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Improvement of Macrophage Dysfunction by Administration of Anti-transforming Growth Factor-β Antibody in EL4-bearing Hosts

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An experimental therapy for improvement of macrophage dysfunction caused by transforming growth factor-β (TGF-β) was tried in EL4 tumor-bearing mice. TGF-β was detected in cell-free ascitic fluid from EL4-bearers, but not in that from normal mice, by western blot analysis. The ascites also showed growth-suppressive activity against Mv1Lu cells, and the suppressive activity was potentiated by transient acidification. To investigate whether the functions of peritoneal macrophages were suppressed in EL4-bearers, the abilities to produce nitric oxide and tumor necrosis factor-α (TNF-α) upon lipopolysaccharide (LPS) stimulation were measured. Both abilities of macrophages in EL4-bearing mice were suppressed remarkably on day 9, and decreased further by day 14, compared with non-tumor-bearing controls. TGF-β activity was abrogated by administration of anti-TGF-β antibody to EL4-bearing mice. While a large amount of TGF-β was detected in ascitic fluid from control EL4-bearers, little TGF-β was detectable in ascites from EL4-bearers given anti-TGF-β antibody. Furthermore, while control macrophages exhibited little or no production of nitric oxide and TNF-α on LPS stimulation *in vitro*, macrophages from EL4-bearers administered with anti-TGF-β antibody showed the same ability as normal macrophages. These results clearly indicate that TGF-β contributes to macrophage dysfunction and that the administration of specific antibody for TGF-β reverses macrophage dysfunction in EL4-bearing hosts.

Key words: TGF-β — Ascitic fluid — EL4 — Anti-TGF-β antibody — Immunosuppression

Tumor-bearing hosts fail to reject tumor cells in experimental animal models, as occurs in clinical cancer patients. Tumor cell destruction by immune effector cells can be prevented by tumor-induced immunosuppressor cells and/or factors. There is much evidence that tumor-induced immunosuppression is mediated by humoral factors and that many malignant cells produce immunosuppressive factors to escape from immune surveillance.[1-3] EL4-bearing C57BL/6 mice have been used as a tumor-induced immunosuppressive model to examine the effects of tumor progression on a variety of cytolytic effector cells.[4] The results indicated that the inhibition of cytotoxic T lymphocytes (CTL), lymphokine-activated killer (LAK) cells, and splenic macrophage responses occurred in the late tumor-bearing state, but the inhibition mechanisms remain unclear.

Transforming growth factor-β (TGF-β) was originally named and purified on the basis of its ability to stimulate the growth of normal rat kidney (NRK) fibroblast colonies in soft agar in the presence of epidermal growth factor.[5] It is now apparent that TGF-β shows potent regulatory effects on the growth, differentiation and functions of immune effector cells. Most of the reported effects of TGF-β on cells in the immune system have been "suppressive," including the inhibition of thymocyte proliferation,[6] T cell proliferation and function,[7-10] B cell proliferation and production of IgM or IgG,[11] NK cell activity,[12] and LAK cell activity.[13] In macrophages, TGF-β displays both stimulatory and inhibitory effects. Chemotaxis of macrophages was enhanced by TGF-β,[14] but most of the macrophage functions were suppressed. For example, production of inorganic oxidants with cytotoxic effects on tumor cells and other targets, such as reactive oxygen intermediates,[15] and nitric oxide,[16,17] and generation of inflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α),[18,19] were strongly inhibited by TGF-β.

In the present study, we analyzed the role of TGF-β in EL4-bearing hosts, especially in relation to macrophage functions. The results indicated that TGF-β has immunosuppressive activity on macrophages, and inhibition of TGF-β activity by the administration of anti-TGF-β antibody released macrophages from immunosuppression in EL4-bearing mice.

MATERIALS AND METHODS

Mice Inbred 6- to 10-week-old female C57BL/6 mice were purchased from the Charles River Japan, Inc. (Kanagawa), and were maintained under specific-pathogen-free conditions.

Cell lines and cell culture EL4 cells, a mouse thymoma cell line, were maintained by i.p. passage of 1×10^7 cells

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every 7 days in 8- to 10-week-old syngeneic C57BL/6 mice. Mv1Lu cells, a mink lung epithelial cell line, were purchased from ATCC (American Type Culture Collection, Rockville, MD). The cells were maintained in Eagle's minimal essential medium (MEM, Flow Laboratories, Irvine, Scotland), supplemented with 10% fetal bovine serum (FBS, Flow) and 1 m*M* L-glutamine (GIBCO Laboratories, Grand Island, NY).

Reagents Recombinant human TGF-β1 (TGF-β1) was purchased from King Brewing Co. (Kakogawa, Hyogo). Neutralizing anti-human TGF-β1 antibody and control chicken IgY were purchased from R&D Systems, Inc. (Minneapolis, MN). Bacterial lipopolysaccharide (LPS), prepared by phenol extraction from *Escherichia coli* 055: B5, was purchased from Sigma (St. Louis, MO).

Preparation of ascitic fluid from EL4-bearing mice and acid-activation EL4 cells were inoculated s.c. (1×10^4, into the right breast) into normal C57BL/6 mice using a plastic 1-ml syringe with a 26-gauge needle. The ascitic fluids were collected on day 14 after tumor inoculation. The cell-free ascitic fluid was obtained by centrifugation (250*g*, 10 min) and filtered through a 0.22 μm filter (Millipore, Bedford, MA) to remove contaminating cells, and then concentrated in a Centriprep-10 (Grace Japan Co., Tokyo). The test preparations of ascitic fluid (400 μl) were activated by treatment with 10 μl of 1 *M* HCl at room temperature for 1 h, and returned to neutral pH by adding 5–10 μl of 1 *M* NaOH before addition to the assay mixture. As a control, ascitic fluid from normal mice was used. The cell-free ascitic fluid was stored at -20°C before being used for the assays.

SDS-PAGE and western blotting Serial concentrations of ascitic fluids were solubilized in SDS sample buffer without β-mercaptoethanol. After SDS-PAGE in 10% polyacrylamide gels, proteins were transferred electrophoretically to 0.2 μm pore size nitrocellulose membranes (Amersham, Arlington Heights, IL) using 20% methanol, 25 m*M* Tris, and 192 m*M* glycine, pH 8.3. The membrane was blocked for 1 h at room temperature with 5% dry milk in Tris-buffered saline containing Tween-20 (TBST: 25 m*M* Tris, pH 7.5, 150 m*M* NaCl, 0.2% Tween-20). The membrane was then washed 4 times in TBST and incubated with chicken anti-TGF-β antibody (R&D) at 1:1000 dilution for 1 h. After washing, the membrane was incubated for 1 h with a 1:5000 dilution of rabbit anti-chicken IgG antibody conjugated to horseradish peroxidase (Cappel, Durham, NC), followed by detection with the ECL western blotting detection system (Amersham). The molecular weight of TGF-β (25 kD) was calculated by comparison with molecular weight markers (Amersham) applied on the same gel.

Mv1Lu cell growth inhibition assay The growth inhibition assay was performed with slight modifications of the method of Like and Massague.[20] Briefly, Mv1Lu cells (5×10^3) were cultured in MEM medium containing 5% FBS with the test preparation of ascitic fluid in 96-well culture plates (Corning Glass Works, NY) for 24 h in a CO_2 incubator. The cells were pulse-labeled with 0.5 μCi/well of ^{3}H-thymidine (NEN, Boston, MA) for the last 2 h, and the incorporated radioactivity was measured by using a Beta plate (Wallac, Turku, Finland). All test results were averaged from duplicate cultures.

Collection and activation of peritoneal macrophages from non-tumor-bearing (NTB) or EL4-tumor-bearing (TB) mice The peritoneal resident macrophages were collected by peritoneal lavage from C57BL/6 NTB mice or TB mice on the indicated day. The peritoneal cells were centrifuged at 200*g* for 5 min, resuspended in serum-free medium, and plated (1×10^6/well) into 24-well tissue culture plates (Corning). After incubation for 4 h, non-adherent cells were removed by vigorous washing and fresh Dulbecco's-MEM (GIBCO) containing 5% FBS was added to the cell monolayer, followed by TGF-β (5 ng/ml) and/or LPS (2, 10 μg/ml). After 24 h activation with LPS, the supernatants of macrophage culture were collected, and cell-free supernatants were obtained by centrifugation. The supernatants were stored at -20°C until use.

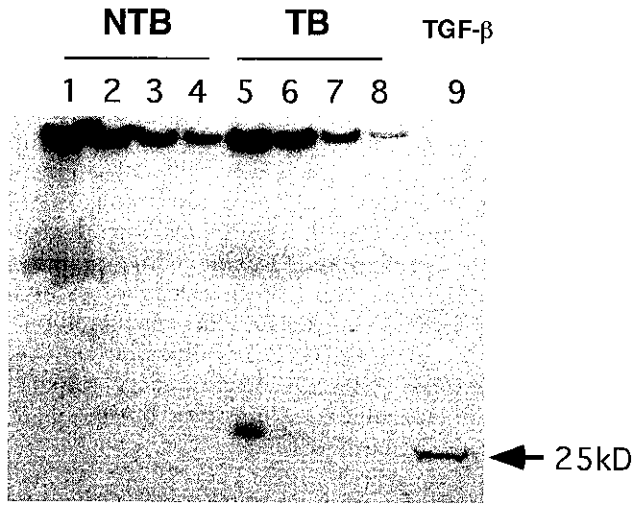

Fig. 1. Western blot analysis of ascitic fluids from EL4-bearing mice with anti-TGF-β antibody. The ascitic fluids from NTB and TB mice were concentrated and subjected to electrophoresis on a 10% SDS-polyacrylamide gel. After completion of SDS-PAGE, the proteins were transferred to a nitrocellulose membrane, followed by immunostaining with anti-TGF-β antibody prior to amplification with an ECL detection kit. Lanes 1–4, ascites from normal mice concentrated 12, 6, 3 and 1.5-fold, respectively; lanes 5–8, ascites from EL4-bearing mice concentrated 12, 6, 3 and 1.5-fold, respectively; lane 9, recombinant human TGF-β1 (5 ng).

TNF quantitation by ELISA and assay for nitric oxide The total amount of TNF-α in the macrophage supernatant was determined by using murine TNF-α ELISA system (Endogen, Boston, MA). Accumulation of nitric oxide in the supernatant was measured by an automated colorimetric assay based on the Griess reaction.[21] Briefly, samples were reacted with 1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% H_3PO_4 at room temperature for 10 min, and the NO_2^- concentration was determined by measuring the absorbance at 550 nm in comparison with sodium nitrite standards.

Antibody administration to TB mice C57BL/6 female mice were injected s.c. with EL4 cells (1×10^4) and given i.p. chicken anti-TGF-β IgY antibody (500 μg/day/mouse; 2 mg in total, R&D) from day 10 (ten days after tumor inoculation) for 4 consecutive days. In controls, saline or control IgY antibody was given by the same route, schedule and dose. The day after the last administration (day 14), the peritoneal macrophages and ascites were collected from mice of each group. The ascites were used in western blot analysis, and the macrophages were tested for their ability to produce nitric oxide and TNF-α by the methods described above. Five mice were used for each group.

RESULTS

Western blot analysis of the ascitic fluid from TB mice using anti-TGF-β antibody Studies with various tumor and non-tumor models have shown immune suppression to be mediated through the secretion of TGF-β.[22] Thus, by using western blot analysis, we investigated if TGF-β was present in the ascitic fluid from TB mice on day 14 after tumor inoculation. Serial concentrations of the acid-activated ascites were fractionated by SDS-PAGE, followed by blotting onto nitrocellulose membrane. After treatment with anti-TGF-β antibody, immunoreactive TGF-β (25 kD under non-reducing conditions) was detected in the ascites from TB mice (Fig. 1, lanes 5–8: concentrated 12, 6, 3 and 1.5-fold, respectively), but not in those of NTB mice (Fig. 1, lanes 1–4: concentrated 12, 6, 3 and 1.5-fold, respectively).

Effect of ascitic fluid from TB mouse on TGF-β-sensitive Mv1Lu cell growth The TGF-β activity in the ascites from TB mice was investigated by growth inhibition assay of Mv1Lu mink lung epithelial cells. Mv1Lu cells were cultured for 24 h in the presence of various concentrations of ascites and pulsed with ^{3}H-thymidine for the last 2 h. ^{3}H-Thymidine incorporation into the cells was then measured. Generally, TGF-β is known to be secreted in a latent form from the producing cells, and therefore the test preparations should be activated for measuring whole TGF-β activity. Thus, acid-activated ascites samples were also assayed. Fig. 2 shows that the suppressive activities of the ascites on the growth of Mv1Lu cells increased in a dose-dependent manner and were potentiated by acid-treatment. Ascitic fluid from NTB mice exhibited no inhibitory effect on Mv1Lu cell growth (data not shown). These results suggested that TGF-β was present in the ascites from TB mice in both active and latent forms.

Abilities of macrophages from TB mice to produce nitric oxide and TNF-α If TGF-β produced in TB mice induced immunosuppression in the host, the functions of peritoneal macrophages might be suppressed. Therefore, we investigated the abilities of macrophages from TB mice to produce nitric oxide and TNF-α when stimulated by LPS. The peritoneal macrophages were harvested on day 9 or 14 after EL4 tumor inoculation. As shown in Fig. 3A, the accumulations of nitric oxide in the supernatants of macrophages on days 9 and 14 were markedly suppressed with or without LPS stimulation to less than 50% of the control.

Similarly, the capacity to produce TNF-α of macrophages from TB mice was strongly suppressed (Fig. 3B). The ability of macrophages from NTB mice to produce TNF-α was induced by LPS in a dose-dependent manner (maximal TNF-α production was over 200 pg/ml of TNF-α in this assay), but EL4-bearer macrophages on day 9 showed far lower productivity, less than 50 pg/ml of TNF-α. Furthermore, macrophages from EL4-bearers on day 14 were unable to produce TNF-α on LPS stimulation, even at a high dose. These results suggested that peritoneal resident macrophages of TB mice, correspond-

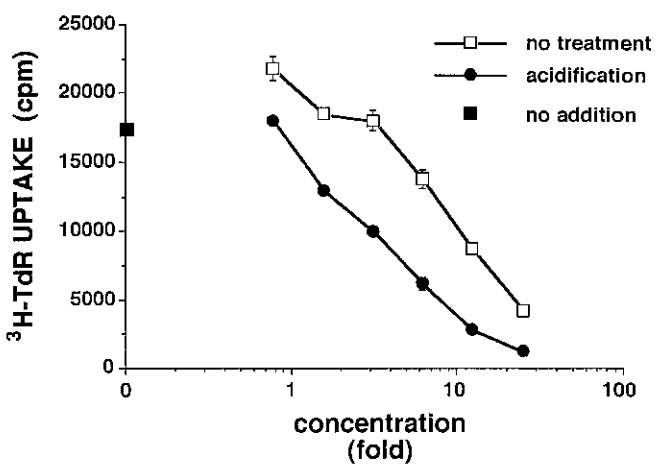

Fig. 2. Growth inhibition assay of Mv1Lu cells by ascitic fluid from EL4-bearing mice. Mv1Lu cells were cultured with the indicated concentrations of the ascites with or without acidification for 24 h, and pulsed with ^{3}H-thymidine for the last 2 h. Data are the means$\pm$SD.

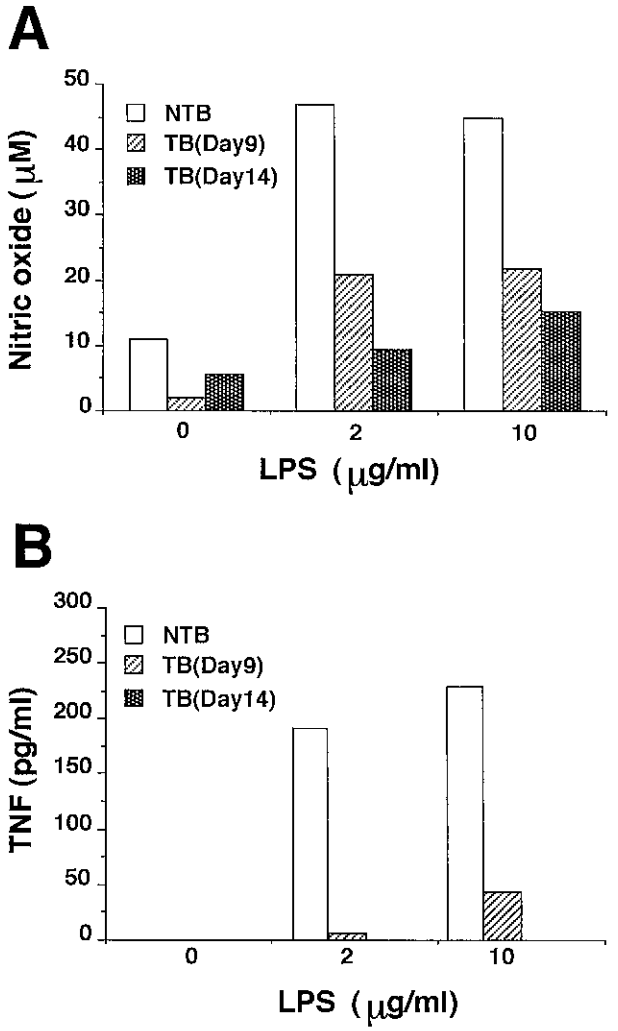

Fig. 3. Nitric oxide (A) and TNF-α (B) production of macrophages from NTB or TB mice. Peritoneal macrophages from mice were treated with LPS (0, 2, 10 μg/ml) for 24 h, and the supernatant was collected to measure the accumulation of nitric oxide and TNF-α.

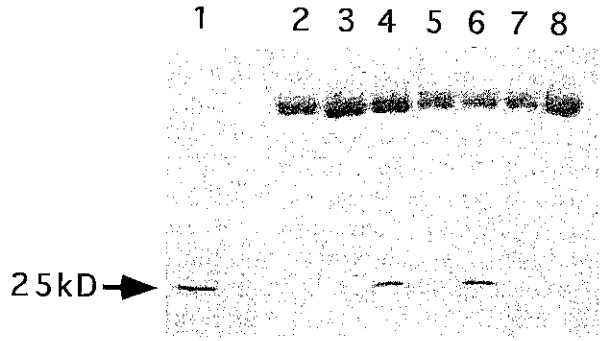

Fig. 4. Western blot analysis of the ascitic fluids from EL4-bearing mice treated with anti-TGF-β antibody. The ascitic fluids were obtained from EL4-bearing mice given saline, pre-immune IgY antibody (control chicken IgY) or anti-TGF-β antibody for 4 consecutive days. After 12-fold concentration of the ascites, TGF-β was detected by western blot analysis with anti-TGF-β antibody. Lane 1, recombinant human TGF-β1 (5 ng); lane 2, ascites from NTB mice; lanes 3 and 4, ascites from TB mice treated with saline; lanes 5 and 6, control IgY; lanes 7 and 8, anti-TGF-β antibody. Acid-activated samples were applied on lanes 2, 4, 6 and 8, respectively.

ing to macrophages of NTB mice suppressed with TGF-β, showed reduced abilities to produce nitric oxide and TNF-α on stimulation with LPS.

Effect of administration of anti-TGF-β-antibody on macrophage activity If the down-regulation of macrophage activity of TB mice were caused by TGF-β, release from the suppressive activity of TGF-β might lead to improvement of the macrophage function. Therefore, we tried an experimental therapy with anti-TGF-β antibody administered i.p. to TB mice (500 μg/day/mouse) from day 10 of inoculation for 4 consecutive days. The ascitic fluids and peritoneal macrophages were harvested, and the amounts of TGF-β in the ascites and abilities of macrophages to produce nitric oxide and TNF-α were measured. Mice of control groups were given saline or control chicken IgY with the same route, schedule and dose.

Western blot analysis revealed elevated TGF-β levels in ascitic fluids from TB control mice treated with saline or control IgY on day 14 (Fig. 4, lanes 4 and 6, respectively). In contrast, there was no detectable TGF-β in ascitic fluid of mice treated with anti-TGF-β antibody (Fig. 4, lane 8), as in control mice (lane 2). These results suggest that TGF-β produced in TB mice was neutralized by the administration of anti-TGF-β antibody *in vivo*. When the exposure time was prolonged, a slight band was detectable in lane 8, suggesting that a small amount of TGF-β remained in the ascites (data not shown).

As shown in Fig. 5A, macrophages from both TB control groups showed impaired nitric oxide production on LPS stimulation, in contrast to the NTB control. However, macrophages from TB mice treated with anti-TGF-β antibody exhibited nitric oxide production comparable to that of the NTB control. In the case of TNF-α production, similar results were obtained (Fig. 5B). Macrophages treated with anti-TGF-β antibody showed potent TNF-α-producing activity, at the same level as that of the NTB control, and far lower activities were seen in the TB control. The anti-TGF-β antibody itself had no effect on macrophage activation or proliferation *in vitro* (data not shown). These results indicate that down-regulation of macrophage activities in TB mice was mainly due to TGF-β.

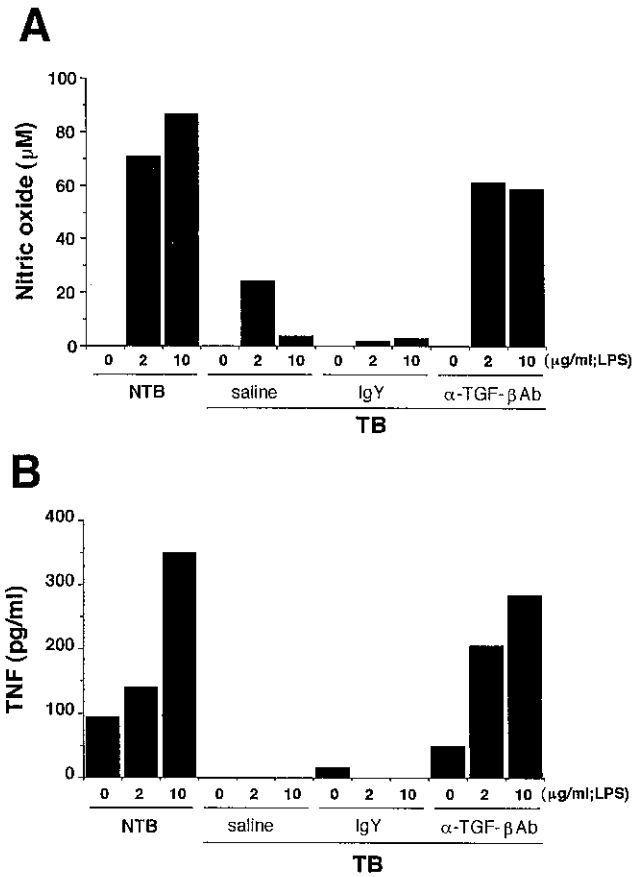

Fig. 5. Improvement of the abilities to produce nitric oxide and TNF-α of macrophages from TB mice treated with anti-TGF-β antibody. The peritoneal macrophages were harvested from TB mice given i.p. saline, preimmune IgY antibody (IgY) or anti-TGF-β antibody (α-TGF-β Ab), and the abilities to produce nitric oxide (A) and TNF-α (B) on LPS stimulation were examined. Macrophages from NTB mice were also tested.

DISCUSSION

The aim of this study was to find a means of improving the severe immunosuppression in tumor-bearing hosts. Immune effectors in the EL4-C57BL/6 model have been characterized, and all of them were found to be inhibited by multiple suppressor mechanisms.[4,23] It was proposed that the immunosuppression in TB mice was mediated by TGF-β, but little evidence for this has been presented. We tried to abolish the immuno-suppressive activity of TGF-β by using a specific antibody, and the effectiveness of this approach was examined by analysis of macrophage function in TB mice.

In the present study, we showed that ascitic fluid from TB mice has growth-inhibitory activity against Mv1Lu cells, and demonstrated the existence of TGF-β in the ascites by western blot analysis. Previous reports indicated that immune effector cells, T cells or macrophages, were down-regulated by TGF-β in tumor-bearing hosts.[24] We have tried to analyze splenocytes and thymocytes in TB mice, but could not obtain pure immune effector cells because of EL4 metastasis, which was observed in spleen and thymus of the tumor bearers even on day 5. Furthermore, we could not investigate the functions of crude populations of splenocytes or thymocytes *in vitro*, because the growth rate of EL4 cells was much faster than that of the host immune effector cells (data not shown).

It has been reported that TGF-β can deactivate macrophages by reducing their capacity to release H_2O_2[15] and nitric oxide,[16] their cytotoxic activity,[25,26] class II expression[27] and their production of TNF-α and IL-1.[28] Therefore, we focused on the correlation between TGF-β and macrophage function in TB mice, and investigated the abilities of macrophages to produce nitric oxide and TNF-α on days 9 and 14. In contrast to NTB, macrophages from TB mice exhibited only slight abilities to produce such mediators. The concentration of TGF-β was about 5 ng/ml in the whole peritoneal lavage of TB mice, enough to exhibit a suppressive effect on macrophages. It is possible that EL4 tumor cells could escape from immuno-surveillance by downregulation of macrophage ability to produce nitric oxide and TNF-α, because nitric oxide and TNF-α are potent cytolytic mediators.[29] As a control, peritoneal resident macrophages in NTB mice were used in place of the induced macrophages. Because macrophages induced with irritants, such as thioglycolate, are known to exhibit higher sensitivity to some macrophage stimulants, they were not thought to appropriate for use in the experiments on immunosuppression.

Recent reports have shown that $CD4^+$ T cells were predominantly affected by the suppressive effect of TGF-β in tumor-bearing mice,[30] so the possibility remained that helper T cell function was reduced at early stages in TB mice. Further studies are needed to clarify the correlation between macrophages and T cells in TB mice.

It has been reported that there is a relationship between TGF-β and tumor metastasis. The excessive secretion of TGF-β by Chinese hamster ovary cells transfected with TGF-β1 gene is linked to the development of a metastatic phenotype.[31] TGF-β may be an important regulator of the invasive phenotype *in vivo*. TGF-β1 could influence cellular recognition of extracellular matrix components, leading to increased invasive potential.[32] These reports suggested that TGF-β might modulate the metastatic potential of mammary tumor cells by controlling their ability to penetrate the basement-membrane barrier. Therefore, it is possible that the metastatic

potential in EL4 cells is potentiated by TGF-β via autocrine and/or paracrine mechanisms.

Finally, we tried to prevent immunosuppression in TB mice by administration of anti-TGF-β antibody. As shown in Fig. 4, TGF-β disappeared in the ascites from anti-TGF-β antibody-treated mice. Furthermore, the ability of LPS-stimulated macrophages to produce nitric oxide and TNF-α was restored to the NTB level (Fig. 5). The antibody used in the experiment showed no direct effect on macrophage activation *in vitro* or *in vivo* (data not shown). Several observations have correlated TGF-β expression with tumorigenesis. Increased expression of TGF-β has been associated with adhesive properties of tumor cells.[33] In another report, the constitutive expression of TGF-β antisense mRNA in tumor cells reduced tumorigenicity *in vivo*.[34] However, in spite of numerous reports which show that TGF-β suppresses some functions of immune effectors *in vitro*, little is known about reversal of the severe immunosuppression caused by TGF-β in tumor-bearing hosts. Our investigation has demonstrated a correlation between TGF-β and immunosuppression in TB mice, and we confirmed that administration of anti-TGF-β antibody is effective for immunotherapy against EL4 thymoma. Although further studies are needed, the data obtained here suggest that anti-TGF-β antibody may be available for the treatment of other TGF-β-producing tumors.

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