T Cell Costimulation by B7/BB1 Induces CD8 T Cell-dependent Tumor Rejection: An Important Role of B7/BB1 in the Induction, Recruitment, and Effector Function of Antitumor T Cells

By Lakshmi Ramarathinam, Madalyn Castle, Yan Wu, and Yang Liu

From the Michael Heidelberger Division of Immunology, Department of Pathology and Kaplan Comprehensive Cancer Center, New York University Medical Center, New York 10016

Summary

A successful antitumor T cell immune response involves induction, recruitment, and effector function of T cells. While B7/BB1 is known as a major costimulatory molecule in the induction of T cell responses, its role in T cell recruitment and effector function is still unclear. In this study, we show that introducing a major costimulatory molecule B7/BB1 into a major histocompatibility complex class II–negative tumor cell line, J558, results in a drastic reduction of its tumorigenicity. The tumor rejection depends on CD8 T cells but not CD4 T cells. However, unlike the previous reports on melanoma cell lines, B7/BB1-transfected J558 cells fail to induce cross-protection against parental J558 cells. The B7/BB1-transfected (J558-B7), but not untransfected J558 cells (J558-Neo) induce a CD8 T cell–dominant inflammatory response, and the T cells isolated from the tumor infiltrating lymphocytes (TIL) are polyclonal in terms of their T cell receptor Vβ usage. Most surprisingly, the freshly prepared TIL have a potent, CD8 T cell–mediated cytotoxicity on tumor cells without any in vitro stimulation. The cytotoxic T lymphocyte (CTL) activity can be blocked by anti-CD8 monoclonal antibody (mAb). Interestingly, the CTL lyse J558-B7 about 10- to 80-fold more efficiently than untransfected J558-Neo cells. This preferential lysis cannot be attributed to recognition of B7/BB1-derived antigen by the T cells. This finding, together with the lack of the cross-protection between the J558-B7 and J558-Neo, suggests that B7/BB1 can also function at the effector phase of CTL responses. This notion is confirmed by our findings that the lysis of J558-B7 can be blocked by anti-B7 mAbs. Taken together, our results indicate that not only can the B7/BB1 molecule function as a costimulatory molecule at the initiation of immune response, it can also play a major role in T cell recruitment and effector function. This conclusion has significant implications for immunotherapy of tumors.

T cells are the main effector cells in antitumor immune responses (1). Activation of tumor-specific T cells is therefore crucial for immune intervention against tumors. Recent studies have demonstrated that two types of signals are needed to initiate a T cell response (2–6). Signal one is derived from the interaction of TCR with peptides presented by MHC class I and class II molecules, whereas signal two is derived from the costimulatory pathway. Failure to deliver either of these two signals may allow tumors to evade the immune system. Several tumors have been reported to evade immune surveillance by downregulation of MHC molecules, or other molecules critical for antigen presentation (7–9). For this category of tumors, reintroduction of MHC molecules or cytokines which upregulate the expression of MHC molecules, induces antitumor immune responses and rejection of the tumor (7–9).

A significant proportion of tumors, however, express a normal level of MHC antigen and present antigen normally. Over 15 yr ago, Talmage et al. first reported that a tumor cell line failed to induce an allogeneic T cell response despite its apparently normal expression of MHC molecules (10). This original observation illustrated the concept that expression of antigen alone is not sufficient to activate T cells. This category of tumors, much like many nonhemapoietic cells, lacks costimulatory activity. It is therefore of great interest to test if it is possible to augment tumor immunogenicity by enhancing their costimulatory activity.

Recent studies have demonstrated that the B7 molecule

L. Ramarathinam and M. Castle contributed equally to this study.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/04/1205/10 $2.00
Volume 179 April 1994 1205–1214

is a costimulatory molecule for T cells. Thus B7 binds to CD28 which has been demonstrated to transduce signals important for T cell clonal expansion (11–15). In addition, fibroblasts transfected with B7/BB1 induce clonal expansion of T cells (12, 16, 17). Antibodies or chimeric molecules that bind B7/BB1 block T cell clonal expansion induced by anti-CD3, allogeneic MHC, and peptide/self-MHC (16–20). Furthermore, the Fab fragment of anti-CD28 antibody 9.3 induces hyporesponsiveness in alloreactive T cells (21), while intact anti-CD28 mAb rescues clonal anergy induced by fixed APCs pulsed with specific peptides (15). Taken together, these results demonstrate that B7/BB1 fulfills the function originally assigned to costimulators. It is thus feasible to enhance antitumor immune responses by introducing the B7/BB1 molecule into the tumor cells.

Recently, three laboratories reported that transfection of B7/BB1 molecule can successfully induce rejection of a MHC class II–positive melanoma (22, 23) and a sarcoma transfected with MHC class II (24). In those studies, B7/BB1 was shown to function at the induction phase of T cell response. In this report, we investigated the effect of B7/BB1 on the induction, recruitment, and effector function of CD8 T cells by introducing B7/BB1 into a MHC class II–negative plasmacytoma, J558. Our results demonstrate that costimulatory molecules are required for efficient induction, recruitment, and effector function of antitumor CD8 T cell responses.

Materials and Methods

Cell Lines and Experimental Animals. Plasmacytoma J558, thymoma EL4 cells, and a macrophage cell line P388D1 (American Type Culture Collection, Rockville, MD) were cultured in RPMI medium containing 5% FCS and 100 μg/ml penicillin and streptomycin. BALB/cByJ and CBA/CaJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Male mice between 6 and 12 wk old were used in this study.

Antibodies. Anti-B7 mAbs 1D5, 3A12, and 7A5 were produced in this laboratory and have been described fully (25); 16.10A1 (26) was kindly provided by Dr. Hans Reiser (Dana-Farber Cancer Institute, Boston, MA). Other antibodies used were: Anti-PC.1 (4G6, a kind gift from Dr. Dumont, Merck, Sharp & Dohme Research Laboratories, Rahway, NJ, reference 27); anti-lymphocyte function–associated antigen 1 (LFA-1)[1] (M1/17, reference 28); anti-MHC class I (K44, reference 29); anti-MHC class II I-A^d (MDK6, reference 30); anti-Vβ antibodies Vβ2 (B20.6, provided by Dr. Kappler and Dr. Marrack, National Jewish Center for Immunology and Respiratory Diseases, Denver, CO), 3 (KJ-25, reference 31), 6 (PR4-7, reference 32), 7 (TR310, reference 33), 8 (F23.1, reference 34), 11 (PR3-15, references 35, 36), 14 (14-2, reference 37); anti-CD4 (GK1.5, reference 38); anti-CD8 (53.6.7, reference 39; and 2.43, reference 40).

Plasmid Construction and Transfection of J558 Cells and EL4 Cells. PSRα vector containing resistance gene for neomycin (G418) was kindly provided by Dr. Norvak of Yale University (New Haven, CT). The murine B7/BB1 gene in pLN vector was provided by Dr. Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). pLN-B7/BB1 was linearized with XbaI and inserted into pSRα at the XbaI site, resulting in a pSRα–B7/BB1 construct. 5×10^6 J558 cells were transfected with 50 μg of pSRα–B7/BB1 or pSRα plasmids by electroporation. The transfected cells were selected at 48 h after electroporation with RPMI-FCS medium containing G418 (0.6 mg/ml; Sigma Chemical Co., St. Louis, MO). The surviving colonies were stained with anti-B7 antibodies and positive clones amplified.

Tumorigenicity Assay. Given numbers of J558-B7 or J558-Neo cells were suspended in 100 μl of PBS and injected subcutaneously in the inguina. The tumor incidence was observed every other day. Tumors >3 mm in diameter were scored as positive tumors.

Cross-protection Experiment. Two different protocols were used to analyze the cross-protection between J558-B7 and J558-Neo. First, in coinjection experiments, 5×10^6 J558-Neo cells were mixed with either 5×10^6 or 10×10^6 J558-B7 cells and injected subcutaneously into syngeneic BALB/cByJ mice; the tumor incidence of the coinjected mice was compared with mice injected with either J558-Neo or J558-B7 alone. 15 mice were used for each group. Second, in challenge experiments, BALB/c mice were injected subcutaneously with either PBS, 5×10^6 J558-B7 cells, or 5×10^6 J558-Neo in the left inguen. 1 wk later, all mice were challenged with 5×10^6 J558-Neo tumor cells subcutaneously in the right inguen. These distinct sites allow identification of the origin of the tumor. The tumor incidence was calculated from each group of 10 mice.

Analysis of Tumor-infiltrating Lymphocytes by Flow Cytometry. J558-B7 and J558-Neo tumors were excised from mice that were inoculated subcutaneously with J558-B7 or J558-Neo tumor cells, respectively. Single cell suspensions were prepared by grinding tumors with frosted glass slides. Viable cells were isolated by centrifugation through a bed of Ficoll-Hypaque solution. These viable cells were analyzed by single or two-color flow cytometry.

Single color assay. pSRα-neo or pSRα–B7/BB1-transfected J558 cells were incubated with either rat or hamster mAbs at 4°C for 30 min. Unbound mAbs were washed away by three consecutive washes with cold PBS containing 1% newborn bovine serum and 0.1% sodium azide. The bound mAbs were detected with either FITC-labeled mouse anti–rat IgG (Accurate Chemical & Science Corp., Westbury, NY) or FITC-labeled goat anti–hamster IgG (Caltag Laboratories, S. San Francisco, CA). The fluorescence was analyzed using FACScan® (Becton Dickinson & Co., Mountain View, CA).

Two-color flow cytometry. The percentage of CD4 and CD8 T cells was determined using a mixture of FITC-labeled anti-CD4 mAb and phycoerythrin-labeled anti-CD8 mAb (PharMingen, San Diego, CA). The data shown are the percentage of certain populations of T cells among total lymphocytes gated on the basis of forward scatter and side scatter. To analyze the Vβ usage of the CD8 T cells from tumor or from spleen, we used phycoerythrin-labeled anti-CD8 mAb to label CD8 T cells. The cells stained with anti-Vβ mAbs and second-step reagents were washed three times and were incubated with normal rat Ig (1 mg/ml; Chemicon International Inc., Temecula, CA) for 30 min to block the unsaturated binding sites of goat anti–hamster Ig or mouse anti–rat Ig. Phycoerythrin-labeled anti-CD8 mAb was added at 1:100 dilution, incubated for 30 min, and washed three times. The data shown are the percentages of CD8 T cells that bound specific anti-Vβ antibodies, with that of the nonspecific binding in the absence of anti-Vβ antibodies subtracted. All samples were fixed with 1% paraformaldehyde before analysis by FACS®.

Cytotoxic T Cell Assay. The cytotoxicity of T cells was determined in a 6-h ^{51}Cr release assay (39). Two populations of CD8

[1] *Abbreviations used in this paper:* MFC, mean fluorescence channel; LFA-1, lymphocyte function–associated antigen 1; TIL, tumor-infiltrating lymphocytes.

T cells were isolated for CTL assay. First, CD8 T cells from normal BALB/cByJ spleens by two rounds of treatment with complement and a cocktail of antibodies, including anti-B220 mAb RA3-3A1/6.1, anti-CD4 mAb 2B6.2D8, anti-Mac-1 mAb M1/70.15.11.5H, and anti-HSA mAb J11d. The CD8 T cell preparation contained >80% CD8 T cells and no detectable CD4 T cells, or B cells. The second group of effector cells were isolated from tumors. Briefly, a single cell suspension prepared from J558-B7 tumors (2×10^7/ml) was incubated with 1:200 dilution of the anti-PC.1 ascites for 45 min. Unbound mAb was washed away and the antibody-coated cells were incubated with goat anti-rat IgG (10 μg/ml) plus 1:6 dilution of low-tox rabbit complement (Accurate Chemical & Science Corp.) for 45 min. P388D1, J558-Neo, and several independent lines of B7/BB1 transfected J558 cells were labeled with ^{51}Cr and used as target cells. The released ^{51}Cr was determined using a 1205 β-plate counter (Pharmacia LKB, Piscataway, NJ). Briefly, 50 μl of each supernatant was mixed with 250 μl scintillation fluid in a 96-well T-tray (Cat. No. 1205-451; Pharmacia LKB) and sealed by T-tray tape sealer (Cat. No. 1205-452; Pharmacia LKB). This method is generally ~10-fold more sensitive than the conventional gamma-counter. The percentage of specific release was calculated as described (41).

In Vivo Depletion of T Cells. Mice were injected with 300 μg/mouse of either normal rat Ig, anti-CD4 mAb GK1.5, or anti-CD8 mAb 2.43 at 48 h before, 48 h after, and 6 d after tumor inoculation. The efficiency of depletion was analyzed by flow cytometric analysis of CD4 and CD8 T cells in the spleen.

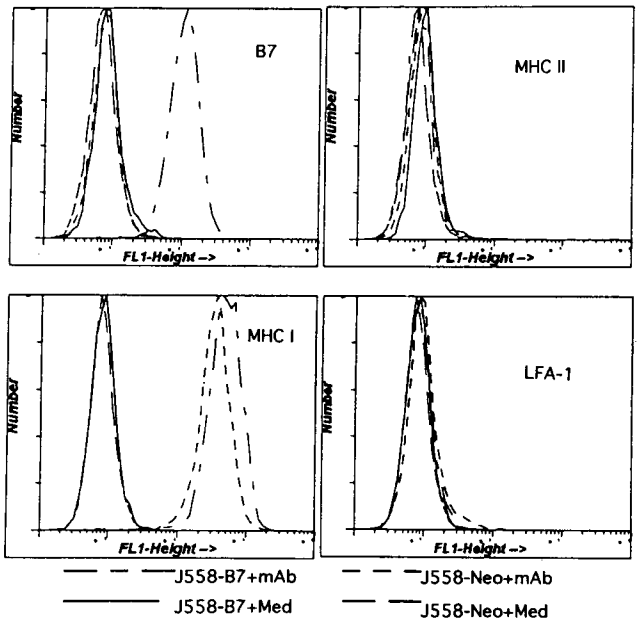


Figure 1. Analysis of cell surface phenotype of J558-B7 and J558-Neo by flow cytometry. The tumor cells were incubated with anti-B7 anti-MHC class I, class II, and LFA-1 mAbs. The binding of these mAbs was detected by FITC-labeled second-step reagents. 5,000 events were analyzed for each sample.

Results

Transfection of J558 Cells with B7/BB1. J558 cells were transfected with either pSRα–B7/BB1 or parental vector pSRα. The cell surface expression of B7/BB1 in G418-resistant clones was determined by flow cytometry using anti-B7 mAbs. Of 17 clones that expressed B7/BB1, one clone, J558-B7, was characterized and the cell surface expression of a number of cell surface antigens was compared with J558 cells transfected with vector alone, J558-Neo, by flow cytometry. As shown in Fig. 1, J558-B7 expresses a significant amount of B7/BB1, whereas J558-Neo does not express any detectable amount of B7/BB1. J558-B7 and J558-Neo express identical levels of MHC class I. They both lack detectable MHC class II and LFA-1. Further experiments showed that CD45$^-$ tumor cells from freshly excised tumors fail to express MHC class II and all MHC class II$^+$ cells in the tumor are CD45$^+$ inflammatory cells (data not shown). J558-B7 is also significantly more competent in stimulating allogeneic T cell responses than J558-Neo cells (data not shown). Thus, J558-B7 expresses significant amounts of B7/BB1 and has costimulatory activity for CD8 T cells.

B7-transfected J558 Cells Induce a CD8 T Cell–dependent, CD4 T Cell–independent Tumor Rejection. To test whether expression of B7 will reduce the tumorigenicity of J558 cells, we compared the tumor incidence in syngeneic mice that were injected with J558-B7 or J558-Neo. As shown in Fig. 2, J558-B7 gives a significantly reduced tumor incidence and delayed tumor onset when injected into syngeneic BALB/c ByJ mice, as compared with J558-Neo. Mice injected with J558-Neo developed tumors between 1 and 2 wk; the tumor incidence correlated with the number of tumors cells injected. In contrast, the tumor incidence in mice injected with J558-B7 showed a reciprocal relationship with the number of tumor cells injected. Mice that received 5×10^6 J558-B7 tumor cells had a significantly lower tumor incidence than mice that received 1.25×10^6 J558-B7 tumor cells. 50% of mice that received 5×10^6 J558-B7 never developed tumors during the 8-mo study. This reciprocal relationship is consistent with the notion that J558-B7 is a immunogen, and the reduced tumor incidence was due to antitumor immune responses.

To test the role of CD4 and CD8 T cells in the rejection of J558-B7 tumor, we injected anti-CD4 or anti-CD8 mAbs to eliminate CD4 and CD8 T cells in vivo and compared the incidence in mice depleted of either CD4 or CD8 T cells. As shown in Fig. 3, injection of anti-CD8 mAb significantly increased the tumor incidence in mice that received J558-B7, whereas injection of anti-CD4 antibody failed to do so (Fig. 3). This result indicates that tumor rejection depends on CD8 but not CD4 T cells. This result also rules out the possibility that the reduced tumor incidence of J558-B7 is due to the intrinsic growth characteristic of J558-B7.

Expression of B7/BB1 on Tumor Cells Induces Selective CD8 T Cell Recruitment into the Tumors. We have phenotyped the tumor-infiltrating lymphocytes (TIL) by flow cytometry. As shown in Fig. 4, the J558 cells and spleen cells can be differentiated by their forward scatter and side scatter. This makes it possible to quantify the lymphocyte infiltration in the tumors. As shown in Fig. 4 *a*, substantial numbers (35%) of viable cells recovered from J558-B7 tumors are lymphocytes, whereas only about 7% of the viable cells recovered from J558-Neo tumors are lymphocytes. It is interesting to

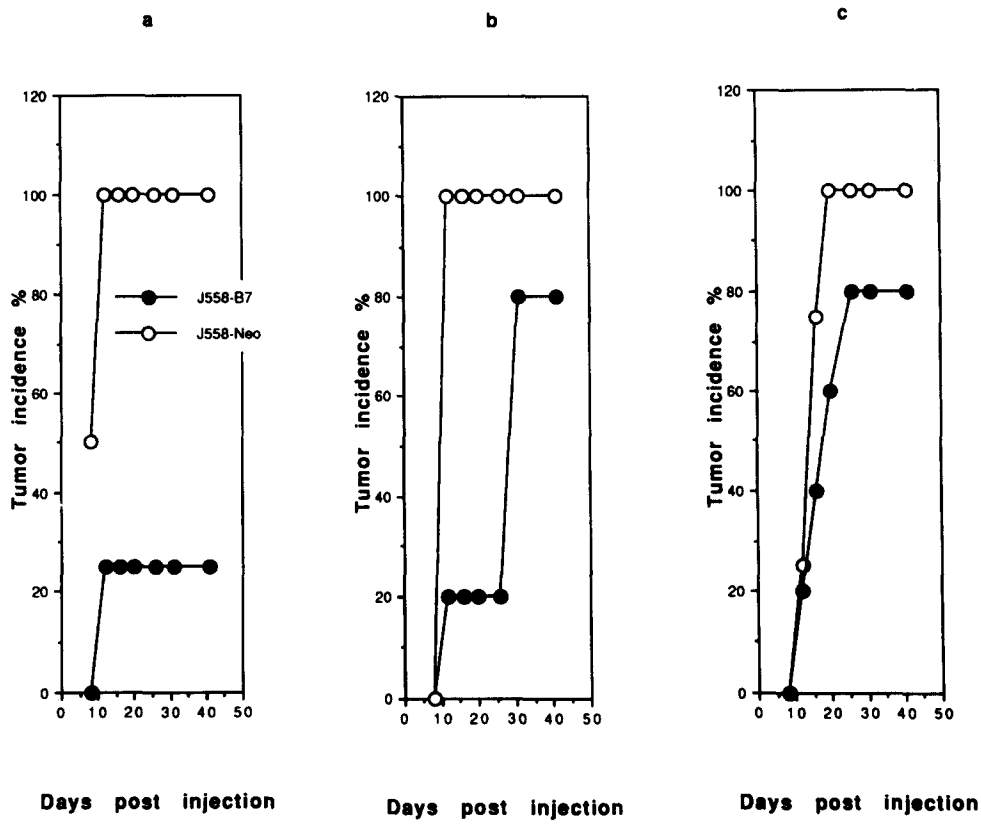


Figure 2. Tumor incidence in syngeneic BALB/cByJ mice injected with 5×10^6 (*a*), 2.5×10^6 (*b*), or 1.25×10^6 (*c*) of J558-B7 (*solid circles*) or J558-Neo (*open circles*). The tumor incidence was calculated from groups of four or five mice. Tumor cells were injected subcutaneously and tumors were scored by physical examination; tumors that were 3 mm or above in diameter were scored positive. All positive tumors scored at an early stage eventually grew into large visible tumors of >20 mm in diameter.

note that CD8 T cells preferentially infiltrated the J558-B7 tumors. TIL from the J558-B7–derived tumor have a CD4/CD8 ratio of 0.35; spleen T cells in the same mice have a ratio of 3; the TIL from J558-Neo–derived tumor have a CD4/CD8 ratio close to that of spleen cells (Fig. 4 *b*).

Despite this selective recruitment of CD8 T cells, the CD8 T cells are polyclonal in terms of their Vβ usage (Table 1). The TIL are slightly enriched for $V\beta8^+$ T cells and reduced for Vβ2, 11, and 14 positive T cells. Nevertheless, most of the Vβ tested are well represented among the TIL.

Table 1. *Vβ Usage of CD8 T Cells in Spleen of J558-B7 Tumor-bearing Mice and in the TIL*

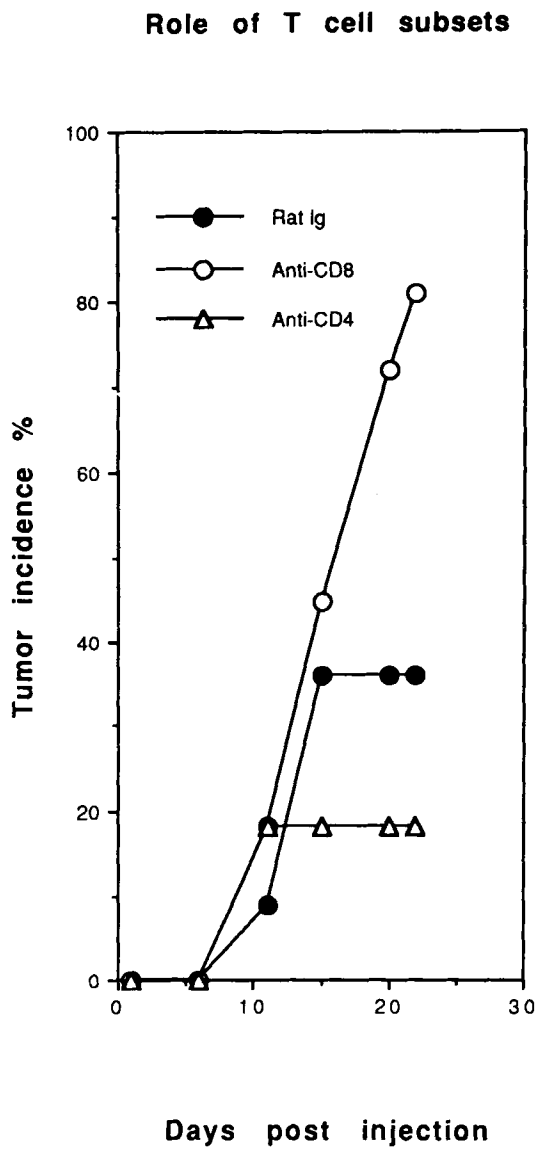
Vβ	TIL CD8 T cells	Spleen CD8 T cells
	%	
2	2.3	6.7
3	7.4	8.2
6	12.2	10.0
7	4.4	9.3
8	45.5	37.8
11	2.2	5.0
14	1.9	6.0
Total	75.9	80.0

Spleen cells or tumor cell suspensions were dually stained with phycoerythrin-labeled anti-CD8 mAb and anti-Vβ mAbs detected by a FITC-labeled second-step reagent. The data shown are percentages of $V\beta^+$ T cells among CD8 T cells. 20,000 events were analyzed per sample.

Table 2. *Cross-protection Between J558-Neo and J558-B7*

		Tumor Incidence	
Primary	Secondary	Primary	Secondary
Expt. 1: Coinjection			
J558-Neo	–	14/15	–
J558-B7	–	3/15	–
J558-B7 + J558-Neo	–	15/15	–
Expt. 2: Preimmunization			
J558-Neo	J558-B7	10/10	2/10
J558-Neo	J558-Neo	10/10	9/10
J558-B7	J558-Neo	2/10	8/10
J558-Neo	–	10/10	–
J558-B7	–	2/10	–

For primary injection, 5×10^6 tumor cells were injected subcutaneously in the left inguen, and 8 d later, the second inoculation of equal number of tumor cells were injected subcutaneously in the right inguen. The data shown in Expt. 1 are the tumor incidence on day 19 post injection. In Expt. 2, the tumor incidence shown was scored at 19 d after secondary injection and 27 d after primary injection.



Expression of B7/BB1 on the Tumor Cell Surface Is Required for Host Rejection of Tumors. The J558-B7 cells do not induce protection against the challenge injection of J558-Neo. As shown in Table 2, in coinjection experiments which have 15 mice/group, tumor incidence in the group that was injected with J558-Neo is comparable with the group that was coinjected with equal amount of J558-B7 and J558-Neo. Similarly, in another experiment, mice coinjected with 10^7 J558-B7 and 5×10^6 J558-Neo have a similar tumor incidence as the group injected with 5×10^6 J558-Neo cells (data not shown). To ascertain whether the tumor was derived from J558-Neo, we injected mice first with J558-B7 cells and subsequently with J558-Neo at a distant site. As shown in Table 2, preinjection of J558-B7 also failed to protect against a subsequent injection of J558-Neo. Thus, all three experiments fail to show any cross-protection of J558-Neo by J558-B7.

B7/BB1 Acts as an Accessory Molecule at the Effector Phase in CTL Response. To understand the mechanism of this B7-dependent rejection, we isolated TIL by depleting tumor cells with complement plus an mAb specific for PC.1, a plasma cell antigen which is expressed on J558 tumor cell surface. The freshly prepared TIL were tested for their cytotoxicity to the tumor cells. As shown in Fig. 5 *a*, the TIL have a very potent cytotoxicity against J558-B7 cells. However, the lysis of J558-Neo cells is about 80-fold less efficient than J558-B7. In five independent experiments (data not shown), the

Figure 3. Requirement of CD8 but not CD4 T cells for tumor rejection. BALB/cByJ mice were injected on day −2, and day 2, and day 6 with 300 μg/mouse of either normal rat Ig, anti-CD4 mAb GK1.5, or anti-CD8 mAb 2.43, intraperitoneally. On day 0, 5×10^6 J558-B7 cells were injected subcutaneously. Tumor incidences were calculated from groups of 11 mice each. The efficacy of the depletion of T cell subsets was monitored by flow cytometry on day 21 when all groups of mice were sacrificed. A similar depletion of CD4 and CD8 T subsets was achieved by their respective antibodies.

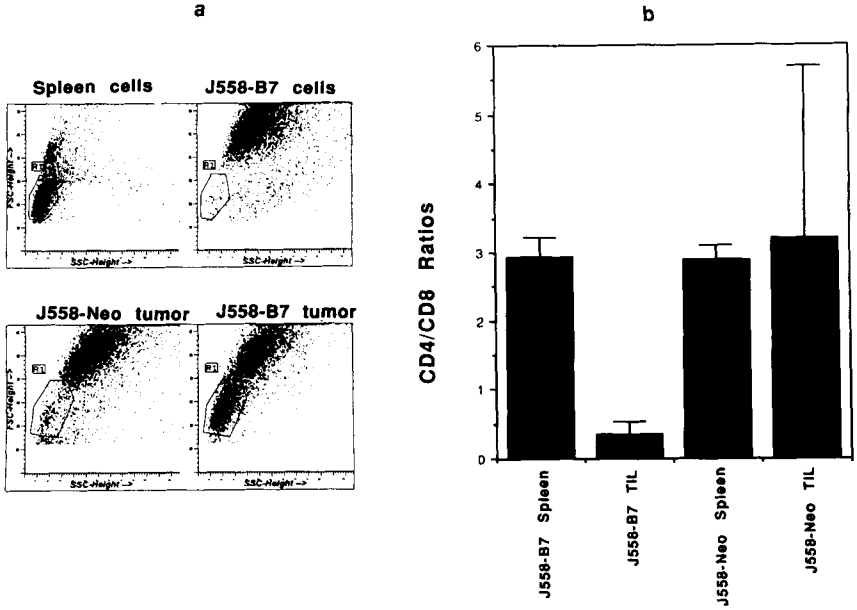


Figure 4. Analysis of inflammatory cells by flow cytometry. (*a*) Quantitation of tumor-infiltrating lymphocytes in J558 tumors. Viable cells were isolated from tumors by grinding with frosted glass slides followed by centrifugation through Ficoll-Hypaque gradient. The lymphocytes were gated on the basis of forward scatter and side scatter. The tumor derived from J558-Neo is composed of 7% infiltrating lymphocytes, whereas that derived from J558-B7 is composed of 35% infiltrating lymphocytes. The normal spleen cells and cultured J558-B7 cells were used as controls. (*b*) The CD4/CD8 T cell ratio in J558-B7 or J558-Neo derived tumors. Spleen cells or tumor cell suspensions were incubated with a mixture of phycoerythrin-labeled anti-CD8 and FITC-labeled anti-CD4 antibodies. The percentages of CD4 or CD8 T cells were measured by two-color flow cytometry. TIL were gated based on the side scatter and forward scatter. The data shown are means of CD4/CD8 ratios from four individual tumor-bearing mice in each group.

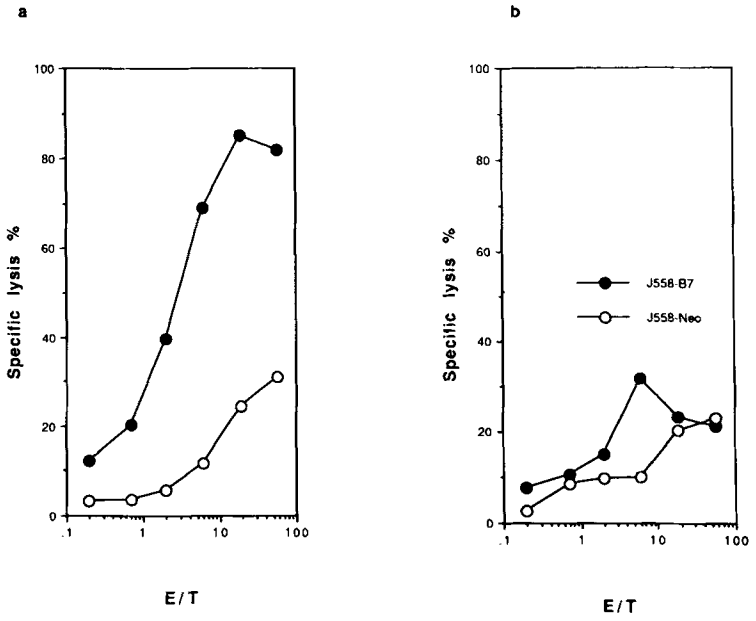


Figure 5. The TIL preferentially lyse B7/BB1 transfected J558 cells. (*a*) Lysis of J558-B7 and J558-Neo by the TIL. (*b*) CTL activity of normal CD8 T cells. TIL were isolated by depleting tumor cells with anti-PC.1 mAb followed by goat anti-rat IgG plus complement. Normal CD8 T cells were isolated from BALB/c mouse spleens as described in Materials and Methods. CTL activity was determined by a 6-h ^{51}Cr release assay.

TIL lysed J558-B7 between 10- and 80-fold more efficiently than J558-Neo. This lysis requires prior priming of CD8 T cells because CD8 T cells isolated from syngeneic mice that did not receive a tumor injection have very low cytotoxicity

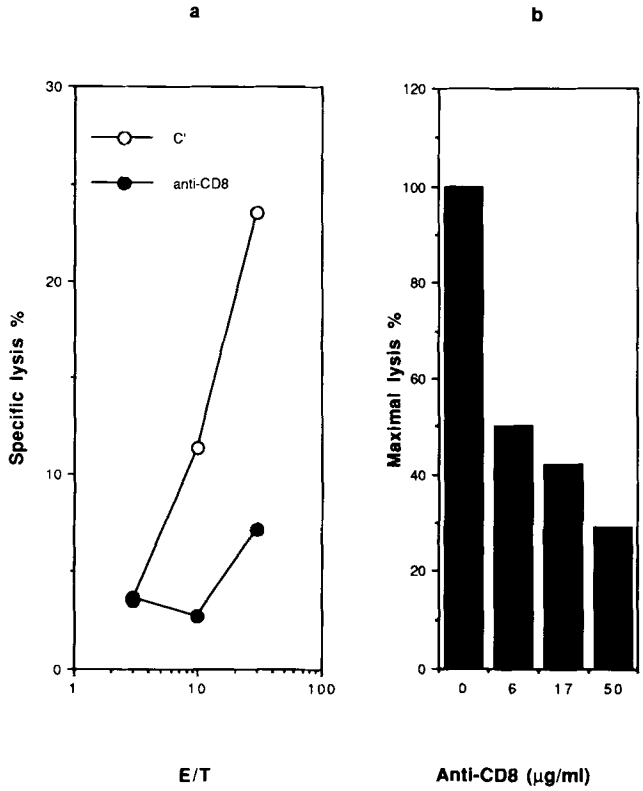


Figure 6. Characterization of the cytotoxicity of TIL. (*a*) The cytotoxicity is mediated by CD8 T cells. The TIL were treated with either C' or C' plus anti-CD8 mAb and used as effector T cells. The number of effectors was calculated based on the number of TIL before depletion. (*b*) Blocking of TIL cytotoxicity by anti-CD8 mAb 2.4.3. The maximal lysis (61%) was the specific lysis of J558-B7 at E/T = 10.

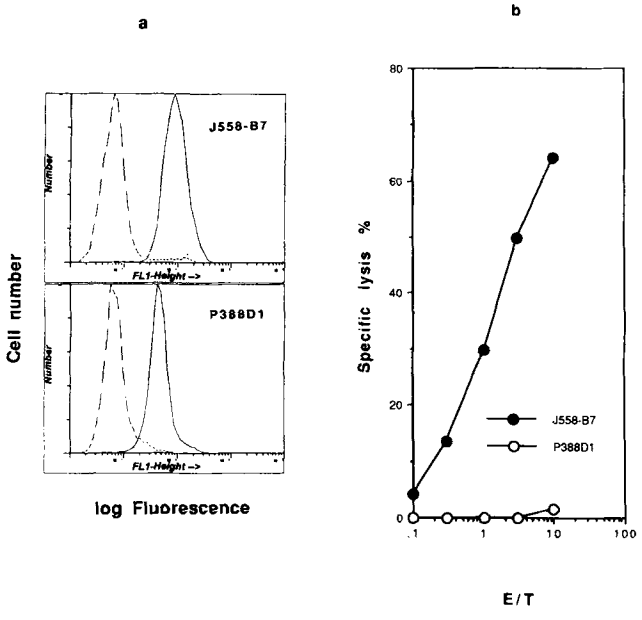


Figure 7. B7/BB1 is not the target antigens recognized by the antitumor CTL. (*a*) Expression of B7/BB1 in J558-B7 (*Top*) and P388D1 (*bottom*). The tumor cells were stained with either second-step reagent alone (*broken lines*) or anti-B7 mAb 3A12 (*solid lines*). (*b*) Lysis of P388D1 and J558-B7 by TIL isolated from J558-B7–derived tumors as determined in a 6-h ^{51}Cr release assay.

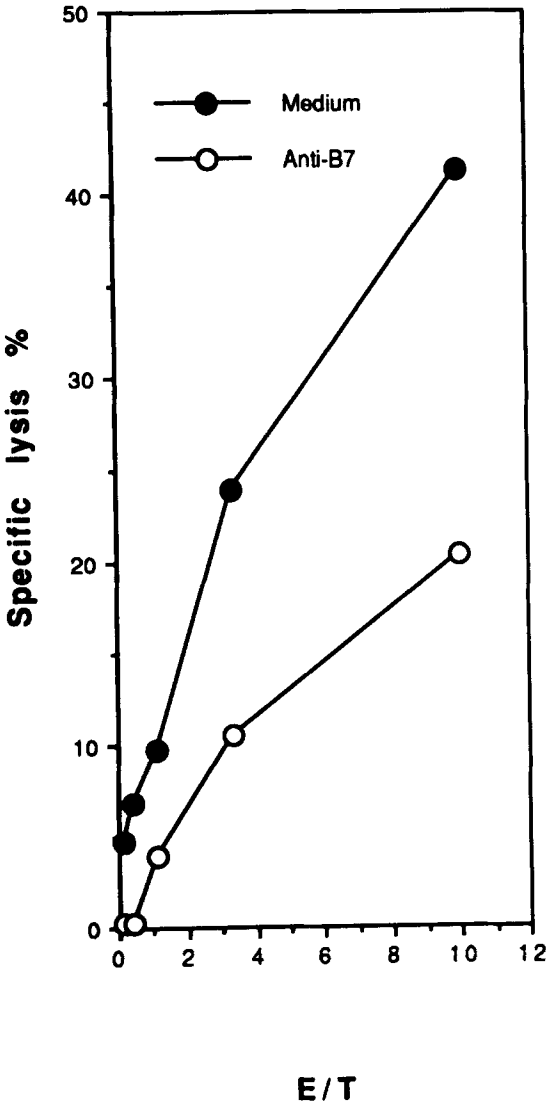


Figure 8. Anti-B7/BB1 mAbs blocks CTL lysis of J558-B7. ^{51}Cr-labeled J558-B7 were incubated with TIL isolated from J558-B7–derived tumors in the presence or absence of anti-B7 mAb 10.16A.1 (10 μg/ml) and the CTL lysis was determined in a 6-h assay.

for J558-B7 cells (Fig. 5 *b*). The cytotoxicity measured is predominantly mediated by CD8 T cells because treatment with anti-CD8 plus complement depletes most of the cytotoxicity (Fig. 6 *a*). In addition, the cytotoxicity depends on CD8 molecules expressed on the cell surface, because anti-CD8 mAb blocks the cytotoxicity in the absence of complement (Fig. 6 *b*). Thus expression of B7/BB1 appears to be required for the efficient recognition of the target by the antitumor CTL.

B7/BB1 can either act as the antigen recognized by CTL or act as an accessory molecule at the effector phase of T cell recognition. Our results ruled out the possibility that B7 donates peptides recognized by the CTL. As shown in Fig. 7, P388D1, an H-2^d macrophage tumor line that expresses significant B7/BB1 on the cell surface cannot be lysed by the CTL, suggesting that B7/BB1 per se, or a peptide derived from B7/BB1, was not the target antigen recognized by the CTL. Another formal possibility is that a mutation in B7/BB1 leads to generation of a mutant peptide that can be recognized by the CTL. Because the B7/BB1 cDNA we used for transfection has the correct sequence as determined by DNA sequencing, the mutation(s) had to occur during the amplification of the transfectants. To rule out this possibility, we tested five independent B7/BB1-transfectants for their lysis by the CTL isolated from the TIL. All five transfectants are lysed ~10-fold more efficiently than J558-Neo (Table 3). As it is highly improbable to have the same mutation in all five independently derived transfectants, we are confident that the antigen recognized by the CTL is not derived from mutated B7.

To directly demonstrate the role of B7/BB1 as an accessory molecule in the recognition of J558 by CTL, we tested whether the lysis of J558-B7 is significantly blocked by anti-B7 mAb. As shown in Fig. 8, anti-B7 mAb 10.16A.1 inhibits CTL lysis of J558-B7 by >90%. This result demonstrates that B7/BB1 can act as an accessory molecule for CTL effector function.

Discussion

A successful antitumor T cell response involves induction, recruitment, and effector function of T cells. Whereas the role of a major costimulatory molecule, B7/BB1, in induction of T cell responses is well documented (11–21), the role of B7/BB1 in recruitment and effector function of T cells is still not clear. Our studies reported here show that B7/BB1 also plays an important role in the recruitment and effector function of antitumor T cells.

Table 3. *Susceptibility of Five Independent B7/BB1-transfected J558 Cells to TIL Prepared from J558-B7–derived Tumors*

J558 clones	B7 expression (delta MCF)	Effector/target 20	6.6	2.2	0.7	0.2	0.07
B7/BB1-transfectants							
J558-B7	132	90.4	93.9	67.3	40.5	21.3	4.7
1aC5	113	72.6	87.9	62.9	36.9	18.9	7.2
1cH1	75	70.2	83.0	49.5	25.9	12.7	4.4
1cD7	69	69.3	71.0	54.6	30.7	16.1	5.2
1bH2	41	78.7	79.0	60.6	34.6	15.7	4.8
Control							
J558-Neo	0	49.8	37.8	14.6	7.4	2.4	1.5

Five independent B7/BB1-transfected J558 clones and control J558-Neo were tested for their lysis by the TIL in a 6-h ^{51}Cr release assay. The spontaneous releases of the six clones were all between 10.2 and 17.7%. The expression of B7 was analyzed with an anti-B7 mAb 3A12, by flow cytometry. The data shown, the delta mean fluorescence channel numbers (MFC) was calculated by subtracting MFC of the average of five negative clones from the MFC of the clones indicated.

In agreement with two recent reports on B7/BB1-transfected melanoma (22, 23), B7/BB1-transfected plasmacytoma induces a potent tumor rejection response. This rejection is dependent on CD8 but not CD4 T cells. However, in contrast to the two reports, we have been unable to demonstrate cross-protection between J558-B7 and J558-Neo, either in coinjection experiments or in preimmunization experiments. This lack of cross-protection is consistent with the poor lysis of J558-Neo by the CTL induced by J558-B7. We have demonstrated that B7/BB1, either as an intact molecule or as a donor of peptides, was not recognized by the CTL as the antigen in two ways. First, P388D1, a macrophage cell line ($H\text{-}2^d$) which expresses MHC class I and class II plus significant amount of B7, was not lysed by the CTL. Second, the better recognition of J558-B7 was not due to mutant B7 peptides being recognized by CTL. As DNA sequencing revealed that the construct used for transfection has the wild-type B7 sequence, the mutation in B7/BB1, if any, had to occur during and/or after transfection. Were it so, mutation would be expected to be random and should occur at a very low frequency. We showed here that five independent B7/BB1 transfectants are lysed to a similar degree by the CTL, making it highly unlikely for a peptide derived from a B7-mutant to be the antigen.

We have directly demonstrated that B7/BB1 acts as an accessory molecule in the effector phase of the anti-tumor immune responses by showing that the lysis of J558-B7 by the CTL can be blocked by the anti-B7 antibodies. Our finding that B7/BB1 is required at the effector phase of T cell responses is in contrast to what has been reported by Chen et al. (22) and Harding and Allison (42) who showed that CTL induced by B7/BB1-transfected tumor lysed target cells with or without B7/BB1 equally well. This difference might be due to the amount and quality of adhesion molecules expressed on tumor cells. The J558 tumor cells express undetectable levels of LFA-1 molecule. The B7/BB1 expressed in large quantity may enhance the interaction between T cells and target cells, much as adhesion molecules do. B7 binds CD28 with an affinity (2×10^{-7} M) comparable with that of LFA-1 intercellular adhesion molecule 1 (ICAM-1) interaction. This interaction would be necessary for the interaction of T cells with J558 which express undetectable LFA-1. Consistent with the notion that B7/BB1 acts as an accessory molecule for CTL lysis in this model, we observed that in the tumors caused by J558-B7, an overwhelming majority of the tumor cells have lost cell surface B7 expression during the course of tumor growth (data not shown). The loss of B7/BB1 allows these tumor cells to multiply even in the presence of potent CTL activity.

The mechanism by which of B7/BB1 functions at the effector phase of T cell response has not been clearly defined. For anti-CD3 mAb redirected cytotoxicity, Lanier et al. have suggested that B7/CD28 interaction may provide a critical signal in inducing cytolytic activity of freshly isolated T cells (43, 44). It is possible that B7/CD28 interaction may provide a crucial signal to activate cytolytic activity of T cells, as well as enhancing T cell/target cell adhesion.

Quantitative analysis of TIL by flow cytometry revealed a role of B7/BB1 in the recruitment of T cells in the tumors. Thus J558-B7–derived tumors have a significantly higher proportion of lymphocyte infiltrate than the J558-Neo tumors. More interestingly, CD8 T cells are preferentially recruited into the tumor. The ratio of CD4/CD8 T cells in spleen cells from both normal and tumor bearing mice is close to 3:1; whereas the CD4/CD8 ratio in the tumor infiltrating lymphocytes in J558-B7–derived tumor is 0.35. In contrast, the infiltrate in J558-Neo–derived tumor consist of more CD4 T cells than CD8 T cells. The preferential recruitment of CD8 T cells into tumor sites resembles some earlier studies by Ceredig et al. (45) and Liu et al. (46) on inflammatory cells induced by viral infection of the central nervous system. This selective recruitment of CD8 T cells in this tumor model and in the virus-infected central nervous system is at least in part due to the lack of MHC class II expression. We have been unable to detect any MHC class II in J558 cells maintained in tissue culture or J558 cells freshly prepared from tumors. In the lymphocytic choriomeningitis virus–infected central nervous system, Allen et al. showed that the T cell recruitment is restricted by MHC class I antigens (47). Thus lymphocyte infiltration into tissues seems to depend on both a costimulatory signal and a MHC antigen on the target tissue. One possible interpretation of this intriguing finding is that the tumor-infiltrating T cells are derived from local clonal expansion of T cells specific for tumor antigens, since clonal expansion of T cells requires these two signals. This hypothesis would predict that all T cells in the tissues are specific for the antigen presented. This appears inconsistent with previous findings by Allen et al. (47) who showed that in the inflammatory cells isolated from the virus-infected central nervous system, only 1 of 100 of the T cells are specific for the viral antigen; most of the T cells are bystanders. In addition, we show in this paper that all seven $V\beta$ tested are well represented, which makes it very unlikely that T cells in the tumor are derived from clonal expansion of a few antigen-specific T cells. We favor the hypothesis that the initiation of the inflammation requires a specific T cell response. This response will lead to production of cytokine(s) that specifically attract T cells of the same subset. Recently, Tanaka et al. (48) reported that macrophage inflammatory factor (MIP-1β) selectively induces CD8 T cell chemotaxis and adhesion by binding to Pgp-1.

Finally, it is worth mentioning that the TIL from the J558-B7-derived tumors show a remarkable CTL activity in the absence of any in vitro stimulation. To our knowledge, such a "primary CTL" against syngeneic tumors has not been reported before. This is certainly due to the enhanced immunogenicity of tumor cells by transfecting costimulatory molecule B7/BB1. Such potent in vivo anti-tumor CTL responses would be very useful for the analysis of the specificity and regulation of anti-tumor CTL responses in vivo.

Taken together, a successful anti-tumor T cell immune response involves at least three steps: recruitment of antigen-specific T cells into tumors; activation of T cells into effector T cells; and elimination of tumor cells by the effector T cells.

Costimulatory molecules expressed on tumor cells can enhance T cell responses at each of these three steps. It is worth noting that for tumors that lack molecules critical for the effector function of T cells, a potent CTL response against the tumor transfectant–expressing costimulatory molecule may not be sufficient to eliminate the parental tumor.

We thank Dr. Charles Janeway for helpful discussions; Dr. Stanislav Vukmanovic and Dr. Allen Frey for critical reading of the manuscript; Dr. Peter S. Linsley and Dr. Tom Norvak for plasmids; Dr. Hans Reiser for anti-B7 mAb 16.10A; Dr. John Hess for flow cytometry; and Mr. Roger Rose for help in preparation of the manuscript.

This study is supported by National Institutes of Health (NIH) grant CA-58033, The Lucille P. Markey Trust, the Searle Scholar program, and NIH grant CA-16087 for the Kaplan Comprehensive Cancer Center. M. Castle was supported by NIH training grant 09161-18.

Address correspondence to Dr. Yang Liu, Michael Heidelberger Division of Immunology, Department of Pathology, Kaplan Comprehensive Cancer Center, NYU Medical Center, 550 First Avenue, New York, NY 10016.

Received for publication 8 June 1993 and in revised form 15 November 1993.

References

1. Greenberg, P.D. 1991. Adoptive T cell therapy of tumors: mechanism operative in the recognition and elimination of tumors. *Adv. Immunol.* 49:281.
2. Lafferty, K.J., S.J. Prowse, and C.J. Simeonovich. 1983. Immunobiology of tissue transplantation: a return to passenger leukocyte concept. *Annu. Rev. Immunol.* 1:143.
3. Jenkins, M.K., and R.H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* 165: 302.
4. Liu, Y., and C.A. Janeway, Jr. 1990. Interferon γ plays a critical role in induced cell death of effector T cell: a possible third mechanism of self-tolerance. *J. Exp. Med.* 172:1735.
5. Liu, Y., and P.S. Linsley. 1992. Costimulation of T cell growth. *Curr. Opin. Immunol.* 4:265.
6. Weaver, C.T., and E.R. Unanue. 1990. The costimulatory function of antigen-presenting cells. *Immunol. Today.* 11:49.
7. Janeway, C.A., Jr. 1992. The immune system is evolved to discriminate infectious nonself from noninfectious self. *Immunol. Today.* 13:11.
8. Restifo, N.P., F. Esquivel, A.L. Asher, H. Stotter, R.J. Barth, J.R. Bennink, J.J. Mulé, J.W. Yewdell, and S.A. Rosenberg. 1991. Defective presentation of endogenous antigens by murine sarcoma: implication for the failure of an anti-tumor immune response. *J. Immunol.* 147:1453.
9. Restifo, N.P., P.J. Spiess, S.E. Karp, J. Mulé, and S.A. Rosenberg. 1992. A nonimmunogenic sarcoma transduced with the cDNA for interferon γ elicits $CD8^+$ T cells against the wild-type tumor: correlation with antigen presentation capacity. *J. Exp. Med.* 175:1423.
10. Talmadge, D.W., J.A. Woolnough, H. Hemmingsen, L. Lopez, and K.J. Lafferty. 1977. Activation of cytotoxic T cells by nonstimulating tumor cells and spleen cell factor(s). *Proc. Natl. Acad. Sci. USA.* 74:4610.
11. Linsley, P.S., E.A. Clark, and J.A. Ledbetter. 1990. The T cell antigen, CD28, mediates adhesion with B cells by interacting with the activation antigen B7/BB1. *Proc. Natl. Acad. Sci. USA.* 87:5031.
12. Linsley, P.S., W. Brady, L. Grosmaire, A. Aruffo, N.K. Damle, and J.A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* 173:721.
13. Jenkins, M.K., P.S. Taylor, S.D. Norton, and K.B. Urdhl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* 147:2461.
14. June, C.H., J.A. Ledbetter, P.S. Linsley, and C.B. Thompson. 1990. Role of CD28 in T cell activation. *Immunol. Today.* 11:211.
15. Harding, F.A., J.G. McArthur, J.A. Cross, D.H. Raulet, and J.P. Allison. 1992. CD28-mediated signalling costimulates murine T cells and prevents induction of anergy in T cell clones. *Nature (Lond.).* 356:607.
16. Liu, Y., B.A. Jones, W. Brady, C.A. Janeway, Jr., and P.S. Linsley. 1992. Costimulation of CD4 T cell growth: cooperation between B7 and the heat-stable antigen. *Eur. J. Immunol.* 22:2855.
17. Razi-Wolf, Z., G.J. Freeman, F. Galvin, B. Benacerraf, L. Nadler, and H. Reiser. 1992. Expression and function of the murine B7-antigen, the major costimulatory molecule expressed by peritoneal exudate cells. *Proc. Natl. Acad. Sci. USA.* 89:4210.
18. Tan, P., C. Anasetti, J.A. Hansen, J. Melrose, M. Brunvand, J. Bradshaw, J.A. Ledbetter, and P.S. Linsley. 1993. Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. *J. Exp. Med.* 177:165.
19. Lenschow, D.J., Y. Zeng, J.R. Thistlethwaite, A. Montag, W. Brady, P.S. Linsley, and J.A. Bluestone. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig. *Science (Wash. DC).* 257:789.
20. Turka, A., P.S. Linsley, H. Lin, W. Brady, J.M. Leiden, R.-Q. Wei, M.L. Gibson, X.-G. Zheng, S. Myrdal, D. Gordon, T. Bailey, S.F. Bolling, and C. Thompson. 1992. T-cell activation by CD28 ligand B7 is required for cardiac allograft rejection

in vivo. *Proc. Natl. Acad. Sci. USA.* 89:11102.
21. Koulova, L., E.A. Clark, G. Shu, and B. Dupont. 1991. The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of $CD4^+$ T cells. *J. Exp. Med.* 173:759.
22. Chen, L.P., S. Ashe, W.A. Brady, I. Hellstrom, K.E. Hellstrom, J.A. Ledbetter, P. McGowan, and P.S. Linsley. 1992. Costimulation of anti-tumor immunity by the B7 counter receptor for the T lymphocyte molecules CD28 and CTLA4. *Cell.* 71:1093.
23. Townsend, S., and J.P. Allison. 1993. Tumor rejection after direct costimulation of $CD8^+$ T cells by B7-transfected melanoma cells. *Science (Wash. DC).* 259:368.
24. Baskar, S., S. Ostrand-Rosenberg, N. Nabavi, and L. Glimcher. 1993. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated MHC class II molecules. *Proc. Natl. Acad. Sci. USA.* 90:7015.
25. Wu, Y., Y. Guo, and Y. Liu. 1993. A major costimulatory molecule on antigen-presenting cells, CTLA4 ligand A, is distinct from B7. *J. Exp. Med.* 178:1789.
26. Razi-Wolf, Z., G.J. Freeman, F. Galvin, B. Benacerraf, L. Nadler, and H. Reiser. 1992. Expression and function of the murine B7 antigen, the major costimulatory molecule expressed by peritoneal exudate cells. *Proc. Natl. Acad. Sci. USA.* 89:4210.
27. Dumont, F.J., R.C. Habbrtsett, L.C. Coker, E.A. Nichols, and E.A. Treffinger. 1985. High level expression of the plasma cell antigen PC.1 on the T cell subset expanding in MRL/MpJ-*lpr/lpr* mice: detection with a xenogeneic monoclonal antibody and allo antisera. *Cell. Immunol.* 96:327.
28. Sanchez-Madrid, F., P. Simon, S. Thompson, and T.A. Springer. 1983. Mapping of antigenic and functional epitopes on the α- and β-subunits of two related mouse glycoproteins involved in cell interaction, LFA-1 and Mac-1. *J. Exp. Med.* 158:586.
29. Ozato, Y., Y.-J. Wan, and B. Orrison. 1985. Mouse major histocompatibility class I gene expression begins at midsomite stage and is inducible in early stage embryos by interferon. *Proc. Natl. Acad. Sci. USA.* 82:2427.
30. Kappler, J.W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2–restricted interleukin 2–producing T cell hybridomas: lack of independent H-2 and antigen recognition. *J. Exp. Med.* 153:1198.
31. Pullen, A., P. Marrack, and J. Kappler. 1988. The T cell repertoire is heavily influenced by tolerance to polymorphic self antigens. *Nature (Lond.).* 335:796.
32. Kanagawa, O., E. Palmer, and J. Bill. 1989. The T cell receptor Vβ6 domain imparts reactivity to Mls1a antigen. *Cell. Immunol.* 119:412.
33. Okada, C., B. Halzmann, C. Guidos, E. Palmer, and I. Weissman. 1990. Characterization of a rat monoclonal antibody specific for a determinant encoded by the Vβ7 gene segment. *J. Immunol.* 144:3473.
34. Staerz, V., H. Rammansee, J. Benedetto, and M. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotypic determinant of the T cell receptor. *J. Immunol.* 134:3994.
35. Bill, J., O. Kanagawa, D.L. Woodland, and E. Palmer. 1989. The MHC molecule I-E is necessary but not sufficient for clonal deletion of Vβ11-bearing T cells. *J. Exp. Med.* 169:1405.
36. Gao, E.-K., O. Kanagawa, and J. Sprent. 1989. Capacity of unprimed $CD4^+$ and $CD8^+$ T cells expressing Vβ11 to respond to I-E alloantigens in vivo. *J. Exp. Med.* 170:1947.
37. Lian, N.-S., J. Maltzman, and D.H. Raulet. 1989. Positive selection determines T cell receptor Vβ14 gene usage by $CD8^+$ T cells. *J. Exp. Med.* 170:135.
38. Dialynas, D.P., Z.S. Quan, K.A. Wall, A. Pieres, J. Quantans, M. Pieres, and F.W. Fitch. 1983. Characterization of murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity to human Leu3/T3 molecule. *J. Immunol.* 131:2445.
39. Ledbetter, J., R. Rouse, S. Micklem, and L. Herzenberg. 1980. T cell subsets defined by expression of Lyt-1, 2, 3 and Thy 1 antigen. *J. Exp. Med.* 152:280.
40. Sarmiento, M., A.L. Glasebrook, and F.W. Fitch. 1985. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt-2 antigen block T cell mediated cytolysis in the absence of complement. *J. Immunol.* 125:2665.
41. Liu, Y., and A. Müllbacher. 1989. Activated B cell can replace CD4 T cells in providing immunological help for CD8 T cells. *Proc. Natl. Acad. Sci. USA.* 86:4629.
42. Harding, F.A., and J.P. Allison. 1993. CD28–B7 interactions allow the induction of $CD8^+$ cytotoxic T lymphocytes in the absence of exogenous help. *J. Exp. Med.* 177:1791.
43. Azuma, M., M. Cayabayab, D. Buck, J.H. Philips, and L.L. Lanier. 1992. CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. *J. Exp. Med.* 175:353.
44. Azuma, M., M. Cayabayab, J.H. Philips, and L.L. Lanier. 1993. Requirement of CD28-dependent T-cell mediated cytotoxicity. *J. Immunol.* 150:2091.
45. Ceredig, R., J.E. Allan, Z. Tabi, F. Lynch, and P.C. Doherty. 1987. Phenotypic analysis of the inflammatory exudate in murine lymphocytic choriomeningitis. *J. Exp. Med.* 165:1539.
46. Liu, Y., R.V. Blanden, and A. Müllbacher. 1989. Identification of cytolytic lymphocytes in West Nile virus-infected central nervous system. *J. Gen. Virol.* 70:565.
47. Allen, J.E., J.E. Dixon, and P.C. Doherty. 1987. Nature of the inflammatory responses in the central nervous system of mice infected with lymphocytic choriomeningitis virus. *Curr. Top. Microbiol. Immunol.* 134:131.
48. Tanaka, Y., D. Adams, S. Hubscher, H. Hirano, U. Siebenlist, and S. Shaw. 1993. T cell adhesion induced by proteoglycan-immobilized cytokine-1β. *Nature (Lond.).* 361:79.