Combination treatment with IL-2 and anti-IL-2 mAbs reduces tumor metastasis via NK cell activation

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Abstract

Combination treatment consisting of IL-2 together with anti-IL-2 mAbs results in markedly larger increases in the numbers of CD8⁺ T cells, dendritic cells (DCs) and NK cells in vivo compared with the results observed with injections of IL-2 or the antibodies alone. We previously showed that this combination treatment overcomes the problems associated with the short half-life of IL-2 in vivo. Importantly, the combination treatment but not IL-2 or the anti-IL-2 mAbs alone protected the mice against tumor metastases in the lungs. Here we have investigated which cell types are responsible for this protective immunity against tumors. We analyzed tumor metastases in mice that were depleted of DCs, CD8⁺ T cells or NK cells. DC-deficient, diphtheria toxin receptor-expressing mice as well as B cell- and T cell-deficient RAG-2-knockout mice were protected against tumors after they were administered the combination treatment. On the other hand, mice that were depleted of NK cells using anti-asialo-GM1 antibodies did not exhibit the anti-tumor activity after treatment with IL-2 combined with anti-IL-2 mAbs. Thus, these data demonstrate that NK cells, but not DCs, or CD8⁺ T cells mediate the anti-tumor effect induced by this combination treatment. Therefore, combining neutralizing anti-IL-2 mAbs with IL-2 may be clinically useful to effectively enhance IL-2-mediated NK cell activities.

Introduction

IL-2—a cytokine that is primarily produced by activated T cells or a subset of CD4⁺ T cells under normal conditions—is a potent growth factor for immune cells, including T cells and NK cells in vitro (1, 2). Because treatment with exogenous IL-2 produces immunostimulatory effects in animals and humans, IL-2 immunotherapy has been repeatedly examined for potential clinical applications (1, 3–5). The half-life of IL-2 in vivo, however, has been reported to be rather short followed by induction of unwanted side effects if we get immunostimulatory effects by high-dose treatment of IL-2, which has prompted a number of studies that have tried to enhance the efficacy of IL-2-mediated T cell and NK cell stimulation in vivo (4, 6). On the other hand, other studies have suggested that endogenous IL-2 may have immunosuppressive and autoimmunity-repressing activities in vivo, as demonstrated by the autoimmune diseases observed in IL-2- and IL-2 receptor-deficient mice (7, 8).

Using S4B6 anti-IL-2 mAbs, we previously found that the number of CD8⁺ memory T cells significantly increased after IL-2 depletion in vivo (9, 10). The increased level of cell divisions was associated with a reduction in the number of CD25⁺CD4⁺ regulatory T cells (11), was IL-15 independent and required CD122 signaling in CD8⁺ memory T cells (10). This dependence on CD122 in IL-2-depleted IL-15-knockout (KO) mice led us to hypothesize that an unknown CD122-targeted cytokine mediates the increased proliferation of CD8⁺ memory T cells (10). Our group as well as Boyman et al. recently noticed that S4B6 anti-IL-2 mAbs appeared to enhance the biological activities of IL-2 in vivo (12, 13). Indeed, only five-time injections of mice with anti-IL-2 antibodies increased the number of memory CD8⁺ T cells for >200 days after the treatment (12). These studies also demonstrated that the S4B6 mAbs potentiated the effects of IL-2 in vivo, when IL-2 was present (12, 13). Importantly, we found that combination treatment with anti-IL-2 mAbs in addition to IL-2 elicited effective anti-tumor immunity in mice (12). Additionally, Kamimura and Bevan (14) also showed that functional CD8⁺ memory T cell responses increased after treatment with IL-2 plus anti-IL-2 antibodies. Therefore, combination treatment consisting of IL-2 and anti-IL-2 mAbs...
may be able to overcome the short half-life of IL-2 in clinical applications (15).

Although significant increases in the numbers of cells believed to function in tumor immunosurveillance, including NK.11.1$^{+}$$^{+}$TCR$^{+}$ classical NK cells and NK.1.1$^{+}$CD44$^{+}$CD8$^{+}$ T cells, were observed after the combination treatment (16, 17), the specific cell populations that were critical for the resulting anti-tumor immunity in the treated mice were unclear. Therefore, as a first step for the clinical application of this combination treatment, we attempted to identify the cell populations that mediated the anti-tumor effect of the combination treatment. Here, we demonstrate that populations of not only NK cells and CD8$^{+}$ memory T cells but also CD11c$^{+}$CD11b$^{+}$ myeloid-type dendritic cells (DCs) increased in response to the combination treatment in vivo. Importantly, we clearly show that the anti-tumor effect induced by the combination treatment was dependent on NK cells. Thus, our results suggest that treatment with IL-2 plus anti-IL-2 mAbs is a promising method to enhance anti-tumor immunity in vivo, particularly against MHC class I$^{+}$ target cells via an activation of NK cells.

Materials and methods

Mice

C57BL/6J (B6) mice were purchased from Japan SLC (Hamamatsu, Japan). B6.SJL (CD45.1) mice were obtained from Taconic (Germantown, NY, USA). RAG-2-KO mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). B6.CD11c-diphtheria toxin receptor (DTR) mice were kindly provided by D. Littman (New York University). All mouse strains were kept under specific pathogen-free conditions in the animal facility of the Osaka University Medical School. All animal experiments were performed following the guidelines of the Institutional Animal Care and Use Committees in Graduate School of Frontier Bioscience and Graduate School of Medicine, Osaka University.

Bone marrow chimeras

Because administration of diphtheria toxin (DT) causes lethal body irradiation, and the mice were exposed to a single lethal dose of 950 rad total body irradiation, and the mice were reconstituted with 2$^{10^6}$ donor bone marrow cells from CD11c-DTR transgenic mice via tail vein injections. The mice were allowed to rest for 8 weeks before they were used in experiments.

Reagents and mAbs for flow cytometry

The mAbs used for flow cytometry were as follows: FITC- or PE-labeled anti-CD4 mAb (GK1.5), PE-labeled or PE-carbocyanin 5 (Cy5)-labeled anti-CD8 mAb (53-6.7), PE-Cy5-labeled anti-CD11b (M1/70), allophycocyanin (APC)-labeled anti-CD11c mAb (N418), FITC-labeled or biotinylated anti-CD19 mAb (MB19-1), PE-labeled anti-CD25 (PC61.5), PE-Cy5- or APC-labeled anti-CD44 mAb (IM7), biotinylated anti-CD45.1 mAb (A20), PE- or APC-labeled anti-CD45R (B220) mAb (RA3-6B2), FITC- or PE-Cy5-labeled anti-Vbeta TCR mAb (H57-597), FITC-labeled anti-NK1.1 mAb (PK136), biotinylated anti-mouse pan-NK cell mAb (DX5), biotinylated antimouse KLRG1 mAb (2F1) and PE-labeled anti-IFN-γ (XM1.2). PE-Cy5-conjugated streptavidin was used to detect the biotinylated mAbs. These mAbs and streptavidin reagents were obtained from BD Biosciences (Tokyo, Japan), eBioscience (San Diego, CA, USA) or BioLegend (San Diego, CA, USA). For staining of CD11c$^{+}$ DCs, we put a dump staining, anti-TCR, anti-CD10 and anti-NK1.1 antibodies, to discriminate a possibility to count activated T cells and NK cells, which were known as CD11c$^{+}$. For staining of NK.1.1$^{+}$ NK cells, we put a dump staining, anti-TCR and anti-CD19 antibodies, and detected NK cells as CD11c$^{+}$. For staining of CD4$^{+}$ and CD8$^{+}$ T cells, we put a dump staining; anti-CD19 and anti-MHC class II antibodies.

Intracellular staining

After staining for surface, cells were fixed and permeabilized with fixation/permeabilization buffer (BD Biosciences) at 4°C for 30 min. After washing once with perm/wash buffer (BD Biosciences), cells were incubated with PE-labeled anti-granzyme B (eBioOMAK-D, eBioscience) or PE-labeled anti-IFN-γ (XM1.2). PE-Cy5-conjugated streptavidin was used to detect the biotinylated mAbs. These mAbs and streptavidin reagents were obtained from BD Biosciences (Tokyo, Japan), eBioscience (San Diego, CA, USA) or BioLegend (San Diego, CA, USA). For staining of CD11c$^{+}$ DCs, we put a dump staining, anti-TCR, anti-CD10 and anti-NK1.1 antibodies, to discriminate a possibility to count activated T cells and NK cells, which were known as CD11c$^{+}$. For staining of NK.1.1$^{+}$ NK cells, we put a dump staining, anti-TCR and anti-CD19 antibodies, and detected NK cells as CD11c$^{+}$. For staining of CD4$^{+}$ and CD8$^{+}$ T cells, we put a dump staining; anti-CD19 and anti-MHC class II antibodies.

Preparation of anti-IL-2 mAb

Preparation of the protein G-purified anti-IL-2 mAb (S4B6) was performed as described previously (12).

Preparation of the anti-IL-2 mAb/recombinant IL-2 mixture

The mixtures of anti-IL-2 mAbs and recombinant IL-2 were prepared as described previously (12). Briefly, 1 mg of anti-IL-2 mAbs or rat IgG and 2 μg of recombinant mouse IL-2 (rmIL-2; PeproTech, Rocky Hill, NJ, USA) were mixed in vitro and incubated overnight at 4°C with rotation. The resulting mixture was intra-peritoneally (i.p.) injected into wild-type or bone marrow chimeras of DTR transgenic or RAG-2-KO mice.
Depletion of NK cells and DCs

We depleted NK cells in vivo essentially as previously described (19). Mice received an intravenous (i.v.) injection of anti-asialo-GM1 antibodies (20 μl per mouse; Wako Pure Chemical, Osaka, Japan) every fifth day. For depletion of DCs, CD11c-DTR chimera mice were i.p. injected with 4 ng of DT per gram body weight every other day. The DT injections were continued until the animals were sacrificed. DT was obtained from Funakoshi (Tokyo, Japan).

A model of B16 cell-derived lung metastases

B16 melanoma cells were kindly provided by Shin-ichiro Fujii (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). B16 cells (2.5 × 10⁵ cells per mouse) were i.v. injected on day 0. Tumor-bearing mice received a mixture of rmIL-2 (2 μg per mouse) and anti-IL-2 mAbs (1 mg per mouse) or rat IgG (1 mg per mouse) by i.p. injection on day 2. Sometimes the tumor-bearing mice received an i.v. injection of an IL-2 expression plasmid (6.25 μg per mouse) with or without an i.p. injection of anti-IL-2 mAbs (1 mg per mouse) on day 2. The number of B16 nodules in the lungs was counted on day 14.

Statistical analysis

Student's t-tests were used to assess the significance of differences between two groups.

Results

Populations of DCs as well as CD8⁺ T cells and NK cells increased after combination treatment with IL-2 plus anti-IL-2 mAbs in vivo

We previously reported that in vivo treatment with IL-2 together with anti-IL-2 mAbs increased the numbers of NK1.1high NK cells and CD8⁺ memory T cells (12). To test the potential effects of this treatment on other immune cells, we examined cell populations in the spleen 5 days after administration of the combination treatment. After treatment with IL-2 and anti-IL-2 mAbs in vivo, we confirmed that the splenic cell percentages of NK1.1high NK cells and CD8⁺ T cells increased, as was reported previously (12). We also found that the percentage of splenic DCs increased after the treatment (data not shown). The total number of splenocytes also increased by >2-fold, and the actual numbers of the NK cell, CD8⁺ T cell and CD11c⁺ DC populations increased significantly (Fig. 1A). We performed the kinetic analysis to investigate the cell accumulation in spleen after the combination treatment in detail. We showed that IL-2 plus anti-IL-2 antibody treatment first increased CD8⁺ T cells followed by NK cells and CD11c⁺ DCs (Fig. 1B). We investigated percentages and numbers of matured KLRG1⁺ NK cells and immatured KLRG1⁻ NK cells (20) after the combination treatment. Both percentages and numbers of whole NK cells, matured KLRG1⁺ NK cells and immatured KLRG1⁻ NK cells peaked at day 5 after IL-2 plus anti-IL-2 mAb stimulation (Fig. 1C and D and data not shown), indicating that the combination treatment induced maturation of NK cells. Moreover, we showed that the protein level of granzyme B and perforin expression increased in NK cells after the combination treatment, suggesting that the combination treatment induced not only maturation of NK cells but also their activation (Fig. 1E). Consistent with these, it was reported that the NK cells are more cytotoxic after IL-2 plus anti-IL-2 mAb stimulation (21). We hypothesized that at least one of these three populations, DCs, CD8⁺ T cells and NK cells, induced the anti-tumor effect observed following combination treatment.

The combination treatment-mediated anti-tumor effect was not dependent on DCs in vivo

We first examined the DC population, because DCs, and in particular activated DCs, enhance immune responses via the activation of T cells (22) and NK cells (23, 24). We employed CD11c-DTR transgenic mice that were depleted of DCs using injections of DT (25) in the presence or absence of IL-2-anti-IL-2 mAb combination treatment. We confirmed that the number of DCs was markedly reduced following injections of DT as described previously (25) (Fig. 2A). We, however, observed that the DC-depleted mice showed an enhanced suppression of tumor metastases after they were treated with IL-2 and anti-IL-2 mAbs (Fig. 2B). These results demonstrated that the combination treatment-mediated anti-tumor effect was not dependent on DCs in vivo.

The IL-2-anti-IL-2 mAb-mediated anti-tumor effect was not dependent on CD8⁺ T cells in vivo

We next employed RAG-2-KO mice to investigate whether CD8⁺ T cells or CD4⁺ T cells contributed to the IL-2-anti-IL-2 mAb treatment-mediated anti-tumor effect in mice. We found that the combination treatment decreased the numbers of B16 nodules in the lungs of RAG-2-KO mice (Fig. 3). These results demonstrated that the anti-tumor effect mediated by IL-2 and anti-IL-2 mAbs was induced even without CD8⁺ T cells or CD4⁺ T cells in vivo, although we did not completely exclude a possibility that T cells also contribute to the anti-tumor responses enhanced by this combination therapy.

The IL-2-anti-IL-2 mAb treatment-mediated anti-tumor effect was dependent on NK cells in vivo

We noticed that the combination treatment, the efficacy of which was monitored by the number of nodules in the lungs, was significantly more effective in the RAG-2-KO mice compared with the results observed in wild-type control mice (Figs 2 and 3). Because it has been reported that NK cell activity is enhanced in RAG-2-KO mice (26) and NK cells play a role for B16 tumor elimination in vivo (27), we hypothesized that NK cells were critical for the induction of the anti-tumor effect after the IL-2-anti-IL-2 mAb treatment. Therefore, we analyzed the phenotypes of the NK cells after the combination treatment in detail. We have previously reported that CD69 expression increases on NK cells after treatment with IL-2 and anti-IL-2 mAbs (12). Moreover, we found that the combination treatment caused NK cells to express more CD25 and IFN-γ than was observed in control cells (Fig. 4A). These results suggested that NK cells are critical for the anti-tumor activity induced by treatment with IL-2 and anti-IL-2 mAbs in vivo.
To definitively determine whether or not NK cells contributed to the combination treatment-mediated anti-tumor effect in mice, we prepared anti-asialo-GM1 antibodies, which can be used to efficiently deplete mice of NK cells as described previously (19). We confirmed that the anti-asialo-GM1 antibodies dramatically reduced the number of NK cells in vivo (data not shown). Furthermore, we found that treatment with anti-asialo-GM1 antibodies abrogated the anti-tumor effect induced by IL-2 and anti-IL-2 mAbs in RAG-2-KO (Fig. 4B) and wild-type mice (Fig. 4C). These results demonstrated that the anti-tumor effect induced by the combination treatment was crucially dependent on NK cells in vivo.

Discussion

We recently demonstrated that the in vivo activities of IL-2 and the resultant anti-tumor effects were enhanced by S4B6 anti-IL-2 mAbs. Indeed, combination treatment with anti-IL-2 mAbs plus IL-2 markedly enhanced antigen-specific CTL activity in vivo and protected mice from metastatic tumors in
the lungs, compared with the results observed with anti-IL-2 mAbs or IL-2 treatment alone (12). In the present study, we attempted to identify cell populations that are critical for the anti-tumor effect induced by this combination treatment. The results demonstrated that NK cells play a role in the observed anti-tumor activity.

We previously observed that the numbers of NK cells and CD44^high CD8^+ T cells dramatically increased in mice treated...
with IL-2 and anti-IL-2 mAbs, which led us to hypothesize that both NK cells and CD44^{high}CD8^+ T cells might contribute to the anti-tumor effect of the combination treatment (12). Moreover, detailed analysis indicated that the number of CD11c^+ DCs also increased after the combination treatment. The number of myeloid-type DCs expressing CD11b increased, with a particular augmentation observed in the spleen. The expanded population of DCs after IL-2-anti-IL-2 mAb treatment expressed low levels of CD86, suggesting that they were not fully activated by the treatment. In fact, depletion of the DCs using the DTR system did not affect the enhanced anti-tumor effect induced by the combination treatment. How does IL-2 and anti-IL-2 mAbs increase DC number in vivo? Because myeloid DCs directly isolated from spleens under normal conditions as well as those induced to differentiate from bone marrow cells in vitro using granulocyte macrophage colony-stimulating factor (GM-CSF) did not show enhanced proliferation or survival in response to IL-2 or IL-2 plus anti-IL-2 mAb treatment, we hypothesized that the treatment with IL-2 and anti-IL-2 mAbs indirectly produces growth factors for myeloid DCs in vivo. One candidate would be GM-CSF, which was expressed by activated CD4^+ T cells in response to the combination treatment (data not shown). However, this might not be a case, because IL-2 expression in RAG-deficient mice where no CD4^+ T cells exist significantly increased CD11c^+ DCs (data not shown). Further examinations should reveal whether factors from CD4^+ T cells or from non-T cells play a role in the IL-2-mediated expansion of the myeloid DC population.

Our data demonstrated that NK cells are a critical cell population that suppresses B16 tumor metastases in the lung in response to the combination treatment, whereas deficiency of CD8^+ T cells and CD4^+ T cells did not affect the anti-tumor activity. These results are supported by the fact that antigen specificity is critical for CD8^+ T cell-mediated anti-tumor activities (28, 29) and increases in cytokine-induced CD8^+ T cell activation were not entirely mediated by tumor-specific CD8^+ T cells. It, however, is possible that injection of IL-2 together with anti-IL-2 mAbs may not only expand the population of CD8^+ memory T cells but also increases the number of clones, which may include tumor-specific CD8^+ T cells. Therefore, the combination treatment may be effective against some types of MHC class I^low tumors, in particular tumors infected by viruses. On the other hand, IL-2-anti-IL-2 mAb-mediated NK cell activation directly induced an anti-tumor effect. Indeed, activation markers, such as CD25, CD69 and intracellular expression of perforin, granzyme B and IFN-γ, increased in NK cells in response to the treatment. Consistent with these results, Prlic et al. (21) demonstrated that NK cell killing is increased after IL-2 complex treatment. Furthermore, increased NK cells and CD8^+ T cells via IL-15 and soluble IL-15 receptor-α treatment resulted in resistance to B16 metastasis (30), suggesting that similar NK cell-mediated mechanism acts there. Thus, our observations clearly indicate that the combination treatment activates NK cells to attack tumor cells in vivo. Additionally, it should be pointed out that Granucci et al. (31) demonstrated that NK cells do not produce IFN-γ upon only IL-2 stimulation but produce IFN-γ upon IL-2 stimulation in the presence of activated DCs in vitro. We hypothesize two possibilities to explain why IFN-γ is produced in NK cells by the combination treatment demonstrated here: (i) excess IL-2 stimulation by the IL-2 and anti-IL-2 mAb treatment directly or indirectly induced a certain level of DC activation and/or (ii) another factor such as type I IFNs and IL-15 expressed by the combination treatment induces IFN-γ expression in NK cells (30, 31).

NK cell activity is a key component of the in vivo tumor surveillance system (32, 33). In fact, we found that mice subjected to anti-asialo-GM1 antibody-mediated depletion of their NK cells exhibited dyspnoea caused by heavy tumor growth in the lungs as well as metastases in the liver, lymph nodes and skin after injection of B16 cells (data not shown). It is also well known that NK cells recognize MHC class I^low cells, including virus-infected cells as well as tumor cells. We observed that B16 cells express low levels of MHC class I molecules on their surfaces (34) (data not shown). Therefore, since virus infections sometimes reduces surface expression of MHC class I molecules, it is possible that combined treatment with IL-2 and anti-IL-2 mAbs targets virus-infected MHC class I^low cells as well as MHC class I^low tumor cells via activation of NK cells. Additionally, it will be of great interest to determine if combination treatment using humanized IL-2-anti-IL-2 mAb suppresses recurrences or metastases of MHC class I^low tumors after surgery and prevents virus infections, which reduce surface level of MHC class I expression in cells infected.

In summary, we have demonstrated that NK cells but not CD8^+ T cells or DCs contribute to the IL-2-anti-IL-2 mAb treatment-induced inhibition of tumor metastasis. Combination treatment using humanized IL-2-anti-IL-2 mAb offers a novel potential therapeutic strategy to enhance IL-2-mediated NK cell activities, which may prevent tumor recurrences or metastases after surgery and some types of virus infections.

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**Abbreviations**

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<th>Abbreviation</th>
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<td>APC</td>
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References


