Antimetastatic Effect of an Immunomodulatory Arabinomannan Extracted from *Mycobacterium tuberculosis* Strain Aoyama B, Z-100, through the Production of Interleukin-12

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In this study, the role of interleukin (IL)-12 on the antimetastatic effect of Z-100 was investigated using wild-type C57BL/6 mice or IL-12p40 knockout (IL-12p40 KO) mice inoculated with highly metastatic B16F10 melanoma. When C57BL/6 mice were inoculated with B16F10 melanoma (2×10^5 cells/mouse i.v.), Z-100 (10 mg/kg i.p.) significantly suppressed the pulmonary metastasis of B16F10 melanoma 14 d after tumor inoculation. On the other hand, the antimetastatic effect of Z-100 was not observed in IL-12p40 KO mice inoculated with B16F10 melanoma. These results indicate that IL-12 is essentially required for the appearance of the antimetastatic effect of Z-100. Since helper T (Th) 2 cell responses have been reported to have a role in tumor metastasis, the regulatory effect of Z-100 on the immune balance of Th1/Th2 cell responses was investigated. In both C57BL/6 mice and IL-12p40 KO mice bearing B16F10 melanoma, Th1 cytokine production (IL-2, interferon-γ) was significantly suppressed as compared with those in normal mice. On the other hand, Th2 cytokine production (IL-4, IL-10) in these mice was increased. The administration of Z-100 (10 mg/kg i.p.) in C57BL/6 mice bearing B16F10 melanoma improved the balance of Th1/Th2 cell responses from the Th2-dominant state to the normal state. However, the improvement of Th1/Th2 cell responses by Z-100 was not observed in IL-12p40 KO mice bearing the same tumors. In addition, Z-100 significantly increased IL-12 production by macrophages in a concentration-dependent manner, while Z-100 significantly decreased IL-10 production by these cells *in vitro*. These results suggested that up-regulation of IL-12 production and down-regulation of IL-10 production by Z-100 are related to the improvement of Th1/Th2 cell responses from the Th2-dominant state to the normal state, which resulted in suppression of tumor metastasis.

Key words *Mycobacterium tuberculosis*; Z-100; interleukin-12; helper T cell response; immunomodulator

Interleukin (IL)-12 was originally identified and isolated as a natural killer (NK) cell stimulatory factor.[1] Compared with other cytokines, it has a unique 70-kDa heterodimeric structure composed of two covalently linked p35 and p40 subunits, both of which are required for biological activity.[1,2] IL-12 is produced by antigen-presenting cells such as monocytes, macrophages,[3] dendritic cells,[4] and Langerhans cells.[5] The immunomodulatory activity of IL-12 has been intensively studied. IL-12 has been shown to enhance lymphocyte proliferation,[6,7] to augment the cytolytic activities of T cells and NK cells,[7,8] and to induce interferon (IFN)-γ production from these cells.[1,9] Furthermore, it plays an important role in regulating the balance between helper T (Th) 1 and Th2 responses.[3,10,11] Recombinant IL-12 or IL-12 gene transfer has shown to have potent antitumor activity against several murine tumors.[12—15] In other instances, a T cell-mediated antitumor response of IL-12 has been shown to be dependent on the function of $CD4^+$ and $CD8^+$ T cells.[16] In addition, the antitumor activity of IL-12 might also depend on the cytotoxic action of NK/NKT cells against tumors.[17] Those reports suggested that IL-12 plays an important role in antitumor immunity.

Z-100, extracted from *Mycobacterium tuberculosis* strain Aoyama B, is an immunomodulator containing arabinomannan as the main component.[18] It is clinically used in patients with leukopenia caused by radiation therapy in Japan. In preclinical experiments, Z-100 was shown to have various immunopotentiating activities including enhancement of protective activity against *Pseudomonas aeruginosa* infection,[19] and antiviral activities against LP-BM5 murine leukemia virus,[20] and herpes virus.[21] Z-100 exhibited inhibition of tumor growth[22] and metastasis, prolongation of survival time,[23] and protection against opportunistic infection[24] in syngeneic murine tumor models. In addition, Z-100 has been shown to exhibit these antimetastatic activities *via* suppression of Th2 cytokine production by tumor-associated Th2 cells.[25] Furthermore, Z-100 improves the balance of Th1/Th2 cell responses in Meth-A tumor cell-bearing mice through both up-regulation of IL-12 production from macrophages and interferon (IFN)-γ production from $CD4^+$ T cells.[26]

In the present study, the role of IL-12 in the antimetastatic effect of Z-100 was investigated using wild-type C57BL/6 mice and IL-12p40 knockout (IL-12p40 KO) mice inoculated with metastatic B16F10 melanoma cells.

MATERIALS AND METHODS

Animals Six- to 7-week-old male C57BL/6 mice were purchased from Charles River Japan. Five- to 6-week-old male C57BL/6-IL12b⟨tm1Jm⟩ (IL-12p40 KO) mice were purchased from Jackson Laboratory, U.S.A. The mice were maintained in aluminum cages with Paper Clean (Japan SLC) bedding under specific pathogen-free conditions in an animal room at controlled temperature and humidity (23±3 °C, 55±20%, respectively). Mice were given CRF-1 feed (Oriental Yeast) and water *ad libitum*. All animal experiments were approved by the Animal Care and Use Committee of the Central Research Laboratories of Zeria Pharmaceutical Co., Ltd.

Z-100 Z-100 was produced by Zeria Pharmaceutical Co., Ltd., Tokyo, Japan. Z-100 (10 mg/kg) was administered

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intraperitoneally once daily for 14 d beginning immediately after tumor inoculation. As a control, saline was injected intraperitoneally on the same schedule.

Reagents RPMI 1640 medium (Nissui Seiyaku), minimum essential medium (MEM, Invitrogen), Dulbecco's phosphate-buffered saline (PBS, Sigma), fetal bovine serum (FBS, JRH Biosciences and Hyclone), 0.25% trypsin and 1 mM EDTA (Invitrogen), IFN-γ, IL-2, IL-4, and IL-10 enzyme-linked immunoabsorbent assay (ELISA) kits (R&D Systems), IL-12 (p40 and p70) ELISA kits (Amersham Biosciences Biotech), anti-CD3 monoclonal antibody (mAb) (clone 145-2C11, Pharmingen), *Bacille Calmette-Guérin* (BCG, Japan BCG), and whole-blood erythrocyte lysing kits (R&D Systems) were used in the experiments.

Implantation of B16F10 Melanoma in C57BL/6 Mice or IL-12p40 KO Mice B16F10 melanoma, a highly metastatic strain of B16 melanoma cells, were grown *in vitro* with RPMI 1640 medium supplemented with 10% heat-inactivated FBS, antibiotics, and 2 mM L-glutamine (complete medium). Cells in a log growth phase were detached from tissue culture flasks using a mixture of 0.25% trypsin and 1 mM EDTA. The cells were washed with PBS and then 1×10^6 cells/ml of B16F10 melanoma was suspended in PBS immediately before implantation. C57BL/6 mice or IL-12p40 KO mice were inoculated with the cultured B16F10 melanoma (2×10^5 cells/mouse i.v.). Fourteen days after tumor inoculation, lung tissues were removed from these mice and fixed in 10% formaldehyde solution. The number of metastatic colonies on the lungs was counted under a dissecting microscope.

Induction and Measurement of Th1/Th2 Cytokine Production Splenocytes were prepared from C57BL/6 mice and IL-12p40 KO mice 14 d after tumor inoculation. Single-cell suspensions were passed through nylon mesh (Becton Dickinson) and washed with RPMI-1640 medium by centrifugation for 5 min at 1200 rpm. Then the erythrocytes were removed with a whole-blood erythrocyte lysing kit and then washed twice with medium. To prepare $CD4^+$ T cells, the splenocytes were passed through a $CD4^+$ T cell subset column (Cytovax Biotechnologies). This procedure resulted in a population of $>84\%$ of $CD4^+$ T cells, as assayed by flow cytometry. The $CD4^+$ T cells were suspended in complete medium. The number of viable cells was determined using the trypan blue dye-exclusion method. Then 2×10^6 cells/ml of these cells was stimulated with anti-CD3 mAb (2.5 μg/ml) in a 96-well tissue culture plate and incubated for 48 h at 37 °C in 5% CO_2. Culture fluids harvested were stored at -80 °C until use and assayed for the amounts of IL-2, IFN-γ, IL-4, and IL-10 using an ELISA kit.

Preparation of Macrophages from C57BL/6 Mice Treated with BCG C57BL/6 mice were administered BCG (1 mg/mouse i.v.). Fourteen days after administration, peritoneal exudate cells (PECs) were collected from the peritoneal cavity of these mice by washing twice with MEM 5 ml. PECs were washed and resuspended in MEM supplemented with 10% heat-inactivated FBS. The cells (2×10^6 cells/well) in 500-μl aliquots of complete culture medium were cultured in Biocoat 24-well plates (Becton Dickinson). To prepare adherent cells as macrophages, these cells were cultured for 30 min at 37 °C in 5% CO_2, then washed twice with warmed MEM (37 °C). The macrophages were stimulated with various concentrations of Z-100 (25, 50, 100, 200 μg/ml) for 24, 48, and 72 h at 37 °C in 5% CO_2. Culture fluids harvested were stored at -80 °C until use and assayed for the amount of IL-12 (p40 and p70) and IL-10 using ELISA kits.

Statistical Analysis Data are expressed as mean±standard error (S.E.). Statistical analysis was performed using Student's *t*-test, Dunnett's test, and the Tukey test. p values less than 0.05 were considered significant.

RESULTS

Role of IL-12 in Antimetastatic Activity of Z-100 IL-12p40 KO mice were previously shown to have dysfunctional IL-12 activities, resulting in the breakdown of Th1 cell responses and the augmentation of Th2 cell responses.[27] Therefore IL-12p40 KO mice are appropriate experimental animals for examining the function of IL-12.

To determine the role of IL-12 in the antimetastatic activity of Z-100, C57BL/6 mice and IL-12p40 KO mice were inoculated with B16F10 melanoma (2×10^5 cells/mouse) and then those mice were administered Z-100 (10 mg/kg i.p.). The number of pulmonary metastases was counted 14 d after tumor inoculation. As shown in Fig. 1a, administration of Z-100 (10 mg/kg) significantly suppressed the pulmonary metastasis of tumors in C57BL/6 mice inoculated with B16F10 melanoma, as compared with that in tumor control mice (suppression rate: 80.6%, $p<0.01$). In IL-12p40 KO mice inoculated with the same tumors, Z-100 did not suppress pulmonary metastasis (suppression rate: 19.1%) (Fig. 1b). These results indicate that Z-100 exhibits its antimetastatic activity through the function of IL-12. On the other hand, Z-100 (200 μg/ml) did not suppress the growth of B16F10 melanoma cells *in vitro*. The results indicate that Z-100 dose not have a direct tumoricidal effect but acts through a host-mediated mechanism against tumor cells.

Regulatory Effect of Z-100 on the Balance of Th1/Th2

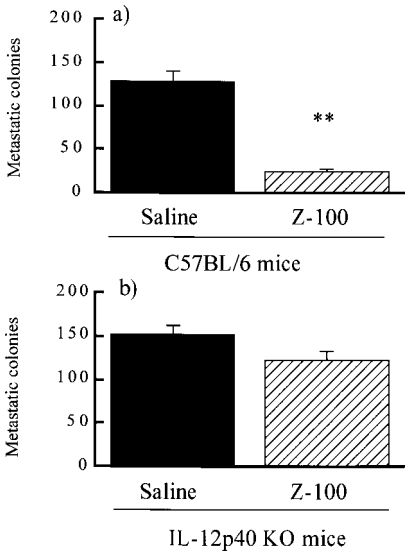

Fig. 1. Role of IL-12 in the Antimetastatic Activity of Z-100

C57BL/6 mice (a) or IL-12p40 KO mice (b) were inoculated with B16F10 melanoma (2×10^5 cells/mouse i.v.) on day 0. The mice were administered Z-100 (10 mg/kg i.p., hatched column) or saline (closed column) from 0 to 13 d. Results are expressed as mean±S.E. (number of mice=5). ** $p<0.01$, compared with control mice treated with saline (Student's *t*-test).

Cell Responses in C57BL/6 Mice Inoculated with B16F10 Melanoma The regulatory effect of Z-100 on the balance of Th1/Th2 cell responses was examined. C57BL/6 mice bearing B16F10 melanoma were administered Z-100 (10 mg/kg i.p.), and then the production of Th1 cytokines (IL-2, IFN-γ) and Th2 cytokines (IL-4, IL-10) by $CD4^+$ T cells prepared from the splenocytes of those mice were measured 14 d after tumor inoculation. IL-2 and IFN-γ production was significantly decreased in C57BL/6 mice bearing B16F10 melanoma, as compared with that of normal mice ($p<0.01$, Figs. 2a, b). IL-4 and IL-10 production was significantly increased in C57BL/6 mice bearing the same tumors, as compared with that in normal mice ($p<0.01$, Figs. 2c, d). These results indicate that the Th cell responses in C57BL/6 mice were shifted to the Th2-dominant state by inoculation with B16F10 melanoma. On the other hand, the administration of Z-100 significantly increased IL-2 and IFN-γ production in C57BL/6 mice bearing B16F10 melanoma ($p<0.01$, Figs. 2a, b). In addition, the administration of Z-100 significantly suppressed IL-4 and IL-10 production in those mice ($p<0.05$, Figs. 2c, d). These results indicate that Z-100 improved the balance of Th1/Th2 cell responses from the Th2-dominant immune responses to the normal state in C57BL/6 mice bearing B16F10 melanoma.

Regulatory Effect of Z-100 on the Balance of Th Cell Responses in IL-12p40 KO Mice Inoculated with B16F10 Melanoma To determine the role of IL-12 in the regulatory effect of Z-100 on the balance of Th1/Th2 cell responses, IL-12p40 KO mice bearing B16F10 melanoma were used. These mice were administered Z-100 (10 mg/kg i.p.), and IL-2, IFN-γ, IL-4, and IL-10 from the $CD4^+$ T cells prepared from splenocytes of those mice were measured 14 d after tumor inoculation. IL-2 and IFN-γ production was significantly decreased in IL-12p40 KO mice bearing B16F10 melanoma, as compared with that in normal IL-12p40 KO mice (IL-2, $p<0.01$; IFN-γ, $p<0.05$, Figs. 3a, b). IL-4 and IL-10 production was significantly increased in IL-12p40 KO mice bearing B16F10 melanoma, as compared with that in normal IL-12p40 KO mice ($p<0.01$, Figs. 3c, d). These results indicate that the Th cell responses in IL-12p40 KO mice bearing B16F10 melanoma also shifted to the Th2-dominant state, as in wild-type C57BL/6 mice bearing these tumors. However, administration of Z-100 did not affect Th1 cytokine production or Th2 cytokine production in IL-12p40 KO mice bearing B16F10 melanoma. These results suggest that Z-100 improved the balance of Th1/Th2 cell responses from Th2-dominant immune responses to the normal state through the activities of IL-12 as observed in C57BL/6 mice bearing B16F10 melanoma.

IL-12 Production from Macrophages Stimulated with Z-100 Macrophages have been shown to produce IL-12 and to be the primary target cells for immunological activity of Z-100. Therefore the effects of Z-100 on IL-12 production by macrophages were determined. The macrophages prepared from BCG-treated mice were cultured in the presence of saline or Z-100 (200 μg/ml) for 24, 48, and 72 h at 37 °C in 5% CO_2. The amounts of IL-12 (p40 and p70) in the culture fluid harvested were measured using an ELISA kit (Fig. 4a). After 24, 48, and 72 h incubation, the amounts of IL-12 (p40 and p70) produced by macrophages treated with saline were 150, 220, and 230 pg/ml, respectively. On the other hand, the amounts of IL-12 (p40 and p70) produced by cells treated with Z-100 (200 μg/ml) were 320, 420, and 460 pg/ml 24, 48, and 72 h after stimulation, respectively. These results indicate that Z-100 significantly increases IL-12 production by macrophages ($p<0.01$). To examine the concentration dependency of Z-100 on the induction of IL-12 from macrophages, these cells were stimulated *in vitro* with Z-100

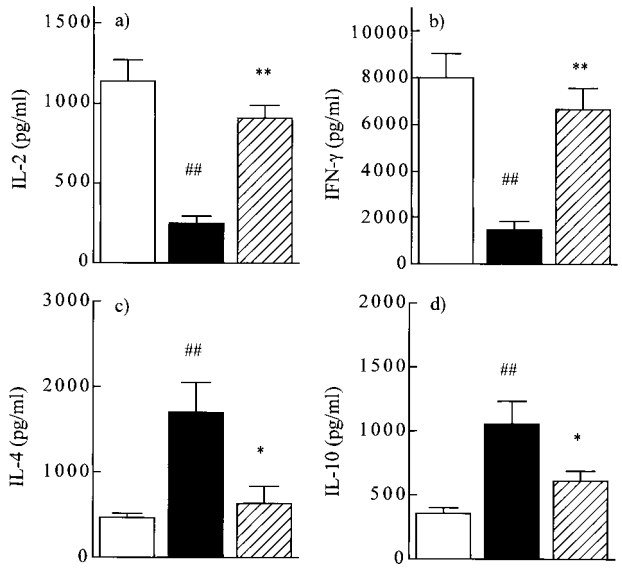

Fig. 2. Regulatory Effect of Z-100 on the Balance of Th Cell Responses in C57BL/6 Mice Inoculated with B16F10 Melanoma

C57BL/6 mice were inoculated with B16F10 melanoma (2×10^5 cells/mouse i.v.) on day 0. The mice were administered Z-100 (10 mg/kg i.p., hatched column) or saline (closed column) from 0 to 13 d. The open column shows untreated C57BL/6 mice. Amounts of IL-2 (a), IFN-γ (b), IL-4 (c), and IL-10 (d) in culture fluids harvested were measured using ELISA. Results are expressed as mean±S.E. (number of mice=5). $^{\#\#}p<0.01$ compared with normal mice, $^{*}p<0.05$, $^{**}p<0.01$, compared with tumor control mice (Tukey test).

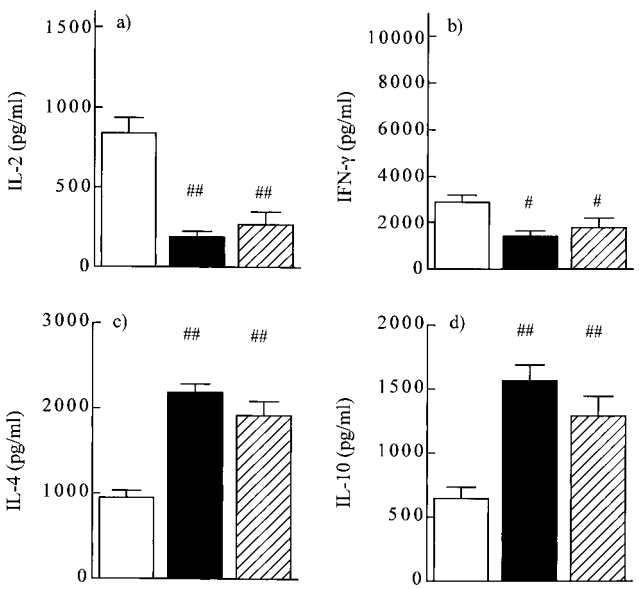

Fig. 3. Regulatory Effect of Z-100 on the Balance of Th Cell Responses in IL-12p40 KO Mice Inoculated with B16F10 Melanoma

IL-12p40 KO mice were inoculated with B16F10 melanoma (2×10^5 cells/mouse i.v.) on day 0. The mice were administered Z-100 (10 mg/kg i.p., hatched column) or saline (closed column) from 0 to 13 d. The open column shows untreated IL-12p40 KO mice. Amounts of IL-2 (a), IFN-γ (b), IL-4 (c), and IL-10 (d) in culture fluids were measured using ELISA. Results are expressed as mean±S.E. (number of mice=5). $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with normal mice (Tukey test).

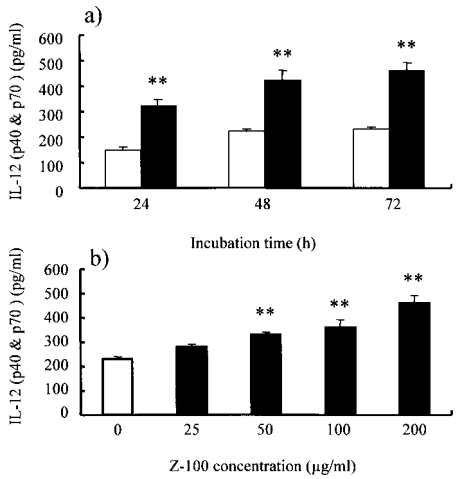

Fig. 4. IL-12 Production by Macrophages Stimulated with Z-100

(a) The macrophages were stimulated without (opened columns) or with Z-100 at a concentration of 200 μg/ml (closed columns) for 24, 48, and 72 h. The results are expressed as mean±S.E. (n=5). ** p<0.01 compared with control at each time (Student's t-test). (b) The macrophages were stimulated without (opened columns) or with Z-100 at concentrations of 25, 50, 100, and 200 μg/ml (closed columns) for 72 h. IL-12 (p40 and p70) production in the culture fluids was determined using ELISA. The results are expressed as mean±S.E. (n=5). ** p<0.01 compared with control (Dunnett's test).

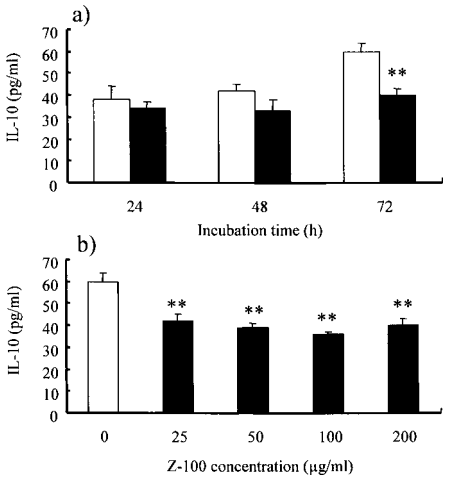

Fig. 5. IL-10 Production by Macrophages Stimulated with Z-100

(a) The macrophages were stimulated without (opened columns) or with Z-100 at concentration of 200 μg/ml (closed columns) for 24, 48, and 72 h. The results are expressed as mean±S.E. (n=5). ** p<0.01 compared with control at each time (Student's t-test). (b) The macrophages were stimulated without (opened columns) or with Z-100 at concentrations of 25, 50, 100, and 200 μg/ml (closed columns) for 72 h. IL-10 production in the culture fluids was determined using ELISA. The results are expressed as mean±S.E. (n=5). ** p<0.01 compared with control (Dunnett's test).

(0, 25, 50, 100, or 200 μg/ml) for 72 h, and the amounts of IL-12 (p40 and p70) in the culture fluid harvested were measured. As shown in Fig. 4b, the amounts of IL-12 (p40 and p70) from macrophages stimulated with saline, and 25, 50, 100, or 200 μg/ml of Z-100 were 230, 280, 330, 360, and 460 pg/ml, respectively. These results indicate that Z-100 significantly increase IL-12 production by macrophages in a concentration-dependent manner at concentrations of 50 μg/ml or more (p<0.01).

IL-10 Production by Macrophages Stimulated with Z-100 IL-10 is a Th2 cytokine that inhibits Th1 cytokine production by Th1 cells.[28] It also inhibits IL-12 production induced by subsequent stimulation.[29] Reports indicated that IL-10 suppresses Th1 immune responses. Since macrophages have been shown to induce IL-12 and IL-10, macrophages may have an important role in the generation of Th cell responses. Therefore the effects of Z-100 on IL-10 production by macrophages prepared from BCG-treated mice were determined. The macrophages were cultured in the presence of saline, as the control group, or Z-100 (200 μg/ml) for 24, 48, and 72 h, and the amounts of IL-10 in the culture fluids were measured using an ELISA kit (Fig. 5a). After 24, 48, and 72 h of incubation, amounts of IL-10 in the control group were 38, 42, and 60 pg/ml, respectively. Amounts of IL-10 in the Z-100-treated group were 34, 33, and 40 pg/ml, respectively. After 72-h incubation, Z-100 significantly decreased IL-10 production by macrophages, compared with that in the control group (p<0.01). In addition, to determine the effect of Z-100 on the suppression of IL-10 production, macrophages were stimulated with Z-100 at concentrations of 0, 25, 50, 100, and 200 μg/ml for 72 h and the amounts of IL-10 in the culture fluids were measured using an ELISA kit. As shown in Fig. 5b, the amounts of IL-10 in cultures of macrophages stimulated with Z-100 (0, 25, 50, 100, or 200 μg/ml) were 60, 42, 39, 36, and 40 pg/ml, respectively. These results indicated that Z-100 significantly decreases IL-10 production by macrophages at concentrations of 25 μg/ml or more (p<0.01).

DISCUSSION

It has been reported that immunomodulators, such as Streptococcal preparation OK-432[30,31] and 1,3-β-D-glucan,[32,33] increase IL-12 production from antigen-presenting cells. Since IL-12 can regulate the Th1/Th2 immune balance, IL-12 may be an important factor in the tumor immunity of immunomodulators. In the present study, we investigated the role of IL-12 in the antimetastatic activity of Z-100, an immunomodulator extracted from *M. tuberculosis*. As a result, Z-100 was shown to suppress significantly the metastasis of tumors in wild-type C57BL/6 mice bearing B16F10 melanoma. However, the antimetastatic activity of Z-100 was not observed in IL-12p40 KO mice bearing the same tumors. These results suggest that IL-12 is an important cytokine for the expression of the antimetastatic activity of Z-100.

Mosmann *et al.* reported that helper T cells could be divided into two subpopulations, Th1 and Th2 cells, according to the differences in their cytokine expression profiles.[34] IFN-γ, secreted from Th1 cells, is known to induce the differentiation of Th0 to Th1 cells and to inhibit the proliferation of Th2 cells.[35] IL-4 and IL-10, secreted from Th2 cells, are known to induce the differentiation of Th0 to Th2 cells and to inhibit the function of Th1 cells.[36,37] Thus, Th1 or Th2 cytokines control the differentiation and function of Th cells. It was recently reported that the Th cell responses shifted to Th2-dominant status depending on the malignancy stage.[38,39] In addition, the regulation of the immune balance of Th1/Th2 cell responses has been shown to be critically important for antitumor immune responses, such as inhibition of tumor growth and metastasis, and survival rate.[40—42] As described above, the balance of Th1/Th2 cell responses is regulated by Th1 and Th2 cytokines. IL-12 produced from antigen-presenting cells, such as macrophages, play been shown to induce Th1 cell responses. Since macrophages play

an important role as primary immune cells in the generation of tumor-specific immunity, cytokines induced from these cells may be related to tumor rejection. In this study, we investigated the role of IL-12 in the regulatory effect of Z-100 on the balance of Th1/Th2 cell responses. Z-100 improved the balance of Th1/Th2 cell responses from Th2-dominant immune responses, as observed in C57BL/6 mice bearing B16F10 melanoma, to the normal level. However, the regulatory effect of Z-100 was not observed in IL-12p40 KO mice bearing the same tumors. Moreover, Z-100 increased IL-12 production and decreased IL-10 production by macrophages. It was suggested that the increase in IL-12 production and decrease in IL-10 production from macrophages by Z-100 may contribute to the improvement of Th1/Th2 cell responses from Th2-dominant immune responses to the normal state, as observed in C57BL/6 mice bearing B16F10 melanoma. In this study, we found that Z-100 increased mouse IL-12 production by activated macrophages prepared from BCG-treated mice. Kobayashi *et al.* also reported that Z-100 induced IL-12 production by splenocytes of BCG-treated mice.[43] On the other hand, it was reported that alveolar macrophages play an important role in the suppression of pulmonary tumor metastases by immunomodulators.[44,45] However, the effects of Z-100 on IL-12 production by alveolar macrophages from normal and tumor-bearing mice were not investigated. Recently, we have found that Z-100 increases IL-12 production by mouse bone marrow-derived adherent cells generated by granulocyte-macrophage colony stimulating factor or by human peripheral blood mononuclear cell-derived adherent cells (data not shown). Based on that knowledge, the effects of Z-100 on IL-12 production by these macrophages are considered to be very important for mechanism analysis of the suppression of pulmonary metastases by Z-100.

IFN-γ has immunomodulatory activities such as the augmentation of Th1 cell activity, macrophage tumoricidal activity, and NK cell cytotoxicity and can suppress tumor growth and pulmonary metastasis.[46,47] Adoption of transferred tumor-specific T cells demonstrated that secretion of IFN-γ plays an essential role in tumor rejection.[48,49] We have already investigated the role of IFN-γ in the antimetastatic activity of Z-100.[50] We demonstrated that Z-100 suppresses the pulmonary metastasis of B16F10 melanoma through the induction of IFN-γ production and improved Th cell responses from the Th2-dominant state to the Th1-dominant state. However, the antimetastatic activity of Z-100 did not depend on IFN-γ alone. The activity of other cytokines in the antimetastatic activity of Z-100 was also assumed from these data. Furthermore, it has been reported that Z-100 increases IL-12 and IFN-γ mRNA levels and improves the balance of Th1/Th2 cell responses from Th2-dominant immune responses to the normal state in mice bearing Meth-A tumor cells.[25] These findings indicate that the interaction of Th1-inducible cytokines such as IL-12 and IFN-γ are required for the antitumor effects of Z-100.

Recently, it has been reported that Th1 cells suppress the metastasis of MCA205 tumor cells.[51] It has also been reported that dendritic cells transduced with tyrosinase-related protein-2 known to be a tumor antigen, suppresses the metastasis of B16 melanoma through the activation of $CD4^+$ T cells.[52] These reports suggest the importance of $CD4^+$ T cells in antimetastatic activities. On the other hand, it has been reported macrophages activated by Th1 cells suppress tumor growth.[53] In addition, it has been demonstrated that natural killer (NK) cells and NKT cells are important effector cells for the antimetastatic effect of IL-12 and/or Th1 cells.[54,55] These reports indicate that a variety of effector cells such as NK/NKT cells, macrophages, and Th1 cells are involved in the antimetastatic effects of IL-12. Moreover, our results and the above reports suggest that the cooperation of antigen-presenting cells, NK/NKT cells and Th1 cells appears necessary for the anti-metastatic activity of Z-100.

In conclusion, we demonstrated that Z-100 increases IL-12 production, decreases IL-10 production, and improves Th1/Th2 cell responses from the Th2-dominant state to the normal state. In addition, the present findings indicate that the antimetastatic activity of Z-100 is exerted through host-mediated immune systems. Since these studies were carried out in mice, the clinical relevance of the results is unknown, but we suggest that Z-100 treatment of patients with cancer could prevent metastasis *via* above the host immune system.

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REFERENCES

1) Kobayashi M., Fitz L., Ryan M., Hewick R. M., Clark S. C., Chan S., Loudon R., Sherman F., Perussia B., Trinchieri G., *J. Exp. Med.*, **170**, 827—845 (1989).
2) Wolf S. F., Temple P. A., Kobayashi M., Young D., Dicig M., Lowe L., Dzialo R., Fitz L., Ferenz C., Hewick R. M., Kelleher K., Herrmann S. H., Clark S. C., Azzoni L., Chan S. H., Trinchieri G., Perussia B., *J. Immunol.*, **146**, 3074—3081 (1991).
3) Hsieh C. S., Macatonia S. E., Tripp C. S., Wolf S. F., O'Garra A., Murphy K. M., *Science*, **260**, 547—549 (1993).
4) Macatonia S. E., Hosken N. A., Litton M., Vieira P., Hsieh C. S., Culpepper J. A., Wysocka M., Trinchieri G., Murphy K. M., O'Garra A., *J. Immunol.*, **154**, 5071—5079 (1995).
5) Kang K., Kubin M., Cooper K. D., Lessin S. R., Trinchieri G., Rook A. H., *J. Immunol.*, **156**, 1402—1407 (1996).
6) Perussia B., Chan S. H., D'Andrea A., Tsuji K., Santoli D., Pospisil M., Young D., Wolf S. F., Trinchieri G., *J. Immunol.*, **149**, 3495—3502 (1992).
7) Robertson M. J., Soiffer R. J., Wolf S. F., Manley T. J., Donahue C., Young D., Herrmann S. H., Ritz J., *J. Exp. Med.*, **175**, 779—788 (1992).
8) Gately M. K., Wolitzky A. G., Quinn P. M., Chizzonite R., *Cell Immunol.*, **143**, 127—142 (1992).
9) Chan S. H., Perussia B., Gupta J. W., Kobayashi M., Pospisil M., Young H. A., Wolf S. F., Young D., Clark S. C., Trinchieri G., *J. Exp. Med.*, **173**, 869—879 (1991).
10) Trinchieri G., *Immunol. Today*, **14**, 335—338 (1993).
11) Manetti R., Parronchi P., Giudizi M. G., Piccinni M. P., Maggi E., Trinchieri G., Romagnani S., *J. Exp. Med.*, **177**, 1199—1204 (1993).
12) Brunda M. J., Luistro L., Warrier R. R., Wright R. B., Hubbard B. R., Murphy M., Wolf S. F., Gately M. K., *J. Exp. Med.*, **178**, 1223—1230 (1993).
13) Boggio K., Nicoletti G., Di Carlo E., Cavallo F., Landuzzi L., Melani C., Giovarelli M., Rossi I., Nanni P., De Giovanni C., Bouchard P., Wolf S., Modesti A., Musiani P., Lollini P. L., Colombo M. P., Forni G., *J. Exp. Med.*, **188**, 589—596 (1998).
14) Cavallo F., Signorelli P., Giovarelli M., Musiani P., Modesti A., Brunda M. J., Colombo M. P., Forni G., *J. Natl. Cancer Inst.*, **89**, 1049—1058 (1997).
15) Schultz J., Pavlovic J., Strack B., Nawrath M., Moelling K., *Hum. Gene Ther.*, **10**, 407—417 (1999).
16) Nastala C. L., Edington H. D., McKinney T. G., Tahara H., Nalesnik M. A., Brunda M. J., Gately M. K., Wolf S. F., Schreiber R. D.,

Storkus W. J., Lotze M. T., *J. Immunol.*, **153**, 1697—1706 (1994).
17) Cui J., Shin T., Kawano T., Sato H., Kondo E., Toura I., Kaneko Y., Koseki H., Kanno M., Taniguchi M., *Science*, **278**, 1623—1626 (1997).
18) Kobatake H., Suekane T., Murakami Y., Niwa S., Okahira A., Kushida H., *Yakugaku Zasshi*, **101**, 713—722 (1981).
19) Kawamura I., Mitsuyama M., Nomoto K., *Immunopharmacol. Immunotoxicol.*, **12**, 331—343 (1990).
20) Sasaki H., Kobayashi M., Pollard R. B., Suzuki F., *Pathobiology*, **69**, 96—103 (2001).
21) Kobayashi M., Herndon D. N., Pollard R. B., Suzuki F., *Immunol. Lett.*, **40**, 199—205 (1994).
22) Sasaki H., Schmitt D. A., Kobayashi M., Hayashi Y., Pollard R. B., Suzuki F., *Nat. Immun.*, **12**, 152—164 (1993).
23) Emori Y., Sasaki H., Hayashi Y., Nomoto K., *Biotherapy*, **9**, 249—256 (1996).
24) Sasaki H., Kobayashi M., Emori Y., Ohya O., Hayashi Y., Nomoto K., *Biotherapy*, **10**, 139—143 (1997).
25) Kobayashi M., Pollard R. B., Suzuki F., *Anti Cancer Drugs*, **8**, 156—163 (1997).
26) Oka H., Emori Y., Ohya O., Kobayashi N., Sasaki H., Tanaka Y., Hayashi Y., Nomoto K., *Immunol. Lett.*, **70**, 109—117 (1999).
27) Magram J., Connaughton S. E., Warrier R. R., Carvajal D. M., Wu C. Y., Ferrante J., Stewart C., Sarmiento U., Faherty D. A., Gately M. K., *Immunity*, **4**, 471—481 (1996).
28) Fiorentino D. F., Zlotnik A., Vieira P., Mosmann T. R., Howard M., Moore K. W., O'Garra A., *J. Immunol.*, **146**, 3444—3451 (1991).
29) Du C., Sriram S., *J. Leukoc. Biol.*, **64**, 92—97 (1998).
30) Okamoto M., Ohe G., Oshikawa T., Furuichi S., Nishikawa H., Tano T., Ahmed S. U., Yoshida H., Moriya Y., Saito M., Sato M., *Cancer Immunol. Immunother.*, **50**, 408—416 (2001).
31) Fujimoto T., Duda R. B., Szilvasi A., Chen X., Mai M., O'Donnell M. A., *J. Immunol.*, **158**, 5619—5626 (1997).
32) Suzuki Y., Adachi Y., Ohno N., Yadomae T., *Biol. Pharm. Bull.*, **24**, 811—819 (2001).
33) Hirata N., Tsuzuki A., Ohno N., Saita M., Adachi Y., Yadomae T., *Zentralbl. Bakteriol.*, **288**, 403—413 (1998).
34) Mosmann T. R., Cherwinski H., Bond M. W., Giedlin M. A., Coffman, R. L., *J. Immunol.*, **136**, 2348—2357 (1986).
35) Gajewski T. F., Schell S. R., Nau G., Fitch F. W., *Immunol. Rev.*, **111**, 79—110 (1989).
36) Fiorentino D. F., Bond M. W., Mosmann T. R., *J. Exp. Med.*, **170**, 2081—2095 (1989).
37) Swain S. L., Weinberg A. D., English M., Huston G., *J. Immunol.*, **145**, 3796—3806 (1990).
38) Maeda H., Shiraishi A., *J. Immunol.*, **156**, 73—78 (1996).
39) Sredni B., Tichler T., Shani A., Catane R., Kaufman B., Strassmann G., Albeck M., Kalechman Y., *J. Natl. Cancer Inst.*, **88**, 1276—1284 (1996).
40) Ghosh P., Komschlies K. L., Cippitelli M., Longo D. L., Subleski J., Ye J., Sica A., Young H. A., Wiltrout R. H., Ochoa A. C., *J. Natl. Cancer Inst.*, **87**, 1478—1483 (1995).
41) Kobayashi M., Kobayashi H., Pollard R. B., Suzuki F., *J. Immunol.*, **160**, 5869—5873 (1998).
42) Takeuchi T., Ueki T., Sasaki Y., Kajiwara T., Li B., Moriyama N., Kawabe K., *Cancer Immunol. Immunother.*, **43**, 375—381 (1997).
43) Kobayashi M., Nakajima N., Pollard R. B., Suzuki F., *Proc. AACR*, **36**, 467 (1995).
44) Kinoshita A., Suzuki I., Sakurai T., Yadomae T., *Int. J. Immunopharmacol.*, **14**, 205—211 (1992).
45) Sakurai T., Hashimoto K., Suzuki I., Ohno N., Oikawa S., Masuda A., Yadomae T., *Int. J. Immunopharmacol.*, **14**, 821—830 (1992).
46) Maekawa R., Kitagawa T., Hojo K., Wada T., Sato, K., *J. Interferon Res.*, **8**, 227—239 (1988).
47) Saiki I., Murata J., Saito S., Higashi N., Azuma I., *Mol. Biother.*, **1**, 261—267 (1989).
48) Barth R. J., Jr., Mule J. J., Spiess P. J., Rosenberg S. A., *J. Exp. Med.*, **173**, 647—658 (1991).
49) Tuttle T. M., McCrady C. W., Inge T. H., Salour M., Bear H. D., *Cancer Res.*, **53**, 833—839 (1993).
50) Oka H., Emori Y., Sasaki H., Shiraishi Y., Yoshinaga K., Kurimoto T., *Microbiol. Immunol.*, **46**, 343—351 (2002).
51) To W. C., Seeley B. M., Barthel S. W., Shu S., *Laryngoscope*, **110**, 1648—1654 (2000).
52) Steitz J., Bruck J., Knop J., Tuting T., *Gene Ther.*, **8**, 1255—1263 (2001).
53) Stout R. D., Bottomly K., *J. Immunol.*, **142**, 760—765 (1989).
54) Kodama T., Takeda K., Shimozato O., Hayakawa Y., Atsuta M., Kobayashi K., Ito M., Yagita H., Okumura K., *Eur. J. Immunol.*, **29**, 1390—1396 (1999).
55) Shinomiya Y., Harada M., Kurosawa S., Okamoto T., Terao H., Matsuzaki G., Shirakusa T., Nomoto K., *Immunobiology*, **193**, 439—455 (1995).