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Inhibition of pulmonary metastasis by Z-100, an immunomodulatory lipid-arabinomannan extracted from *Mycobacterium tuberculosis*, in mice inoculated with B16 melanoma

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The anti-metastatic effect of Z-100, an immunomodulatory arabinomannan extracted from *Mycobacterium tuberculosis*, was investigated in mice bearing B16 melanoma cells. Treatment of BF10 mice implanted with high metastatic B16F10 melanoma cells with a 10 mg/kg dose of Z-100 resulted in the reduction of experimental pulmonary metastasis as compared with that of BF10 mice treated with saline. The number of pulmonary metastatic colonies in BF1 mice (mice implanted with low metastatic B16F1 melanoma cells) was greatly increased after the inoculation of $CD4^+$ $CD11b^+$ $CD28^+$ $TCR\alpha\beta^+$ type 2 T cells (F10-T_h2 cells) derived from BF10 mice, while only a few metastatic colonies were demonstrated in lungs of BF1 mice inoculated with naive $CD4^+$ T cells. However, the numbers of metastatic colonies in BF1 mice were not increased when they were inoculated with the F10-T_h2 cell fraction derived from Z-100-treated BF10 mice and the generation of F10-T_h2 cells in BF10 mice was effectively suppressed by the Z-100 treatment. These results suggest that Z-100 inhibits pulmonary metastasis of B16 melanoma through the regulation of tumor-associated T_h2 cells, which are a key cell in the acceleration of tumor metastasis.

Key words: Arabinomannan, B16 melanoma, immunotherapy, pulmonary metastasis, T_h2 cells.

Introduction

Type 1 T cell response is an up-regulatory cellular immune response associated with increased levels of type 1 cytokines interleukin-2 (IL-2) and interferon (IFN)-γ and the generation of cytotoxic T lymphocytes (CTLs), a typical type 1 effector cell. It has been well recognized that antitumor immunotherapy is established, in certain cases, through the activation of type 1 T cell responses and the suppression of these T cell responses has been shown to lead the accelerating tumor growth.[1–3] Type 1 T cell responses are suppressed by T helper type 2 cells (T_h2 cells) and $CD8^+$ type 2 T cells, generated in response to the growth of tumors.[1,2] Type 2 cytokines (IL-4 and IL-10) release from type 2 T cells have been suggested as effector cytokines for the inhibition of type 1 T cell responses.[4–6] Therefore, type 2 T cells and their type 2 cytokine products may lead to unsuccessful cancer immunotherapies in patients with malignancies. In this meaning, the elimination of type 2 T cells and their type 2 cytokine products from tumor-bearing hosts may cause successful anti-cancer immunotherapies and restore natural antitumor resistance.

In Japan a lipid-arabinomannan (Z-100) extracted from *Mycobacterium tuberculosis*[7] has been used clinically against leukopenia induced by X-irradiation. Antitumor effects of Z-100 have also been demonstrated in patients and animals with malignancies.[8,9] The antitumor activity of Z-100 was not demonstrated in mice depleted of $CD4^+$ T cells.[9] IL-3 was demonstrated in culture fluids of $CD4^+$ T cells stimulated with Z-100 *in vivo* and *in vitro*,[10,11] and the tumor growth was inhibited when mice bearing Meth-A solid tumors were treated intraleasionally with IL-3-containing culture fluids of $CD4^+$ T cells derived from Z-100-treated mice. These data suggest that the anti-tumor activity of Z-100 may be expressed, in part, through a function of $CD4^+$ T cells or their cytokine products (IL-3). Recently Z-100 has been shown to inhibit the generation of $CD8^+$ type 2 T cells in spleens of mice exposed to thermal injury.[12] Since the susceptibility of thermally injured mice to herpesvirus infections was correlated on the appearance of burn-associated $CD8^+$ type 2 T cells,[13] the resistance of these mice to the infection was effectively improved after the treatment with Z-100.[12] The production of type 2 cytokines from burn-associated $CD8^+$ type 2 T cells was also

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reduced in thermally injured mice treated with Z-100.[12]

In the present paper the anti-metastatic activity of Z-100 in mice bearing B16 melanoma cells was studied. In our preliminary studies the experimental pulmonary metastasis of B16 melanoma was closely related to its abilities to induce type 2 T cells, because the metastasis was developed in mice inoculated with B16F1 melanoma cells, a low metastatic clone of B16 melanoma cells, when these mice were inoculated with splenic lymphocytes (type 2 T cells) from mice bearing B16F10 melanoma cells, a high metastatic clone of B16 melanoma cells.

Materials and methods

Animals

Eight-week-old C57BL/6 and BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used in these experiments. All animal experiments were approved by the Animal Care and Use Committee of the University of Texas Medical Branch at Galveston (ACUC approval no.: 89-03-066).

Regents

Cytokines (rIL-2, rIL-4 and rIL-10) and mAbs (anti-IFN-γ, anti-IL-2, anti-IL-4, anti-IL-10, anti-CD11b, anti-CD28, anti-TCR$\alpha\beta$ and anti-TCR$\gamma\delta$) were purchased from PharMingen (San Diego, CA). Anti-CD3 mAb was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Anti-L3T4 and anti-Lyt 2.2 mAbs were obtained from Accurate Chemical and Scientific (Westbury, NY). Anti-mouse immunoglobulin (anti-Ig) antiserum (Cappel Laboratory, Cochranville, PA) and Low-Tox-M rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) were also used in these experiments.

Z-100

Z-100, a lipid-arabinomannan extracted from the Aoyama B strain of *M. tuberculosis*,[7] was supplied from Zeria Pharmaceutical (Tokyo, Japan). Z-100 was dissolved in saline at a concentration of 1 mg/ml and diluted to appropriate concentrations by saline when it was used in animal experiments. In the majority of experiments Z-100 was administered i.p. to mice at a dose of 10 mg/kg, 1, 3, 5 and 7 days after the tumor inoculation.[12]

Preparation of splenic lymphocytes

Mononuclear cells (SMNC) were prepared from spleens of tumor-bearing mice treated with or without Z-100.[9] To prepare whole T cells, $CD4^+$ T cells and $CD8^+$ T cells, SMNC (1×10^7 cells/ml) were passed through a T cell enrichment column (R&D Systems, Minneapolis, MN), CD4 T cell subset column (R&D Systems) and CD8 T cell subset column (R&D Systems), respectively.[12,13] After washing with column buffer (R&D Systems), the eluted cells were used for the experiments. When whole T cells obtained in this procedure were treated with anti-Ig antiserum and complement, only a 3% reduction in viable cells was demonstrated, whereas treatment of these cells with anti-CD3 mAb followed by complement caused a 98% reduction in the number of viable cells. When $CD4^+$ or $CD8^+$ T cells obtained in the above procedures were treated with anti-L3T4 mAb followed by complement, 96 or 3% of viable cells were lysed. When they were treated with anti-Lyt 2.2 mAb followed by complement, 2 or 97% of viable cells were lysed, respectively. These results suggested that the purity of these three cell preparations (whole T cells, $CD4^+$ T cells and $CD8^+$ T cells) was more than 96%. In order to determine the phenotypic properties of tumor-associated type 2 T cells, $CD4^+$ T cells from tumor-bearing mice were further treated with various mAbs (4°C, 30 min) followed by complement (1:30 dilution, 37°C, 30 min), as described previously,[12,13] and residual cells were assayed for their type 2 cytokine-producing abilities.

Experimental pulmonary metastasis

A low metastatic strain (B16F1 cells) and high metastatic strain (B16F10 cells) of B16 melanoma cells,[14] provided by Tohoku University School of Medicine, Sendai, Japan, were grown *in vitro* with RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), antibiotics and 1% L-glutamine, and store at −70°C. B16F1 cells or B16F10 cells, maintained six to 12 times after regrowth from frozen stocks, were inoculated i.v. to mice. Just before inoculation to mice, cells in a log growth phase were detached from tissue culture flasks with a mixture of 0.25% trypsin and 0.03% EDTA. The cells harvested were washed twice and suspended in phosphate-buffered saline. Then 0.2 ml of the suspension was injected i.v. into mice (2×10^5 cells/mouse). C57BL/6 mice inoculated i.v. with B16F1 cells or B16F10 cells were designated as BF1 mice or

BF10 mice, respectively. As required, Z-100 at doses ranging from 0.1 to 100 mg/kg was administered i.p. to these mice 1, 3, 5 and 7 days after tumor inoculation.[12] Fourteen days after the tumor inoculation mice were sacrificed and lung tissues of these mice were fixed with 10% formaldehyde solution.[14] The numbers of metastatic colonies in the lungs were counted under a dissecting microscope.[14] Except for lungs, the metastatic colonies were not observed in any organs of BF10 mice 2 weeks after the tumor cell inoculation.[14] In some experiments, various numbers of splenic lymphocytes from BF1 mice or BF10 mice treated with or without Z-100 were adoptively transferred i.v. to BF1 mice (3 days after the tumor inoculation). The pulmonary metastasis of recipient mice 11 days after the adoptive transfer was calculated as described above. Each assay was performed a minimum of three times and representative data was indicated.

Assay of suppressor cell activity

As described previously,[15] the suppressor cell activity of various lymphocyte preparations was measured in a one-way mixed lymphocyte reaction (MLR). Responders (SMNC from naive C57BL/6 mice, 5×10^4 cells/well) and stimulators (SMNC from naive BALB/c mice, 5×10^4 cells/well) were co-cultured with or without putative suppressors (lymphocytes from tumor-bearing mice, 2.5×10^5 cells/well) in a 96-well round-bottomed microtiter plate for 5 days at 37°C in 5% CO_2. Before being utilized to the MLR, all cells except responders were treated with mitomycin C, as described previously.[15] [^{3}H]Thymidine (0.5 μCi/well) was added to the plate for the last 12 h of the incubation and the uptake of [^{3}H]thymidine by responders was measured. The suppressor cell activity was calculated using the following formula: suppression (%) = [1 − (c.p.m. in the presence of putative suppressor cells/c.p.m. in the absence of putative suppressor cells)] × 100.[16]

Measurement of cytokine activities

For *in vitro* induction of IL-4 and IL-10, 2×10^6 cells/ml of whole T cells from BF1 mice or BF10 mice, which were previously treated with or without Z-100, were stimulated with anti-CD3 mAb (2.5 μg/ml) for 24–72 h at 37°C.[16] Culture supernatants harvested from these cultures were assayed for their IL-4 and IL-10 activities by the growth of CTLL-2 cells, an IL-2- and IL-4-dependent T cell line.[17] Briefly, 5×10^3 CTLL-2 cells were suspended in 100 μl of media and plated in 96-well microtiter plates in triplicate. Then, 100 μl of serially diluted assay samples was added to each well in the presence of either anti-IL-2 mAb (20 μg/ml) or anti-IL-4 mAb (10 μg/ml), and the plates were incubated for 18 h at 37°C in 5% CO_2. [^{3}H]Thymidine (0.5 μCi/well) was added to each well 4 h before being harvested. The incorporation of [^{3}H]thymidine into CTLL-2 cells was measured by a liquid scintillation counter. As a control, various concentrations of rIL-2 or rIL-4 were added to the appropriate wells to get standard curves. The IL-10 activity was assayed by an ELISA technique using anti-IL-10 mAb.[18] The assay was performed three times and the results were expressed as the mean of these three tests. The IFN activities were measured by means of a plaque reduction assay utilizing L-Galveston cells infected with vesicular stomatitis virus, as described previously.[9] The IFN titer was determined by the reciprocal of the greatest dilution of the test sample that reduced virus plaques by 50%. The IFN titer obtained was standardized to international units using reference murine IFN-γ (G-002-904-511).

Statistical analysis

The number of pulmonary metastasis and the titer of cytokines were statistically analyzed by the Student's *t*-test. If $p < 0.05$, the result obtained was considered significant.

Results

Effect of Z-100 on experimental pulmonary metastasis in BF10 mice

In the first experiment the inhibitory effect of Z-100 on experimental pulmonary metastasis of B16F10 melanoma was investigated in mice (BF10 mice). As shown in Figure 1, metastatic colonies were first demonstrated in lungs of BF10 mice 6 days after inoculation with 2×10^5 cells/mouse of B16F10 melanoma cells, while it was first detected in Z-100-treated BF10 mice 9 days after the tumor inoculation. Up to 250 colonies on average were observed in lungs of BF10 mice 14 days after tumor inoculation, although, a 70% reduction in numbers of metastatic colonies in BF10 mice treated i.p. with a 10 mg/kg dose of Z-100 was demonstrated. The dose-dependent anti-metastatic effect of Z-100 is shown in Figure 2. Doses of Z-100 greater than

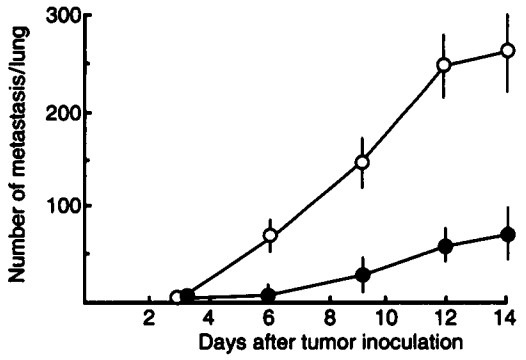

Figure 1. Pulmonary metastasis in BF10 mice treated with Z-100. One hundred mice inoculated i.v. with 2×10^5 cells/mouse of B16F10 cells (BF10 mice) were divided into 10 small groups. A half of them (●) were treated i.p. with 10 mg/kg of Z-100 1, 3, 5 and 7 days after tumor inoculation. As a control, the other (○) was treated with saline (0.2 ml/mouse). Various days (shown in the figure) after the tumor inoculation, lungs were removed from each group of 10 mice and numbers of pulmonary metastatic colonies were determined under a dissecting microscope. Data is shown as the mean number of metastatic colonies ± SEM.

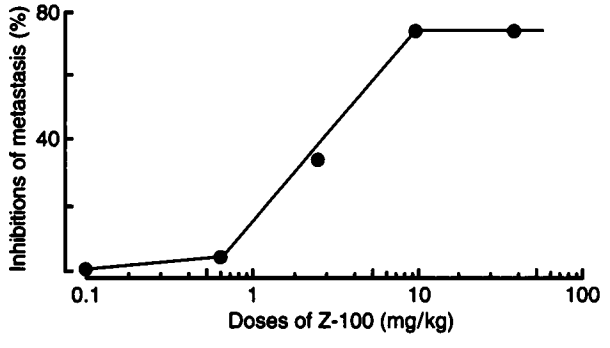

Figure 2. A dose-dependent inhibitory effect of Z-100 on the pulmonary metastasis in BF10 mice. BF10 mice (10 mice each) were treated i.p. with various doses of Z-100 1, 3, 5 and 7 days after tumor inoculation. The pulmonary metastasis in these mice was determined 14 days after the tumor implantation, as described in Figure 1.

10 mg/kg expressed the maximum inhibitory activity (greater than 70% inhibition of the pulmonary metastasis). However, the inhibitory activity of Z-100 on the metastasis was not observed when it was administered to BF10 mice at a dose of 0.6 mg/kg or less. Since 10–1000 μg/ml concentrations of Z-100 did not show any cytotoxic activities on the growth of B16F10 melanoma cells *in vitro* (data not shown), this anti-metastatic effect of Z-100 in BF10 mice may be displayed through the host's antitumor immune functions stimulated with the compound.

Anti-metastatic mechanisms of Z-100

Significant numbers of metastatic colonies were not demonstrated in lungs of BF1 mice (mice inoculated with low metastatic B16F1 melanoma cells) inoculated with naive splenic T cells (Figure 3). However, the pulmonary metastasis in BF1 mice was developed, as the same levels observed in BF10 mice (see Figure 1), when BF1 mice were inoculated i.v. with 2×10^7 cells/mouse of splenic whole T cells from BF10 mice (Figure 3). These results indicated that whole T cells from BF10 mice have a very important function in the development of lung metastasis in BF1 mice.

These whole T cells derived from BF10 mice were identified as type 2 T cells because they expressed suppressor cell activities in the MLR and produced only type 2 cytokines when they were stimulated with anti-CD3 mAb (Table 1). However, the generation of type 2 T cells was not demonstrated in BF1 mice, because splenic whole T cells from these mice did not express their suppressor cell activities and type 2 cytokine-producing abilities. Whole T cells from BF1 mice produced type 1 cytokines into their culture fluids when they were stimulated with anti-CD3 mAb (Table 1). In the next experiment these type 2 T cells generated in BF10 mice were characterized phenotypically. When various T cell preparations (5×10^6 cells/ml) derived from whole T cells of BF10 mice were stimulated with anti-CD3 mAb for 48 h at 37°C, original whole T cells and

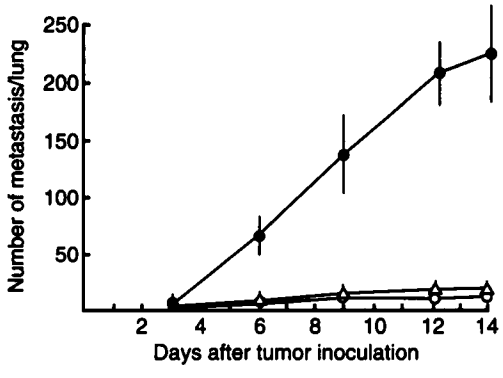

Figure 3. Pulmonary metastasis in BF1 mice inoculated with whole T cells from BF10 mice. About 2×10^7 cells/mouse of whole T cells from BF10 mice (●) or naive mice (△) were adoptively transferred into mice 3 days after inoculation of B16F1 cells. BF1 mice treated with saline serve as a control (○). Various days after tumor implantation, pulmonary metastasis in these mice was determined, as described in Figure 1. Data is shown as the mean colony numbers obtained from 10 mice ± SEM.

Table 1. Suppressor cell activities and cytokine-producing abilities of whole T cells from mice inoculated with high-metastatic B16F10 melanoma cells (BF10 mice) or low-metastatic B16F1 melanoma cells (BF1 mice)

Whole T cell from[a]	Suppression of MLR[b] (%)	Cytokine production[c]			
		IL-2 (U/ml)	IFN-γ (U/ml)	IL-4 (U/ml)	IL-10 (ng/ml)
BF1 mice	0	170	320	< 5	< 0.1
BF10 mice	82	< 5	< 10	300	15

[a]Splenic T cells were prepared from BF1 or BF10 mice 14 days after the tumor inoculation (2×10^5 cells/mouse, i.v.).
[b]Responders (MNC from C57BL/6 mice, 5×10^4 cells/well) and stimulators (MNC from BALB/c mice) were co-cultured with splenic T cells from BF1 or BF10 mice for 5 days in the MLR at a cell ratio of 1:1:5.
[c]Splenic T cells (5×10^6 cells/ml) from BF1 or BF10 mice were stimulated with anti-CD3 mAb (2.5 μg/ml) for 48 h. Cytokine activities in the culture fluids of these cells were assayed by as described in the text.

their $CD4^+$ T cell fraction produced IL-4 (whole T cells, 151 U/ml; $CD8^+$ T cells, 218 U/ml) and IL-10 (whole T cells, 20 ng/ml; $CD8^+$ T cells, 28 ng/ml) into their culture fluids. However, $CD8^+$ T cells purified from whole T cells of BF10 mice did not produce type 2 cytokines even though they were stimulated with anti-CD3 mAb (Table 2, experiment 1). When $CD4^+$ T cells purified from whole T cells of BF10 mice were treated with anti-TCR$\gamma\delta$ mAb followed by complement, their type 2 cytokine-producing abilities were unchanged (Table 2, experiment 2). However, type 2 cytokines were not produced by these cells treated with anti-CD11b mAb, anti-CD28 mAb or anti-TCR$\alpha\beta$ mAb followed by complement (Table 2, experiment 2). These results indicated that $CD4^+$ $CD28^+$ $CD11b^+$ TCR$\alpha\beta^+$ IL-4- and IL-10-producing T cells (F10-T_h2 cells) were generated in spleens of BF10 mice. F10-T_h2 cells may play a role on the development of the pulmonary metastasis in BF10 mice, because the lung metastasis was developed in BF1 mice inoculated with these type 2 T cells.

Therefore, in the next experiment a role of type 2 cytokines produced by F10-T_h2 cells on the pulmonary metastasis in BF10 mice was explored. In this experiment a mixture of anti-IL-4 (200 μg/mouse) and anti-IL-10 mAbs (200 μg/mouse) was administered to mice (0.2 ml/mouse) 1, 3 and 5 days after the inoculation of 2×10^5 cells/mouse of B16F10 melanoma cells. Various days after the tumor inoculation, the lungs were removed from these mice (10 mice each) and the metastatic colonies in the organs

Table 2. Characterization of type 2 T cells generated in spleens of BF10 mice

Cytokine producer cells[a]	Cytokine production[b]	
	IL-4 (U/ml)	IL-10 (ng/ml)
Experiment 1		
T cells from naive mice	< 5	< 0.1
whole T cells from BF10 mice	151 ± 12[c]	20 ± 4[c]
$CD4^+$ T cells from BF10 mice	218 ± 39[c]	28 ± 3[c]
$CD8^+$ T cells from BF10 mice	< 5	< 0.1
Experiment 2		
T cells from BF10 mice (effector cells)	250 ± 42	31 ± 3
effector cells depleted of $CD11b^+$ cells	12 ± 5[c]	0.7 ± 0.2[c]
effector cells depleted of $CD28^+$ cells	8 ± 3[c]	< 0.1[c]
effector cells depleted of TCR$\alpha\beta^+$ cells	< 5[c]	< 0.1[c]
effector cells depleted of TCR$\gamma\delta^+$ cells	238 ± 30	26 ± 4

[a]SMNC were prepared from mice 14 days after the inoculation of B16F10 melanoma cells (2×10^5 cells/mouse, i.v.). Whole T cells, $CD4^+$ T cells or $CD8^+$ T cells were purified from these SMNC by affinity columns (see text). $CD4^+$ T cells from BF10 mice were further treated with various mAbs plus complement, as described in the text.
[b]All cell preparations were stimulated with anti-CD3 mAb for 48 h for induction of cytokine production. Culture fluids harvested were assayed for their cytokine activities, as described in Table 1.
[c]Student's *t*-test, $p < 0.001$ versus original T cell fraction.

were determined, as described in Methods. As shown in Figure 4, there were no metastatic colonies in lungs of BF10 mice treated with the mixture of anti-type 2 cytokine mAbs, while more than 250 pulmonary colonies were demonstrated in control mice 14 days after the tumor inoculation. This suggests that the experimental pulmonary metastasis of B16F10 melanoma cells may be strongly influenced by type 2 cytokines (IL-4 and IL-10) produced by F10-T_h2 cells.

The pulmonary metastasis of B16 melanoma was developed in BF1 mice when F10-T_h2 cells prepared from BF10 mice were adoptively transferred to these mice (Table 3). However, the metastatic colonies of B16 melanoma were not demonstrated in BF1 mice inoculated with the F10-T_h2 cell preparations derived from Z-100-treated BF10 mice (Table 3). Also, the type 2 T cell activity was not demonstrated in the F10-T_h2 cell preparations from Z-100-treated BF10 mice. When 84% of [^{3}H]thymidine incorporation into responder cells was suppressed by F10-T_h2 cells from BF10 mice in the MLR, only 13% of the MLR was suppressed by the same cell preparation derived from Z-100-treated BF10 mice (Table 4). When splenic $CD4^+$ T cells (F10-T_h2 cells) from BF10 mice were stimulated with anti-CD3 mAb for 48 h, type 2 cytokines were produced in their culture fluids (IL-4, 280 U/ml; IL-10, 25 ng/ml). However, the same cell population from BF10 mice previously treated with Z-100 did not produce significant amounts of type 2 cytokines into their culture fluids (Table 5). These results suggested that T_h2 cells associated with B16F10 melanoma cells

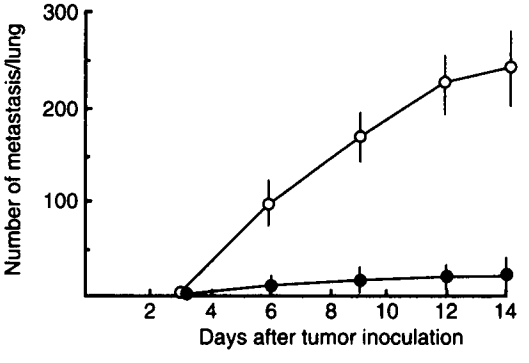

Figure 4. Pulmonary metastasis in BF10 mice treated with a mixture of anti-type 2 cytokine mAbs. A mixture of anti-IL-4 mAb (200 μg/mouse) and anti-IL-10 mAb (200 μg/mouse) was given i.p. to mice 1, 3 and 5 days after the inoculation of B16F10 cells (●). BF10 mice treated with saline (○) serve as a control. Various days after the tumor inoculation, numbers of pulmonary colonies in these mice (10 mice each) were determined, as described in Figure 1.

Table 3. Pulmonary metastasis in BF1 mice inoculated with $CD4^+$ T cells from Z-100-treated BF10 mice

BF1 mice received[a]	Number of metastasis/lung (mean ± SEM)
Saline (Control)	13 ± 4
Z-100	14 ± 2
$CD4^+$ T cells from BF1 mice	12 ± 2
$CD4^+$ T cells from BF10 mice	> 250
$CD4^+$ T cells from BF10 mice treated with Z-100	31 ± 8[b]

[a]Mice, 1 day after implantation of B16F1 melanoma cells (2×10^5 cells/mouse, i.v.), were inoculated with 5×10^6 cells/mouse of $CD4^+$ T cells from BF10 mice treated with or without Z-100 (10 mg/kg, i.p.). Fourteen days after the tumor inoculation, numbers of pulmonary metastatic colonies in these mice (10 mice each) were determined under a dissecting microscope.
[b]Student's *t*-test, $p < 0.001$ versus recipients inoculated with $CD4^+$ T cells from BF10 mice.

Table 4. The suppressor cell activity of $CD4^+$ T cells from of BF10 mice treated with Z-100

MLR was conducted with $CD4^+$ T cells from[a]	c.p.m.[b] (mean ± SEM)	Suppression (%)
Naive mice	12596 ± 1717	—
BF10 mice treated with saline	2016 ± 134	84
BF10 mice treated with Z-100	10959 ± 988[c]	13

[a]Responder cells and stimulater cells were co-cultured with various sources of $CD4^+$ T cells in the one-way MLR, as described in Table 1. $CD4^+$ T cells were prepared from spleens of BF10 mice treated with or without Z-100 (10 mg/kg, i.p.).
[b][^{3}H]Thymidine incorporation into responder cells in the MLR.
[c]Student's *t*-test, $p < 0.001$ versus BF10 mice treated with saline.

Table 5. The production of type 2 cytokines by $CD4^+$ T cells from BF10 mice treated with Z-100

$CD4^+$ T cells from[a]	Cytokine production	
	IL-4 (U/ml)	IL-10 (ng/ml)
BF10 mice treated with saline	280 ± 34	25 ± 3[b]
BF10 mice treated with Z-100	10 ± 2[b]	0.8 ± 0.1[b]

[a]Splenic $CD4^+$ T cells (5×10^6 cells/ml) were prepared from BF10 mice treated with or without Z-100 (10 mg/kg, i.p.). The culture supernatants harvested 48 h after the cultivation were assayed for their cytokine activities as described in Table 1.
[b]Student's *t*-test, $p < 0.001$ versus BF10 mice treated with saline.

and their type 2 cytokine products participated in the development of pulmonary metastasis of B16 melanoma cells. Through the regulation of melanoma cell-associated type 2 T cells (F10-T_h2 cells), Z-100 may exhibit its anti-metastatic activities in BF10 mice and BF1 mice inoculated with F10-T_h2 cells.

Discussion

The development of tumor metastasis is biologically complex and depends on the interplay between host elements and the intrinsic factors of tumor cells.[20] It has been described in previous papers that granulocyte macrophage colony stimulating factor,[21] growth factors [fibroblast growth factor and transforming growth factor (TGF)-β][22] and adhesion molecules[23,24] increase the metastasis of various tumors. Also, it has been reported[25] that pulmonary metastasis of murine melanoma cells was markedly reduced in mice treated with IL-12. Since IL-12 has been shown to induce type 1 T cell responses from predominant type 2 T cell responses,[26] this fact suggests that the tumor metastasis may be influenced by the type 2 T cell responses. It was described in recent studies[27–29] that immunosuppression in mice bearing EL-4 thymoma was induced by tumor-produced TGF-β through the enhancement of IL-10 production by macrophages and inhibition of IFN-γ production by T cells. Further, T cells from these mice produced only type 2 cytokines when they were stimulated with anti-CD3 mAb *in vitro*. These data indicate that the predominance of type 2 T cell responses associated with the tumor growth plays a very important role on the further growth of tumors and the host's antitumor resistance. In fact the pulmonary metastasis of B16F10 melanoma was clearly reduced when tumor-bearing mice were treated with a mixture of anti-IL-4 mAb and anti-IL-10 mAb (see Figure 4).

Z-100 has various pharmacological activities including: (i) protective activities against *Pseudomonas*[30] and herpesvirus[12] infections in immunocompromised hosts, (ii) immunostimulatory activities on the reticuloendothelial systems,[31] and (iii) stimulating activities on the production of IL-3 and IFN-γ from T_h1 cells.[11,32] In our previous studies, the concomitant immunity in mice immunized with Meth-A tumors cells and *Corynebacterium pervum* was prolonged when these mice were treated with Z-100,[15] because the generation of tumor-associated $CD8^+$ type 2 T cells in immunized mice was suppressed by the compound. Also, Z-100 improved the resistance of thermally injured mice exposed to a lethal amount of herpes simplex virus type 1, through the inhibition of generating burn-associated $CD8^+$ type 2 T cells, which play a key role in the resistance of these mice to the infection.[12]

In the present study, the anti-metastatic effect of Z-100 on pulmonary metastasis of B16 melanoma was examined in mice inoculated with a high metastatic strain (B16F10 cells) or a low metastatic strain (B16F1 cells) of B16 melanoma cells. Administration of a 10 mg/kg dose of Z-100 to BF10 mice (mice inoculated with B16F10 melanoma cells) resulted in a 70% inhibition in numbers of pulmonary metastatic colonies as compared with that of BF10 mice treated with saline. $CD4^+$ $CD11b^+$ $CD28^+$ $TCR\alpha\beta^+$ IL-4- and IL-10-producing T cells (F10-T_h2 cells), demonstrated in BF10 mice but not in BF1 mice (mice inoculated with B16F1 melanoma cells), were identified as B16 melanoma-associated type 2 T cells. Although only little pulmonary metastasis was demonstrated in BF1 mice, the pulmonary tumor metastasis in these mice was developed, to levels observed in BF10 mice, when BF1 mice were inoculated with F10-T_h2 cells. However, the pulmonary metastasis was not demonstrated in BF1 mice inoculated with the F10-T_h2 cell preparation from BF10 mice treated with Z-100. Also, Z-100 inhibited the pulmonary metastasis in BF1 mice inoculated with F10-T_h2 cells. Further, $CD4^+$ T cells from BF10 mice treated with Z-100 did not show any suppressor cell activities and did not produce type 2 cytokines into their culture fluids. These results suggest that Z-100 may inhibit pulmonary metastasis in mice inoculated with B16 melanoma cells through the suppression of tumor-associated type 2 T cells, which are a key cell in the metastasis of B16 melanoma.

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