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Effects of macrophage-colony-stimulating factor on cyclophosphamide-injected mouse NK1.1$^+$ cell activity

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Abstract We injected cyclophosphamide into mice and examined their natural killer (NK) activity both in vitro and in vivo. Cyclophosphamide injection temporarily abrogated the lung clearance activity of Yac-1 lymphoma cells, which is considered to be an index of NK activity in vivo. However, administration of recombinant human macrophage-colony-stimulating-factor (rhM-CSF) to cyclophosphamide-injected mice restored the lung clearance activity. To clarify whether the administration of rhM-CSF activated NK cells, we purified NK1.1$^+$ cells from mice treated with cyclophosphamide and/or rhM-CSF and examined their functions (cytotoxicity, proliferation, and interferon γ production) in vitro. Cyclophosphamide injection decreased the number, but did not suppress the functions of NK1.1$^+$ cells. The numbers of NK1.1$^+$ cells in cyclophosphamide-injected mice restored by rhM-CSF administration. And the functions of NK1.1$^+$ cells from both saline-injected and cyclophosphamide-injected mice were accelerated by rhM-CSF administration. These results suggested that the temporary abrogation of NK activity in vivo caused by cyclophosphamide injection was due to a decrease in the number and not to suppression of the functions of NK1.1$^+$ cells. The injection of cyclophosphamide into mice increased the number of tumor (B16 melanoma) nodules formed in the lungs and liver. However, treatment with rhM-CSF recovered the anti-metastatic activity in the lungs of cyclophosphamide-injected mice. These results show that administration of rhM-CSF restores NK activity suppressed by cyclophosphamide injection in vivo.

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Introduction

Natural-killer(NK)-cell-dependent antitumor and antifungal effects are important in cancer therapy. However, the administration of some chemical anticancer drugs impairs blood-forming functions (e.g. the generation of neutrophils, NK cells etc.) that are important to maintain the defense systems of the patient. As a result, chemotherapy may accelerate the risk of tumor metastases and fungal infections. Metastasis of tumor cells is a serious concern, causing deterioration in the condition of patients receiving cancer therapy [1, 21, 25, 31]. Some cytokines are now used in combination to avoid these side-effects. Macrophage-colony-stimulating factor (M-CSF) is now commercially available, and treatment with M-CSF has been reported to reduce the average duration of pyrexia, the number of parenteral antibiotic injections required, and the average number of total platelet units transfused as compared with control courses [14, 16, 20, 22].

M-CSF is known to have many roles in vivo, and a number of studies of its hematological effects and roles in cholesterol homeostasis, bone remodeling and the maintenance of pregnancy have been conducted [10, 17, 23, 26, 31, 36, 40]. The effects of M-CSF on macrophage proliferation, differentiation and activation have been studied for many years [1–3, 6, 8, 33]. M-CSF also affects the generation of NK cells in vitro and in vivo [13, 18, 29]. These findings indicated that M-CSF plays an important role in the maintenance of the biophylaxis systems.

In our previous study, we demonstrated that the administration of recombinant human (rh)M-CSF to mice increased the number and activated the functions of NK1.1$^+$ cells in vivo. These effects contributed to the anti-metastatic activity of rhM-CSF in a mouse experimental metastasis model [28, 29].

On the basis of these observations, we studied the effects of rhM-CSF treatment on NK activity in cyclophosphamide-injected mice in vivo. We report here that administration of rhM-CSF restored the level of $NK1.1^+$ cells and increased NK activity in cyclophosphamide-injected mice. Furthermore, these effects contributed to the recovery of antitumor activity, which was abrogated by cyclophosphamide injection, in the experimental metastasis model.

Materials and methods

Animals

Six-week-old male mice of the C57 BL/6 strain were purchased from Charles River Japan Inc. (Atsugi, Kanagawa, Japan) and were used at 8–10 weeks of age.

Reagents

rhM-CSF was purified to protein homogeneity from serum-free medium, conditioned by CHO-3ACSF-69 cells, by the same procedure as used for the purification of native M-CSF, and had a specific activity of 2×10^8 U/mg protein [9]. This purified rhM-CSF was dissolved in saline containing 0.1% heat-inactivated mouse serum at a concentration of 12.5 μg/ml. The endotoxin content of the rhM-CSF preparations was less than 0.01 ng/ml as measured by the *Limulus* assay (*Limulus* HS-Single Test; Wako Pure Chemicals, Osaka, Japan).

Recombinant mouse interleukin-2 (rmIL-2) and recombinant mouse interleukin-12 (rmIL-12) were purchased from Genzyme Co. (Cambridge, Mass.). Goat anti-(mouse IgG2a) fluorescein-isothiocyanate-labeled (FITC; Caltag), and mouse anti-(mouse NK-1.1) phycoerythrin(PE)-labeled (Pharmingen) were used to identify cell populations by flow cytometry. A monoclonal antibody against NK1.1 (from hybridoma PK136, ATCC HB191) was prepared as ascites in BALB/c mice and purified by ammonium persulfate precipitation and protein A column chromatography (mAb Trap GII, Pharmacia Biotech, Sweden).

In vivo assay of mouse NK cell activity

The in vivo assay of mouse NK activity was carried out by the "lung clearance assay", as previously described, using [^{125}I]iododeoxyuridine(^{125}I-dUrd)-labeled Yac-1 cells [24]. Yac-1 cells were incubated for 20 h in complete medium containing 0.5 μCi/ml ^{125}I-dUrd then harvested and washed at least three times with phosphate-buffered saline (PBS). Aliquots of 10^5 cells in 0.2 ml PBS were injected into each mouse via the tail vein. Three hours after injection, the mice were sacrificed and the radioactivity in the lungs was measured with a gamma counter (Auto-Gamma model 5650). The relative NK activity was expressed as the mean percentage of the control lung radioactivity.

Purification of $NK1.1^+$ cells

The rhM-CSF (500 μg/kg), or saline was injected i.v. into four mice daily for 3 days from the day after injection of cyclophosphamide (40 mg/kg). On day 6, spleen cells were collected from each group and $NK1.1^+$ cells were purified from these cells as previously described [29]. The spleen cells were labeled with anti-NK1.1 mAb and anti-(mouse IgG2a + b) microbeads (Miltenyi Biotec, Germany). The magnetically labeled cells were then collected with by passage through a MACS VS^+ column (Miltenyi Biotec, Germany). The purity of the collected cells was tested by flow cytometry (EPICS-Profile II, Coulter, Japan). We used $NK1.1^+$ cells of over 95% purity for all in vitro experiments.

$NK1.1^+$ cell-mediated cytotoxic assay

Various numbers of $NK1.1^+$ cells ($3000–10^5$) from vehicle- or rhM-CSF-treated mouse spleen cells were seeded in quadruplicate in round-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) in 100 μl complete medium (RPMI-1640, 7.5% fetal calf serum). Target cells were then plated in the wells at a concentration of 5000 cells/100 μl and incubated at 37 °C in a humidified atmosphere flushed with 5% CO_2 for 5 h. Lysis of the target cells was assessed by measuring the activity of lactate dehydrogenase, which was released into the culture medium by the lysed cells [12]. The CytoTox96 assay (Promega, Wis., USA) was used in this experiment. The percentage cytotoxic activity was calculated from the following formula:

Cytotoxicity (%) $= (a - b - c)/(d - c) \times 100$, a = experimental lactate dehydrogenase release, b = effector spontaneous release, c = target spontaneous release, d = target maximum release.

IL-2-dependent proliferation of $NK1.1^+$ cells

The purified $NK1.1^+$ cells were seeded (5×10^4/well) in quadruplicate in flat-bottomed 96-well microtiter plates (NUNC) in 200 μl complete medium containing rmIL-2 (10–250 U/ml). The plates were incubated at 37 °C in a humidified atmosphere flushed with 5% CO_2 for 48 h. During the final 6 h of incubation, 20 μl (18.5 kBq) [^{3}H]methylthymidine was added to each well. Then the cells were harvested and thymidine incorporation was measured by a scintillation counter. Data are expressed as the mean [^{3}H]methylthymidine uptake (cpm) by quadruplicate cultures plus the standard deviation.

Interferon γ (IFNγ) production by $NK1.1^+$ cells

The $NK1.1^+$ cells were cultured (5×10^4/well) in flat-bottomed 96-well microtiter plates (NUNC) in 200 μl of complete medium. To stimulate the production of IFNγ, rmIL-2 (1–100 U/ml) or rmIL-12 (0.1–10 ng/ml) was added to the culture and the plates were incubated at 37 °C in a humidified atmosphere flushed with 5% CO_2 for 24 h. The culture supernatant was then collected and stored at −80 °C until use.

Cytokine assay

IFNγ was assayed by enzyme-linked immunosorbent assay (ELISA; Endogen, Cambridge, Mass.).

Induction of lung and liver metastases

Lung and liver metastases were induced by i.v. injection of B16 melanoma cells via the lateral tail vein. Cyclophosphamide (40 mg/kg) or saline was injected (i.p.) on day 0. Then, saline or rhM-CSF (500 μg/kg) was injected (i.v.) every day for 3 days. On day 4, B16 melanoma cells (10^5/mouse) were injected intravenously and the mice were sacrificed 21 days later. The lungs and livers were excised and fixed in 15% formaldehyde, and the tumor nodules were counted under a dissecting microscope.

Statistical analysis

All statistical analyses were performed by the two-tailed Student's *t*-test.

Results

Effects of rhM-CSF on the lung clearance assay

We previously reported that administration of rhM-CSF enhanced lung clearance activity in normal mice. In this

study, we injected cyclophosphamide (40 mg/kg, the appropriate dose for cancer chemotherapy of cyclophosphamide-sensitive tumors in mice) or saline into the mice intraperitoneally on day 0 (Fig. 1). From the next day, saline or rhM-CSF (500 μg/kg) was injected intravenously daily for 3 or 4 days. NK activity in these mice was measured on day 0, 2, 4, 5 and 6 by lung clearance assay. As shown in Fig. 1, the NK activity (clearance of Yac-1 cells) was reduced temporarily by cyclophosphamide injection. However, administration of rhM-CSF (500 μg/kg) produced a partial recovery of the NK activity of the cyclophosphamide-injected mice on days 4 and 5.

Blocking of the effects of rhM-CSF on the lung clearance assay

The injection of anti-NK1.1 antibody depleted NK1.1$^+$ cells in vivo [28]. Anti-NK1.1 antibody was then injected into both cyclophosphamide-treated and untreated mice and their NK activity was measured by the lung clearance assay (as in Fig. 1). As shown in Fig. 2, anti-NK1.1 antibody injection markedly reduced the clearance activity of Yac-1 cells in vivo, even though M-CSF was administered. The same results were also observed in the cyclophosphamide-injected mice.

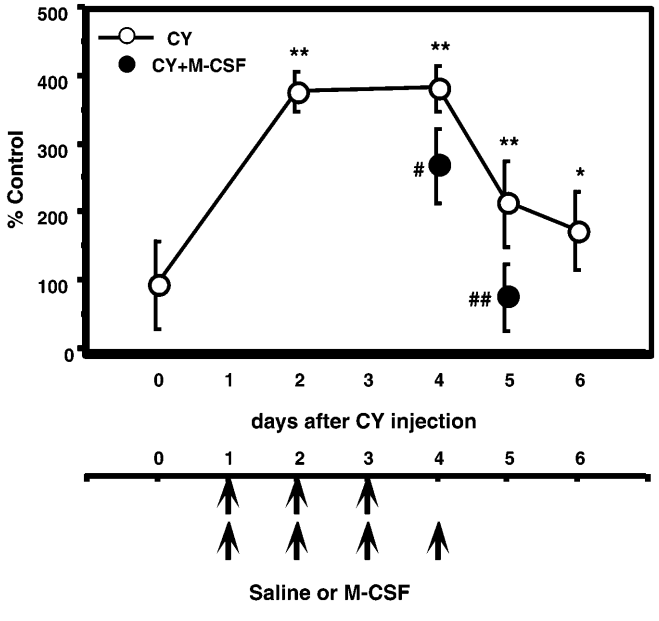

Fig. 1 Effects of recombinant human macrophage-colony-stimulating factor (*rhM-CSF*) on the lung clearance assay. Cyclophosphamide (*CY*, 40 mg/kg) was injected (i.p.) on day 0. Then saline or rhM-CSF (500 μg/kg) was injected (i.v.) every day for 3 or 4 days (*arrows*). Natural killer (NK) activity in vivo was tested by the lung clearance assay on days 0, 2, 4, 5 and 6. The target Yac-1 cells were labeled with ^{125}I and inoculated intravenously; 3 h later, the Yac-1 cells remaining in the lungs were enumerated with a gamma counter. The relative NK activity is expressed as the mean percentage of the lung radioactivity of the control group. $^{**}P < 0.001$, $^{*}P < 0.01$ (CY + saline versus control); $^{\#\#}P < 0.005$, $^{\#}P < 0.03$ (CY + saline versus CY + M-CSF)

Effects of rhM-CSF on NK1.1$^+$ cell number in the spleen

To investigate the mechanism responsible for the reduction (and recovery) of NK activity, we first examined the number of NK1.1$^+$ cells in the mice in each group (Fig. 3). As we reported previously, the administration of rhM-CSF increased the number of NK1.1$^+$ cells in the spleen. By contrast, administration of cyclophosphamide caused a decrease in the number of NK1.1$^+$ cells in the spleen. However, injection of

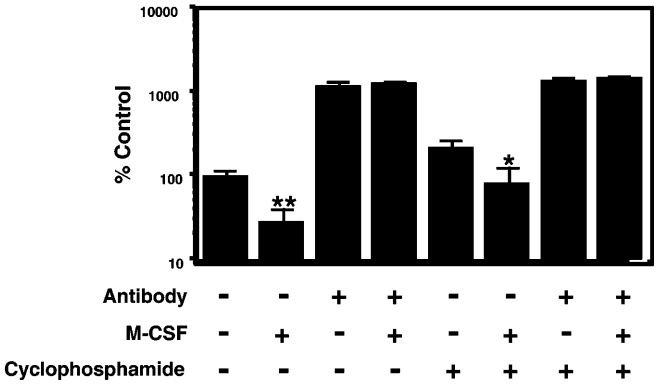

Fig. 2 Blocking of the effects of rhM-CSF on lung clearance assay. Cyclophosphamide (40 mg/kg) was injected (i.p.) on day 0. Then saline or rhM-CSF (500 μg/kg) was injected (i.v.) every day for 4 days. Anti-NK1.1 antibody was injected (2 mg/mouse, i.p.) on days 1 and 3. NK activity in vivo was tested by the lung clearance assay on day 4. The relative NK activity is expressed as the mean percentage of the lung radioactivity of the control group. $^{**}P < 0.005$ (M-CSF versus saline); $^{*}P < 0.01$ (CY + M-CSF versus CY)

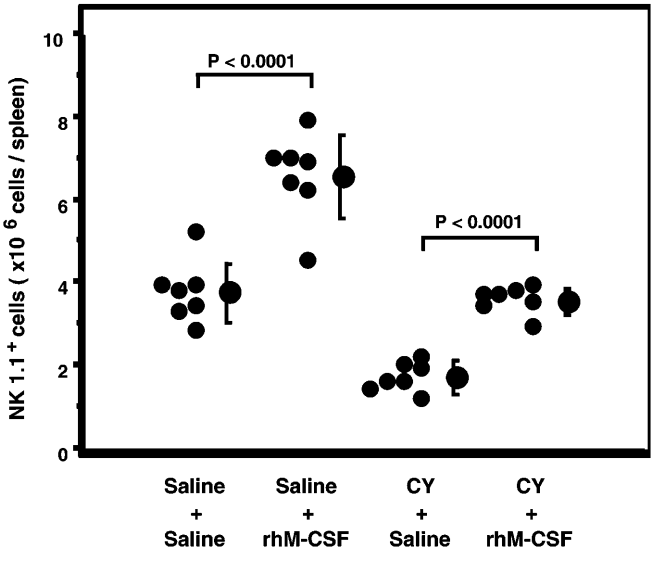

Fig. 3 Effects of rhM-CSF on NK1.1$^+$ cell number in the spleen. Cyclophosphamide (40 mg/kg) or saline was injected (i.p.) on day 0. Then saline or rhM-CSF (500 μg/kg) was injected (i.v.) every day for 3 days. On day 4, spleen cells were collected from each mouse, and NK1.1$^+$ cells were stained with phycoerythrin-conjugated anti-NK1.1 antibody. The percentages of NK1.1$^+$ cells were analyzed by flow cytometry and the numbers of NK1.1$^+$ cells were calculated

rhM-CSF after cyclophosphamide administration resulted in the NK1.1$^+$ cells recovering to almost the normal level. These results suggested that the rise in NK activity in cyclophosphamide-injected mice was due to an increase in the effector/target (E/T) ratio in vivo.

Cytotoxic activity of NK1.1$^+$ cells

We next examined whether the administration of cyclophosphamide suppressed the function of NK1.1$^+$ cells. NK1.1$^+$ cells were prepared from the mice in each group (saline + saline, saline + rhM-CSF, CY + saline and, CY + rhM-CSF) by MACS and their cytotoxic activities against Yac-1 cells were examined in vitro. As shown in Fig. 4, injection of cyclophosphamide did not suppress the cytotoxic activities of NK1.1$^+$ cells, and the administration of rhM-CSF augmented their cytotoxic activity.

IL-2-dependent growth of NK1.1$^+$ cells

NK cells proliferate in response to IL-2 in vitro. We prepared NK1.1$^+$ cells as described above for the cytotoxicity assay and cultured them with IL-2 (0–250 U/ml) for 48 h. The uptake of [^{3}H]dT by NK1.1$^+$ cells was expressed as an index of proliferation. Regardless of administration of cyclophosphamide, NK1.1$^+$ cells proliferated in response to the addition of IL-2 in vitro (Fig. 4). And administration of rhM-CSF enhanced the proliferative responsiveness of the NK1.1$^+$ cells to IL-2. (The full scale of the vertical axis of Fig. 5a is indicated by a dotted line in Fig. 5b.)

IFNγ production by NK1.1$^+$ cells

Secretion of IFNγ is an essential function of NK cells in defense systems in vivo. Therefore, we examined the NK1.1!$^+$ cells (prepared as described above) for production of IFNγ in vitro. The NK1.1$^+$ cells were stimulated by adding IL-2 and/or IL-12 to the culture medium followed by incubation for 24 h. The IFNγ levels in the culture supernatants were then measured by ELISA. As shown in Fig. 6, NK1.1$^+$ cells from mice treated with either saline + rhM-CSF or cyclophosphamide + rhM-CSF exhibited high levels of IFNγ production.

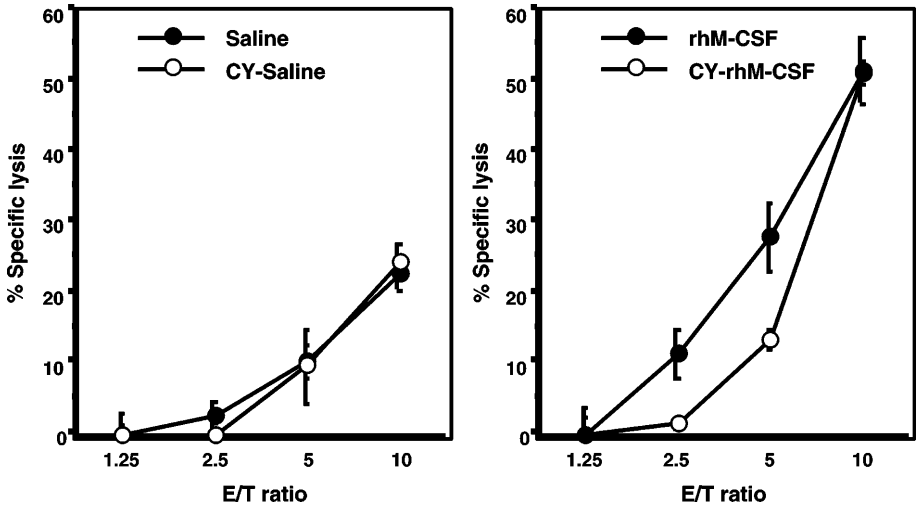

Fig. 4 Cytotoxic activity of NK1.1$^+$ cells. Cyclophosphamide (40 mg/kg) or saline was injected (i.p.) on day 0. Then saline or rhM-CSF (500 μg/kg) was injected (i.v.) every day for 3 days. On day 4, spleen cells were collected from each mouse, and NK1.1$^+$ cells were purified by MACS. Target Yac-1 cells (5×10^3/well) and purified NK1.1$^+$ cells were cultured at various effector/target (*E*/*T*) ratios. The specific cytotoxicity was estimated by measuring the lactate dehydrogenase released from the target cells

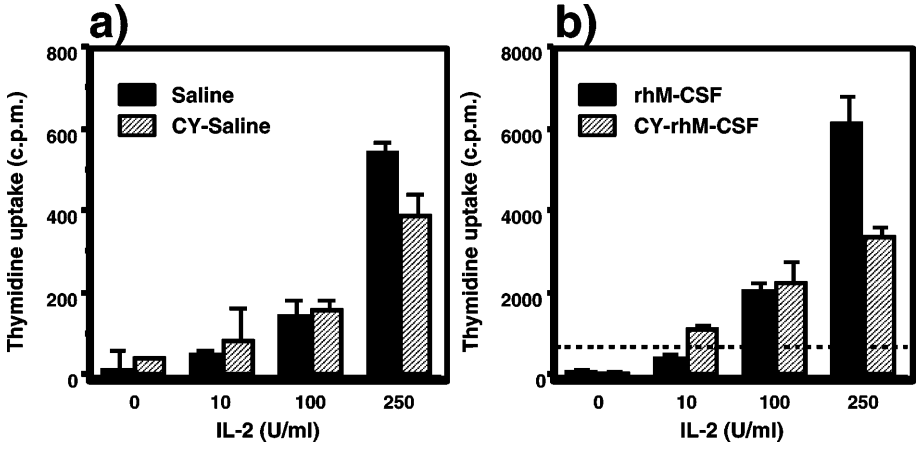

Fig. 5a, b Interleukin-2(*IL-2*)-dependent growth of NK1.1$^+$ cells. NK1.1$^+$ cells were purified by MACS as described in Fig. 4. Purified NK1.1$^+$ cells were cultured (5×10^4/well) with recombinant murine (rm) IL-2 (10–250 U/ml) for 48 h. Cell proliferation was assessed by [^{3}H]methylthymidine uptake in the last 6 h of culture. (The full scale of the vertical axis of **a** is indicated by a dotted line in **b**)

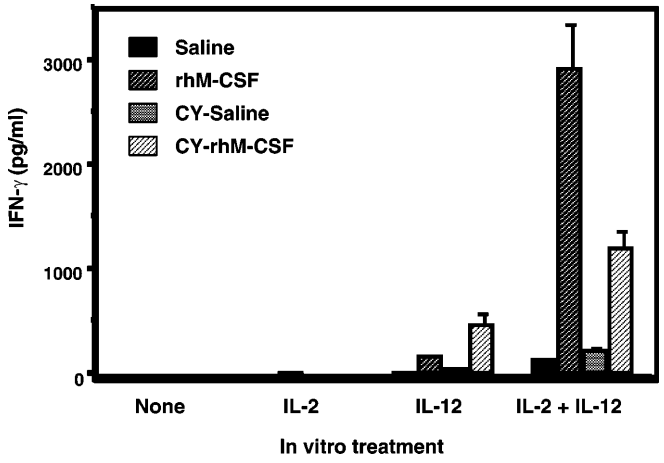

Fig. 6 Interferon γ (*IFN*γ) production of NK1.1$^+$ cells. NK1.1$^+$ cells were purified by MACS as described in Fig. 4. Purified NK1.1$^+$ cells were cultured (5×10^4/well) with rmIL-2 (250 U/ml) and rmIL-12 (1 ng/ml) for 24 h. The concentration of IFNγ in the culture supernatant was assayed by enzyme-linked immunosorbent assay

These results demonstrated that the administration of cyclophosphamide did not suppress the functions (cytotoxicity, proliferation, and IFNγ production) of NK1.1$^+$ cells and that rhM-CSF treatment activated NK1.1$^+$ cells mice treated with saline + rhM-CSF or with cyclophosphamide + rhM-CSF.

Anti-metastatic effect of rhM-CSF on B16 melanoma

Finally, we applied the above findings to the mouse tumor metastasis model (Table 1). B16 melanoma cells (10^5/mouse) were inoculated via the lateral tail vein 4 days after saline or cyclophosphamide injection. In the mice pretreated with saline, the administration of rhM-CSF suppressed the formation of tumor nodules in the lungs and liver. On the other hand, pretreatment with cyclophosphamide increased the numbers of tumor nodules in the lungs and liver. Under these conditions, administration of rhM-CSF significantly suppressed the formation of tumor nodules in the lungs, but not in the liver.

Table 1 Anti-metastatic effect of recombinant human macrophage-colony-stimulating factor (*rhM-CSF*) on B16 melanoma metastasis. Cyclophosphamide (*CY*, 40 mg/kg) or saline was injected (i.p.) on day 0. Then saline or rhM-CSF (500 μg/kg) was injected (i.v.) every day for 3 days. On day 4, B16 melanoma cells (10^5/mouse) were injected intravenously. The numbers of tumor nodules in lungs and liver were counted 21 days after tumor inoculation

Treatment (n = 13)	Number of tumor nodules	
	Lung	Liver
Saline + saline	82 ± 46	14 ± 4
Saline + rhM-CSF	41 ± 18*[1]	4 ± 6*[2]
CY + saline	192 ± 63	46 ± 30
CY + rhM-CSF	118 ± 41*[3]	36 ± 30*[4]

*[1] $P < 0.0005$, *[2] $P < 0.003$ (saline + saline versus saline + rhM-CSF), *[3] $P < 0.0005$, *[4] $P < 0.4$ (CY + saline versus CY + rhM-CSF)

Discussion

To examine the effects of rhM-CSF administration on the NK activity of cyclophosphamide-injected mice, we examined the number of NK1.1$^+$ cells in the spleen, tested the clearance of Yac-1 cells in vivo and tested the functions of NK1.1$^+$ cells in vitro. Injection of cyclophosphamide caused a temporary reduction in NK activity in vivo. In this study, we demonstrated that the reduction of NK activity in vivo was due to a decrease in the number of NK1.1$^+$ cells and not to suppression of their functions. In addition, activation of NK1.1$^+$ cells by rhM-CSF administration was not arrested by cyclophosphamide injection.

The NK1.1$^+$ cells recovered to the normal level after rhM-CSF administration (Fig. 4); however, we could not completely restore the clearance activity of Yac-1 cells to the normal level (Fig. 1). We suppose that the incomplete recovery of clearance ability in Yac-1 cells was due to other damage caused by cyclophosphamide injection; in fact, the numbers of Mac-1$^+$ and B220$^+$ cells in the spleen were also decreased by cyclophosphamide injection, and were partially restored by rhM-CSF injection (data not shown).

The administration of rhM-CSF not only increased the number but stimulated the functions of NK1.1$^+$ cells [29]. As shown in Figs. 4–6, the cyclophosphamide injection did not suppress the functions of NK1.1$^+$ cells, and administration of rhM-CSF activated NK1.1$^+$ cells of both saline-injected and cyclophosphamide-injected mice. However, NK1.1$^+$ cells from mice injected with cyclophosphamide + rhM-CSF were somewhat less active than those from mice receiving saline + rhM-CSF. This tendency was observed in every experiment (Figs. 4–6) so we supposed that cyclophosphamide injection damaged the cascade that was maintaining the activation of NK1.1$^+$ cells in vivo. For all that, the up-regulation of the proliferative response to IL-2 in vitro (following rhM-CSF administration) may be beneficial for generating lymphokine-activated killer cells after the chemotherapy.

Previously we demonstrated that the rate of clearance of B16 melanoma cells in vivo was an important factor for anti-metastatic activity. That is, the administration of rhM-CSF facilitated the clearance of B16 melanoma cells and suppressed the formation of tumor nodules in the lungs and liver. Furthermore, injection of anti-NK1.1 antibody (depletion of NK1.1$^+$ cells in vivo) markedly increased the metastasis of B16 melanoma [28]. Here, we have reported that injection of cyclophosphamide reduced the number of NK1.1$^+$ cells and that this effect induced a temporary reduction of NK activity in vivo. As expected from these results, the injection of cyclophosphamide also increased metastasis of B16 melanoma (Table 1). We examined the anti-metastatic effect of rhM-CSF in this experimental metastasis

model and found the formation of tumor nodules to be suppressed in the lungs of CY-injected mice. However, the administration of rhM-CSF did not suppress the formation of tumor nodules in the liver. Thus, there appears to be an additional antimetastatic mechanism independent of NK cells (e.g. macrophages), and these other effectors were also destroyed by cyclophosphamide injection. We therefore supposed that, although the administration of rhM-CSF partially restored NK activity, the anti-metastatic activity of the liver could not be recovered completely.

Dyshematopoiesis is a serious side-effect of chemotherapy. In particular, decreases in white blood cell numbers result in a fatal crisis in the maintenance of the biophylaxis system. Therefore, administration of CSF is thought to act beneficially by stimulating hematopoiesis. It has been reported that administration of cyclophosphamide augments immune responses and improves the therapeutic efficacy of IL-2 and IL-15 [5, 11, 35]. However, a transient decrease in blood cell number is an unavoidable side-effect of chemotherapy. These observations led us to examine M-CSF with cyclophosphamide. Many CSF and cytokines that contribute to hematopoiesis have been discovered. For example, G-CSF is now widely used in the treatment of leukopenia in cancer chemotherapy. However, granulocytes activated by G-CSF have been reported to suppress NK activity [15]. Furthermore, high serum levels of G-CSF have been reported to depress NK activity in patients with aplastic anemia [34]. IL-15 and IL-2 bind to IL-2 receptor β and γ and induce the differentiation and proliferation of NK cells [7, 38], Flt-3L can promote the generation of NK cell progenitors [30, 41], and IL-12 and IL-18 are strong activators of NK cells [37, 39]. NK activity is a key factor in the maintenance of biophylaxis systems, therefore combinations of these cytokines with M-CSF should be examined to find beneficial applications for cancer therapy. We are now studying the use of combinations of IL-2 and M-CSF.

In this study, we induced a decrease of NK activity by injecting cyclophosphamide into mice. Our results suggested that the reduction of NK activity in vivo was due to a decrease in the number of NK cells rather than to suppression of their functions. The administration of rhM-CSF restored the number and activated the functions of $NK1.1^+$ cells in saline and cyclophosphamide-treated mice. These effects contributed to the recovery of antimetastatic ability in lung reduced by cyclophosphamide injection. Further studies of the effects of M-CSF administration on $NK1.1^+$ cell activity and number will help the search for beneficial applications of M-CSF for cancer chemotherapy.

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