

B7-2 Expressed on EL4 Lymphoma Suppresses Antitumor Immunity by an Interleukin 4-dependent Mechanism

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Summary

For T cells to become functionally activated they require at least two signals. The B7 costimulatory molecules B7-1 and B7-2 provide the "second signal" pivotal for T cell activation. In this report, we studied the relative roles of B7-1 and B7-2 molecules in the induction of antitumor immunity to the T cell thymoma, EL4. We generated EL4 tumor cells that expressed B7-1, B7-2, and B7-1+B7-2 by transfecting murine cDNAs. Our results demonstrate that EL4-B7-1 cells are completely rejected in syngeneic mice. Unlike EL4-B7-1 cells, we find that EL4-B7-2 cells are not rejected but progressively grow in the mice. A B7-1- and B7-2-EL4 double transfectant was generated by introducing B7-2 cDNA into the EL4-B7-1 tumor line that regressed in vivo. The EL4-B7-1+B7-2 double transfectant was not rejected when implanted into syngeneic mice but progressively grew to produce tumors. The double transfectant EL4 cells could costimulate T cell proliferation that could be blocked by anti-B7-1 antibodies, anti-B7-2 antibodies, or hCTLA4 immunoglobulin, showing that the B7-1 and B7-2 molecules expressed on the EL4 cells were functional. In vivo, treatment of mice implanted with double-transfected EL4 cells with anti-B7-2 monoclonal antibody resulted in tumor rejection. Furthermore, the EL4-B7-2 and EL4-B7-1+B7-2 cells, but not the wild-type EL4 cells, were rejected in interleukin 4 (IL-4) knockout mice. Our data suggests that B7-2 expressed on some T cell tumors inhibits development of antitumor immunity, and IL-4 appears to play a critical role in abrogation of the antitumor immune response.

Key words: T lymphoma • B7-2 • immune suppression • interleukin 4 • interleukin 10

The activation of T lymphocytes for optimal proliferation and induction of effector functions requires at least two distinct signals (1). The antigen-specific "first signal" is delivered by the engagement of the T cell antigen receptor with the peptide-MHC on APCs. The "second signal," or costimulatory signal, is antigen independent and provided by soluble factors, such as IL-2, or through a set of surface molecules expressed on professional APCs (2). T cell antigen receptor signaling by antigen without proper costimulation may induce a state of antigen-specific unresponsiveness termed anergy (3). Of the known costimulatory molecules expressed on APCs, the B7 family delivers the most potent costimulatory signal. Two B7 molecules have been identified: B7-1 (CD80; 4) and B7-2 (CD86; 5, 6), both belonging to the Ig gene superfamily (7). Although these two proteins share the ability to costimulate T cells through CD28, they show only 25% identity to each other (6, 8). Both B7-1 and B7-2 are usually found on APCs such as dendritic cells and B lymphocytes and can also be

expressed on T cells. They often show differences in temporal expression after activation, with B7-2 appearing first and B7-1 later (9, 10). The B7 molecules interact with CD28 and CTLA4 receptors that are expressed on T cells. Studies with CTLA4-Ig, a soluble fusion protein of the extracellular domain of CTLA4 and the Fc portion of an IgG1 molecule, indicate that both B7 family members bind to CTLA4 with a 20-200-fold greater affinity than to CD28 (11, 12). CD28 is the most important costimulatory receptor constitutively expressed on the surface of T cells. Ligation of CD28 by B7-1 or B7-2 results in T cell activation and induction of effector function (13). CTLA4 shows a 31% identity in amino acid sequence to CD28 and appears on the cell surface after B7 engages CD28 (14). In contrast to CD28, CTLA4 appears to be a negative regulator of T cell activation (15), indicating that the B7 pathway may also negatively affect T cell responses (16).

The majority of tumor cells are of low immunogenicity and generally lack expression of costimulatory molecules

(17). According to the two-signal hypothesis of T cell activation, absence of B7-CD28 interaction would result in T cell anergy due to the lack of costimulation. This leads to the idea that if tumor cells could be made to express costimulatory molecules, they would become better APCs and generate tumor-specific CTLs, promoting tumor regression. By transfection of the murine B7-1 gene into different tumor cells, protective and sometimes curative immunity against wild-type tumors has been induced in several mouse models (17, 18). The protective effects appear to vary depending on the tumor system studied. In particular, the immunogenicity of the tumor is one of the factors that determines the success of B7-1-mediated tumor regression (19).

Since B7-2 was discovered, the effects of B7-2 on the induction of antitumor immunity have been studied and compared with those of B7-1. More recently, B7-2 has been transfected into a number of tumor cell lines with differing results. Although B7-2 expression on the tumor cell surface induces rejection of BSC-1 (20) and P815 mastocytoma cells (21), it was found to be ineffective with EL4 thymoma cells (22) and MCA 102 fibrosarcoma cells (21). Matulonis et al. have reported that B7-1 is superior to B7-2 in its capacity to provide protection to wild-type leukemia cell challenge and in its ability to regress existing tumors (23). Gajewski has also reported that B7-1-, but not B7-2-transfected P815 cells, were able to generate alloantigen-specific CTL activity and to costimulate proliferation of CD8⁺ lymphocytes in the presence of low doses of anti-CD3 mAb (24).

We have investigated the roles of B7-1 and B7-2 on the immune system utilizing B7-1- and B7-2-transfected (B7-1-EL4 and B7-2-EL4) and B7-1+B7-2 double-transfected EL4 (B7-1+B7-2-EL4) cells in an *in vivo* tumor model. In this report, we describe the role of B7 costimulatory molecules B7-1 and B7-2 in the induction of the antitumor response. We have previously demonstrated that EL4-B7-1 cells are rejected in syngeneic C57BL/6 mice and that EL4-B7-2 cells produce tumors that grow as well as the wild-type EL4 (EL4-wt)¹ tumors (22). In this report, we show that B7-1+B7-2 double-transfected EL4 cells were not rejected but progressively grew to produce tumors. Our data suggests that the effects of B7-2 are dominant in that, when expressed on EL4 tumors along with B7-1, B7-2 inhibits the development of antitumor immunity. Suppression of the antitumor immune response by B7-2 on the EL4 cells was IL-4-dependent because IL-4-deficient mice rejected B7-2-expressing EL4 cells.

Materials and Methods

Mice. Female C57BL/6 mice 6–8 wk of age were purchased from Harlan Sprague Dawley, Inc. Female C57BL/6 (nu/nu) mice were purchased from Taconic Farms, Inc. IL-4 and IL-10 gene-disrupted C57BL/6 mice were purchased from The Jackson Laboratory. The mice were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical

School and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, as stated in the Department of Health and Human Services publication 85-23 (revised 1985).

Cell Lines. The tumor cell lines used in this study were obtained from the American Type Culture Collection (ATCC). The carcinogen-induced lymphoma EL4 is of C57BL/6 (H-2^b) origin. The EL4-B7-1 cells were provided by Dr. James Allison (University of California, Berkeley, CA). EL4-B7-2 cells were generated by introducing murine B7-2 cDNA into a pCDM8 vector by electroporation into EL4-wt cells (ATCC) as described (22). The transfectants were selected in the presence of 1 mg/ml G418 (Life Technologies). In another series, EL4-B7-1 cells (obtained from Dr. James Allison) were transfected with murine B7-2 cDNA to produce EL4-B7-1+B7-2 as described above. These transfectants were selected in 800 µg/ml hygromycin B (Boehringer Mannheim). The mock transfectant EL4 line (EL4-neo/hygro) was derived similarly but transfected only with the genes for the selection antibiotics (G418 and hygromycin B). After 3–4 wk, cells growing in the presence of the drugs were sorted for B7-2-expressing cells with anti-mB7-2 mAb (GL1) using a Becton Dickinson FACS VantageTM cell sorter. The B7-2-expressing cells were then subcloned and clones showing stable B7-2 expression were used in the experiments. The clones were screened for B7 expression once per week. All cell lines were maintained at 37°C in 10% CO₂ in DMEM (Life Technologies) containing 10% FCS (Fetalclone I; Hyclone), 1 mg/ml G418 (Life Technologies), and/or 800 µg/ml hygromycin B (Boehringer Mannheim).

Antibodies. Anti-B7-1 antibody (1G10) was provided by Dr. Nasrin Nabavi (Hoffmann-LaRoche Research Center, Nutley, NJ; 28). Anti-B7-2 antibody (GL1) was obtained from the GL1 hybridoma line (ATCC). The antibodies were purified from ascitic fluid on protein G columns (LKB/Pharmacia). The anti-B7-1 and anti-B7-2 antibodies are both of rat IgG2a isotype. The anti-murine CD3 hybridoma line 145-2C11 was provided by Dr. Jeffrey Bluestone (Ben May Institute, University of Chicago, Chicago, IL). Human CTLA4-Ig was provided by Dr. Peter Linsley (Bristol Myers Squibb, Seattle, WA).

Flow Cytometry. Spleen cells and tumor cells from mice or those growing in culture were harvested and washed three times with cold 1% BSA/PBS, pH 7.2, and then incubated with either supernatant from antibody-producing hybridomas or purified antibody (5 µg/ml) diluted in 1% BSA/PBS for 30 min at 4°C. The cells were then washed two to three times with the 1% BSA/PBS solution before incubating with FITC- or PE-conjugated goat anti-mouse Ig, goat anti-rat Ig, or goat anti-human Ig secondary antibodies (Zymed). The secondary antibodies were diluted 1/50 in 1% BSA/PBS and then incubated with the cells for 30 min at 4°C in the dark. After this incubation, the cells were washed three times with PBS and then fixed with an equal volume of 1% paraformaldehyde/PBS solution. Analysis was performed using a FACScanTM (Becton Dickinson). FITC- and PE-conjugated antibodies used for direct staining were obtained from PharMingen and included the following: anti-B7-1-FITC (clone 16.10A1), anti-B7-2-PE (clone GL1), and anti-CD95L-PE (clone KAY-10). For binding studies with hCTLA4-Ig, PE-conjugated goat F(ab')₂ anti-human IgG (Southern Biotechnology, Inc.) was used for indirect staining.

Costimulation Assay. T lymphocytes freshly isolated from C57BL/6 mouse spleen cells were positively selected using MicroBeads bound with anti-Thy1.2 (CD90) on an appropriate column (MACS; Miltenyi Biotec). This purification method yielded

¹Abbreviation used in this paper: wt, wild-type.

>95% enriched T cells. The positively selected T lymphocytes were used in costimulation assays as responder cells at a concentration of 10^5 cells/50 μ l/well of 96-well tissue culture plates (Costar Corp.). Untransfected and transfected EL4 cells were treated overnight with 40 μ g/ml mitomycin C (Sigma Chemical Co.) and then washed thoroughly and added as stimulator cells at 10^5 cells/25 μ l/well. Supernatant from anti-CD3-secreting hybridoma 145-2C11 cultures was added at 1/100 final dilution/100 μ l/well. In some experiments, anti-B7-1 antibody, anti-B7-2 antibody, or hCTLA4-Ig was added at a final concentration of 2 μ g/ml per 100 μ l/well for blocking in vitro costimulation. The plates were then incubated at 37°C in a humidified CO₂ incubator for 48 h. The plates were then pulsed with 1 μ Ci of ³H-TdR per well for 16 h and harvested using a Tomtec Mach II 96 cell harvester and counted on a 1205 Betaplate liquid scintillation counter (Wallac, Inc.).

Generation of T Cell Lines. To generate long-term T cell lines specific for each of the EL4 tumor cells, spleen cells ($0.5\text{--}1 \times 10^6$ /well) from mice implanted with each of the tumors were removed on days 10 to 12 and stimulated with the corresponding mitomycin C-fixed (40 μ g/ml overnight) EL4 cells (EL4-B7-1, EL4-B7-2, or EL4-B7-1+B7-2 cells; 2×10^5 cells/well) together with gamma-irradiated (5,000 rads) syngeneic spleen cells. The T cells were restimulated every 12–14 d with the corresponding mitomycin C-treated tumor cells that had been used to stimulate them in vivo, resulting in the generation of long-term T cell lines. The T cell lines were grown in DMEM supplemented with sodium pyruvate, l-glutamine, penicillin, streptomycin, gentamycin sulfate, nonessential amino acids (0.1 mM), MEM vitamin mixture (1 \times ; BioWhittaker), asparagine (0.1 mM), folic acid (0.1 mg/ml), 2-ME (5×10^{-5} M; Sigma Chemical Co.), 10% FBS (Hyclone), and 2% T cell growth factor (T-STIM; Collaborative Biomedical Products). The T cell lines thus generated were tested for cytotoxicity, cytokine production, and in vivo functional effects on tumor growth.

⁵¹Cr Release Assay to Test Cytolytic Activity. Effector cells were harvested, washed, and adjusted to 10^6 cells/ml, and varying numbers of effector cells (Ficoll-purified if necessary) were added to 5×10^3 ⁵¹Cr-labeled target cells in 150 μ l of culture medium in 96-well v-bottomed plates. After a 4-h incubation, 50 μ l culture supernatant was collected and measured in a gamma counter. The mean percentage specific lysis of triplicate wells was calculated as follows: % specific lysis = [(cpm experimental release – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release)] \times 100. The spontaneous release of the ⁵¹Cr-labeled target cells was <20% in all experiments.

In Vitro Cytokine Assay. Supernatants were collected from T cells 40 h after activation in vitro with the corresponding EL4 cell line in the presence of syngeneic spleen cells as APCs (see described generation of T cell lines). The concentrations of IL-2, IL-4, IL-10, IFN- γ , and TNF- α were measured by quantitative capture ELISA according to the guidelines of the manufacturers (PharMingen). In brief, purified rat mAb to mouse IL-2 (clone JES6-1A12), IL-4 (clone BVD4-1D11), IL-10 (clone JES5-2A5), IFN- γ (clone R4-6A2), and TNF- α (clone MP6-XT22) were obtained from PharMingen and used to coat ELISA plates (Immulon 4; Dynatech Laboratories, Inc.). Recombinant mouse cytokines (IL-2, IL-4, IL-10, IFN- γ , and TNF- α ; PharMingen) were used to construct standard curves, and biotinylated rat mAb to mouse IL-2 (clone JES6-5H4), IL-4 (clone BVD4-24G2), IL-10 (clone SXC-1), and IFN- γ (clone XMG1.2; all PharMingen) were used as the second Ab. Detection of TNF- α was performed with biotinylated polyclonal rabbit IgG (PharMingen). Plates were developed with

TMB microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Inc.) and read after the addition of stop solution at 450 nm using a microplate reader (model 3550; Bio-Rad Laboratories).

Animal Studies. C57BL/6 syngeneic mice were prepared for intradermal EL4 cell implantation by shaving their hind flank regions followed by depilation with Nair (Carter Wallace, Inc.) 24 h before intradermal implantation of tumor cells. EL4-wt and transfected EL4 tumor cells were harvested in log phase growth from tissue culture flasks and washed four times with PBS (Bio-Whittaker) and resuspended at 4×10^7 cells/ml in PBS for implantation. Each intradermal injection consisted of 2×10^6 cells in 50 μ l PBS and was performed using a 1-ml syringe fitted with a 27-gauge needle. 5–7 d after implantation, a tumor could be observed at the implantation site. The mice were scored for tumor growth three times per week and tumor size was documented by direct measurement in three perpendicular directions using a Max-Cal caliper (Cole Parmer Instrument Co.) and a plastic ruler. The experiments were terminated when the tumors reached 20–22 mm in diameter, if severe ulceration and bleeding had developed, or the mice had died. The measurements were recorded as tumor volumes (mm³) from groups of five mice each. For blocking of the B7 pathway in vivo, mice were injected with 2×10^6 EL4-B7-1+B7-2 cells that had been premixed with 50 μ g of murine anti-B7-1 or anti-B7-2 antibodies for 10 min at 4°C. Mice were treated with anti-B7 antibody every other day for 20 d by i.p. injection at 150 μ g/mouse following tumor implantation. Tumor growth was measured every other day.

Adoptive Transfer of Spleen Cells and T Cells. To test the biological effects of the T cells on tumor growth, a T cell line generated from EL4-B7-1+B7-2-bearing mice (5×10^6 /mouse) were intravenously injected into EL4-B7-1-bearing mice 24 h after tumor implantation. To test the direct effect of the unmanipulated spleen cells from mice (4×10^6 cells/mouse) that were bearing EL4-B7-2 tumors for 12 d, spleen cells from these mice were also intravenously injected into EL4-B7-1-bearing mice 24 h after tumor implantation. The control group received either PBS or 4×10^6 spleen cells from normal C57BL/6 mice intravenously.

Results

Expression of Murine B7 Molecules on EL4 Tumor Cell Lines. The wild-type tumor, the vector-only-transfected EL4, EL4-B7-1, and EL4-B7-2, and the double transfectant EL4-B7-1+B7-2 were screened for surface expression of B7-1, B7-2, and CD95L. B7-1+B7-2 double transfectants were generated by retransfection of EL4-B7-1 cells with B7-2 cDNA. Cell surface expression was determined by flow cytometry after indirect immunofluorescent staining using specific mAbs to B7-1 (16.10A1), B7-2 (GL1), or the soluble ligand hCTLA4-Ig, which binds to both B7-1 and B7-2. Only clones showing stable expression of B7 molecules were selected for further experimentation. The EL4-wt cells and the vector neo/hygro-transfected EL4 cells did not show any detectable expression of B7-1 or B7-2 (Fig. 1 A). EL4-B7-1 cells showed a strong expression level for B7-1 but no detectable B7-2, as demonstrated by binding of anti-B7-1 and CTLA4-Ig but not anti-B7-2 (Fig. 1 B). B7-2-transfected EL4 cells stained brightly with anti-B7-2 antibodies and CTLA4-Ig but not with anti-

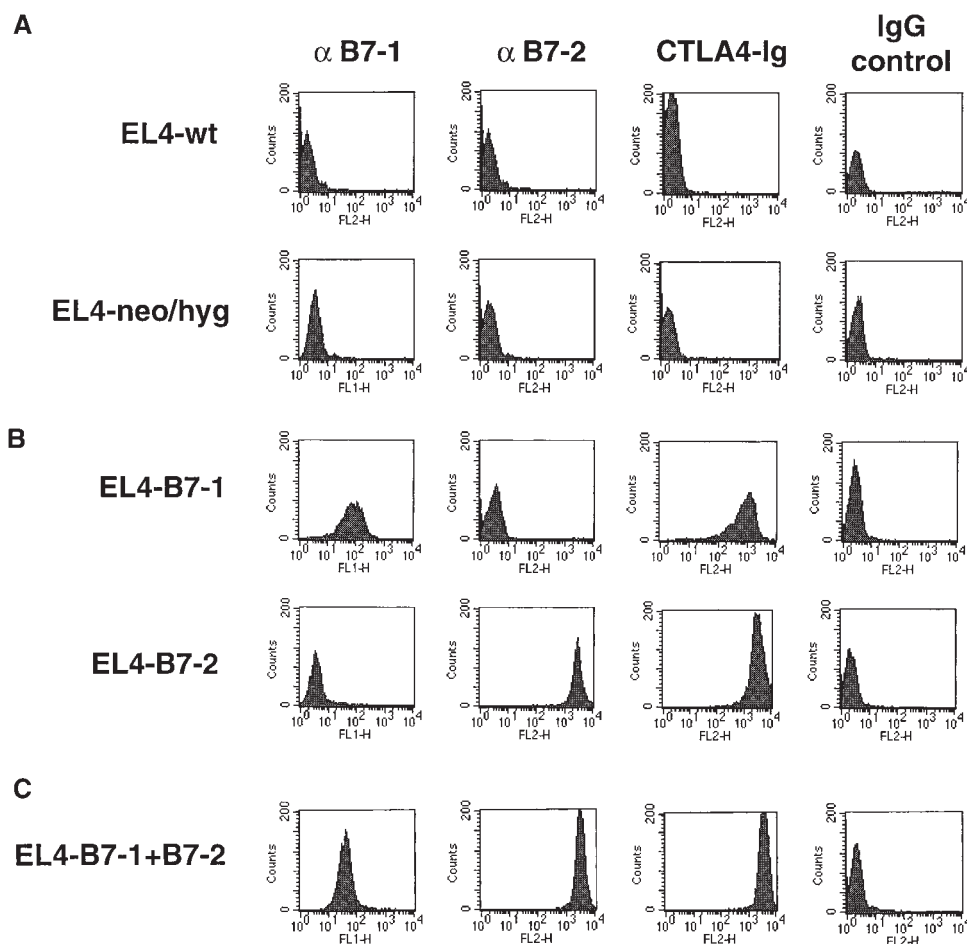


Figure 1. Expression of B7 molecules on transfected tumor cell lines. EL4-wt and EL4-neo/hygro (A), B7-1- and B7-2-single-transfected cells (B), and B7-1-B7-2-double-transfected cells (C) from in vitro were stained with direct PE- or FITC-conjugated mAbs specific for α B7-1 (16.10A1) or α B7-2 (GL1) and with hCTLA4-Ig (followed by a secondary PE-conjugated goat anti-human antibody). Ig controls included staining with normal rat Ig, hamster Ig, goat Ig, and human Ig, of which a representative histogram was selected to show the negative staining. A total of 10,000 cells were analyzed per sample by flow cytometry.

B7-1 antibody (Fig. 1 B). The double-transfected EL4 cells showed a fairly equivalent expression for both B7-1 and B7-2 when compared to the expression levels found on the single transfectants (Fig. 1 C). None of the cells expressed CD95L (data not shown).

B7-1+B7-2 Double Transfectants Induce Tumors In Vivo. The tumor growth potential of EL4 cells was assessed by intradermal implantation of 2×10^6 cells into syngeneic C57BL/6 mice. EL4-wt cells showed aggressive tumor growth, producing visible tumors in the mice after only 5–9 d. No significant differences were observed between tumors produced from vector-only-transfected cells (EL4-neo/hygro) and EL4-wt cells (data not shown), demonstrating that the transfection process and vector did not have any effect on the ability of EL4 cells to produce tumors in syngeneic mice (data not shown). In the same experiment, the tumorigenicity of transfected EL4-B7-1, EL4-B7-2, and double-transfected EL4-B7-1+B7-2 cells were tested by implanting 2×10^6 cells into syngeneic C57BL/6 mice. B7-1-transfected EL4 cells completely regressed by 13–16 d after implantation (Fig. 2 A). In contrast, the EL4-B7-2 cells continued to grow at a rate similar to that of the EL4-wt cells. The most aggressive tumor growth was observed with the double-transfected EL4-B7-1+B7-2 cells, which reached experimental limits between days 13 and 17, 5–6 d earlier

than the EL4-wt control group (Fig. 2 A). Cumulative data from all the experiments is presented in Table I. The majority of EL4-B7-1 tumors were rejected, whereas the B7-2-transfected and B7-1+B7-2 double-transfected EL4 cells were not rejected by syngeneic mice. Furthermore, the difference between EL4-B7-1 and EL4-B7-2 or EL4-B7-1+B7-2 in the incidence of tumor rejection and tumor volume was highly significant ($P < 0.0001$). In nude mice, all of the EL4 cell lines (wild type and transfected) showed similar tumor growth rates (Fig. 2 B). Therefore, the regression of EL4-B7-1 tumors was not due to different rates of growth of the transfected tumor cells but required the presence of T cells for the regression to occur.

Expression of B7-1 and B7-2 on EL4 Cells Is Stable In Vivo. One of our major concerns in these experiments was whether the expression level of B7 molecules on the surfaces of the transfected cells was maintained in vivo for the complete term of the experiment or whether the cells lost or lowered their B7 surface expression. If the B7 expression of the transfectant was to decrease, it could result in an increased growth rate of the tumors in vivo. Progressively growing tumors from the syngeneic C57BL/6 mice were removed (explanted) after 20 d, made into single-cell suspensions, and analyzed by flow cytometry to determine their surface expression levels of B7. Mice implanted with

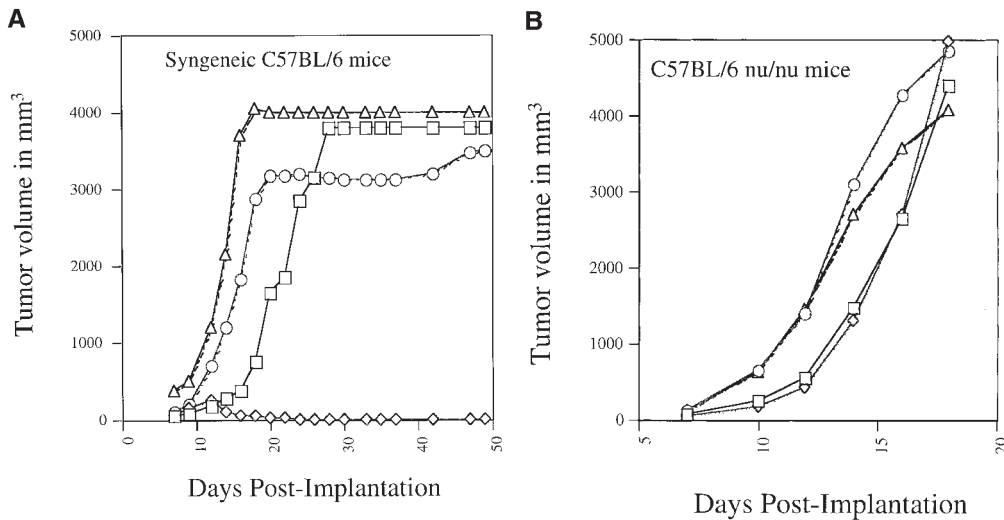


Figure 2. Tumorigenicity of B7-transfected EL4 cells in vivo. B7-transfected EL4 and EL4-wt cells (2×10^6 cells/mouse) were intradermally implanted into syngeneic C57BL/6 mice (A) or C57BL/6 nu/nu mice (B) in groups of five mice each. Tumor growth was assessed every 2–3 d by measuring in three perpendicular directions. The results are expressed as tumor volumes in mm³. □, EL4-wt; ◇, EL4-B7-1; ○, EL4-B7-2; △, EL4-B7-1+B7-2.

EL4-B7-1 cells did not have tumors available to explant due to complete tumor regression. The explanted tumor cells were then stained for B7 expression immediately after explantation and also after 4 d in tissue culture (Fig. 3). The explanted EL4-B7-2 cells showed the same expression as before implantation, and expression was stable when the cells were maintained in cell culture (Fig. 3). The explanted EL4-B7-1+B7-2 cells also showed stability for the expression of both B7 molecules on its surface (Fig. 3). The B7-1 expression level of the double-transfected tumor cells was the same as before implantation. This clearly demonstrates that, although the EL4-B7-1+B7-2 cells still expressed B7-1 on their surfaces, B7-1 was no longer able to induce regression and tumor growth killed the mouse.

B7-2 Molecules on the B7-1 and B7-2 Double Transfectants Are Functional and Costimulate T Cell Responses In Vitro. To determine the mechanisms for the different immune responses to EL4-B7-1 and B7-2 tumors, we tested the transfectants for their ability to costimulate T cell proliferation. We have previously reported that, although EL4-B7-1

costimulates T cell proliferation, EL4-B7-2 does not costimulate an anti-CD3-induced T cell response. In this series of experiments, single- and double-transfected EL4 cells were used as costimulators in a proliferation assay using submitogenic concentrations of soluble anti-CD3 antibody. The data presented in Fig. 4 confirmed that EL4-B7-1 cells costimulated significant amounts of T lymphocyte proliferation. This proliferation was almost completely blocked by the addition of anti-B7-1 antibodies or hCTLA4-Ig but was unaffected by the addition of anti-B7-2 antibodies (Fig. 4). EL4-wt, vector-only-transfected EL4, and EL4-B7-2 cells were unable to costimulate T cell proliferation (Fig. 4). The EL4-B7-1+B7-2 double transfectants were able to costimulate T cell proliferation as well as the EL4-B7-1 cells. This costimulation could be 60% blocked by the addition of anti-B7-1 antibodies but more than 90% blocked with anti-B7-2 antibody or CTLA4-Ig (Fig. 4). EL4-B7-1+B7-2 cells explanted after 20 d from tumors growing in mice also retained the same costimulatory activity as the in vitro-cultured EL4-B7-1+B7-2 cells.

Table I. Tumor Growth of EL4-wt and Transfected Tumor Cells In Vivo

	Incidence of tumors	Incidence of tumor rejection	Tumor volume on day 14	Maximal tumor volume	Percent tumor-free on	
					day 20	day 30
			<i>mm³ ± SEM</i>		<i>%</i>	
EL4-wt	20/20	0/20	1,092 ± 584	4,208 ± 883	0	0
EL4-B7-1	30/30	28/30	88 ± 38	542 ± 343	60	93
EL4-B7-2	23/23	1/23*	2,216 ± 319 [‡]	3,873 ± 396 [‡]	4	4
EL4-B7-1+B7-2	30/30	5/30*	2,563 ± 257 [‡]	3,707 ± 343 [‡]	0	10

B7-transfected EL4 and EL4-wt cells (2×10^6 cells/mouse) were intradermally implanted into syngeneic C57BL/6 mice. The tumor volumes in mm³ are the average tumor volumes from all mice in the experiment groups. Maximal tumor volume is calculated as average tumor volume in each mouse on day of largest size.

*Significantly different from EL4-B7-1 group ($P < 0.0001$) by chi-square test.

[‡]Significantly different from EL4-B7-1 group ($P < 0.0001$) by unpaired *t* test.

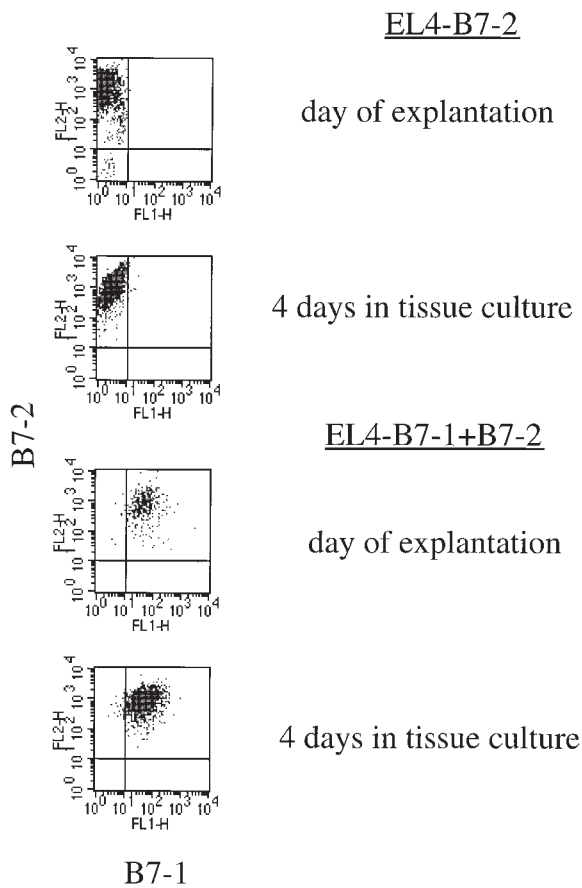


Figure 3. Stability of B7-2 expression on transfected cells in vitro and in vivo. Tumors from mice implanted with EL4-B7-2 and EL4-B7-1+B7-2 cells were removed from the mice 20 d after implantation, made into a single-cell suspension, and stained with FITC-conjugated anti-B7-1 mAb (16.10A1) and PE-conjugated anti-B7-2 mAb (GL1). The B7 expression was determined by flow cytometry for explanted EL4-B7-2 and EL4-B7-1+B7-2 tumor cells immediately after explantation and after maintaining the explanted cells for 4 d in tissue culture. A total of 10,000 cells were analyzed by flow cytometry for each sample.

Blocking B7-2 Molecules In Vivo Results in Inhibition of Tumor Growth of the B7-1+B7-2 Double-transfected EL4 Tumors. To study whether the lack of antitumor immunity induced by the double transfected was due to a dominant negative immunoregulation induced by the B7-2 molecule, we implanted syngeneic C57BL/6 mice with the double-transfected EL4-B7-1+B7-2 cells and tested the effect of blocking either B7-1 or B7-2 molecules with monoclonal antibodies in vivo. Groups of mice were intradermally implanted with EL4-B7-1+B7-2 cells that were premixed with excess (50 μ g) anti-B7-1 or anti-B7-2 antibody to block the B7 molecules expressed on the cell surface. The mice were further treated with i.p. injections of 150 μ g of the anti-B7 antibody every other day for 20 d after implantation of EL4 cells. Anti-B7-1 and anti-B7-2 antibodies are of the same isotype and thus control for each other. A control group was injected i.p. every other day with 200 μ l PBS. In the anti-B7-1 antibody-treated group, the EL4-B7-1+B7-2 tumor grew as quickly as the PBS-treated control group (Fig. 5 A), reaching experimental limits between days 15 and 22. In other experiments, treatment with anti-B7-1 antibody made the tumors grow more quickly than the PBS treatment (data not shown). In contrast, in the anti-B7-2 antibody-treated group, tumors grew slowly or began to regress by day 15, indicating that when the B7-2 molecule on the surface of the EL4 cell was blocked, the signal provided by the B7-1 molecule became dominant and induced regression. The experiment was discontinued at day 21, as the tumors in the PBS control and anti-B7-1 antibody groups had reached experimental limits. The in vivo effects of anti-B7-2 antibody in inducing tumor regression could be due to its modification of the immune response of the host and not just due to blocking of B7-2 molecules on the surfaces of tumor cells. To address this issue, we implanted EL4-wt cells into C57BL/6 host mice that were treated with anti-B7-1 antibodies, anti-B7-2 antibodies, or a control Ig to determine if the antibody treatments themselves were affecting the experi-

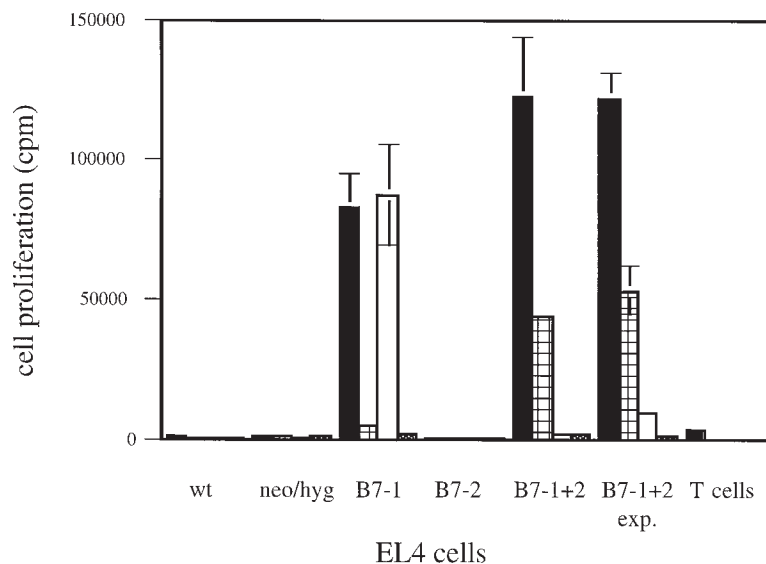


Figure 4. Costimulation of T cell proliferation in response to submitogenic doses of anti-CD3 mAb by B7-transfected EL4 and EL4-wt cells. T lymphocytes (5×10^4 /well) were isolated from syngeneic C57BL/6 mice and combined with EL4 stimulator cells (5×10^4 /well) that had been pretreated with 50 μ g/ml mitomycin C overnight. The cells were incubated for 48 h at 37°C, pulsed with 1 μ Ci/well 3 H-TdR, incubated for an additional 14–16 h, harvested, and the amount of TdR incorporation was determined. The costimulatory effect of the B7 molecules on T cell proliferation was blocked by the addition of 2 μ g/well of anti-B7-1 (1G10) mAb, anti-B7-2 (GL1) mAb, or hCTLA4-Ig. The background proliferation of T cells treated only with anti-CD3 antibody was <2,000 cpm in this assay. Mitomycin C-treated tumor cells, without T cells, did not show any significant proliferation (100–300 cpm; data not shown). B7-1+2 exp. represents double-transfected EL-4 cells obtained from in vivo-explanted tumors. Treatments: ■, alone; hatched box, anti-B7-1; □, anti-B7-2; dotted box, hCTLA4-Ig.

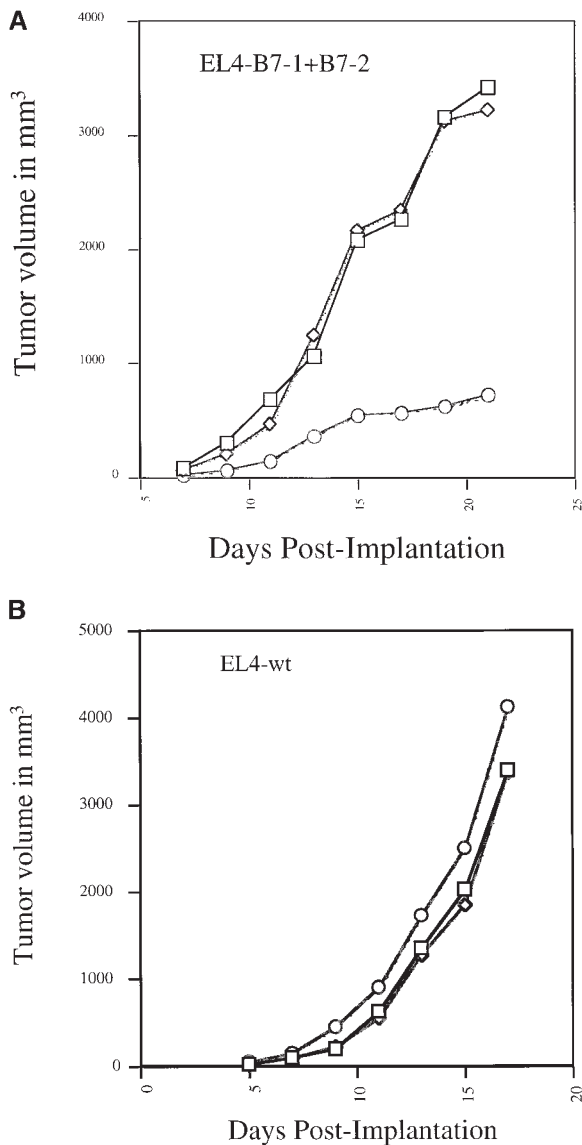


Figure 5. Effect of injection of anti-B7 antibodies on the tumorigenicity of EL4-B7-1+B7-2 or EL4-wt cells in vivo. Groups composed of five syngeneic C57BL/6 mice each were intradermally implanted with 2×10^6 EL4-B7-1+B7-2 cells (A) or EL4-wt cells (B) and injected i.p. with 150 $\mu\text{g}/\text{mouse}$ of anti-B7-1 mAb (\diamond), anti-B7-2 mAb (\circ), or PBS (\square) every other day. The cells had been premixed with 50 μg of anti-B7-1 or anti-B7-2 mAbs before implantation. Treatment was stopped at day 20. Tumor size was assessed every 2–3 d by measuring in three perpendicular directions. The results are expressed as tumor volumes in mm^3 .

mental results. During the 20-d course of the experiment, none of the antibodies had any significant effect on the growth of the EL4-wt tumors in vivo (Fig. 5 B). Furthermore, in vitro treatment of EL4-wt, EL4-B7-1, EL4-B7-2, and EL4-B7-1+B7-2 cells with anti-B7-1 or anti-B7-2 antibodies did not inhibit or slow the in vitro growth rate of the cells (data not shown).

Transfer of Immune Suppression by a T Cell Line and Spleen Cells from EL4-B7-bearing Mice. To examine the role of T cells in tumor rejection and immune suppression, T cell lines from EL4-B7-1, EL4-B7-2, and EL4-B7-1+B7-2

tumor-bearing mice were established. The T cell lines were derived from spleens of mice bearing tumors implanted 10–12 d earlier. Because of the lack of costimulatory activity of EL4-wt and EL4-B7-2 cells, we have not been successful in establishing long-term T cell lines against these tumors. However, we were successful in establishing long-term T cell lines against EL4-B7-1 and EL4-B7-1/2 double transfectant tumor cells. Three cell lines were established from three mice bearing the EL4-B7-1 tumor, and each of the three T cell lines were CD8^+ (>95%). All of these T cells showed the same cytokine profile as demonstrated for the T cell line No. 4 (Fig. 6 B), and all of them lysed EL4-wt and EL4-B7-1 tumor cells in a CTL assay. Five T cell lines were established from six mice bearing the EL4-B7-1+B7-2 tumor. Of these five lines following four in vitro restimulations, four T cell lines were >93% CD4^+ T cells. The fifth cell line was 65% CD4^+ and 35% CD8^+ . This cell line was positive in a CTL assay for EL4-B7-1 and the double-transfected tumor cells, but over time it lost the CD8^+ T cells and CTL activity. Detailed analysis of two prototypic T cell lines, No. 4 generated against the EL4-B7-1 and No. 1 generated against EL4-B7-1/2 double transfectants, is shown (Fig. 6). The No. 4 T cell line, generated against EL4-B7-1, was CD8^+ (>98%) and showed a very strong and specific CTL activity for the different EL4 cells, including the EL4-wt cells, but did not lyse a control tumor target R1.1-B7-1 (this cell line is also B7-1-transfected; Fig. 6 A). After specific activation of T cell No. 4 with EL4-B7-1, it produced high levels of $\text{IFN-}\gamma$, low levels of IL-10, and no IL-2, IL-4, or $\text{TNF-}\alpha$ (Fig. 6 B). This is completely opposite to the response of T cell line No. 1, which was derived from a mouse bearing the B7-1/2 double-transfected tumor. This T cell No. 1 (>99% CD4^+) showed no CTL activity at all (Fig. 6 A) and produced high amounts of IL-4 and IL-10 but no IL-2, $\text{IFN-}\gamma$, or $\text{TNF-}\alpha$ in response to EL4-B7-1+B7-2 (Fig. 6 B). Transfer of the T cell line No. 1 i.v. into mice with a EL4-B7-1 tumor resulted in tumor growth in 9/16 animals (Fig. 6 C). Furthermore, the intravenous transfer of spleen cells from mice with EL4-B7-2 tumors resulted in growth of EL4-B7-1 tumors (Fig. 6 C). Intravenous injection of PBS or normal C57BL/6 spleen cells did not affect the tumor growth of EL4-B7-1 cells. In contrast to the EL4-B7-1-expressing tumor cells, which induce CTL responses, the data suggest that expression of B7-2 on the EL4 cells results in the induction of CD4^+ T cells that predominantly produce Th2 cytokines. To further confirm that it is CD4^+ cells that are responsible for suppression of anti-tumor immunity, we also tested the growth of EL4-B7-1+B7-2 in B6 mice depleted of CD4^+ cells. Groups of C57BL/6 mice were either treated with control Ig or anti- CD4 mAb (GK1.5) and then transplanted with EL4-B7-1+B7-2 double transfectants. The results showed that in the control Ig-treated group, all mice developed tumors of large size; all mice had to be killed because of the large tumor size. In contrast, in the anti- CD4 -treated group, average tumor size was much smaller. In the latter group, 75% of mice were still alive on day 24 and 25% of mice were actively rejecting tumors.

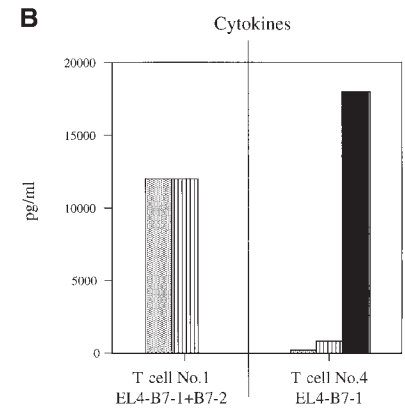
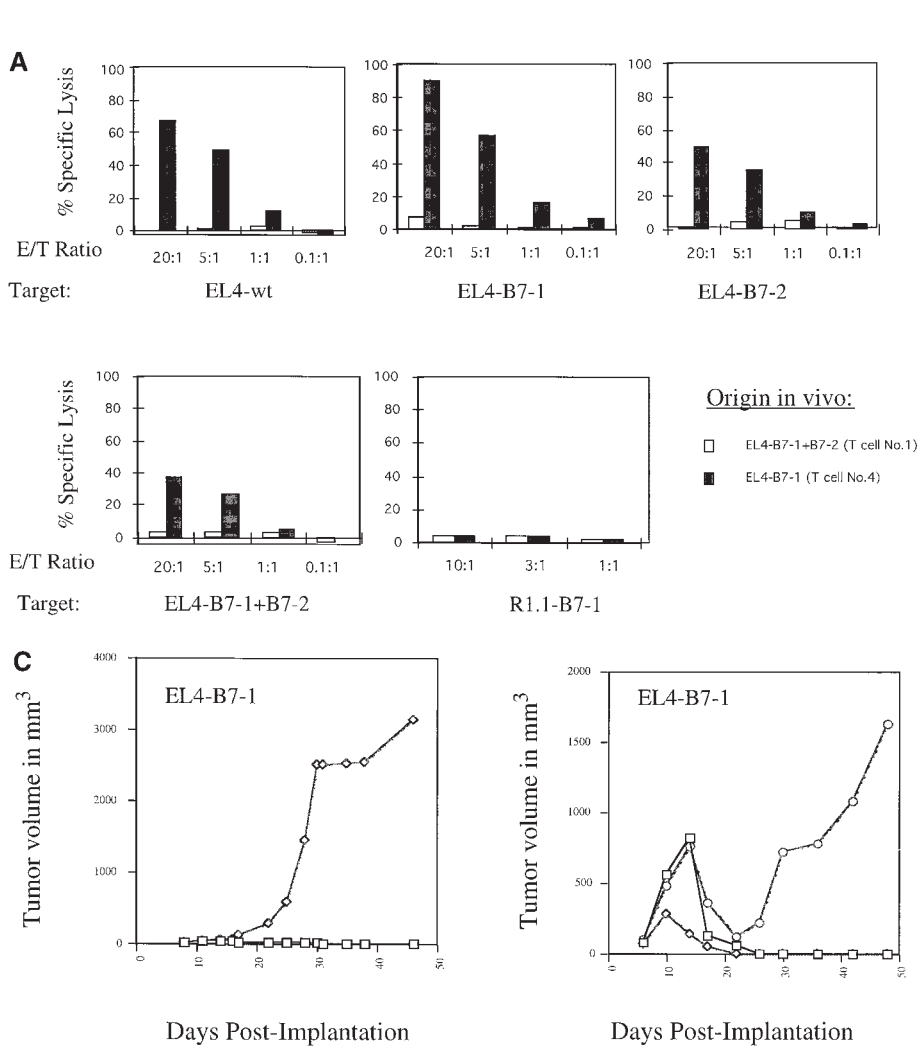


Figure 6. In vitro and in vivo functions of T cells derived from EL4-B7-1 or EL4-B7-1+B7-2 tumor-bearing mice. The long-term T cell lines were established from spleens of mice which had the EL4-B7-1 (T cell No. 4) or the double-transfected tumor (T cell No. 1) for 12 d. (A) Cytolytic responses of the two T cell lines to different ⁵¹Cr-labeled targets at different E/T ratios. The data are presented as % specific lysis. Similar results were obtained in a total of six independent experiments. (B) Cytokine production of the T cell lines after 40 h of activation with the corresponding EL4 transfectant. The cytokines were measured by an ELISA. Dotted bar, IL-4; hatched bar, IL-10; ■, IFN- γ . One of five independent experiments is shown. (C) Effect of transfer of $4-5 \times 10^6$ cells from T cell line No. 1 (generated from EL4-B7-1+B7-2-bearing mice) or spleen cells from normal mice or mice bearing EL4-B7-2 tumors into mice which were implanted with EL4-B7-1 cells 24 h earlier. Tumor size was assessed every 2-3 d

by measuring in three perpendicular directions. The results are expressed as mean tumor volumes in mm³. □, alone; ◇ (left), T cell line No. 1; ◇ (right), spleen C57BL/6; ○, spleen EL4-B7-2.

These data directly demonstrate that CD4⁺ cells play an active role in suppressing antitumor immunity.

EL4-B7-2 and B7-1+B7-2 Cells Are Rejected in IL4^{-/-} Mice. Previous studies have reported that B7-2 may preferentially induce Th2 responses (25, 26). B7-2 expression on EL4 cells probably results in the induction of IL-4-producing Th2 or NK1.1 responses, which may in turn suppress antitumor responses. To further explore whether IL-4 and IL-10 were involved in the suppression of antitumor immunity by EL4-B7-2 and EL4-B7-1+B7-2 tumor cells, we assessed the tumor growth of EL4-wt and transfectants by intradermal implantation of 2×10^6 cells into syngeneic IL-4^{-/-}, IL-10^{-/-}, and normal C57BL/6 mice.

EL4-wt and EL4-B7-1 tumor cells showed the same tumor growth or tumor rejection in IL-4- or IL-10-deficient mice as in normal C57BL/6 mice (Fig. 7, A and B). EL4-wt cells produced visible tumors in the mice after 5-9 d (Fig. 7 A). B7-1-transfected EL4 cells were rejected in all three groups by 13-20 d after implantation (Fig. 7 B). In contrast, EL4-B7-2-transfected tumor cells progressively grew

in normal C57BL/6 and IL-10^{-/-} mice but were completely rejected in IL-4^{-/-} C57BL/6 mice (Fig. 7 C). The B7-1+B7-2 double-transfected EL4 cells showed aggressive tumor growth in the C57BL/6 control group, whereas in the IL-4^{-/-} and IL-10^{-/-} mice, the EL4-B7-1+B7-2 tumor cells were rejected (Fig. 7 D). These data demonstrate that host-derived IL-4 and/or IL-10 plays a critical role in abrogation of antitumor immunity induced by EL4-B7 transfectants.

Discussion

We have examined the costimulatory molecules B7-1 and B7-2 on EL4 thymoma cells for their ability to induce antitumor immune responses. Our studies using B7-2-transfected EL4 cells confirm the previously reported results that B7-1 and B7-2 expressed on EL4 tumor cells differ in the way they affect antitumor immunity and tumor growth in vivo (22). Transfecting B7-2 into EL4 cells does not induce antitumor immunity but results in more vigor-

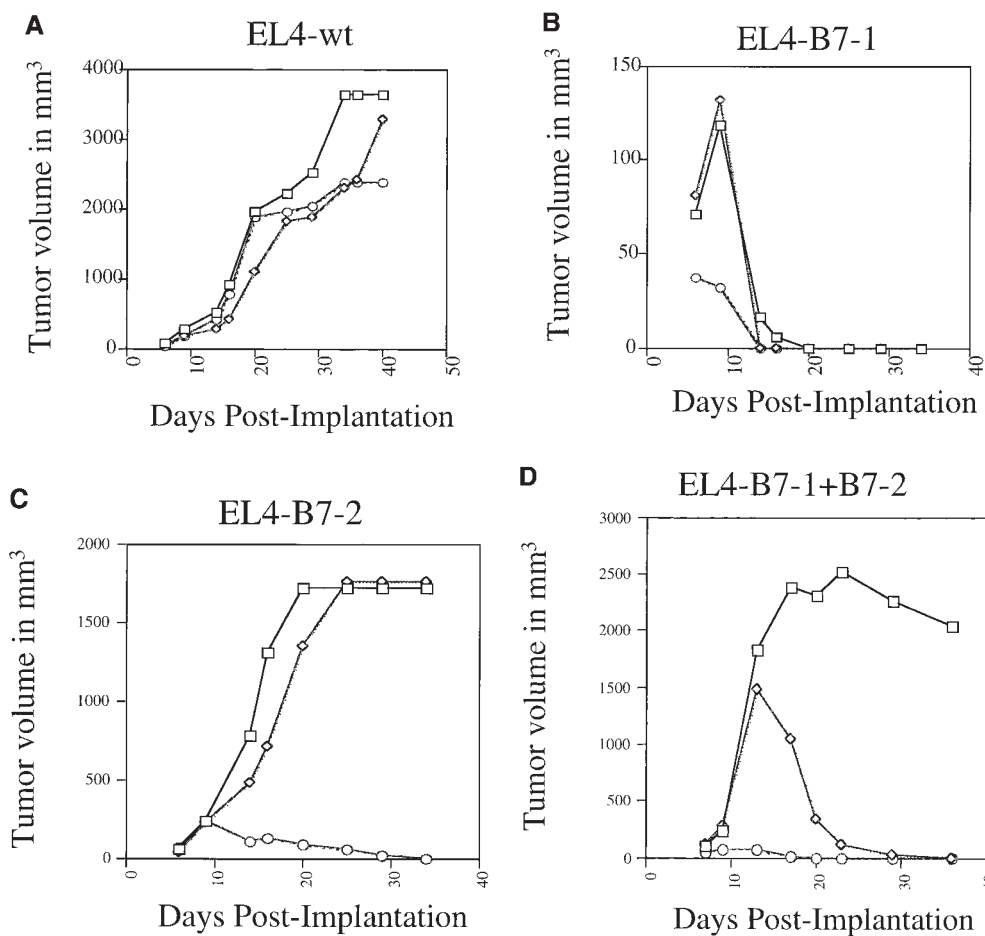


Figure 7. Tumor growth of EL4-wt and B7-transfected EL4 cells in C57BL/6 (□) and IL-4^{-/-} (○) and IL-10^{-/-} (◇) C57BL/6 mice. EL4-wt (A), B7-1-transfected EL4 (B), EL4-B7-2 (C), and EL4-B7-1+B7-2 cells (D) were intradermally implanted into syngeneic C57BL/6 mice and IL-4^{-/-} and IL-10^{-/-} deficient C57BL/6 mice (2×10^6 cells/mouse). Tumor growth was assessed every 2–3 d by measuring in three perpendicular directions. The results are expressed as tumor volumes in mm³.

ous tumor growth. This is in sharp contrast to the complete regression observed with the B7-1-transfected EL4 cells. Interestingly, we observed in this study that expression of B7-2 in B7-1-transfected EL4 cells does not result in tumor regression but rather more aggressive growth of the tumor cells. This suggests that the presence of B7-2 on the cell surface was dominant, as it could suppress or negate the ability of B7-1 to induce antitumor immunity and elimination of the tumor.

Although some investigators have reported that both B7-1 and B7-2 have equivalent costimulatory abilities (27), increasing evidence suggests that the functional outcome of B7-1- and B7-2-mediated signaling appears to have both distinct and overlapping functions (26, 28, and 29). We and others have recently demonstrated that expression of B7-1 on the surfaces of malignant tumor cells results in their rejection and can reduce tumor burden and eliminate established metastases (17, 18). B7-2 has been transfected into a number of different types of tumor cells with conflicting results. Transfecting B7-2 into P815 mastocytoma cells (21) or malignant melanoma tumors (30) resulted in tumor regression. In contrast are the results obtained by Yang et al. for the fibrosarcoma MCA 102 (21) and Gajewski (24), who have reported that B7-1, but not B7-2, can efficiently costimulate CD8⁺ T lymphocytes. Leong et al. have found that B7-1, but not B7-2, can induce immunity to murine-

malignant mesothelioma (31). Matulonis et al. have also reported that B7-2 is less potent than B7-1 in inducing anti-tumor immunity and tumor regression in myeloid cells (23). Our results are consistent with the Leong et al. and Matulonis et al. reports.

Why would the two B7 molecules behave differently, even though they bind to the same receptors on the surfaces of T cells? There is some indirect evidence that suggests that B7-1 is quantitatively superior to B7-2 in providing costimulation. The differences in affinity/avidity and length of interaction (on/off rates) between B7 molecules and the CD28/CTLA4 receptors may affect the intracellular signaling events induced in T cells (29). Mechanisms which could explain the B7-2 effect on the immune system include the induction of anergy by preferentially engaging CTLA4, activation of the fas/fas ligand apoptotic pathway, or the recruitment of regulatory T cells producing inhibitory cytokines. The induction of anergy through the CTLA4 pathway does not appear to be an important mechanism of the observed B7-2 effect, as *in vivo* blocking of CTLA4 using a specific monoclonal antibody (whole or Fab) did not result in the rejection of the EL4-B7-2 tumors (data not shown).

Th2 cells have been shown to be induced by B7-2 signaling, leading to the downregulation of Th1 cells and their associated cytokines. Kuchroo et al. (25) and Freeman et al. (26) initially suggested that B7-1 and B7-2 may have

differential roles in T cell differentiation. Whereas B7-1 was suggested to induce Th1 differentiation, more compelling evidence has been reported for the role of B7-2 in inducing Th2 differentiation. Although they are controversial, a number of studies have now confirmed this initial observation (28, 29, and 33) and have also shown the importance of B7-2 for IL-10 production (29). It is not yet clear whether B7-2 expressed on the EL4 cells is inducing differentiation of naive T cells into a Th2/Tc2 pathway or whether it is expanding a memory Th2/Tc2 population. If B7-2 expression does not directly result in Th2 differentiation of the naive T cells, EL4-B7-2 cells may preferentially expand a preexisting memory Th2/Tc2 cell population that is cross-reactive with the EL4 cells. Although EL4 tumor cells are MHC class II⁻, they induce CD4⁺ T cells, probably by indirect presentation in that the tumor antigen of the EL4 cells is presented by host APCs. The rejection of EL4-B7-2 and EL4-B7-1+B7-2 tumor cells in IL-4-deficient mice demonstrates the important role of this Th2 cytokine in the regulation of an antitumor response in this system. Thus, if B7-2-expressing EL4 cells predominantly induced a Th2 response, these EL4-specific Th2 cells may inhibit antitumor immune responses. The importance of CD4⁺ and not CD8⁺ T cells for the immune-suppressive effect of the EL4-B7-1+B7-2 cells could be further demonstrated in mice depleted of CD4⁺ cells, as described in the Results section: mice depleted of CD4 cells could reject tumors or showed slower tumor growth when compared with control Ig-treated mice. This data, together with the results from the IL-4-deficient mice, which still have functional CD4 compartments, supports the idea that Th2 cytokines produced by CD4 cells induced by B7-2-bearing tumor cells are responsible for suppression of antitumor immunity in vivo. A few earlier studies have shown that preferential induction of Th2 responses may suppress specific antitumor immunity. Ghosh et al. have suggested that Th2 cells dominate in progressive tumor-bearing animals, reducing the number of Th1 cells and their associated cytokines and thus allowing the tumor to grow in the host (34). IL-10 has been implicated in reducing the ability of CD8⁺ T cells (CTLs) to eliminate tumors in vivo (35, 36). The in vivo data for IL-4^{-/-} C57BL/6 mice further supports the hypothesis that B7-2 may be delivering a dominant costimulatory signal which induces IL-4, producing Th2-like responses. It would then follow that IL-4 and IL-10 would be produced by these cells, leading to inhibition of Th1 cells and CTL activity and favoring tumor growth. Our data further supports this hypothesis, in that transfer of T cells from B7-1+B7-2 tumor-bearing mice inhibited antitumor immunity in mice challenged with EL4-B7-1 cells. This may be part of the explanation for the B7-2 effect we have observed.

Functional differences in the effect of B7-2 expression on different cell types might be due to quantitative differences in surface expression, cell-specific posttranslational modifications in the B7-2 molecule, different isoforms of B7-2 used for transfection, or mutation in the cDNA. The

same murine B7-2 cDNA preparation was transfected into cells of different tissue types such as CHO, 3T3, and BW1100 cells. We have found that only the EL4 cells expressing B7-2 failed to costimulate, eliminating the possibility that the cDNAs are different (22). Freshly isolated splenic T cells from a number of strains of mice were also examined and found to be unable to costimulate T cells in *in vitro* allo-MLRs, although they all expressed low levels of B7-2 as determined by flow cytometry. These two pieces of data argue against there being a mutation in the B7-2 cDNA used for transfection. Quantitative differences in B7-2 expression on the surfaces of the cells is also not a likely explanation for the observed inability of EL4-B7-2 and EL4-B7-1+B7-2 cells to induce antitumor immunity. We tested low and high B7-2-expressing EL4 cells, and all showed similar tumor growth *in vivo* (data not shown). Also, in the case of EL4-B7-1+B7-2 double transfectants, there is high expression of both costimulatory molecules yet tumors grow progressively in the host. Quantitatively lower expression of B7 may not, therefore, be responsible for the observed effect. Furthermore, overexpression of B7-2 on EL-4 cells does not lead to induction of antitumor immunity, suggesting that B7-2 expressed on EL-4 or other T cells may be qualitatively different. In support of this hypothesis, Höllsberg et al. (37) have reported that human T cells express a hypoglycosylated form of B7-2 that exhibits a reduced ability to bind CD28 and does not costimulate T cell proliferation. This also indicates that B7-2 expressed on the surfaces of T cells may be qualitatively different. It is possible that B7-2 exists in different forms in various cell types and that posttranslational modifications can affect the binding and functions of the B7-2 molecule.

The conflicting reports concerning B7-2 effects on the antitumor response may be due in part to sequence differences in the B7-2 construct used for transfection. Comparison of the published sequences of B7-2 by Freeman et al. (12) and Azuma et al. (5) indicates the presence of six additional residues at the amino terminus of the B7-2 sequence isolated by Freeman et al. It is possible that these six additional amino acids, which are encoded by a separate exon (38), can affect the function of the B7-2 molecule or shunt it into different intracellular processing pathways within the cell. This could result in conformational changes in protein folding or posttranslational modifications (e.g., glycosylation), leading to alterations in the function of the B7-2 molecule in some cell types, such as tumors of T cell origin.

In summary, data presented here demonstrates that, whereas B7-1 and B7-2 enhance immune responses by providing a potent costimulatory signal to the T cell, B7-2 may serve to inhibit immune responses under some circumstances. The Th2 cytokine, IL-4 appears to play an important role in abrogating the antitumor response induced by the B7-2 molecule on EL4 cells. B7 molecules may thus have evolved with the capability to both enhance and regulate the immune response, depending on which receptor they engage (CD28 vs. CTLA4) and the cell type in which they are expressed (professional APC vs. T cell).

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