B7–CD28 Costimulation Unveils the Hierarchy of Tumor Epitopes Recognized by Major Histocompatibility Complex Class I–restricted CD8$^+$ Cytolytic T Lymphocytes

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Summary

Immunization of mice with tumors genetically engineered to express the B7 costimulatory molecules amplifies the antitumor immune response mediated by CD8$^+$ cytolytic T lymphocytes (CTL). In this report, we examined the effect of B7–CD28 costimulation on the hierarchy of tumor epitopes. Using a combination of affinity chromatography/reversed-phase high performance liquid chromatography and CTL cloning, we show that major histocompatibility complex (MHC) class I molecules from EL4 lymphoma cells can present at least six distinct CTL epitopes presented by MHC class I molecules. Nevertheless, mice immunized with wild-type B7-negative EL4 cells develop CTL only to one immunodominant epitope. In contrast, immunization with B7-transduced EL4 cells led to not only the amplification of the CTL response to this immunodominant epitope, but also to the recognition of five otherwise silent subdominant epitopes. The adoptive transfer of a CTL clone against such a subdominant epitope cured mice bearing EL4 lymphoma growing as an ascites tumor. The fact that CTL response can be spread to normally silent epitopes as a result of B7–CD28 costimulation suggests a novel approach to manipulate the hierarchy of CTL epitopes and offers an opportunity to explore novel targets for T cell–mediated cancer therapy.

For an efficient T cell activation to occur, both a specific signal delivered through the TCR by antigenic peptides–MHC and a costimulatory signal mediated by molecules of the B7 family through their ligand CD28 on T cells are required (1, 2). TCR signaling by antigen without proper costimulation may cause antigen-specific unresponsiveness (3). By transfer of the *B7-1* or *B7-2* genes into mouse tumor cells, protective and sometimes curative immunity against untransfected wild-type (wt)[1] tumors has been generated in several mouse models (4–7). Immune responses amplified by B7 costimulation are mainly mediated by CD8$^+$ CTL (8, 9), although CD4$^+$ T cells may also play a role in some tumor models (10, 11).

Many potential antigenic peptides are presented by a tumor or virus, but only a few immunodominant epitopes elicit a response whereas others are silent (12, 13). There are several possible mechanisms for this epitope hierarchy. The "hole" in the T cell repertoire may exist because of a deletion of T cell clones in the thymic or peripheral development (13). In addition, there is growing evidence that silent epitopes are produced with very low efficiency by normal routes of antigen processing and presentation (14–16). Proteasome-mediated proteolysis may contribute to the selection process by either destroying the epitopes or changing the cleavage pattern (17). The strong MHC class I–binding of most immunodominant epitopes argues that a peptide's affinity plays a role in determining its immunogenicity, although not all strongly binding peptides are immunodominant (18–20).

The beneficial effect of B7-CD28 costimulation on the immune response to tumors could be caused by an expansion of CTL clones recognizing immunodominant tumor antigens due to the increased production of lymphokines (21) which prevents T cell anergy caused by exposure to antigen in the absence of a second signal (22, 23). However, costimulation by B7–CD28 interaction may also make possible the recognition of additional, otherwise silent antigens by either increasing the tumor–CTL interaction or by amplifying the signaling to TCR. To be able to distinguish between these possibilities, we have employed a previously studied mouse EL4 lymphoma model (24). The EL4 lymphoma is highly tumorigenic in syngeneic C57BL/6 mice and does not express molecules of the B7 family whereas it expresses a high level of MHC class I molecules.

[1] *Abbreviations used in this paper:* BC-SC, bulk cultured spleen cells; TAP, transporter-associated protein; wt, wild type.

It can both process and present antigens (24). Whereas immunization of syngeneic mice with wt EL4 cells induces a weak CTL response and, at best, a partial protective immunity against challenge with wt tumor (24–26), immunization with EL4 cells transduced with a recombinant retrovirus containing mouse B7-1 cDNA ($B7^+$ EL4) induces a strong protective immunity against wt tumor. Moreover, mice bearing a small established s.c. EL4 lymphoma can be cured by repeated injection with $B7^+$ EL4 cells (24).

In the current study, we first established a panel of CTL clones and long-term CTL lines from mice immunized with $B7^+$ EL4 cells and used them as probes to identify the CTL epitopes presented by EL4 cells. The peptides associated with MHC class I molecules of EL4 cells were isolated and subjected to HPLC fractionation. Individual peptides were then loaded into transporter-associated protein (TAP)-deficient RMA-S cells to examine the reactivity of CTL. We demonstrate that at least six distinct CTL epitopes are naturally processed and presented by the EL4 lymphoma. Using this system, we were able to trace the activation status of CTL to the corresponding epitopes in polyclonal populations, and to study the effect of B7-CD28 costimulation in the hierarchy of tumor epitopes recognized by $CD8^+$ CTL.

Materials and Methods

Mice and Cell Lines. Female C57BL/6 mice, 4–8 wk old, were purchased from Taconic Farms, Inc. (Germantown, NY). MHC class II–deficient female C2D mice (27), which originated from C57BL/6 mice, were purchased from GenPharm International (Mountain View, CA). The EL4 and TIMI.4 lymphomas are of C57BL/6 ($H\text{-}2^b$) origin, the P815 mastocytoma is of DBA/2 ($H\text{-}2^d$) origin, and the NK cell–sensitive lymphoma YAC-1 is of A/Sn origin. The $B7^+$ EL4 line had been generated previously in our laboratory by infection of EL4 cells with pLXSN recombinant retrovirus containing murine B7-1 cDNA (24). The mock.EL4 line was derived similarly, but infected with parental retrovirus (24). The P815.K^b and P815.D^b clones are derived from P815 cells transfected with pBR327 plasmids containing H-$2K^b$ or H-$2D^b$ genomic DNA (28), respectively. RMA-S lymphoma, which is deficient in the TAP-2 peptide transporter (29), is of C57BL/6 ($H\text{-}2^b$) origin. All lines were examined in a mouse antibody production test (Microbiological Associates, Inc., Rockville, MD) and shown to be free of Mycoplasma and the following viruses: Sendai pneumonia virus, mouse hepatitis virus, pneumonia virus of mice, reovirus type 3, mouse adenovirus, Theiler's mouse encephalitis virus, ectromelia virus, minute virus of mice, polyoma virus, lactic dehydrogenase virus, epizootic diarrhea of infant mice, mouse thymic virus, Hantaan virus, K virus, mouse orphan parvo virus, mouse cytomegalovirus, and lymphocytic choriomeningitis virus.

Generation and Assay of CTL. Methods for the immunization and assay of EL4-reactive CTL in bulk-cultured spleen cells (BC-SC) have been described (30). Briefly, mice were injected subcutaneously with live $B7^-$ EL4 (wt or mock transduced) or live $B7^+$ EL4 cells at 5×10^4/mouse or, in some experiments, at 10^6/mouse. 10–14 d later, spleen cell suspensions were prepared and cocultivated for 5–7 d at 5×10^6/well with γ-irradiated (10,000 rad) $B7^+$ EL4 cells (10^5/well) in 24-well plates. BC-SC were then tested in a standard 4-h ^{51}Cr release assay as described. In six experiments, irradiated $B7^-$ EL4 cells were injected at $3–5 \times 10^6$/mouse s.c. or i.p. and the mice were boosted twice with the same number of cells at 2-wk intervals. All immunization methods gave similar results. In cell depletion assays, BC-SC from mice immunized with either $B7^-$ EL4 or $B7^+$ EL4 were pretreated as described (30) with mAbs to murine TCR-α/β (H57-597), TCR-γ/δ (GL3) (PharMingen, San Diego, CA), CD4 (GK1.5), CD8 (HO2.2), or human MHC class II (L227) as a control at 20 μg/ml plus rabbit complement before the CTL assay.

Generation of CTL Lines and Clones. To generate long-term CTL lines, ~1–5 × 10^5/well BC-SC were restimulated every 7–14 d with 10^5/well irradiated (10,000 rad) $B7^+$ EL4 cells together with 5×10^6 irradiated (3,000 rad) syngeneic spleen cells in the presence of human recombinant IL-2 at 10 U (Cetus)/ml (1 cetus unit = 6 international units). CTL clones were obtained by limiting dilution of CTL lines in 96-well plates.

Isolation and Purification of MHC Class I–associated Peptides. Purified peptides were prepared from MHC proteins as described (31), with modifications. Briefly, 20–40 g cell pellets (~2.7–5.5 × 10^{10}) of cultured $B7^-$ or $B7^+$ EL4 cells were washed with PBS and mixed with an equal volume of solubilization buffer containing 2% NP-40, 0.8 M NaCl, 20 mM Na_2EDTA, 2 mM PMSF, 100 μM iodoacetamide, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 5 μg/ml aprotinin in PBS. The cells were gently agitated for 30 min at 4°C, nuclei removed by low-speed centrifugation at 1,500 *g* for 5 min at 4°C, and the total protein concentration adjusted to 2 mg/ml. The cell lysate was clarified by centrifugation at 100,000 *g* for 90 min. The H-$2K^b$ and H-$2D^b$ complexes were then purified by immunoaffinity chromatography using mAb (the K^b-specific mAb, Y-3, and the D^b-specific mAb, 28-14-8S) coupled to CNBr-activated Sepharose 4B. MHC complexes were eluted with 1% TFA. Peptides were recovered by ultrafiltration using a stirred cell (Amicon Corp., Beverly, MA) with a 10,000 molecular weight cut-off and were concentrated by vacuum centrifugation. Peptides were purified on a narrow-bore HPLC system (model 130A; Applied Biosystems, Inc., Foster City, CA) using a 100-mm column (model C18; Vydac, Hesperia, CA) at a flow rate of 100 μl/min at 40°C. Linear acetonitrile gradients from solvent A (0.1% TFA) to solvent B (0.085% TFA in acetonitrile) were used for elution.

Peptide Sensitization of RMA-S Cells. Individual peptide fractions from $B7^-$ or $B7^+$ EL4 cells were reconstituted in 200 μl PBS and 1% aliquots were used for each well. For peptide sensitization, RMA-S cells were preincubated at 22°C for 48 h, labeled with ^{51}Cr at 22°C for 2 h, incubated with eluted peptides in 96-well V-shaped plates at 37°C for 1.5 h, and the cells were subjected to a 4-h ^{51}Cr release assay.

Animal Studies. Our method for in vivo depletion of T cell population has been described (8). Briefly, mice were injected intraperitoneally twice with purified mAb to CD4 (GK1.5) or CD8 (2.43) at 1 mg/mouse on the day of and 14 d after tumor inoculation. Subsequently, the mice were injected on the shaved back with 5×10^4 $B7^+$ EL4 cells. They were killed 4–5 wk later and spleen cell suspensions were prepared and subjected to FACS analysis to determine the efficiency of depletion (8). For blocking of the B7–CD28 interaction in vivo, the mice were injected with 5×10^4 $B7^+$ EL4 cells that had been premixed with 50 μg of murine CTLA4Ig (32). CTLA4Ig treatment was continued for a total of 2 wk by i.v. injection every other day at 50 μg per treatment. Mice treated with an equal amount of mAb to human CD5 (clone 10.2) were used as controls. Tumor growth was monitored weekly as described (8). For adoptive immunotherapy,

C57BL/6 mice, in groups of five, were administered intraperitoneally with 10^4 wt EL4 cells. 3 d later, the mice were injected with 5×10^6 cells i.p. of a T cell clone Cl29 or the control CTL line followed on day 4 by injection of 1,500 U of recombinant human IL-2 i.p. (Cetus Corp., Emeryville, CA) every day for 3 d. A C57BL/6-derived anti-H-2^d CTL line was generated as described (33) and used as a control. The mice were monitored daily for survival.

Results

Costimulation by B7 of the Cytolytic T Cell Response to EL4 Lymphoma. We reported previously that EL4 cells transduced with a recombinant retrovirus containing murine B7-1 cDNA lost their tumorigenicity in syngeneic mice and induced a strong CTL response (24). To determine which T cell population mediates the regression of $B7^+$ EL4, mice were treated with anti-CD4 or anti-CD8 mAbs to deplete different T cell subpopulations, a procedure that removed >95% of the respective T cell population without affecting other cells (8, 30). As shown in Fig. 1 *A*, treatment with a mAb to CD8, but not to CD4 or a control mAb, prevented the rejection of tumors induced by $B7^+$ EL4 cells, indicating that $CD8^+$ CTL play a key role in B7-mediated tumor rejection in this system, similar to others studied in the past (8). Treatment with CTLA4Ig, which blocks the interaction between B7 and CD28/CTLA-4 (32), abrogated the regression of tumors induced by $B7^+$ EL4 cells (Fig. 1 *B*) in syngeneic mice. Furthermore, the CTL activity of BC-SC against EL4 from mice treated with CTLA4Ig was significantly decreased (Fig. 2 *A*), indicating that induction of CTL by $B7^+$ EL4 cells was dependent on costimulation mediated by B7–CD28 interaction.

The cytolytic activity of BC-SC from $B7^+$ EL4-immunized mice appears to be restricted by MHC class I molecules and specific for EL4 since CTL did not lyse the syngeneic lymphoma TIMI.4, the allogeneic mastocytoma P815, P815 cells transfected to express K^b (P815.K^b) or D^b (P815.D^b), or the NK-sensitive lymphoma YAC-1 (Fig. 2 *B*). Experiments in which T cell subsets of BC-SC were depleted with specific mAb to TCR-α/β or γ/δ or to the CD4 or CD8 markers indicated that the cytolytic activity was mediated by TCR^+CD8^+ α/β T cells (Fig. 2 *C*). BC-SC generated from wt EL4-immunized mice had similar characteristics although the cytolytic activity was much lower (Fig. 2, *B* and *C*). Taken together, the findings demonstrate that a B7-1–costimulated $CD8^+$ CTL response can effectively destroy $B7^-$ and $B7^+$ EL4 lymphoma cells in vitro and suggest that a similar response is involved in tumor rejection.

At least Six Distinct CTL Epitopes Are Presented by MHC Class I Molecules of wt EL4 Cells. The strong CTL response detected after immunization with $B7^+$ EL4 cells offered an opportunity to establish long-term CTL cultures and clones. A panel of >50 $CD3^+CD8^+$ CTL clones and long-term CTL lines specific for EL4 cells was established from BC-SC of $B7^+$ EL4-immunized mice. Similar to their parent BC-SC, these clones and lines lyse EL4 cells; their CTL

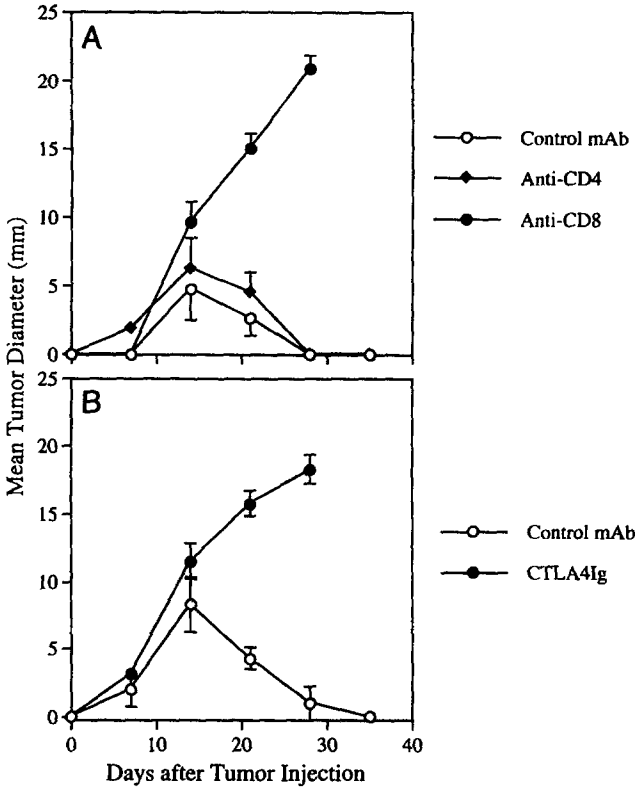

Figure 1. Regression of $B7^+$ EL4 lymphoma is dependent on B7-costimulated $CD8^+$ CTL. (*A*) Rejection of $B7^+$ EL4 tumor is mediated by $CD8^+$ T cells. C57BL/6 mice were injected with purified anti-CD4 (GK1.5) or anti-CD8 (2.43) antibodies at 1 mg/mouse i.p. The same amount of the anti–human CD5 antibody (10.2) was injected as a control. Mice were then inoculated with 5×10^4 $B7^+$ EL4 cells s.c., and tumor growth was assessed as previously described (24). (*B*) CTLA4Ig blocks rejection of $B7^+$ EL4 cells. Mice were injected on the shaved back with 5×10^4 $B7^+$ EL4 cells that had been premixed with 50 μg of murine CTLA4Ig. CTLA4Ig treatment was continued for a total of 2 wk by i.p. injection every other day at 50 μg/treatment. Mice treated with an equal amount of mAb to human CD5 (10.2) were used as controls. Tumor growth was monitored weekly.

activity appears to be restricted by either K^b or D^b molecules since inclusion of mAbs specific to K^b or D^b (see Materials and Methods) in the assay abrogated their cytolytic activity (data not shown).

To identify EL4 cell–derived antigens, peptides associated with MHC class I molecules were purified from cell lysates of in vitro–cultured EL4 cells. Peptide–MHC class I complexes were purified by affinity chromatography using specific mAbs to either K^b or D^b and peptides were released by acid treatment and quantitated by Edman analysis. Sequencing of the peptide pools demonstrated that the K^b-associated peptides were eight amino acids long and the D^b-associated peptides were nine amino acids long, sharing the consensus motifs described (31). Incubation of TAP-deficient RMA-S cells at 37°C with H-2^b–associated peptides prevented loss of both K^b and D^b from the cell surface, as detected by flow cytometry with specific mAbs (data not shown).

Eluted peptides were further fractionated by reversed-

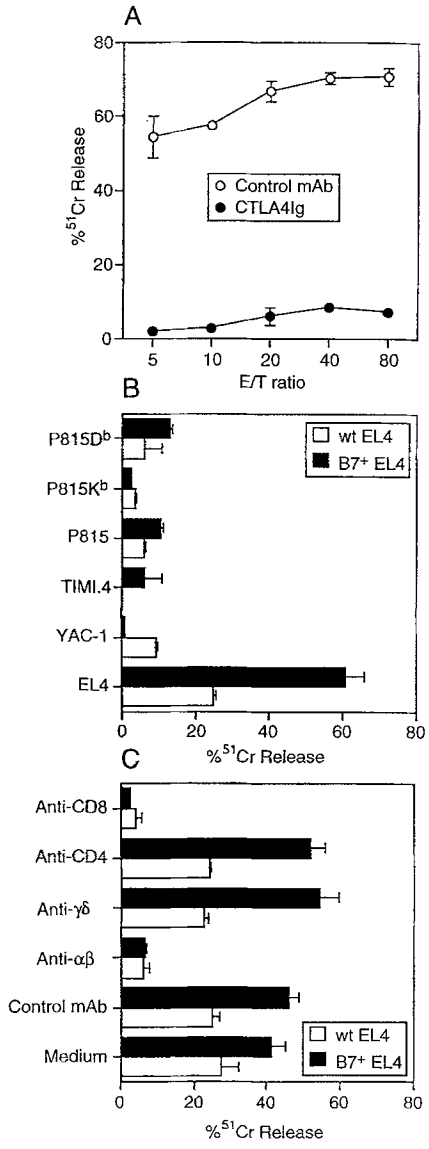

Figure 2. Characteristics of EL4-specific CTL. (*A*) C57BL/6 mice, in groups of five, were injected with 5×10^4 $B7^+$ EL4 cells s.c. and then treated with CTLA4Ig at 50 μg/mouse i.v. every other day for a total of 2 wk. The same concentration of mAb to human CD5 (10.2) was used as a control. 3 wk after injection of $B7^+$ EL4 cells, spleen cells were prepared and cocultured with irradiated $B7^+$ EL4 for 5 d. BC-SC were then tested for cytolytic activity on wt EL4 cells at the indicated E/T ratios. (*B*) The indicated tumor lines were labeled with 250 μCi ^{51}Cr at 37°C for 1 h and incubated at 5,000 cells/well for 4 h with BC-SC from mice immunized with either wt EL4 (*unfilled bar*) or $B7^+$ EL4 (*filled bar*) at an E/T ratio of 10:1 at 37°C. The supernatant was collected and assayed in a gamma counter and the percent cytolytic activity was calculated. (*C*) BC-SC from mice immunized with either wt EL4 (*unfilled bar*) or $B7^+$ EL4 (*filled bar*) were treated with mAbs to murine αβ TCR (H57-597), γδ TCR (GL3), CD4 (GK1.5), or CD8 (HO2.2) at 20 μg/ml plus complement, and then incubated with ^{51}Cr-labeled wt EL4 cells at E/T ratios of 10:1. The same concentration of mAb to human MHC class II (L227; American Type Culture Collection, Rockville, MD) was used as a control.

phase HPLC. The separation profiles of K^b- (Fig. 3 *A*, *left*) and D^b-derived peptides (Fig. 3 *A*, *right*) are shown. Individual peptide fractions were then loaded to sensitize RMA-S cells for CTL lysis (Fig. 3 *B*). Clone 4D9 reacted with RMA-S cells pulsed with K^b fraction 9 and clone Cl2 reacted with K^b fractions 15 and 16, indicating that at least two different K^b-restricted tumor peptides are recognized by two different CTL clones. The CTL line C2D recognized D^b fraction 8, indicating that this fraction contained a peptide not present in any of the other fractions. Similarly, clone 1A7 recognized D^b fraction 14, and clone 1B5 reacted with D^b fraction 9 only, whereas clone Cl29 recognized D^b fractions 16. We conclude that at least six distinct peptides are presented by MHC class I molecules of EL4 cells.

Immunization with $B7^-$ EL4 Cells Cannot Induce CTL against Subdominant Tumor Antigens. The peptide-specific retention time of eluted peptides (Fig. 3 *A*) allowed us to explore the specificity of individual T cell clones in polyclonal CTL. BC-SC generated from mice immunized with wt EL4 or mock.EL4 (both are referred to as $B7^-$ EL4) were tested against RMA-S cells loaded with individual HPLC fractions (Fig. 4, *A–C*). BC-SC from $B7^-$ EL4-immunized mice lysed wt EL4 cells (Fig. 4 *A*) and reacted to K^b-derived peptides in HPLC fractions 16 significantly (Fig. 4 *B*) but did not react with D^b-derived peptides (Fig. 4 *C*). These results indicate that K^b-restricted peptides in fractions 16 may contain an immunodominant peptide. The weaker CTL responses seen by immunizing mice with $B7^-$ as compared to $B7^+$ EL4 cells (see below) is not caused by insufficient exposure to tumor antigens since two different immunization methods, multiple injections of large numbers of irradiated EL4 cells or excision of growing tumor nodules (see Materials and Methods), gave comparable results. We conclude that immunization with $B7^-$ EL4 cells induced a CTL response against an epitope presented by the H-$2K^b$ molecule, which is, therefore, referred to as immunodominant, as compared to subdominant epitopes, which were not recognized by CTL raised with the wt EL4 lymphoma.

Activation of CTL against Subdominant Tumor Antigens by Immunization with $B7^+$ EL4 Cells. An increased activity of costimulated CTL can be caused by (*a*) an increased expansion of CTL clones against immunodominant antigens; (*b*) an activation of CTL clones reactive to subdominant epitopes; and (*c*) a combination of these two events. As an attempt to examine these possibilities, the CTL activity of BC-SC from mice immunized with $B7^+$ EL4 cells was tested against RMA-S cells loaded with individual peptide fractions. The costimulated BC-SC more efficiently lysed wt EL4 cells (Fig. 4 *D*) and RMA-S cells loaded with HPLC fractions 9, 10, 15, and 16 of the K^b peptides (Fig. 4 *E*) as compared to noncostimulated BC-SC (Fig. 4 *B*), indicating that the B7–CD28 interaction expanded CTL clones reactive to dominant EL4 antigens. The costimulated BC-SC displayed a strong CTL reactivity also against D^b peptides in fractions 8, 9, and 14 and a weaker CTL response against D^b peptides in fractions 11, 13, and 16 (Fig. 4 *F*). Since fractions 9 of K^b and 8, 9, 14, and 16 of D^b contained antigenically different peptides (Fig. 3 *B*) that did not induce any CTL activation when the mice were immunized with $B7^-$ EL4 cells, we conclude that immuni-

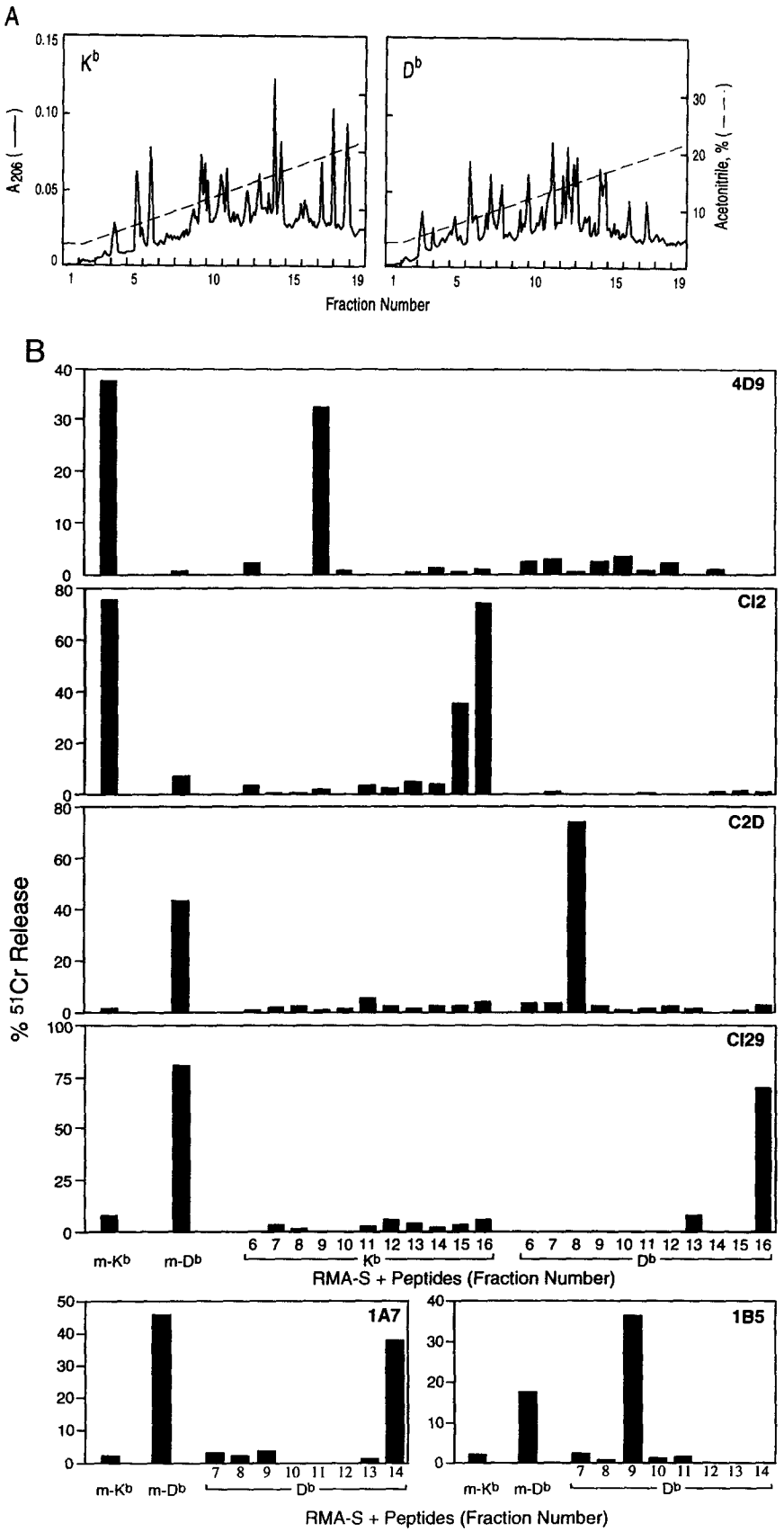

Figure 3. CTL epitopes in HPLC-fractionated peptides from EL4 cells. (*A*) Reversed-phase HPLC separation of K^b- and D^b-associated peptides from EL4. Peptides eluted from MHC of wt EL4 cells were chromatographed on a 100-mm C18 Vydac column. Elution patterns of (*left*) 1,600 pmol K^b peptides; (*right*) 1,620 pmol D^b peptides from a 27-g ($\sim$1.6 $\times$ 10^{10}) wt EL4 cell pellet. Fractions were collected at 2-min intervals. (*B*) Peptide specificity of CTL lines and clones against EL4 cells. The T cell line C2D and clones 4D9, 1A7, Cl2, Cl29, and 1B5 were tested for cytolytic activity on RMA-S cells pulsed with individual HPLC fractions from either K^b or D^b. Individual HPLC fractions were reconstituted in 200 μl PBS and 1% aliquots used to pulse RMA-S target cells as described in Materials and Methods. Fraction 6 represents the pool of HPLC fractions 1–6, fractions 15, 16, and 17 were collected at 5-min intervals. E/T ratios used for assays: (clone *4D9*) 1:1; (*C2D* line) 2:1; (clone *1A7*) 1.5:1; (clone *Cl2*) 2:1; (clone *Cl29*) 2:1; and (clone *1B5*) 2:1. The unfractionated K^b- (*m-K*^{b}) or Db- (*m-D*^{b}) derived peptide pools at 40 nM were also used for pulsing of RMA-S cells to determine K^b or D^b restriction of CTL clones.

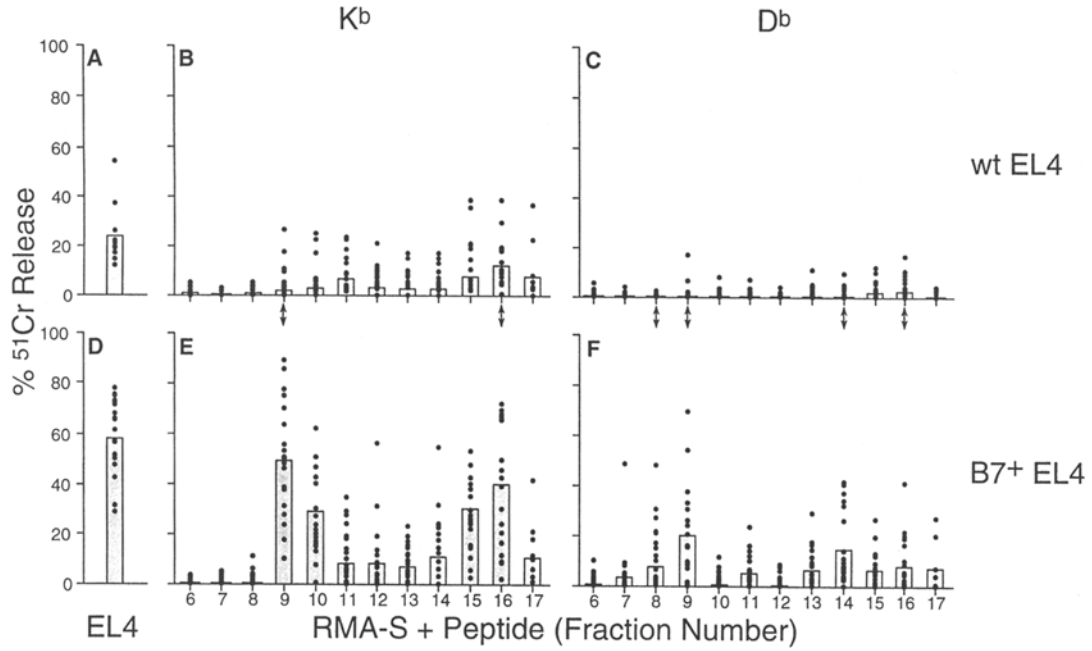

Figure 4. Recognition of tumor epitopes by BC-SC from mice immunized with B7 or B7$^+$ EL4 cells. BC-SC from mice immunized with either B7$^-$ (*A–C*) or B7$^+$ EL4 (*C–E*) were generated and tested for cytolytic activity on wt EL4 (*A* and *D*) and on RMA-S cells pulsed with individual HPLC fractions from either K^b (*B* and *E*) or D^b (*C* and *F*). An E/T ratio of 40:1 was used in all experiments. Three similar preparations of HPLC-purified peptides, quantitated by serial dilution using BC-SC or CTL clones in a ^{51}Cr release assay, were used for all experiments presented in this report. The results are expressed as the individual (*dot*) and the mean (*horizontal line*) of percent ^{51}Cr release from RMA-S pulsed with peptides from 20 independent experiments with BC-SC from B7$^-$ EL4-immunized mice and 20 experiments with SC from B7$^+$ EL4-immunized mice. (*Arrows*) Fractions of peptide recognized by CTL clones (see Fig. 3 *B*). The background ^{51}Cr release in the control group (incubation of BC-SC with RMA-S cells and no peptides) was subtracted from counts of the experimental group.

zation with B7$^+$ EL4 cells activates CTL clones recognizing not only immunodominant but also subdominant EL4 antigens.

These findings cannot be interpreted on the basis of a different expression level of MHC class I molecules in B7$^+$ and B7$^-$ EL4 cells, since these two types of cells express similar levels of K^b and D^b molecules as determined both by MHC class I–peptide complex quantitation and peptide purification after reversed-phase HPLC (data not shown) and by flow cytometry with specific mAbs (24). In addition, there are no quantitative differences in the lysis of B7$^+$ and B7$^-$ EL4 cells by either BC-SC or CTL clones (data not shown). To demonstrate whether B7$^+$ and B7$^-$ EL4 cells express similar levels of antigenic peptides, we prepared peptides from both wt EL4 and B7$^+$ EL4 cells and compared their ability to sensitize RMA-S for lysis by CTL. As shown, fraction 9 of K^b (Fig. 5 *A*), fraction 8 and 9 of D^b (Fig. 5, *B* and *C*) prepared from either wt EL4 or B7$^+$ EL4 cells were equally efficient on sensitization of RMA-S cells for CTL lysis. We conclude that the difference in the ability of B7$^-$ EL4 and B7$^+$ EL4 cells to induce a CTL response is due to the ability of the latter cells to provide B7-CD28 costimulation.

In Vivo Exposure to B7$^-$ EL4 Cells Does Not Induce Tolerance/Anergy of CTL against Subdominant Epitopes. To examine whether subdominant antigens induce tolerance in vivo, we first injected B7$^-$ EL4 cells into mice to induce tumor and then injected these mice with B7$^+$ EL4 cells at a distant site either at the same time or 1, 2, 3 wk later. Growth of tumor nodules induced by B7$^+$ EL4 cells was monitored 2–4 wk after challenge with B7$^+$ EL4 cells. As shown in Fig. 6, *A–D*, tumor nodules induced by B7$^+$ EL4 cells regressed rapidly in all the mice regardless of the size of the B7$^-$ EL4 tumor they were bearing, indicating that

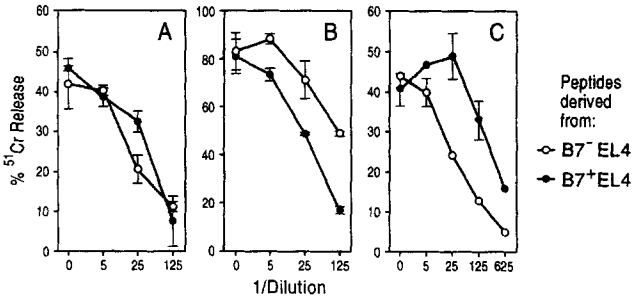

Figure 5. Comparison between the ability of HPLC-fractionated peptides from wt EL4 and B7$^+$ EL4 to sensitize RMA-S cells. Equivalent amounts of HPLC-fractionated peptides from either wt EL4 or B7$^+$ EL4 were prepared and used for pulsing RMA-S cells. The sensitivity of peptide-pulsed cells was examined by coculture with 4D9 clone, CD2 line, or the BC-SC. (*A*) Cytolytic activity of 4D9 clone on RMA-S cells pulsed with 1 μl of HPLC fraction 9 of K^b. E/R ratio was 1:1. (*B*) Cytolytic activity of C2D line on RMA-S cells pulsed with 1 μl of HPLC fraction 8 of D^b. E/T ratio was 10:1. (*C*) Cytolytic activity of BC-SC from a B7$^+$ EL-4-immunized mouse on RMA-S cells pulsed with 1 μl of HPLC fraction 14 of D^b. E/T ratio was 40:1.

an immune response against EL4 cells is not affected by the tumor-bearing status.

The CTL activity against EL4 tumor in tumor-bearing mice was also examined. There were no significant changes after challenge with $B7^+$ EL4 cells in the CTL response of mice bearing wt EL4 for 0, 1, or 2 wk (Fig. 6 *E*), although a significant decrease of CTL activity was seen in mice bearing large 3-wk EL4 tumors. Moreover, several subdominant epitopes presented by both K^b (fraction 13) and D^b (fractions 12, 13, and 14) molecules can be recognized by BC-SC from mice bearing wt EL4 for 2 wk followed by challenge with $B7^+$ EL4 cells (Fig. 6 *F*). Since the CTL as-

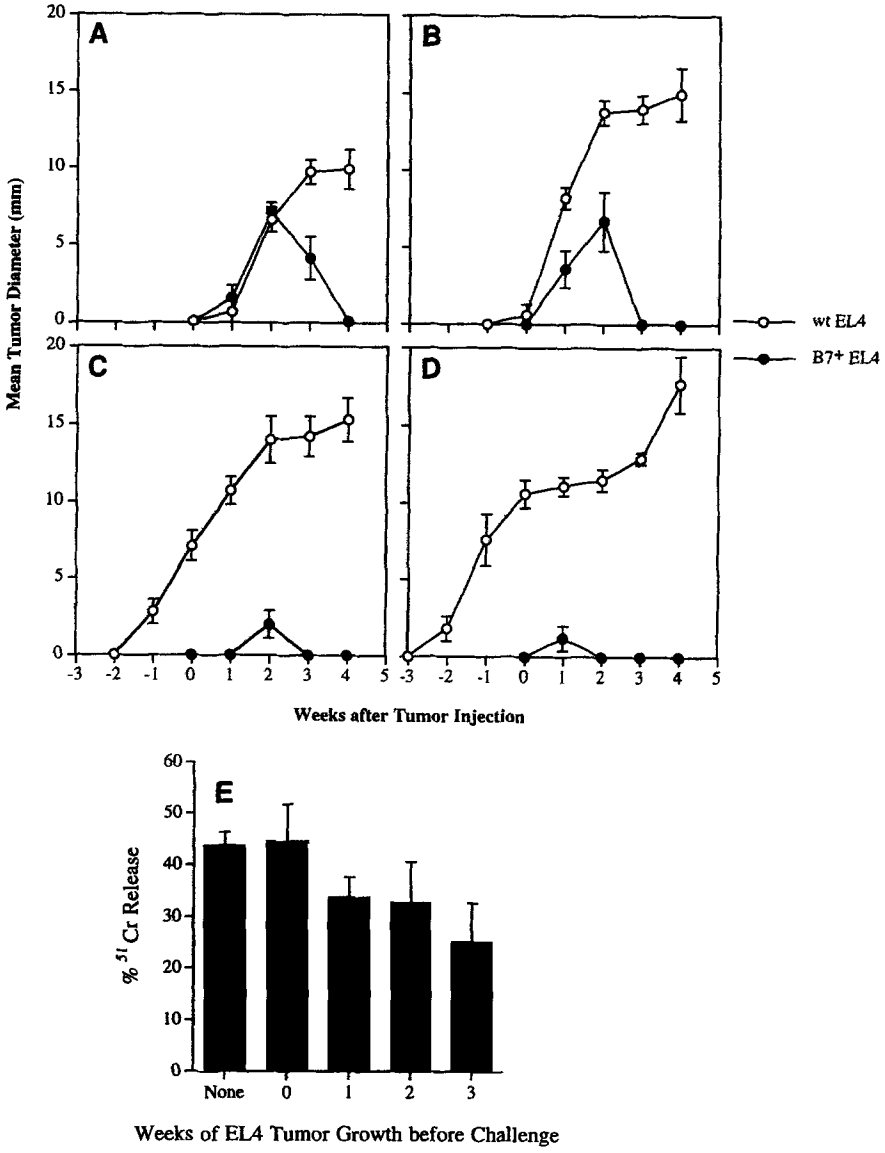

Figure 6. CTL reactivity to $B7^+$ EL4 cells in mice exposed to $B7^-$ EL4 cells. C57BL/6 mice, in groups of 5, were injected with 5×10^4 wt EL4 cells s.c. at 3, 2, and 1 wk before or at the same time and were then challenged on the distant site with 5×10^4 $B7^+$ EL4 cells. Tumor growth at both sites was assessed and is presented as mean tumor diameters in *D*, *C*, *B*, and *A*, respectively. (*E*) 2 wk after injection of $B7^+$ EL4 cells, spleen cells were prepared and cocultured with irradiated $B7^+$ EL4 for 5 d after which BC-SC were tested for cytolytic activity on wt EL4 cells at a ratio 80:1. (*F*) BC-SC from three mice immunized with wt EL4 at 2 wk before challenge with $B7^+$ EL4 cells were tested for CTL activity on RMA-S cells pulsed with 1 μl of HPLC-fractionated peptides.

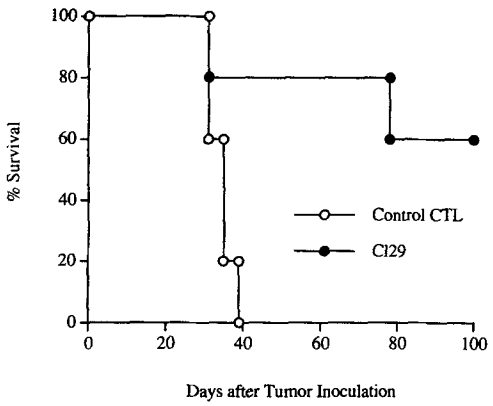

Figure 7. Treatment of mice with EL4 ascites tumor by adoptive transfer of a CTL clone specifically recognizing a subdominant epitope. C57BL/6 mice, in groups of five, were injected with 10^4 wt EL4 cells i.p. 3 d later, they were injected with 5×10^6 cells i.p. of T cell clone Cl29 followed the next day by injection with 1,500 U of IL-2 i.p.; injection of IL-2 was repeated once a day for three consecutive days. Control groups were injected with IL-2 combined with a control (C57BL/6 against H-2^d) CTL line. The mice were monitored daily for survival and the experiment was terminated at day 100 after tumor inoculation.

says in Fig. 4 were all performed 2 wk after injection of wt EL4, we conclude that the absence of a CTL response to subdominant EL4 epitopes was not caused by CTL tolerance or anergy in mice bearing wt EL4 tumor.

Treatment of EL4 Ascites Tumor by Adoptive Transfer of a CTL Clone Recognizing a Subdominant Tumor Epitope. To determine whether a CTL clone recognizing a subdominant epitope can be employed for treatment of an established EL4 tumor, we treated C57BL/6 mice that had been injected intraperitoneally with wt EL4 cells 3 d previously and had EL4 growing as an ascites tumor. Two of five mice treated with the CTL clone Cl29 plus IL-2 died at days 31 and 78 without visible ascites, whereas the three remaining mice were alive without sign of tumor when the experiment was terminated on day 100 (Fig. 7). In contrast, mice treated with IL-2 combined with a control anti-H-2^d CTL line all died with ascites within 40 d of tumor cell injection.

Discussion

We have demonstrated that at least six distinct tumor peptides are processed and presented by MHC class I molecules of the EL4 lymphoma cells in such a way that they can serve as targets for recognition by CTL clones in vitro and/or in vivo. However, the majority of these CTL epitopes are silent, i.e., they fail to induce an efficient CTL response unless the *B7-1* gene has been transfected into the EL4 tumor used as immunogen. They are, therefore, referred to as subdominant. Our findings thus indicate that even when a tumor expresses a multitude of potential target epitopes, and is able to present them, its immunogenicity may still be low.

One may speculate that exposure of CTL to tumor antigens presented by a B7-negative tumor, such as the EL4 lymphoma, may induce antigen-specific tolerance. In the present study, however, several lines of evidence do not support this mechanism: (*a*) mice bearing a large EL4 lymphoma were resistant to challenge with $B7^+$ EL4 cells (2, and Fig. 6, *A–D*); (*b*) the CTL activity which could be induced by immunization with $B7^+$ EL4 cells was not significantly affected until after 3 wk of tumor-bearing status (Fig. 6 *E*); and (*c*) the BC-SC from mice bearing 2-wk-old EL4 tumor could still react to several subdominant epitopes (Fig. 6 *F*). Although a single injection of $B7^+$ EL4 cells is not sufficient to induce regression of established large EL4 tumors (Fig. 6, *A–D*), small tumors can be cured if multiple injections of $B7^+$ EL4 cells are initiated at an early stage of tumor growth (24). It is noteworthy that signaling through the B7-CD28 pathway has been shown to prevent the induction of T cell anergy in vitro (22, 23) whereas it does not break already formed T cell anergy (2, 5). Our findings thus indicate that there was no T cell tolerance/anergy at least during the first 2 wk of growth of wt EL4 tumor.

The requirements for sensitizing a target cell for lysis by an already activated CTL clone and for activating CTL clones in a naive animal are different with presentation of much more antigenic peptides being required in the latter case (34–36). It is thus possible that CTL clones "ignore" a peptide encoding a tumor antigen unless it is expressed at a certain level needed for activation. EL4 cells are capable of presenting subdominant epitopes (Fig. 3) but probably at low level. It is possible that one of the roles of costimulation mediated by B7-1 is to decrease such a minimal "threshold" requirement for antigenic peptides to activate CTL.

Our findings have several implications for the immunotherapy of cancers. Whereas a tumor can express multiple antigens recognized by CTL simultaneously, the CTL response to tumor usually is directed to one or, at best, a few antigens, a situation that may facilitate the selection of antigen-negative escape variants (12, 37, 38). We have shown that immunization with a tumor engineered to express B7 can stimulate multiple CTL clonal expansions against an expanded array of antigens, and this should make tumor escape less likely. The generation of CTL against subdominant antigens will also offer opportunities to explore novel targets for T cell–mediated cancer therapy.

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References

1. June, C.H., J.A. Bluestone, L.M. Nadler, and C.B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol. Today.* 15:321–332.
2. Linsley, P.S., and J.A. Ledbetter. 1993. The role of the CD28 receptor during T cell responses to antigen. *Annu. Rev. Immunol.* 11:191–212.
3. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.* 7:445–480.
4. Chen, L., P.S. Linsley, and K.E. Hellström. 1993. Costimulation of T cells for tumor immunity. *Immunol. Today.* 14.482–486.
5. Allison, J.P. 1994. CD28–B7 interactions in T cell activation. *Curr. Opin. Immunol.* 6:414–419.
6. Ostrand-Rosenberg, S. 1994. Tumor immunotherapy: the tumor cell as an antigen-presenting cell. *Curr. Opin. Immunol.* 6:722–727.
7. Hellström, K.E., I. Hellström, and L. Chen. 1995. Can costimulated tumor immunity be therapeutically efficacious? *Immunol. Rev.* 145:123–145.
8. Chen, L., S. Ashe, W.A. Brady, I. Hellström, K.E. Hellström, J.A. Ledbetter, P. McGowan, and P.S. Linsley. 1992. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell.* 71:1093–1102.
9. Townsend, S.E., and J.P. Allison. 1993. Tumor rejection after direct costimulation of $CD8^+$ T cells by B7-transfected melanoma cells. *Science (Wash. DC).* 259:368–370.
10. Baskar, S., S. Ostrand-Rosenberg, N. Nabavi, L.M. Nadler, G.J. Freeman, and L.H. Glimcher. 1993. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. *Proc. Natl. Acad. Sci. USA.* 90:5687–5690.
11. Li, Y., P. McGowan, I. Hellström, K.E. Hellström, and L. Chen. 1994. Costimulation of tumor-reactive $CD4^+$ and $CD8^+$ T lymphocytes by B7, a natural ligand for CD28, can be used to treat established mouse melanoma. *J. Immunol.* 153:421–428.
12. Urban, J.L., and H. Schreiber. 1992. Tumor antigen. *Annu. Rev. Immunol.* 10:617–644.
13. Sercarz, E.E., P.V. Lehmann, A. Ametani, G. Benichou, A. Miller, and K. Moudgil. 1994. Dominance and cripticity of T cell antigenic determinants. *Annu. Rev. Immunol.* 11:729–766.
14. Sheil, J.M., S.E. Shepherd, G.F. Klimo, and Y. Paterson. 1992. Identification of an autologous insulin B chain peptide as a target antigen for H-$2K^b$–restricted cytotoxic T lymphocytes. *J. Exp. Med.* 175:545–552.
15. Houbiers, J.G., H.W. Nijman, S.H. van der Burg, J.W. Drijfhout, P. Kenemans, C.J. wan de Welde, A. Brand, F. Momburg, W.M. Kast, and C.J. Melief. 1993. In vitro induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53. *Eur. J. Immunol.* 23: 2072–2077.
16. Oukka, M., N. Riche, and K. Kosmatopoulos. 1994. A nonimmunodominant nucleoprotein-derived peptide is presented by influenza A virus–infected H-2^b cells. *J. Immunol.* 152:4843–4851.
17. Niedermann, G., S. Nutz, H.G. Ihlenfeldt, R. Grimm, M. Lucchiari, H. Hoschützky, G. Jung, B. Maier, and K. Eichmann. Contribution of proteasome-mediated proteolysis to the hierarchy of epitopes presented by major histocompatibility complex class I molecules. *Immunity.* 2:289–299.
18. Jameson, S.C., and M.J. Bevan. 1992. Dissection of major histocompatibility complex (MHC) and T cell receptor contact residues in a K^b-restricted ovalbumin peptide and an assessment of the predictive power of MHC-binding motifs. *Eur. J. Immunol.* 22:2663–2667.
19. Ressing, M.E., A. Sette, R.M. Brandt, J. Ruppert, P.A. Wentworth, M. Hartman, C. Oseroff, H.M. Grey, C.J. Melief, and W.M. Kast. 1995. Human CTL epitopes encoded by human papillomavirus type 16 E6 and E7 identified through in vivo and in vitro immunogenicity studies of HLA-A*0201-binding peptides. *J. Immunol.* 154:5934–5943.
20. Sette, A., A. Vitiello, B. Reherman, P. Fowler, R. Nayersina, W.M. Kast, C.J. Melief, C. Oseroff, L. Yuan, J. Ruppert, et al. 1994. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J. Immunol.* 153:5586–5592.
21. Schwartz, R.H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell.* 71:1065–1068.
22. Harding, F.A., J.G. McArthur, J.A. Gross, D.H. Raulet, and J.P. Allison. 1992. CD28-mediated signaling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature (Lond.).* 356:607–609.
23. Gimmi, C.D., G.J. Freeman, J.G. Gribben, G. Gray, and L.M. Nadler. 1993. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. *Proc. Natl. Acad. Sci. USA.* 90:6586–6590.
24. Chen, L., P. McGowan, S. Ashe, J. Johnston, Y. Li, I. Hellström, and K.E. Hellström. 1994. Tumor immunogenicity determines the effect of B7 costimulation on T cell–mediated tumor immunity. *J. Exp. Med.* 179:523–532.
25. Benjamini, E., S. Fong, C. Erickson, C.Y. Leung, D. Rennick, and R.J. Scibienski. 1977. Immunity to lymphoid tumors in syngeneic mice by immunization with mitomycin C–treated cells. *J. Immunol.* 118:685–93.
26. Visseren, M.J.W., M. Koot, E.I.H. van der Voort, L.A. Gravestein, H.J. Schoenmakers, W.M. Kast, M. Zijlstra, and C.J.M. Melief. 1994. Production of interleukin-2 by EL4 tumor cells induces natural killer cell– and T-cell–mediated immunity. *J. Immunother.* 15:119–128.
27. Grusby, M.J., R.S. Johnson, V.E. Papaioannou, and L.H. Glimcher. 1991. Depletion of $CD4^+$ T cells in major histocompatibility complex class II–deficient mice. *Science (Wash.*

DC). 253:1417–1420.
28. Allen, H., D. Wraith, P. Pala, B. Askonas, and R.A. Flavall. 1984. Domain interactions of H-2 class I antigens alter cytotoxic T-cell recognition sites. *Nature (Lond.).* 309:279–281.
29. Ljunggren, H.-G., and K. Kärre. 1985. Host resistance directed selectively against H-2–deficient lymphoma variants. Analysis of the mechanism. *J. Exp. Med.* 162:1745–1752.
30. Chen, L., M.T. Mizuno, M.C. Singhal, S.L. Hu, D.A. Galloway, I. Hellström, and K.E. Hellström. 1992. Induction of cytotoxic T lymphocytes specific for a syngeneic tumor expressing the E6 oncoprotein of human papillomavirus type 16. *J. Immunol.* 148:2617–2621.
31. Falk, K., O. Rötzschke, S. Stevanovic, G. Jung, and H.G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature (Lond.).* 351:290–296.
32. Finck, B.K., P.S. Linsley, and D. Wofsy. 1994. Treatment of murine lupus with CTLA4Ig. *Science (Wash. DC).* 265:1225–1227.
33. Yang, G., K.E. Hellström, M. Mizuno, and L. Chen. 1995. In vitro priming of tumor-reactive cytolytic T lymphocytes by combining interleukin-10 with B7-CD28 costimulation. *J. Immunol.* 155:3897–3903.
34. Koeppen, H., M. Acena, A. Drolet, D.A. Rowley, and H. Schreiber. 1993. Tumors with reduced expression of a cytotoxic T lymphocyte recognized antigen lack immunogenicity but retain sensitivity to lysis by cytotoxic T lymphocytes. *Eur. J. Immunol.* 23:2770–2776.
35. De Bruijn, M.J.H., T.N.M., Schumacher, J.D. Nieland, H.L. Ploegh, W.M. Kast, and C.J.M. Melief. 1991. Peptide loading of empty major histocompatibility complex molecules on RMA-S cells allows the induction of primary cytotoxic T lymphocyte responses. *Eur. J. Immunol.* 21:2963–2968.
36. Nonacs, R., C. Humborg, J.P. Tam, and R.M. Steinman. 1992. Mechanisms of mouse spleen dendritic cell function in the generation of influenza-specific, cytolytic T lymphocytes. *J. Exp. Med.* 176:519–529.
37. Lehmann, F., M. Marchand, P. Hainaut, P. Pouillart, X. Sastre, H. Ikeda, T. Boon, and P.G. Coulie. 1995. Differences in the antigens recognized by cytolytic T cells on two successive metastases of a melanoma patient are consistent with immune selection. *Eur. J. Immunol.* 25:340–347.
38. Weinhold, K.H., D. Miller, and E.F. Wheelock. 1979. The tumor-dormant state: comparison of L5178Y cells used to establish dormancy with those that emerge following its termination. *J. Exp. Med.* 419:745–757.