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Authors

Azuma, M
Cayabyab, M
Buck, D
et al.

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CD28 Interaction with B7 Costimulates Primary Allogeneic Proliferative Responses and Cytotoxicity Mediated by Small, Resting T Lymphocytes

By Miyuki Azuma,* Mark Cayabyab,* David Buck,‡ Joseph H. Phillips,* and Lewis L. Lanier*

*From the *Department of Immunology, DNAX Research Institute for Molecular and Cellular Biology, Palo Alto, California 94304; and the ‡Research Department, Becton Dickinson Immunocytometry Systems, San Jose, California 95131*

Summary

Engagement of the CD3/T cell antigen receptor complex on small, resting T cells is insufficient to trigger cell-mediated cytotoxicity or to induce a proliferative response. In the present study, we have used genetic transfection to demonstrate that interaction of the B7-BB1 B cell activation antigen with the CD28 T cell differentiation antigen costimulates cell-mediated cytotoxicity and proliferation initiated by either anti-CD2 or anti-CD3 monoclonal antibody (mAb). Moreover, a B7-negative Burkitt's lymphoma cell line that fails to stimulate an allogeneic mixed lymphocyte response is rendered a potent stimulator after transfection with B7. The mixed leukocyte reaction proliferative response against the B7 transfectant is inhibited by either anti-CD28 or B7 mAb. We also demonstrate that freshly isolated small, resting human T cells can mediate anti-CD3 or anti-CD2 mAb–redirected cytotoxicity against a murine Fc receptor–bearing mastocytoma transfected with human B7. These preexisting cytotoxic T lymphocytes in peripheral blood are present in both the CD4 and CD8 subsets, but are preferentially within the $CD45RO^+$ "memory" population. While small, resting T cells apparently require costimulation by CD28/B7 interactions, this requirement is lost after T cell activation. Anti-CD3 initiates a cytotoxic response mediated by in vitro cultured T cell clones in the absence of B7 ligand. The existence of functional cytolytic T cells in the small, resting T cell population may be advantageous in facilitating rapid responses to immune challenge.

T lymphocytes recognize antigen via the CD3/TCR complex. Binding of anti-CD3 or anti-TCR mAb to T cells results in a rapid increase in intracellular $[Ca^{2+}]$ and the generation of inositol triphosphate (IP_3) (1–4). Although crosslinking of CD3/TCR alone is often sufficient to induce inositolphosphate pathway activation, other signals are apparently necessary to induce functions such as cytokine secretion and proliferation (4). Both soluble factors and interaction with cell surface receptors have been implicated as costimulators of CD3/TCR-mediated activation. For example, allo-antigen-specific helper T cells fail to produce cytokines in response to HLA-DR7 antigen expressed in murine L cells. However, cotransfection of HLA-DR7 and CD54 (intracellular adhesion molecule 1 [ICAM-1]), a cellular ligand for CD11/18 leukocyte function-associated molecule 1 [LFA-1]), restores T cell responsiveness (5). Thus, antigen-specific recognition alone may be insufficient to trigger effector functions since additional accessory molecules are required for an efficient response.

CD28 is a disulfide-linked homodimer that is expressed on the majority of human peripheral blood T cells (6, 7). mAbs against CD28 in conjunction with phorbol ester induce T cell proliferation (7) and augment proliferation induced by anti-CD3 or anti-CD2 mAb (8–12). Anti-CD28 mAb–induced proliferation is IL-2 dependent (7, 8) and possibly results from stabilization of messenger RNA for IL-2 and other cytokines (13).

Recently, it has been demonstrated that a B cell activation antigen, B7 (14) or BB1 (15), is a natural ligand for CD28 and that this receptor/ligand interaction mediates heterotypic adhesion (16). Interaction of CD28 and B7 results in augmentation of T cell proliferation and cytokine production, and antibodies against CD28 or B7 inhibit alloantigen and mitogen-induced proliferative responses (16, 17). B7 is weakly expressed on resting B cells and monocytes, and is elevated after stimulation with pokeweed mitogen, anti-Ig, anti-HLA class II, and EBV (14, 15, 17). Therefore, it is likely that T cell activation via CD28 will depend on the capacity of the APC to upregulate expression of B7. The importance of the CD28/B7 interaction in polyclonal mitogenic responses prompted us to investigate whether the binding of B7 to CD28

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costimulates the generation of a proliferative response against alloantigen and can augment the generation of T cell–mediated cytotoxicity in small, resting T lymphocytes.

Materials and Methods

Preparation of T Lymphocytes. Human peripheral blood was obtained from the Stanford Blood Center (Palo Alto, CA). Mononuclear cells were isolated by density gradient centrifugation using Ficoll/Hypaque. Monocytes and B cells were depleted by plastic adherence and passage through nylon wool columns (18). Nonadherent lymphocytes were fractionated by centrifugation on discontinuous gradients consisting of 30% and 40% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) in PBS containing 10% FCS (18). High buoyant density lymphocytes enriched for small, resting T cells were isolated from the bottom of the Percoll gradients. Thymocytes from 1-yr-old children undergoing cardiac surgery were obtained from Stanford Medical Center (Palo Alto, CA). The biopsies were minced with scissors, the single cell suspension was passed through a nylon mesh, and mononuclear cells were isolated by Ficoll/Hypaque density centrifugation. Umbilical cord blood was collected from healthy full-term neonates immediately after vaginal delivery. High buoyant density cord blood $CD3^+$ T lymphocytes were obtained as for peripheral blood.

T Cell Clones. T cell clones were established from normal PBL or fetal liver, as described (19). The following clones were used: 1375b2.22 is a TCR-γ/δ^+ CTL clone established from fetal liver; 1360b.1 and 1320.3 are TCR-α/β^+ CTL clones established from fetal liver. Jp28a.6 is a TCR-α/β^+ CTL clone established from normal PBL. Antigen specificity of these T cell clones has not been determined.

mAbs. BB1 mAb (15) was generously provided by Dr. Ed Clark (University of Washington, Seattle, WA). L293 is a murine IgG1 mAb directed against CD28 that was generated by immunizing BALB/c mice with the HPB-ALL T cell line and fusing immune splenocytes with Sp2/0 myeloma cells. L303 (IgG2a, κ) and L304 (IgG1, κ) are murine mAbs directed against CD2 and CD2R, respectively, that were generated by immunizing BALB/c mice with anti-CD3-activated PBL and fusing immune splenocytes with Sp2/0 myeloma cells. Other mAbs were generously provided by Becton Dickinson Immunocytometry Systems (San Jose, CA). $F(ab')_2$ fragments of anti-CD3, CD28 and CD16 mAbs (all IgG1 isotype) were prepared using immobilized pepsin (Pierce Chemical Co., Rockford, IL). Purity of $F(ab')_2$ fragments was verified by SDS-PAGE analysis.

Transfection. P815, a murine mastocytoma cell line, and Ramos, a human Burkitt's lymphoma cell line, were obtained from American Type Culture Collection (Rockville, MD). Human B7 cDNA (generously provided by Dr. J. Allison, University of California, Berkeley) was subcloned into the pBJ expression vector (20). 10^7 P815 and Ramos cells were transfected with 15 μg pBJ/B7 plasmid by electroporation (2.0 KV, 25 μFD) using a Gene Pulser (Bio-Rad Laboratories, Richmond, CA) and selected in culture medium (RPMI 1640 [M. A. Bioproducts, Walkersville, MD] + 10% FCS [JR Scientific, Woodland, CA], 1 mM sodium pyruvate, 1 mM L-glutamine, 1% penicillin-streptomycin) supplemented with 2 mg/ml G418 (Gibco Laboratories, Grand Island, NY). After drug selection, B7-transfected P815 and Ramos cells were cloned and selected for high cell surface B7 expression by flow cytometry. Transfectants were maintained in culture medium containing 1 mg/ml G418.

Anti-CD3-induced Redirected Cytotoxicity Assays. Cytotoxicity was measured in a 4-h ^{51}Cr-release assay (18). Anti-CD3 or anti-CD2 mAbs were added to ^{51}Cr-labeled P815 or $B7^+$ P815 target cells 30 min before addition of effector T cells, unless indicated otherwise. In mAb blocking studies, all mAbs were used at a final concentration of 5 μg/ml and were added at initiation of the assay.

Proliferation Assays. Irradiated (80 Gy) P815 and $B7^+$ P815 (3×10^4 cells/well) were plated with anti-CD3 (anti-Leu-4; 5 μg/ml) or anti-CD2 (L303 and L304, 1.25 μg/ml) mAbs in flat-bottomed 96-well microtiter plates (Falcon Labware, Lincoln Park, NJ). After incubation for 30 min at room temperature, high buoyant density T cells were added at 10^5 cells/well. Cultures were incubated at 37°C with 5% CO_2 in a humidified atmosphere for 72 h. For the mixed lymphocyte response, 10^5 high buoyant density T cells and 10^4 irradiated (80 Gy) Ramos or $B7^+$ Ramos cells were added to microtiter plate wells with or without mAbs and were incubated for 7 d. All cultures were labeled for the final 18 h with 1 μCi/well [^3H]thymidine (New England Nuclear, Boston, MA) and were harvested on a 96-well plate harvester (LKB Instruments Inc., Gaithersburg, MD). Incorporated radioactivity was measured in a beta plate scintillation counter (LKB Instruments, Inc.). Each value is the mean of triplicate wells. Standard mean error of the triplicates was <10%.

Immunofluorescence and Flow Cytometry. Methods of immunofluorescence and flow cytometry have been described previously (21, 22). Flow cytometry was performed using a FACScan® or FACStar^{PLUS}® (Becton Dickinson Immunocytometry Systems).

Results and Discussion

CD28/B7 Costimulates Both CD2- and CD3-dependent T Cell Proliferation. Human B7 cDNA (23) was subcloned into a mammalian expression vector, transfected into an FcR-bearing murine mastocytoma cell line, P815, and stable transfectants expressing high levels of surface B7 were established. A representative transfectant is shown in Fig. 1. To determine whether the $B7^+$ P815 cells were functionally competent to activate CD28, purified high buoyant density peripheral blood T cells were cocultured with irradiated $B7^+$ P815 or parental P815 cells in the presence or absence of anti-CD3 mAb. Con-

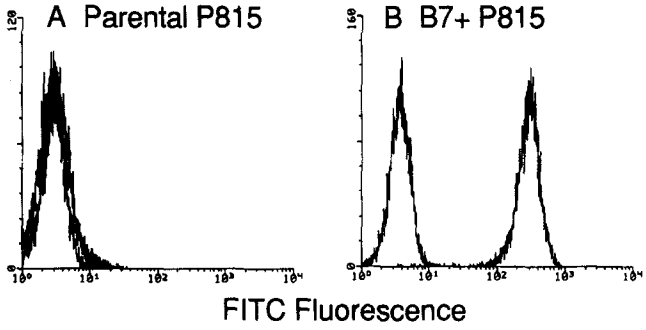

Figure 1. $B7^+$ P815 transfectants. Parental P815 (*A*) and $B7^+$ P815 (*B*) transfectants were stained with control or BB1 mAb, followed by FITC-conjugated goat anti-mouse Ig. Samples were analyzed by flow cytometry. The *x*-axis represents fluorescence (four-decade log scale) and the *y*-axis represents the relative cell number. Histograms from cells stained with control mAb (nearest the ordinate) are superimposed over the histogram of cells stained with BB1.

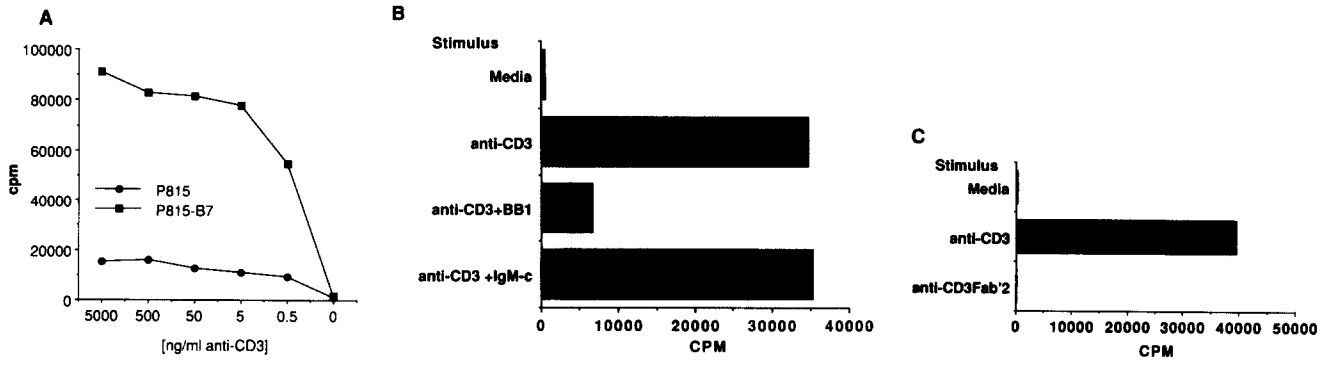

Figure 2. B7 costimulation of anti-CD3-induced T cell proliferation. (*A*) Proliferation of small, resting peripheral blood T cells cocultured with irradiated P815 (●) or B7+ P815 (■) in the presence of anti-CD3 mAb. (*B*) BB1 mAb, but not IgM control mAb Leu-7, inhibits B7+ P815 augmentation of anti-CD3-induced T cell proliferation. Note that BB1 mAb is a murine IgM and does not interfere with binding of anti-CD3 mAb to IgG Fc receptors on P815. (*C*) Intact but not F(ab')$_2$ fragments of anti-CD3 mAb stimulate proliferation of small, resting T cells in the presence of B7+ P815.

sistent with prior findings (24), freshly isolated, small peripheral blood T cells obtained from high buoyant density lymphocytes were minimally responsive to triggering via anti-CD3. However, when T cells were stimulated by coculture with B7+ P815, but not parental P815, we observed a pronounced effect on anti-CD3-induced proliferation (Fig. 2 *A*). Anti-CD3-induced proliferation costimulated by B7+ P815 was substantially inhibited by anti-B7 mAb BB1 (IgM) but not isotype-matched anti-CD57 mAb Leu-7 (IgM) (Fig. 2 *B*). Similarly, the addition of anti-CD28 F(ab')$_2$ fragments resulted in the inhibition of T cell proliferation (not shown). This indicated that the response was dependent upon the CD28/B7 interaction. Anti-CD3-induced proliferation was Fc dependent, in that no proliferation was observed when T cells and B7+ P815 were cultured with F(ab')$_2$ anti-CD3 fragments (Fig. 2 *C*).

mAbs directed against certain CD2 epitopes induce T cell proliferation (25). To determine whether the CD28/B7 interaction could also stimulate T cell proliferation triggered via CD2, we examined the effect of anti-CD2 mAbs on B7-dependent T cell proliferation (Fig. 3). Saturating concentrations of L303 (CD2) and/or L304 (CD2R) mAbs never induced substantial proliferation of small resting T cells in

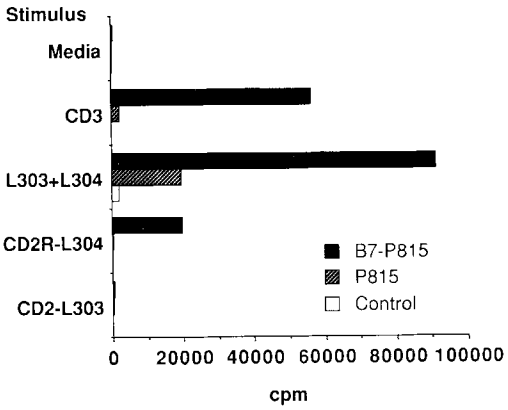

Figure 3. B7 costimulation of anti-CD2-induced T cell proliferation. Small, resting peripheral blood T cells were cocultured with irradiated parental P815 (▩), B7+ P815 (■), or without P815 cells (▨) in the presence or absence of anti-CD3, anti-CD2 (L303), and/or anti-CD2R (L304) mAb.

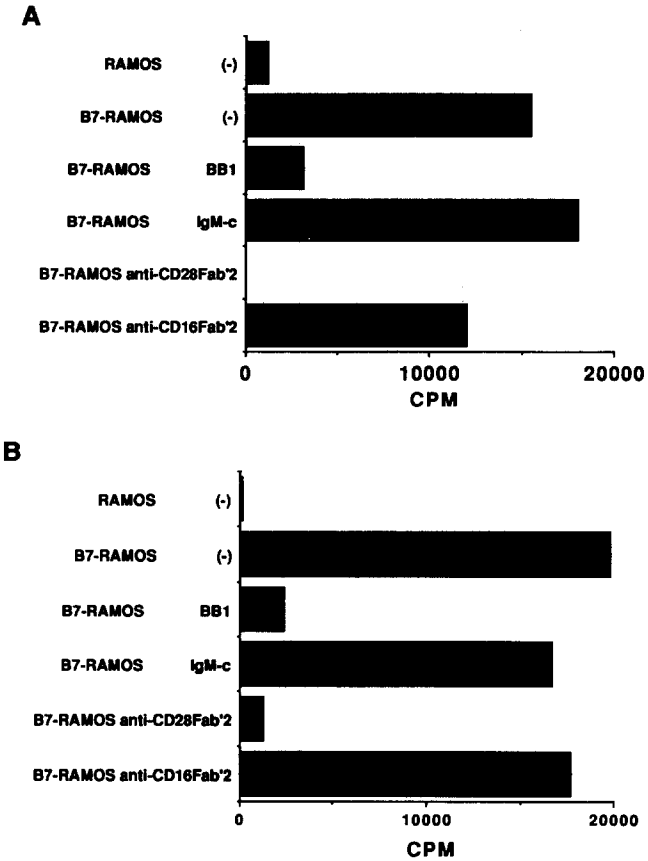

Figure 4. B7 expression augments an allogeneic MLR. Small, resting peripheral blood T cells from two donors (*A* and *B*) were cocultured with irradiated Ramos or B7+ Ramos in the presence or absence of BB1 mAb, anti-CD28 F(ab')$_2$ fragments, control IgM mAb Leu-7, or control anti-CD16 F(ab')$_2$ fragments, as indicated.

the presence of P815. However, when resting T cells were costimulated with B7+ P815, L303 and L304 together induced significant proliferation, which was inhibited by BB1 mAb (not shown). These results suggest that the binding of B7 to CD28 costimulates both the CD3 and CD2 activation pathways.

CD28/B7 Interactions Augment Primary Allogeneic MLR. EBV-transformed B cell lines are known to serve as potent stimulators of alloantigen-induced T cell proliferation. B lymphoblastoid cell lines have been reported to express high levels of cell surface B7 (15), suggesting the possibility that B7 expression is necessary for the generation of a primary MLR. Ramos is an EBV-negative American Burkitt's lymphoma cell line that expresses high levels of class I and II MHC antigens, but does not express B7. Preliminary experiments demonstrated that this cell line was a poor stimulator of allogeneic MLR. Ramos was transfected with B7 cDNA, and stable transfectants expressing high levels of B7 were selected. Fig. 4 shows the proliferative response of small, resting T cells from two donors cocultured with irradiated parental Ramos and B7-transfected Ramos. Parental B7-negative Ramos failed to stimulate small, resting T cells. By contrast, small, resting T cells cocultured with B7-transfected Ramos mediated substantial proliferative responses. These proliferative responses were substantially inhibited by either BB1 or anti-CD28 $F(ab')_2$ fragments, but not isotype-matched anti-CD57 or anti-CD16 $F(ab')_2$ fragments.

CD28/B7 Interactions Augment Cell-mediated Cytotoxicity Triggered through CD3 and CD2. CTL clones can lyse Fc receptor–bearing targets in the presence of anti-CD3 mAb in a "redirected" cytotoxicity assay (26–29). Unlike CTL clones, freshly isolated small resting T lymphocytes do not mediate anti-CD3 redirected lysis (29, 30), although a small subset of CD56+ or CD57+ T cells that may represent in vivo activated T cells do mediate this function (30, 31). Thus, engagement of CD3/TCR alone is insufficient to trigger a cytolytic response in the majority of resting peripheral blood T lymphocytes. It is possible that small, resting T lymphocytes lack the cellular machinery necessary for cytotoxicity. Alternatively, additional signals may be necessary to generate a cytolytic response in resting T cells that are not required for stimulation of activated T cells. The ability of combinations of hetero-bifunctional anti-CD3/antitumor and anti-CD28/antitumor mAbs to induce cytotoxicity suggested that interaction of CD28 with its natural ligand may provide the required costimulatory signal for generation of CTL from resting peripheral blood T cells (32). As shown in Fig. 5, freshly isolated small resting peripheral blood T cells demonstrated potent anti-CD3-redirected cytotoxicity against B7+ P815, but not parental P815, detectable using a 4-h ^{51}Cr release assay. Comparable results were obtained using three independently derived B7+ P815 transfectants; however, P815 transfectants expressing high levels of CD16-II were not lysed in the presence of anti-CD3 (not shown). Lysis of B7+ P815 transfectants was inhibited by BB1 or anti-CD28 $F(ab')_2$ fragments (Fig. 6 *A*). Anti-CD3-induced cytotoxicity was Fc dependent since anti-CD3 $F(ab')_2$ fragments failed to trigger lysis against B7+ P815 (Fig. 6 *B*).

mAbs against CD4 (Leu-3a), CD5 (Leu-1), CD7 (Leu-9), CD8 (Leu-2a), CD11a (L7), CD18 (L130), and CD38 (Leu-17) failed to induce redirected lysis against parental or B7+ P815 cells (not shown). However, as with the proliferative response, appropriate combinations of anti-CD2 mAb (L303 + L304) could trigger cytotoxicity against B7+ P815, but not parental P815 (Fig. 7). Anti-CD2-induced cytotoxicity was inhibited by BB1 antibody, but not isotype-matched control mAb (not shown). These data indicate that binding of B7 to CD28 costimulates CD3/TCR- and CD2-dependent T cell–mediated cytotoxicity and demonstrates that small, resting T cells are capable of cytotoxic function.

The mechanism whereby expression of B7 on the target cell enables small, resting T cells to initiate lysis has not been defined. It is possible that the CD28/B7 interaction increases or stabilizes heterotypic cellular adhesion between the effector and target cells. However, in the present experimental system this seems unlikely since effectors and Fc receptor–bearing targets are already efficiently bridged by anti-CD3 or anti-CD2 mAbs. No obvious differences were observed in conjugate formation between parental or B7+ P815 cells and effector T cells in the presence of anti-CD3 mAb (unpublished observation). Alternatively, interaction of CD28 with B7 on the target may provide a cosignal to the T cell that is necessary to mobilize the cellular machinery necessary for cytotoxicity. Preliminary studies have indicated that cytotoxicity is unlikely to be mediated by a soluble cytotoxic factor, since no bystander cytotoxicity was observed when ^{51}Cr-labeled parental P815 cells were admixed with unlabeled B7+ P815 cells in the presence of effector T cells and anti-CD3 mAb (unpublished observation). Further studies will be necessary to elucidate the mechanism of cell-mediated cytotoxicity initiated by small, resting peripheral blood T cells.

Effectors of CD28/B7-dependent Cytotoxicity. Using B7+ P815 targets, anti-CD3-redirected cytotoxicity could also be

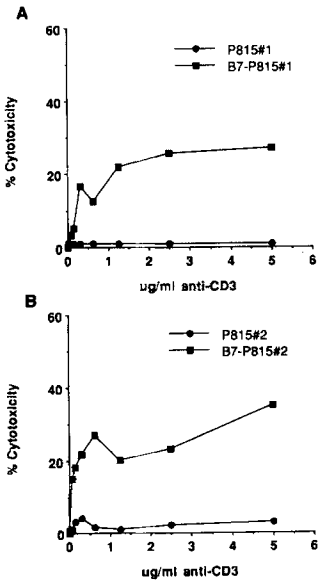

Figure 5. B7-dependent anti-CD3-redirected cytotoxicity. ^{51}Cr-labeled P815 (●) or B7+ P815 (■) target cells were added to plates with titered amount of anti-CD3 (Leu-4) mAb. After incubation for 30 min at room temperature, freshly isolated small, resting peripheral blood T cells were added and the assay was harvested after 4 h. Results from experiments using two different T cell donors (*A* and *B*) are shown. E/T ratio was 20:1.

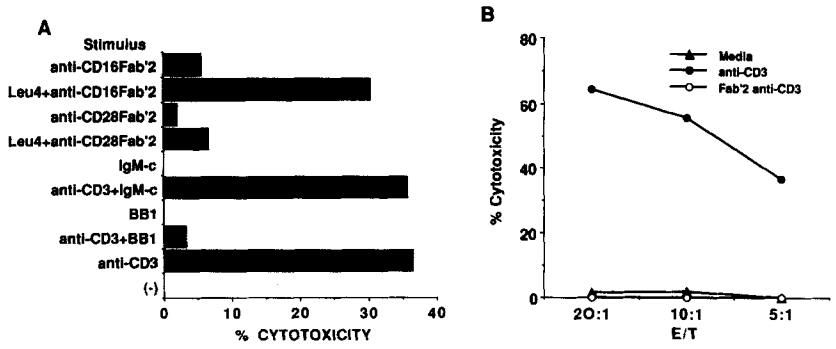

Figure 6. Inhibition of B7-dependent anti-CD3-redirected cytotoxicity. (*A*) Anti-CD28 $F(ab')_2$ fragments and BB1 (IgM) mAb, but not control anti-CD16 $F(ab')_2$ fragments or control Leu-7 (IgM) mAbs, inhibit anti-CD3-redirected cytotoxicity against $B7^+$ P815. E/T ratio was 20:1. (*B*) Intact (●), but not $F(ab')_2$ (○) anti-CD3, induces redirected cytotoxicity against ^{51}Cr-labeled $B7^+$ P815 targets. (▲) Cytotoxicity against $B7^+$ P815 cells in the absence of anti-CD3 mAb. All mAbs were used at a final concentration of 5 μg/ml and were added at the start of the assay.

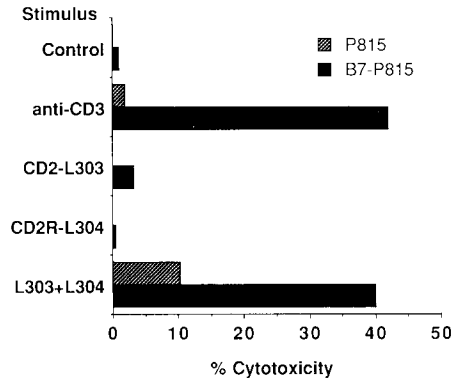

Figure 7. B7-dependent anti-CD2-redirected cytotoxicity. Anti-CD2 mAb–redirected cytotoxicity mediated by small, resting peripheral blood T cells against P815 (▩) or $B7^+$ P815 (■). All mAbs were used at a final concentration of 5 μg/ml and were added at the start of the assay. E/T ratio was 20:1.

demonstrated using freshly isolated thymocyte and cord blood T cell effectors, although the activity was lower than that observed using adult small, resting T cells (Fig. 8 *A*). Both small resting peripheral blood $CD56^-$,$CD4^+$ and $CD56^-$, $CD8^+$ T cells mediated equivalent levels of anti-CD3-induced cytotoxicity (Fig. 8 *B*). The ability of both CD4 and CD8 T cells to mediate cytotoxicity is consistent with the ability of both CD4 and CD8 T cell clones to mediate anti-CD3-redirected cytotoxicity, demonstrating that both major subsets of T lymphocytes possess the necessary cellular components for cytolytic function (27, 33, 34).

Prior studies have indicated that differential immune responses can be mediated by subsets of T cells distinguished based on expression of CD45 isoforms (35, 36). "Virgin" or "naive" T cells are characterized by expression of high levels of CD45RA and the absence of CD45RO, whereas "memory" T cells express CD45RO and lower levels of CD45RA. When small, resting peripheral blood T lymphocytes were separated into $CD45RO^-$ and $CD45RO^+$ fractions, $CD45RO^+$ T cells demonstrated a significantly higher level of cytotoxicity against $B7^+$ P815 targets when stimulated with either anti-CD3 (Fig. 9) or anti-CD2 mAbs (not shown). Comparable results were obtained using T cells isolated from four different individuals. It is likely that the B7-costimulated anti-CD3-redirected killing observed in cord blood T cells is accounted for by the presence of a low number of $CD45RO^+$ cells (9%) in this population. As shown in Fig. 10, both $CD45RO^-$ and $CD45RO^+$ T cells express CD28 on the cell surface, demonstrating that this differential responsiveness cannot be explained simply by lack of CD28 expression in the $CD45RO^-$ T cell population.

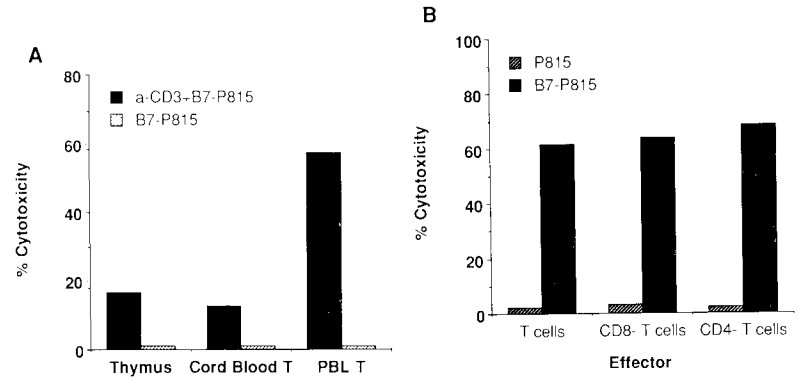

Figure 8. T subsets demonstrating B7-dependent T cell–mediated cytotoxicity. (*A*) Cytotoxicity mediated by thymocytes, cord blood T cells, and adult small, resting peripheral blood T cells against $B7^+$ P815 in the presence (■) or absence (▩) of anti-CD3. Note that high buoyant density cord blood $CD3^+$ T lymphocytes were 87% $CD4^+$, 12% $CD8^+$, and 9% $CD45RO^+$. T cells failed to demonstrate any cytotoxicity against parental P815 in the presence of anti-CD3 mAb (not shown). (*B*) Anti-CD3-redirected cytotoxicity mediated by $CD4^-$ and $CD8^-$ small, resting peripheral blood T cells against P815 (▩) or $B7^+$ P815 (■). High buoyant density T lymphocytes were stained with PE-conjugated anti-Leu-19 (CD56) and FITC-conjugated anti-Leu-2 (CD8) or anti-Leu-3 (CD4) mAbs. $CD8^+$,$CD56^-$; $CD8^-$,$CD56^-$; $CD4^+$,$CD56^-$, and $CD4^-$,$CD56^-$ subsets were isolated to >95% purity by flow cytometry. Data are presented for the $CD4^-$,$CD56^-$ (i.e., $CD8^+$) T cell fraction and the $CD8^-$,$CD56^-$ (i.e., $CD4^+$) T cell fraction. Positively sorted $CD8^+$,$CD56^-$ and $CD4^+$,$CD56^-$ T cells were also tested and demonstrated equivalent cytotoxicity against B7-P815 targets in the presence of anti-CD3 (not shown). E/T ratio was 20:1.

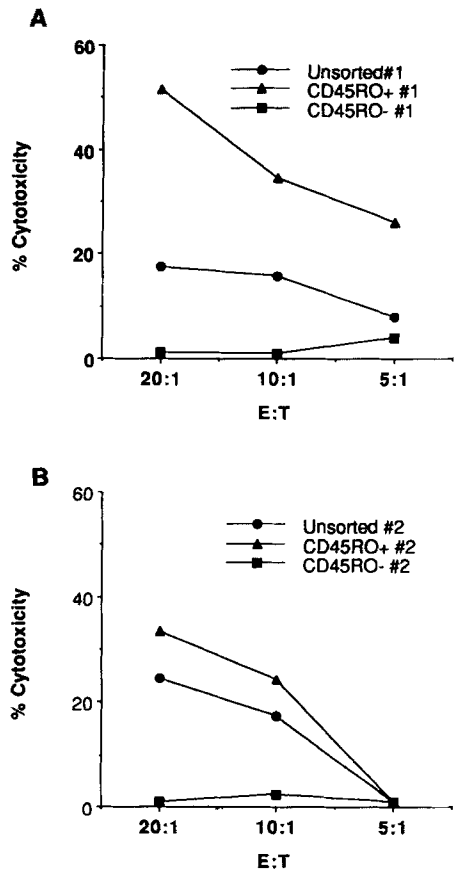

Figure 9. B7-dependent cytotoxicity mediated by memory T cells. High buoyant density T lymphocytes from two individuals (*A* and *B*) were stained with PE-conjugated anti-CD45RO (UCHL-1) and were sorted by flow cytometry. Purity of the sorted subsets was >98%. Unseparated T cells (●), CD45RO$^-$ T cells (■), and CD45RO$^+$ T cells (▲) were assayed for anti-CD3-redirected cytotoxicity against ^{51}Cr-labeled B7$^+$ P815 cells. Staining with anti-CD45RO mAb did not affect levels of anti-CD3-redirected lysis compared with unstained cells (not shown).

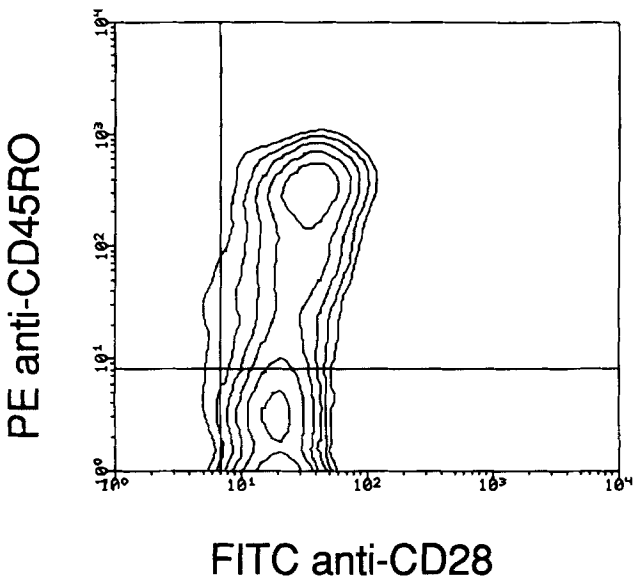

Figure 10. Coexpression of CD28 on CD45RO$^-$ and CD45RO$^+$ small, resting peripheral blood T cells. High buoyant density T lymphocytes were stained with FITC-conjugated anti-CD28 (9.3), PE-conjugated anti-CD45RO, and PerCP-conjugated anti-CD3, or appropriate control mAb. Samples were analyzed on a FACScan®. Markers were positioned to include >98% of cells stained with FITC- and PE-conjugated isotype control antibodies in the lower left quadrant (not shown). An electronic gate was set on PerCP-stained CD3$^+$ T cells, and correlated fluorescence of FITC (CD28) and PE (CD45RO) were displayed as a contour map (four-decade log scales). Analysis of CD45RO expression in small, resting T cells isolated from four individuals revealed that the proportion of CD45RO$^+$ T cells ranged from 48 to 78%.

memory T cell population is primarily responsible for this activity and both CD4 and CD8 T cells are capable of this function. It is unlikely that this cytotoxicity is mediated by in vivo activated T lymphocytes, since highly purified, small,

B7-independent Cytotoxicity Mediated by T Cell Clones. The above results suggest that small, resting T cells require CD28/B7 costimulation to induce CD2- or CD3-dependent cell-mediated cytotoxicity. In accordance with prior observations (26–29), TCR-α/β^+ and TCR-γ/δ^+ CTL clones mediated anti-CD3-redirected cytotoxicity against parental P815 (Fig. 11). Thus, while resting T cells apparently require CD28/B7 interactions to trigger cell-mediated cytotoxicity, this is no longer essential for stimulation of activated T lymphocytes. However, even with the T cell clones, we consistently observed that the presence of B7 on P815 slightly augmented cytotoxicity compared with parental P815 targets (Fig. 11).

In summary, interaction of CD28 with its natural ligand B7 costimulates the induction of cell-mediated cytotoxicity, as well as proliferation, of small, resting T lymphocytes. Both CD2 and CD3 activation pathways cooperate with CD28 as a consequence of CD28 binding its natural ligand B7. With respect to induction of cytotoxicity, the CD45RO$^+$

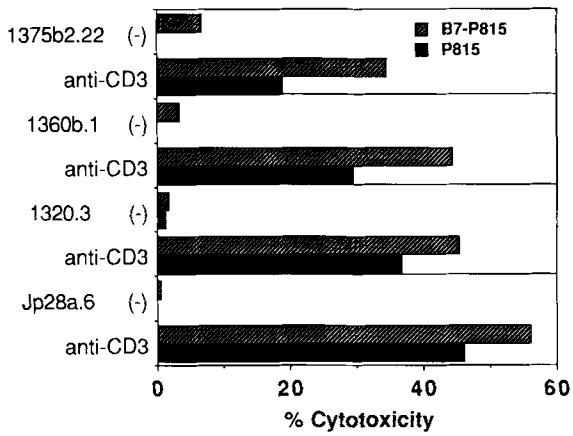

Figure 11. B7-independent anti-CD3-redirected cytotoxicity mediated by T cell clones. TCR-α/β^+ (Jp28a.6, 1360b.1, and 1320.3) and TCR-γ/δ^+ (1375b2.22) T cell clones were incubated with ^{51}Cr-labeled B7$^+$ P815 (▩) or P815 (■) in the presence or absence of anti-CD3 mAb, as indicated. E/T ratio was 10:1.

resting T cells isolated by density gradient centrifugation were used for these experiments. Moreover, cytotoxicity was only observed using the $B7^+$ P815 transfectant and not with the parental P815 cells. Since in vitro cultured CTL, as well as the presumably in vivo activated $CD56^+$ T cells mediate redirected lysis against parental P815, it seems likely that the CD28/B7 interaction is necessary only for costimulation of small, resting T cells and is no longer required after activation. Consistent with this concept, we have previously demonstrated that TCR-γ/δ^+ T lymphocytes that mediate potent anti-CD3-redirected cytotoxicity downregulate CD28 expression after stimulation with IL-2 (37). Moreover, the subset of $CD28^-$ peripheral blood T cells that coexpress CD11b in vivo are predominantly granular, low buoyant density T cells that may represent activated T cells that have undergone clonal expansion (38, 39). Therefore, the CD28/B7 interaction may be most critical in the early generation of a cell-mediated immune response, consistent with a possible role of CD28/B7 in the stimulation of immature thymocytes (40, 41). The existence of functional cytolytic T cells in the small, resting T cell population may be advantageous in facilitating rapid responses to immune challenge.

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Address correspondence to Dr. Lewis L. Lanier, Department of Immunology, DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304

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References

1. Imboden, J.B., and J.D. Stobo. 1985. Transmembrane signalling by the T cell antigen receptor: perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *J. Exp. Med.* 161:446.
2. Weiss, A., J. Imboden, D. Shoback, and J. Stobo. 1984. Role of T3 surface molecule in human T-cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium. *Proc. Natl. Acad. Sci. USA.* 81:4169.
3. Weiss, M.J., J.F. Daley, J.C. Hodgdon, and E.L. Reinherz. 1984. Calcium dependency of antigen-specific (T3-Ti) and alternative (T11) pathways of human T-cell activation. *Proc. Natl. Acad. Sci. USA.* 81:6836.
4. Weiss, A., J. Imboden, K. Hardy, B. Manger, C. Terhorst, and J. Stobo. 1986. The role of the T3/antigen receptor complex in T-cell activation. *Annu. Rev. Immunol.* 6:593.
5. Altmann, D.M., N. Hogg, J. Trowsdale, and D. Wilkinson. 1989. Cotransfection of ICAM-1 and HLA-DR reconstitutes human antigen-presenting cell function in mouse L cells. *Nature (Lond.).* 338:512.
6. Hansen, J.A., P.J. Martin, and R.C. Nowinski. 1980. Monoclonal antibodies identifying a novel T-cell antigen and Ia antigens of human lymphocytes. *Immunogenetics.* 10:247.
7. Hara, T., S.M. Fu, and J.A. Hansen. 1985. Human T cell activation II. A new activation pathway used by a major T cell population via a disulfide-bonded dimer of a 44 kilodalton polypeptide (9.3 antigen). *J. Exp. Med.* 161:1513.
8. Martin, P.J., J.A. Ledbetter, Y. Morishita, C.H. June, P.G. Beatty, and J.A. Hansen. 1986. A 44 kilodalton cell surface homodimer regulates interleukin 2 production by activated human T lymphocytes. *J. Immunol.* 136:3282.
9. Yang, S.Y., S.M. Denning, S. Mizuno, B. Dupont, and B.F. Haynes. 1988. A novel activation pathway for mature thymocytes: costimulation of CD2 (Tp50) and CD28 (Tp44) induces autocrine interleukin 2/interleukin 2 receptor-mediated cell proliferation. *J. Exp. Med.* 168:1457.
10. Damle, N.K., L.V. Doyle, L.S. Grosmaire, and J.A. Ledbetter. 1988. Differential regulatory signals delivered by antibody binding to the CD28 (Tp44) molecule during the activation of human T lymphocytes. *J. Immunol.* 140:1753.
11. van Lier, R.A., M. Brouwer, and L.A. Aarden. 1988. Signals involved in T cell activation. T cell proliferation induced through the synergistic action of anti-CD28 and anti-CD2 monoclonal antibodies. *Eur. J. Immunol.* 18:167.
12. Pierres, A., M. Lopez, C. Cerdan, J. Nunes, D. Olive, and C. Mawas. 1988. Triggering CD28 molecules synergize with CD2 (T11.1 and T11.2)-mediated T cell activation. *Eur. J. Immunol.* 18:685.
13. Lindsten, T., C.H. June, J.A. Ledbetter, G. Stella, and C.B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science (Wash. DC).* 244:339.
14. Freedman, A.S., G. Freeman, J.C. Horowitz, J. Daley, and L.M. Nadler. 1987. B7, a B cell-restricted antigen that identifies preactivated B cells. *J. Immunol.* 139:3260.
15. Yokochi, T., R.D. Holly, and E.A. Clark. 1982. B lymphoblast antigen (BB-1) expressed on Epstein-Barr virus-activated B cell blasts, B lymphoblastoid cell lines, and Burkitt's lymphomas. *J. Immunol.* 128:823.
16. 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.

17. 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.
18. Phillips, J.H., and L.L. Lanier. 1986. Dissection of the lymphokine-activated killer phenomenon: relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. *J. Exp. Med.* 164:814.
19. Yssel, H., J.E. De Vries, M. Koken, W. van Blitterswijk, and H. Spits. 1984. Serum-free medium for the generation and the propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods.* 72:219.
20. Lin, A.Y., B. Devaux, A. Green, C. Sagerstrom, J.F. Elliott, and M.M. Davis. 1990. Expression of T cell antigen receptor heterodimers in a lipid-linked form. *Science (Wash. DC).* 249:677.
21. Lanier, L.L., A.M. Le, J.H. Phillips, N.L. Warner, and G.F. Babcock. 1983. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. *J. Immunol.* 131:1789.
22. Lanier, L.L., and D.J. Recktenwald. 1991. Multicolor immunofluorescence and flow cytometry. *Methods, A Companion to Methods Enzymol.* 2:192.
23. Freeman, G.J., A.S. Freedman, J.M. Segil, G. Lee, J.F. Whitman, and L.M. Nadler. 1989. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J. Immunol.* 143:2714.
24. Ledbetter, J.A., C.H. June, P.J. Martin, C.E. Spooner, J.A. Hansen, and K.E. Meier. 1986. Valency of CD3 binding and internalization of the CD3 cell-surface complex control T cell responses to second signals: distinction between effects of protein kinase C, cytoplasmic free calcium, and proliferation. *J. Immunol.* 136:3945.
25. Meuer, S.C., R.E. Hussey, M. Fabbi, D. Fox, O. Acuto, K.A. Fitzgerald, J.C. Hodgdon, J.P. Protentis, S.F. Schlossman, and E.L. Reinheiz. 1984. An alternative pathway of T-cell activation: a functional role for the 50 kD T11 sheep erythrocyte receptor protein. *Cell.* 36:897.
26. Spits, H., H. Yssel, J. Leeuwenberg, and J.E. DeVries. 1985. Antigen-specific cytotoxic T cell and antigen-specific proliferating T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3. *Eur. J. Immunol.* 15:88.
27. Mentzer, S.J., J.A. Barbosa, and S.J. Burakoff. 1985. T3 monoclonal antibody activation of nonspecific cytolysis: a mechanism of CTL inhibition. *J. Immunol.* 135:34.
28. Siliciano, R.F., J.C. Pratt, R.E. Schmidt, J. Ritz, and E.L. Reinherz. 1985. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. *Nature (Lond.).* 317:428.
29. Leeuwenberg, J.F.M., H. Spits, W.J.M. Tax, and J.A. Capel. 1985. Induction of nonspecific cytotoxicity by monoclonal anti-T3 antibodies. *J. Immunol.* 134:3770.
30. Phillips, J.H., and L.L. Lanier. 1986. Lectin-dependent and anti-CD3 induced cytotoxicity are preferentially mediated by peripheral blood cytotoxic T lymphocytes expressing Leu-7 antigen. *J. Immunol.* 136:1579.
31. Garrido, M.A., P. Perez, J.A. Titus, M.J. Valdayo, D.F. Winkler, S.A. Barbieri, J.R. Wunderlich, and D.M. Segal. 1990. Targeted cytotoxic cells in human peripheral blood lymphocytes. *J. Immunol.* 144:2891.
32. Jung, G., J.A. Ledbetter, and H.J. Muller-Eberhard. 1987. Induction of cytotoxicity in resting human T lymphocytes bound to tumor cells by antibody heteroconjugates. *Proc. Natl. Acad. Sci. USA.* 84:4611.
33. Hayward, A., A. Boylston, and P. Beverley. 1988. Lysis of CD3 hybridoma targets by cloned human CD4 lymphocytes. *Immunology.* 64:87.
34. Rotteveel, F.T.M., I. Kokkelink, R.A.W. van Lier, B. Kuenen, A. Meager, F. Miedema, and C.J. Lucas. 1988. Clonal analysis of functionally distinct human $CD4^+$ T cell subsets. *J. Exp. Med.* 168:1659.
35. Sanders, M.E., M.W. Makgoba, and S. Shaw. 1988. Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets. *Immunol. Today.* 9:195.
36. Beverley, P.C.L. 1990. Is T-cell memory maintained by cross-reactive stimulation? *Immunol. Today.* 11:203.
37. Testi, R., and L.L. Lanier. 1989. Functional expression of CD28 on T cell antigen receptor γ/δ-bearing T lymphocytes. *Eur. J. Immunol.* 19:185.
38. Morishita, Y., H. Sao, J.A. Hansen, and P.J.Martin. 1989. A distinct subset of human CD4+ cells with a limited alloreactive T cell receptor repertoire. *J. Immunol.* 143:2783.
39. Yamada, H., P.J. Martin, M.P. Braun, P.G. Beatty, K. Sadamoto, and J.A. Hansen. 1985. Monoclonal antibody 9.3 and anti-CD11 antibodies define reciprocal subsets of lymphocytes. *Eur. J. Immunol.* 15:1164.
40. Turka, L.A., J.A. Ledbetter, K. Lee, C.H. June, and C.B. Thompson. 1990. CD28 is an inducible T cell surface antigen that transduces a proliferative signal in CD3+ mature thymocytes. *J. Immunol.* 144:1646.
41. Turka, L.A., P.S. Linsley, R. Paine III, G.L. Schieven, C.B. Thompson, and J.A. Ledbetter. 1991. Signal transduction via CD4, CD8, and CD28 in mature and immature thymocytes: Implications for thymic selection. *J. Immunol.* 146:1428.