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## Enhanced Antitumor Effect of Oncolytic Adenovirus Expressing Interleukin-12 and B7-1 in an Immunocompetent Murine Model

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**Abstract Purpose:** We investigated whether an armed viral platform, where lytic property of a viral infection is coupled to viral-mediated delivery of therapeutic genes, could increase the therapeutic potential of a viral-based therapy.

**Experimental Design:** We generated interleukin (IL)-12-expressing oncolytic adenovirus (YKL-IL-12) and IL-12- and B7-1-expressing (YKL-IL12/B7) oncolytic adenovirus. Therapeutic efficacy of these newly engineered adenoviruses was then evaluated *in vivo* using an immunocompetent mouse bearing murine melanoma B16-F10 tumors. Overall survival was assessed with the Kaplan-Meier method. The induction of immune cell cytotoxicity was assessed by CTL assay, IFN- $\gamma$  enzyme-linked immunospot assay, and immunohistochemical studies.

**Results:** YKL-IL12/B7 oncolytic adenovirus, expressing both IL-12 and B7-1, showed a higher incidence of complete tumor regression compared with the analogous oncolytic adenovirus, YKL-1, or IL-12-expressing, YKL-IL12. Significant survival advantage was also seen in response to YKL-IL12/B7. Moreover, IL-12 and IFN- $\gamma$  levels produced in tumors treated with YKL-IL12/B7 was significantly greater than those treated with YKL-IL12. The enhanced survival advantage was mediated by the induction of immune cell cytotoxicity. In agreement with these results, massive infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells into tissues surrounding the necrotic area of the tumor was observed following *in situ* delivery of YKL-IL12/B7.

**Conclusion:** Combination of oncolysis and the enhancement of antitumor immune response by oncolytic adenovirus expressing both IL-12 and B7-1 elicits potent antitumor effect and survival advantage.

Oncolytic adenoviruses are currently being developed as anticancer biological agents (1). Creating an armed therapeutic viral platform, where the lytic property of viral infection coupled to viral-mediated transgene delivery, may enhance viral-based therapeutic potential. A clear advantage is the amplification of a therapeutic gene, as replicating viruses would be able to infect and deliver the gene of interest to neighboring cells, ultimately enhancing the potential of the viral-based therapy to deal with the complexity of a human tumor.

Recently, interleukin (IL)-12 has shown to be one of the most effective and promising antitumor cytokines (2, 3). IL-12 is a heterodimeric protein consisting of disulfide-linked 40- and 35-kDa subunits, and coexpression of both subunit cDNAs is necessary for the production of biologically active protein. Produced by activated macrophages, monocytes, dendritic cells, and stimulated B lymphocytes, IL-12 has been shown to augment the proliferation and cytolytic potential of both CTL and natural killer (NK) cells (4). In general, IL-12 stimulates the production of IFN- $\gamma$  production from T and NK cells. Local expression of IL-12 has been shown previously to render tumor cells susceptible to T-cell-mediated cytotoxicity, resulting in inhibition of tumor growth and in some cases establishment of a systemic immunity (5–7). Moreover, Lui et al. (8) reported that systemic administration of naked DNA encoding IL-12 using hydrodynamics-based liver gene delivery induces significant antitumor effect. However, to date, the full therapeutic effect, particularly in pre-established tumors, has been limited mostly due to gene transfection of only one cytokine.

To achieve full T-cell activation, in addition to engaging its receptor to the MHC, T cells require the interaction of CD28 on its surface with a costimulatory molecule, such as B7-1 (CD80) and B7-2 (CD86), located on professional antigen-presenting cells (9, 10). Unfortunately, however, most tumor cells do not express B7 family on its cell surface, thus resulting in T-cell anergy (11, 12). Along this line, if cancer cells are engineered to express B7-1, then enhanced T-cell proliferation and activation

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is seen. Using this approach, there has been several reports describing successful induction of antitumor immune response, in which B7-1-transfected tumor cells were used as a vaccine (13, 14). It has also been shown that B7-1-expressing tumor vaccines have been implicated to elicit CD8<sup>+</sup> CTLs directly (15). Interestingly, Guckel et al. (16) reported that expression of IL-12 results in improvement in generating tumor-reactive T cells. Further, Kubin et al. (17) showed that IL-12 expression leads to a synergistic antitumor effect with a helper T (Th1) pattern cytokine production. This synergistic relationship, in which the effect of the combined costimuli is greater than their additive effect, has been exploited with some success in the generation of CTL against solid tumors (18–20).

In this study, we have used a combinatorial approach to generate IL-12-expressing oncolytic adenovirus and IL-12- and B7-1-coexpressing oncolytic adenovirus. We show that high levels of IL-12 and B7-1 are expressed by these newly engineered oncolytic adenoviruses in infected murine B16-F10 cells. In addition, we show that intratumoral delivery of oncolytic adenoviruses elicited significant antitumor effect as well as prolonging survival in an immunocompetent murine melanoma model. These antitumor effects were mediated by the induction of immune cell-mediated cytotoxicity as shown by intratumoral infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, CTL assay, and IFN- $\gamma$  enzyme-linked immunospot (ELISPOT) assay. These results highlight the potential of oncolytic adenovirus platform-based vectors in delivering synergistically interacting cytokines for use in cancer gene therapy.

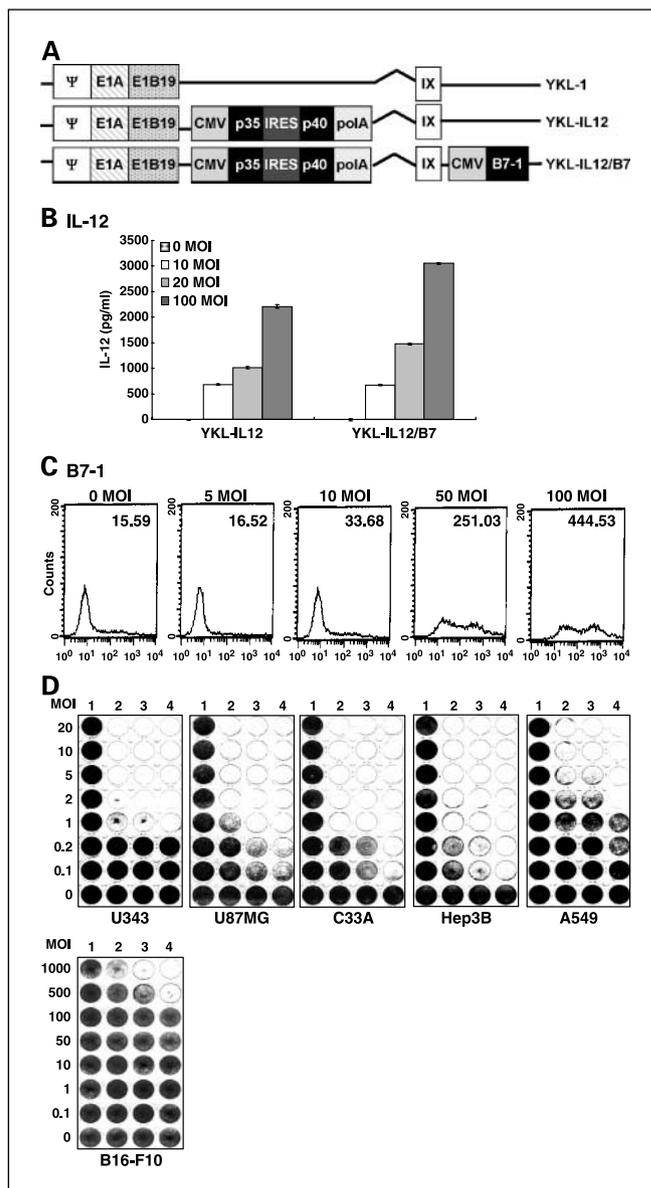
## Materials and Methods

**Cell lines and animals.** All cell lines with the exception of Hep3B and B16-F10, which were maintained in MEM (Life Technologies, Grand Island, NY) and RPMI 1640 (Life Technologies), respectively, were cultured in DMEM (Life Technologies) supplemented with fetal bovine serum (10%; Life Technologies), L-glutamine (2 mmol/L), penicillin (100 IU/mL), and streptomycin (50  $\mu$ g/mL). Human embryonic kidney cell line expressing the adenoviral E1 region (HEK293), brain cancer cell lines (U343 and U87MG), cervical cancer cell line (C33A), liver cancer cell line (Hep3B), non-small lung cancer cell line (A549), murine melanoma cell line (B16-F10), and mouse fibroblast cell line (NIH3T3) were purchased from the American Type Culture Collection (Manassas, VA). All cell lines were maintained 37°C in a humidified atmosphere at 5% CO<sub>2</sub>.

For *in vivo* study, male C57BL/6 mice (6–8 weeks of age) were purchased from Charles River Laboratories International, Inc. (Wilmington, MA). All mice were maintained in a laminar air-flow cabinet at a room kept at 24  $\pm$  2°C temperatures and 40% to 70% humidity with a 12-hour light/dark cycle under specific pathogen-free conditions. All facilities are approved by the Association for Assessment and Accreditation of Laboratory Animal Care, and all animal experiments were conducted under the institutional guidelines established for the Animal Core Facility at Yonsei University College of Medicine (Seoul, South Korea).

**Generation of adenoviruses expressing IL-12 and B7-1.** To generate IL-12-expressing oncolytic adenovirus, we first constructed an E1 shuttle vector expressing murine IL-12 composed of p35 and p40. The IRES region was excised from pcDNA3.1 (Invitrogen, Carlsbad, CA) using *Eco*RI and subcloned into pcDNA3.1-p35 (Cytokine Bank, Chonbuk University, Chunju, South Korea), generating a pcDNA3.1-p35/IRES. The p40 gene was then excised from pcDNA3.1-p40 (Cytokine Bank, Chonbuk University) using *Pme*I and *Xho*I and subcloned into

pcDNA3.1-p35/IRES predigested with *Eco*RV, resulting in pcDNA3.1-IL-12. To produce IL-12-expressing E1 shuttle vector, *Pme*I-*Xho*I fragments containing IL-12 expression cassette was excised from pcDNA3.1-IL-12 and subcloned into pCA14/E1AE1B19 (21) predigested



**Fig. 1.** Characterization of oncolytic adenoviruses. **A**, schematic representation of oncolytic adenoviruses used in this study. YKL-1 is a type 5 adenovirus with normal E1A and E1B 19 kDa but E1B 55 kDa deleted; YKL-IL12 is composed of the IL-12 gene driven by the cytomegalovirus (CMV) promoter inserted into the E1 region of YKL-1; YKL-IL12/B7 is composed of the IL-12 and B7-1 genes driven by the CMV promoter inserted into the E1 and E3 region of YKL-1, respectively. **B**, murine IL-12 expression level in B16-F10 cells transduced with YKL-IL12 or YKL-IL12/B7. Cell culture supernatant was collected at 48 hours after infection, and the amount of IL-12 was quantified by conventional ELISA kit. Results are representative of three independent experiments. Columns, mean of quadruplicate experiments; bars, SD. **C**, murine B7-1 expression level in B16-F10 cells transduced with YKL-IL12/B7. Cells were incubated with anti-B7-1 antibody. Solid line, phycoerythrin fluorescence. Values are the mean fluorescence of B7-1-positive cells compared with negative control. Data are representative fluorescence histograms of three independent experiments. **D**, *in vitro* cytopathic effect. Monolayers grown in 24-well plates were infected with Ad- $\Delta$ E1 (1), YKL-1 (2), YKL-IL12 (3), or YKL-IL12/B7 (4) at various MOIs, and cell killing was allowed to proceed for 6 to 10 days followed by crystal violet staining to detect live cells. Replication-incompetent Ad- $\Delta$ E1 served as a negative control. Results are representative of experiment done three times. The expression of IL-12 and B7-1 did not compromise the viral ability to induce classic cytopathic effect.

with *EcoRV* and *Sall*, generating a pCA14/E1AE1B19-IL-12 E1 shuttle vector. The newly constructed pCA14/E1AE1B19/IL-12 E1 shuttle vector was then linearized with *NdeI* digestion, and the adenoviral vector pvm1324BstBI was linearized with *BstBI* digestion. The linearized pCA14/E1AE1B19/IL-12 E1 shuttle vector was then cotransformed into *Escherichia coli* BJ5183 along with the *NdeI*-digested pvm1324BstBI for homologous recombination, generating a pYKL-IL12 adenoviral vector (Fig. 1A). To generate an oncolytic adenovirus expressing IL-12 and B7-1 at the E1 and E3 region, respectively, we first constructed an E3 shuttle vector expressing B7-1. The murine *B7-1* gene was excised from pLNCX-B7-1 (a kind gift from Dr. Richard Junghans, Harvard Medical School, Boston, MA) using *BstBI* and *ClaI* and subcloned into the adenovirus E3 shuttle vector, pSP72-E3 (22), creating a pSP72-E3/CMV-B7-1. The newly constructed pSP72-E3/CMV-B7-1 shuttle vector was then linearized with *XmnI* digestion and then cotransformed into *E. coli* BJ5183 together with the *SpeI*-digested pYKL-IL12 for homologous recombination, generating a pYKL-IL12/B7 adenoviral vector. To verify the respective homologous recombinants, the plasmid DNA purified from overnight *E. coli* culture was digested with *HindIII*, and the digestion pattern was analyzed. The proper homologous recombinant adenoviral plasmid DNA was then digested with *PacI* and transfected into 293 cells to generate YKL-IL12 and YKL-IL12/B7 oncolytic adenoviruses. E1-deleted replication-incompetent adenovirus (Ad-ΔE1) and E1B 55 kDa deleted oncolytic adenovirus (YKL-1) were also prepared. All viruses were propagated in 293 cells and purified by CsCl density purification, dissolved in storage buffer (10 mmol/L Tris, 4% sucrose, 2 mmol/L MgCl<sub>2</sub>), and stored at -80°C. Viral particle numbers were calculated from measurements of absorbance at 260 nm ( $A_{260}$ ), where 1 absorbency unit is equivalent to 10<sup>12</sup> viral particles/mL, and infectious titers (plaque-forming units per milliliter) were determined by limiting dilution assay on 293 cells. The multiplicity of infection (MOI) was calculated from viral particle numbers. The particle/plaque-forming unit ratio for YKL-1, YKL-IL12, and YKL-IL12/B7 were 40:1, 39:1, and 42:1, respectively.

**ELISA for IL-12 expression.** IL-12 expression was determined using an ELISA according to the manufacturer's instructions (Endogen, Woburn, MA). The flat-bottomed 96-well microtiter plates (Nunc Maxisorp, Montgomery, TX) were coated with a rat anti-IL-12 antibody (10 mg/mL) at 4°C overnight and blocked with bovine serum albumin (4%) in PBS for 2 hours at 37°C. B16-F10 melanoma cells were plated onto six-well plates at 1 × 10<sup>4</sup> per well and then infected with YKL-IL12 or YKL-IL12/B7 adenovirus at MOIs of 10 to 100. At 48 hours after infection, supernatants were harvested and transferred to the wells of anti-IL-12 antibody-coated microtiter plates. After incubation at 37°C for 1 hour, the plates were then washed several times, and rat anti-IL-12 antibody conjugated with biotin was added. Color was developed using an avidin-conjugated horseradish peroxidase and o-phenylenediamine dihydrochloride substrate (Sigma, St. Louis, MO), and the reaction was terminated by the addition of 2 N H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at 490 and 540 nm with a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The amounts of IL-12 were quantified by interpolation of a standard curve generated using known amounts of standard recombinant IL-12 (Endogen).

**Fluorescence-activated cell sorting analysis for B7-1 expression.** B16-F10 melanoma cells were seed onto 25-T culture flask at 1 × 10<sup>6</sup> per well and then infected with YKL-IL12/B7 adenovirus at MOIs of 5 to 100. At 48 hours after infection, cells were detached with cell dissociation solution (Sigma) and washed with PBS. Phycoerythrin-conjugated hamster anti-mouse CD80 monoclonal antibody (PharMingen, San Diego, CA) was subsequently added at 4°C for 45 minutes. After PBS washing, 500 μL PBS was added to do flow cytometric analysis. Cells were analyzed on a FACScan (Becton Dickinson, Sunnyvale, CA) using CellQuest software (Becton Dickinson). Live cells were gated by forward and side scatter.

**Cytopathic effect assay.** To evaluate the cytopathic effect of oncolytic adenoviruses, cells were plated onto 24-well plates at about 30% to 80% confluence and then infected with Ad-ΔE1, YKL-1, YKL-IL12, or

YKL-IL12/B7 adenovirus at MOIs of 0.1 to 1,000. Their killing effect was monitored daily under a microscope. At the moment that cells infected with any one of the virus exhibited complete cell lysis at low MOIs, the dead cells were washed out, and cells on the plate were then stained with 0.5% crystal violet in 50% methanol.

**Antitumor effects of IL-12 and B7-1-expressing oncolytic adenovirus.** Tumors were implanted s.c. on the abdomen of C57BL/6 mice by injecting B16-F10 murine melanoma cells (5 × 10<sup>5</sup>) in 100 μL HBSS (Life Technologies). When tumors reached a range of 80 to 100 mm<sup>3</sup>, animals were randomized into four groups (PBS, YKL-1, YKL-IL12, and YKL-IL12/B7) of 7 to 13 animals each, and treatment was initiated. First day of treatment was designated as day 1. Adenoviruses or PBS was given intratumorally (5 × 10<sup>8</sup> plaque-forming units per tumor in 20 μL PBS) on days 1, 3, 5, 7, and 9. Tumor growth delay was assessed by taking measurements of the length (*L*) and width (*w*) of the tumor on a 2- to 3-day interval. Tumor volume was calculated using the following formula: volume = 0.523*Lw*<sup>2</sup>. Tumor responses to each treatment were compared using a log-rank test analysis (StatView software, Abacus Concepts, Inc., Berkeley, CA). The percentage of surviving mice was determined by monitoring the tumor growth-related events (tumor size, >3,000 mm<sup>3</sup>) over a period of 40 days. Survival curve was then plotted against time after treatment. Differences in survival were considered statistically significant when *P* < 0.05.

**Measurement of IL-12 and IFN-γ expression in tumor tissue.** Tumor tissues were removed from mice treated with PBS, YKL-1, YKL-IL12, or YKL-IL12/B7 and snap frozen in liquid nitrogen. Tissues were homogenized (ART-MICCRA D-8, ART-moderne Labortechnik, Munich, Germany) and treated with protein inhibitor cocktail (Sigma). Homogenates were centrifuged in a high-speed microcentrifuge for 10 minutes and analyzed for total protein content using a bicinchoninic acid protein assay reagent kit (Bio-Rad, Hercules, CA). Levels of IL-12 and IFN-γ in supernatant were determined by ELISA (Endogen). Cytokine results were normalized relative to the total protein concentration in each tumor and were calculated as picograms per milligram of total protein.

**Fluorescence-activated cell sorting analysis of splenocytes.** To assess T-cell and dendritic cell population, spleens were harvested 3 to 5 days after last viral injection into the B16-F10 tumor-bearing mice. Spleens were minced into single-cell suspensions in PBS plus 2% FCS and treated with EDTA-ammonium chloride solution (0.15 mol/L NH<sub>4</sub>Cl, 1 mmol/L KHCO<sub>3</sub>, and 0.1 mmol/L EDTA) to remove RBC. To assess T-cell population, fluorescence-activated cell sorting was done on a FACScan after labeling 1 × 10<sup>6</sup> splenocytes with FITC-conjugated hamster anti-mouse CD3ε monoclonal antibody (145-2C11, DiNonA, Seoul, South Korea), phycoerythrin-conjugated rat anti-mouse CD4/L3T4 monoclonal antibody (GK1.5, DiNonA), or phycoerythrin-conjugated rat anti-mouse CD8/Lyt-2 monoclonal antibody (53-6.7, DiNonA). To determine dendritic cell population, splenocytes were stained with rat anti-mouse CD11b/Mac-1 (3A33, Southern Biotech, Birmingham, AL), hamster anti-mouse FITC-labeled CD11c (HL3, PharMingen), rat anti-mouse CD80 (RMMP-1, Serotec, Oxford, United Kingdom), or rat anti-mouse CD86 (GL-1, Southern Biotech) as a primary antibody and goat anti-rat IgG-phycoerythrin (Southern Biotech) as a secondary antibody. Cells were then fixed with 1% paraformaldehyde in PBS for fluorescence-activated cell sorting analysis. Live cells were gated using forward and side scatter. Isotype-matched control antibodies were used to set voltages and positive controls were used to set compensation.

**Cytotoxicity assay.** The cytolytic activity of CTL was determined by standard 4-hour <sup>51</sup>Cr release assays. Spleens were removed aseptically 3 to 5 days after last viral injection into the B16-F10 tumor-bearing mice, and unicellular splenocytes were prepared as same as in the method of fluorescence-activated cell sorting analysis of spleen cells. RBC-depleted splenocytes were then cocultured with irradiated B16-F10 or NIH3T3 cells (5,000 rad) for 5 days in the presence of recombinant human IL-2 (100 units/mL). Following this coculture, splenocytes as effector cells were recovered and incubated in triplicate with <sup>51</sup>Cr-labeled B16-F10 or NIH3T3 cells (1.5 × 10<sup>6</sup>) as target cells at four

different E:Ts (10:1, 20:1, 30:1, and 50:1). After 4 hours of incubation at 37°C, the supernatant was harvested and radioactive release was measured. The percentage of cytotoxicity was calculated as [(cpm experimental release – cpm spontaneous release) / (cpm maximum release – cpm spontaneous release)] × 100. The extent of maximal release of the incorporated chromium was determined by detergent lysis of the labeled cells, and spontaneous release was determined by incubation of the labeled cells without effector cells. The cytolytic activity of CTL was also visualized by staining live B16-F10 cells after coculture with splenocytes. B16-F10 cells (target) were first plated onto 24-well plates at  $3 \times 10^4$  per well and then mixed with splenocytes (effector) derived from B16-F10 tumor-bearing mice at three different E:Ts (10:1, 30:1, and 100:1). The mixtures of target and effector cells were then incubated for 4 to 8 days at 37°C followed by staining with 0.5% crystal violet in 50% methanol.

**IFN- $\gamma$  ELISPOT assay.** To determine the frequency of antigen-specific cytokine-producing cells, ELISPOT assay was carried out. RBC-depleted splenocytes were first restimulated by coculturing with irradiated B16-F10 (5,000 rad) tumor cells for 3 days in the presence of recombinant human IL-2 (100 units/mL). Serial dilutions of the stimulated splenocytes ( $3 \times 10^4$ ,  $6 \times 10^4$ ,  $3 \times 10^5$ ,  $6 \times 10^5$ ,  $1.2 \times 10^6$ , and  $2.4 \times 10^6$ ) were then incubated in anti-IFN- $\gamma$  monoclonal antibody-coated plates. Each experimental condition was plated in triplicate and incubated at 37°C/5% CO<sub>2</sub> for 24 hours. Cells were removed, and the plates were washed three times with PBS containing 1% fetal bovine serum and 0.05% Tween 20. Biotinylated secondary anti-IFN- $\gamma$  monoclonal antibody (50  $\mu$ L) was added to each well and incubated in the dark at room temperature for 2 hours. The plates were washed with PBS containing 1% fetal bovine serum and 0.05% Tween 20, and then 50  $\mu$ L streptavidin-horseradish peroxidase was added to each well and incubated at room temperature in the dark for 1 hour. Washing was repeated and a colorimetric reaction was initiated by the addition of substrates from a commercial kit (Endogen). The colored spots, representing IFN- $\gamma$ -producing cells, were counted with a KS ELISPOT (Zeiss-Kontron, Jena, Germany) and confirmed by the computer-based immunospot system (AID Elispot Reader System, version 3.4, Autoimmun Diagnostika GmbH, Strassberg, Germany). The results were similar for manual and computer-based counting. All wells were averaged and normalized by comparing the ratio of antigen-specific spots to negative control spots.

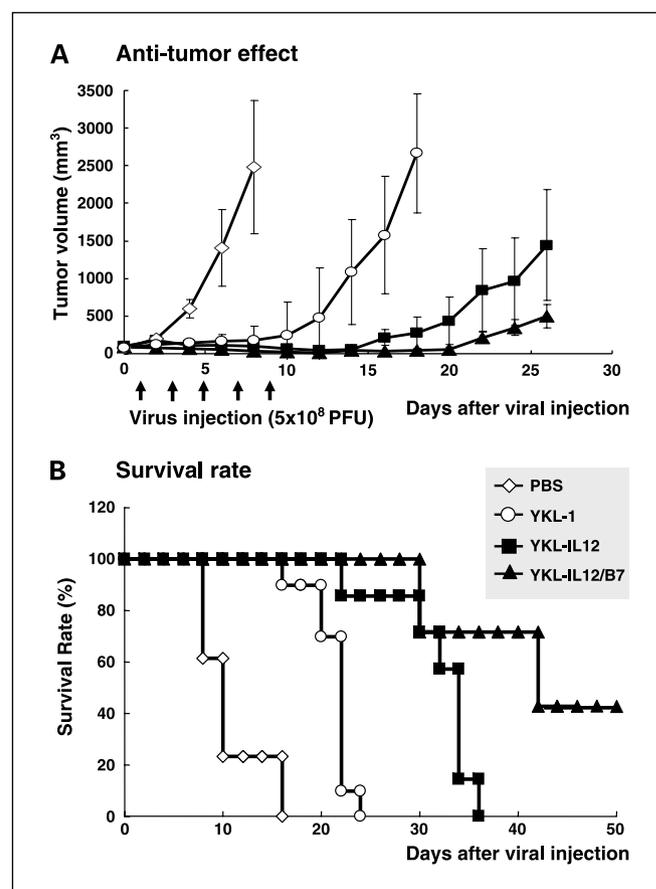
**Histology and immunohistochemistry.** Tumor tissues for histologic examination were fixed in 10% neutral buffered formalin, processed to paraffin embedment, and cut into 4- $\mu$ m sections. Representative sections were stained with H&E and examined by light microscopy. To identify lymphocytes, tumor tissues were frozen in OCT compound (Sakura Finetec, Torrance, CA) and cut into 10- $\mu$ m sections. The cryosections were blocked with 1% bovine serum albumin in PBS and stained with purified rat anti-mouse CD4 monoclonal antibody (PharMingen) or purified rat anti-mouse CD8 monoclonal antibody (PharMingen) as a primary antibody and then with biotin-conjugated goat anti-rat IgG (PharMingen) as a secondary antibody. Diaminobenzidine/hydrogen peroxidase (DAKO, Carpinteria, CA) was used as the chromogen substrate. All slides were counterstained with Meyer's hematoxylin. The average number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells per field was determined under a light microscope at  $\times 400$  magnification.

**Statistical analysis.** The data were expressed as mean  $\pm$  SE. Statistical comparison was made using StatView software and the Mann-Whitney test (nonparametric method). The criterion for statistical significance was taken as  $P < 0.05$ .

## Results

**Oncolytic adenovirus-mediated IL-12 and B7-1 expression.** To generate the two oncolytic adenoviruses expressing either IL-12 alone or IL-12 plus B7-1, murine IL-12 and B7-1 genes

were placed in the E1 and E3 region of E1B 55 kDa deleted oncolytic adenovirus, YKL-1, respectively, generating YKL-IL12 and YKL-IL12/B7 adenoviruses (Fig. 1A). To determine the level of IL-12 expression from these adenoviruses, B16-F10 melanoma cells were infected with YKL-IL12 and YKL-IL12/B7 at different MOIs. The results showed a significant expression of the virally transduced IL-12 gene, whose levels increased in a dose-dependent manner up to MOI of 100 (Fig. 1B). Notably, the IL-12 expression from YKL-IL12/B7 infection at an MOI of 20 and 100 were  $1,472 \pm 13$  and  $3,050 \pm 13$  pg/mL, respectively, whereas those from YKL-IL12 were  $1,012 \pm 20$  and  $2,209 \pm 39$  pg/mL. Thus, additional expression of B7-1 in the E3 region did not affect IL-12 expression. Next, the expression level of B7-1 was assessed by flow cytometric analysis 48 hours after infection with YKL-IL12/B7 in B16-F10 cells. As shown in Fig. 1C, B7-1 was also strongly expressed on the cell surface of B16-F10



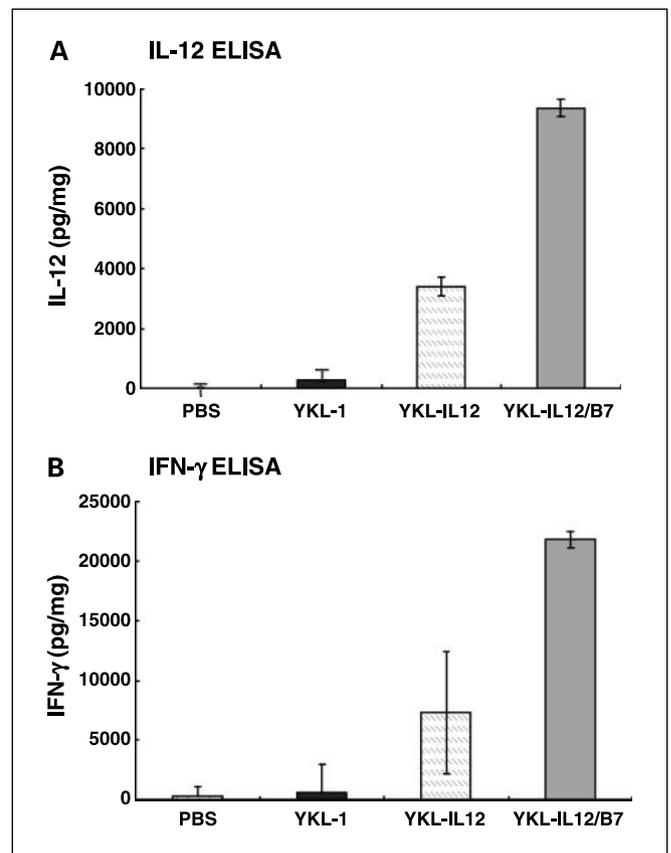
**Fig. 2.** Evaluation of therapeutic effect on established tumor model. **A**, tumor growth response following intratumoral injection of PBS, YKL-1, YKL-IL12, or YKL-IL12/B7. B16-F10 cells ( $5 \times 10^5$ ) were injected s.c. into the abdomen of male C57BL/6 mice. When tumor size reached to about 80 to 100 mm<sup>3</sup>, tumors were injected with the following: PBS ( $n = 13$ ), YKL-1 ( $n = 10$ ), YKL-IL12 ( $n = 7$ ), or YKL-IL12/B7 ( $n = 7$ ). Adenoviruses [ $5 \times 10^8$  plaque-forming units (PFU)] were injected intratumorally five times every other day. Tumor growth was then evaluated by microcaliper measurements done two to three times weekly. The difference between all experimental and PBS control groups is statistically significant (day 9;  $P < 0.001$  for all three adenoviruses). **B**, long-term survival of mice bearing B16-F10 tumors following treatment with PBS, YKL-1, YKL-IL12, or YKL-IL12/B7. The percentage of mice viability was determined by monitoring the tumor growth-related events (tumor size  $>3,000$  mm<sup>3</sup>) over a period of 40 days. The survival advantage for the YKL-IL12- and YKL-IL12/B7-treated animals was statistically significant compared with PBS and YKL-1 ( $P < 0.001$ ). Results are representative data from one of two independent experiments.

infected with YKL-IL12/B7, whose level of expression was directly proportional to the MOIs used for infection, indicating that the B7-1 protein expressed by this oncolytic adenovirus was properly presented on the cell surface.

**IL-12 and B7-1 expression does not inhibit viral replication.** To determine whether IL-12 and B7-1 expression would alter viral replication, YKL-IL12 and YKL-IL12/B7 oncolytic adenoviruses were examined for their ability to induce viral cytopathic effects in a variety of cell lines. In general, murine cells tended to be less sensitive or even resistant to adenoviral infections compared with human cells. Thus, we used five different types of human cancer cell lines (U343, U87MG, C33A, Hep3B, and A549) from varying histologic types, in addition to the murine melanoma cell line (B16-F10) in the cytopathic effect assay. Cells were infected with YKL-1 (cognate oncolytic adenovirus), YKL-IL12, or YKL-IL12/B7 adenovirus along with Ad- $\Delta$ E1 as a negative control. Next, the subjected cells were treated with crystal violet to show relative extent of cell lysis. As seen in Fig. 1D, YKL-IL12- and YKL-IL12/B7-induced cytopathic effect appeared to be slightly faster in U87MG and Hep3B compared with YKL-1. This result was repeated over several independent experiments, indicating that IL-12 and B7-1 expression does not inhibit viral replication. Similarly, the cytopathic effect induced by YKL-IL12/B7 was comparable with that induced by YKL-IL12, showing that viral replication of oncolytic adenovirus expressing both IL-12 and B7-1 was not decreased compared with oncolytic adenovirus expressing IL-12 alone. This observation is consistent with the result of IL-12 expression level of YKL-IL12 and YKL-IL12/B7 oncolytic adenoviruses.

**Comparison of the relative antitumor effect of YKL-IL12 and YKL-IL12/B7.** We next evaluated the relative antitumor effect of oncolytic adenovirus expressing either IL-12 alone or IL-12 plus B7-1 in B16-F10 melanoma model established in C57BL/6 mice. As seen in Fig. 2A, control tumors which received PBS showed robust growth, resulting in a tumor volume of  $2,481 \pm 885 \text{ mm}^3$  by 9 days after initial injection. Subsequently by 20 days after treatment, all mice in the PBS control group were not viable (Fig. 2B). In marked contrast, the tumor growth was severely suppressed in mice injected with YKL-IL12 ( $P < 0.01$  versus PBS or YKL-1 group) or YKL-IL12/B7 ( $P < 0.01$  versus PBS or YKL-1 group). More specifically, the average tumor volume at 20 days after injection for YKL-IL12 and YKL-IL12/B7 groups were  $274 \pm 211$  and  $46 \pm 22 \text{ mm}^3$ , respectively, whereas tumors treated with YKL-1 grew progressively large, reaching to an average size of  $2,666 \pm 793 \text{ mm}^3$ . Further, 3 of the 7 animals that received YKL-IL12/B7 remained viable 50 days after initial treatment (Fig. 2B). In contrast, no animal in YKL-1 and YKL-IL12 group was viable in the same time period. The survival advantage conferred by YKL-IL12/B7 therapy was statistically significant when compared with either of the YKL-1 and YKL-IL12 groups ( $P < 0.001$  versus YKL-1 group and  $P < 0.05$  versus YKL-IL12).

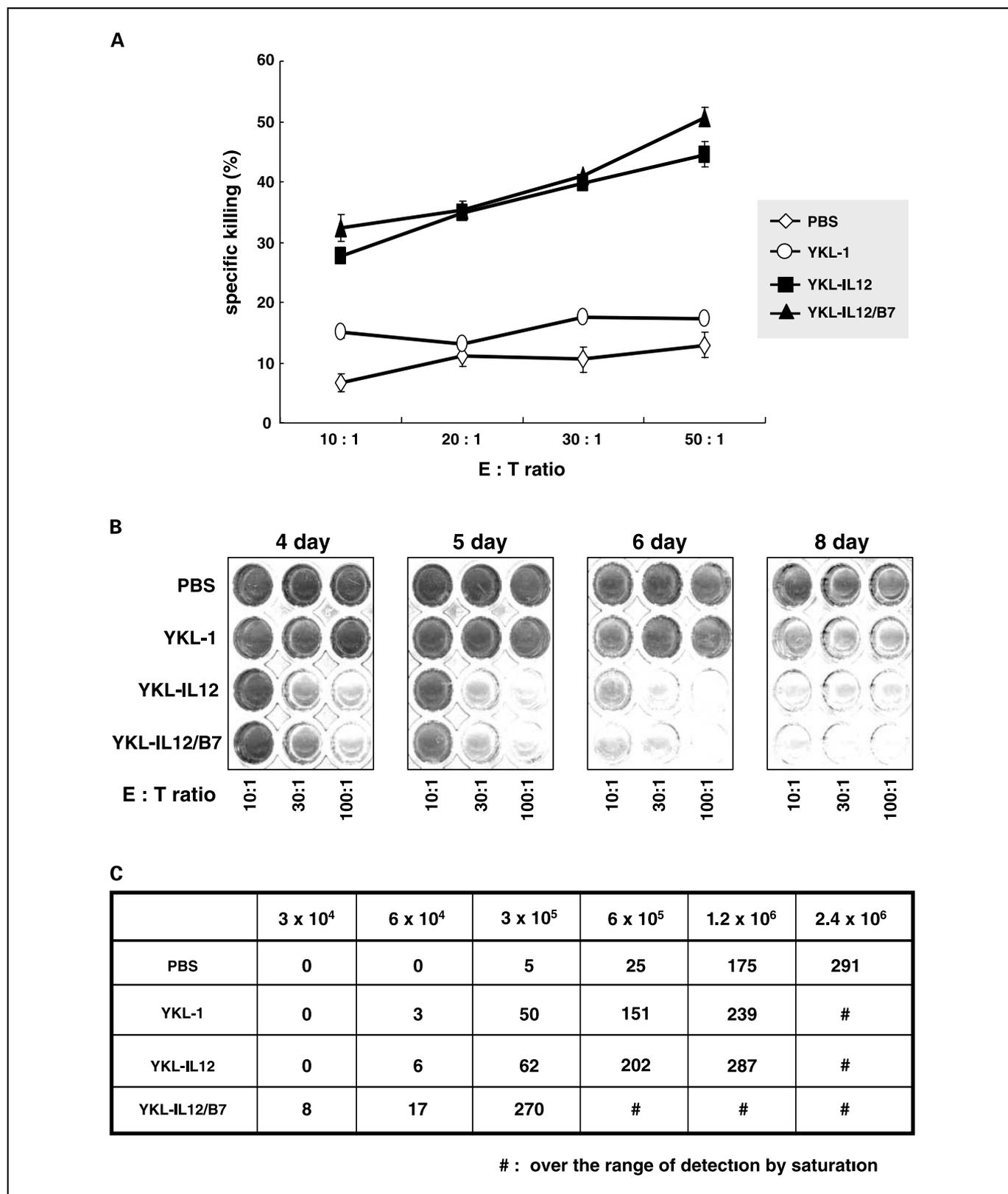
**In vivo expression of IL-12 and IFN- $\gamma$ .** To evaluate the level of IL-12 and IFN- $\gamma$  produced in YKL-IL12- or YKL-IL12/B7-treated mice, tumor tissues were obtained 5 days after final viral injection. Figure 3 shows IL-12 and IFN- $\gamma$  in picogram per milligram of total protein from three independent experiments. In PBS-treated and YKL-1 adenovirus-treated tumor cells, only a trace amount of IL-12 was detected ( $21.1 \pm 144.1$  and  $249.4 \pm 40.7 \text{ pg/mg}$ , respectively). In comparison, YKL-



**Fig. 3.** Cytokine production after treatment of PBS, YKL-1, YKL-IL12, or YKL-IL12/B7. Tumor tissue was collected 5 days after final viral injection, and ELISA was done to determine the levels of IL-12 (A) and IFN- $\gamma$  (B) in tumor tissues. Data are representative of three independent experiments.

IL12 adenovirus-treated or YKL-IL12/B7 adenovirus-treated tumors exhibited high concentration of IL-12 ( $3,412.2 \pm 316.5 \text{ pg/mg}$  for YKL-IL12 and  $9,364.8 \pm 276.7 \text{ pg/mg}$  for YKL-IL12/B7; Fig. 3A). Similarly, YKL-IL12- or YKL-IL12/B7-treated mice produced significantly greater level of IFN- $\gamma$  ( $7,289.8 \pm 5,130.3 \text{ pg/mg}$  for YKL-IL12 and  $21,800.0 \pm 694.6 \text{ pg/mg}$  for YKL-IL12/B7) compared with PBS-treated mice of  $259.8 \pm 857.0 \text{ pg/mg}$  and YKL-1-treated mice of  $573.6 \pm 2,393.1 \text{ pg/mg}$  (Fig. 3B). Finally, in relative comparison, YKL-IL12/B7-treated tumors produced significantly higher level of both IL-12 and IFN- $\gamma$  than YKL-IL12.

**Generation of tumor-specific CTL.** To establish that tumor regression is associated with the generation of tumor-specific immune response,  $^{51}\text{Cr}$  release assay was carried out. Splenocytes obtained from YKL-IL12- or YKL-IL12/B7-treated mice showed potent B16-F10-specific lytic activity on day 5 after exposure. CTL killing of splenocytes from mice treated with YKL-IL12 or YKL-IL12/B7 was 39.8% and 41.0% at an E:T of 30:1 and 44.5% and 50.6% at an E:T of 50:1, respectively, with no measurable background (Fig. 4A). At the same time, however, splenocytes from mice treated with PBS or YKL-1 did not show any significant B16-F10-specific lytic activity. The CTL activity of splenocytes from mice treated with YKL-IL12 or YKL-IL12/B7 was specific to a given tumor, as no CTL activity could be detected when NIH3T3 cells were used as target cells (data not shown).



**Fig. 4.** Generation of antitumor specific immune response. Tumor-bearing mice received three intratumoral injections of PBS, YKL-1, YKL-IL12, or YKL-IL12/B7. **A**,  $^{51}\text{Cr}$  release from B16-F10 target cells was measured after incubation with *in vitro* – stimulated splenocytes from mice treated with PBS, YKL-1, YKL-IL12, or YKL-IL12/B7. Points, mean of quadruplicate experiments; bars, SD. Results are representative of three independent experiments. **B**, visualization of tumor cell killing effect of CTL. Splenocytes (effector) obtained from mice injected with YKL-IL12 or YKL-IL12/B7 showed a significantly higher lysis of B16-F10 cells (target). However, most of cells remained viable when those of PBS- and YKL-1-treated mice were cocultured with B16-F10 target cells in the same time period. **C**, ELISPOT assay for IFN- $\gamma$ . Adenoviruses-infected animals were sacrificed 3 days after final viral injection. Results are presented as number of spots assayed in the condition as described in Materials and Methods.

The cytolytic activity of CTL was also visualized by staining live B16-F10 cells after coculture with splenocytes. B16-F10 cells (target) were mixed and cocultured for 4 to 8 days with splenocytes (effector) derived from B16-F10 tumor-bearing mice treated with PBS, YKL-1, YKL-IL12, or YKL-IL12/B7 at three different E:Ts (10:1, 30:1, and 100:1). As seen in Fig. 4B, splenocytes derived from mice treated with YKL-IL12 or YKL-IL12/B7 exerted higher tumor cell killing potency over those from YKL-1-treated mice. Specifically, when cocultured for 6 days, splenocytes from mice treated with YKL-IL12 or YKL-IL12/B7 induced rapid target cell killing, resulting in complete eradication of cells on the plate. However, most cells remained viable and stained with crystal violet when those of PBS- and

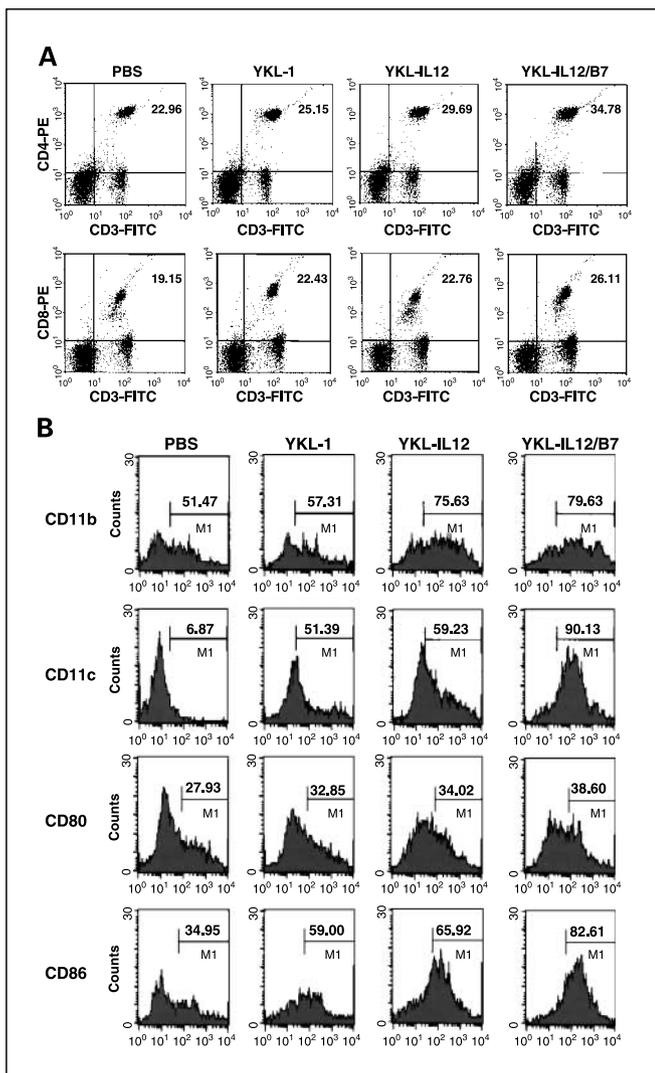
YKL-1-treated mice were cocultured with B16-F10 target cells in the same time period.

To further delineate the tumor-specific immune responses in mice, number of cells expressing IFN- $\gamma$ , a cytokine secreted by CTL, was evaluated through IFN- $\gamma$  ELISPOT assay. Splenocytes were isolated aseptically on day 3 after final viral injection from C57BL6 mice treated as described above and were serially seeded at a concentration from  $3 \times 10^4$  to  $2.4 \times 10^6$  cells per well into the anti-IFN- $\gamma$  antibody-coated 96-well plate. The number of IFN- $\gamma$ -secreting cells was then subsequently analyzed. As presented in Fig. 4C, the frequency of IFN- $\gamma$ -secreting immune cells recovered from mice injected with YKL-IL12/B7 was significantly greater than that rescued from mice given YKL-IL12 or YKL-1. Specifically, the number of IFN- $\gamma$ -secreting cells obtained from mice injected with YKL-IL12/B7 at a concentration of  $3 \times 10^5$  splenocytes was about 4.4- and 5.4-fold higher than that of YKL-IL12 and YKL-1, respectively. These results suggest that animals treated with YKL-IL12/B7 generated higher tumor-specific immune responses than those treated with YKL-IL-12 or YKL-1, showing synergy of IL-12 with B7-1 in promoting the IFN- $\gamma$  production.

**Alteration in splenic CD4<sup>+</sup>/CD8<sup>+</sup> T-cell, macrophage, and dendritic cell populations.** Purified splenocytes from mice at 3 to 5 days following final viral injection were first analyzed to determine the population of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell. Analysis revealed a slight elevation in the frequency of CD4<sup>+</sup> (29.69% and 34.78%) T cells in the YKL-IL12- and YKL-IL12/B7-treated mice, respectively, compared with YKL-1-treated mice (25.15%; Fig. 5A). Similarly, the frequency of CD8<sup>+</sup> T cells was also marginally increased in the mice treated with YKL-IL12/B7 (26.11%) compared with those treated with YKL-1 (22.43%). These results show the increased number of T lymphocytes capable of inducing cancer-specific immune response in response to coexpression of IL-12 and B7-1. To further evaluate macrophage and dendritic cell population, which are the most potent antigen-presenting cells, splenocytes were also analyzed for their expression of CD11b, CD11c, CD80, and CD86 by flow cytometry. As shown in Fig. 5B, YKL-IL12- and YKL-IL12/B7-treated splenocytes expressed increased level of activation/costimulatory molecules when compared with PBS- or YKL-1-treated splenocytes. In particular, the expression level of CD11c and CD86 of YKL-IL12/B7-treated splenocytes was notably higher than those of YKL-IL12-treated splenocytes. These results suggest that adenovirus coexpressing IL-12 and B7-1 induced more efficient antigen-presenting cell maturation *in vivo* than adenovirus expressing IL-12 alone.

**Increased CD4<sup>+</sup> and CD8<sup>+</sup> T-cell infiltration in YKL-IL12 and YKL-IL12/B7 adenovirus-treated tumors.** Histologic analysis with H&E staining was carried out to study lymphocyte infiltration into tumor tissues. As seen in Fig. 6A, YKL-IL12- and YKL-IL12/B7-treated tumors showed visibly smaller size with significantly higher immune cell infiltration than YKL-1-treated controls. In particular, for tumors treated with YKL-IL12/B7, comparatively extensive lymphocytic infiltration intermingled with nuclear debris was observed not only around but also inside the tumor tissue. Moreover, in the YKL-IL12- and YKL-IL12/B7-treated group, almost all of the remaining tumor tissues appeared to be necrotic.

The involvement of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte subsets in the antitumor response was further studied using antibodies with specificities for CD4<sup>+</sup> and CD8<sup>+</sup> T-cell markers. Higher



**Fig. 5.** Assessment of T-cell and dendritic cell population. *A*, fluorescence-activated cell sorting analysis after double staining by immunofluorescence with anti-CD3/anti-CD4 and CD3/anti-CD8 monoclonal antibodies of splenocytes from mice whose s.c. B16-F10 tumors had been treated with PBS, YKL-1, YKL-IL12, or YKL-IL12/B7. Top right quadrants, percentages of the CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T-cell population. Results are representative of several independent experiments, in which proportions of CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> T cells were consistent.

*B*, splenocytes were analyzed for dendritic cell phenotype (CD11b, CD11c, CD80, and CD86) by flow cytometry after treatment with PBS, YKL-1, YKL-IL12, or YKL-IL12/B7. Cells were stained with indicated monoclonal antibodies or isotype controls and analyzed on an open gate for phenotype by fluorescence-activated cell sorting. Values are the relative mean fluorescence of stained cells over controls.

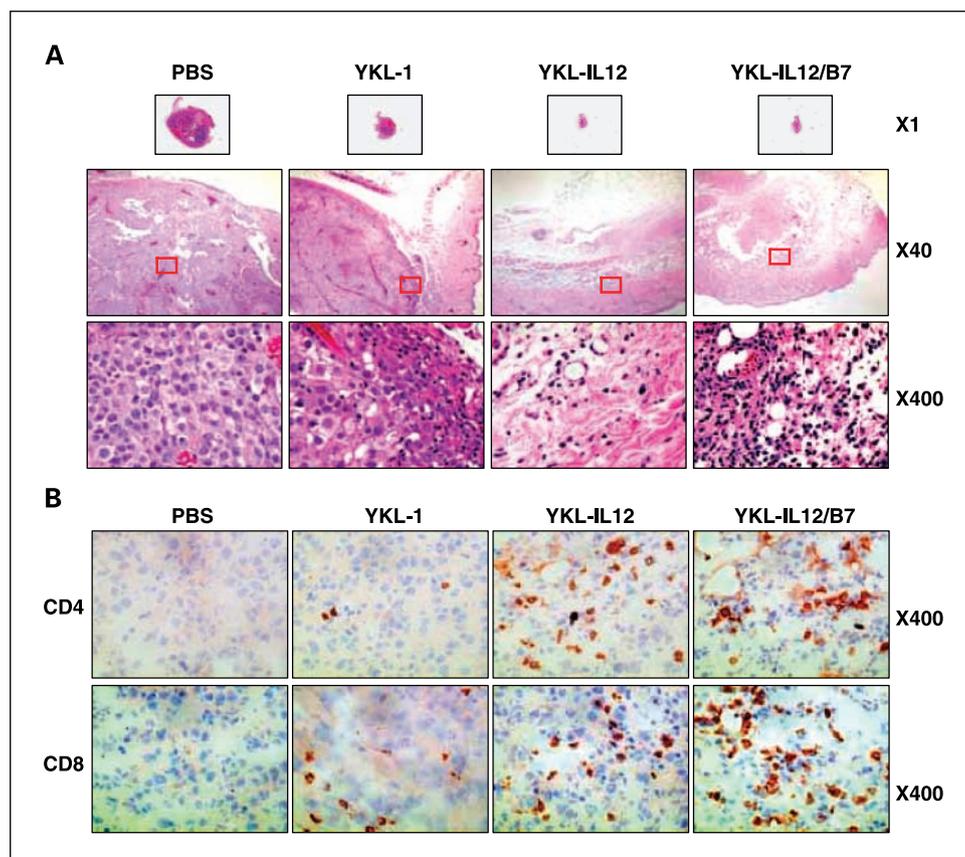
frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were observed in both the center and the border zones of the tumors treated with YKL-IL-12/B7 when compared with YKL-IL12-treated tumors (Fig. 6B). In contrast, the tumors of the YKL-1 control groups contained only minor levels of CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

## Discussion

Direct administration of immunostimulatory cytokine genes to modify cytokine profile *in situ* has been an attractive and effective approach in inhibiting tumor growth. Several cytokines, including IL-2, IL-12, IL-18, granulocyte macrophage colony-stimulating factor, IL-12, IFN- $\alpha$ , and IFN- $\gamma$  have shown significant antitumor effects (23–26). Among these, IL-12 has become the most promising cytokine for immunotherapy of many cancers due to its pleiotropic effects showed both *in vitro* and *in vivo*. IL-12 is believed to promote antitumor immunity by stimulating Th1 immune response by increasing MHC expression and up-regulating the cytotoxic activity of NK and CD8<sup>+</sup> T cells (27, 28). B7-1, on the other hand, provides potent antigen-independent costimulatory signal to T cells and lowers the threshold for T-cell activation, resulting in an increase in the production of Th1-associated cytokines, including IL-2, IL-12, and IFN- $\gamma$ , important in promoting antitumor CTL responses (17, 29). Murphy et al. (20) reported that proliferation by terminally differentiated Th1 clones, in contrast to naive T cells, requires stimulation via membrane-bound B7-1 and IL-12. Moreover, it has been also shown that coexpression of IL-12 and B7-1 synergistically improves T-cell activation and the production of Th1 pattern cytokines (17).

In the present study, we examined the antitumor efficacy potential of a single oncolytic adenovirus vector expressing both IL-12 and B7-1. IL-12 and B7-1 expression was first examined in B16-F10 cells as murine cells are resistant to infection by human adenovirus. As shown in Fig. 1, virally induced IL-12 and B7-1 genes were expressed in this murine cell line in a dose-dependent manner. Further, IL-12 and B7-1 expression did not compromise viral ability to induce classic cytopathic effect in infected cells or abolish the ability to produce progeny virus. The therapeutic efficacy of YKL-IL12 or YKL-IL12/B7 adenovirus was then evaluated in immunocompetent mice bearing murine melanoma B16-F10 tumors. Significant inhibition of tumor growth resulted in mice treated with YKL-IL12 or YKL-IL12/B7 adenovirus. More specifically, YKL-IL12/B7 oncolytic adenovirus showed a higher incidence of complete tumor regression compared with the analogous vector, YKL-1, or YKL-IL12. Significant survival advantage was also seen in response to YKL-IL12/B7. These results suggest that using an oncolytic adenovirus in combination with expression of IL-12 and B7-1 result in greater potent antitumor effect than either YKL-1 or YKL-IL12.

To investigate whether the generation of tumor-specific T-cell-mediated immune responses was responsible for the observed antitumor effect, CTL assay and IFN- $\gamma$  ELISPOT assay were carried out. Activation of CD8<sup>+</sup> T cells and stimulation of CTL activity are involved in antitumor effect induced by IL-12 in different animal tumor models (30, 31). In our system, we observed that cytolytic activity of CTLs cultured from splenocytes of mice treated with YKL-IL12 or YKL-IL12/B7 was significantly elevated against inoculated tumor compared with



**Fig. 6.** Histologic and immunohistochemical analysis. Formalin-fixed tumor tissues were embedded in paraffin and sectioned. **A**, H&E-stained tumor sections. Original magnifications,  $\times 1$ ,  $\times 40$ , and  $\times 400$ . **B**, frozen tumor sections were stained with anti-CD4 or anti-CD8 antibodies. Immunohistochemical analysis of these sections showed enhanced infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in tumor tissue in YKL-IL12/B7-treated mice compared with YKL-1- or YKL-IL12-treated tumor. Original magnification,  $\times 400$ .

that with PBS or YKL-1. Moreover, the frequencies of IFN- $\gamma$ -positive cells measured with the IFN- $\gamma$  ELISPOT assay correlated with the corresponding tumor cell-specific killing measured by  $^{51}\text{Cr}$  release assay. In agreement with these results, the histologic and immunohistochemical studies also showed massive infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells into the tissues surrounding the necrotic area of the tumor after *in situ* delivery of YKL-IL12/B7 than those in the tumor tissue treated with YKL-1 or YKL-IL12. These results suggest that IL-12 and B7-1 play a critical and interactive role in promoting tumor-specific immunity.

In several systems, it has been shown that IFN- $\gamma$  is the main downstream mediator of the IL-12 antitumor efficacy (32–34). IL-12-activated cells, such as antigen-presenting cells, T cells, or NK cells, produce IFN- $\gamma$ , and IFN- $\gamma$  in turn induces effects not only on the immune system but also on tumor cells, making them susceptible to be lysed by CTLs (35). Antitumor effects of IL-12 were reported in a variety of cancer models. In a hepatocellular carcinoma model, ~50% of mice infected intratumorally with  $5 \times 10^9$  plaque-forming units of IL-12 expressing adenovirus showed complete regression after 2 weeks of treatment, accompanied by local secretion of IL-12 and IFN- $\gamma$  (36). Consistent with these findings, we found that high levels of IL-12 and IFN- $\gamma$  are produced in mice after injection with YKL-IL12 or YKL-IL12/B7. The high IFN- $\gamma$  induction following treatment with YKL-IL12 or YKL-IL12/B7 supports the notion that IL-12 produced by tumor cells is biologically active and exerts its characteristic immunoregulatory functions. It is interesting note that the level of IFN- $\gamma$  expression by YKL-IL12 (7,289 pg/mg) or YKL-IL12/B7 (21,800 pg/mg) is far greater than what was reported previously by Mazzolini et al. (200 pg/mg; ref. 31). These data indicate that, compared with conventional non-replicating vector systems, these newly engineered oncolytic adenoviruses are much more efficient in driving cytokine expressions, leading potent antitumor immune responses. Moreover, IL-12 and IFN- $\gamma$  levels produced in tumors treated with YKL-IL12/B7 was significantly greater than those treated with YKL-IL12, suggesting that the expression of IL-12 and B7-1 at the site of the tumor plays an important role in inducing antitumor responses. This observation is consistent with the reported synergistic effect between IL-12 and B7-1 as reported in murine mammary adenocarcinoma model showing that coinjection of two different adenovirus vectors expressing either IL-12 or B7-1 induced higher antitumor effect than injection with either vector alone (18). Likewise, it has been reported that the effectiveness of IL-12 in a poorly immunogenic pancreatic tumor model that lack

endogenous B7-1 expression can be significantly potentiated by B7-1 costimulation (37).

Higher doses of IL-12 not only elicit potent antitumor effect but also induce dose-dependent toxicity, including ascites, dry mucous membranes, lack of grooming, and occasional death (<5%). Fatal toxicity in a phase II human clinical trial using systemic administration of recombinant IL-12 has also been reported (38). Thus, the use of IL-12 as an antitumoral agent by means of systemic administration has been hampered by these cases of serious side effects. In an orthotopic hepatocellular carcinoma and a bladder carcinoma models, intratumoral injection with IL-12 expressing adenovirus resulted in very potent antitumor effect, but no detectable serum level of IL-12 or IFN- $\gamma$  was documented (36, 39). Our data are consistent with these observations, as we found no evidence of increased IL-12 or IFN- $\gamma$  serum levels at 3 days after intratumoral injection of YKL-IL12 or YKL-IL12/B7 oncolytic adenovirus nor was there any evident toxicity. Thus, we find that local delivery of IL-12 by oncolytic adenovirus would endow higher local synthesis of IL-12 in tumor bed, resulting in potent antitumor effects without accompanying toxicities.

Taken collectively, our data suggest that combination of oncolysis and the enhancement of antitumor immune response by oncolytic adenovirus expressing both IL-12 and B7-1 elicits potent antitumor effect. The YKL-IL12/B7 adenovirus showed augmented and prolonged effect in suppressing tumor growth *in vivo*, in contrast to the YKL-1 adenovirus that only showed limited and temporary antitumor effect. Moreover, YKL-IL12/B7 adenovirus elicited higher survival advantage compared with YKL-IL12. The therapeutic advantage of YKL-IL12/B7 would be greatly enhanced in a human system as human tumor cells are much more permissive to adenoviral infection and replication. Because the therapeutic value of YKL-IL12/B7 adenovirus has been verified to be very high, further studies are being planned to investigate the application of this adenovirus for cancer therapy, especially in preventing metastasis and secondary tumorigenesis.

In conclusion, the findings of this study show the effectiveness of augmenting the immune response against tumors with oncolytic adenovirus expressing both IL-12 and B7-1. The present study provides further support for the use of oncolytic adenovirus in immunogene therapy in combating cancer and particularly for cancer-specific delivery of cooperatively interacting immunomodulatory genes in a single vector. Overall, given the results shown in this study, oncolytic adenovirus-mediated IL-12 and B7-1 gene transfer may open promising avenues to effective and better tolerated immunogene therapy in combating cancer.

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