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ORIGINAL ARTICLE

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Early-appearing tumor-infiltrating natural killer cells play an important role in the nitric oxide production of tumor-associated macrophages through their interferon production

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Abstract Both natural killer (NK) cells and macrophages are thought to be the main effectors responsible for early antitumor defense. In this study, we investigated the role of tumor-infiltrating NK cells in initiating nitric oxide (NO) production by tumor-associated macrophages (TAM). The in vivo depletion of NK cells prior to the i.p. inoculation of melanoma cells resulted in a significant decrease in the NO production of the TAM prepared from the peritoneal exudate cells (PEC). Such prior NK cell depletion also decreased the ability of TAM to show any antitumor activity in vitro. The addition of N^G-monomethyl-L-arginine (Me-L-Arg) to the culture partially inhibited the ability of TAM to suppress the proliferation of melanoma cells and also decreased their cytolytic activity against melanoma cells. These results suggest that the TAM exhibited both cytostatic and cytolytic activities through their NO production. In an in vivo assay, the administration of Me-L-Arg permitted the more rapid growth of i.p. inoculated melanoma cells compared with the control. On the other hand, the decreased NO production of TAM, resulting from the prior NK cell depletion, was restored by the i.p. administration of interferon γ (IFNγ). In addition, the in vivo administration of anti-IFNγ mAb into mice inoculated i.p. with melanoma cells also significantly decreased the NO production of TAM in peritoneal exudate cells. Furthermore, the tumor-infiltrating NK cells produced a considerable level of IFNγ. Overall, these results indicate that early-appearing tumor-infiltrating NK cells play an important role in the NO production of TAM through their IFNγ production.

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Introduction

Natural killer (NK) cells are large granular lymphocytes that demonstrate cytotoxic activity against tumor cells and virus-infected cells [20, 43]. Since they show such activity without any prior sensitization or major histocompatibility complex (MHC) restriction, they are thought to play a crucial role in the early defense against malignant transformation [18, 25, 41] and viral infection [4, 44]. As for the role of NK cells in the early antitumor defense, we previously demonstrated that NK cells infiltrate the site of tumor development at an early stage [29] and that such NK cells play a critical role in the subsequent generation of tumor-specific cytotoxic T lymphocytes (CTL) [30].

Nitric oxide (NO) has recently been given much attention as an important molecule with tumoricidal and microbicidal activities [21, 24, 27, 40]. NO is mainly produced by macrophages and generated from oxidation of the terminal guanidino nitrogen atom of L-arginine by inducible NO synthase, an enzyme known to be induced by interferon γ (IFNγ), tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and lipopolysaccharide [8, 9, 32, 34, 37, 39]. In tumor immunology, many reports have revealed macrophages to show an antitumor activity, mainly through their NO production [6, 10, 15, 22, 28]. NO production by macrophages is also induced by stimulation with tumors both in vivo and in vitro [2, 23]. In addition, NO is revealed to be a factor regulating immunological responses. It was reported that activated macrophages contribute to the suppression of antigen-specific T cell proliferation through their NO production during primary listerial infection [17]. In addition, Alleva et al. reported that peritoneal macrophages from tumor-bearing mice produce more NO than do those from normal mice, while tumor cells also promote the macrophages to suppress tumor-specific T cell proliferation through their NO production [2]. Furthermore,

NO is reported to be involved in peripheral T cell tolerance [33] and T cell development in the thymus [12].

Both NK cells and macrophages are important components responsible for the antitumor early defense until antitumor-specific T cell immunity can be evoked. Concerning their interaction, several studies have revealed that either IL-12 or TNFα secreted by macrophages can activate NK cells [7, 36, 42], while it still remains unclear what effect the tumor-infiltrating NK cells could exert on the tumor-associated macrophages (TAM). In this study, we designed experiments to investigate the influence of tumor-infiltrating NK cells on TAM, focusing on NO production. Our results demonstrated that early-appearing tumor-infiltrating NK cells play an important role in inducing TAM to produce NO and thereby show a tumoricidal activity. Such NK cells were suggested to induce TAM to produce NO through their IFNγ production. The implications of these findings are discussed.

Materials and methods

Mice

Female C57BL/6 (B6) mice were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan) and kept in a specific-pathogen-free animal facility at our institution. They were used in experiments at 8–11 weeks of age. In some experiments, to deplete NK cells in vivo, the mice were injected i.v. with 100 μg anti-NK1.1 mAb either 1 day before or 2 days after the i.p. inoculation of tumor cells. This treatment completely depleted the NK1.1$^+$ cells and the NK activity of the peritoneal exudate cells (PEC) for at least 4 days, as confirmed by flow cytometry and ^{51}Cr-release assay respectively (data not shown).

Reagents

To prepare anti-NK1.1 mAb, PK136 hybridoma cells were grown in serum-free medium 101 (kindly provided by the Nissui Pharmaceutical Co. Ltd, Tokyo, Japan). The supernatant was collected and the mAb was concentrated by 50% ammonium sulphate precipitation. Anti-(heat-stable antigen) mAb (J11D; rat IgM) and anti-Thy1.2 mAb (HO13-4; mouse IgM) were obtained from ATCC, Rockville, Md. Recombinant IFNγ (rIFNγ) was purchased from Gibco, Grand Island, N.Y. Anti-IFNγ mAb (rat IgG1) was obtained from Upstate Biotechnology Incorporated, Lake Placid, N.Y. An irrelevant rat IgG1 was used as an isotype-matched control antibody. Greiss reagent, N^G-monomethyl-L-arginine (Me-L-Arg) and Me-D-Arg were purchased from Wako Pure Chemicals, Inc., Osaka, Japan.

Tumor

The B16 melanoma used in this study was derived from B6 mice. B16 melanoma cells were maintained in vitro in a complete culture medium, which consists of RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 40 μg/ml gentamicin, 2 mM L-glutamine, 10 nM HEPES buffer, 10% heat-inactivated fetal calf serum (Interoen company, N.Y.), 50 μM 2-mercaptoethanol and 0.2% sodium bicarbonate.

Preparation of TAM

To inactivate B16 melanoma cells, they were treated with mitomycin C (MMC; Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan) at a dose of 100 mg/ml for 90 min and then washed with complete medium three times. The viable or MMC-treated B16 melanoma cells (1×10^6) were inoculated i.p. into syngeneic B6 mice, which were injected i.v. with 100 μg anti-NK1.1 mAb either 1 day before or 2 days after the i.p. inoculation of melanoma cells. On day 3 after the tumor inoculation, peritoneal lavage was performed with 10 ml complete culture medium. After five washes, the PEC were collected. To enrich the macrophages obtained from the PEC, both the T cells and B cells were depleted by treatment with anti-Thy1.2 mAb and anti-(heat-stable antigen) mAb plus Low-Tox-M rabbit complement (Cedarlane Laboratories, Ontario, Canada). The resulting macrophage-enriched cells consisted of more than 82% Mac-1$^+$ cells and fewer than 1% NK1.1$^+$ cells, and were used as the TAM in this study.

Assay of NO production

The level of NO in the culture supernatants was estimated by measuring the concentration of nitrite in each sample, according to the method reported by Green et al. using Greiss reagent [16]. The titer was determined by a standard curve generated by the absorbance of serial dilution of $NaNO_2$.

Assay of cytostatic activity

The TAM (1×10^5 cells) were cultured with 1×10^6 viable melanoma cells in 200 μl complete culture medium in 96-well flat-bottom tissue-culture plates. In some experiments, the indicated doses of Me-L-Arg or Me-D-Arg were added to the cultures. After 48 h, 100 μl samples of the culture supernatants were collected to determine the level of NO. Thereafter, each well was pulsed with 1 μCi [^{3}H]dThd 6 h before the harvesting. The incorporated [^{3}H]dThd was determined by a Beta Plate system (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Assay of cytolytic activity

The TAM (5×10^4 cells) were incubated with 1×10^4 ^{51}Cr-labelled B16 melanoma cells in 200 μl complete culture medium for 18 h in a 96-well round culture plate (Nunc, Roskilde, Denmark). After incubation, 100 μl samples of the supernatants were harvested. The radioactivity of the supernatants was measured by a gamma counter and the percentage of specific ^{51}Cr release was calculated according to the formula:

$$\frac{(\text{experimental release} - \text{spontaneous release}) \times 100}{\text{maximal release} - \text{spontaneous release}}$$

The spontaneous release was determined by the sample of target cells incubated without effector cells. The maximal release was determined of the sample of target cells incubated with 10% Triton-X (Wako Chemical Industries Ltd.). The spontaneous release was less than 20% of the maximal release. All samples were assayed in quadruplicate and the values were shown as the means ± SD.

In vivo administration of either rIFNγ or anti-IFNγ mAb

In some experiments, the mice were administered i.p. 2500 U rIFNγ twice daily from day 1 to day 2 after the i.p. inoculation of tumor cells. As a control, the complete medium was injected. In other experiments, the mice were injected i.p. with 250 μg anti-IFNγ mAb once daily from day 1 to day 2 after the i.p. inoculation of tumor cells. As a control, phosphate-buffered saline (PBS) was also injected.

Assay of IFNγ production

The mice were inoculated i.p. with either 1×10^6 viable B16 melanoma cells or MMC-treated B16 melanoma cells on day 0. On day 2, some mice were injected i.v. with 100 μg/ml NK1.1 mAb to deplete NK cells

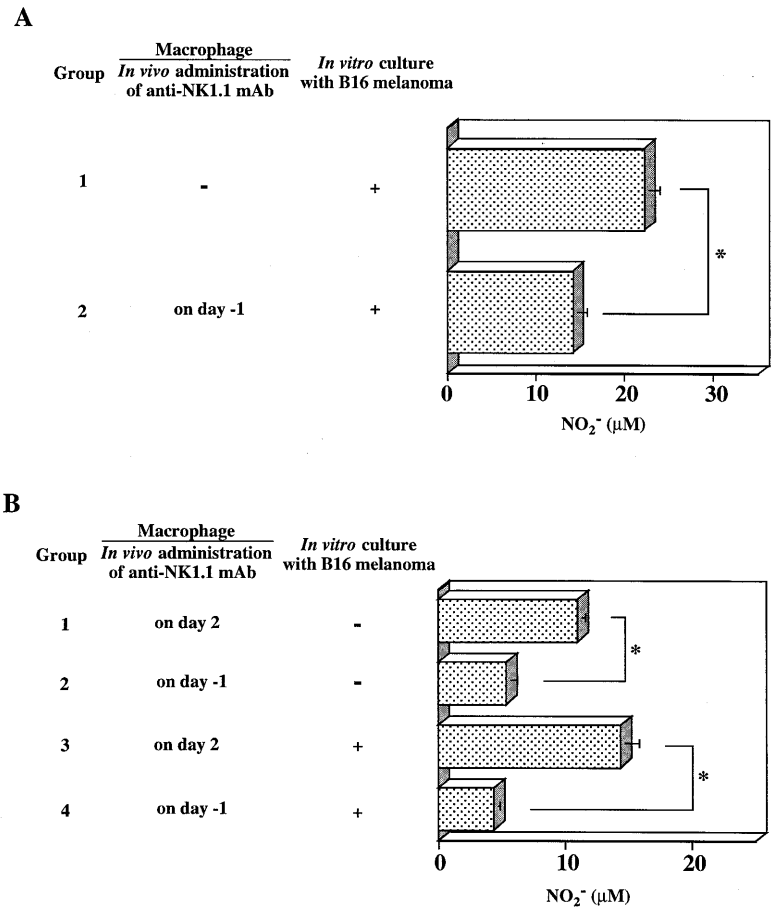

Fig. 1A, B The effect of natural killer (NK) cell depletion on the NO production of the tumor-associated macrophages (TAM). **A** In group 1, the B6 mice were inoculated i.p. with 1×10^6 mytomycin-C (MMC)-treated B16 melanoma cells. In group 2, B6 mice were administered i.v. 100 μg anti-NK1.1 mAb 1 day before the i.p. inoculation of 1×10^6 MMC-treated B16 melanoma cells. **B** In groups 1 and 3, B6 mice were inoculated i.p. with 1×10^6 MMC-treated B16 melanoma cells on day 0 and administered i.v. 100 μg anti-NK1.1 mAb on day 2. In groups 2 and 4, B6 mice were administered i.v. 100 μg anti-NK1.1 mAb on day –1 and inoculated i.p. with 1×10^6 MMC-treated B16 melanoma cells on day 0. In all cases, the peritoneal exudate cells (PEC) were collected on day 3 after the tumor inoculation and the TAM were enriched, as described in Materials and methods. The TAM (1×10^5 cells) were cultured either with or without 2×10^4 viable B16 melanoma cells in 96-well culture plates. After 48 h, the culture supernatants were collected and their nitrite levels were determined. The data are representative of three experiments and are expressed as the mean of the quadruplicate values ± SD, *A significant difference ($P<0.05$)

in vivo. On day 3, the PEC were collected and further enriched for the non-adherent cells after incubation on plastic culture plates for 60 min at 37 °C. Such non-adherent cells (1×10^5 cells) were cultured with 2×10^4 viable B16 melanoma cells for 48 h in a 96-well flat plate. The culture supernatants were harvested and the levels of IFNγ determined by two-site sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, microplates (EIA/RIA plate, Costar, Cambridge, Mass.) were coated with anti-IFNγ mAb (1.5 μg/ml, 100 μl) in 0.1 M sodium phosphate buffer, pH 7.2 for 12 h at 4 °C, and than blocked with 100 μl complete medium, pH 7.2, for 30 min at room temperature. The samples and mouse r IFNγ, as a standard, were diluted in 0.05% PBS/Tween 20, incubated in anti-IFNγ-coated plates, washed with PBS/Tween 20 and further incubated with biotin-conjugated-anti-IFNγ mAb (5 μg/ml, 50 μl, PharMingen) at room temperature. After 60 min, the plates were washed and incubated with streptavidin-b-galactosidase (Gibco BRL) in PBS/Tween 20 for 30 min at room temperature. The plates were washed three times with PBS/Tween 20 and 4-methylumbelliferyl β-D-galactoside (Wako) in 0.01 M phosphate buffer containing 0.1 M NaCl, 0.1% bovine serum albumin, 1 mM $MgCl_2$ and 0.1% NaN_3 (0.2 mM, 100 μl), pH 7.0, was added and incubated for 60 min at room temperature. The reaction was terminated by adding 100 μl 0.1 M glycine/NaOH (pH 10.2) and absorbance was measured and monitored with a fluorescence microplate reader (MTP-32, Corona Co. Ltd., Ibaragi, Japan), which was calibrated for excitation at 360 nm and emission at 460 nm. The values for IFNγ were calculated from a standard curve of rIFNγ.

Counting the tumor cell number in the peritoneal cavity

B6 mice were inoculated i.p. with 1×10^6 B16 melanoma cells on day 0. Some mice were injected i.p. with 10 mg of either Me-L-Arg, Me-D-Arg or PBS on days 1 and 2. On day 3, the tumor cells in the peritoneal cavity were harvested by the same method as was used for the collection of the PEC. The number of B16 melanoma cells was counted by using trypan blue.

Statistics

The statistical significance of the data was determined by Student's *t*-test. A *P*-value of less than 0.05 was considered to be significant.

Results

The tumor-infiltrating NK cells were essential for the optimal NO production by the TAM

We previously reported that NK cells significantly infiltrate into tumors on day 3 after the i.p. inoculation of syngeneic tumor cells [29]. In this study, to investigate the role of such early-appearing tumor-infiltrating NK cells in the NO production by TAM, we first prepared macrophages from the PEC of either the mice that had been inoculated i.p. with B16 melanoma cells or the mice that had been inoculated i.p. with B16 melanoma cells 1 day after the i.v. injection of anti-NK1.1 mAb. The PEC were collected on day 3 after the i.p. inoculation of melanoma cells and the

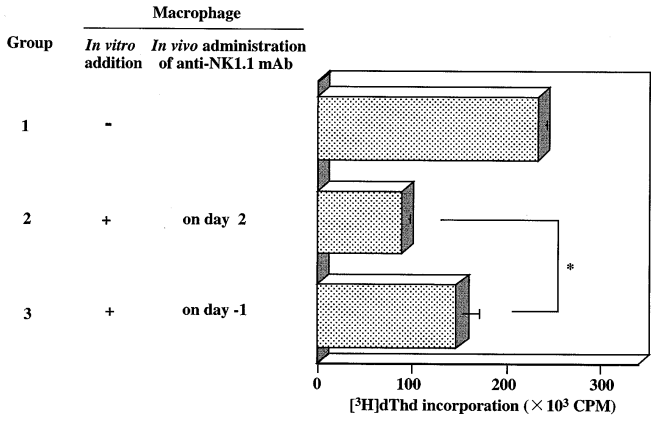

Fig. 2 Tumor-infiltrating NK cells were essential for the optimal antitumor activity of the TAM. The TAM from B6 mice, which were inoculated i.p. with 1×10^6 MMC-treated B16 melanoma cells either before or after the in vivo depletion of NK cells, were prepared by the same method described in Fig. 1B. The TAM (1×10^5 cells) were cultured with 2×10^4 viable B16 melanoma cells for 48 h and [^{3}H]dThd was added to each well 6 h before the harvesting. The data are representative of three experiments and are expressed as the mean of the quadruplicate values ±SD. *A significant difference ($P<0.05$)

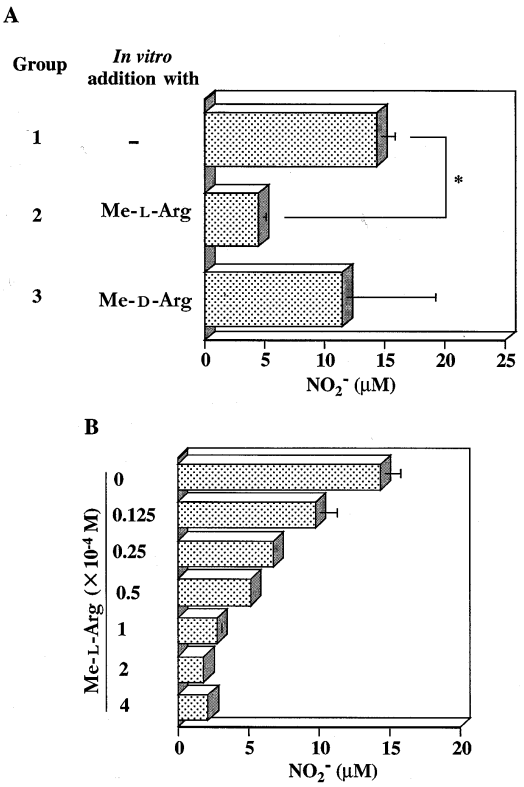

Fig. 3A, B The NO production of the TAM was specifically inhibited by the addition of N^G-monomethyl-L-arginine (Me-L-Arg), but not (Me-D-Arg). **A** B6 mice were inoculated i.p. with 1×10^6 MMC-treated B16 melanoma cells on day 0, and administered i.v. 100 μg anti-NK1.1 mAb on day 2. On day 3 after the tumor inoculation, the PEC were collected and the TAM were prepared. The TAM (1×10^5 cells) were cultured with 2×10^4 viable B16 melanoma cells either with or without 0.1 mM Me-L-Arg or Me-D-Arg. After 48 h, the culture supernatants were collected and their nitrite levels were determined. The data are representative of three experiments and are expressed as the mean of the quadruplicate values ±SD. * A significant difference ($P<0.05$). **B** The TAM (1×10^5 cells) were cultured with 2×10^4 viable B16 melanoma cells either with or without the indicated doses of Me-L-Arg. After 48 h, the culture supernatants were collected and their nitrite levels were determined. The data are representative of two experiments and are expressed as the mean of the quadruplicate values ±SD

enriched macrophages (the TAM), were examined for the levels of nitrite in the culture supernatants. Figure 1A shows that NK cell depletion prior to the tumor inoculation resulted in a significant decrease in NO production by the TAM. Although this result suggests that the tumor-infiltrating NK cells influenced the NO production by the TAM in our system, there is also a possibility that NK cells in the NK-cell-non-depleted group participated in the NO production by the TAM during the in vitro culture through their production of IFNγ and NO [5, 45]. To exclude this possibility, by the removal of any NK cells before the in vitro culture, anti-NK1.1 mAb was injected either 1 day before or 2 days after the preparation of TAM (on day 2). As shown in Fig. 1B, NK cell depletion prior to the tumor inoculation resulted in a significant decrease in the NO production by the TAM, compared with that after the tumor inoculation (groups 1 and 2). A similar result was also observed when melanoma cells were added during the in vitro culture (groups 3 and 4). These results thus indicate that early-appearing tumor-infiltrating NK cells were necessary for optimal NO production by TAM.

TAM showed both cytostatic and cytolytic activities against B16 melanoma cells through their NO production

On the basis of previous demonstrations that NO is an important molecule responsible for tumoricidal effects [6, 10, 15, 22, 28], we next examined the antitumor activity of the TAM from mice that had been depleted of NK cells either before or after to i.p. inoculation of melanoma cells, as shown in Fig. 2. As a result, the TAM from mice that were NK-cell-depleted after the tumor inoculation suppressed the in vitro proliferation of B16 melanoma cells more strongly than did those from mice that were NK-cell-depleted before the tumor inoculation. These results suggest that tumor-infiltrating NK cells also had an influence on the antitumor activity of TAM.

To investigate the mechanisms whereby TAM to show the antitumor activity in vitro, we tested the possibility that this was through their NO production. Before using either Me-L-Arg, as an NO synthase inhibitor, or Me-D-Arg, as a control, we confirmed their specificity and also identified the size of the dose sufficient to suppress in vitro NO production by TAM. As shown in Fig. 3A, the NO production of TAM was significantly suppressed by the addition of 0.1 mM Me-L-Arg, but not Me-D-Arg. In addition, as shown in Fig. 3B, doses of even less than 0.1 mM Me-L-Arg were sufficient to inhibit TAM from producing NO in vitro. Based on these results, the following experiments were performed at a dose of 0.1 mM Me-L-Arg.

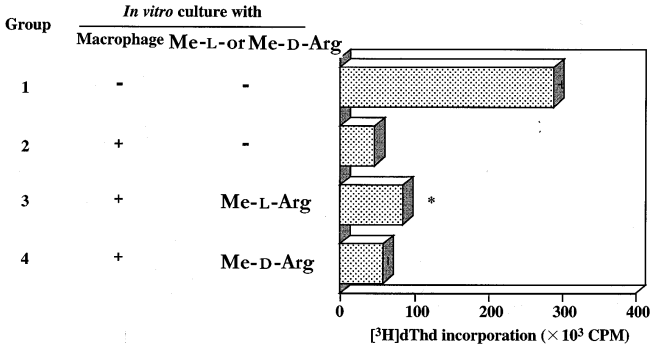

Fig. 4 The addition of Me-L-Arg partially restored the in vitro proliferation of melanoma cells. B6 mice were inoculated i.p. with 1×10^6 MMC-treated B16 melanoma cells on day 0, and administered i.v. 100 μg anti-NK1.1 mAb on day 2. On day 3 after the tumor inoculation, the PEC were collected and the TAM were prepared. The TAM (1×10^5 cells) were cultured with 2×10^4 viable B16 melanoma cells either with or without 0.1 mM Me-L-Arg or Me-D-Arg for 48 h. [^{3}H]dThd was added to each well 6 h before harvesting. The data are representative of three experiments and are expressed as the mean of the quadruplicate values ±SD. * (Group 3) a significant difference ($P<0.05$) compared with groups 2 and 4

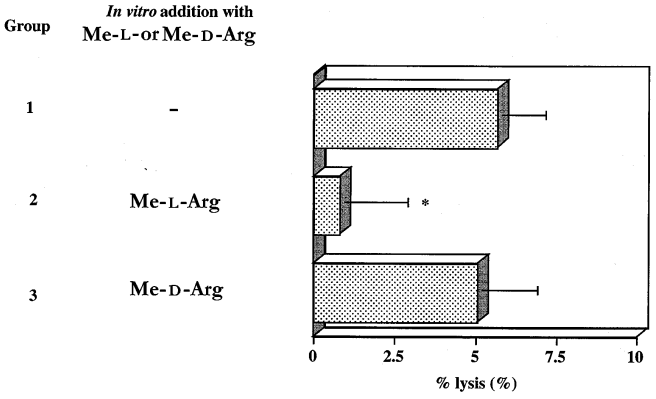

Fig. 5 The cytolytic activity of TAM against B16 melanoma was inhibited by the addition of Me-L-Arg. B6 mice were inoculated i.p. with 1×10^6 MMC-treated B16 melanoma cells on day 0, and administered i.v. 100 μg anti-NK1.1 mAb on day 2. On day 3 after the tumor inoculation, the PEC were collected and the TAM were prepared. The TAM were examined for their cytolytic activity against B16 melanoma at an E/T ratio of 5/1 with an 18-h ^{51}Cr-release assay. In some groups, either 0.1 mM Me-L-Arg or Me-D-Arg was added to each well at the initiation of the assay. The data are representative of two experiments and are expressed as the mean of the quadruplicate values ±SD. * A significant difference ($P<0.05$) compared with the other groups

We then determined whether or not the addition of Me-L-Arg to the in vitro culture of TAM could decrease their cytostatic activity against the in vitro proliferation of B16 melanoma cells. Figure 4 shows that the in vitro blockade of NO production by TAM resulted in an increased proliferation of B16 melanoma cells, though the effect was small. We therefore further examined the other possibility that the TAM showed a cytolytic activity through their NO production. We then next examined the cytolytic activity of TAM by an 18-h ^{51}Cr-release assay, as shown in Fig. 5. TAM showed a low but significant level of cytolytic activity against B16 melanoma, while their cytolytic activity was significantly inhibited by the addition of Me-L-Arg, but not Me-D-Arg. Taken together, these results thus indicate that TAM, prepared from the PEC 3 days after the i.p. inoculation of B16 melanoma, showed both cytostatic and cytolytic activities through their NO production.

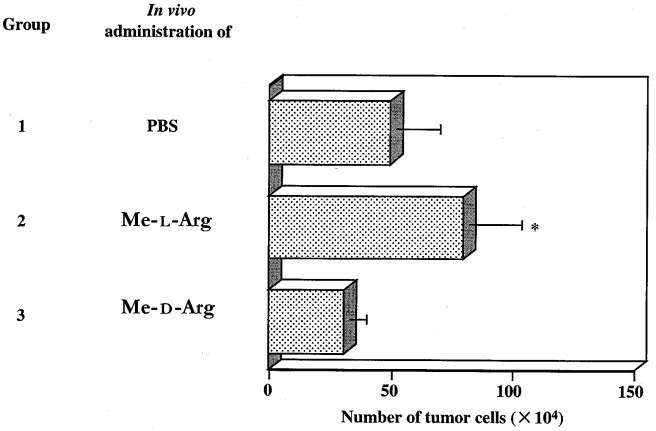

Fig. 6 The in vivo administration of Me-L-Arg accelerated the tumor growth of i.p. inoculated B16 melanoma cells. B6 mice were inoculated i.p. with 1×10^6 viable B16 melanoma cells on day 0. On days 1 and 2, such mice were administered i.p. either phosphate-buffered saline, 10 mg of Me-L-Arg or 10 mg Me-D-Arg in a volume of 0.5 ml. On day 3, the cells in the peritoneal cavity were harvested and the tumor cells were counted. The data are representative of two experiments and are expressed as the mean ±SD of four mice. * A significant difference ($P<0.05$) compared with the other groups

The in vivo inhibition of NO production resulted in a more rapid growth of i.p. inoculated B16 melanoma cells

We further determined whether or not the in vivo inhibition of NO production could have any influence on the in vivo growth of tumor cells, as shown in Fig. 6. We examined the number of tumor cells in the PEC of mice, that had been inoculated i.p. with viable B16 melanoma cells 3 days earlier and followed by the i.p. administration of either Me-L-Arg or Me-D-Arg. The in vivo administration of Me-L-Arg resulted in an increased cell number of B16 melanoma cells compared with that following Me-D-Arg administration. These results therefore indicate that NO is involved in the in vivo antitumor activity against the i.p. inoculated melanoma cells.

The in vivo administration of rIFNγ could restore the decreased NO production of the TAM from NK-cell-depleted mice subsequently inoculated i.p. with B16 melanoma

It has been reported that the NO production of macrophage is regulated by a number of factors, including cytokines [8, 9, 32, 37, 39]. Among them, IFNγ is regarded as the main factor inducing macrophages to produce NO [9, 14, 46]. On the other hand, NK cells are also well known to be IFNγ producers [38] and we also previously reported that, in a

Group	Macrophage		*In vivo* administration of IFN γ
	In vitro addition	*In vivo* administration of anti-NK1.1 mAb	
1	+	-	-
2	+	-	+
3	+	on day 2	-
4	+	on day -1	-
5	+	on day -1	+

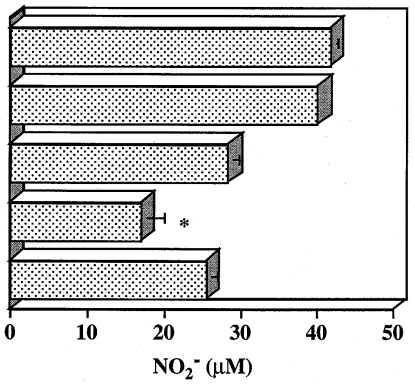

Fig. 7 The in vivo administration of interferon γ (IFNγ) restored the NO production of the TAM prepared from the mice depleted of NK cells and inoculated i.p. with B16 melanoma. In groups 1 and 2, B6 mice were inoculated i.p. with 1×10^6 MMC-treated B16 melanoma cells on day 0 without any administration of anti-NK1.1 mAb. In group 3, B6 mice were inoculated i.p. with 1×10^6 MMC-treated B16 melanoma cells on day 0, and administered i.v. 100 μg anti-NK1.1 mAb on day 2. In groups 4 and 5, B6 mice were administered i.v. 100 μg anti-NK1.1mAb on day –1, and inoculated i.p. with 1×10^6 MMC-treated B16 melanoma cells on day 0. In groups 2 and 5, 1250 U IFNγ was administered i.p. twice daily on days 1 and 2. In all groups, the PEC were harvested and the TAM were prepared on day 3. Such TAM (1×10^5 cells) were cultured with 2×10^4 viable B16 melanoma cells for 48 h. The nitrite levels in the culture were determined. The data are representative of three experiments and are expressed as the mean of the quadruplicate values ±SD. * A significant difference compared with the other groups ($P<0.05$)

3LL carcinoma/B6 system, tumor-infiltrating NK cells produce IFNγ [29]. These observations led us to test the possibility that IFNγ, derived from tumor-infiltrating NK cells, might play a pivotal role in inducing TAM to produce NO. We therefore initially determined whether the in vivo administration of rIFNγ could restore the decreased NO production by the TAM caused by the prior depletion of NK cells, as shown in Fig. 7. The TAM from mice that had been injected i.v. with anti-NK1.1 mAb and then inoculated i.p. with melanoma cells (group 4) showed a decrease in their NO production compared with those from mice that had been inoculated i.p. with melanoma cells and then i.v. injected with anti-NK1.1 mAb (group 3), and the data were similar to those shown in Fig. 1. However, the i.p. injection with 1250 U rIFNγ twice daily on days 1 and 2 after the i.p. inoculation of tumor cells significantly augmented the NO production by TAM (group 5). When the TAM were prepared from mice that had not been injected with anti-NK1.1 mAb, the in vivo administration of rIFNγ showed no definite influence on the level of NO production by the TAM, probably because there was sufficient IFNγ produced by the infiltrating NK cells (groups 1 and 2). These results imply that the in vivo administration of IFNγ could restore the decreased NO production of TAM from NK-cell-depleted mice subsequently inoculated i.p. with melanoma cells, and also indirectly suggest that IFNγ treatment could substitute for the role of early-appearing tumor-infiltrating NK cells in activating TAM to produce NO. We directly addressed this hypothesis by the in vivo neutralization of IFNγ, as shown in Fig. 8. The in vivo administration of anti-IFNγ mAb to the mice induced the TAM to produce less NO. In addition, this treatment caused TAM to induce significantly less antitumor activity in vitro (data not shown). These results thus suggest the participation of IFNγ in inducing TAM to produce NO in vivo after the i.p. inoculation of B16 melanoma cells.

The NK cells were mainly responsible for the IFNγ production of the PEC after the i.p. inoculation of melanoma cells

Although IFNγ was shown to play a central role in inducing TAM to produce NO, this does not mean that this IFNγ was derived from the tumor-infiltrating NK cells. We therefore finally determined to what degree the IFNγ production by the PEC after i.p. inoculation of melanoma cells depended on the tumor-infiltrating NK cells. Figure 9 shows that the PEC, prepared on day 3 after the i.p. inoculation of B16 melanoma cells, produced a detectable level of IFNγ. However, IFNγ production by the PEC was significantly diminished by the i.v. injection of anti-NK1.1 mAb 1 day before the PEC were harvested. When mice were inoculated i.p. with non-treated B16 melanoma cells, the level of IFNγ in the culture of TAM decreased about 50% (groups 3 and 4), whereas in the case of mitomycin-C-treated B16 melanoma cells, the depletion of NK cells prior to the in vitro culture completely abolished the capacity of the PEC to produce IFNγ (groups 5 and 6). This difference is probably due to the fact that non-treated B16 melanoma cells could survive longer than mitomycin-treated melanoma cells after an i.p. inoculation. Collectively these results indicate that IFNγ, which was essential for the optimal NO production by the TAM, mainly derived from the early-infiltrating tumor-infiltrating NK cells.

Discussion

Both NK cells and macrophages are thought to be the main effector cells responsible for the early antitumor defense [1,

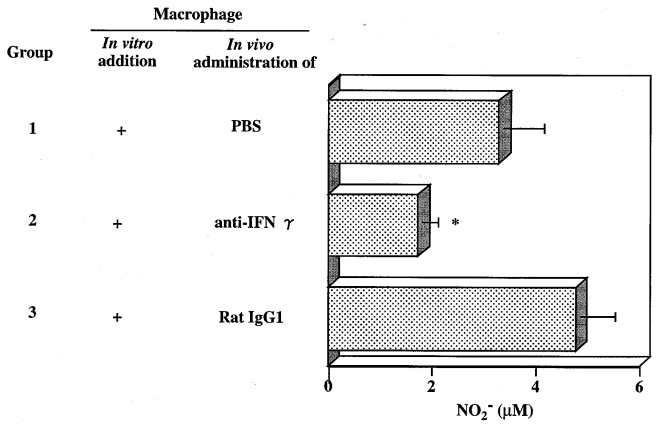

Fig. 8 The in vivo neutralization of IFNγ decreased the capacity of the TAM to produce NO. B6 mice were inoculated i.p. with 1×10^6 MMC-treated B16 melanoma cells on day 0. On days 1 and 2, some mice were injected i.p. with either 250 μg anti-IFNγ mAb or rat IgG1, as an isotype-matched control antibody. On day 2, all mice were administered i.v. 100 μg anti-NK1.1 mAb. On day 3, the PEC were collected and the TAM were prepared. The TAM (1×10^5 cells) were cultured with 2×10^4 viable B16 melanoma cells for 48 h. The nitrite levels in the culture were determined. The data are representative of two experiments and are expressed as the mean of the quadruplicate values ±SD. * (Group 2) a significant difference ($P<0.05$) compared with the other groups

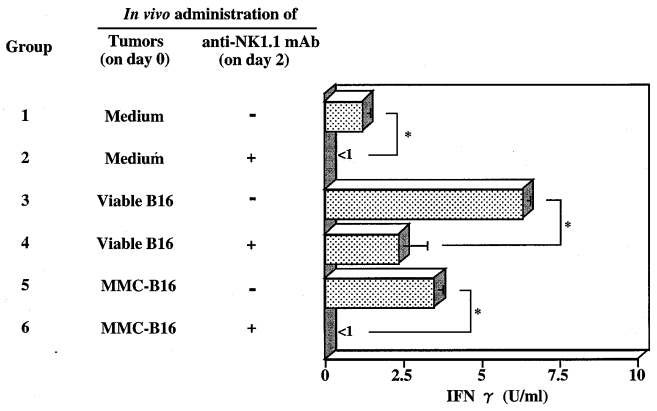

Fig. 9 The tumor-infiltrating NK cells were the cells mainly responsible for the IFNγ production of the PEC after the i.p. inoculation with B16 melanoma cells. B6 mice were inoculated i.p. with either 1×10^6 viable or MMC-treated B16 melanoma cells on day 0. To deplete NK cells before the in vitro culture, some B6 mice were injected i.v. with 100 μg anti-NK1.1 mAb on day 2. On day 3, the PEC were collected and adherent cells were removed. Thereafter, the non-adherent cells (1×10^5 cells) were cultured with 2×10^4 viable B16 melanoma cells for 48 h. The levels of IFNγ in the culture supernatants were determined by the two-site sandwich enzyme-linked immunosorbent assay. The data are representative of three experiments and are expressed as the mean of the quadruplicate values ±SD. * A significant difference ($P<0.05$)

18, 21, 25, 26, 41, 43]. In contrast to many reports suggesting their individual roles in antitumor immunity, there have been few reports discussing their interaction [26, 48]. In particular, the effects that tumor-infiltrating NK cells might have on TAM have yet to be elucidated. On the other hand, while analyzing PEC after an i.p. inoculation of tumor cells, we previously demonstrated that NK cells infiltrate the tumor-developing site at an early stage [29] and that such NK cells are also essential for the subsequent generation of tumor-specific CTL [30]. In this study, we further extended our experimental protocol to determine whether or not such NK cells could show any influence on the TAM, particularly on their NO production. Our results indicate that early-appearing tumor-infiltrating NK cells play an important role in the NO production of the TAM through their IFNγ production.

To investigate the role of NK cells in inducing the TAM to produce NO, we compared the levels of NO in the culture supernatant of the TAM, which were prepared either from the NK-cell-depleted mice that were subsequently inoculated i.p. with melanoma or from the mice that were inoculated i.p. with melanoma and subsequently NK-cell-depleted. In both cases, the NK cells were evenly depleted before the in vitro culture of TAM. The results showed that NK cell depletion prior to the i.p. inoculation of melanoma cells significantly decreased the capacity of the TAM to produce NO (Fig. 1A, B). Regarding the mechanism by which NK cells initiated NO production by the TAM, while taking into account the potential of NK cells to produce IFNγ [11, 47], we supposed that the following possibility was the most plausible: the tumor-infiltrating NK cells produced IFNγ and thereby induced the TAM to produce NO. In support of this hypothesis, the NO production by the TAM, decreased by the prior NK cell depletion, was then restored by the i.p. administration of IFNγ (Fig. 7). In addition, we also reported that the early-appearing tumor-infiltrating NK cells, after the i.p. inoculation of syngeneic 3LL lung carcinoma cells, produced a high level of IFNγ [29] while, in the B16 melanoma system, such NK cells strongly expressed the mRNA of IFNγ [30]. To directly address this issue, we performed an experiment using anti-IFNγ mAb. The in vivo neutralization of IFNγ significantly decreased the capacity of the TAM to produce NO (Fig. 8). Together, these findings strongly suggest that the early-appearing tumor-infiltrating NK cells induced the TAM to produce NO through their IFNγ production.

Although we showed that the in vivo administration of anti-IFNγ mAb decreased the capacity of TAM, which were prepared on day 3 after the i.p. inoculation of melanoma cells, to produce NO, this treatment did not necessarily neutralize only NK-cell-derived IFNγ. For instance, IFNγ-producing T cells, such as γ/δ T cells and heat-shock-protein-reactive α/β T cells, have also been reported to respond to antigen stimulation more quickly than conventional T cells [13, 19]. We therefore tried to determine to what degree the IFNγ production of PEC, after the i.p. inoculation of melanoma cells, could be ascribed to the tumor-infiltrating NK cells, and found that a considerable degree of IFNγ production by the PEC depended on the existence of tumor-infiltrating NK cells (Fig. 9). It could therefore be concluded that IFNγ, which induce TAM to produce NO, was mainly derived from the early-appearing tumor-infiltrating NK cells.

In this study, we demonstrated that TAM could show antitumor activity through their NO production. The in vitro suppressive activity of TAM on the proliferation of B16

melanoma cells was partially decreased by the addition of Me-L-Arg, but not Me-D-Arg (Fig. 4). The addition of Me-L-Arg diminished the cytolytic activity of the TAM against B16 melanoma cells (Fig. 5). These results indicate that TAM showed both cytostatic and cytolytic activities through NO production, that the existence of some other mechanism can not be ruled out. Cui et al. demonstrated that macrophage-derived NO induces apoptosis in P815 mastocytoma but that there are other NO-independent mechanisms to induce cell death in L929 fibroblasts [6]. Many previous reports have shown that TNFα has the ability to induce the apoptosis and/or necrosis of susceptible targets and thus suggested the participation of TNFα in killing such target cells [3, 31]. To investigate this possibility, we performed an experiment using anti-TNFα mAb, whereas the in vitro addition of anti-TNFα mAb to the culture of macrophages before the cytolytic assay resulted in no significant change (data not shown). It is likely that many other factors, such as IL-1 [35] and serine protease [1], could also potentially induce TAM to kill the tumor cells.

Although the main producers of NO are macrophages, recent reports indicate that human NK cells [45] and lymphokine-activated killer cells [5] can also produce NO and that their tumoricidal activity is in part, dependent on the NO synthase pathway. These findings might suggest the possibility that the more rapid growth of tumor cells after the in vivo administration of NO inhibitor was partially dependent on the inhibition of NO production by infiltrating NK cells and activated T cells. However, the adherent PEC did not contain any NK1.1$^+$ cells and we also detected no NO production by the non-adherent PEC, which had all the macrophages removed from them, 3 days after the i.p. inoculation of melanoma cells (data not shown). Therefore, we conclude that macrophages were important NO producers in the PEC after i.p. tumor inoculation.

In conclusion, we tried to investigate the influence of tumor-infiltrating NK cells on the TAM and demonstrated that such NK cells play a crucial role in NO production by TAM through their IFNγ production. Our results thus seem to demonstrate an important interaction between the two cell populations, both of which are responsible for the innate immunity against tumor development. We hope that an analysis of the antitumor early defense could thus provide helpful information for the subsequent antitumor T cell immunity.

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