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Adenovirus Vector-Mediated *in Vivo* Gene Transfer of OX40 Ligand to Tumor Cells Enhances Antitumor Immunity of Tumor-Bearing Hosts

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ABSTRACT

OX40 ligand (OX40L), the ligand for OX40 on activated $CD4^+$ T cells, has adjuvant properties for establishing effective T-cell immunity, a potent effector arm of the immune system against cancer. The hypothesis of this study is that *in vivo* genetic engineering of tumor cells to express OX40L will stimulate tumor-specific T cells by the OX40L-OX40 engagement, leading to an induction of systemic antitumor immunity. To investigate this hypothesis, s.c. established tumors of three different mouse cancer cells (B16 melanoma, H-2^b; Lewis lung carcinoma, H-2^b; and Colon-26 colon adenocarcinoma, H-2^d) were treated with intratumoral injection of a recombinant adenovirus vector expressing mouse OX40L (AdOX40L). In all tumor models tested, treatment of tumor-bearing mice with AdOX40L induced a significant suppression of tumor growth along with survival advantages in the treated mice. The *in vivo* AdOX40L modification of tumors evoked tumor-specific cytotoxic T lymphocytes in the treated host correlated with *in vivo* priming of T helper 1 immune responses in a tumor-specific manner. Consistent with the finding, the antitumor effect provided by intratumoral injection of AdOX40L was completely abrogated in a $CD4^+$ T cell-deficient or $CD8^+$ T cell-deficient condition. In addition, *ex vivo* AdOX40L-transduced B16 cells also elicited B16-specific cytotoxic T lymphocyte responses, and significantly suppressed the B16 tumor growth in the immunization-challenge experiment. All of these results support the concept that genetic modification of tumor cells with a recombinant OX40L adenovirus vector may be of benefit in cancer immunotherapy protocols.

INTRODUCTION

One major mechanism that underlies the escape of tumors from immunological surveillance is a deficiency in the way tumor-specific T cells are optimally activated within the local microenvironment of lymphoid organs (1, 2). For an effective T-cell response, signals from costimulatory receptors are required in addition to T-cell receptor-mediated recognition of antigen in the form of peptide-MHC expressed by antigen-presenting cells (APCs; Refs. 3 and 4). OX40 (also referred to as CD134) is a member of such costimulatory receptors and has a unique pattern of expression, which is, for the most part, restricted to $CD4^+$ T cells and is induced by T-cell receptor engagement by peptide antigen in the context of MHC class II (5–8). The ligand for OX40 (OX40L; also referred to as gp34) is a M_r 34,000 type II membrane protein expressed on professional APCs such as dendritic cells, B cells, and macrophages (9–11). The interaction of OX40 with OX40L provides antigen-specific $CD4^+$ T cells with costimulatory signals that drive them to proliferate, augment effector functions such as cytokine secretions, and increase cell survival through inhibition of activation-induced cell death (7, 11–18).

Based on our understanding of the OX40L-OX40 function in immune responses, we hypothesized that *in vivo* genetic modification of tumor cells to express OX40L would trigger OX40 on tumor-responding $CD4^+$ T cells to develop effective antitumor immunity, thus suppressing the growth of the tumor. To test this hypothesis, we have constructed an $E1^-$ recombinant adenovirus vector expressing OX40L (AdOX40L) to transfer the OX40L gene to tumor cells *in vivo*. The data demonstrate that *in vivo* OX40L-transduced tumor cells will elicit tumor-specific T helper 1 (Th1) immune responses and subsequently generate antitumor immunity mediated by cytotoxic T lymphocytes (CTLs) in the treated host, leading to inhibition of the tumor growth.

MATERIALS AND METHODS

Mice. Female C57Bl/6 (H-2^b) and BALB/c (H-2^d) mice, 6–8 weeks of age, were purchased from Japan Charles River (Atsugi, Japan). Female $CD4^+$ T cell-deficient (B6.129S2-*Cd4*^{tm1Mak}; Ref. 19) and $CD8^+$ T cell-deficient (B6.129S2-*Cd8a*^{tm1Mak}; Ref. 20) mice that had been backcrossed to the C57Bl/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed under specific pathogen-free conditions in accordance with the guidelines of the institutional Animal Care and Use Committee.

Cell Lines. B16-F10 melanoma (B16; H-2^b), Lewis lung carcinoma (LLC; H-2^b), Colon-26 colon adenocarcinoma (H-2^d), and BALB/3T3 fibroblast (H-2^d) cell lines were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). E.G7-OVA, the mouse lymphoma cell line EL-4 (H-2^b) modified to express chicken ovalbumin (OVA), was obtained from the American Type Culture Collection (Manassas, VA). B16, Colon-26, and BALB/3T3 cells were cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. E.G7-OVA cells were grown in complete RPMI 1640 containing 0.4 mg/ml G418 (Invitrogen, Carlsbad, CA). Dendritic cells were generated from mouse bone marrow precursors as described previously (21–23). LLC cells were maintained in complete DMEM (Sigma).

Adenovirus Vectors. AdOX40L and AdNull are replication-deficient serotype 5-based adenovirus vectors with E1 and E3 deletions in which the mouse OX40L cDNA (11) and no transgene, respectively, are under transcriptional control of the cytomegalovirus immediate-early enhancer and promoter. The recombinant viruses were amplified, purified using cesium chloride gradient ultracentrifugation, and titered as described previously (23–25). All vectors were free of replication competent adenovirus.

Reverse Transcription-PCR. To confirm the expression of OX40L mRNA mediated by AdOX40L, total cellular RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) from the transduced B16 cells or B16 tumors. cDNA synthesized from the RNA by reverse transcription (Takara Shuzo, Kyoto, Japan) was amplified at 94°C for 2 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s, using primers specific for OX40L or control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts: for OX40L, 5′-TGCCAAGAGTGACGTGTCCA-3′ or 5′-GTGGTACTTGGTTCACAGTG-3′; for GAPDH, 5′-ATGGTGAAGGTCGGTGTGAACGGA-3′ or 5′-TTACTCCTTGGAGGCCATGTAGGC-3′. The PCR products were run on 1% agarose gel and stained with 0.5 μg/ml ethidium bromide.

Flow Cytometric Analysis. To assess OX40L expression on transduced B16 cells, cells were stained for 30 min at 4°C with phycoerythrin-conjugated antimouse OX40L monoclonal antibody (mAb; clone RM134L; eBioscience,

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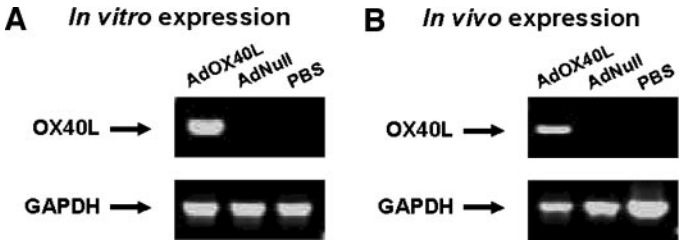

Fig. 1. AdOX40L-mediated OX40L expression assessed by reverse transcription-PCR. *A, in vitro* expression of OX40L mRNA. B16 cells were transduced with AdOX40L or AdNull (multiplicity of infection 50, 3 h) or PBS (mock transduction), and 48 h later, total cellular RNA was extracted from the transduced cells. *B, in vivo* expression of OX40L mRNA. Three days after treatment of B16 tumor-bearing C57Bl/6 mice with intratumoral injection of 10^9 pfu of AdOX40L or AdNull or PBS, total cellular RNA was isolated from the treated tumors. For both panels, cDNA generated from the extracted RNA was amplified with the primers for OX40L mRNA (*top*) or control GAPDH mRNA (*bottom*). PCR products were resolved in 1% agarose gel and stained with ethidium bromide.

San Diego, CA) or phycoerythrin-conjugated isotype-matched control antibody (BD Biosciences PharMingen, San Jose, CA). Stained cells were analyzed on an EPICS XL cytometer with EXPO32 ADC software (Beckman Coulter, Miami, FL). To determine the percentage of stained cells above the control staining, 1% of false positive events was accepted in the isotype-matched antibody.

Tumor Therapy Models. Tumor cells (3×10^5 B16, 5×10^5 LLC, 2×10^5 Colon-26, or 5×10^5 E.G7-OVA) were injected s.c. in the right flank of mice at day 0. When the tumors became palpable (*i.e.,* approximately 4 mm in diameter; day 8 for B16, day 7 for LLC, day 5 for Colon-26, and day 7 for E.G7-OVA), they received injections of 50 μl of 10^9 plaque-forming unit (pfu) of AdOX40L or AdNull in PBS or PBS alone. The size of each tumor was measured using calipers every other day, and the tumor volume was calculated as length $\times$ width^2 $\times$ 0.52. When the mice became moribund or the diameter of the tumors reached 15 mm, they were sacrificed, and this was recorded as the date of death for the survival studies.

Tumor Challenge Model. For immunization, 10^6 B16 cells transduced with AdOX40L or AdNull at a multiplicity of infection of 50 for 3 h or PBS and irradiated (5000 rad) were injected s.c. into the left flank of mice twice at a 1-week interval. Fourteen days after the last immunization, the mice were challenged with s.c. injection of 3×10^5 B16 in the right flank. The size of tumors in the right flank was measured as described above.

CTLs. Ten days after the intratumoral injection of adenovirus vectors in the tumor therapy model, or 10 days after the tumor challenge after the immunization in the tumor challenge model, splenocytes were isolated and restimulated at 3×10^6 cells/ml with mitomycin C-treated tumor cells (10^6 cells/ml) in 24-well culture plates. After 5 days, viable cells were harvested as effector cells and tested for their ability to lyse target cells using the lactate dehydrogenase cytotoxicity assay kit (Promega, Madison, WI). The percentage of cytotoxicity was calculated as 100 $\times$ [(experimental release $-$ spontaneous release)/(maximal release $-$ spontaneous release)] (26). Spontaneous or maximal release was obtained from target cells incubated in medium alone or in lysis buffer included in the kit, respectively.

Immunohistochemistry. Three days after the intratumoral injection of adenovirus vectors in the tumor therapy model, the tumors were removed, and 5-μm-thick frozen sections were prepared and fixed in acetone. After blocking nonspecific staining and endogenous peroxidase, sections were incubated with 0.31 μg/ml antimouse CD4 mAb (clone RM4–5; BD Bioscience PharMingen), 10 μg/ml antimouse CD8 mAb (clone KT15; Serotec, Kidlington, United Kingdom), 0.5 μg/ml antimouse OX40L mAb (clone RM134L; eBioscience), 10 μg/ml antimouse OX40 mAb (clone OX86; Serotec), or 5 μg/ml isotype-matched rat IgG2a (BD Bioscience PharMingen) overnight at 4°C. After washing, the samples were then incubated with 2.5 μg/ml biotinylated rabbit antirat immunoglobulins (DakoCytomation, Glostrup, Denmark) for 15 min at room temperature. Signals were visualized with horseradish peroxidase-conjugated streptavidin and 3,3′-diaminobenzidine chromogen/substrate mixture (Nichirei, Tokyo, Japan). The specimens were then incubated with 2.5% methyl green for nuclear counterstaining. Sections were assessed by counting the number of positive cells in 10 randomly selected high-power fields (hpf; magnification, $\times$400).

CD4$^+$ T-Cell Responses. E.G7-OVA tumor-bearing C57Bl/6 mice were treated with adenovirus vectors as described in tumor therapy model. Ten days after the intratumoral injection, splenic CD4$^+$ T cells were magnetically isolated using antimouse CD4 magnetic beads (clone L3T4; Miltenyi-Biotec, Auburn, CA). In 96-well plates, 5×10^5 CD4$^+$ T cells were then cocultured with 5×10^4 irradiated dendritic cells (3000 rad) with or without 50 μg/ml OVA. After 4 days, the supernatant was analyzed for the mouse IFN-γ and interleukin 4 (IL-4) contents by ELISA (BioSource International, Camarillo, CA), and cell proliferation was measured using a WST-1 cell proliferation assay system (Takara Shuzo). The percentage of proliferation was calculated as 100 $\times$ [(experimental absorbance) $-$ (background absorbance)]/[(absorbance at start of the coculture) $-$ background absorbance].

Statistical Analysis. Statistical comparison was made using the two-tailed Student's *t* test, and a value of $P < 0.05$ was accepted as indicating significance. Survival evaluation was carried out using Kaplan-Meier analysis.

RESULTS

***In Vitro* and *in Vivo* Expression of OX40L Mediated by AdOX40L.** AdOX40L-mediated *in vitro* and *in vivo* expression of OX40L was confirmed by reverse transcription-PCR and flow cytometric analyses (Fig. 1; Fig. 2). reverse transcription-PCR analysis demonstrated that 0.7-kb fragments corresponding to the OX40L cDNA were amplified with total RNA from AdOX40L-transduced B16 cells, but not with that from AdNull-transduced B16 cells and nontransduced B16 cells (Fig. 1*A*). The integrity of the RNA was shown by amplification of GAPDH cDNA in AdOX40L-transduced B16 cells and control cells (Fig. 1*A*). Similar results were achieved in transduced B16 tumors *in vivo* (Fig. 1*B*). Reverse transcription-PCR reaction produced 0.7-kb OX40L cDNA fragments in the total RNA sample only from AdOX40L-transduced B16 tumors (Fig. 1*B*). Control GAPDH reverse transcription-PCR products were detected in RNA samples from the Ad OX40L- and AdNull-transduced B16 tumors as well as the nontransduced B16 tumors (Fig. 1*B*). The *in vitro* OX40L expression on AdOX40L-tranduced B16 cells was further confirmed by flow cytometric analysis; 79.2% of AdOX40L-

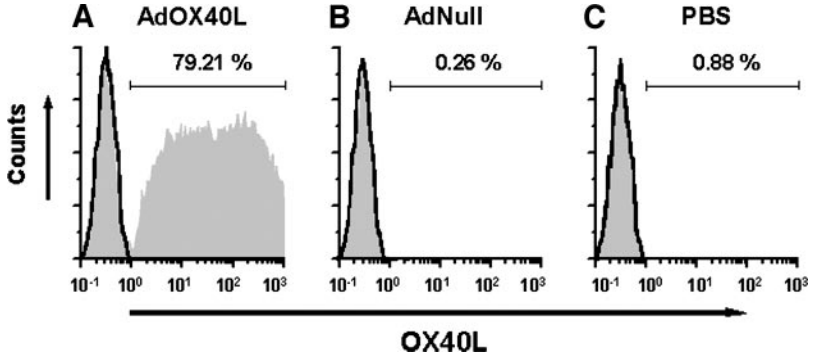

Fig. 2. Flow cytometric analysis of AdOX40L-transduced tumor cells. B16 cells were transduced with AdOX40L or AdNull (multiplicity of infection 50, 3 h) or PBS, washed, and cultured for 48 h. The transduced cells were analyzed by flow cytometry for the expression of OX40L (*gray, filled*). The overlay histogram (*bold line*) in each panel depicts isotype control staining. The percentage of OX40L$^+$ cells is shown in each panel.

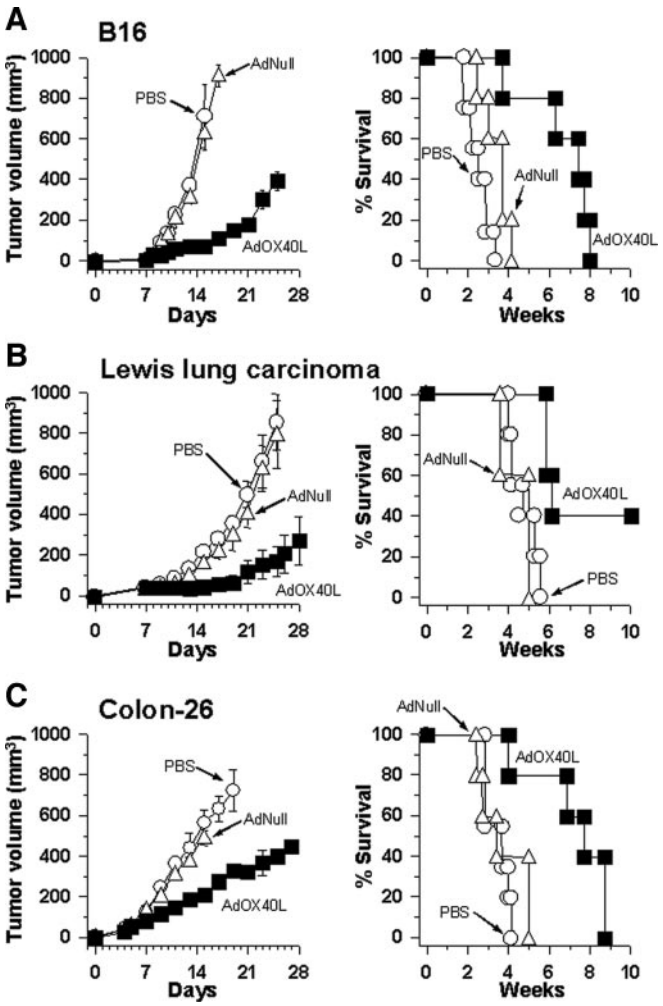

Fig. 3. Antitumor effect of intratumoral administration with AdOX40L. *A,* B16 tumors, C57Bl/6 mice. C57Bl/6 mice received injections in the right flank s.c. with B16 cells (day 0). On day 8, established B16 tumors were treated with intratumoral administration of 10^9 pfu AdOX40L (■) or AdNull (△) or PBS (○). *B,* LLC tumors, C57Bl/6 mice. This study is similar to that in *A,* but 7-d established LLC tumors were treated. *C,* Colon-26, BALB/c mice. This study is also similar to that in *A,* but BALB/c mice with 5-d established Colon-26 tumors received injections. For all panels, the size of each tumor was assessed every other day and is reported as the average tumor volume ± SE (*left*). The survival is presented as the percentage of surviving animals (*right*). Five mice were included in each group.

transduced B16 cells were positive cells for the OX40L expression, whereas AdNull-transduced B16 cells and nontransduced B16 cells displayed no expression of OX40L (Fig. 2).

Antitumor Effects of Intratumoral Administration of AdOX40L. Treatment of the well-established tumors with intratumoral injection of AdOX40L induced inhibition of the tumor growth, resulting in improved survival of the treated mice (Fig. 3). Intratumoral administration of AdOX40L to 8-day established B16 tumors in C57Bl/6 mice ($H\text{-}2^b$) induced a significant suppression of tumor growth and prolonged survival compared with AdNull or PBS (tumor volume days 9–15, $P < 0.05$; survival, $P < 0.05$; Fig. 3*A*). The growth of less immunogenic LLC tumors in C57Bl/6 was also restrained significantly by intratumoral administration of AdOX40L, and 40% of treated mice survived for 10 weeks, at which time the experiment was terminated (tumor volume days 13–25, $P < 0.05$ to all other groups; survival, $P < 0.05$ to all other groups); whereas LLC tumors that received injections of AdNull or PBS grew similarly, and none of the LLC tumor-bearing mice survived (Fig. 3*B*). In the Colon-26 tumor model in BALB/c mice ($H\text{-}2^d$), AdOX40L significantly suppressed the growth of 5-day established Colon-26 tumors and prolonged the survival of the treated mice when injected intratumorally, in contrast with AdNull or PBS (tumor volume days 7–15, $P < 0.05$; survival, $P < 0.05$; Fig. 3*C*).

Tumor-Specific CTL Responses Induced by Intratumoral Administration of AdOX40L. Direct administration of AdOX40L to B16 or Colon-26 tumors elicited tumor-specific CTL activity (Fig. 4). C57Bl/6 mice bearing B16 tumors received injections intratumorally of AdOX40L, AdNull, or PBS (Fig. 4, *A* and *B*). Splenocytes were obtained from the treated mice 10 days after the injection and restimulated by *in vitro* culture with mitomycin C-treated B16 cells. Effector cells generated from splenocytes of AdOX40L-treated mice exhibited the cytotoxic response against B16 cells, but effector cells from the control groups of mice treated with AdNull or PBS could not lyse B16 target cells (Fig. 4*A*). No apparent lysis of irrelevant but syngenic LLC target cells was observed regardless of the treatment, confirming the AdOX40L-induced tumor-specific cytotoxicity against B16 cells (Fig. 4*B*). Similar to B16 tumors in C57Bl/6 mice, the intratumoral administration of AdOX40L exhibited Colon-26-specific CTL responses in Colon-26 tumor-bearing BALB/c mice (Fig. 4, *C* and *D*). The cytotoxic response against Colon-26 cells was obtained using splenocytes only from AdOX40L-treated mice (Fig. 4*C*). In contrast, no apparent lysis was achieved against irrelevant but syngenic BALB/3T3 fibroblasts with any treatments in the Colon-26 tumor-bearing mice (Fig. 4*D*).

Intratumoral T-Cell Infiltration Augmented by AdOX40L. Immunohistochemical analyses of Colon-26 tumors that received injections of AdOX40L demonstrated that OX40L expression in the tumors enhanced an intratumoral infiltration of both $CD8^+$ T cells and $CD4^+$ T cells compared with control tumors that received injections of AdNull or PBS [$CD4^+$ T cells/10 hpf: AdOX40L, 395; AdNull, 37; and PBS, 29 (Fig. 5, *A–C*); $CD8^+$ T cells/10 hpf: AdOX40L, 225;

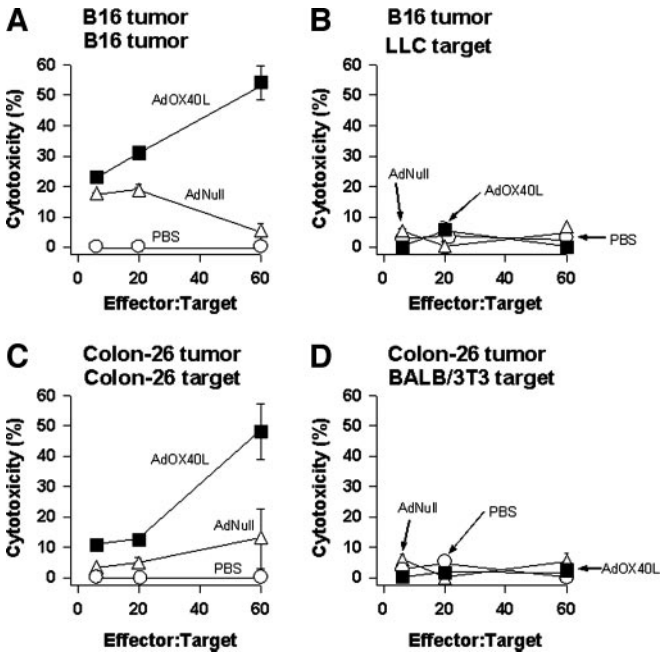

Fig. 4. Tumor-specific cytotoxic T cells induced by intratumoral administration of AdOX40L. *A* and *B,* B16 tumors, C57Bl/6 mice. Eight-day established s.c. B16 tumors were treated with intratumoral injection of 10^9 pfu AdOX40L (■) or AdNull (△) or PBS (○). Ten days after the treatment, spleen cells were isolated and restimulated with mitomycin C-treated B16 for 5 days. The restimulated effector cells were then assayed for cytolytic function by using B16 (*A*) or LLC (*B*) cells as targets. *C* and *D,* Colon-26 tumors, BALB/c mice. Conditions were similar to those in *A* and *B,* but 5-day established Colon-26 tumors in BALB/c mice were treated. Colon-26 (*C*) or BALB/3T3 (*D*) cells were used as targets. All results are shown as mean ± SE ($n = 3$ per data point).

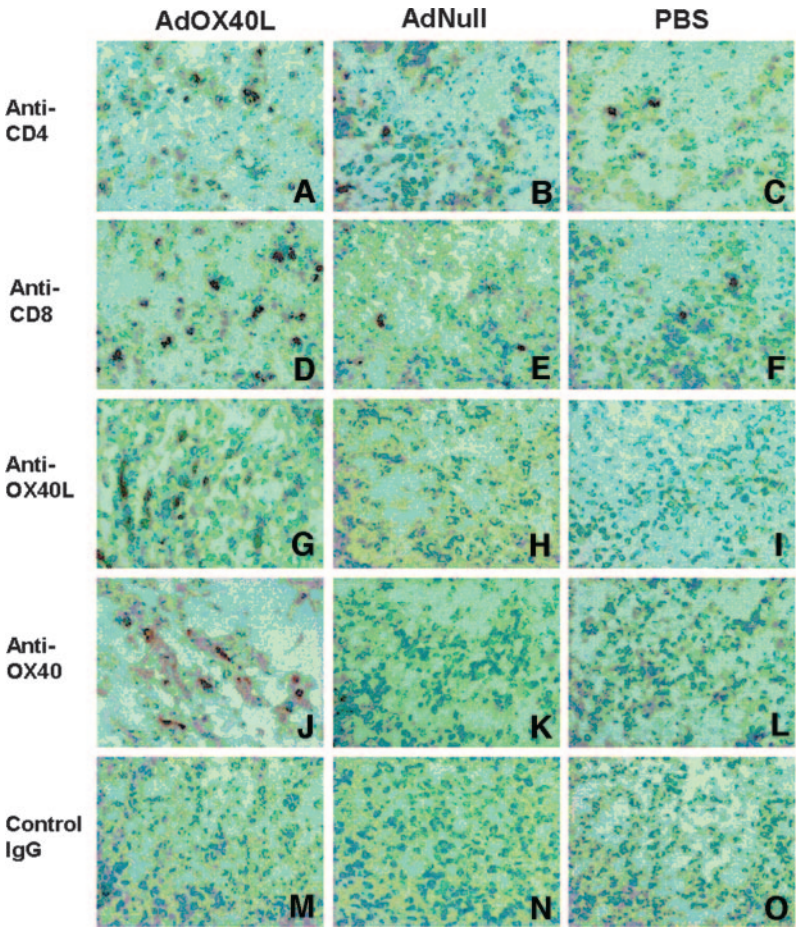

Fig. 5. Immunohistochemical evaluation of tumors treated with intratumoral administration of AdOX40L. Day-5 established Colon-26 tumors were treated with intratumoral injection of 10^9 pfu AdOX40L (*A, D, G, J,* and *M*) or AdNull (*B, E, H, K,* and *N*) or PBS (*C, F, I, L,* and *O*). Three days after the injection, the treated tumors were dissected, and frozen tumor sections were stained with antimouse CD4 mAb (*A–C*), antimouse CD8 mAb (*D–F*), antimouse OX40L mAb (*G–I*), antimouse OX40 mAb (*J–L*), or control rat IgG2a (*M–O*). Signals were amplified by secondary biotinylated rabbit antirat immunoglobulins and detected by peroxidase-conjugated streptavidin and 3,3′-diaminobenzidine. Nuclei of sections were counterstained with methyl green.

AdNull, 33; and PBS, 45 (Fig. 5, *D–F*); and $OX40L^+$ cells/10 hpf: AdOX40L, 353; AdNull, 26; and PBS, 20 (Fig. 5, *G–I*)]. Interestingly, increased numbers of OX40-expressing cells were observed only in the AdOX40L-transduced tumors, suggesting that $CD4^+$ T cells and $CD8^+$ T cells infiltrating the AdOX40L-treated tumors described above were activated [$OX40^+$ cells/10 hpf: AdOX40L, 219; AdNull, 17; and PBS, 19 (Fig. 5, *J–L*)]. With isotype-matched control IgG, only minimal staining was detected in these tumors treated with AdOX40L, AdNull, or PBS (Fig. 5, *M–O*).

Requirements of $CD4^+$ and $CD8^+$ T Cells for AdOX40L-Mediated Antitumor Effects. To delineate the role of $CD4^+$ T cells and $CD8^+$ T cells in the antitumor responses elicited by intratumoral administration of AdOX40L, B16 tumors established in wild-type, $CD4^+$ T cell-deficient (CD4−/−), or $CD8^+$ T cell-deficient (CD8−/−) C57Bl/6 mice were treated by intratumoral injection with 10^9 pfu of AdOX40L (Fig. 6). The growth of B16 tumors in CD4−/− and CD8−/− mice was not diminished by the AdOX40L treatment compared with that in wild-type mice receiving the identical treatment, and it was similar to the growth of PBS-treated B16 tumors in wild-type mice (days 7–15, $P < 0.05$, CD4−/− and CD8−/− mice compared with AdOX40L-treated wild-type mice; $P > 0.05$ or $P > 0.05$, CD4−/− or CD8−/− mice compared with PBS-treated wild-type mice, respectively; Fig. 6). As an additional control, PBS-treated B16 tumors in CD4−/− and CD8−/− mice grew progressively as those in wild-type mice ($P > 0.5$ or $P > 0.2$, CD4−/− or CD8−/− mice compared with wild-type mice, respectively; not shown).

***In Vitro* Responses of $CD4^+$ T Cells from AdOX40L-Treated Mice.** *In vivo* AdOX40L-mediated modification of tumors to express OX40L developed tumor-specific $CD4^+$ T cells that proliferated in a tumor antigen-specific manner and predominantly secreted the Th1-associated cytokine IFN-γ *in vitro* (Fig. 7). In this context, C57Bl/6 mice bearing E.G7-OVA tumors expressing OVA as a model tumor antigen were treated with intratumoral injection of AdOX40L, AdNull, or PBS on day 7. Ten days after the treatment, splenic $CD4^+$ T cells were isolated and assayed for their proliferating reactivity to OVA in the presence of syngenic bone marrow-derived dendritic cells as APCs (Fig. 7*A*). Although a similar proliferative response to OVA was achieved in each $CD4^+$ T-cell group as compared with the corresponding OVA depletion culture (AdOX40L, 2.6-fold,

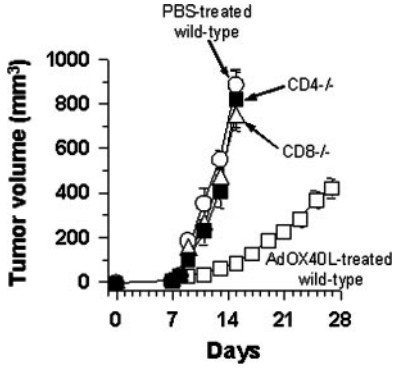

Fig. 6. The role of $CD4^+$ and $CD8^+$ T cells in suppressing tumor growth by intratumoral administration of AdOX40L. Day-8 established B16 tumors in $CD4^+$ T cell-deficient (■), $CD8^+$ T cell-deficient (△), or wild-type (□) C57Bl/6 mice were treated with intratumoral injection of 10^9 pfu AdOX40L. The size of each tumor was assessed every other day and is reported as the average tumor volume ± SE. This study included five mice per group and tumor-bearing wild-type mice treated with intratumoral injection of PBS as controls (○).

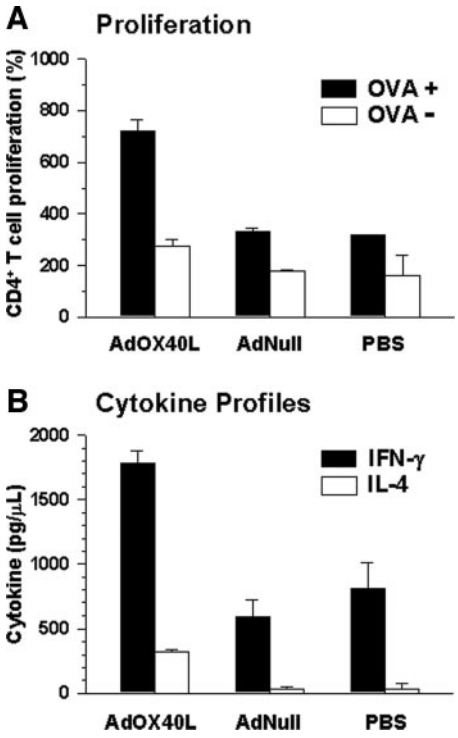

Fig. 7. Responses of $CD4^+$ T cells from tumor-bearing mice treated with intratumoral administration of AdOX40L. *A,* antigen-specific proliferation. Day 7 established EG.7-OVA tumors in C57Bl/6 mice were treated with intratumoral administration of 10^9 pfu AdOX40L or AdNull or PBS. Ten days after the treatment, 5×10^5 $CD4^+$ T cells isolated from spleens of the treated mice were cocultured with (■) or without (□) OVA, in the presence of 5×10^4 irradiated dendritic cells for in 96-well culture plates. Four days later, the number of viable cells was determined by the WST-1 cell proliferation assay. The data are presented as the percentage increase over baseline on the initiation of the coculture. *B,* cytokine profile. The levels of IFN-γ (■) and IL-4 (□) in the coculture medium described above were determined by ELISA. For both panels, results are shown as mean ± SE ($n = 3$ per data point).

$P < 0.001$; AdNull, 2.2-fold, $P < 0.001$; PBS, 1.4-fold, $P < 0.001$), the level of the $CD4^+$ T-cell proliferation to OVA in the AdOX40L-treated group was greater than that of any other control group (767% proliferation, $P < 0.005$ compared with AdNull or PBS; Fig. 7*A*).

The level of IFN-γ or IL-4 secreted from the $CD4^+$ T cells cultured with OVA was then analyzed to determine which of the Th1 or T helper 2 (Th2) immune responses to the tumor antigen was predominantly induced in the treatment of E.G7-OVA tumor-bearing mice (Fig. 7*B*). A significant enhancement of IFN-γ production from $CD4^+$ T cells was observed with intratumoral injection of AdOX40L compared with AdNull or PBS ($P < 0.005$, both comparisons; Fig. 7*B*). IL-4 production from $CD4^+$ T cells was also significantly enhanced by the AdOX40L treatment ($P < 0.005$, compared with AdNull or PBS alone), but the AdOX40L-enhanced level of IL-4 was only modest as compared with that of IFN-γ ($P < 0.001$; Fig. 7*B*).

Antitumor Effect of *ex Vivo* AdOX40L-Transduced Tumor Cells. To avoid the confounding effects by the *in vivo* genetic modification of the tumors, we examined whether *ex vivo* AdOX40L-transduced tumor cells could induce antitumor immunity to suppress the tumor growth in the immunization-challenge experiments (Fig. 8). In this context, C57Bl/6 mice were immunized with AdOX40L-transduced and irradiated B16 cells twice at a 1-week interval and 2 weeks after the last immunization, challenged s.c. with B16 cells (day 0). The AdOX40L-mediated immunization resulted in a significant slowing of the tumor growth with enhanced survival of the immunized mice, in contrast to the control immunization with AdNull-transduced and mock-transduced B16 cells (tumor volume days 13–17, $P < 0.05$; survival, $P < 0.05$; Fig. 8*A*). The protective effect induced by the AdOX40L immunization has correlated to the tumor-specific CTL responses *in vivo* primed by AdOX40L-transduced tumor cells (Fig. 8*B*). Effector cells were generated from splenocytes of mice immunized and challenged as described above, and assayed for their cytotoxic function against the parental B16 tumor cells. Cells from mice immunized with AdOX40L-transduced B16 cells exhibited a 42.2% lysis of B16 target cells at an effector/target ratio of 100/1, whereas those from mice immunized with AdNull-transduced or mock-transduced B16 cells led to only 2.1% or 0.5% lysis, respectively, at the identical effector/target ratio (Fig. 8*B, left part*). As a control for the specificity of the detected B16 lysis, no apparent lysis was observed against irrelevant but syngenic LLC cells regardless of the immunization regimen, and the lysis of control LLC targets was within 5% in all groups (Fig. 8*B, right part*).

DISCUSSION

This study is based on the hypothesis that Ad vector-mediated *in vivo* gene transfer of the coding sequence of OX40L to tumor cells will develop a tumor-specific cellular immune response that can suppress the tumor growth and increase the survival of the tumor-bearing host. All of our experimental data support this hypothesis. Intratumoral injection of an adenovirus vector expressing mouse OX40L to three different types of mouse tumors inhibited the growth of the established tumors and prolonged the survival of the treated mice. The direct administration of AdOX40L into established tumors mediated the induction of tumor-specific Th1 and CTL immune responses that are essential for antitumor immunity. Indeed, the anti-

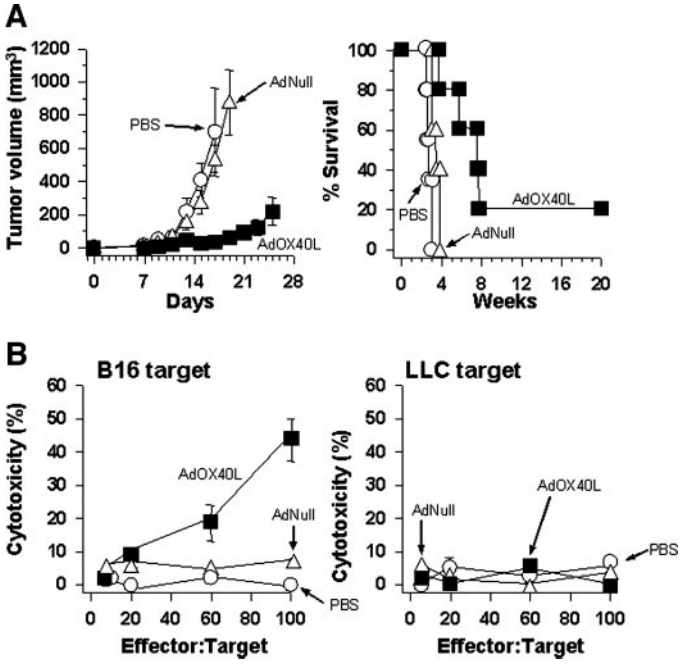

Fig. 8. Specific antitumor effect induced by AdOX40L-transduced tumor cells. *A,* suppression of tumor growth by immunization with AdOX40L-transduced tumor cells. C57Bl/6 mice were immunized by injecting s.c. irradiated B16 cells that had been transduced with AdOX40L (■) or AdNull (△; multiplicity of infection 50, 3 h) or PBS (○) in the left flank twice at 1-week interval. Two weeks after the last immunization, immunized mice were challenged with s.c. administration of 3×10^5 B16 cells in the right flank (day 0). The size of each tumor was assessed every other day and is reported as the average tumor volume ± SE ($n = 5$ per group; *left*). The survival is presented as the percentage of surviving animals (*right*). *B,* tumor-specific cytotoxic T-cell responses. C57Bl/6 mice were immunized and challenged identically to those described in *A,* and splenocytes taken 10 days after the tumor challenge were restimulated for 5 days with mitomycin C-treated B16 cells. Cytolytic activity was then measured using B16 cells (*left*) or LLC cells (*right*) as targets. Results are presented as means ± SE ($n = 3$ per data point).

tumor effect of AdOX40L was markedly impaired in a $CD4^+$ T cell-deficient or $CD8^+$ T cell-deficient condition.

One important mechanism by which functional immune systems fail to eliminate tumor cells is a failure of adequate tumor-specific T-cell activation (1, 2). The induction of potent and long-lasting T-cell responses against antigens requires at least two major signals delivered by APCs (3, 4). The first signal alone that is brought by a T-cell receptor triggering by peptides bound to MHC class I or class II products is not sufficient to elicit competent T-cell responses against antigens. The second signal that is provided by the engagement of costimulatory receptors by their ligands, together with the first signal, is needed for an effective T-cell response specific for the antigen (3, 4). The costimulatory receptors expressed on T cells can be divided into two groups: the immunoglobulin superfamily (*e.g.,* CD28) and the tumor-necrosis factor receptor superfamily (4). OX40 is involved in the latter. OX40 is preferentially expressed on $CD4^+$ T cells after the T-cell receptor engagement by peptide antigen in the context of MHC class II, and the OX40 triggering with the ligand, OX40L, enhances the effector T-cell function by augmenting the number of antigen-reactive T cells, up-regulating the production of cytokines, and increasing the life span of effector T cells (7, 11–18). Activated T cells expressing OX40 have been found *in vivo* not only in T-cell zones of spleen or lymph nodes, but also in peripheral inflammatory sites including those of growing tumors (7, 8, 27). Based on these considerations, we envisioned a scenario in which *in vivo* genetic modification of tumor cells to express OX40L would costimulate tumor-reactive $OX40^+$ T cells within the tumor through OX40L-OX40 interactions, enabling helper $CD4^+$ T cells to facilitate the tumor-specific cellular immunity associated with $CD8^+$ killer T cells.

Consistent with this concept, *in vivo* genetic modification of tumor cells to express OX40L efficiently induced tumor-relevant CTL immune responses, resulting in a suppression of the tumor growth and prolonged survival of the tumor-bearing mice. Additional evidence for this hypothesis comes from the observations that the antitumor immunity mediated by intratumoral administration of AdOX40L was not generated in a $CD4^+$ T cell-deficient or $CD8^+$ T cell-deficient condition and that $CD4^+$ T cells from tumor-bearing mice treated with intratumoral injection of AdOX40L proliferated *in vitro* and secreted a Th1 cytokine, IFN-γ, rather than a Th2 cytokine, IL-4, in a tumor antigen-specific manner, suggesting that tumor-specific type 1 immune responses were elicited by the AdOX40L treatment. Moreover, the AdOX40L-augmented $CD4^+$ T-cell *in vitro* proliferation with a tumor antigen was confirmed by the *in vivo* observation of a marked intratumoral infiltration of $OX40^+$ and $CD4^+$ T cells in the AdOX40L-injected tumors, indicating that *in vivo* genetic modification of tumor cells to express OX40L increased the number of tumor-reactive $CD4^+$ T cells in the tumor-bearing hosts.

With regard to the Th1 *versus* Th2 immune responses of the OX40-OX40L interactions, early *in vitro* experiments suggested the preferential involvement of Th2 responses (28), and this was substantiated by *in vivo* experiments using murine models of *Leishmania major* infection in BALB/c mice and allergic lung inflammation (29, 30). However, recent *in vivo* studies of OX40- and OX40L-deficient mice have provided greater insight and demonstrated that these animals show reductions in both Th1 and Th2 cytokine responses (7, 11). Furthermore, studies using experimental disease models such as multiple sclerosis, rheumatoid arthritis, and colitis have demonstrated that *in vivo* blockade of OX40-OX40L interactions decreases the disease severity by inhibiting the associated inflammatory processes including Th1 cytokine production (31–35). Taken together, it appears that OX40 engagement with OX40L participates in both Th1 and Th2 responses, and that the preferential participation of either type 1 or type 2 immune responses is influenced by a variety of associated factors, notably inflammatory and microbial products (4). The present study has demonstrated that the *in vivo* adenovirus vector-mediated expression of OX40L in tumor cells led to the priming of $CD4^+$ T cells, which could predominantly produce IFN-γ in response to the tumor antigen, resulting in tumor-specific CTL generation. The AdOX40L-induced tumor-specific Th1 polarity may be due to the immunological state of the treated hosts (*i.e.,* tumor-bearing), the manner by which OX40 is engaged (*i.e.,* gene transfer of OX40L), or the gene delivery system (*i.e.,* adenovirus vector). In this regard, the cytokine profile of $CD4^+$ T cells from treated mice suggested that Th2 cells also have something to do with the antitumor immunity induced by AdOX40L.

Current knowledge concerning the critical costimulatory role of OX40-OX40L interactions in T-cell immunity has been applied to facilitate antitumor immunity by using agonist reagents to OX40 or forced expression of OX40L in mouse tumor models. Morris *et al.* (36) and Weinberg *et al.* (37) showed that an i.p. administration of agonist OX40L:immunoglobulin fusion protein or anti-OX40 antibody after s.c. tumor inoculation resulted in prolonged survival in treated mice compared with untreated controls. Similarly, Kjærgaad *et al.* (38, 39) and Pan *et al.* (40) i.p. injected agonist anti-OX40 antibody alone or together with immunostimulatory cytokines (*i.e.,* interleukin 2 or interleukin 12) to tumor-inoculated mice and proved the therapeutic efficacy of these treatments. Differing from such strategies to engage OX40 nonspecifically, one recent study was aimed at triggering OX40 for tumor-specific T-cell immune responses. In this study, Gri *et al.* (41) demonstrated that, when injected s.c., retrovirally OX40L and GM-CSF double-transduced C26 mouse carcinoma cells mounted CTL responses against C26 cells, with the result that the s.c. vaccination with *ex vivo* OX40L and GM-CSF double-transduced C26 cells improved the survival of mice bearing C26 lung metastasis. The present study directly expands the concept of OX40 engagement in a tumor-specific manner to an *in vivo* strategy involving intratumoral injection of a recombinant adenovirus vector to express OX40L alone. Although additional studies will be needed to define the detailed mechanisms whereby *in vivo* genetic modification of tumor cells with AdOX40L induced tumor-specific cellular immunity (*e.g.,* the role of APCs in developing the immunity), *in vivo* OX40L transduction using a recombinant adenovirus vector may be a potent tumor immunotherapy.

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