# Tumor Immunogenicity Determines the Effect of B7 Costimulation on T Cell–mediated Tumor Immunity

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**Summary**

A costimulatory signal through B7 to its counter-receptor CD28 on T cells enhances T cell activation. We have generated recombinant retroviruses containing cDNA for murine B7 and transduced a panel of murine tumor lines with varying immunogenicity to study the effect of B7 costimulation on antitumor immunity. In contrast to the progressive outgrowth of all wild-type ($B7^-$) tumors in unimmunized syngeneic mice, four immunogenic tumors, lymphoma RMA, EL4, mastocytoma P815, and melanoma E6B2, regressed completely when transduced with the B7 gene. In contrast, four nonimmunogenic tumors, sarcomas MCA101, MCA102, and Ag104, and melanoma B16, remained tumorigenic after transduction of the B7 gene. Immunization with B7-tranduced immunogenic tumors enhanced protective immunity and increased specific cytotoxic T lymphocyte (CTL) activity against the respective wild-type tumors as compared to immunization with nontransduced or mock-transduced tumors. Moreover, cocultivation of CTL with B7-transduced EL4 cells augmented the specificity of tumor-reactive CTL in long-term cultures. Treatment by injection of B7-transduced tumor cells cured 60% of mice with established wild-type EL4 lymphoma. In contrast, immunization with nonimmunogenic tumors transduced with B7 did not provide protective immunity and did not increase specific CTL activity. Our results show that tumor immunogenicity is critical to the outcome of costimulation of T cell–mediated tumor immunity by B7.

Many experimentally induced animal tumors are immunogenic, i.e., they express tumor-specific transplantation antigens as evidenced by their rejection after transplantation to syngeneic animals immunized against the respective tumors (1). Other tumors are nonimmunogenic, i.e., they are not rejected when similarly tested.

Rejection of immunogenic tumors is primarily mediated by T cells (1) with both $CD8^+$ CTL and $CD4^+$ Th cells playing important roles (2, 3). Growing evidence indicates that the T cells require at least two signals to become activated (4, 5). Signal one is generated by the interaction between an antigen-specific receptor on the T lymphocytes and antigenic peptide–MHC complexes on APCs, e.g., tumor cells or macrophages. Signal two is delivered by costimulatory molecules on the APCs through their counter-receptors on the T lymphocytes (4). Without costimulation, exposure of T cells to an antigen may cause unresponsiveness or anergy (4).

There are several molecules that can provide costimulation (3). Particular interest has recently been given to B7, which is the natural ligand for the CD28 and CTLA-4 counter-receptors on T cells (6). B7 is a member of the Ig superfamily (7, 8) and is expressed on the majority of APCs, such as dendritic cells, activated macrophages, and activated B cells. The interaction of B7 with CD28/CTLA-4 results in an increased stability of mRNA for several lymphokines, including IL-2 (for a review see reference 6). Treatment of mice with a CTLA4Ig fusion protein, which binds to B7 with high affinity, strongly inhibits T cell–dependent antibody responses (9), and makes possible the long-term acceptance of xenografted pancreatic islets (10).

Costimulation of T cells by B7 also plays an important role in eliciting antitumor immunity, and an inadequate costimulation has been suggested to contribute to the progressive growth of tumors in immunocompetent host (11, 12). We have previously shown that an immunogenic mouse melanoma E7C3, which contains the transfected E7 gene of human papillomavirus (HPV)[1]-16, grew progressively in immunocompetent syngeneic mice, but no longer grew after transfection with the murine B7 gene, whereas its growth in im-

[1] *Abbreviations used in this paper:* HPV, human papillomavirus; MiTD, minimal tumorigenic dose.

munodeficient (nu/nu) mice was not impaired. The immune response was primarily mediated by $CD8^+$ T cells and the rejection of tumors did not require the expression of B7 on the target cells. Treatment by injection of $B7^+$ E7C3 cells cured 40% of mice with micrometastasis in their lungs established from intravenously injected E7C3 wild-type cells (12). Townsend and Allison (13) reported that the mouse melanoma K1735, after transfection with the B7 gene, was rejected when transplanted into immunocompetent syngeneic mice, and that these mice rejected a subsequent transplant of wild-type KI735 melanoma. Baskar et al. (14) generated a protective immunity towards the mouse Sal sarcoma subsequent to coexpression of B7 with truncated MHC class II molecules that lacked the cytoplasmic domain.

Providing B7 costimulation does not always lead to augmentation of tumor immunity, however. As was shown previously in our experiments, mouse melanoma K1735-M2 did not become immunogenic by transfection of B7 (12), a finding distinct from that obtained by Townsend and Allison (13) whose experimental conditions were slightly different. Likewise, B7-transfected mouse sarcoma Sal did not regress in the absence of cotransfected MHC class II molecule (13).

Based on the two signal theory of T cell activation (15–17), efficient induction of T cell immunity against a tumor requires, first, the existence of tumor antigen(s) which can be processed, transported to, and presented by MHC class I and/or II molecules on the APCs, and second, sufficient costimulatory molecule(s) on the tumor cells or other APCs. A prediction of this theory is that, in the presence of costimulatory molecules, the expression of tumor antigen and its presentation will be factors limiting the generation of effective T cell–mediated tumor immunity (11). Anomalies exist in antigen processing and presentation, as evidenced by tumors with downregulated MHC class I expression (18) or defective MHC-encoded proteasome components (19). Therefore, transfection of tumor cells with B7 may be used to probe the existence of tumor antigen and/or the ability of tumor cells to present such antigen.

In this report, we describe the effect of transduction of the B7 gene into a variety of tumors with high, low, or nondetectable immunogenicity when tested in tumor rejection assays. Retroviruses containing the murine B7 gene were prepared and used to transduce the B7 gene into the various tumors, whose growth in immunocompetent, syngeneic mice, as well as their capacity to augment T cell–mediated tumor immunity in vivo and in vitro was then investigated.

## Materials and Methods

*Mice.* Female C3H/HeN and BALB/c (nu/nu) mice, 4–8-wk-old, were bought from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Female C57BL/6, 4–8-wk-old, were purchased from Taconic Farms Inc. (Germantown, NY). Female DBA/2 mice, 4–6-wk-old, were purchased from The Jackson Laboratory (Bar Harbor, ME).

*Cell Lines.* The tumor lines used for this study are listed in Table 1. The EL4 and TIMI.4 lymphomas and the B16 melanoma are of C57BL/6 ($H\text{-}2^b$) origin, and the P815 mastocytoma and L5178Y lymphoma came from DBA/2 ($H\text{-}2^d$) mice. YAC-1 is a NK-sensitive lymphoma. A20, ABE-8.1/2, CH1, RAW8.1, WEHI-231, and 2PK-3 are B lymphoma lines. The Meth A fibrosarcoma originated in BALB/c ($H\text{-}2^d$). All these tumor lines were obtained from American Type Culture Collection ([ATCC] Rockville, MD). RMA lymphoma (from Dr. P. Höglund, Karolinska Institute, Stockholm, Sweden) is of C57BL/6 origin (20), as are 3-Methylcholanthrene–induced sarcomas MCA101, MCA102 (21), and colon carcinoma MC38 (22) (from Dr. J. J. Mulé, the National Cancer Institute, National Institutes of Health, Bethesda, MD). The K1735-M2 melanoma (23) (from Dr. I. J. Fidler, M.D. Anderson Cancer Research Center, Houston, TX), its HPV-16 E6 transfectant E6B2 (24), and the Ag104 sarcoma (25) (from Dr. H. Schreiber, University of Chicago, Chicago, IL) are of C3H/HeN ($H\text{-}2^k$) origin. The M109 lung carcinoma (from Dr. P. Wallace, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) came from a BALB/c ($H\text{-}2^d$) mouse (26). Once acquired, all tumors were briefly expanded in vitro and frozen to decrease experimental variation. All cells were maintained in vitro at 37°C in DMEM containing 10% FCS (HyClone Laboratories, Logan, UT) for no longer than 1 mo before in vivo experiments.

*Plasmid Construction.* A DNA fragment encoding the entire open reading frame of murine B7 was amplified by PCR from plasmid pmB7 (12) or by reverse transcription-coupled PCR from RNA prepared from Con A–activated murine spleen cells (27). The sense primer (5′CGATGACGATCGCCTCGAGAAGCTTATGGCTT-GAAATTGT-3′) consists of an oligonucleotide corresponding to 1–15 nucleotides of murine B7 plus a 6-bp consensus sequence and a restriction site for XhoI. The antisense primer (5′-GACTAG-AGCTATCCTCGAGCTAAAGGAAGACGGTCTGTTC-3′) corresponds to 901-921 nucleotides of murine B7 plus a site for XhoI. PCR was performed as described (27) and the PCR product was directly cloned into the pCR™II vector (Invitrogen, San Diego, CA) and subcloned into the retroviral vectors pLXSN and pLNSX (28) (from Dr. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA). The structures of the resulting constructs pLXSNmB7 and pLNSXmB7 are shown (see Fig. 1).

*Retrovirus Preparation and Infection of Tumor Lines.* Recombinant viruses were generated as described previously (24, 29). Briefly, plasmids were a murine B7 insert were transfected by electroporation into the PA317 amphotropic packaging line, and viruses produced from the PA317 cells were used to infect the $\psi$-2 ecotropic packaging cell line in the presence of 8 $\mu$g/ml of polybrene (Sigma Chemical Co., St. Louis, MO). After infection, the $\psi$-2 cells were selected in DMEM containing G418 (1 mg/ml) (GIBCO BRL, Gaithersburg, MD). Viruses generated from G418-resistant lines of $\psi$-2 were used to infected tumor lines according to previously described procedures (24, 29). Cell lines infected with the recombinant retroviruses were cloned by limiting dilution or sorted by FACS® (Becton Dickinson & Co., Mountain View, CA) and selected for expression of B7. All lines were tested by a marker rescue assay (30) and found to be free of helper virus. The pLXSN and pLNSX vectors without B7 insert were used for the preparation of mock viruses by the same procedure.

*FACS® Analysis.* For detection of B7 expression, cells were incubated for 30 min at 4°C with either a control Ig, chimeric L6, or with CTLA4Ig (9, 12) at 10 $\mu$g/ml. They were then washed and incubated or an additional 1 h at 4°C with FITC-conjugated goat anti–human Ig C$\gamma$ serum (Tago Inc., Burlingame, CA). Stained cells were analyzed by FACS®. For detection of MHC class I molecules, the following FITC- or biotin-labeled mAbs were used: AF3-12.1 for $H\text{-}2K^k$; AF6-88.5 for $K^b$; KH95 for $D^b$; SF1.1 for $K^d$; and 34-2-12 for $D^d$ (Pharmingen, San Diego, CA). Purified mAb from 15-5-5S hybridoma (ATCC) was used for staining of $H\text{-}2D^k$.

*Generation of CTL.* The methods for inducing and assaying for a CTL response to tumor cells have been described (24) and were used with minor modification. Briefly, mice were injected subcutaneously with mock- or B7-transduced cells. Tumor nodules were removed surgically at day 10. 2 wk after injection, spleen cells were prepared and cocultivated with γ-irradiated tumor cells (5,000 rad) in 24-well plates (Costar, Cambridge, MA) for 5 d. For the generation of long-term T cells lines, spleen cell cultures were restimulated every 7–10 d in the presence of human recombinant IL-2 (Cetus Corp., Emeryville, CA) at 10 U/ml as described (24). The cytolytic activity of lymphocytes was examined in a standard 4-h $^{51}$Cr-release assay with different E/T ratios as indicated in the figures.

*Animal Studies.* Tumor cells were injected into the shaved right back of mice in a 0.1-ml volume via a 26-gauge needle and using a 1-ml syringe. The minimal tumorigenic doses (MiTD) of tumor cells required for outgrowth in 100% of syngeneic mice were first determined (see Table 2) and were used except when indicated differently in the legends of the figures and tables. The mice were scored for tumor growth once a week and tumor size was documented by measuring two perpendicular diameters in millimeters using a caliper. Two protocols were used for immunization: (*a*) Mice were injected with irradiated (5,000 rad) tumor cells and subsequently challenged with wild-type tumor cells 3 wk later. The B7$^+$ EL4 and B7$^+$ P815 lines were not irradiated as they regressed in immunocompetent mice. (*b*) Mice were injected with liver tumor cells and surgically removed tumor nodules at day 10 after injection and 2 wk after tumor removal, the mice were challenged with wild-type tumor cells.

## Results

*Expression of B7 in Murine Tumor Lines.* The expression of B7 on the surface of cells from 18 murine tumor lines derived from different tissues, including melanomas, sarcomas, lymphomas, carcinomas, and a mastocytoma, was examined by FACS® using CTLA4Ig immunostaining. Four out of six B cell lymphoma lines were positive, whereas B7 was not detectable in any of the other tumors (Table 1).

*Determination of Tumor Immunogenicity.* Eight B7$^-$ tumor lines were selected for further experiments based on their immunogenicity as determined by tumor immunization and challenge assay as described in the reports listed in Table 2. The tumorigenicity and MiTD of each line were determined by s.c. inoculation of serially diluted tumor cell suspensions into syngeneic mice. A single injection of irradiated RMA or E6B2 at dose of 1× MiTD or 10× MiDT completely protected syngeneic mice from challenge of the respective tumors at dose of 1× MiTD. The same injection of EL4 or P815 did not provide any significant protection against subsequent chal-

**Table 1.** *Expression of B7 on Murine Tumor Lines*

| Cell line | Tissue type | Strain | Transformation | B7 expression* |
|---|---|---|---|---|
| K1735 | Melanoma | C3H/HeN | UV | – |
| B16 | Melanoma | C57BL/6 | Spontaneous | – |
| MCA101 | Fibrosarcoma | C57BL/6 | Chemical carcinogen | – |
| MCA102 | Fibrosarcoma | C57BL/6 | Chemical carcinogen | – |
| Ag104 | Fibrosarcoma | C3H/HeN | Spontaneous | – |
| MethA | Fibrosarcoma | BALB/c | Chemical carcinogen | – |
| EL4 | T Lymphoma | C57BL/6 | Chemical carcinogen | – |
| RMA | T Lymphoma | C57BL/6 | Virus | – |
| L5178Y | T Lymphoma | DBA/2 | Chemical carcinogen | – |
| A20 | B Lymphoma | BALB/c | Spontaneous | – |
| ABE-8.1/2 | B Lymphoma | BALB/c | Virus | – |
| CH1 | B Lymphoma | B10.H-2$^a$H-4$^b$ | Unknown | + + |
| RAW8.1 | B Lymphoma | BALB/c | Virus | + |
| WEHI-231 | B Lymphoma | BALB/c × NZB | Chemical carcinogen | + |
| 2PK-3 | B Lymphoma | BALB/c | Chemical carcinogen | + + |
| P815 | Mastocytoma | DBA/2 | Chemical carcinogen | – |
| MC38 | Carcinoma | C57BL/6 | Chemical carcinogen | – |
| M109 | Carcinoma | BALB/c | Spontaneous | – |

* The expression of B7 was determined by indirect immunofluorescence and FACS® analysis by using CTLA4Ig as described in Materials and Methods. (+) 2–10-fold above background fluorescence; (+ +) >10-fold above background fluorescence.

**Table 2.** *Tumor Lines Used for This Study*

| Cell line | H-2 type | Transplantation immunogenicity | MiTD ($\times 10^4$) | Reference no. |
|---|---|---|---|---|
| RMA | b | +* | 0.1‡ | 20 |
| E6B2 | k | + | 200.0 | 24 |
| P815 | d | + | 5.0 | 40 |
| EL4 | b | + | 5.0 | 41, 42 |
| MCA101 | b | – | 20.0 | 21 |
| MCA102 | b | – | 20.0 | 21 |
| Ag104 | k | – | 100.0 | 25 |
| B16 | b | – | 10.0 | 31 |

* Transplantation immunogenicity is recorded based on results from immunization and challenge experiments as described in the publications indicated and confirmed by our own studies.
‡ MiTD was determined as described in Materials and Methods.

lenge. However, complete protection against the outgrowth of EL4 and P815 in syngeneic mice could be achieved if multiple boosts were performed after the initial immunization. Therefore, these four tumors were scored as immunogenic. Four other tumors, i.e., MCA101, MCA102, Ag104, and B16 were nonimmunogenic in similar tests (Table 2). These results agree with the published immunogenicity of the various tumor lines.

*Transduction of the Murine B7 Gene into B7⁻ Tumor Lines.* Two vectors containing the murine B7 gene were used to prepare retroviruses (Fig. 1). In pLXSNmB7, the B7 open

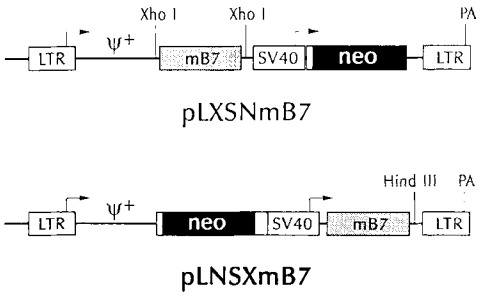
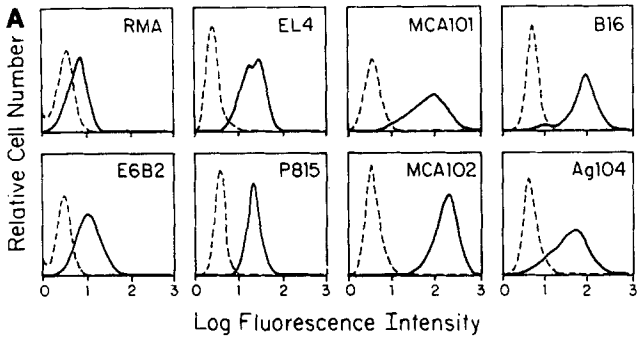



**Figure 1.** Structure of retroviral vectors containing the murine B7 cDNA.

reading frame is driven by the murine leukemia virus enhancer–promoter LTR. This vector also contains the neomycin-resistance gene (*neo*) for drug selection. The second vector, pLNSXmB7, has the same backbone as pLXSNmB7 except that the SV40 promoter is used for driving B7 and the LTR promoter for neo. High titer stocks of ecotropic virus were prepared as described (24, 29). The tumor lines listed in Table 2 were transduced by the pLXSN-based retrovirus except for P815 and B16 which were transduced by the pLNSX-based retrovirus because of a low efficiency of transduction by the pLXSN-based retrovirus in these two lines.

Tumor lines infected with retrovirus were propagated for 7–10 d in selection medium and cloned. Clones obtained from each line were screened by FACS® for expression of B7 by binding of CTLA4Ig. Three-to-five clones from each tumor line which stably expressed highest B7 on their surface were picked for expansion. After in vivo tumorigenicity tests (see Fig. 3), one clone from each line was selected for further ex-



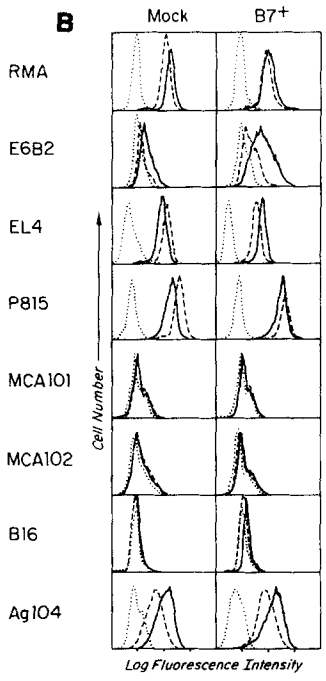




**Figure 2.** (*A*) Expression of B7 on transduced tumor lines. Cells were stained with either CTLA-4Ig (*solid line*) or the control Ig chimeric L6 (*dashed line*) followed by FITC-conjugated goat anti-human Ig Cγ serum as described in Materials and Methods. A total of 5,000 cells was analyzed for each sample. (*B*) Expression of MHC class I molecules on transduced tumor lines. The mock-transduced (*Mock*) or B7-transduced (*B7⁺*) cells were stained with control mAb (*dotted line*), FITC-, or biotin-labeled mAbs to H-2K (*dashed line*) or H-2D (*solid line*) and subjected to FACS® analysis. A total of 5,000 cells was analyzed for each sample.

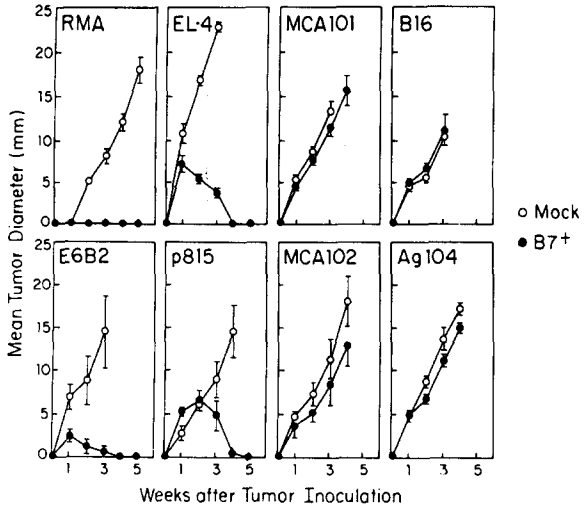



**Figure 3.** Growth of tumors induced by mock-transduced and B7+ tumor cells in syngeneic mice. Mice were injected subcutaneously with the indicated cells at MiTD (see Table 2). Tumor size was assessed weekly by measuring perpendicular diameters with a caliper. The experiments were terminated when the tumors reached 20–30 mm in diameter, severe ulceration and bleeding had developed, or the mice had died. The results are expressed as mean diameter (in millimeters) of tumors from groups of five mice each. Error bars represent the standard deviation of the mean. Similar results were obtained in at least three experiments with each cell line.

periments. The levels of B7 expression on the selected tumor clones are summarized in Fig. 2 *A*.

The expression of MHC class I molecules on the various tumor lines was also examined with or without B7 transduction. EL4, P815, RMA, and Ag104 all expressed high level of MHC class I molecules. On the other hand, expression of MHC class I was either low or not detectable on the other four tumor lines, i.e., E6B2, MCA101, MCA102, and B16. There are no significant changes on the level of MHC I expression by B7 transduction (Fig. 2 *B*).

*Transduction of the B7 Gene Eliminates the Tumorigenicity of Immunogenic Tumors but Does Not Affect Nonimmunogenic Tumors.* B7+ cells from each of the eight transduced tumor lines were injected subcutaneously into syngeneic, immunocompetent mice, and mock-transduced cells from the respective tumors served as controls. The B7− control tumors grew progressively in all mice inoculated. In contrast, four B7+ immunogenic tumors, i.e., RMA, E6B2, EL4, and P815, either did not grow at all (RMA) or completely regressed after a transient growth (E6B2, EL4, and P815) in the mice (Fig. 3, Table 3). B7+ clones from RMA, E6B2, and P815 grew progressively in BALB/c nude mice, indicating that T cell–mediated immunity was responsible for their rejection in immunocompetent mice. On the other hand, B7+ EL4 regressed also in the nude mice (Table 3). No regressions were seen in immunocompetent mice injected with B7+ clones derived from the nonimmunogenic tumors that were nonimmunogenic.

*B7-transduced Immunogenic Tumors, but Not Nonimmunogenic Tumors, Can Induce Systemic Immunity to Wild-type Tumors.* The ability of both immunogenic and nonimmunogenic tumors to induce systemic immunity was examined. Preimmunization of mice by a single injection of irradiated EL4 or P815 did not protect them from challenge with the respective wild-type cells. In contrast, the same dose of nonirradiated B7+ EL4 or P815 cells completely protected mice from challenge with the respective wild-type tumors (Fig. 4 *A*). To exclude that this difference is due to a radiation effect on

**Table 3.** *Tumorigenicity of B7-transduced Tumor Lines in Syngeneic and in BALB/c (nu/nu) Mice*

| | Tumor incidence* | | | | | |
|---|---|---|---|---|---|---|
| | Syngeneic mice‡ | | | BALB/c nude mice | | |
| Cell line | WT | Mock | B7+ | WT | Mock | B7+ |
| RMA | ND | 10/10 | 0/10§ | ND | ND | ND |
| E6B2 | 18/20 | ND | 0/10§ | 5/5 | 10/10 | 10/10 |
| P815 | 10/10 | 10/10 | 0/10§ | ND | 10/10 | 10/10 |
| EL4 | 20/20 | 20/20 | 0/30§ | ND | 10/10 | 0/10§ |
| MCA101 | 15/15 | 15/15 | 15/15 | ND | 5/5 | 5/5 |
| MCA102 | 15/15 | 10/10 | 14/14 | 5/5 | 5/5 | 5/5 |
| Ag104 | 15/15 | 15/15 | 15/15 | ND | 5/5 | 5/5 |
| B16 | 10/10 | 10/10 | 15/15 | ND | ND | ND |

* Mice were injected with tumor cells subcutaneously at 1 × MiTD (RMA at 100 × MiTD). Tumor size was assessed by measuring two perpendicular diameters in millimeters by a caliper weekly for each animal. Mice with tumors <2 mm in diameter at week 6 after injection were scored as negative.
‡ Mouse strains syngeneic to the corresponding tumor lines are listed in Table 1. (*WT*) wild type; (*Mock*) mock transduced; (*B7+*) B7-transduced tumor cells.
§ Significantly different from the WT and Mock groups ($p < 0.05$).

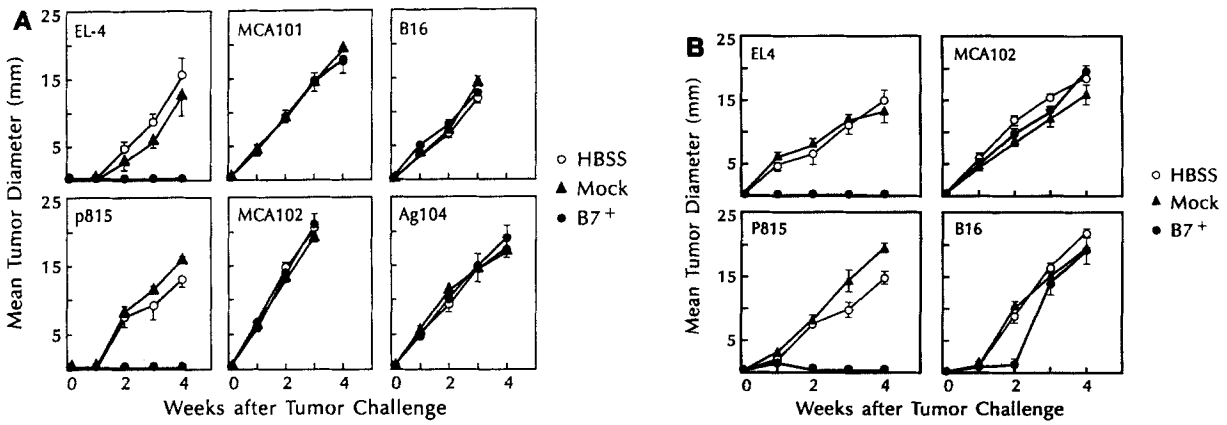



**Figure 4.** B7+ immunogenic tumors, but not nonimmunogenic tumors, induce protective immunity against wild-type tumors. Mice were injected with (*A*) HBSS, irradiated, mock- or B7-transduced tumor cells (B7+ *EL4* and B7+ *P815* were nonirradiated) intraperitoneally at 2× MiTD. 3 wk later, the mice were challenged subcutaneously in the right back with the respective wild-type tumor cells at 1× MiTD. (*B*) Alternatively, all mice were also injected with live tumor cells. 10 d later, tumor nodules were removed surgically. 2 wk after tumor removal, mice were challenged with wild-type tumor cells at 1× MiTD. Tumor size was assessed weekly by measuring perpendicular diameters with a caliper. The results are expressed as mean diameters (in millimeters) of tumors from groups of five mice. Error bars represent the standard deviation of the mean. The same results were obtained in two experiments with each cell line.

the wild-type EL4 and P815 cells, experiments were also performed in which mice were immunized by s.c. injection of live tumor cells that were either B7+ or B7− followed, 10 d later, by surgical removal of the tumor nodules (Fig. 4 *B*). The results were similar. No recurrence was seen in mice immunized with B7+ cells during a follow-up period of 3 mo after challenge. Preimmunization with B7+ clones from the four nonimmunogenic tumors, i.e., from B16, Ag104, MCA101, or MCA102, did not confer any protective immunity against challenge with the respective wild-type cells (Fig. 4).

We also investigated whether immunization with B7+ cells could be used to treat already growing wild-type tumors using the EL4 lymphoma, which was the most aggressive of our four immunogenic tumors. Mice were injected s.c. with wild-type EL4 and were repeatedly injected intraperitoneally with B7+ EL4 cells starting 4 d after tumor inoculation. This treatment cured 60% of mice (Fig. 5), whereas injection of irradiated wild-type or mock-transduced EL4 cells did not affect the progressive growth of the EL4 lymphoma.

*B7+ Immunogenic Tumors, but Not Nonimmunogenic Tumors, Are Superior to B7− Tumors to Augment the Antitumor Response and Specificity of CTL.* To test whether the increased immunogenicity of tumors transduced with B7 correlated with an increased CTL activity, we compared the ability of B7+ and B7− cells from mastocytoma P815 and lymphoma EL4 to induce tumor-specific CTL as assayed in vitro. Mice were immunized by injection of either B7- or mock-transduced cells followed by surgical removal of tumor nodules 10 d after injection. Splenocytes were harvested and further stimulated in vitro by cocultivation with irradiated wild-type cells. Subsequently, they were tested for CTL activity in a standard $^{51}$Cr-release assay. Mice were also immunized according to an alternative method, i.e., by the injection of nonirradiated B7+ or irradiated mock-transduced cells. Similar results were observed by both immunization methods: mice immunized by B7+ cells generated significantly higher CTL activity against the respective wild-type tumor cells than mice immunized by B7− cells (Fig. 6). The CTL from B7+ EL4-immunized mice were tumor specific in that they did not lyse syngeneic, Con A–induced spleen blasts, syngeneic TIMI.4 lymphoma cells, or allogeneic P815 cells (data not shown). Similar results were obtained in the P815 tumor model. In contrast, immunization with B7-transduced MCA102 or B16 did not induce any specific CTL response to the corresponding wild-type tumors (Fig. 6).

T cell lines were generated by repeated stimulation with either irradiated B7+ or B7− EL4 cells in order to examine

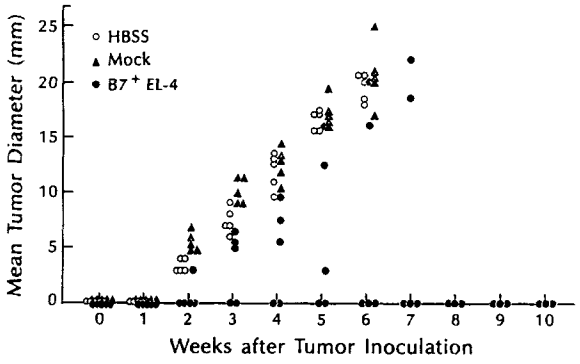



**Figure 5.** B7+ EL4 cells induce systemic immunity against existing wild-type tumors. C57BL/6 mice, five per group, were injected subcutaneously with 5 × $10^4$ wild-type EL4 tumor cells. Mice were then treated 4 d later by i.p. injection with HBSS, irradiated, mock-transduced or B7+ EL4 at 5 × $10^5$. The same injections were subsequently repeated seven times at 5 d intervals. Tumor size was assessed weekly by measuring perpendicular diameters with a caliper. The experiments were terminated when the tumors reached 20–30 mm in diameter, severe ulceration and bleeding had developed, or the mice had died. The results are expressed by tumor size of individual mouse. The same results were obtained in two other experiments.

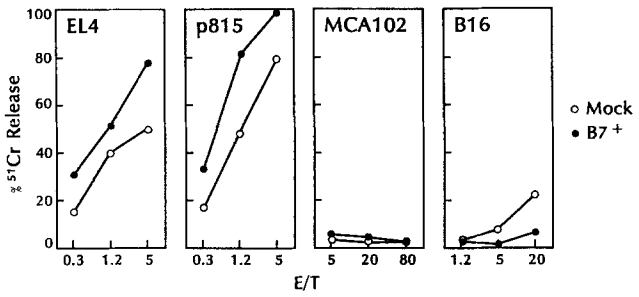



**Figure 6.** Comparison of CTL activity generated from mice immunized with either B7- or mock-transduced tumor cells. The mock (O) or B7-transduced tumor cells (●) at $10^6$ were injected subcutaneously into syngeneic mice. 10 d later, tumor nodules were removed surgically. Spleen cells were prepared 14 d after injection, and were cocultivated with γ-irradiated wild-type cells for 5 d. CTL activity against $^{51}$Cr-labeled wild-type cells was measured as described in Materials and Methods. There are significantly differences between mock and B7$^+$ groups in every E/T ratio ($p < 0.05$).

whether the presence of B7 on the stimulator cells would augment a tumor-specific cytolytic activity. In both cases, irradiated EL4 cells were used for stimulation of the T cells every 7–10 d in the presence of a low dose of IL-2 (10 U/ml). 1 mo later, the T cell lines were tested for phenotypic markers and assayed for specific lytic activity towards wild-type EL4 cells. In both types of cultures, >95% of the cells were CD8$^+$, and they were highly cytolytic for EL4 cells but did not lyse the syngeneic TIMI.4 lymphoma line, when tested in a standard $^{51}$Cr-release assay (Fig. 7). However, the T cells stimulated with B7$^-$ EL4 had high NK activity as demonstrated by the lysis of NK-sensitive YAC-1 cells, whereas T cells stimulated with B7$^+$ EL4 did not (Fig. 7). Several CD8$^+$ CTL clones, which are K$^b$ restricted and highly specific for EL4, have recently been established from the T cell lines stimulated with B7$^+$ EL4 (Chen, L., unpublished data). These results suggest that antigen presentation together with B7 costimulation augments the specificity of antitumor CTL maintained in vitro.

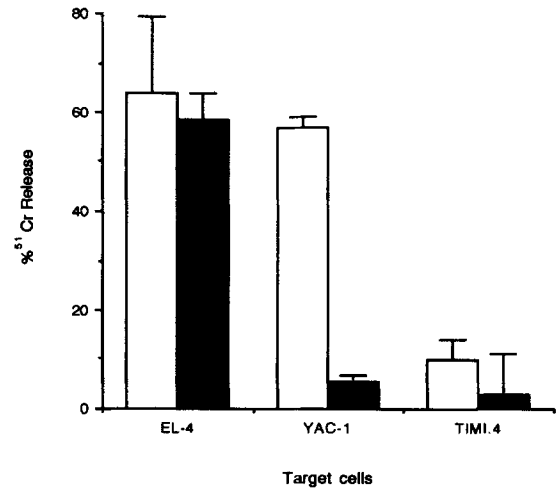



**Figure 7.** Specificity of CTL line to EL4 tumor cells. B7-transduced EL4 cells, $10^6$ per mouse, were injected subcutaneously into C57BL/6 mice. Spleen cells were prepared 14 d after injection and were repeatedly stimulated in vitro with γ-irradiated, mock-transduced EL4 cells (*open columns*) or with B7$^+$ EL4 cells (*solid columns*) every 7–10 d in the presence of irradiated syngeneic spleen cells and 10 U/ml of human IL-2 (Cetus Corp.). CTL activity against $^{51}$Cr-labeled target cells was measured as described in Materials and Methods. This assay was done at passage 8. E/T ratio = 0.1.

## Discussion

Our results indicate that the immunogenicity of tumors, as determined by immunization and challenge experiments, is critical to the effect of B7 costimulation on tumor immunity. The capacity of tumor antigens to induce immune responses, even when weak, could be amplified by the expression of B7 in tumors so as to preclude their growth in syngeneic, immunocompetent hosts. In contrast, nonimmunogenic tumors continued to grow progressively after transfer of the B7 gene. These data support the view that both an antigen-specific signal 1 and a nonspecific, costimulatory signal 2 are required for the amplification of T cell immunity.

A single injection of syngeneic mice with B7$^+$, but not with B7$^-$, EL-4, or P815 cells protected them from challenge with the respective wild-type cells (Fig. 4), indicating that a weak immune response against tumor antigens was amplified. In these experiments, the B7$^+$ tumors were either allowed to form tumor nodules that regressed within a 2–4-wk period, or such nodules were surgically removed at day 10 after inoculation. The latter procedure allowed for a better comparison since both the B7$^+$ and B7$^-$ tumors had grown to approximately the same size.

Similar to what we have reported for the highly immunogenic E7C3 line (12), transfer of B7 into tumor cells increased their ability to induce a strong, tumor-specific cytolytic response mediated by CD8$^+$ T cells (Fig. 6). The finding that wild-type EL4 and P815 cells were effectively lysed by CTL in vitro may explain why a systemic immune response towards wild-type tumors was seen in vivo. Furthermore, a therapeutic effect, causing the regression of small, established tumors, was seen in mice bearing wild-type EL4 cells and subsequently treated by injection of the respective B7$^+$ cells. These results, obtained with tumors of weak immunogenicity, extend our previous findings using the highly immunogenic E7C3 line (12).

It is noteworthy that the degree of NK cell reactivity against YAC-1 target cells was much less when the CTL had been cocultivated with B7$^+$ EL4 cells than when B7$^-$EL4 cells were used (Fig. 7). Since highly specific reactivity of in vitro–expanded polyclonal T cells is desirable in adoptive cellular immunotherapy, the fact that sensitization with tumor cells expressing B7 augments CTL activity in vivo, as well as CTL specificity as assayed in vitro, should be taken into account.

We found that B7-transduced EL4 regressed in BALB/c nude mice (Table 3), as well as in NK-deficient beige mice, and that they were resistant to NK cell–mediated lysis in vitro (Chen, L., unpublished data). Regression started 7–10 d after

a transient tumor growth (Fig. 3), suggesting that it was due to a host response. A possible explanation of the regression seen in nude mice is that B7-transduced EL4 may produce some cytokines which induce an antitumor response.

The finding that transduction of B7 into nonimmunogenic tumors failed to make them immunogenic can have several explanations. It is possible that nonimmunogenic tumors, such as the four studied by us, either lack molecules that can serve as tumor antigens recognized by T cells, or are deficient in the processing, transportation, or presentation of such molecules, i.e., that the tumor cells do not deliver signal 1. It is relevant, therefore, that MHC class I molecules are undetectable in B16, MCA101, and MCA102 (31, 32, and Fig. 2 *B*) and that Tanaka et al. (31) could induce a protective immunity against challenge with wild-type B16 by immunization with B16 cells transfected with the MHC class I $K^b$ gene. Likewise, in vitro treatment of MCA101 sarcoma cells by IFN-γ (32) or transfection by IFN-γ cDNA (33), which upregulate the expression of MHC class I, enhanced their sensitivity to lysis by CTL in vitro and induced the infiltration of tumors by $CD8^+$ lymphocytes in vivo; systemic immunity against tumors was not examined, however. Furthermore, Dranoff et al. (34) have recently reported that B16-F10 melanoma cells transduced with a gene encoding GM-CSF could elicit protective immunity against challenge with wild-type B16-F10. The latter finding may be explained by the production of some cytokines or costimulatory molecules other than B7 induced by GM-CSF. Taken together, these results suggest that lack of immunogenicity of B16 and MCA101 was not related to the absence of putative tumor rejection antigens and one may speculate that coexpression of B7 with either MHC class I molecules or IFN-γ will provide the double signaling needed for effective T cell activation against some nonimmunogenic tumors.

We like to point out, however, that the in vitro expression of MHC class I molecules as detected by FACS® analysis is not always a reliable indicator of tumor immunogenicity. Whereas the nonimmunogenic Ag104 tumor expresses a high level of MHC class I molecules, two of our highly immunogenic tumor lines, E6B2 (Fig. 2 *B*) and E7C3 (12) displayed low or undetectable levels of MHC class I, a finding which we have discussed previously (12).

It is also possible that costimulatory molecules other than B7 or B7-like molecules, such as intercellular adhesion molecules (ICAMs), vascular cell adhesion molecule 1 (VCAM-1), lymphocyte function–associated antigen 3 (LFA-3) and heat-stable antigen (HSA), are also needed to induce the rejection of some neoplasms since greater $CD4^+$ T cell responses have been seen when coexpressing ICAM-1 (35), HSA (36) or VCAM-1 (37) with B7 on APCs. This possibility is also suggested by the fact that some B lymphomas of human (7) and mouse origin (Table 1) express both high levels of B7 and MHC class I antigens.

Although it remains controversial to what extent human cancers are immunogenic (1), T cell–mediated responses to human tumor cells (38), and in some cases, to well-defined human tumor antigens, such as the MAGE antigen of melanomas, have been demonstrated (39). Therefore, augmentation of T cell costimulation by transfer of the B7 gene may be therapeutically beneficial in selected human cancers. Further investigations in which genes encoding B7 and/or other costimulatory molecules, as well as genes encoding MHC class I molecules, are transduced into tumor cells, will probably extend the group of murine and human tumors to which rejection responses can be obtained.

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