TCR$\beta^+$ population (12). Before culture (day 0), populations of NK1.1$^+$ TCR$\beta^+$ (NK cells), NK1.1$^+$ TCR$\beta^+$ cells, CD11d tetramer$^+$ TCR$\beta^+$ cells (NKT cells), NK1.1$^+$ TCR$\beta^+$ (conventional T cells), and NK1.1$^+$ TCR$\beta^+$ cells between naive and B16-bearing mice were not significantly different (Fig. 1A). In the culture of splenocytes from naive mice, the NK1.1$^+$ TCR$\beta^+$ population expanded well by day 7 (Fig. 1B). In contrast, this expansion of NK1.1$^+$ TCR$\beta^+$ 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$^+$ TCR$\beta^+$ 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 that than 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 hyporesponsiveness 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 (Fig. 3C, left). 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 (l-NMMA) and stimulated them with α-GalCer. There was no significant difference in IL-4 production between l-NMMA-treated and l-NMMA-untreated
spleenocytes obtained from naive mice (Fig. 4A). However, the IL-4 production by spleenocytes from cancer-bearing mice was almost completely restored by the pretreatment with L-NMMA (Fig. 4A).

To determine whether the NO-mediated hyporesponsiveness to α-GalCer was due to the increased CD11b+ Gr-1+ cells, we pretreated isolated Gr-1+ cells with L-NMMA and added them into the culture of α-GalCer-stimulated naive spleenocytes. This pretreatment of Gr-1+ cells derived from cancer-bearing mice significantly

Figure 1. α-GalCer-mediated cell expansion and cytokine production. A, proportion of NK1.1+ TCRγδ+ (A), CD1d tetramer+ TCRγδ+ (B), and NK1.1+ TCRγδ+ NK1.1+ TCRγδ+ 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+ TCRγδ+ cells by α-GalCer. Spleenocytes obtained from naive or cancer-bearing mice were cultured with α-GalCer (100 ng/mL) in the presence of recombinant IL-2 in 24-well plates for the indicated duration. Fraction of NK1.1+ TCRγδ+ cells in the cultured cells were estimated by FACS. Absolute number of NK1.1+ TCRγδ+ cells. Points, mean of triplicate cultures; bars, SD. *, P = 0.0062; **, P = 0.0071 versus naive. C, α-GalCer-induced cytokine production. Top, naive and cancer-bearing mice were i.p. injected with α-GalCer (2 μg) and serum samples were obtained at the indicated time points; bottom, splenocytes obtained from naive or B16-bearing mice were stimulated with α-GalCer (100 ng/mL) for the indicated duration in vitro, and culture supernatants were collected. Concentrations of IFN-γ 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. α-GalCer-mediated cytotoxicity and antimetastatic effect. A, naive and B16-bearing mice were i.p. injected with vehicle or α-GalCer (2 μg/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 α-GalCer in vivo. Naive and cancer (3LL or B16)-bearing mice were i.v. injected with 2 × 10^5 corresponding cancer cells (left, 3LL; right, B16) via tail vein. α-GalCer (2 μg/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 antimetastatic 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. Moreover, 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

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**Figure 3. 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+ cells measured by flow cytometry from naive (left) and B16-bearing (right) mice. 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 μg/mL) was added. The culture duration was 72 hours. The experiments were repeated twice with similar results.

**Figure 4. 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 × 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-VαVβ T cells in the immature myeloid cell population known to be increased in vitamin A–deficient mice or other vitamin A derivatives, which could reduce the size of immature myeloid cell population known to be increased in cancer patients (9).

conceivable that the immature myeloid cells may directly contact and affect NKT cells when presenting α-GalCer on their CD1d. A study examining this possibility is now under way.

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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