Interleukin-18 and the Costimulatory Molecule B7-1 Have a Synergistic Anti-Tumor Effect on Murine Melanoma; Implication of Combined Immunotherapy for Poorly Immunogenic Malignancy

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Interleukin-18 has been described recently as a cytokine secreted primarily by Kupffer cells. Furthermore, it has been shown that it has significant anti-tumor effects, which are mediated by T cells and natural killer cells, in a manner similar to interleukin-12. Here, we report the evaluation of the effects of the systemic administration of interleukin-18 in combination with B7-1 (CD80) expressed on tumor cells [interleukin-18 + B7-1] on the growth of murine B16 melanoma *in vivo*. After the subcutaneous inoculation of B16 melanoma, B16 tumors grew progressively in immunocompetent syngeneic C57BL/6 mice. Mice treated with either interleukin-18 or immunized with B7-1-transduced B16 did not demonstrate significant anti-tumor effect. The combination of the two treatments, however, resulted in dramatic suppression of melanoma formation, tumor growth, and a significant improvement in survival. Inhibitory effects of [interleukin-18 + B7-1] on lung metastasis in mice were also detected. Additionally, mice treated with [interleukin-18 + B7-1] showed an increase of natural killer cytotoxicity and interferon-γ production *in vivo*. Unlike [interleukin-18 + B7-1], [interleukin-12 + B7-1] did not have a strong anti-tumor effect against B16 melanoma. Histologic characterization after the [interleukin-18 + B7-1] treatment confirmed the infiltration of natural killer cells into the tumor, suggesting that natural killer cells may be involved in the [interleukin-18 + B7-1]-induced anti-tumor effect. This finding was confirmed by showing that depletion of NK1.1$^+$ cells before immunization inhibits the [interleukin-18 + B7-1]-induced anti-tumor effect. Depletion of CD3$^+$ cells *in vivo* also decreased the anti-tumor effect of [interleukin-18 + B7-1], suggesting the importance of CD3$^+$ T cells. Collectively, combination with interleukin-18 and B7-1 expression has synergistic anti-tumor effects against B16 murine melanoma. ***Key words: anti-tumor effect/B7-1/immunotherapy/interleukin-18/melanoma. J Invest Dermatol 114:928–934, 2000***

The development of tumors in the environment of an intact immune system, indicates that the host cannot offer a protective immunity. Therefore, the primary goal of cancer immunotherapy is to enhance the host's immune system to a point where it rejects tumor growth. One approach taken involves the systemic administration of cytokines. A number of cytokines, such as interleukin (IL)-2, interferon (IFN)-γ, and IL-12, have been investigated to control tumor development and metastasis (Rosenberg *et al*, 1985; Giovarelli *et al*, 1986; Nastala *et al*, 1994). Recently, IL-18 has been shown to have potent anti-tumor effects which are mediated by T cells and natural killer cells (Tadashi *et al*, 1998).

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Abbreviation: rIL-18, recombinant interleukin-18.

IL-18 is a recently discovered cytokine that was described in accord with its ability to induce IFN-γ secretion from natural killer and T cells. It was therefore named "IFN-γ-inducing factor" (Okamura *et al*, 1995). IL-18 has significant immunoregulatory functions on both T and natural killer cells. In particular, IL-18 augments the proliferation of T cells, enhances cytotoxic activity of natural killer cells, induces secretion of granulocyte-macrophage colony-stimulating factor from both natural killer and T cells, and synergizes with IL-12 in terms of IFN-γ production (Okamura *et al*, 1995; Micallef *et al*, 1996). Because of its ability to induce IFN-γ, IL-18 appears to play an important part in the development of the Th1 immune response (Kohno *et al*, 1997). IL-18 is structurally related to IL-1. In an analogous manner to IL-1, IL-18 is produced via a biologically inactive precursor, which lacks a signal peptide. The precursor IL-18 is cleaved into the bioactively mature IL-18 by IL-1β-converting enzyme, also known as caspase-1 (Okamura *et al*, 1995; Gu *et al*, 1997).

An alternative approach to the problem of developing a protective immune response to cancer involves the engineering of tumor cells to make them express costimulatory molecules.

Among these costimulatory species, B7-1 (CD80) has been shown to be effective in the treatment of murine tumors (Li *et al*, 1994). The functional significance of B7-1 molecules was described first in terms of T cell activation. B7-1 molecules expressed on the antigen-presenting cells recognize CD28 molecules (its natural ligand) on the T cells. The resulting inter-reaction prevents the induction of anergy in T cells and indicates that B7-1/CD28 play a crucial part in the mechanism of cell-mediated immune responses (Chen *et al*, 1992; Linsely and Ledbetter, 1993). In addition, the importance of the B7-1/CD28 interaction in the cell-mediated immune system has been reported in the context of natural killer cell response regulation during infection. The interaction of CD28 positive natural killer cells with B7-1 molecules enhances the production of IFN-γ from natural killer cells, leading to a resistance against infection (Chambers *et al*, 1996; Geldhof *et al*, 1998).

In this study, we evaluated the effects of IL-18 and B7-1 molecules on tumorigenesis using B16 murine melanoma, which is highly metastatic and poorly immunogenic. IL-18 administration or B7-1 expression, when applied separately, to B16 melanoma merely delayed tumor development in C57BL/6 mice. Combined immunotherapy by the administration of IL-18 and molecular expression of B7-1 on tumor cells [IL-18 + B7-1], however, significantly suppressed the growth of B16 melanoma in mice, suggesting a new approach to immunotherapy against cancer.

MATERIALS AND METHODS

Recombinant cytokines Murine rIL-18 was purchased from Endogen (Woburn, MA) and murine rIL-12 was purchased from R&D Systems (Minneapolis, MN).

Tumor cells and mice B16 murine melanoma cells, obtained from C57BL/6 mice, were cultured in Dulbecco's modified Eagle's medium supplemented 10% heat-inactivated fetal bovine serum, 4 mM L-glutamine, and 2 mM sodium pyruvate. Female C57BL/6 mice were purchased from Charles River Japan (Kanagawa, Japan) and used for experiments when they were 8–10 wk old.

B7-1 gene transfer and expression To clone the mouse B7-1 gene, total cytoplasmic RNA from concanavalin A-activated C57BL/6 mouse spleen cells were reverse-transcribed into cDNA and amplified by polymerase chain reaction (PCR) using specific oligonucleotides encompassing the entire open reading frames. The following PCR primers were used: mouse B7-1 sense 5′-CTGATAAGCTTATGGCTT-GCAATTGTCAGTT-3′, mouse B7-1 anti-sense 5′-GAAGGGTCGAC-CTAAAGGAAGACGGTCGTGTT-3′. After denaturation at 95°C for 5 min, PCR was performed with the following profile; 30 s at 95°C, 1 min at 57°C, and 2 min at 72°C for 25 cycles. The PCR products were cloned in Bluescript SK^+ vectors containing *Hin*dIII and *Xho*I sites, and sequenced to determine possible Taq polymerase errors. The mouse B7-1 cDNA inserts were subcloned into pcDNA3neo vector (Invitrogen, San Diego, CA) on *Hin*dIII and *Xho*I sites.

Prior to transfection, 1×10^6 B16 cells were plated on 100 mm culture dishes. Then, the pcDNA3neo/mB7-1 vector DNA was transduced into the B16 cells by the calcium-phosphate transfection system (Life Technologies, Gibco-BRL, Rockville, MD). Cells were incubated at 37°C in a humidified 5% CO_2 incubator for 36 h. Cells were harvested and analyzed by flow cytometry. As control, cells were also transduced with pcDNA3neo vector without the mouse B7-1 insert, these are referred to as "mock-transduced". For fluorescence-activated cell sorter analysis, mouse B7-1 transduced cells were incubated with rat anti-mouse B7-1 monoclonal antibody (MoAb) (4 μg per ml, Pharmingen, San Diego, CA) for 30 min at 4°C and washed twice with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Boehringer Mannheim, Mannheim, Germany). Subsequently, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rat IgG (4 μg per ml, Pharmingen) for 30 min at 4°C. After two washes, the cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ)

Animal experiments To evaluate the tumor growth, survival, and metastases in experimental animals the design grouping described in **Table I** was adopted. Eight mice were allocated to each group. After injection of tumor cells, five mice were used to measure the tumor growth and survival, whereas three mice were used to investigate *in vivo* immune status and metastases. Briefly, mock or B7-1 transduced melanoma cells (1×10^5 cells in 0.1 ml of PBS) were injected subcutaneously (s.c.) into right backs of the mice (day 0). One microgram of IL-18 or 0.5 μg of IL-12 were administered intraperitoneally (i.p.) for seven consecutive days from day 0 to day 6. Each group of mice was observed daily to identify the onset of tumor formation and survival. The largest radius of the tumor was measured every 2–3 d, using a caliper, and this was used to calculate the tumor volume using the formula; $4/3\pi r^3$. On day 25, tumor, spleen, and lungs of each group mice (n = 3) were obtained to perform immunohistologic analysis, cellular cytotoxicity assay and to investigate the lung metastases. Serum was also collected to determine the level of IFN-γ. The statistics concerning tumor volumes were analyzed by two-way ANOVA using Duncan's multiple range test and survivals were calculated by the Kaplan–Meier method using the log-rank test. $p < 0.05$ was regarded as significant.

Table I. IFN-γ production *in vivo*

Groups	Transduction	Treatment		Serum concentration[a] (pg per ml, mean ± SD)
		IL-18	IL-12	
I	Mock	–	–	900 ± 27.3
II	Mock	+	–	1000 ± 25.6
III	B7-1	–	–	1125 ± 45.2
IV	B7-1	+	–	1900 ± 100.4
V	B7-1	–	+	1466 ± 141.6

[a]At day 25 after tumor inoculation, serum of mice (n = 3) concentrations of IFN-γ were measured in triplicate by enzyme-linked immunosorbent assay.

Depletion of $NK1.1^+$ or $CD3^+$ cells To deplete $NK1.1^+$ or $CD3^+$ cells *in vivo*, mice (five mice per group) were injected intravenously with 100 μg of MoAb against NK1.1 or CD3 (PharMingen) on days –1, 0, and 1. The mice were then treated with i.p. injection of PBS or IL-18 (1 μg per mouse) for 7 d. Treated mice was observed daily to investigate the development of tumor growth. On day 25, lungs and spleen were dissected out to evaluate metastases and natural killer cell cytotoxicity. Tumor specimens were also obtained to detect the infiltration of immune cells to the tumor sites.

Natural killer and cytotoxic T lymphocyte cytotoxicity To examine natural killer activity *in vivo*, spleens were excised under sterile conditions on day 25. Splenic mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation. Erythrocytes were lyzed by incubating mononuclear cells for 5 min at 37°C, using red blood cell lysing buffer (Sigma, St Louis, MO). Adherent cells were removed by a plastic adherent step (1 h, 37°C), and nonadherent cells were used as effector cells for the analysis of natural killer cytotoxicity against murine natural killer-sensitive target cells, YAC-1. For the analysis of CTL activity against B16 melanoma cells, the nonadherent cells, obtained with the procedure described above, were collected and plated in 24-well plates at 4×10^6 cells per well with 2×10^5 irradiated B16 melanoma cells. Recombinant IL-2 (40 U per ml; Genzyme, Cambridge, MA) was added after 3 d. Four days later, the nonadherent cells were used as effector cells for the analysis of CTL cytotoxicity against the wild-type B16 melanoma cells. A standard 4 h ^{51}Cr-release assay was performed to measure natural killer and CTL activity. Briefly, effector cells were added to the U-bottomed 96-well plates in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units per ml penicillin, 100 μg per ml streptomycin, and 10% heat-inactivated fetal bovine serum. ^{51}Cr-labeled target cells (YAG-1, 1×10^4 cells per well; B16 melanoma cells, 5×10^3 cells per well) were then added to the wells, this was performed in triplicate for each sample. They were then incubated for a period of 4 h in a 37°C, 5% CO_2 humidified incubator. After incubation, target cell lysis was detected by the measurement of ^{51}Cr release. Results are expressed as a percentage of specific release based upon the formula: % specific release = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

IFN-γ production IFN-γ concentration in the serum of the mice treated with IL-18 and/or B7-1 was measured using enzyme-linked immunosorbent assay. Mouse IFN-γ enzyme-linked immunosorbent assay kits were purchased from Endogen (Cambridge, MA).

Immunohistologic analysis Tumors and lungs were harvested on day 25 after tumor challenge. To investigate the infiltration of immune cells at the tumor tissue, tumors were immediately frozen and embedded in OCT compound. Serial 7 μm tissue sections were incubated with biotinylated antibodies to CD3, CD19, and NK1.1 (PharMingen). Sections were visualized by streptavidin–alkaline phosphatase reacting with naphthol AS-MX phosphate-Fast Blue (Sigma). For the evaluation of metastases, lung tissues were stained with hematoxylin and eosin and examined by optical microscopy.

RESULTS

Effect of [IL-18 + B7-1] on tumor formation, growth, and survival B16 melanoma cells do not express B7-1 molecules at the cell surface. In order to enhance tumor cell immunogenicity and induce protective immune response, the murine B7-1 gene was transduced to B16 melanoma cells. The expression of B7-1 on the B16 melanoma cells was detected in about 47% of cells by flow cytometry analysis (**Fig 1**). C57BL/6 mice inoculated with the B7-1-transduced B16 melanoma were compared with the mock-transduced B16 melanoma for tumor growth. Although tumors in mice injected with the B7-1-transduced B16 melanoma grew slower than those in mice which had received mock-transduced B16 melanoma, there was no difference in the mean survival rate of these two groups (**Fig 2**). This result suggests that the expression of B7-1 was not sufficient to induce protective immunity against B16 melanoma.

To evaluate the effects of IL-18 on B16 tumorigenesis, tumor inoculated mice were treated with IL-18 i.p. injection for 7 d. **Figure 2** shows that the tumor growth and mean survival in the IL-18-treated group were similar to those of the mock-transduced group, which indicated that IL-18 treatment was not effective.

Next, we evaluated whether there were any synergistic anti-tumor effect by combining the systemic administration of IL-18 and B7-1 expression [IL-18 + B7-1] upon B16 melanoma. As can be seen from the data in **Fig 2**, [IL-18 + B7-1] significantly suppressed tumor growth and enhanced mean survival days in C57BL/6 mice ($p < 0.05$). It is known that B7-1 expression on tumor cells and systemic administration of IL-12 [IL-12 + B7-1] can produce a complementary anti-tumor effect by different mechanisms (Coughlin *et al*, 1995). Based on this information, the synergistic anti-tumor effect, in terms of tumor growth and survival, in mice treated with [IL-18 + B7-1] was compared with the results obtained from mice that were treated with [IL-12 + B7-1]. Although mice exposed [IL-12 + B7-1] also were significantly suppressed the tumor growth, the treatment did not improve the mean survival days (**Fig 2**). These results suggest that [IL-18 + B7-1] might be an effective treatment for melanoma.

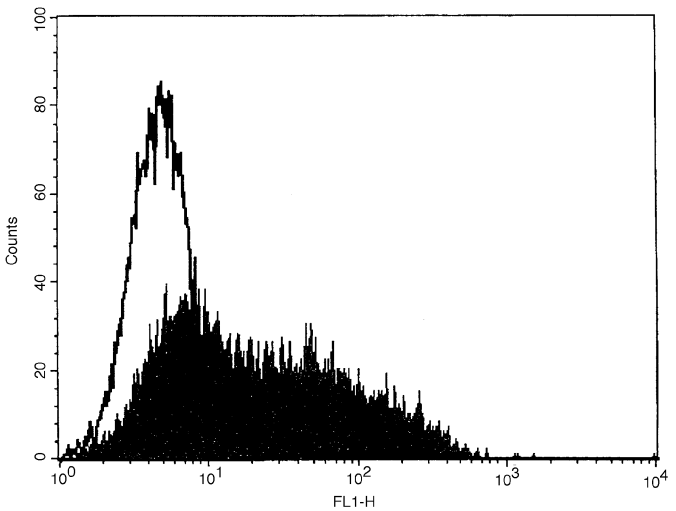

Figure 1. Cell surface expression of B7-1 molecules on transduced B16 melanoma. The B7-1 transduced B16 melanoma cells were incubated with either the control MoAb (open histogram) or rat anti-mouse B7-1 MoAb (closed histogram). Surface expression was then determined with fluorescein isothiocyanate-conjugated goat anti-rat IgG using a flow cytometry. A total of 10,000 cells was analyzed in each sample. Percentage of gated cells was 46.99. Similar results were obtained in three other experiments.

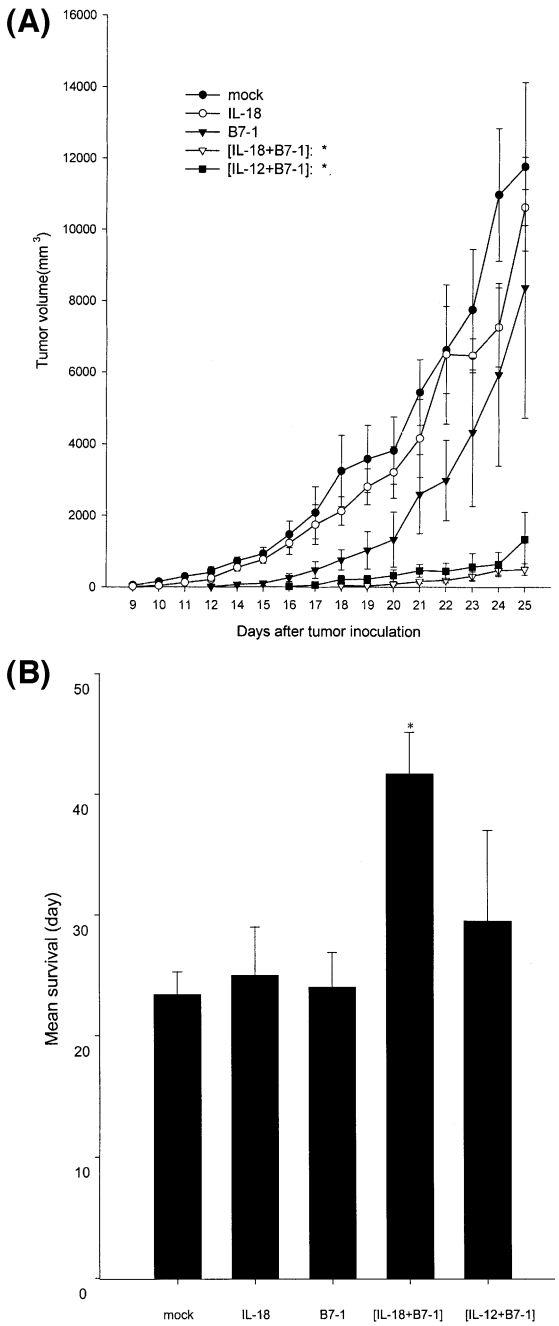

Figure 2. Significant effects of [IL-18 + B7-1] on B16 melanoma growth and survival. Groups of five mice were inoculated subcutaneously with mock or B7-1-transduced B16 melanoma, and then received daily i.p. injections of PBS, 1 μg of IL-18, or 0.5 μg of IL-12 for 7 d, as presented in **Table I**. (*A*) Tumor volume was measured every 2–3 d as described in *Materials and Methods* and the volume was presented as the mean ± SD, n = 5. Significant suppression of B16 melanoma growth was detected only in [IL-18 + B7-1] and [IL-12 + B7-1] (*$p < 0.05$). (*B*) After tumor inoculation, mice were monitored daily for survival. Treatment with [IL-18 + B7-1] significantly increased survival days (*$p < 0.05$), but treatment with IL-18 or B7-1 separately did not positively effect in survival. Additionally, mice treated with [IL-12 + B7-1] did not show significant anti-tumor effect in terms of survival. Bars represent mean ± SD. Similar results were obtained in two other experiments.

Effect of [IL-18 + B7-1] on tumor metastasis As [IL-18 + B7-1] was found to be a potent inhibitor of tumor growth and to offer a substantial improvement in survival, we hypothesized that its anti-tumor effect could lead to the inhibition of lung metastasis of the B16 melanoma. The data shown in **Fig 3** demonstrate that mice treated with [IL-18 + B7-1] were without lung metastases, whereas lung metastases were found in the other treatment group including [IL-12 + B7-1]-treated mice. This result might explain why [IL-12 + B7-1] failed to promote survival, although it did significantly inhibit tumor growth.

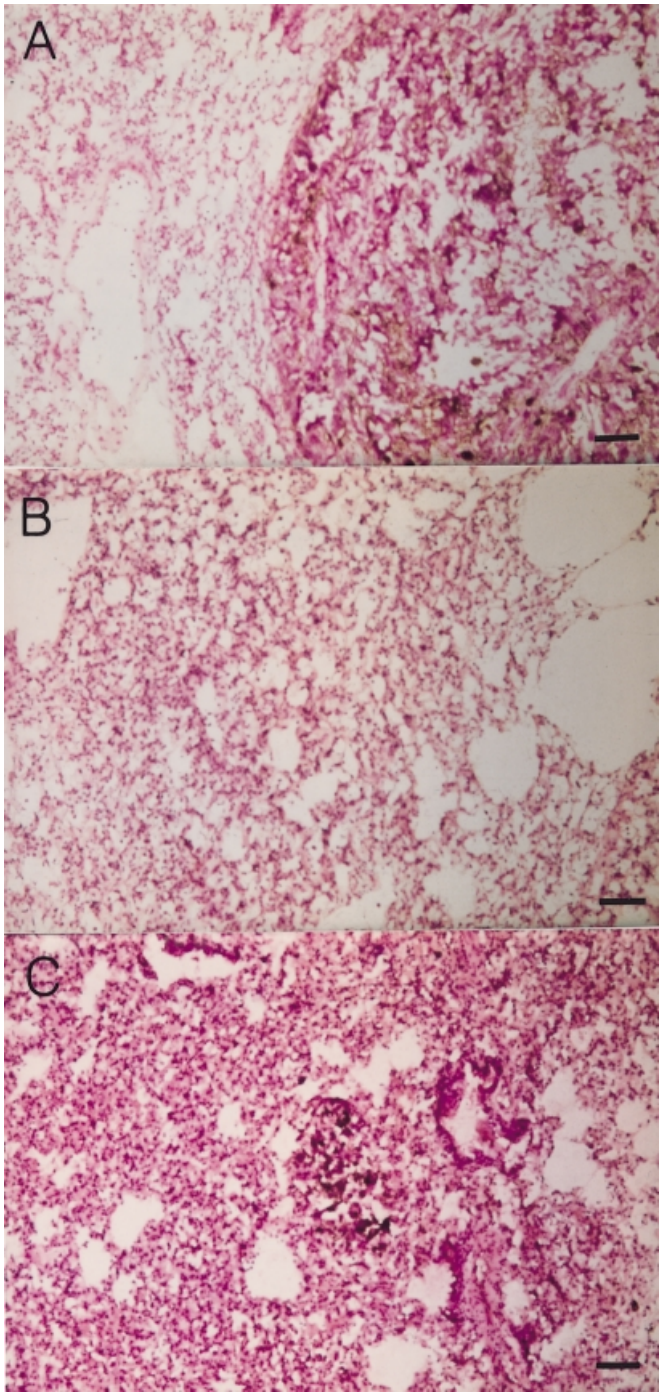

Figure 3. Inhibition of lung metastases in mice treated with [IL-18 + B7-1]. Mice were injected s.c. with mock or B7-1 transduced tumor cells (day 0). Then, mice were injected i.p. with PBS (*A*), IL-18 (*B*), or IL-12 (*C*) for 7 d. On day 25, lungs of three mice in each group were collected, and evaluated the metastases through serial sectioned histologic analysis (hematoxylin and eosin stain). Representative photos of mock control (*A*), [IL-18 + B7-1] (*B*), and [IL-12 + B7-1] (*C*) are shown. Similar results were obtained in two other experiments. *Scale bar*: 50 μm.

Enhancement of natural killer cytolytic activity by [IL-18 + B7-1] *in vivo* Previous reports have shown that both natural killer cells and T cells may be involved in the rejection of tumors treated with cytokines or B7-1 gene transfection (Coughlin *et al*, 1995; Zheng *et al*, 1996; Tadashi *et al*, 1998). To characterize the effector cells involved in the anti-tumor effect of [IL-18 + B7-1], immunohistochemical analysis was performed to detect invading the immune cells into the tumor tissues. Cells expressing natural killer cell markers ($NK1.1^+$) were not detected in the tumor tissues of mice treated with mock-transfected B16 melanoma (**Fig 4*A***), but many $NK1.1^+$ cells were found in the tumor tissues of [IL-18 + B7-1] (**Fig 4*B***). To determine whether natural killer cells contribute to the synergistic anti-tumor effect [IL-18 + B7-1] *in vivo*, we evaluated the natural killer cytolytic activity of splenic lymphocytes. A significant enhancement of natural killer cytolytic activity was observed from mice treated with [IL-18 + B7-1] (**Fig 5*A***). We could not detect a significant augmentation of natural killer cytotoxicity from the other groups of mice. Additionally, we tested CTL activity, but failed to detect enhancement of CTL cytolysis by [IL-18 + B7-1], suggesting that CTL may not be induced in [IL-18 + B7-1] of B16 melanoma (**Fig 5*B***).

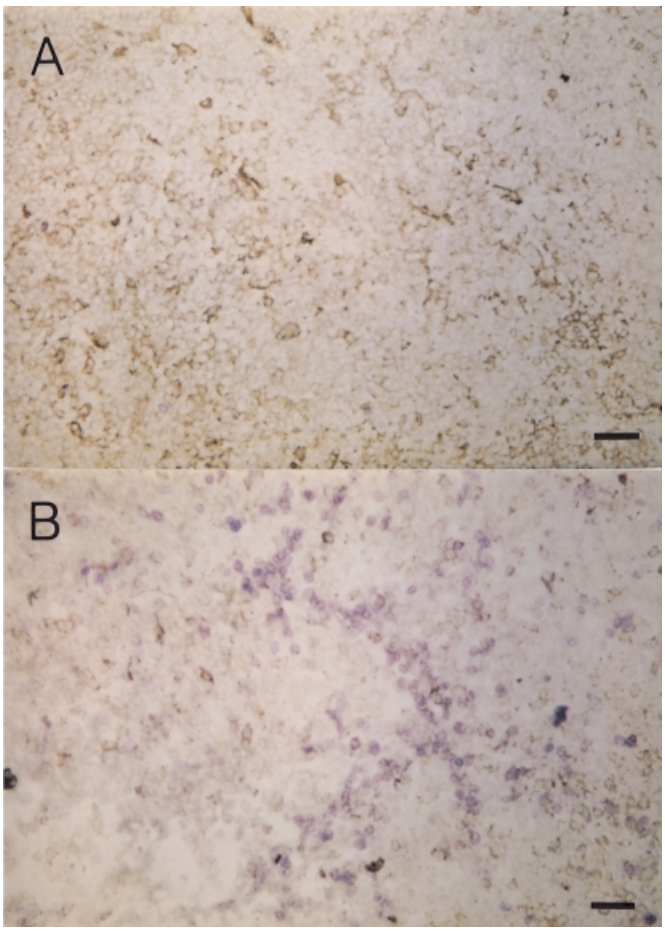

Figure 4. Infiltration of $NK1.1^+$ cells at tumor tissue in mice treated with [IL-18 + B7-1]. Mice were inoculated with 1×10^5 mock or B7-1 transduced tumor cells, then were injected i.p. with PBS, IL-18 or IL-12 for 7 consecutive days. Tumors (three mice in each group) were excised on day 25 and the infiltration of natural killer cells by immunohistochemical staining was evaluated with anti-NK1.1 MoAb. Representative photos of mock control (*A*) and [IL-18 + B7-1] (*B*) are shown. A dense infiltration of $NK1.1^+$ cells was only detected in the tumor of [IL-18 + B7-1]. The same results were obtained in two other experiments. *Scale bar*: 50 μm.

It is well known that activated natural killer cells can produce various cytokines, including IFN-γ (Trinchieri, 1989). To analyze whether [IL-18 + B7-1] induces productions of IFN-γ *in vivo*, we tested the serum levels of IFN-γ in mice using enzyme-linked immunosorbent assay. Whereas mice of other groups did not produce a measurable IFN-γ in serum, [IL-18 + B7-1] and [IL-12 + B7-1] induced significant levels of IFN-γ. [IL-18 + B7-1] produced higher levels of serum IFN-γ than [IL-12 + B7-1] (**Table I**). The difference in natural killer cytotoxicity and IFN-γ levels *in vivo* between [IL-18 + B7-1] and [IL-12 + B7-1] might be the reason why [IL-12 + B7-1] did not effectively inhibit lung metastasis of mice. Collectively, these data suggest that the natural killer cells have a fundamental role upon the anti-tumor effect of [IL-18 + B7-1] in B16 melanomas.

Requirement of NK1.1$^+$ and CD3$^+$ cells for the synergistic anti-tumor effect of [IL-18 + B7-1] In order to determine the nature of natural killer cells responsible for the synergistic anti-tumor effect of [IL-18 + B7-1] *in vivo*, mice were depleted of NK1.1$^+$ cells by injecting anti-NK1.1 MoAb before immunization of [IL-18 + B7-1]. As expected, this group of mice showed suppression of natural killer cytotoxicity, enhancement of tumor growth, inhibition of natural killer cell infiltration to the tumor site, and metastatic deposition of melanoma in the lungs (**Figs 6**, **7**). Thus, we believe that natural killer cells are key effector cells involved in the synergistic anti-tumor effect of [IL-18 + B7-1]. To characterize further the role of subsets lymphocytes in the anti-tumor effect of [IL-18 + B7-1], mice were injected with MoAb specific for the CD3 to deplete T cells selectively *in vivo* before immunization of [IL-18 + B7-1]. Removal of CD3$^+$ T cells reduced natural killer cytotoxicity and infiltration of natural killer cells to the tumor site. Micrometastases in the lung was also detected, whereas mice treated with [IL-18 + B7-1] were absent

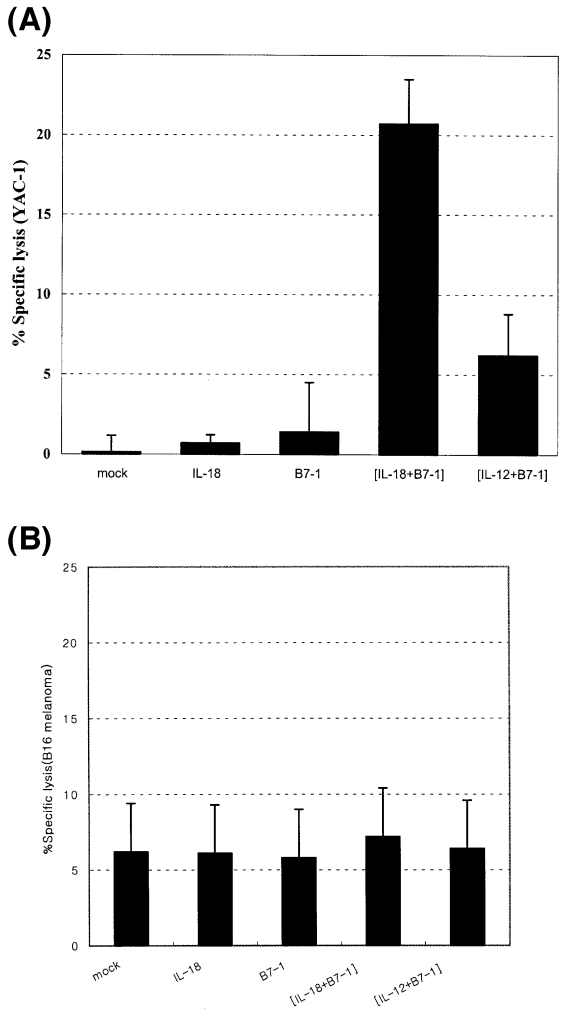

Figure 5. Comparison of natural killer cytotoxicity and CTL activity in mice treated with [IL-18 + B7-1]. The mock or B7-1 transduced tumor cells (1×10^5 cells) were injected subcutaneously into C57BL/6 syngeneic mice (day 0). After tumor inoculation, mice were treated with PBS, IL-18 or IL-12 for 7 d. Twenty-five days later, spleen cells were harvested. (*A*) Natural killer cytotoxicity was determined by the 4 h ^{51}Cr-release assay. YAC-1 cells were used as targets and the effector to target (E/T) ratio was 200:1. (*B*) To test CTL activity, spleen cells were cocultivated for 7 d with γ-irradiated wild-type B16 melanoma cells. CTL activity against ^{51}Cr-labeled wild-type B16 melanoma cells measured in a standard 4 h ^{51}Cr-release assay (E/T ratios = 20:1). Error bars represent the SD of the mean. This datum is a representative of three experiments.

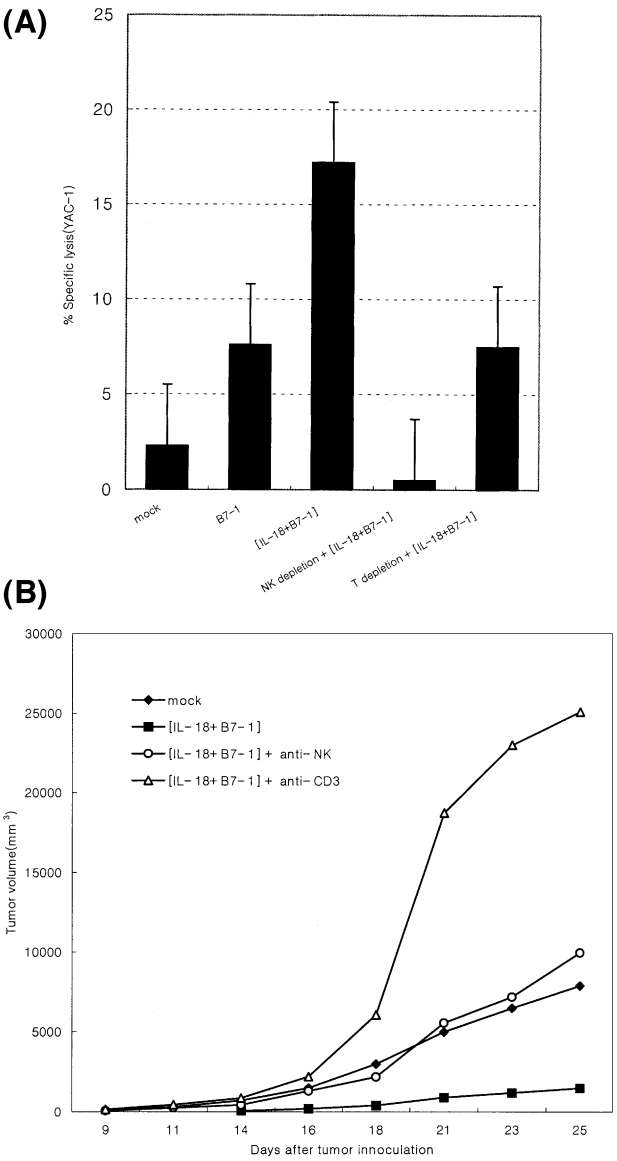

Figure 6. Natural killer cytotoxicity and tumor development in mice depleted of NK1.1$^+$ and CD3$^+$ cells. Mice (n = 5 per group) were injected i.v. with purified mouse anti-NK1.1 or CD3 MoAb at days –1, 0, and 1. On day 0, the mice were inoculated s.c. with mock or B7-1 transduced tumor cells. After then, mice were injected i.p. with PBS, or IL-18 (1 μg) for 7 d. (*A*) On day 25, spleen cells were harvested and used to measure natural killer cytotoxicity with ^{51}Cr-labeled YAC-1 target cells (E/T = 200:1). (*B*) After tumor inoculation, mice were monitored every 2–3 d for the measurement of tumor growth in each group of mice (n = 5). Error bars represent the SD of the mean. Similar results were detected in two other experiments.

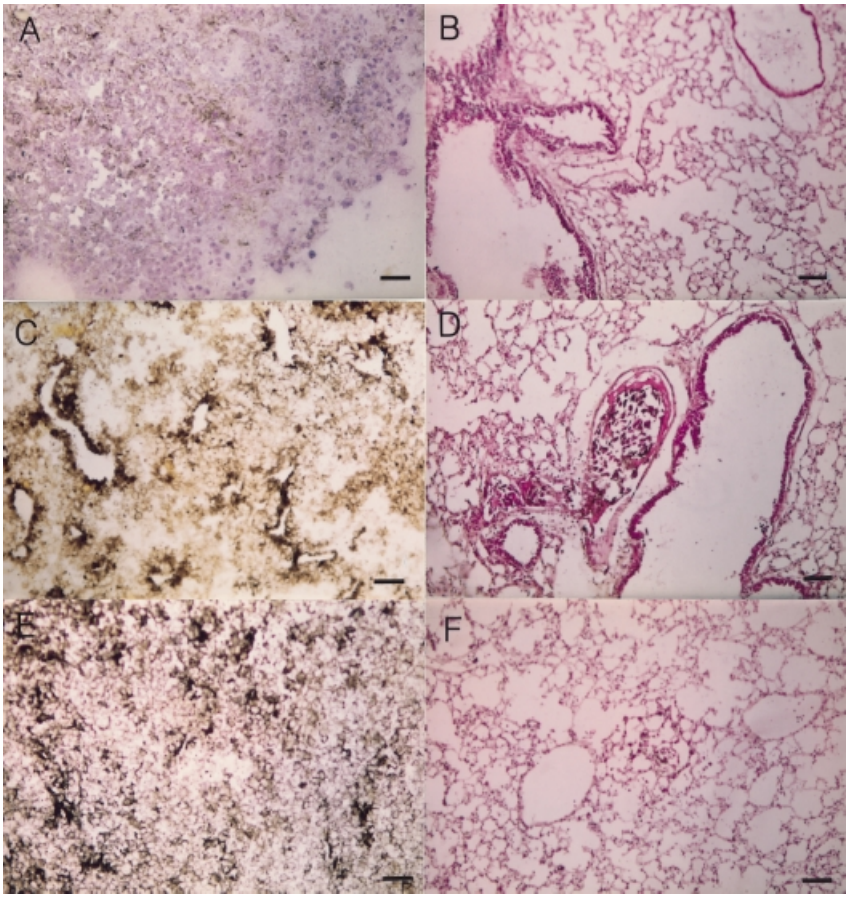

Figure 7. Effects of depleting NK1.1$^+$ or CD3$^+$ cells before [IL-18 + B7-1] immunization on the infiltration of natural killer cells and lung metastases. To deplete NK1.1$^+$ or CD3$^+$ cells *in vivo*, mice (n = 5 per group) were injected i.v. with 100 μg of anti-NK1.1 or CD3 MoAb on days −1, 0, and 1. After inoculation of B7-1 transduced B16 melanoma cells (day 0), mice were treated i.p. with IL-18 (1 μg per mouse) for 7 d. On day 25, tumors and lungs were excised and used to study the infiltration of natural killer cells and lung metastases as described in *Materials and Methods*. (*A*, *C*, *E*) Representative photos of tumors immunostained with anti-NK1.1 MoAb. (*B*, *D*, *F*) Representative photos of lungs stained with hematoxylin and eosin. (*A*, *B*, control mice; *C*, *D*, mice depleted of NK1.1$^+$ cells; *E*, *F*, mice depleted of CD3$^+$ cells.) *Scale bar*: 50 μm.

lung metastases (**Figs 6*a*, 7**). The effect of depleting CD3$^+$ T cells was most evident in the development of tumor growth. Mice that were depleted CD3$^+$ T cells dramatically increased tumor growth and the enhancement was much higher compared with natural killer cell-depleted mice (**Fig 6*b***). In addition to natural killer cells, these results suggest that CD3$^+$ T cells are largely responsible for the delay in tumor development seen with the synergistic anti-tumor effect of [IL-18 + B7-1].

DISCUSSION

In this study, we have evaluated the synergistic ability of the systemic administration of IL-18 and the expression of B7-1 molecules on tumor cells to promote the regulation of B16 melanoma in C57BL/6 mice. The data demonstrate that [IL-18 + B7-1] induced significant anti-tumor immunity against B16 melanoma. In addition, we showed that [IL-18 + B7-1] induced natural killer cell infiltration into tumor and enhanced natural killer cytolytic activity *in vivo*. The augmented natural killer cytolytic function correlated well with an enhanced level of IFN-γ the suppression of lung metastases, and an improvement in survival. Additionally, depletion of NK1.1$^+$ cells *in vivo* confirmed the importance of natural killer cells in the synergistic anti-tumor effect of [IL-18 + B7-1].

IL-18 was recently shown to have significant anti-tumor effect against CL8–1, a mouse melanoma cell line (Tadashi *et al*, 1998), and the anti-tumor effect found to be mediated by natural killer cells and CD4$^+$ T cells. In addition, a combination of IL-18 and IL-12 significantly enhanced anti-tumor activity in mice inoculated with CL8–1 cells. This study, however, shows that IL-18 did not induce any protective immunity against B16 melanoma, as shown in **Fig 1**. This difference may due to tumor cell characteristics. It is well known that B16 melanoma is poorly immunogenic (Sun *et al*, 1992). Therefore, treatment with IL-18 may not be enough to induce protective immunity against B16 melanoma, even though the systemic injection of IL-18 is effective against CL8–1 tumor. It is also possible that other factors may explain this difference, such as different dose levels of the cytokine, routes and times of injection, or differences in the mouse strain.

The ability of B7-1 costimulatory molecules on antigen-presenting cells to enhance natural killer and CTL cytolytic activity suggests that this molecule should be therapeutically beneficial. Although it has been shown that B7-1 has a potent anti-tumor effect, Chen *et al* (1994) demonstrated that the anti-tumor effects of B7-1 is dependent upon the immunogenicity of the tumor cells, indicating that the expression of the costimulatory B7-1 molecules on the surface of tumor cells could not in itself induce adequate protective immune responses against nonimmunogenic malignancies. Indeed, our study on the anti-tumor efficacy of B7-1 showed it only has a capacity to delay tumor development (**Fig 2*A***). It had little or no effect on the suppression of lung metastasis (data not shown), enhancement of natural killer cytotoxicity *in vivo* (**Fig 5**), or the infiltration of natural killer cells into tumor tissue (data not shown). Coughlin *et al* (1995) demonstrated that IL-12 in combination with B7-1 could synergistically induce protective immunity against SCK tumors, a poorly immunogenic mammary carcinoma. In this study, [IL-12 + B7-1] was applied as a positive control. Unexpectedly, [IL-12 + B7-1] treatment did not offer a significant anti-tumor effect on metastasis and survival but delay the formation of tumor (**Figs 2** and **3**). These results suggest that B16 melanoma might be less immunogenic than SCK mammary carcinoma and that IL-18 might be the better choice to induce synergistic anti-tumor effect with B7-1. We cannot exclude the importance of IL-12 against melanoma, however, as the effects of IL-12 are dependent on variable factors such as doses, different times of injections, and different ways of injections, etc.

We examined the possibility that T cells were involved in the synergistic anti-tumor effect of [IL-18 + B7-1]. As mentioned

previously, other researchers reported that IL-18 or B7-1 could augment the cytolytic functions of T cells as well as those of natural killer cells (Chen *et al*, 1992; Linsely and Ledbetter, 1993; Okamura *et al*, 1995; Chambers *et al*, 1996; Geldhof *et al*, 1998). To test the involvement of T cells in the case [IL-18 + B7-1] treatment, the infiltration of T cells into tumor tissue and the induction of CTL *in vivo* were analyzed using immunohistochemical analysis and CTL assay, respectively. We could not detect any infiltration of $CD3^+$ T cells in tumor tissue, and failed to detect CTL activity *in vivo* in mice treated with [IL-18 + B7-1]. Thus, we speculated that the contribution of B7-1 expression by tumor cells is mainly mediated through natural killer cells in the synergistic effect of [IL-18 + B7-1]. $CD3^+$ T cell ablation studies, however, demonstrate that $CD3^+$ T cells are required for the anti-tumor activity of [IL-18 + B7-1]. In fact, the pattern of tumor development in $CD3^+$ T cell-depleted mice was more dramatic than that in $NK1.1^+$ cell-depleted mice. In contrast, the requirement for $CD3^+$ T cells is less absolute in the inhibition of lung metastases. Mice depleted of $CD3^+$ T cell only developed micrometastases in the lungs, whereas deposit of melanoma was detected in the mice-depleted $NK1.1^+$ cells. These data were well correlated with the difference of natural killer cytotoxicity between these two groups (**Fig 6**). Additional studies are clearly needed to define the mechanisms of natural killer and T cells involved in the synergistic anti-tumor effect of [IL-18 + B7-1]. Nevertheless our data strongly suggest that natural killer and T cells are the key effector cells, which are involved in the synergistic anti-tumor effect of [IL-18 + B7-1].

In conclusion, molecular transfection of costimulatory molecule B7-1 on tumor cells and systemic administration of IL-18 enhance host's protective immune system against the poorly immunogenic B16 melanoma. It is hoped that this study may lead the advancement for the treatment of malignant melanoma.

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