Relative contribution of NK and NKT cells to the anti-metastatic activities of IL-12

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Abstract

Conventional T cells, NK cells and NKT cells have been implicated in the anti-tumor activities induced by IL-12. Here we show that IL-12-induced immune responses are partially impaired in T and NKT cell-deficient RAG-2−/− mice, and in NKT cell-deficient CD1−/− mice. In response to a small dose (≤1000 U) of IL-12, RAG-2−/− and CD1−/− mice demonstrated reduced cytotoxicity, serum IFN-γ elevation and anti-metastatic activities; in contrast, in response to a high dose (>2000U) of IL-12, the IL-12-induced immune responses of RAG-2−/− and CD1−/− mice were indistinguishable from wild-type mice. The defective responses to low-dose IL-12 of RAG-2−/− mice were corrected by adoptive transfer of NKT cells but not NK cells. These findings indicate that both NK and NKT cells contribute to the anti-metastatic responses induced by IL-12, and that NKT cells are mostly responsible for the low-dose activities of this cytokine.

Introduction

IL-12, which was originally identified as a stimulator of NK cell activity and a maturation factor for cytotoxic T lymphocytes, plays an important role in the protection against microbial infections and the elimination of tumors (1,2). IL-12 augments the cytotoxic activity of T cells, NK cells and NKT cells against a variety of target cells, and induces the production of IFN-γ by these cells (1–6). In turn, IFN-γ activates macrophages to eliminate microbial agents and tumors, and exerts additional anti-tumor activities by inhibiting angiogenesis (1,2,6). It remains controversial, however, which lymphocyte population is most critically important in mediating the anti-tumor activities of IL-12. Several studies suggested that NKT cells were the major effector cells for IL-12-induced inhibition of experimental tumor metastasis (5,7,8). On the other hand, we also observed that NK cells were sufficient for rejection of tumors by IL-12 (9). To elucidate the relative contribution of NKT cells and NK cells to IL-12-induced immune responses, we analyzed the IL-12-induced cytotoxicity, serum IFN-γ elevation and anti-metastatic activities in RAG-2−/− mice, which lack conventional T cells and NKT cells, and in CD1−/− mice that only lack NKT cells. Our present results indicate that the relative contribution of NKT and NK cells varies depending on the dose of IL-12 administered and that NKT cells are more sensitive to low-doses of IL-12 in vivo.

Methods

Mice

Male C57BL/6 (B6) mice, 6 weeks of age, were purchased from Clea Japan (Tokyo, Japan). B6 RAG-2−/− mice were bred in the Central Institute for Experimental Animals (Kawasaki, Japan). CD1-deficient mice generated by targeted disruption of the CD1d gene have been described (10). Homozygous (−/−) or control (+/+)(B6×129/Sv)F₃ male mice were used at 8 weeks of age. All mice were maintained under specific pathogen-free conditions.
**IL-12**

Recombinant murine IL-12 (4.9×10^6 U/mg) was kindly provided by Genetics Institute (Andover, MA). The preparation was diluted serially from 2000 U (400 ng)/200 μl to 250 U (50 ng)/200 μl in PBS immediately before use.

**Experimental lung metastasis model**

B16-BL6 melanoma cells (10^5), which were MHC class I-as estimated by flow cytometry, were injected through the tail vein. Mice were sacrificed 14 days later and metastatic black nodules on the lung surface were counted macroscopically as previously described (5,7).

**Mononuclear cell (MNC) preparation and adoptive transfer**

Hepatic MNC and splenic MNC were prepared as previously described (5,7). NK cells and NKT cells were isolated from naive B6 hepatic and splenic MNC by the panning method. Briefly, B cells were removed by adherence on plates coated with affinity-purified goat anti-mouse IgG + IgM antibody (Caltag, San Francisco, CA). NK1.1^+^ cells, including both NK and NKT cells, were positively selected by adherence on anti-NK1.1 mAb (PharMingen, San Diego, CA)-coated plates. Then, NK1.1^+^CD3^+^CD5^+^ NKT cells were positively separated from NK1.1^+^CD3^+^CD5^-^ NK cells by adherence on anti-CD5 mAb (PharMingen)-coated plates. Purity of the respective cell fractions was verified by two-color flow cytometry analysis on a FACScan (Becton Dickinson, San Jose, CA) using FITC-conjugated anti-mouse CD3e (145-2C11) and phycoerythrin-conjugated anti-CD122 mAb (PharMingen). The NKT cell fraction contained >90% CD3^+^CD122^+^ NKT cells and <5% CD3^+^CD122^-^ NK cells. The NK cell fraction contained >90% CD3^+^CD122^-^ NK cells and ~5% CD3^-^CD122^+^ NKT cells.

**Cytotoxic assay**

Cytolytic activity was assessed against B16-BL6 (NK-resistant) and YAC-1 (NK-susceptible) target cells by a standard 51Cr-release assay as previously described (5,7).

**ELISA**

Serum IFN-γ levels were evaluated using a specific ELISA kit for mouse IFN-γ (Amersham, Little Chalfont, UK).

**Statistical analysis**

Statistical analysis of the number of metastatic nodules and serum IFN-γ in each group was performed by two-sample t-test.

**Results**

**IL-12-induced immune responses in RAG-2^-/-^ mice**

We recently demonstrated that IL-12 can induce cytotoxic activity of splenic and hepatic MNC, serum IFN-γ elevation, and anti-metastatic effect in RAG-2^-/-^ mice (9). RAG-2^-/-^ mice lack NKT cells that have been reported to play a critical role in IL-12-induced immune responses (5,7,8,11). To further investigate the responsiveness of RAG-2^-/-^ mice to IL-12, we administered various amounts of IL-12 to RAG-2^-/-^ mice, in which only NK cells can be IL-12-responsive effector cells (9), or normal B6 mice, in which both NKT and NK cells can be IL-12-responsive effector cells (5,7), and compared their IL-12-induced immune responses. Hepatic and splenic MNC of RAG-2^-/-^ mice exhibited reduced cytotoxicity against B16 tumor cells as compared to those of normal mice when low doses (500 or 1000 U) of IL-12 were administered (Fig. 1A and B). In contrast, as we reported previously (9), a high-dose of IL-12 (2000 U) induced comparable cytotoxicity. Serum IFN-γ elevation was also partially impaired in RAG-2^-/-^ mice when a low dose (500 U) of IL-12 was administered but not when a high dose (2000 U) was administered (Fig. 1B). Moreover, administration of a low dose of IL-12 (total of 1000 U) over a period of 3 days after tumor injection induced reduced anti-metastatic activities against B16 melanoma cells in RAG-2^-/-^ mice as compared with normal mice; while a high dose (total of 3000 U) induced comparable anti-metastatic activities in these mice (Fig. 1D). These results indicated that RAG-2^-/-^ mice, which have normal or perhaps increased numbers of NK cells but no conventional T cells and NKT cells (12), have normal immune responses induced by a high dose of IL-12, but partially impaired activities induced by a low dose of IL-12.

**IL-12-induced immune responses in CD1^-/-^ mice**

To substantiate the contribution of NKT cells to IL-12-induced immune responses, we injected various doses of IL-12 into CD1^-/-^ mice that selectively lack NKT cells, but with normal numbers of NK cells and conventional T cells as possible IL-12 responsive effector cells (10,11,13,14). As shown in Fig. 2A and B, IL-12-induced cytotoxic activities of hepatic MNC against NK-resistant B16 and NK-susceptible YAC-1 cells were significantly reduced in CD1^-/-^ mice when a low dose (<1000 U) of IL-12 was administered, whereas a high dose (2000 U) of IL-12 induced comparable cytotoxicity in CD1^-/-^ and CD1^-/-^ mice. Similar results were obtained with splenic MNC (data not shown). IL-12-induced serum IFN-γ levels were also reduced in CD1^-/-^ mice when a low dose of IL-12 was administered (Fig. 2C). Moreover, anti-metastatic activities against the experimental lung metastasis of B16 were reduced in CD1^-/-^ mice when a low amount (<2000 U) of IL-12 was injected, while a high amount (3000 U) of IL-12 induced comparable anti-metastatic activities in CD1^-/-^ and CD1^-/-^ mice (Fig. 2D). These results suggested a substantial contribution of NKT cells to low-dose IL-12-induced immune responses in vivo.

**Adoptive transfer of NKT cells restores the low-dose IL-12 defect**

To provide further evidence for the contribution of NKT cells to IL-12-induced immune responses, we tested whether the diminished low-dose IL-12 response of RAG-2^-/-^ mice could be restored by adoptive transfer of NKT cells. NKT cells (2×10^6) or NK cells (2×10^5), isolated from splenic and hepatic MNC of normal B6 mice, were i.v. injected into RAG-2^-/-^ mice prior to administration of a low-dose IL-12. As represented in Fig. 3A and B, the adoptive transfer of NKT cells significantly enhanced the IL-12-induced cytotoxicity of splenic and hepatic MNC against B16 cells, while transfer of NK cells did not. IL-12-induced serum IFN-γ levels were more markedly increased by the adoptive transfer of NKT cells than NK cells (Fig. 3C). Moreover, the adoptive transfer of NKT cells substantially aug-
Contribution of NKT cells to IL-12-induced responses

Fig. 1. RAG-2–/– mice have a partial defect in IL-12-induced immune responses. (A and B) Reduced IL-12-induced cytotoxicity. Normal B6 mice or RAG-2–/– mice were i.p. injected with PBS or the indicated amount of IL-12, and splenic and hepatic MNC were prepared 24 h later. Cytotoxic activity of splenic MNC (A) and hepatic MNC (B) was tested against B16 tumor cells at an E:T ratio of 50. Data shown are representative of three independent experiments with similar results. (C) Impairment in IL-12-induced serum IFN-γ elevation. Serum samples were obtained from normal B6 mice or RAG-2–/– mice 24 h after i.p. injection of the indicated doses of IL-12. Data are shown as the mean ± SD of three mice in each group. Serum IFN-γ in the PBS-injected mice was not detectable (data not shown). (D) Impairment of IL-12-induced anti-metastatic activities. Normal B6 or RAG-2–/– mice were i.v. inoculated with 10^5 B16 cells on day 0, and then i.p. injected with PBS or the indicated total amount of IL-12 on days 0, 1 and 2. All mice were sacrificed on day 14 and metastatic nodules on the lung surface were counted. Data are represented as mean ± SD of five mice in each group. Similar results were obtained in three independent experiments.* P < 0.01.

Discussion

In this study, we demonstrated that IL-12-induced cytotoxicity, serum IFN-γ elevation and anti-metastatic activities were impaired in NKT cell-deficient CD1–/– and RAG-2–/– mice when a low dose of IL-12 was administered. Moreover, adoptive transfer of NKT cells but not NK cells could significantly restore the IL-12 responsiveness of RAG-2–/– mice. These results suggested that NKT cells, as compared with NK cells, are more preferentially responsive to limited amounts of IL-12 in vivo.

NKT cells constitute a unique subset among mature CD4+ and CD8+ T lymphocytes, expressing an invariant TCR α chain composed of Vα14 rearranged to Jα281 and a semi-invariant TCR β chain (Vβ8.2, Vβ7 and Vβ2) (15–17). Although NKT cells were suggested to favor the establishment of a Th2-type immune response through their ability to quickly secrete IL-4 (18), it has been shown that these cells are not absolutely required for the induction of Th2 immune responses (10,13,14). On the other hand, it has been suggested that NKT cells represent the predominant lymphocyte population that responds to IL-12 and that mediates the anti-metastatic effects of this cytokine (5,7,8,11). However, we recently noted that NK cells, by themselves, were able to mediate IL-12-induced cytotoxicity, serum IFN-γ production and anti-metastatic activities in RAG-2–/– mice when a high-dose of IL-12 was administered (9), suggesting that NKT cells are not absolutely necessary for IL-12-induced immune responses. In the present study we clearly demonstrated that NKT cells are more responsive to IL-12 than NK...
Contribution of NKT cells to IL-12-induced responses

Fig. 2. CD1<sup>+/+</sup> mice have a partial defect in IL-12-induced immune responses. (A and B) Reduced IL-12-induced cytotoxicity. CD1<sup>+/+</sup> or CD1<sup>−/−</sup> mice were i.p. injected with PBS or the indicated amount of IL-12 and hepatic MNC were prepared 24 h later. Cytotoxic activity against B16 (A) and YAC-1 (B) tumor cells was tested at an E:T ratio of 50. Data shown are representative of three independent experiments with similar results. Similar results were obtained with splenic MNC (data not shown). (C) Impairment in IL-12-induced serum IFN-γ elevation. Serum samples were obtained from CD1<sup>+/+</sup> or CD1<sup>−/−</sup> mice 24 h after i.p. injection of the indicated dose of IL-12. Data are shown as the mean ± SD of three mice in each group. Serum IFN-γ in the PBS-injected mice was not detectable (data not shown). (D) Impairment in IL-12-induced anti-metastatic activities. CD1<sup>+/+</sup> or CD1<sup>−/−</sup> mice were i.v. inoculated with 10<sup>5</sup> B16 cells on day 0, and then i.p. injected with PBS or the indicated total amount of IL-12 on days 0, 1 and 2. All mice were sacrificed on day 14 and metastatic nodules on the lung surface were counted. Data are represented as mean ± SD of five mice in each group. Similar results were obtained in three independent experiments.*P < 0.01.

cells: NKT cell-deficient RAG-2<sup>−/−</sup> and CD1<sup>−/−</sup> mice were impaired in low-dose but not high-dose IL-12 responses. These findings are consistent with our previous observation that NKT cells expressed higher levels of IL-12 receptors than NK cells (11). Therefore, when a suboptimal dose of IL-12 is administered to NKT cell-deficient mice such as the RAG-2<sup>−/−</sup> and CD1<sup>−/−</sup> mice studied here, or the J<sub>α</sub>281<sup>−/−</sup> mice (8), profound defects in tumor cell rejection may be observed.

Recently, α-galactosylceramide (α-GalCer) presented by CD1d has been identified as a specific ligand for V<sub>α</sub>14 TCR on NKT cells (19). Moreover, it was reported that administration of α-GalCer stimulated NKT cells to produce large amounts of IFN-γ and develop cytotoxic activity, which resulted in potent anti-tumor responses (20). We recently demonstrated that IL-12 produced by dendritic cells plays a critical role in the activation of NKT cells by α-GalCer (21). Therefore, IL-12 may be a critical component of NKT cell activation when these cells respond to their specific ligand(s) in physiological situations.

It should be noted that we investigated here anti-metastatic activities in an experimental model for tumor metastasis as a biological function of IL-12. In this model, NKT cells and/or NK cells are fully responsible for IL-12-induced anti-metastatic activities, since depletion of NK<sub>1</sub><sup>−</sup> cells in normal mice completely abolished these activities (22). In contrast, in models of IL-12-induced solid tumor regression, critical contributions of T cells and IFN-γ-mediated anti-angiogenesis have been demonstrated (3,4,6). A critical role for NKT cells in the production of IFN-γ (23,24) and the inhibition of solid tumor growth (8) in response to IL-12 was also demonstrated. Thus, NKT cells may also play a role in inducing and modulating the anti-tumor activities by conventional T cells. Further studies are needed to address this possibility.
Contribution of NKT cells to IL-12-induced responses

Fig. 3. Adoptive transfer of NKT cells enhances IL-12-induced immune responses in RAG-2−/− mice. (A and B) Enhancement of IL-12-induced cytotoxicity. RAG-2−/− mice were i.p. injected with PBS or IL-12 (1000 U), and splenic and hepatic MNC were prepared 24 h later. Some groups of mice were i.v. injected with 2×10^6 NK or NKT cells prior to injection of PBS or IL-12. Cytotoxic activities of splenic MNC (A) and hepatic MNC (B) were tested against B16 tumor cells at the indicated E:T ratios. Data shown are representative of three independent experiments with similar results. (C) Enhancement of IL-12-induced serum IFN-γ elevation. Serum samples were obtained from RAG-2−/− mice with or without adoptive transfer of NK or NKT cells 24 h after i.p. injection of IL-12 (1000 U). Data are shown as the mean ± SD of three mice in each group. Serum IFN-γ in the PBS-injected mice was not detectable (not shown). (D) Enhancement of IL-12-induced anti-metastatic activities. RAG-2−/− mice with or without adoptive transfer of NK or NKT cells were i.v. inoculated with 10^5 B16 cells on day 0 and then i.p. injected with PBS or 300 U of IL-12 daily from day 0 to day 2. All mice were sacrificed on day 14 and metastatic nodules on the lung surface were counted. Data are represented as mean ± SD of five mice in each group. Similar results were obtained in three independent experiments. *P < 0.05; **P < 0.01.

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Abbreviations

α-GalCer  α-galactosylceramide
MNC  mononuclear cells

References


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