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**Go Ohe · Masato Okamoto · Tetsuya Oshikawa
Sachiko Furuichi · Hidetomo Nishikawa
Tomoyuki Tano · Kayo Uyama · Takashi Bando
Hideo Yoshida · Toru Sakai · Kunisuke Himeno
Mitsunobu Sato · Shinya Ohkubo**

Th1-cytokine induction and anti-tumor effect of 55 kDa protein isolated from *Aeginetia indica* L., a parasitic plant

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Abstract We have isolated a 55 kDa protein from the seed extract of *Aeginetia indica* L. (AIL), a parasitic plant, by affinity chromatography on an N-hydroxysuccinimide-activated Sepharose High Performance column bound with F3, a monoclonal antibody that neutralizes the cytokine-inducing and anti-tumor effect of AIL. In the present study, we examined this protein (AILb-A) for cytokine induction and anti-tumor effects by animal study, using syngeneic Meth-A tumor-bearing BALB/c mice, in which the Th2 response is genetically dominant. AILb-A administration resulted in markedly increased levels of Th1 cytokines [interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-12 and IL-18] in the sera derived from Meth-A-bearing mice. The in vitro re-stimulation with AILb-A of splenocytes derived from AILb-A-primed mice also selectively induced Th1-type cytokines and antigen-specific killer cell activity. The neutralizing test using cytokine-specific antibodies revealed that AILb-A-induced IL-18 plays a most significant role for IFN-γ- and killer cell-inducing activities. Furthermore, IL-12 and IL-18 induced by AILb-A inhibited specifically IL-10 and IL-4 production, respectively. Finally, we examined the anti-tumor effect of AILb-A in both Meth-A-bearing BALB/c mice and Meth-A-bearing nude mice with BALB/c background. AILb-A exhibited a striking anti-tumor effect in normal BALB/c mice inoculated with Meth-A cells. In athymic nude mice, the anti-tumor effect of AILb-A was relatively weak. These findings strongly suggested that AILb-A is a potent Th1 inducer and may be a useful immunotherapeutic agent for patients with malignant diseases.

Key words *Aeginetia indica* L. · Affinity chromatography · Th1 cytokine · Anti-cancer immunity

G. Ohe · M. Okamoto (✉) · T. Oshikawa · S. Furuichi
H. Nishikawa · T. Tano · K. Uyama · T. Bando
H. Yoshida · M. Sato
Second Department of Oral and Maxillofacial Surgery,
Tokushima University School of Dentistry,
3-18-15 Kuramoto-cho, Tokushima 7708504, Japan
e-mail: mokamoto@dent.tokushima-u.ac.jp
Tel.: +81-88-6337354; Fax: +81-88-6337462

T. Sakai · K. Himeno
Department of Parasitology,
Tokushima University School of Medicine,
Tokushima, Japan

S. Ohkubo
Ohkubo Hospital, Tokushima, Japan

Introduction

Aeginetia indica L. (AIL), a plant parasitic on roots of Japanese pampas grasses or sugar canes, has been used as a tonic and an anti-inflammatory herb agent in China and Japan. We previously reported that the butanol extract from seeds of AIL mediates potent anti-tumor immunity in tumor-bearing mice. Anti-tumor immunity induced by AIL seemed to be exerted through activating tumor-antigen-specific $CD4^+$ T cells, while $CD8^+$ T cells did not usually play an essential role in this system [5]. We also found that spleen cells from AIL-treated mice secreted interferon (IFN)-γ, tumor necrosis factor (TNF), interleukin (IL)-2 and IL-6 when re-stimulated in vitro with AIL, and that $CD4^+$ T cells were the main producers of these cytokines [6]. Furthermore, we generated a monoclonal antibody that recognized a 55 kDa protein in AIL (mAb F3), and elimination of the protein from AIL by immunoprecipitation using F3 significantly reduced the anti-tumor ability of AIL in tumor-bearing mice [7]. Recently, we have isolated the 55 kDa protein (AILb-A) by an affinity chromatography on an N-hydroxysuccinimide (NHS)-activated Sepharose High Performance (HP) column bound with mAb F3. The AILb-A protein did not contain any carbohydrate determinants and was found to markedly induced Th1-type cytokines and apoptosis-inducing factors, such as TNF-α, TNF-β, Fas ligand, TNF-related apoptosis-

inducing ligand and perforin, on human peripheral blood mononuclear cells (PBMC) in vitro [19]. Since the findings suggested that AILb-A may be a useful immunotherapeutic agent for patients with malignancies, in the present study we examined the protein for Th1-cytokine induction and anti-tumor effect by animal experiments using syngeneic Meth-A tumor-bearing BALB/c mice, in which the Th2 response is genetically dominant [17].

Materials and methods

Isolation from AIL of the antigen recognized by mAb F3

The antigen recognized by F3 was purified from butanol extract of the seed of AIL (AILb) by affinity chromatography on HiTrap NHS-activated Sepharose HP column (bed volume 5 ml) (Amersham Pharmacia Biotech AB, Upsala, Sweden) bound with F3. Five milliliters of AILb in phosphate-buffered saline (PBS; 1 mg/ml) was layered on the F3-binding HiTrap NHS-activated Sepharose HP column and stood for 2 h at room temperature. After the column was washed with a large volume of PBS, the antigen bound to F3 was eluted with 0.15 M NaCl-NH_3 buffer, pH 11, at a flow rate of 30 ml/h at room temperature. The eluate was dialyzed immediately against PBS and then lyophilized. This preparation, which reacted positively with F3 in ELISA, was designated AILb-A and was used for the present study. The AILb-A protein had a molecular weight of 55 kDa and did not contain any carbohydrate determinants [19]. No LPS contamination in the AILb-A was confirmed by endo-specy test (data not shown).

Cells and media

The mouse fibrosarcoma cell line Meth-A [1] and mouse Molony lymphoma cell line YAC-1 [13] were cultured in RPMI-1640 (Life Technologies, Inc., Gaithersburg, Md., USA) supplemented with 10% fetal calf serum (Bio-Whittaker, Walkersville, Md., USA), 100 mg/ml streptomycin and 100 U/ml penicillin, in a humidified atmosphere of 95% air and 5%CO_2 at 37 °C. The human salivary adenocarcinoma cell line HSG [21] was grown in Eagle's minimum essential medium (Life Technologies, Inc.) supplemented with 10% newborn calf serum (Bio-Whittaker), 2 mM L-glutamine (Wako Pure Chemical Co., Osaka, Japan), 100 μg/ml streptomycin and 100 U/ml penicillin, in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Animal model

Six-week-old female BALB/c mice were inoculated with 1×10^5 Meth-A cells i.p. and were randomly divided into 2 groups consisting of 4 animals each. The animals were primed with 10 μg of AILb-A suspended in 0.2 ml of physiologic saline once on day 7 after the inoculation of Meth-A cells. The mice in the control group were received 0.2 ml of physiologic saline only. On day 8, the sera and the spleen cells from the mice were collected. The spleen cells (1×10^6/ml) were cultured in RPMI-1640 medium containing 10% fetal calf serum in the presence or absence of AILb-A (0.01, 0.1 and 1.0 μg/ml) for 24 h at 37 °C. In some experiments, anti-IFN-γ (10 μg/ml, R & D Systems Inc., Minneapolis, Minn., USA), anti-TNF-α (10 μg/ml, R & D), anti-IL-6 (10 μg/ml, R & D), anti-IL-12 (10 μg/ml, Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA), anti-IL-10 (10 μg/ml, R & D), anti-IL-12 (10 μg/ml, R & D) or anti-IL-18 (10 μg/ml, PeproTech EC LTD., London, England) neutralizing Ab was added into the above cultures. Cytokines in the sera and in the supernatants of these cultures, as well as cytolytic activities of the spleen cells, were measured by following techniques.

To examine the anti-tumor effect of AILb-A, Meth-A-bearing BALB/c mice were randomly divided into 4 groups consisting of 8 animals each. The animals were given 1 μg of AILb-A, 5 μg of AILb-A and 10 μg of AILb-A i.p. every other day, from days 1 through 9, after the inoculation of Meth-A cells. The mice in the control group received 0.2 ml of physiologic saline. Furthermore, Meth-A-bearing athymic nude mice with BALB/c background were randomly divided into 2 groups consisting of 8 animals each. The nude mice were given 10 μg of AILb-A i.p. every other day, from days 1 through 9, after the inoculation of Meth-A cells. The control nude mice received 0.2 ml of physiologic saline.

Cytokine assay

The assay for cytokines was performed using ELISA kits. The ELISA kits for mouse IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10 and IL-12 were purchased from BioSource International (Camarillo, Calif., USA). The ELISA system for mouse IL-18 was established in our laboratory. Polyclonal goat anti-mouse IL-18 (Santa Cruz) as a solid phase Ab and polyclonal rabbit anti-murine IL-18 (PeproTech) as a second Ab were used. The assay detected mouse IL-18 at 10 pg/ml or higher in a linear fashion.

Assay for cytolytic activities

The cytolytic activities of mouse spleen cells were assayed against YAC-1, markedly sensitive target cells for mouse NK cells; against Meth-A cells, sensitive target for the cytotoxic T cells recognizing the antigens expressed in Meth-A, but not destroyed by NK cells; and against HSG, resistant for mouse NK cells in a ^{51}Cr-release test. The ^{51}Cr-release was carried out as described previously [23]. For cell-mediated cytotoxicity assays, 0.5, 1.0, 2.0 or 4.0×10^5 effector cells were mixed in the wells of 96-well microtiter plates (Falcon; Becton Dickinson Labware, Lincoln Park, N.J., USA) with 1.0×10^4 ^{51}Cr-labeled target cells [effector cell/target cell (E/T) = 5, 10, 20 or 40/1] in a total volume of 200 μl medium and incubated at 37 °C for 4 h. The percent specific ^{51}Cr-release was calculated according to the formula: $[(E–S)/(M–S)] \times 100$, where E was experimental ^{51}Cr-release, S spontaneous ^{51}Cr-release, and M maximum ^{51}Cr-release.

Statistical analysis

The data were evaluated by using one-way analysis of variance. Differences in survival time between groups were determined by the Kaplan-Meier method. $P < 0.05$ was considered significant.

Results

Cytokines in the sera derived from syngeneic Meth-A tumor-bearing BALB/c mice treated with or without AILb-A

Meth-A-bearing mice given 10 μg of AILb-A or physiologic saline were killed on day 8 from the inoculation of Meth-A cells and their sera assayed for cytokines. The levels of all of the Th1-type cytokines tested (IFN-γ, TNF-α, IL-2, IL-12 and IL-18) were significantly increased in the sera derived from the animals given AILb-A as compared with those from control animals. In contrast, in the production of Th2-type cytokines (IL-4, IL-6 and IL-10) there was no significant difference in

the sera between AILb-A-treated and untreated mice (Fig. 1).

Cytokine production and killer cell activities by AILb-A-stimulated spleen cells derived from Meth-A-bearing mice primed with or without AILb-A

The spleen cells prepared from tumor-bearing mice primed with or without AILb-A were re-stimulated with AILb-A in vitro. Twenty-four hours later, the supernatants were measured for cytokines. Induction of Th1 cytokines (IFN-γ, TNF-α, IL-2, IL-12 and IL-18) by the in vitro re-stimulation with AILb-A was striking on the spleen cells derived from AILb-A-primed animals, as compared with those from animals given saline (Fig. 2A–C, G and H). Of the Th2 cytokines, IL-4 was not induced by in vitro re-stimulation with AILb-A on the splenocytes from either AILb-A-treated or untreated animals (Fig. 2D). No significant difference on IL-6 secretion by the AILb-A re-stimulated splenocytes was observed between the mice given AILb-A and those given saline (Fig. 2E). Interestingly, IL-10 was not induced by the in vitro re-stimulation with AILb-A on splenocytes derived from animals primed with AILb-A, but was induced by in vitro stimulation of splenocytes from animals not primed with AILb-A (Fig. 2F).

In addition, the killer cell activities of the spleen cells were analyzed. The cytolytic activities of splenocytes derived from AILb-A-primed mice were enhanced markedly by the additional in vitro stimulation with AILb-A, as compared with those of non-primed mice-derived splenocytes. Additional stimulation with AILb-A in vitro accelerated the killer cell activity against Meth-A far better than against YAC-1 or HSG (Fig. 3). Furthermore, the killing activities of the splenocytes from AILb-A-primed mice against Meth-A were significantly increased at a 5/1 E/T ratio, and were further enhanced in a manner dependent on E/T ratio, as compared with those of non-primed animal-derived splenocytes (Fig. 4A). However, the nonspecific killing activities against YAC-1 and HSG were only slightly enhanced at an E/T ratio of 10/1 or 20/1 (Fig. 4B and C).

Effect of neutralizing antibodies against cytokines on Th1/Th2 cytokines and killer cell activities induced by AILb-A

The spleen cells derived from tumor-bearing animals primed with AILb-A were cultured in the presence or absence of AILb-A (1 μg/ml) and the neutralizing Abs were added to the cultures. Twenty-four hours later, cytokines in the supernatants, and cytolytic activities of the splenocytes, were analyzed. The neutralizing Abs against IL-2, IL-12 and IL-18 significantly inhibited IFN-γ production by AILb-A-stimulated splenocytes, and the inhibitory effect of anti-IL-18 Ab was

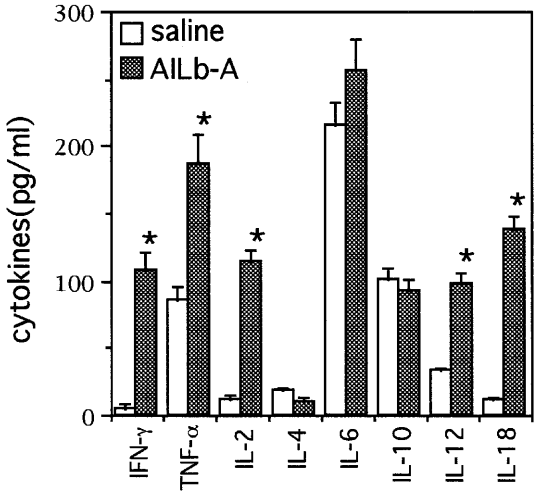

Fig. 1 Cytokines in the sera derived from syngeneic Meth-A tumor-bearing BALB/c mice given AILb-A. The sera collected from Meth-A-bearing BALB/c mice administered with 10 μg of AILb-A or saline were analyzed for cytokines by ELISA. *Bars* denote SD of 4 samples. *, $P < 0.001$, as compared with the respective controls given saline. Similar results were obtained from another independent experiment

the most striking among the Abs tested (Fig. 5A). Production of TNF-α was not influenced by all of the Abs tested (Fig. 5B). IL-2 production was inhibited by the Abs against TNF-α and IL-12 (Fig. 5C). In addition, anti-IL-10-neutralizing Ab further increased IFN-γ and IL-2 production augmented by AILb-A (Fig. 5A and C). Of the Th2-type cytokines, IL-4 production by AILb-A-stimulated splenocytes was significantly increased by addition of anti-IL-18 Ab (Fig. 5D). IL-10 production was accelerated by neutralization of IL-12 (Fig. 5E).

Additionally, the effect of neutralizing Abs against cytokines on the cytotoxic activities of the AILb-A-activated splenocytes was examined. The antigen-specific killing against Meth-A of the splenocytes accelerated by AILb-A was reduced significantly when the neutralizing Ab against IFN-γ, IL-2, IL-12 or IL-18 was added into the culture (Fig. 6A). Non-specific killing activities were also inhibited by neutralization of these cytokines (Fig. 6B and C). Anti-IL-10 Ab accelerated cytotoxic activities against Meth-A and YAC-1 (Fig. 6A and B).

The addition of Abs in the absence of AILb-A showed no significant effect on Th1 cytokine-production and cytotoxic activities of splenocytes as compared with the untreated control (data not shown).

Anti-tumor effect of AILb-A in syngeneic Meth-A tumor-bearing BALB/c mice and in Meth-A-bearing athymic nude mice

Finally, we examined anti-tumor effect of AILb-A in both Meth-A-bearing BALB/c mice and Meth-A-bearing nude mice with BALB/c background. In normal

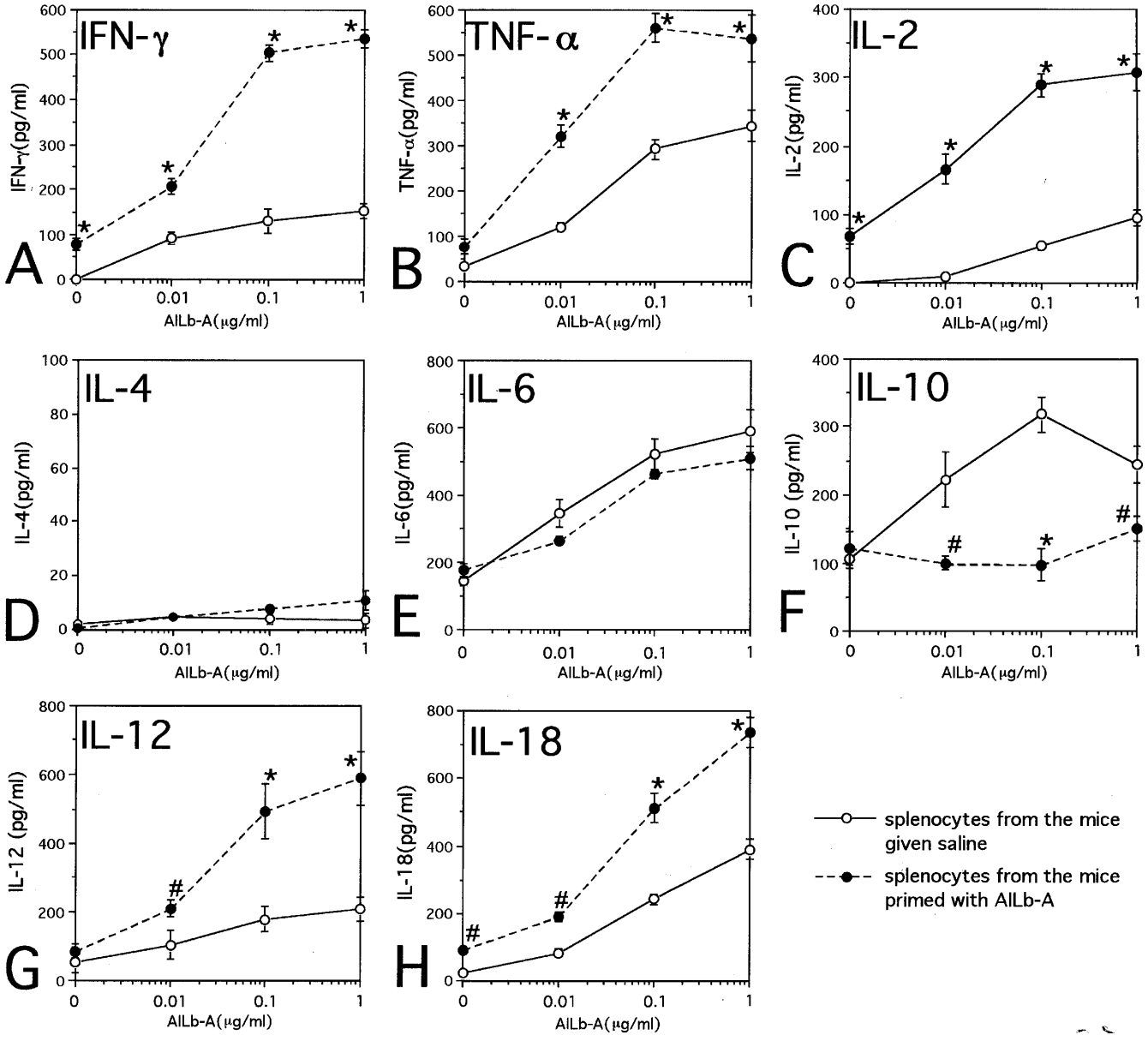

Fig. 2A–H Cytokine induction in spleen cells stimulated with AILb-A. The spleen cells (1×10^6/ml) derived from tumor-bearing mice primed with or without AILb-A (10 μg) were cultured in RPMI-1640 medium containing 10% FCS in the presence or absence of AILb-A (0.01, 0.1 and 1.0 μg/ml) for 24 h at 37 °C. IFN-γ (**A**), TNF-α (**B**), IL-2 (**C**), IL-4 (**D**), IL-6 (**E**), IL-10 (**F**), IL-12 (**G**) and IL-18 (**H**) in the supernatants of these cultures were assayed by ELISA. *Bars* denote SD of 4 samples. *; $P < 0.01$; #; $P < 0.05$, as compared with respective control animals given saline. Similar results were obtained from another independent experiment

BALB/c mice inoculated with Meth-A, the control group given saline died within 23 days from day 12 after transplantation of Meth-A cells. Although the tumor-bearing animals given 1 μg of AILb-A showed no significant effect in the survival time of the animals, the treatment of the mice with 5 μg of AILb-A significantly extended their survival time. Furthermore, the administration of 10 μg of AILb-A markedly extended the survival time of the tumor-bearing animals and 62.5% of the animals (5 of 8) were alive on day 60 (Fig. 7A). The anti-tumor effect of AILb-A was slight, but significant, in tumor-bearing nude mice (Fig. 7B).

Discussion

We have reported previously that the butanol extract from seeds of AIL induces cytokines and mediates potent anti-tumor immunity in tumor-bearing mice [5, 6], and that a protein fraction in AIL may be closely involved in the anti-tumor activity of AIL [7]. Recently, we have succeeded in isolating the 55 kDa protein (AILb-A) by affinity chromatography on an NHS-activated Sepharose HP column bound with F3, a mAb that neutralizes the IFN-γ-inducing and anti-tumor activities of AIL. AILb-A induced strikingly Th1-type cytokines

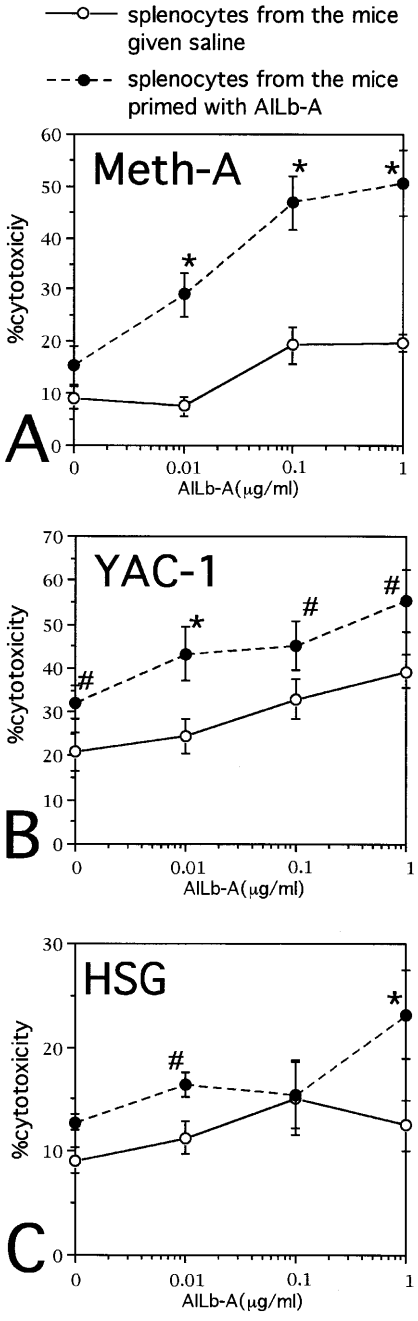

Fig. 3A–C Cytolytic activities of spleen cells stimulated with AILb-A. The spleen cells (1×10^6/m1) derived from tumor-bearing mice primed with or without AILb-A (10 μg) were cultured in RPMI-1640 medium containing 10% FCS in the presence or absence of AILb-A (0.01, 0.1 and 1.0 μg/m1) for 24 h at 37 °C. Cytolytic activities of the spleen cells against Meth-A (**A**), YAC-1 (**B**) and HSG (**C**) were analyzed by ^{51}Cr-release test. *Bars* denote SD of 4 samples. *; $P < 0.01$; #; $P < 0.05$, as compared with respective control animals given saline. Similar results were obtained from another independent experiment

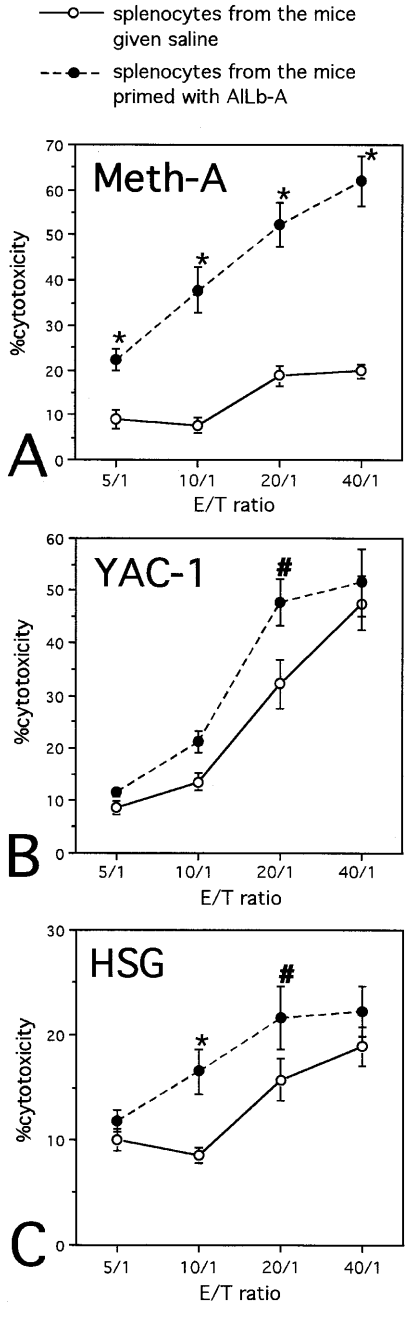

Fig. 4A–C Cytolytic activities of spleen cells stimulated with AILb-A. The spleen cells (1×10^6/ml) derived from tumor-bearing mice primed with or without AILb-A (10 μg) were cultured in RPMI-1640 medium containing 10% FCS in the presence or absence of AILb-A (1.0 μg/ml) for 24 h at 37 °C. Cytolytic activities of the spleen cells against Meth-A (**A**), YAC-1 (**B**) and HSG (**C**) were analyzed at 4 different E/T ratios by ^{51}Cr-release test. *Bars* denote SD of 4 samples. *; $P < 0.01$; #, $P < 0.05$, as compared with respective control animals given saline. Similar results were obtained from another independent experiment

and apoptosis-inducing factors, such as TNF-α, TNF-β, Fas ligand, TNF-related apoptosis inducing ligand and perforin, on human PBMC in vitro [19]. It was strongly suggested that AILb-A might be a useful immunotherapeutic agent for patients with malignancies.

In the current study, AILb-A markedly induced IFN-γ, TNF-α, IL-2, IL-12 and IL-18, and accelerated killer cell activities in splenocytes derived from tumor-bearing mice primed with 10 μg of AILb-A (i.p.). Especially in the killing activity against Meth-A, which is resistant to NK cells and is killed by Meth-A-specific killer T cells in

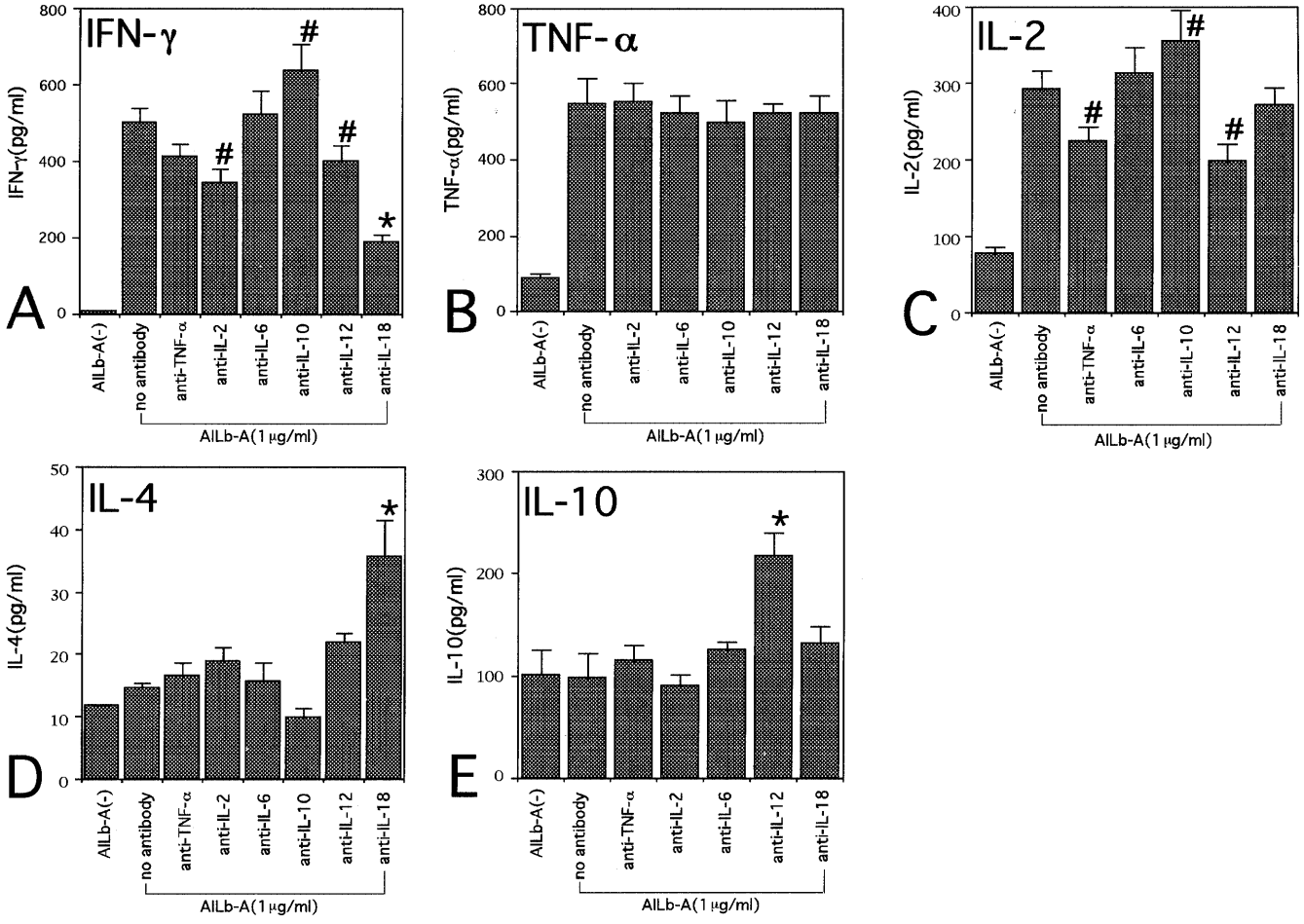

Fig. 5A–E Effect of neutralizing antibodies against cytokines on the Th1/Th2 cytokine-inducing activity of AILb-A. The spleen cells (1×10^6/ml) derived from tumor-bearing mice given AILb-A (10 μg) were cultured in RPMI-1640 medium containing 10% FCS in the presence or absence of AILb-A (1.0 μg/ml) for 24 h at 37 °C. Each neutralizing antibody (10 μg/ml) against cytokines was added into the cultures. The supernatants were analyzed for IFN-γ (**A**), TNF-α (**B**), IL-2 (**C**), IL-4 and (**D**) IL-10 (**E**). *Bars* denote SD of 5 samples. *; $P < 0.01$; #, $P < 0.05$, as compared with respective positive controls treated with AILb-A alone. Similar results were obtained from another independent experiment

the current system, the combined effect of AILb-A-priming of the animals and in vitro re-stimulation with AILb-A was striking compared with the killing activity against YAC-1 and HSG, which are target cells for non-specific killer cells. This indicates that antigen-specific killing might be enhanced by AILb-A-priming and additional in vitro re-stimulation. From this evidence, we speculate the possibility that AILb-A may elicit its effect at two stages: first in the tumor-antigen presentation in vivo by antigen-presenting cells, mainly dendritic cells, and subsequently in the activation of effector cells, such as T cells. This hypothesis is now under investigation in our laboratory.

Interestingly, of the Th2-type cytokines, IL-4 was not induced by AILb-A-treated splenocytes derived from either AILb-A-primed or non-primed animals. IL-6 production was also not influenced by AILb-A-priming. Furthermore, the level of IL-10 production was not increased in AILb-A-stimulated splenocytes derived from AILb-A-primed mice, whereas IL-10 was induced upon in vitro stimulation of splenocytes from animals not primed with AILb-A. Thus, AILb-A selectively induced Th1 cytokines in Meth-A-bearing mice.

The findings from the in vitro neutralizing test using specific Abs against cytokines suggested that IL-18 plays a more significant role for the IFN-γ- and killer cell-inducing activities of AILb-A than IL-12, whereas it was recently reported that stimulation with IL-18 alone, but not in combination with IL-12, increases the production of IL-4 and IL-13 in T cells, NK cells and basophils, and induces a Th2-dominant state [11, 25]. Furthermore, treatment with recombinant IL-12 and IL-18 was effective for the control of tumor cells, but lethal side effects occurred [15]. We believe that the inducing ability of AILb-A for both endogenous IL-12 and IL-18 may be of great advantage in Th1 induction, as well as in the anti-tumor effect. Furthermore, anti-IL-10 Ab significantly accelerated the IFN-γ, IL-2 and killer cell-inducing activities of AILb-A. Although IL-10 production was not increased in AILb-A-primed mice, spontaneously released IL-10 may inhibit these activities. Moreover, anti-IL-18 Ab specifically increased IL-4 production, and anti-IL-12 increased IL-10 specifically. The amount of IL-4 seemed to be increased slightly by addition of anti-IL-12 Ab, but the in-

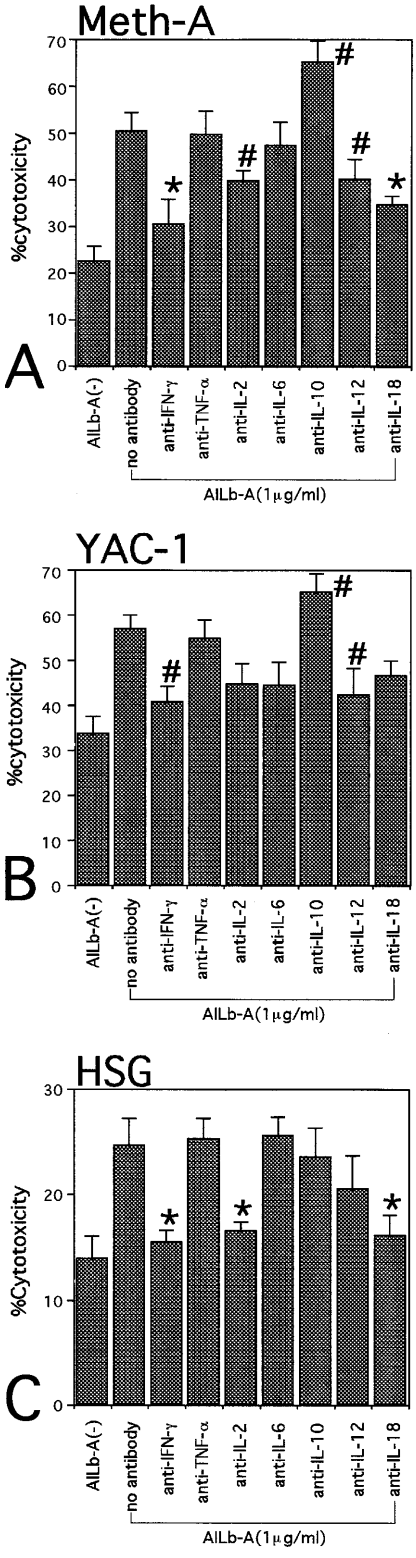

Fig. 6A–C Effect of neutralizing antibodies against killer cell inducing activity of AILb-A. The spleen cells (1×10^6/ml) derived from tumor-bearing mice given AILb-A (10 μg) were cultured in RPMI-1640 medium containing 10% FCS in the presence or absence of AILb-A (1.0 μg/ml) for 24 h at 37 °C. Each neutralizing antibody (10 μg/ml) against cytokines was added into the cultures. The treated spleen cells were assayed for cytolytic activities against Meth-A (**A**), YAC-1 (**B**) and HSG (**C**). *Bars* denote SD of 5 samples. *; $P < 0.01$; #, $P < 0.05$, as compared with respective positive controls treated with AILb-A alone. Similar results were obtained from another independent experiment

crease was not statistically significant ($P = 0.094$). While it has been reported that Th1 and Th2 cytokines regulate the production of each other [4, 9], this is the first report demonstrating the relationships clearly between IL-4 and IL-18, between IL-10 and IL-12, induced by an immunomodulator. Such specific relationships were not demonstrated in experiments using other immunomodulators, including *Bacillus* Calmette-Guerin (BCG), OK-432, a penicillin-killed streptococcal agent, and LTA preparation of *Streptococcus* [10, 20] (Okamoto and Sato, unpublished observations). At least, the endogenous IL-12 and IL-18 induced by AILb-A inhibit specifically IL-10 and IL-4, respectively. Additionally, it has also been reported that IL-12 may increase IL-10 production to limit its own effect [12, 24]; however our own observations, as described above, indicated that a Th1 dominant state was further induced by inhibition of IL-4 and IL-10, at least in the case of AILb-A. As above, AILb-A markedly induced Th1-type cytokines in Meth-A-bearing BALB/c mice, in which the Th2 response is genetically dominant [17], as well as in human PBMC [19]. In addition, AILb-A demonstrated a striking anti-tumor effect in Meth-A-bearing BALB/c mice. It has been reported that cytokine balance shifts to Th2 dominance in patients with malignant diseases and that an inhibition of anti-tumor immunity occurs, as well as tumor cachexia [3, 22]. The results from the in vivo study using BALB/c mice strongly suggest that AILb-A may also be effective for the cancer patients with a Th2 dominant state. AILb-A showed a significant anti-tumor effect in tumor-bearing nude mice, but the effect was relatively weak. Most of the anti-tumor immunity induced by AILb-A may be exhibited by T cells, monocytes/macrophages and possibly dendritic cells.

In conclusion, the 55 kDa protein isolated from AIL (AILb-A), which can induce Th1 cytokines greatly and elicit anti-tumor activity in tumor-bearing BALB/c mice, may be a useful immunotherapeutic agent for patients with malignant diseases. The evidence that AILb-A-priming in vivo is necessary for Th1 induction, and that antigen-specific killing is exhibited in this case, suggests that immunization of effector cells by tumor antigens that have escaped from the host immune response in the tumor-bearing host is essential for the anti-tumor effect, and that AILb-A accelerates the antigen presentation ability, as well as sequential activation, of immunized effector cells. Furthermore, it is also suggested that AILb-A administration once only is not sufficient to cure patients with malignancies in clinical use of AILb-A. We are now cloning the gene encoding the protein. Recently, immuno-gene therapies using cDNAs encoding several cytokines, such as IFN-γ, granulocyte macrophage colony stimulating factor, IL-2, IL-12 and IL-18, were performed against several types of malignancies [2, 8, 14, 16, 18] Whereas it is difficult to cure

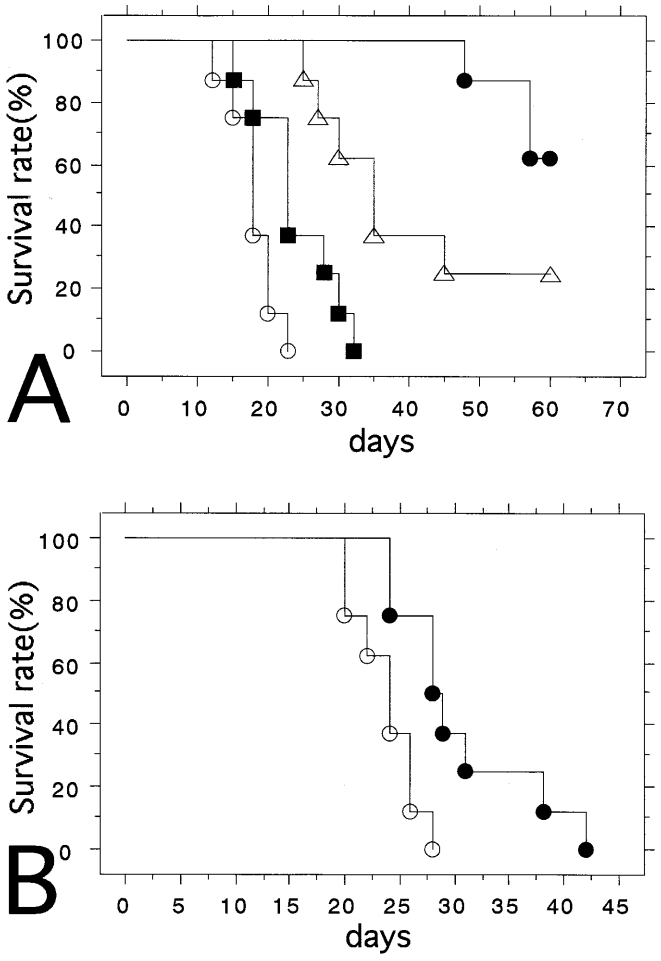

Fig. 7A, B Effect of AILb-A on the survival of syngeneic Meth-A tumor-bearing BALB/c mice (**A**) and of Meth-A-bearing nude mice with BALB/c background (**B**). Meth-A-bearing BALB/c mice were given 0.2 ml of physiologic saline (○), 1 μg of AILb-A (■), 5 μg of AILb-A (△) or 10 μg of AILb-A (●) i.p. every other day from days 1 through 9 after the inoculation of Meth-A cells (**A**). Meth-A-bearing nude mice were also given 0.2 ml of physiologic saline (○) or 10 μg of AILb-A (●) i.p. every other day from days 1 through 9 (**B**). Statistical values: in **A**, (○) and (■), $P = 0.244$; (○) and (△), $P = 0.020$; (○) and (●), $P < 0.001$; in B, (○) and (●), $P = 0.037$

malignant diseases completely by therapy using only one or two exogenous cytokine gene(s), we believe that therapy using the gene encoding the Th1 cytokine-inducing protein, AILb-A, may be a useful strategy for the treatment of cancer patients.

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