

Combination B7-Fc Fusion Protein Treatment and Treg Cell Depletion Therapy

Aihong Liu, Peisheng Hu, Leslie A. Khawli, and Alan L. Epstein

Abstract **Purpose:** A B7.1 fusion protein consisting of the extracellular domains of human B7.1 and the Fc portion of human IgG1, called B7.1-Fc, was generated and evaluated for its antitumor potential when used alone or in combination with regulatory T (Treg) cell depletion.

Methods: A human B7.1-Fc fusion protein was constructed, expressed, purified, and examined for its antitumor activity in experimental mouse tumor models.

Results: Soluble B7.1-Fc showed costimulatory activity of T-cell proliferation *in vitro*, and when given *in vivo*, it induced complete regression of Colon 26 tumors after a 5-day treatment regimen. Parallel studies with human B7.2-Fc gave very similar results in the Colon 26 tumor model. Even in mice with established RENCA and Madison 109 tumors, which are poorly immunogenic, B7.1-Fc treatment slowed tumor growth dramatically. In these models, more potent antitumor activity was achieved when B7.1-Fc was used in combination with Treg depletion by i.p. administration of antibody PC61. Rechallenge experiments done with mice that had sustained complete tumor regressions showed that these mice had immunologic memory by their ability to reject subsequent implants. Histologically, B7.1-Fc treatment induced multiple areas of necrosis and infiltration of CD4⁺ and CD8⁺ T cells in tumors along with a concomitant dramatic increase in T-cell proliferation in tumor-draining lymph nodes.

Conclusions: The B7.1-Fc fusion protein seems to be an effective antitumor agent especially in combination with Treg depletion. Its potency in stimulating immune responses and its human origin suggest that clinical studies may be warranted in the future.

Optimal activation of naive T cells requires both antigen-specific signaling and induction of costimulatory pathways. The absence of costimulatory signaling leads naive T cells to fail to recognize antigens and become tolerant to cancer cells, a possible strategy by which tumors escape from host immunity (1, 2). To break this immune tolerance and elicit effective antitumor responses, many strategies have been used, including the expression of costimulatory molecules B7.1 and B7.2 (3–5), blockade of inhibitory signaling using anti-CTLA-4 antibody (6), and grafting T cells with the stimulatory receptor CD28 in adoptive immunotherapy (7, 8). In the above studies, suppression of tumor growth was achieved by presenting costimulatory signals directly on the tumor cell surface by transfection, gene therapy, and similar approaches (3–5, 9, 10). All these strategies are promising but have only been met

with limited success in experimental therapeutic models. In the past few years, soluble costimulator proteins fused to the Fc portion of antibody have been developed and tested in mouse tumor models, such as murine B7.2-Ig (11–17). Sturmhoefel et al. (12) and Swiniarski et al. (15) have reported that B7.2-Ig therapy as an adjuvant in cancer vaccines could produce complete tumor rejection in some tumor models, including the poorly immunogenic B16/F10 tumor. A study by Zhou et al. (18) showed that i.m. coinjection of B7.1/IgG1 Fc and carcinoembryonic antigen DNA plasmid can protect mice from the same tumor challenge. Based on these promising results, we took a closer look at the therapeutic potential of soluble B7 fusion proteins as potent stimulators of antitumor immunity when used alone and combined with strategies to reverse immune tolerance to tumor antigens.

Regulatory T (Treg) cells have been found to be essential in immune system homeostasis (19, 20). CD4⁺CD25⁺ T cells, also called naturally occurring Treg cells, constitutively express CD25 [α chain of the interleukin (IL)-2 receptor] on their surface and constitute 5% to 10% of CD4⁺ T cells in humans and rodents. Because this small subpopulation has been found to play a key role in immunologic self-tolerance (21, 22), it is not surprising that manipulation of Treg function might have potential therapeutic effects in the treatment of cancer (23–26). Experimentally, *in vivo* depletion of CD25⁺ cells with the anti-CD25 antibody PC61 causes the regression of leukemia and solid tumors in animal models. Moreover, combination therapy seems to be more promising than Treg depletion alone as exemplified by studies in which Treg depletion is used with

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CTLA-4 blockade (27) or with the chemokine fusion protein LEC/chTNT-3 produced in our laboratory (28). Based on these results, we wanted to determine if Treg could improve the therapeutic potential of soluble B7.1-Fc for the immunotherapy of experimental solid tumors. To accomplish this, we describe the construction and characterization of a human B7.1-Fc fusion protein and its use *in vivo* with and without Treg depletion for the immunotherapy of cancer.

Materials and Methods

Mice. Six-week-old female BALB/c mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Gene knockout mice, including IFN- $\gamma^{-/-}$ and perforin $^{-/-}$ mice with H-2^d background, were generously provided by Dr. Stephen Stohlman (Department of Molecular Microbiology, Keck School of Medicine, University of Southern California, Los Angeles, CA; ref. 29). Institutional Animal Care and Use Committee-approved protocols and institutional guidelines for the proper humane care and use of animals in research were followed in all experiments.

Reagents. The Glutamine Synthetase Gene Amplification System, including the expression plasmid pEE12, was purchased from Lonza Biologics (Slough, United Kingdom). The plasmid pBJ, containing the human B7.1 cDNA, was purchased from American Type Culture Collection (Manassas, VA). Restriction endonucleases, T4 DNA ligase, Vent polymerase, and other molecular biology reagents were obtained from New England Biolabs (Beverly, MA). Characterized and dialyzed FCS were purchased from Hyclone Corp. (Logan, UT), and RPMI 1640, Hybridoma Selective Medium without L-glutamine, and MEM nonessential amino acids solution (100 \times) were purchased from Life Technologies (San Diego, CA). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes (Eugene, OR). Human B7.2-Fc fusion protein (12) was generously provided by Dr. Stanley F. Wolf (Department of Inflammation, Wyeth Research Institute, Cambridge, MA).

Antibodies and cell lines. Biotin anti-CD4 (clone GK1.5), biotin anti-CD8 (clone 2.43), FITC anti-CD4 (clone RM4-4), phycoerythrin anti-CD25 (clone 7D4), phycoerythrin anti-CD3e (clone 145-2c-11), and horseradish peroxidase-streptavidin were purchased from BD Pharmingen (San Diego, CA). Hybridomas, including rat anti-mouse CD4 (GK1.5), anti-CD-8 β (H35), anti-CD25 (PC61), and anti-IL-4 (11B11) were purchased from American Type Culture Collection. Horseradish peroxidase-conjugated goat anti-human IgG (Fc specific) and activating antibodies, including purified rat anti-mouse CD3 (17A2) and rat anti-mouse CD28, were purchased from Caltag (Burlingame, CA).

The NS0 murine myeloma cell line was obtained from Lonza Biologics. The Raji African Burkitt's lymphoma, the Colon 26 murine colorectal adenocarcinoma, and the RENCA murine renal carcinoma cell lines were obtained from American Type Culture Collection. The Madison 109 (MAD109) murine lung carcinoma cell line was obtained from the National Cancer Institute (Frederick, MD).

Construction of the B7.1-Fc fusion protein. Expression plasmid encoding the human B7.1 signal and extracellular domains fused to the Fc region of human IgG1 were constructed as follows: cDNA encoding the signal and extracellular domains of human B7.1 was generated by PCR amplification from full-length cDNA purchased from American Type Culture Collection. The cDNA extends from the initiation Met in the signal sequence through Asp²⁴¹ of the total protein sequence. Primary PCR of B7.1 was done with the 5' and 3' primers 5'-TTCTCTA-GAATGGGCCACACACGG-3' and 5'-TGTGTGAGTTTGTATCAG-GAAAATGCTCTTGCTT-3', respectively, to append a *Xba*I restriction site to the 5' end of the human B7.1 cDNA (the protein sequence was identical to that of Genbank accession no. M27533). The hinge-CH2-CH3 portion of human IgG1 was PCR amplified with 5' primer 5'-GAGCATTTTCTGATGACAAAACACTCACACATGCCCA-3' and 3' primer

5'-TGATTAATGATCAATGAATTCTCATTACCCGGAGACAGGGA-3' to append *Eco*RI and *Bcl*I restriction sites to the 3' end of the human IgG1. The gene encoding human B7.1-Fc was produced by assembling the above PCR products with the 5' primer of B7.1 5'-TTCTCTA-GAATGGGCCACACACGG-3' and 3' primer of human IgG1 Fc 5'-TGATTAATGATCAATGAATTCTCATTACCCGGAGACAGGGA-3'. The fusion expression gene was produced by single-step insertion of the B7.1-Fc gene into *Xba*I and *Eco*RI sites in pEE12 vector, resulting in the expression vector pEE12/B7.1-Fc.

Expression and purification of B7.1-Fc. The B7.1-Fc fusion protein was expressed in NS0 murine myeloma cells for long-term stable expression according to the manufacturer's protocol (Lonza Biologics). The highest producing clone was scaled up for incubation in an aerated 3-L stir flask bioreactor using 5% heat-inactivated dialyzed FCS. The fusion protein was then purified from the filtered spent culture medium by sequential protein A affinity chromatography and ion exchange chromatography as described previously (30). Heat-inactivated (68°C for 1 hour) dialyzed FCS was used to prevent the induction of endogenous proteolytic enzymes and the subsequent cleavage of the fusion protein produced in the NS0 cells. The fusion protein was analyzed by SDS-PAGE to show proper assembly and purity.

T-lymphocyte proliferation assay. The bioactivity of the B7.1 moiety was measured by a flow cytometric method with CFSE. Briefly, a spleen was aseptically removed from a healthy BALB/c mouse and a single-cell suspension was isolated by gradient centrifugation. T cells were enriched from the mononuclear cell suspension by negative adhesion on an anti-mouse immunoglobulin antibody-coated Petri dish. Fluorescence-activated cell sorting (FACS) analysis confirmed the purity of T-cell population to be ~85%. After two washes in PBS, cells were incubated in 5 μ g/mL CFSE for 5 minutes at room temperature followed by the addition of prewarmed 20% fetal bovine serum to neutralize unbound CFSE. CFSE-labeled cells (2×10^6) were cultured in a 24-well plate, which was precoated with 1.5 μ g/mL B7.1-Fc and/or 1 μ g/mL anti-CD3 (clone 17A2). After a 72-hour incubation, 10^6 cells were collected and stained with phycoerythrin anti-CD4 and anti-CD8 antibodies for FACS analysis.

Depletion of lymphocyte subsets in vivo. Antibodies were injected on the day of tumor implantation (day 0) for CD25⁺ T-cell depletion or day 5 after tumor implantation for the depletion of other T-cell subsets (CD4⁺ and CD8⁺). To deplete these T-cell subsets, 0.5 mg anti-CD4 antibody (GK1.5), anti-CD8 antibody (H35), or anti-CD25 antibody (PC61) was injected i.p. using a 1 mL inoculum in PBS and repeated every 5 days thereafter. Depletion of specific T-cell subsets was confirmed by FACS analyses of lymph nodes of inoculated mice using antibody clones different from those used for depletion (data not shown).

Immunotherapy studies. Groups ($n = 5$) of 6-week-old female BALB/c mice were injected s.c. in the left flank with a 0.2 mL inoculum containing 5×10^6 Colon 26, RENCA, or MAD109 cells under a university animal care committee-approved protocol. Treatments were started when tumors reached 0.5 cm in diameter. Groups of tumor-bearing mice (with or without lymphocyte subset depletion) were treated i.v. with a 0.1 mL inoculum containing B7.1-Fc or isotype control antibody. B7.2-Fc was given to Colon 26-bearing mice using the same dose as B7.1-Fc to compare their antitumor effects. When the tumors reached 0.5 cm in diameter, all the groups were treated daily $\times 5$, and tumor growth was monitored every other day by caliper measurement in three dimensions for ~19 days after implantation. Tumor volumes were calculated by the formula: length \times width \times height. The results were expressed as the mean \pm SD, and the significance levels (P s) were determined using the Wilcoxon's rank-sum test. In addition, all *in vivo* experiments were repeated two or more times and found to be consistent in nature.

Rechallenge experiments. Three months after completion of the immunotherapy studies, tumor-free mice from previous studies and control naive mice ($n = 5$) were challenged with 10^6 cells of Colon 26 and MAD109 or Colon 26 and RENCA in the left and right flanks, respectively. The injection sites were observed for 3 months. Tumor

dimensions were measured weekly, and tumor volume was calculated as described above.

Immunotherapy studies in knockout mice and with interleukin-4 neutralization. Perforin and IFN- γ knockout mice were s.c. implanted with 5×10^6 Colon 26 tumor cells in the left flank and mice were depleted of CD25⁺ cells with i.p. injection of 0.5 mg PC61 on the same day of tumor implantation. Treatment began on day 5 when tumors reached 0.5 cm in diameter. For these studies, mice were divided into four groups: (a) isotype control antibody (40 μ g), (b) CD25 depletion, (c) B7.1-Fc (40 μ g), or (d) B7.1-Fc (40 μ g) plus CD25 depletion. All the groups were treated daily for 5 consecutive days, starting on day 5 after tumor implantation, and tumor volumes were monitored by caliper every other day as above. An IL-4-neutralizing study was done in 6-week-old BALB/c mice by i.p. injection of 0.5 mg of the anti-IL-4 antibody 11B11 every other day, starting at day 5 after tumor implantation. The schedules of B7.1-Fc treatment and combination treatment were the same as above. The concentration of IL-4 in the IL-4-depleted mouse and control mouse sera was determined using an ELISA kit purchased from Pierce Endogen (Rockford, IL).

T-cell proliferation in tumor-draining lymph nodes. The proliferation of T cells in tumor-draining lymph nodes (TDLN) was measured by CFSE vital staining. Briefly, TDLNs from control and treated mice were removed on days 14 and 21 after tumor implantation. Single-cell suspensions were obtained by mincing the lymph nodes in a Petri dish, and the cells were washed and labeled with CFSE as above. Tumor lysates obtained previously by four repeated freeze/thaw cycles and stored frozen at -80°C were thawed and centrifuged at 1,200 rpm as the source of tumor antigens. Single-cell suspensions (2×10^6 cells) were then plated into 24-well plates and aliquots of tumor lysate were added to a final concentration of 10 μ g/mL. Cells were collected at days 1, 2, 3, and 5 after plating and stained with phycoerythrin anti-CD3e. FACS analyses were done to determine the proliferation of T cells in the presence of tumor antigens.

Morphologic and immunohistochemical studies. Tumors from different groups were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E for histologic examination. To perform immunohistochemical staining, tumors were snap frozen in liquid nitrogen after immersion in OCT compound (Lab-Tek Products, Naperville, IL) for frozen sectioning. Cryostat sections (5 μ m) were cut, air-dried, and stored at -80°C until used. After incubation in PBS containing 10% hydrogen peroxide at room temperature for 30 minutes to block endogenous peroxidase activity, frozen sections were stained with biotinylated anti-CD4 or anti-CD8 monoclonal antibodies. Sections were then incubated with horseradish peroxidase-conjugated streptavidin and developed with 3,3'-diaminobenzidine (KPL, Gaithersburg, MD) before being counterstained with hematoxylin. Microscopic findings were recorded by an Optronix digital camera (Wetzlar, Germany) attached to a Leitz Orthoplan microscope.

Results

Construction, expression, and purification of B7.1-Fc. A schematic of B7.1-Fc and SDS-PAGE of the purified fusion protein under reducing conditions are shown in Fig. 1. The highest B7.1-Fc-producing cell subclone was screened by sandwich ELISA with monoclonal antibodies against human B7.1 or human IgG Fc portion. This subclone was found to produce >100 μ g/mL fusion protein in aerated stir flask cultures. The molecular weight, assembly, and purity of the fusion protein were shown by SDS-PAGE, which also confirmed its dimeric nature. The activity of the fusion protein (see below) was tested and shown to be preserved after storage at -20°C .

Bioactivity of B7.1 moiety of the fusion protein. To determine whether the B7.1 moiety of the fusion protein retained its

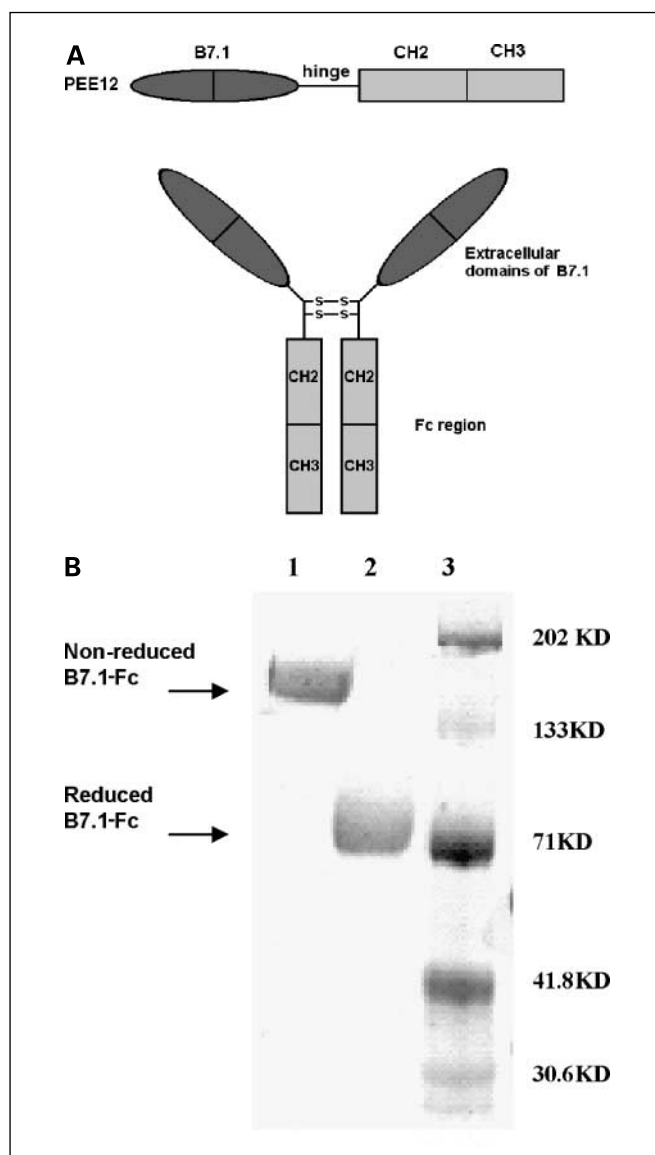
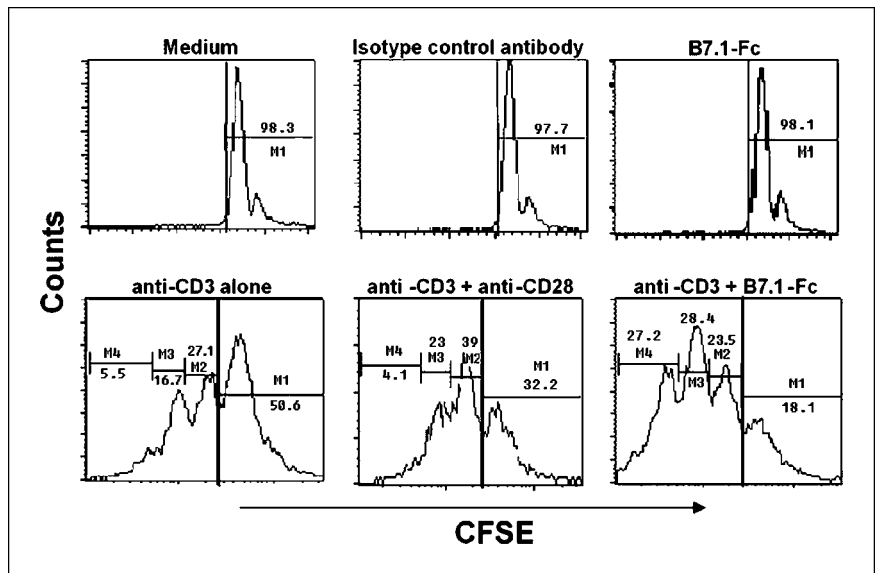


Fig. 1. A, schematic diagram of the construction and final assembly of the human B7.1-Fc fusion protein. B, electrophoretic analysis of the purified B7.1-Fc by SDS-PAGE. Extracellular domains of human B7.1 were fused to the NH₂ terminus of the Fc portion, including hinge, CH2, and CH3 of human IgG1 by PCR to produce a dimeric protein. Coomassie blue-stained 4% to 20% gradient SDS-PAGE gel showed nonreduced B7.1-Fc (1), reduced B7.1-Fc (2), and standard molecular weight markers (3).

biological activity, a mouse T-cell proliferation assay was done. Anti-CD28 antibody (37.51.3 clone) was used in the same concentration as a positive control because it has been shown to activate T cells in the presence of mitogens. As shown in Fig. 2, plate-bound B7.1-Fc enhanced T-cell proliferation dramatically in the presence of anti-CD3, whereas the fusion protein alone was inactive.

B7.1-Fc dosing studies in Colon 26 tumor model. The antitumor activity of the B7.1-Fc and B7.2-Fc was studied at different doses in tumor-bearing BALB/c mice using the Colon 26 tumor model. Five days after tumor implantation, groups of mice were treated i.v. daily $\times 5$ with doses of 40, 20, 10, 5, 1, or 0.5 μ g B7.1-Fc compared with 40 μ g isotype matched antibody control. As shown in Fig. 3A, at a concentration of 40 μ g/d, the

Fig. 2. Stimulation of T-cell proliferation by B7.1-Fc. CFSE-labeled murine T cells were cultured in 24-well plates precoated with anti-CD3, anti-CD3 + B7.1-Fc, anti-CD3 + anti-CD28, or B7.1-Fc alone. After 72-hour incubation, 10^6 cells were collected and CFSE intensity was analyzed by FACS.

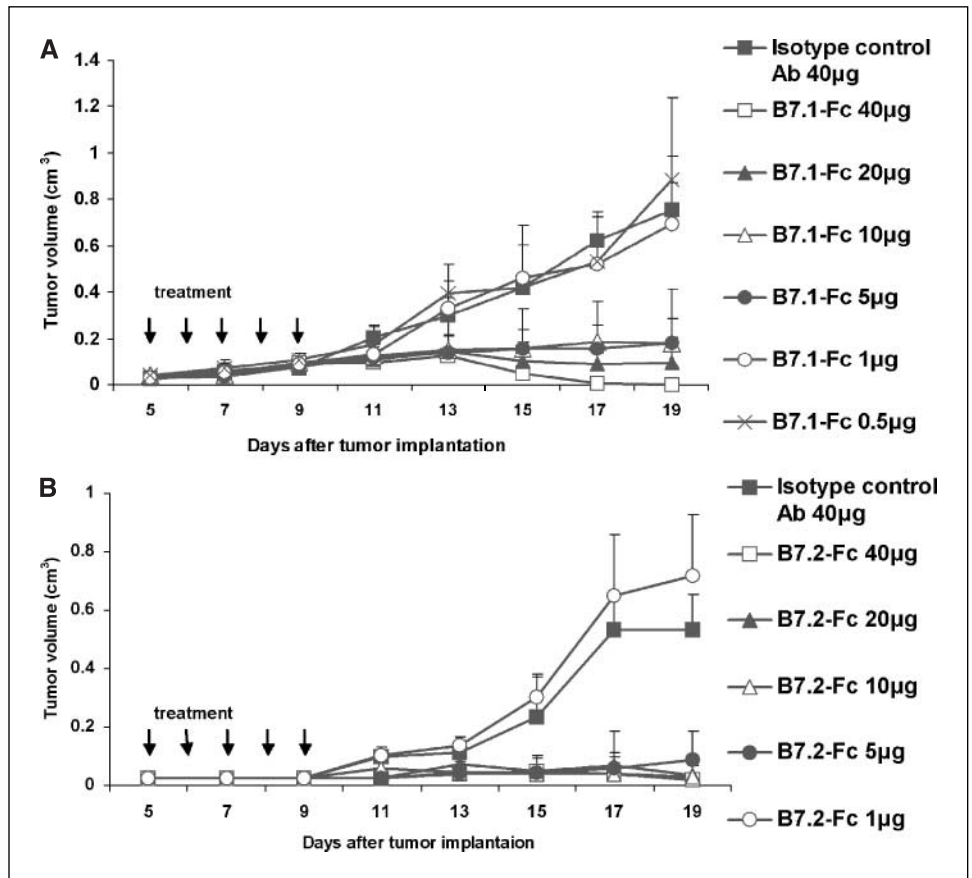


administration of B7.1-Fc induced complete regression of implanted tumors. Mice receiving B7.1-Fc treatment with doses ranging from 5 to 20 $\mu\text{g} \times 5$ also underwent tumor regression from 76% ($P < 0.01$) to 88% ($P < 0.01$) reduction of tumor volume compared with controls, respectively. When the dose was decreased to 1 μg , however, no significant reduction of tumor volume was observed in the treatment groups receiving B7.1-Fc or B7.2-Fc. B7.2-Fc treatment showed

similar results to B7.1-Fc as shown in Fig. 3B, showing compatible activities for these two costimulatory molecules. These studies showed a dose threshold of antitumor effect for B7.1-Fc and B7.2-Fc treatments in this tumor model with a dramatic difference in tumor growth observed between 1 and 5 $\mu\text{g}/\text{dose}$.

Combination B7.1-Fc immunotherapy and T-cell subset depletion. Tumor rejection has been proven to depend on a

Fig. 3. Dose response of B7.1-Fc (A) and B7.2-Fc (B) immunotherapy in Colon 26 tumor-bearing BALB/c mice. Antitumor effects of B7.1-Fc and B7.2-Fc are comparable because both fusion proteins show significant activity at $\geq 5 \mu\text{g}/\text{dose}$. $P < 0.01$, Wilcoxon's rank-sum test.



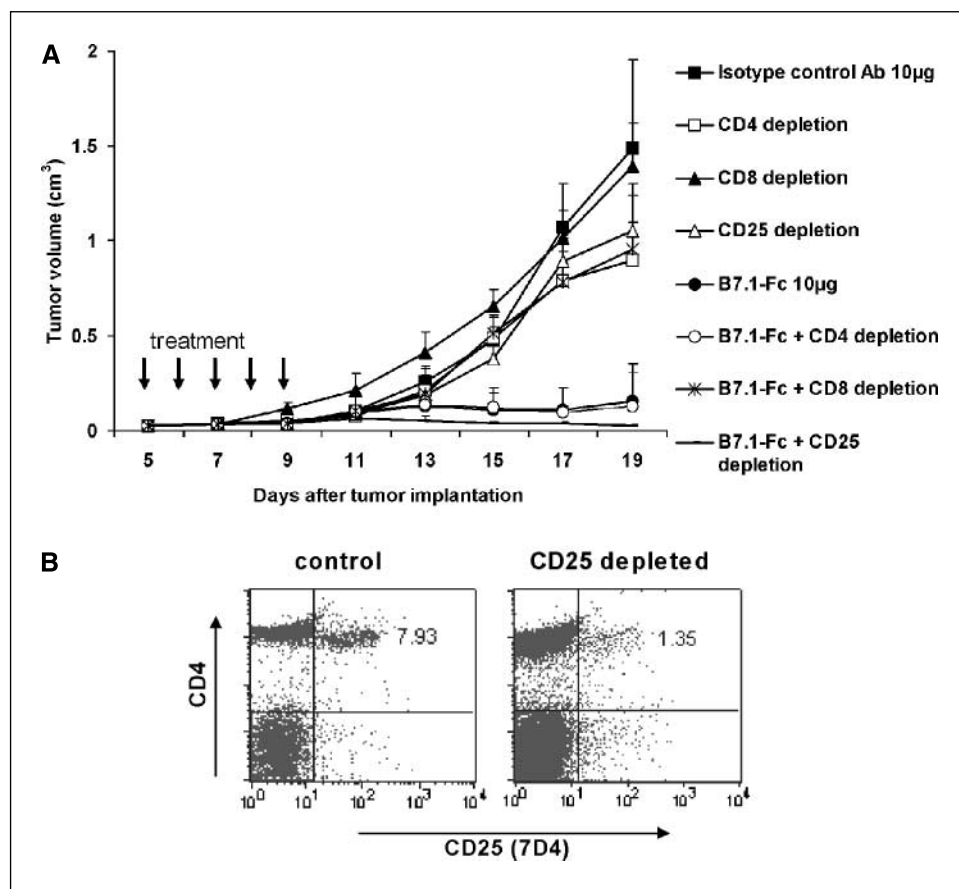


Fig. 4. Combination B7.1-Fc immunotherapy with CD4⁺, CD8⁺, or CD25⁺ T-cell depletion in Colon 26 tumor-bearing BALB/c mice. *A*, tumor growth curves in combination immunotherapy groups. Note that none of the mice in control treated groups showed tumor regression. 0.01 < *P* < 0.05, Wilcoxon's rank-sum test. *B*, FACS analysis of CD4⁺CD25⁺ subset in peripheral lymph nodes in control mice and 5 days after injection of 0.5 mg PC61.

cell-mediated immune response in which CD4⁺ helper T cells and CD8⁺ cytotoxic T cells are critical. To determine the roles of CD4⁺, CD8⁺, and CD25⁺ T cells in human B7.1-Fc immunotherapy, each of these populations was depleted during B7.1-Fc treatment. As shown in Fig. 4A, depletion of the CD8⁺ T-cell subset reversed the antitumor effects of B7.1-Fc, providing supporting data for the critical immunotherapeutic role of this subset. In contrast, CD4⁺ T-cell depletion did not affect the results of B7.1-Fc treatment. As shown in Table 1, both B7.1-Fc treatment alone and combination treatment with anti-CD4⁺ depletion were found to induce 80% (*P* < 0.01) of mice to undergo complete tumor regression by day 23 after tumor implantation. When combined with CD25⁺ T-cell depletion on day 0, B7.1-Fc treatment caused complete tumor remission of all the mice (*P* < 0.01), whereas CD25⁺ T-cell depletion alone did not result in significant tumor suppression (*P* < 0.05). No mice in the control groups were observed to undergo tumor regression. Depletion of CD4⁺CD25⁺ T-cell subsets by PC61 antibody was confirmed by FACS analysis of lymph nodes of normal mice (Fig. 4B).

Combination immunotherapy studies of B7.1-Fc in different tumor models. To determine if combination immunotherapy was also effective in other solid tumor models of the BALB/c mouse, two other solid tumors, RENCA and MAD109, were treated as above with B7.1-Fc and CD25⁺ depletion. As shown in Fig. 5, combination therapy with B7.1-Fc and CD25⁺ T-cell depletion produced the most profound tumor suppression in these tumor models (*P* < 0.05). Unlike combination immunotherapy in Colon 26 model shown in Fig. 4A, however, none

of the mice in these two models underwent complete tumor regression. Due to rapid growth of the tumors in the control groups, these mice were sacrificed by day 25 as specified by the university animal care committee-approved protocol.

Tumor rechallenge experiments. In the above immunotherapy studies using the Colon 26 tumor model, 100% of mice

Table 1. Combination B7.1-Fc immunotherapy with CD4⁺, CD8⁺, or CD25⁺ T-cell depletion in Colon 26 tumor-bearing BALB/c mice

Immunotherapy treatment	Depleted T-cell subset	% Tumor-free mice (complete regression)*
Isotype control antibody (10 µg/dose)	—	0
—	CD4	0
—	CD8	0
—	CD25	0
B7.1-Fc (10 µg/dose) [†]	—	80
B7.1-Fc (10 µg/dose) [†]	CD4	80
B7.1-Fc (10 µg/dose)	CD8	0
B7.1-Fc (10 µg/dose) [†]	CD25	100

*0.01 < *P* < 0.05, Wilcoxon's rank-sum test.

[†]Rechallenged experiments were done with these sets of mice.

receiving combination treatment of B7.1-Fc and CD25⁺ T-cell depletion underwent complete tumor regression with no observable regrowth over a 5-month period. By contrast, no tumor regression was observed in any of the control groups for these experiments. For tumor rechallenge studies, tumor-free (see Table 1) and naive mice were implanted with 10⁶ parental Colon 26 in the left flank and 10⁶ RENCA or MAD109 tumor cells in the contralateral flank. One month after tumor implantation, all of the naive mice were found to have tumors growing in their left (Colon 26) and right (RENCA or MAD109) flanks (Fig. 6), whereas the rechallenged mice rejected Colon 26 and RENCA tumors and only had small MAD109 tumors in the right flank. Caliper measurements of the MAD109 tumors showed that their size was reduced by ~90% compared with those growing in naive mice (data not shown).

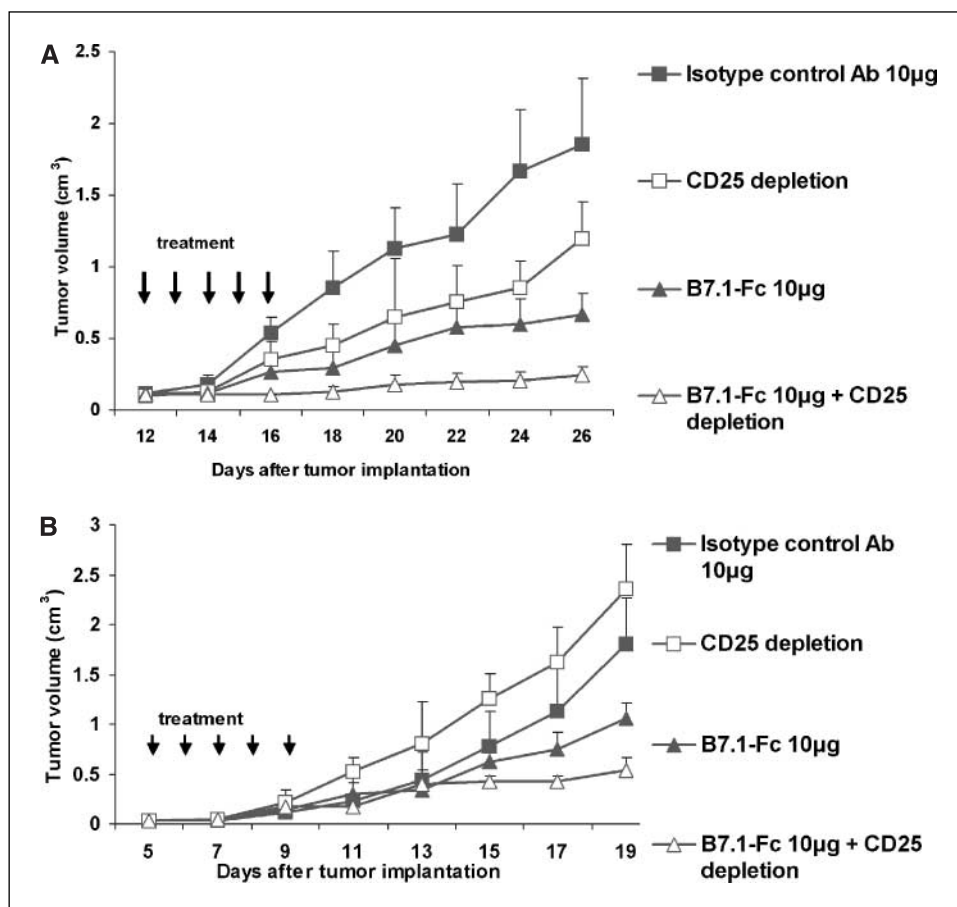
Tumor-draining lymph node T-cell proliferation. It has been proven that B7.1 expressed on antigen-presenting cells can induce T-cell proliferation by providing the required second signal. In fact, we frequently observed that TDLNs from B7.1-Fc treated mice were significantly larger than those from isotype control antibody-treated mice. To determine the effect of B7.1-Fc and CD25⁺ treatments on T-cell activation, a tumor-specific T-cell proliferation assay was done using the vital dye CFSE, which stains viable cells and decreases in fluorescence intensity with each division. Single-cell suspensions prepared from TDLNs were stained with CFSE and incubated with tumor lysates (from same tumor model) for 72 hours before the cells were stained with anti-CD3e for FACS analysis. As shown in

Fig. 7, control groups (isotype antibody group and CD25⁺ depletion group) did not show T-cell proliferation (0.06% and 0.22%, respectively). In contrast, the B7.1-Fc treatment groups (with and without CD25⁺ depletion) showed a dramatic decrease in fluorescence intensity of CFSE. The combination treatment group showed a moderately higher rate of proliferation by these methods.

Histologic studies. Light microscopic and immunohistochemical examination of tumors removed from B7.1-Fc-treated mice and control mice at different time points revealed increased levels of mononuclear cell infiltrations and CD4⁺ and CD8⁺ staining as shown in Fig. 8. It should be noted that tumors from B7.1-Fc-treated mice formed widespread multiple necrotic foci in both peritumoral and intratumoral areas. Such necrosis started early, increased in size with time, and finally led to the eventual disappearance of the tumors (slides at different time points not shown). This kind of tumor degeneration was distinguished from those in control tumors, which usually were found to have only one or two large necrotic areas in the central zones of tumors at advanced stages.

IFN- γ and perforin knockout mice studies and interleukin-4 neutralization. The observation that B7.1-Fc-induced tumor regression was dependent on CD8⁺ T cells provoked us to determine which components of the CD8⁺ CTL pathway were critical. To show this, the effect of B7.1-Fc treatment with and without CD25⁺ Treg depletion in mice lacking IFN- γ , a critical cytokine for CTL differentiation and function, or perforin, a downstream effector for natural killer and CTL function, was

Fig. 5. Combination B7.1-Fc immunotherapy and CD25⁺ T-cell depletion on the growth of RENCA (A) and MAD109 (B) solid tumors. Six-week-old female BALB/c mice were injected with rat anti-mouse CD25 (PC61) antibody on the day of tumor implantation and every 5 days thereafter. $P < 0.05$, Wilcoxon's rank-sum test.



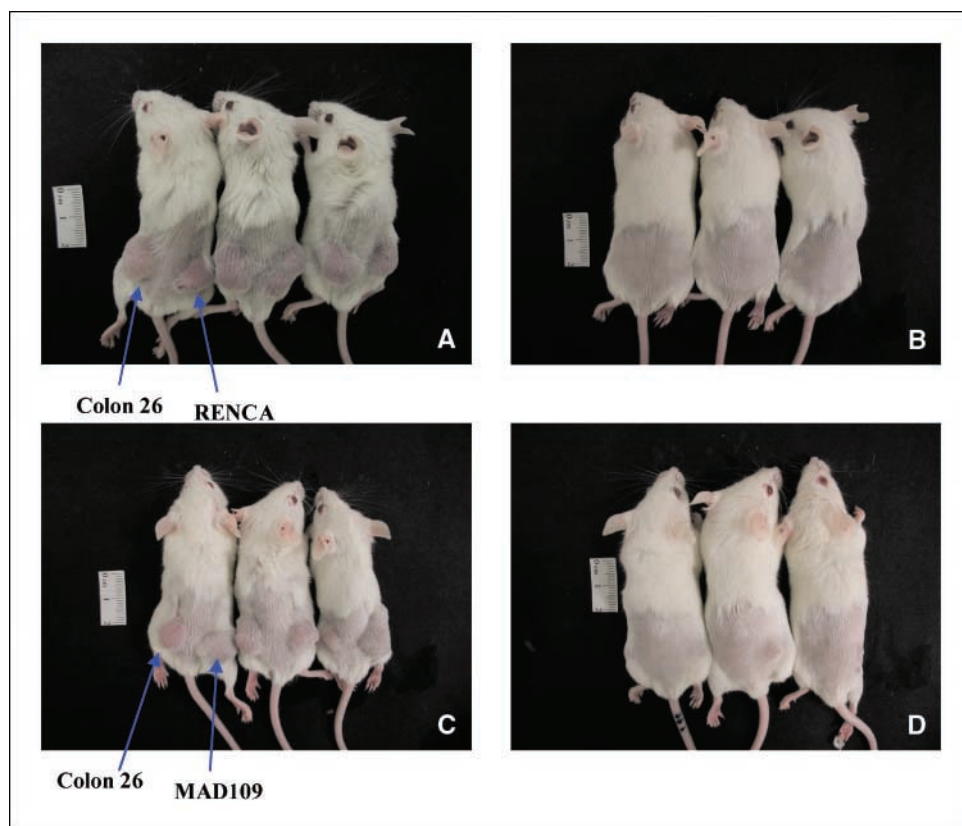


Fig. 6. Rechallenge experiments. *A* and *C*, naive mice (controls). *B* and *D*, tumor-regressed mice from previous studies. Mice in (*A* and *B*) were s.c. implanted with 10^6 Colon 26 cells on the left flank and 10^6 RENCA cells on the right flank. Mice in (*C* and *D*) were s.c. implanted with 10^6 Colon 26 cells on the left flank and 10^6 MAD109 cells on the right flank. Mice in (*B* and *D*) were treated with B7.1-Fc and CD25⁺ depletion in previous studies and remained tumor-free for 3 months before beginning rechallenge experiments.

examined. Figure 9A shows that IFN- γ -deficient mice failed to reject tumors in B7.1-Fc-treated groups, whereas perforin-deficient mice still underwent 50% and 75% reductions in tumor size in B7.1-Fc ($P < 0.05$) and combination treatment ($P < 0.05$) groups, respectively, as shown in Fig. 9B. To test the roles of IL-4 in B7.1-Fc immunotherapy, IL-4 was neutralized by i.p. injection of the anti-mouse IL-4 antibody 11B11. As shown in Fig. 9C, IL-4 neutralization did not alter the antitumor efficacy of B7.1-Fc treatment or anti-Treg combination therapy.

Discussion

In the present study, we describe the construction and characterization of a soluble human B7.1 fusion protein (B7.1-Fc) for the immunotherapy of solid tumors. Some investigators have reported on the antitumor effects of murine B7.2-Ig (B7.2-Fc) when given alone or as an adjuvant in mouse tumor models, but much less has been known about the *in vivo* potential of B7.1-Fc (12–15). The fact that the human B7.1/B7.2 functionally bound to their murine counterreceptors facilitated our studies with murine models. Before embarking on detailed animal studies, we confirmed that human B7.1-Fc could bind to murine T cells and stimulate their proliferation (data not shown). The present studies are the first comprehensive immunotherapy studies of human B7.1-Fc in murine models. We opted to study the B7.1 costimulatory ligand in more detail than B7.2 because it has been shown previously that B7.1 was more potent than B7.2 in inducing CTL activity and favored differentiation along the Th1 pathway (31, 32).

Our *in vitro* and *in vivo* data suggest that human B7.1-Fc can efficiently stimulate an antitumor immune response and induce tumor regression in different mouse tumor models. There have been many approaches involving B7.1 in cancer

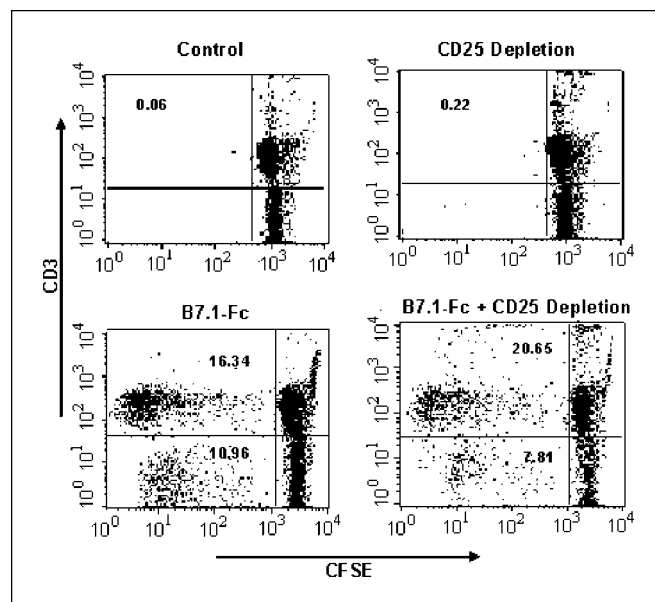


Fig. 7. FACS analysis of T-cell proliferation in TDLNs removed from tumor-bearing mice on day 21. Single-cell suspensions were labeled with 5 $\mu\text{mol/L}$ CFSE and incubated with tumor lysate for 72 hours before staining with phycoerythrin anti-CD3e.

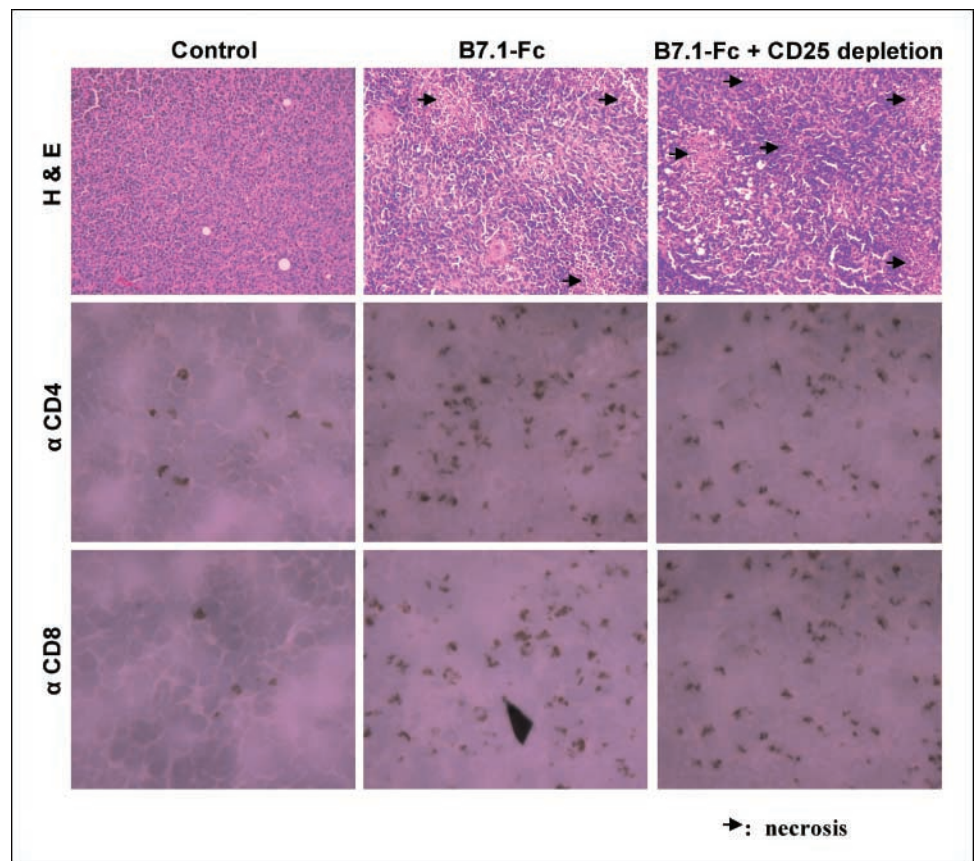


Fig. 8. H&E and immunohistochemical staining of tumors from control and B7.1-Fc-treated mice. Colon 26 tumors were harvested 5 days after completion of treatment and show widespread intratumoral necrosis only in tumors from B7.1-Fc treated mice. Frozen sections of each tumor mass were stained with anti-CD4 and anti-CD8 antibodies. Original magnification, $\times 150$ (H&E) and $\times 400$ (immunohistochemistry). Arrow, necrosis.

immunotherapy other than its use as an Fc fusion protein. These other approaches, including vaccination with B7.1-transfected tumor cells, have only met limited success in treating tumor models (3, 4). The relative ineffectiveness of tumor cells transfected with B7.1 compared with injection of soluble B7.1-Fc in the present study may reflect functional differences between membrane-bound B7.1 and soluble B7.1 (3, 12). Several mechanisms may be involved in the antitumor efficacy of soluble B7.1-Fc. B7.1 has two known receptors. One is CD28, which when engaged by B7.1 triggers a stimulatory signal to activate naive T cells (4). Conversely, engagement of its counterreceptor, CTLA-4, triggers a negative signal to terminate T-cell activation (33). Membrane-bound B7.1 on transfected tumor cells or antigen-presenting cells might not only cross-link CD28 but also cross-link CTLA-4 to trigger a negative signal. In contrast, because B7.1 has a high affinity to CTLA-4, soluble B7.1-Fc may block CTLA-4 signaling instead of cross-linking CTLA-4, thereby sustaining the activation of tumor-specific T cells or preventing their down-regulation (12). In addition, because the stimulation of activated T cells recognizing tumor antigens requires less costimulation, cross-linking of CD28 may be less important than blocking CTLA-4 (34). At this point, soluble B7.1-Fc might have a comparable function to CTLA-4 blocking antibody, which has significant antitumor effects in tumor models (6). Because B7.1-Fc was found to be capable of activating T cells *in vitro* and *in vivo*, it may have dual functions of both triggering stimulatory signals and blocking negative signals. This may provide an explanation of a more effective antitumor response using soluble B7.1-Fc versus B7.1-transfected tumor vaccines. Moreover,

Grohmann et al. (35) reported that soluble CTLA-4-Ig treatment results in elevated levels of the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase in dendritic cells. These data indicate that CTLA-4 engagement of its ligands on dendritic cells promotes inhibitory functions in dendritic cells, whereas in our study CTLA-4 blocking by soluble B7.1-Fc may also protect dendritic cells from negative signals. This might be another explanation for the observed tumor regression induced by B7.1-Fc.

Classically, it has been shown that highly responsive CD8⁺ memory T cells are dependent on CD4⁺ T helper cells by secreting cytokines, such as IL-2 and IL-4. Our results, however, show that CD4⁺ helper T cells are not required for the induction of effective immunotherapy (Fig. 4; Table 1). Consistent with these findings, Di Rosa and Matzinger (36) have likewise shown that tumor immunotherapy by CD8⁺ effector cells is independent of CD4⁺ helper T cells. In addition, Kaech and Ahmed (37) have shown that CD4 help with memory CD8⁺ T-cell responses (i.e., responses between "helped" and "unhelped" memory CD8 T cells in a physiologic setting of infection) are different in quantity but not in quality. The exact mechanism(s) of activation CD8⁺ T cells and the generation of CD8⁺ memory T cells (long-term protection) are currently unknown, but it seems that CD4⁺ cells are not required.

In a separate publication,¹ our laboratory reported recently on the antitumor effects of tumor-targeted B7.1 using the fusion protein B7.1/NHS76, where NHS76 is a tumor-targeted

¹A. Liu et al. B7.1/NHS76: a new costimulator fusion protein for the immunotherapy of solid tumors, submitted for publication.

antibody that binds to necrotic cores of solid tumors (38). Tumor targeting with immunomodulatory molecules is an attractive strategy to enhance the host's antitumor response, but success with them has been limited to date. Challita-Eid et al. found that although B7.1 anti-HER-2/*neu* fusion protein retains antibody specificity and ability to activate via the T-cell costimulatory pathway, there was little *in vivo* antitumor

response (39, 40). Our results indicate that although B7.1/NHS76 is more effective than B7.1-Fc in costimulating T-cell proliferation *in vitro*, B7.1-Fc is a much more efficient antitumor agent *in vivo*. The enhanced antitumor effects of B7.1-Fc may be due to two reasons. First, although B7.1-Fc is not a targeted molecule, it could be trapped in TDLNs where B7.1-Fc efficiently activates T-cell proliferation by binding to its

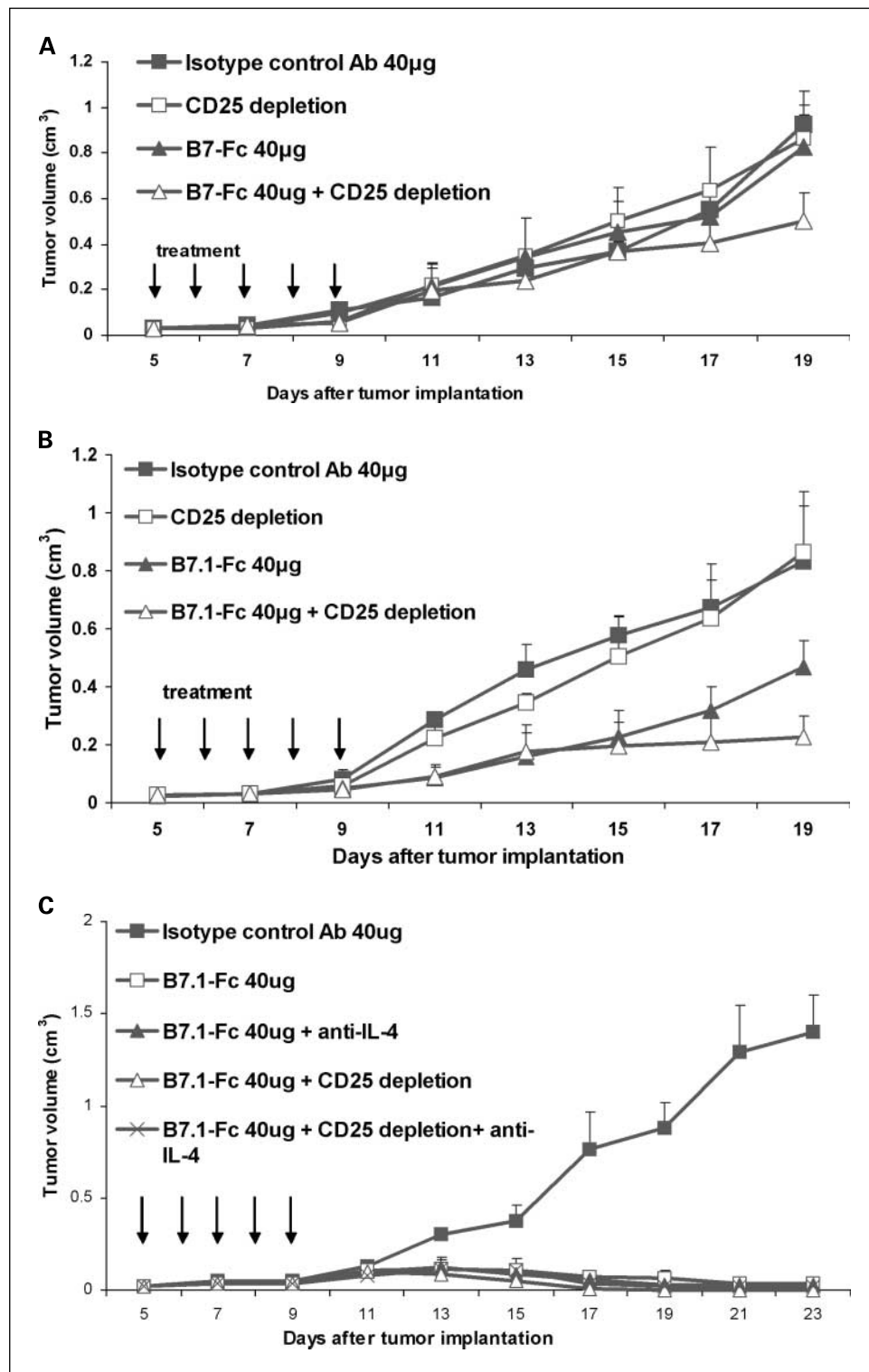


Fig. 9. Combination immunotherapy in IFN- $\gamma^{-/-}$ (A), perforin $^{-/-}$ (B), and IL-4-neutralizing (C) antibody-treated mice. In these studies, CD25 $^{+}$ cells were depleted on the same day as tumor implantation. The antitumor effects of B7.1-Fc were not affected by IL-4 neutralization but were abrogated completely in IFN- γ knockout mice and partially in perforin knockout mice. $P < 0.05$, Wilcoxon's rank-sum test.

counterreceptors on T cells. As shown in Fig. 7, B7.1-Fc treatment induced dramatic proliferation of T cells in TDLNs when stimulated with tumor lysate, whereas B7.1/NHS76 did not share this property possibly because most of the B7.1/NHS76 was targeted to the tumor site. With the exception of TDLNs, other lymphoid tissue did not show T-cell proliferation, possibly because they lacked tumor specific antigen-presenting cells.² TDLNs are very important for tumor immunity because they are sites where effector cells are activated and thereafter undergo clonal expansion with the help of tumor-specific antigen-presenting cells (41). As a reservoir of activated effector T cells, especially CD8⁺ T cells, TDLNs can continue providing effector cells to the tumor site (42). Secondly, B7.1-Fc may block the inhibitory CTLA-4 signaling, which is important because CTLA-4 has a 20-fold higher affinity for B7.1 than CD28 (43). Previous reports described the successful therapeutic treatment of established murine tumors with antibody blocking CTLA-4, which induced the regression of strongly immunogenic tumors (44). B7.1-Fc may also work through blocking CTLA-4 or may exert a dual function in cross-linking CD28 and blocking CTLA-4, a function-targeted B7.1/NHS76 or anti-CTLA-4 may not be able to do.

As shown in Fig. 3, the antitumor effects of B7.1-Fc are comparable with that of B7.2-Fc at the same doses. The mechanism of action of B7.1-Fc, however, may be different from that of B7.2-Ig to some extent. Yamaguchi et al. (17) reported that B7.2-Ig-mediated tumor regression was dependent on type 2 CD8⁺ T cells and IL-4 but not IFN- γ . They showed that B7.2-Ig-induced tumor regression was not observed in IL-4^{-/-} or STAT6^{-/-} mice that were deficient in the development of type 2 T-cell responses, whereas B7.2-Ig treatment was still effective in IFN- γ ^{-/-} or STAT4^{-/-} mice in which type 1 T-cell differentiation is impaired. In our studies, although we also observed that the antitumor effect of B7.1-Fc treatment was CD8⁺ T-cell dependent, the antitumor efficacy was dependent on IFN- γ and perforin but not IL-4. Differences in the activity profiles of B7.1 and B7.2 ligands have been controversial and our data provide proof that B7.1 is more type I oriented. The origin (human versus murine) and/or isotype (IgG1 versus IgG2a) difference of Fc portions may also play a role here because the avidity and signal transduction of Fc

receptors on murine macrophages may be different between these two fusion proteins and thereby induce different functions (45).

T cells, especially CD8⁺ CTLs, are major effector cells in tumor immunotherapy. In our study, B7.1-Fc induced dramatic increases in the infiltration of T cells not only in quantity but also in their proliferation status as seen in the TDLN studies shown in Figures 7 and 8. It was observed in studies that TDLNs from B7.1-Fc-treated mice with and without Treg depletion were significantly bigger in size than those from control mice. In addition, tumors from B7.1-Fc-treated mice showed significant widespread cell death in the tumor, which was different than that seen in control-treated mice usually found to have one or two large areas of central necrosis. These phenomena further support the notion that effector T cells caused the observed tumor regression in B7.1-Fc-treated mice.

Tregs include a variety of subpopulations of cells that have important regulatory functions in immune responses. Naturally occurring Tregs (CD4⁺CD25⁺ cells) have been most intensively investigated (19–23). In our studies, we found that combination treatment with CD25⁺ cell depletion significantly increased the antitumor efficacy of B7.1-Fc in therapeutic experiments. These results are consistent with those of two tumor-targeted fusion proteins generated in our laboratory, B7.1/NHS76 (36) and LEC/chTNT-3 (28), but not in other cytokine fusion proteins (28). Treg depletion alone did not result in highly significant tumor regression, which contrasts with the observations of Sakaguchi (20), who used different tumor models than reported here.

In summary, a B7.1-Fc fusion protein was constructed, expressed, purified, and examined for its potent antitumor activities. The B7.1-Fc fusion protein seems to be an effective antitumor agent when used alone or in combination with methods to deplete Treg cells. Its potency in stimulating immune responses, its human origin, and its ability to induce complete tumor regression of experimental solid tumors together suggest that clinical studies may be warranted.

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² Unpublished observations.

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Combination B7-Fc Fusion Protein Treatment and Treg Cell Depletion Therapy

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