Enhancement of tumoricidal activity of alveolar macrophages via CD40-CD40 ligand interaction

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1 The First Department of Internal Medicine and 2Department of Clinical Preventive Medicine, Nagoya University School of Medicine, Nagoya 466-8550; 2Department of Clinical Laboratory Medicine, Kyoto University Hospital, Kyoto 606-8507; 3Department of Molecular Immunology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871; and 4Department of Immunology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

Imaizumi, Kazuyoshi, Tsutomu Kawabe, Satoshi Ichiyama, Hitoshi Kikutani, Hideo Yagita, Kaoru Shimokata, and Yoshinori Hasegawa. Enhancement of tumoricidal activity of alveolar macrophages via CD40-CD40 ligand interaction. Am. J. Physiol. Lung Cell. Mol. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L49–L57, 1999.—CD40-CD40 ligand (CD40L) interaction was originally defined as important molecules for the development of humoral immunity. Thereafter, some investigations have focused on its essential roles for the induction of cell-mediated immunity in host defenses. Here we investigated the antitumor activity of murine alveolar macrophages through CD40-CD40L interaction. The CD40L gene was transfected into murine lung cancer cells (3LLSA), and CD40L-expressing clones (3LLSA-CD40L) were established. Stimulation of CD40 molecules on the surface of alveolar macrophages with 3LLSA-CD40L cells induced the production of nitric oxide, tumor necrosis factor-α, and interleukin-12 and the tumoricidal activity on alveolar macrophages in the presence of interferon-γ, which increased the surface expression of CD40 molecules on alveolar macrophages. These findings were not observed when alveolar macrophages were obtained from CD40-deficient mice. On the other hand, interleukin-6 production by alveolar macrophages did not depend on CD40-CD40L interaction. We also established a murine melanoma cell line expressing CD40L (B16 4A5-CD40L) that could induce tumoricidal activity of alveolar macrophages. Furthermore, when spleen cells were cocultivated with 3LLSA-CD40L cells, specific cytotoxic T lymphocytes for wild-type 3LLSA cells could be induced. These results suggest that CD40L gene transfer into tumor cells may induce antitumor immunity in a tumor-bearing host and may offer a new strategy for cancer gene therapy.

CD40 is a member of the tumor necrosis factor (TNF)-receptor family of cell surface protein expressed on B cells, dendritic cells, human thymic epithelial cells, human endothelial cells, and several carcinoma cell lines (5, 19). CD40 binds a CD40 ligand (CD40L; CD154), which is an ~35-kDa glycoprotein, a member of the TNF superfamily, and expressed on activated T cells, basophils, and mast cells (5). CD40-CD40L interaction is important for cross talking between T cells and B cells that is essential for B-cell immunoglobulin class switching (5), avoidance of B-cell apoptosis (40), and formation of germinal centers in secondary lymphoid organs (22). A recent study (29) demonstrated the important role of this ligand-receptor pair in cell-mediated immunity in addition to its function in the regulation of humoral immunity. It has been reported (2, 29, 34, 39) that monocytes and macrophages were activated by stimulation through CD40-CD40L interaction and developed tumoricidal activity. CD40 also regulates the activity of antigen-presenting cells (APCs) (29). Recently, several reports demonstrated that CD40-CD40L interaction plays a critical role in the induction of antitumor immunity (26), especially the essential role in the induction of cytotoxic T cells (CTLs) (6, 25, 30, 32). Taking these findings into consideration, we hypothesized that stimulation of macrophages and APCs through CD40 with CD40L-expressing tumor cells could enhance the cytotoxic effect of macrophages and the antitumor immunity of T cells. In the present study, we investigated the antitumor immunity against lung cancer cells because lung cancer cells generally express low antigenicity, and it seems to be difficult to induce the lung cancer-specific cellular immunity, in contrast to melanoma cells. We generated CD40L-expressing lung carcinoma cells by transfection of CD40L cDNA, and we attempted to verify whether these cells could induce alveolar macrophage activation and enhance its tumoricidal effect against wild-type lung carcinoma cells.

MATERIALS AND METHODS

Mice. C57BL/6 mice (H-2b) were supplied by the Institute for Laboratory Animal Research (Nagoya University School of Medicine, Nagoya, Japan). CD40-deficient mice used in this study were generated by a gene-targeting technique as previously reported (22). A CD40(+/−) mouse was produced by backcrossing the originally described CD40(−/−) mouse to a C57BL/6 mouse. The heterozygous littermates were intercrossed to generate CD40(+/+), CD40(+/−), and CD40(−/−) mice. These mice were genotyped by a PCR of genomic DNA obtained from a tail biopsy with primers to identify the rearranged CD40 locus as described previously (22).

Cells. A murine lung carcinoma cell line (3LLSA; Lewis lung cancer cells), which was originally established from the lung of a C57BL/6 mouse bearing a tumor, was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). A murine melanoma cell line, B16 4A5, which also originated from a C57BL/6 mouse (31, 35), was obtained from Riken Cell Bank (Tsukuba, Japan). 3LLSA cells were main-

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tained in RPMI 1640 medium supplemented with 1% l-glutamine, 1% penicillin-streptomycin, and 10% fetal calf serum (RPMI-10% FCS). B16 4A5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) containing 10% FCS (DMEM-10% FCS). Alveolar macrophages were collected from C57BL/6 or CD40-deficient mice by bronchoalveolar lavage. A 26-gauge polystyrene needle was inserted into the mouse trachea, and 1 ml of phosphate-buffered saline (PBS) was injected into the lung. After the inflated lung was gently massaged, the injected fluid was sucked up by the syringe. These procedures were repeated three times in each mouse. The collected fluid was centrifuged, and the cell pellet was suspended in PBS. After being washed, the cells were cultured in RPMI-10% FCS. After 90 min of incubation at 37°C, nonadherent cells were removed by gentle washing, and adherent cells were used as an alveolar macrophage population.

Reagents. Recombinant murine interferon (IFN)-γ was purchased from Gibco BRL (Gaithersburg, MD). Hamster anti-mouse CD40 monoclonal antibody (Mab) IgM (HM40-3) and hamster anti-mouse CD40L MAb IgG (HM40-1) were established as previously described (16, 18). Fluorescein isothiocyanate (FITC)- conjugated goat anti-hamster IgG F(ab’2) fragment was purchased from Caltag Laboratories (San Francisco, CA). FITC-conjugated hamster anti-mouse CD40 antibody (HM40-3) and rat anti-mouse TNF-α antibody (MP6-XT3) were purchased from PharMingen (San Diego, CA). N5-monomethyl-L-arginine (L-NIMMA) was obtained from Calbiochem (La Jolla, CA).

Transfection. Murine CD40L cDNA encoding the entire coding region was prepared by RT-PCR according to the published sequence (4). CD40L cDNA was cloned into the pEF-BO5 mammalian expression vector (27), which contains the promoter lesion of the human elongation factor-1α gene (pBOS-CD40L). pMC1Neo Poly A, which contains the neo gene from Tn5, the herpes simplex thymidine kinase promoter, and the enhancer sequence from the polyoma virus Py F411, was purchased from Stratagene (La Jolla, CA).

The expression vectors pBOS-CD40L and pMC1Neo Poly A were cotransfected into 3LLSA and B16 4A5 cells. Transfections were performed on 100-mm plates with 10 µg plasmid DNA/plate with the lipofection method with Lipofectace reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. After 12 h of exposure, the cells were washed three times with medium and cultured in 10 ml of complete medium. Forty hours after the medium exchange, cells were selected in medium containing 800 µg/ml of G418 (GIBCO BRL, Grand Island, NY). After 2 wk of selection, G418-resistant clones were selected from the surviving colonies and used in the following experiments. Mock-transfected clones (3LLSA-MOCK and B16 4A5-MOCK), which were established by cotransfecting the pEF-BOs plasmid without a cDNA insert and pMC1Neo Poly A, were used in the experiments as a control.

Flow cytometry. After the cells were incubated in PBS with 0.5 mM EDTA for 3 min at 37°C, they were detached by vigorous pipetting. The cells were then harvested into complete medium containing 10% FCS, centrifuged at 1,500 rpm for 3 min at 4°C, and resuspended (5 × 10^5 cells/100 µl) in PBS. To examine the expression of CD40L, the cells were incubated with hamster anti-mouse CD40L IgG (HM40L-1; 25 µg/ml) or an isotype-matched control IgG (25 µg/ml) for 30 min at 4°C. The cells were then washed and stained with an FITC-conjugated goat anti-hamster IgG F(ab’2) fragment (2.5 µg/ml) for 30 min at 4°C. After analyzing the CD40 expression of alveolar macrophages, the cells were cultured with and without IFN-γ (100 U/ml) for 24 h, then incubated with 5% rabbit serum and 2% goat serum in PBS for 30 min at 4°C to block the nonspecific antibody binding for Fc receptors. After being washed with PBS supplemented with 1% FCS and 0.1% NaN₃, the cells were incubated with FITC-conjugated hamster anti-mouse CD40 IgM (2.5 µg/ml) for 30 min at 4°C. Fluorocytometric analysis was done with a Coulter Epics XL equipped with an argon-ion laser (Coulter Electronics, Miami, FL).

RNA isolation and RT-PCR. Total RNA was isolated from the cells with the Isogen RNA extraction kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. For PCR analysis of RNA, cDNA was prepared by RT from 1 µg of each RNA sample in a 20-µl reaction volume containing 0.5 µg of oligo(dT)12-18 primer, 10 mM dithiothreitol, 0.5 mM deoxynucleotide triphosphates, 20 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂, and 200 µM of reverse transcriptase (Superscript II, GIBCO BRL, Gaithersburg, MD). Then the reaction mixture was incubated at 42°C for 50 min and heated at 70°C for 15 min to stop the RT. Amplification of cDNA was carried out with the PCR method with the sense primer 5’-AAG CGA AGC CAA CAG CAG TAA TG-3’ and the antisense primer 5’-GAC AAA CAC AGA AGC ACC AG-3’. These primers result in a 337-bp cDNA encoding murine CD40L. As an internal control, amplification of an 887-bp cDNA encoding murine β-actin was used (the sense primer was 5’-GCA AGA GAT TCC TGA CCC TGA AG-3’, and the antisense primer was 5’-CAT CTG CTG GAA GGT GGA CAG TGA GG-3’). The PCR amplifications were performed in a 20-µl reaction volume containing 1 µg of each cDNA preparation, 0.5 µM each primer, 0.2 mM deoxynucleotide triphosphates, 20 µM Tris-HCl, 50 mM KCl, 200 mM EDTA, 200 µM dithiothreitol, 0.01% Tween 20, 0.01% Nonidet P-40, 1% glycerol solution (final concentration), and 1 U of Taq polymerase (Takara Shuzo, Tokyo, Japan) by 30 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min, and extension at 72°C for 2 min. PCR products were analyzed by electrophoresis on 3% agarose gels and visualized by ethidium bromide staining.

Macrophage-mediated tumor cell lysis assay. Alveolar macrophage-mediated tumor cell lysis was assessed by measuring cytotoxicity against 51Cr-labeled 3LLSA cells as target cells (15, 33). 3LLSA or B16 4A5 cells (10^6 cells) were labeled with 100 µCi of Na^51CrO₄ in 0.5 ml of RPMI-10% FCS for 1 h at 37°C. Then the cells were washed three times with medium and finally resuspended in medium containing 10% FCS. Alveolar macrophages as effector cells obtained from C57BL/6 or CD40-deficient mice were preincubated in round-bottom 96-well microculture plates (1 × 10^5 cells/well) with and without murine IFN-γ (100 U/ml) for 24 h at 37°C and then stimulated via CD40 for 12 h at 37°C. As for the stimulation via CD40, alveolar macrophages were cultured with hamster anti-mouse CD40 IgM (10 µg/ml) or 40 Gy Ir-irradiated murine CD40L cDNA-transduced cells (3LLSA-CD40L and B16 4A5-CD40L cells, respectively; 1 × 10^6 cells/well). 40 Gy-irradiated 3LLSA-MOCK or B16 4A5-MOCK cells (1 × 10^6 cells/well) were used as a control. To inhibit the function of cell surface CD40L, hamster anti-mouse CD40L IgG (20 µg/ml) was added to the culture medium. Radiolabeled target cells (1 × 10^4 cells/well) were placed onto the round-bottom 96-well microculture plates in which the alveolar macrophages were cultured. The radioactivity released during a 36-h incubation was determined with a gamma counter with 100 µl of culture supernatant from each well. Experimental release was determined from the amount of 51Cr released by the target cells when they were incubated with the alveolar macrophages. Spontaneous 51Cr release was determined from
cultures containing target cells alone. Total release was determined from a 1% Nonidet P-40 lysate of the target cells. The percentage of specific $^{51}$Cr release was calculated as:

$$\frac{\text{[experimental release} - \text{spontaneous release]}}{\text{[total release} - \text{spontaneous release}] \times 100.}$$

All tests were performed in triplicate, and mean values were calculated.

**Measurement of nitrite and cytokines.** Alveolar macrophages obtained from C57BL/6 or CD40-deficient mice were incubated in round-bottom 96-well microculture plates ($1 \times 10^5$ cells/well) with and without murine IFN-$\gamma$ (100 U/ml) in combination with the stimulation of CD40 as mentioned in Macrophage-mediated tumor cell lysis assay. After 48 h of incubation at 37°C, the supernatants were removed and applied for measurement of nitrite, TNF-$\alpha$, interleukin (IL)-6, and IL-12 ($p<0.05$). The nitrite concentration was measured with a calorimetric assay based on the Griess reaction previously described (21). Briefly, 100 µl of supernatant were incubated for 10 min at room temperature with an equal volume of Griess reagent containing 0.5% sulfanilamide and 0.05% N-(1-naphthyl)ethyleneamine dihydrochloride in 2.5% phosphoric acid. The optical density at 570 nm was measured with a microtiter plate reader. The NO$_2^-$ contents were quantified by comparison with a standard curve generated with NaNO$_2$ in the range of 0–100 µM. Murine TNF-$\alpha$, IL-6, and IL-12 ($p<0.05$) concentrations were determined with an enzyme-linked immunoabsorbent assay (ELISA) kit with mouse TNF-$\alpha$ ELISA (Endogen, Woburn, MA), mouse IL-6 immunoassay (R&D Systems, Minneapolis, MN), and mouse IL-12 (p70) ELISA kits (Genzyme, Cambridge, MA). All kits were used according to the manufacturer’s instructions. The lower limits of detection were 10 pg/ml for TNF-$\alpha$, 3.1 pg/ml for IL-6, and 5 pg/ml for IL-12.

**CTL assays.** Spleens were removed from 8- to 10-wk-old C57BL/6 mice. After a single-cell suspension was prepared, $2 \times 10^5$ spleen cells were cocultivated with 40 Gy-irradiated 3LLSA-CD40L, 3LLSA-MOCK, or wild-type 3LLSA cells ($2 \times 10^5$ cells) in 2 ml of RPMI-10% FCS containing 1% nonessential amino acids and 1% sodium pyruvate in a six-well tissue culture plate. Five days later, nonadherent cells were harvested, counted, and used as effector cells. Wild-type 3LLSA or B16 4A5 cells ($5 \times 10^5$ cells) were labeled with 100 µCi of Na$_2$Na$_2$CrO$_4$ in 0.5 ml of conditioned medium containing 10% FCS for 1 h at 37°C and used as target cells. Then, labeled target cells ($5 \times 10^5$) were cocultivated for 12 h with effector cells at different effector-to-target cell ratios in wells of round-bottom 96-well microculture plates. The radioactivity released during incubation was determined with a gamma counter with 100 µl of culture supernatant from each well. The percentage of specific $^{51}$Cr release was calculated as described in Macrophage-mediated tumor cell lysis assay.

**RESULTS**

Expression of CD40L in cDNA-transfected murine lung carcinoma cells. Expression of CD40L mRNA was studied by RT-PCR. Total cellular RNA was extracted from the CD40L cDNA-transfected 3LLSA clone (3LLSA-CD40L) and the vector-alone-transfected clone (3LLSA-MOCK). Expression of CD40L mRNA was detected only in 3LLSA-CD40L but not in 3LLSA-MOCK cells (Fig. 1A). Expression of the cell surface protein of CD40L was analyzed by flow cytometry with an anti-CD40L MAb. As shown in Fig. 1B, a high expression of CD40L was obtained in CD40L gene-transfected clones but not in the vector-alone-transfected clone. Thus we used these clones in the following experiments.

CD40 expression on alveolar macrophages. To investigate the activation of alveolar macrophages through CD40, we first studied CD40 expression on the cell surface of murine alveolar macrophages collected by bronchoalveolar lavage. The macrophages were analyzed by flow cytometry with an FITC-labeled anti-CD40 MAb. Freshly isolated macrophages cultured in medium alone for 24 h showed no significant expression of CD40 on the cell surface (Fig. 2). However, macrophages cultured in medium containing recombinant murine IFN-$\gamma$ (100 U/ml) induced a significantly higher expression of cell surface CD40 (Fig. 2). These findings
suggest that IFN-γ will enhance the CD40-CD40L-mediated activation of alveolar macrophages.

Effect of CD40 stimulation on alveolar macrophage tumoricidal activity. When murine alveolar macrophages were incubated with the anti-CD40 IgM antibody or 3LLSA-CD40L cells alone, we could not observe any tumoricidal activity of alveolar macrophages (data not shown). Then we incubated the alveolar macrophages with IFN-γ (100 U/ml) in addition to the stimulation via CD40. IFN-γ activated macrophage tumoricidal activity by itself, and stimulation of anti-CD40 IgM antibody (data not shown) or 3LLSA-CD40L cells with IFN-γ even further enhanced its cytotoxic activity against wild-type 3LLSA cells (Fig. 3A). The enhancement by 3LLSA-CD40L cells was blocked by the anti-CD40L blocking antibody (Fig. 3A). In addition, macrophages obtained from CD40-deficient mice showed no enhancement of tumoricidal activity against wild-type 3LLSA cells when they were incubated with anti-CD40 IgM antibody (data not shown) or 3LLSA-CD40L cells combined with IFN-γ (Fig. 3B).

We also investigated whether the enhancement of tumoricidal activity via CD40-CD40L interaction would be demonstrated in another tissue origin of the tumor cell line. We used the B16 4A5 murine melanoma cell line originated from C57BL/6 mice and established the CD40L gene-transfected clone designated B16 4A5-CD40L, as was the clone for 3LLSA-CD40L. Although parental B16 4A5 cells showed no expression of CD40L on their cell surface, B16 4A5-CD40L cells expressed both CD40L mRNA and the cell surface protein (data not shown). As shown in Fig. 3C, B16 4A5-CD40L could enhance alveolar macrophage tumoricidal activity against the parental wild-type melanoma cells. These results demonstrated that CD40 stimulation activates the alveolar macrophage, resulting in the enhancement of tumoricidal activity.

CD40 stimulation enhances nitric oxide and TNF-α production by alveolar macrophages. It is of interest to know how alveolar macrophages exhibit the tumoricidal effect after CD40 stimulation. We investigated the production of nitric oxide (NO) and cytokines by alveolar macrophages under the stimulation of CD40. As shown in Fig. 4, we detected significant increases in NO
and TNF-α production when alveolar macrophages were stimulated with 3LLSA-CD40L cells in combination with IFN-γ. In contrast, these enhancements were not observed when alveolar macrophages from CD40-deficient mice were used (data not shown). The increase in NO and TNF-α production by stimulation with IFN-γ and 3LLSA-CD40L cells was blocked by the anti-CD40L blocking antibody down to the same level as the production by macrophages stimulated with IFN-γ and 3LLSA-MOCK cells. The production of NO and TNF-α by alveolar macrophages stimulated with IFN-γ and 3LLSA-CD40L cells was much higher than that in macrophages stimulated with IFN-γ and anti-CD40 IgM antibody (data not shown). In addition, 3LLSA-MOCK cells moderately enhanced the production of NO and TNF-α by alveolar macrophages stimulated with IFN-γ (Fig. 4). These findings suggest that 3LLSA cells express some molecules other than CD40L and activate alveolar macrophages to produce NO and TNF-α.

Because the tumoricidal activity and production of NO and TNF-α seem to be mutually related (Figs. 3 and 4), we speculate that NO and TNF-α play important roles in the generation of macrophage tumoricidal activities through CD40-CD40L interaction. To confirm these findings, we used an anti-TNFα antibody and L-NMMA for inhibiting macrophage-mediated tumoricidal activity. As shown in Fig. 5, the anti-TNFα antibody and L-NMMA inhibited the macrophage-mediated tumoricidal activity induced by CD40L. However, the combination of the two agents could not block the tumoricidal activity completely. These findings showed that both NO and TNF-α were major factors contributing to macrophage tumoricidal activity. In addition, we speculated that other molecules, including cell surface proteins or factors except NO and TNF-α, may also contribute to the development of the macrophage-mediated tumoricidal activity induced by tumor cells.

Production of IL-6 by alveolar macrophages does not depend on CD40-CD40L interaction. We also investigated IL-6 production by alveolar macrophages. As shown in Fig. 6, anti-CD40 IgM with IFN-γ could induce IL-6 production by alveolar macrophages in both wild-type and CD40-deficient mice (Fig. 6). Similarly, IL-6 was significantly produced from alveolar macrophages cultured with 3LLSA-CD40L or 3LLSA-MOCK cells with and without IFN-γ, and the anti-CD40L blocking antibody could not inhibit IL-6 production (Fig. 6A). These findings were also confirmed by the results from alveolar macrophages obtained from CD40-deficient mice (Fig. 6B) in which IL-6 production was stimulated by 3LLSA cells with and without CD40L molecules on their cell surfaces. These results indicated that IL-6 production by alveolar macrophages was mainly stimulated through pathways other than CD40-CD40L interaction.

CD40 stimulation enhances production of IL-12. We investigated the effect of CD40-CD40L interaction on IL-12 production by alveolar macrophages stimulated with IFN-γ (Fig. 7). 3LLSA-CD40L cells with IFN-γ but not 3LLSA-MOCK cells enhanced the production of IL-12. Furthermore, the anti-CD40L antibody blocked

Fig. 4. Enhancement of nitric oxide (NO; nitrite; solid bars) and tumor necrosis factor (TNF)-α (open bars) production by alveolar macrophages through CD40 stimulation. Alveolar macrophages from C57BL/6 mice were stimulated with irradiated 3LLSA-CD40L or irradiated 3LLSA-MOCK cells in presence and absence of IFN-γ (100 U/ml). After a 48-h culture, supernatants were removed, and concentrations of nitrite and TNF-α were measured. Data are means ± SE from 3 independent experiments.

Fig. 5. NO and TNF-α play important roles in generation of macrophage tumoricidal activity. Alveolar macrophages from C57BL/6 mice were cultured in presence of murine IFN-γ (100 U/ml) for 24 h and then cultured with irradiated 3LLSA-CD40L cells with and without anti-TNF-α antibody (200 ng/ml) and/or 0.5 mM N,N'-monomethyl-L-arginine (L-NMMA). 51Cr-labeled 3LLSA cells were cultured with stimulated alveolar macrophages for an additional 36 h. Amount of 51Cr released in supernatants was measured. Data are means ± SE from 3 independent experiments. Spontaneous counts released in absence of macrophages were always <30% of maximal counts released by 1% Nonidet P-40 lysate of target cells.
the enhancement of IL-12 production by alveolar macrophages stimulated with 3LLSA-CD40L cells. These findings were confirmed by the results with alveolar macrophages obtained from CD40-deficient mice. Although IFN-γ alone moderately stimulated IL-12 production by alveolar macrophages from CD40-deficient mice, neither anti-CD40 IgM nor 3LLSA-CD40L cells could enhance IL-12 production by macrophages stimulated with IFN-γ (data not shown).

Induction of CTL response against wild-type lung cancer cell in vitro. Because the CD40L gene-transfected lung cancer cells activate macrophages and produce cytotoxic factors or cytokines, we speculate that the activated macrophages or APCs may effectively present the tumor antigen to CTLs and induce tumor cell-specific killer T lymphocytes. Figure 8 shows the CTL assay with mouse spleen cells stimulated by 3LLSA-CD40L, 3LLSA-MOCK, and wild-type 3LLSA cells. CTL activity against wild-type 3LLSA target cells was induced when 3LLSA-CD40L cells were used as the stimulation. CTLs induced by 3LLSA-CD40L cell stimulation did not kill the B16 4A5 target cells (data not shown). These findings suggested that the stimulation of macrophages through CD40 with CD40L-expressing tumor cells could induce tumor-specific CTL activity.

DISCUSSION

We demonstrated in this study that stimulation of CD40 molecules on alveolar macrophages enhanced the production of NO, TNF-α, and IL-12 as well as the tumoricidal activity under the stimulation of IFN-γ.
Enhancement of the tumoricidal activity and NO, TNF-α, and IL-12 production were observed when macrophages were stimulated with CD40L-transfected tumor (3LLSA-CD40L) cells but not with mock-transfected (3LLSA-MOCK) or wild-type tumor (3LLSA) cells. The observed findings were blocked by an anti-CD40L blocking antibody. Furthermore, we could not observe these findings when we used alveolar macrophages obtained from CD40-deficient mice.

Several reports have shown that cross-linking of CD40 molecules by anti-CD40 antibodies or recombinant soluble CD40L activates monocytes (2), splenic macrophages (39), and dendritic cells (8) to produce various proinflammatory mediators and cytokines. These findings suggest important roles for the upregulation of microbicidal activity (7, 17, 36), antigen presentation, and costimulation (29). Thus CD40-CD40L interaction appears to induce multifunctional activating signals in macrophages and monocytes in addition to the regulation of humoral immunity (37). In fact, several reports (14, 20, 26, 28) suggested that CD40-CD40L interaction would be required for the induction of antitumor immunity. However, there have been few reports demonstrating that alveolar macrophages activated by CD40-CD40L signaling showed tumoricidal activity against syngeneic lung cancer cells. Furthermore, we demonstrated that CD40L gene-transfected lung cancer cells could induce tumor-specific CTL activity against the parental lung cancer cells, which generally express low antigenicity. Thus we focused on alveolar macrophages that play a central role for the host defense immunity in the alveolar space and the lung and demonstrated that the expression of CD40L on lung cancer cells enhanced the antitumor immunity of the tumor-bearing host.

Activation of alveolar macrophages through CD40 stimulation required costimulation of IFN-γ for the production of NO and TNF-α and the generation of tumoricidal activity. We observed that surface expression of CD40 on alveolar macrophages was detectable only after the stimulation of IFN-γ, suggesting that IFN-γ increases the number of CD40 molecules on the cell surface of alveolar macrophages and enhances the signaling through CD40-CD40L interaction.

Previous reports showed that CD40 cross-linking induced the production of NO (39), TNF-α, IL-1 (42), IL-6 (2), and IL-12 (3, 23, 34) by macrophages or monocytes. We showed in this report that CD40 stimulation enhanced the production of NO, TNF-α, and IL-12 but not of IL-6 by alveolar macrophages under the stimulation of IFN-γ. CD40L or lymphocyte function-associated antigen-1 on the cell surface of activated T cells enhanced the production of NO by splenic macrophages (39). In fact, 3LLSA-MOCK cells enhanced NO and TNF-α production by itself by alveolar macrophages under the stimulation of IFN-γ. Furthermore, stimulation of CD40 on dendritic cells increased the expression of adhesion molecules on their cell surfaces (8). Therefore, we speculated that CD40 cross-linking could activate alveolar macrophages not only by direct CD40 signaling but also by upregulating other cell surface molecules. Because tumoricidal activity was inhibited by the addition of a specific inhibitor of NO synthesis or an anti-TNF-α antibody, we concluded that NO and TNF-α play an important role in the generation of macrophage tumoricidal activities through CD40-CD40L interaction.

We could not observe the enhancement of IL-6 production by alveolar macrophages with CD40 stimulation, whereas Alderson et al. (2) reported that human monocytes stimulated by CD40L with granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, or IL-3 produced IL-6. Although anti-CD40 IgM slightly enhanced its production, IL-6 produced by alveolar macrophages is mainly induced by the tumor cell itself and is not specific for CD40-CD40L interaction in our experimental conditions. 3LLSA cells produced GM-CSF (12), and GM-CSF induced IL-6 production (38). These results suggest that IL-6 production by alveolar macrophages is enhanced by soluble factors or surface molecules of the tumor cells rather than by CD40-CD40L interaction.

IL-12 plays a critical role in the development of Th1 cells, which are effective inducers of cellular immunity. IL-12, which is mainly produced by monocytes and macrophages, induces the production of IFN-γ by natural killer (NK) or T cells, and enhances the lytic activity of CTLs, NK cells, and lymphokine-activated killer cells. Furthermore, dendritic cells also produce a high amount of IL-12 by CD40 cross-linking (9, 24). These findings suggest that the CD40-CD40L interaction may augment the tumoricidal activity of not only macrophages and monocytes but also of CTLs, NK cells, and lymphokine-activated killer cells. In addition, the CD40-CD40L interaction induces APC activation, including IL-12 production and B7 surface expression (29). These findings also support the fact that the transduction of the CD40L gene into tumor cells may have the advantage for inducing antitumor immunity.

Recent advances in immunology may lead to several strategies for cancer treatment by enhancing antitumor immunity (11), including cytokine gene transfer such as IL-2 (41), IFN-γ (43), and GM-CSF (1, 13), into tumor cells and gene transfer of costimulatory molecules such as B7, which is a counterreceptor for T-cell surface molecules of CD28 and CTLA-4 (10). Taken together, our study may offer the possibility of inducing antitumor immunity by CD40L gene transfer combined with IFN-γ gene transfer into lung cancer cells as one of cancer gene therapy strategies. We are now investigating the induction of antitumor cellular immunity by the CD40L and IFN-γ two-gene transduction into lung cancer cells.
macrophage-colony-stimulating factor gene-modified tumor vac-

cination: comparison of adenovirus- and retrovirus-mediated geneti-


