CD2 Is Involved in Maintenance and Reversal of Human Alloantigen-specific Clonal Anergy

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Summary

Induction and maintenance of a state of T cell unresponsiveness to specific alloantigen would have significant implications for human organ transplantation. Using human histocompatibility leukocyte antigen DR7–specific helper T cell clones, we demonstrate that blockade of the B7 family of costimulatory molecules is sufficient to induce alloantigen-specific T cell clonal anergy. Anergized cells do not respond to alloantigen and a variety of costimulatory molecules, including B7-1, B7-2, intercellular adhesion molecule-1 (ICAM-1), and lymphocyte function–associated molecule (LFA)-3. However, after culture in exogenous interleukin (IL)-2 for at least 7 d, anergized cells can respond to alloantigen in the presence of LFA-3. LFA-3 costimulation subsequently restores responsiveness to alloantigen in the presence of previously insufficient costimulatory signals. Expression of CD2R epitope is downregulated on anergic cells and is restored after 7 d of IL-2 culture. The loss of the CD2R is temporally associated with the inability of anergized cells to respond to LFA-3. These results suggest that in addition to blockade of B7 family members, inhibition of CD2 and, potentially, other costimulatory pathways that might reverse anergy will be necessary to maintain prolonged alloantigen-specific tolerance.

Successful human allogeneic bone marrow and organ transplantation is presently dependent on the chronic administration of toxic nonspecific immunosuppressive agents. However, if long-lasting and potentially irreversible tolerance to alloantigen could be induced, then the associated attendant clinical toxicities might be ameliorated and the eligible donor pool would be increased. Approaches to achieve this objective would be to specifically inhibit the expansion of alloreactive T cells and, more importantly, devise strategies to induce anergy to donor-specific alloantigens.

For clonal expansion and effector function, alloreactive T cells require two signals (1). Signal 1 is both antigen specific and MHC restricted and is delivered to the TCR complex. Signal 2, termed costimulation, is provided by one or more accessory molecules expressed on alloantigen-presenting cells (allo-APCs)[1]. Costimulatory signals, in contrast to signal 1, are neither antigen specific nor MHC restricted, yet they are essential to induce maximal T cell proliferation, cytokine secretion, and induction of effector function (2–10). Engagement of the TCR by alloantigen without requisite costimulation fails to induce an immune response, but rather results in a state of antigen-specific unresponsiveness, termed anergy (11, 12). Although a large number of experimental systems have been developed to examine the mechanism(s) of anergy in both murine and human systems (12–16), little is known about which costimulatory molecules are involved in the induction, maintenance, and reversal of this state.

Of the numerous costimulatory molecules expressed on allo-APCs, preliminary studies demonstrated that B7-mediated costimulation appears to be critical to prevent the induction of anergy (17–22). Two members of the B7 family of costimulatory molecules, B7-1 and B7-2, have been molecularly cloned and are expressed on activated B lymphocytes and a variety of APCs (23–30). CD28, which serves as a major functional B7 family receptor, is constitutively expressed on resting T cells and is upregulated after activation (31). After signal 1 via the TCR, ligation of CD28 by either B7-1 or B7-2 results in IL-2 secretion and T cell proliferation (6, 7, 32, 33). T cells also express a second inducible receptor for the B7 family termed CTLA4 (34), which serves as the high affinity receptor for B7 (35). An immunoglobulin tailed fusion protein of CTLA4 (CTLA4-Ig) has proven to be the most effective reagent to inhibit B7 family member–mediated costimulatory function (19, 21, 32, 35). Recently, the importance of B7-mediated costimulation in the prevention of anergy was demonstrated when the consequences of B7-1– and intercellular adhesion molecule-1 (ICAM-1)–mediated costimulation were directly compared (36). Whereas B7-1 or ICAM-1 costimulatory molecules were equally potent in their ability

[1] *Abbreviations used in this paper:* allo-APC, alloantigen-presenting cell; ICAM-1, intercellular adhesion molecule 1; LBL, lymphoblastoid cell line.

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to efficiently present alloantigen to unprimed human alloreactive $CD4^+$ T cells, only B7-1 costimulation was accompanied by significant IL-2 accumulation, thereby preventing the induction of anergy. In contrast, ICAM-1–mediated costimulation did not induce detectable IL-2 accumulation and did not prevent the induction of human alloantigen–specific anergy.

Although blockade of the B7 costimulatory family appears to be critical for the induction of anergy, a major concern is whether other costimulatory molecules expressed on allo-APCs might reverse the established state of anergy. We and others have observed that anergized T cells can proliferate in response to exogenous IL-2 (12, 18, 36, 37). Moreover, antigen-specific T cell clonal anergy can be reversed by prolonged culture of anergized cells in IL-2 followed by presentation of that same antigen by professional APC (37, 38). The capacity of endogenous IL-2 to reverse anergy would present a significant obstacle to implementing the induction of alloantigen-specific anergy as a clinical strategy. Therefore, we sought to determine how addition of exogenous IL-2 would reverse the state of human alloantigen–specific anergy in vitro, and if so, which costimulatory molecules are involved in this response.

Materials and Methods

Human T Cell Clones. HLA-DR7 alloantigen-specific T cell clones were generated by use of standard methodology (39). Briefly, PBMCs from an HLA-DR7/DR1/DR8–negative individual were stimulated with an HLA-DR7 homozygote EBV-transformed lymphoblastoid cell line (LBL-DR7), which strongly expresses MHC class II, B7-1, B7-2, ICAM-1, and LFA-3 molecules, in 1:1 responder/stimulator ratio at a concentration of 10^6 cells/ml. After three repetitive stimulations, alloactivated T cells were isolated by Percoll gradient in the 40–50% fraction and cloned by limiting dilution (0.3 cells/well) in 96-well flat-bottom plates (Nunc, Roskilde, Denmark). Clones were expanded in 24-well plates with addition of fresh IL-2 (50 U/ml) (Collaborative Biomedical Products, Bedford, MA) every 5 d and restimulation with LBL-DR7 stimulators every 10 d. Before each experiment, T cell clones were rested for 10 d to 2 wk after restimulation. Before use, cells were resuspended in complete culture medium consisting of RPMI 1640 with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml) and gentamicin sulfate (5 μg/ml) and cultured overnight without any stimulus or IL-2.

Monoclonal Antibodies and Fusion Proteins. mAbs were used as purified Ig unless indicated otherwise: anti-B7 (133): IgM (23); BB-1: IgM (40) (Dr. E. Clark, University of Washington, Seattle, WA); anti-MHC class II: 9-49, IgG2a (41) (Dr. R. Todd, University of Michigan, Ann Arbor, MI); anti-LFA-1a: TS1/22, IgG1 (42) and anti-LFA-3: TS2/9, IgG1 (43) (Dr. T. Springer, Center for Blood Research, Boston, MA); anti-CD24 (BA-1,1:100 ascites) (44), anti-CD40 (JRG 12): IgG1, anti-CD72 (J3-109, BU-40, 1:100 ascites) (45); anti-CD2 (T11.1, T11.2, T11.3): IgG1 (46). Construction of human CTLA4-Ig (18) and control fusion protein (control-Ig) (36) was previously described. All (purified) mAbs and fusion proteins were used at 10 μg/ml.

NIH 3T3 and COS Transfected Cells. Alloantigen and/or costimulatory molecules were presented by use of NIH-3T3 stable transfectants (t-) that had been named artificial allo-APCs. The preparation of these allo-APCs has been previously described (36). COS cells were transfected with cDNAs encoding either HLA-DRα and DR7β (DRB1*0701) chains (COS-DR7), B7-1 and B7-2 (COS-B7-1/B7-2), HLA-DRα and DR7β, B7-1 and B7-2 (COS-DR7/B7-1/B7-2), LFA-3 (COS-LFA-3), HLA-DRα and DR7β and LFA-3 (COS-DR7/LFA-3), HLA-DRα and DR7β, LFA-3, B7-1 and B7-2 (COS-DR7/LFA-3/B7-1/B7-2), or pCDNAI vector alone (COS-mock). The expression of DR7 and LFA-3 were assessed by use of 9-49 (41) and TS2/9 (43) mAbs, respectively, followed by FITC-conjugated goat anti–mouse Ig; the expression of B7-1 and B7-2 was assessed by use of biotinylated CTLA4-Ig, followed by phycoerythrin-conjugated streptavidin. The coexpression of LFA-3 and DR7 was assessed by use of TS2/9 (43) followed by FITC-labeled goat anti–mouse Ig and phycoerythrin-conjugated 9-49 (41); it was detectable in 72% of the untransfected cells (data not shown). The coexpression of DR7 and B7-1/B7-2 was assessed by use of FITC-conjugated 9-49 (41) and biotinylated CTLA4-Ig (18) followed by phycoerythrin-conjugated streptavidin; it was detectable in 50% of the transfected cells (data not shown).

Primary and Secondary Stimulation. Alloantigen-specific T cell clones were incubated in a primary culture with either t-DR7, t-DR7/B7-1, LBL-DR7, LBL-DR7 + anti-B7-1 (133) mAb (23), or LBL-DR7 + CTLA4-Ig fusion protein (18) (which blocks costimulation by B7-1 and B7-2). TC-1 and allo-APCs were cultured at the optimal 1:1 ratio (Boussiotis, V. A., unpublished results). After 72 h of primary culture, TC-1 cells were isolated from artificial allo-APCs by Percoll gradient centrifugation and from LBL-DR7 by Ficoll separation, washed extensively, and cultured in media without IL-2 for 24 h. Each population was subsequently rechallenged with a secondary stimulus: (*a*) each one of the allo-APCs (t-DR7, t-DR7/B7, t-DR7/ICAM-1); (*b*) LBL-DR7; and (*c*) IL-2 (100 IU/ml). The secondary stimulation with artificial allo-APCs or LBL-DR7 was also performed at 1:1 ratio (2.5×10^4 cells/well). Samples were cultured in triplicate in flat-bottom 96-well culture plates at 37°C for 72 h in 5% CO_2. Artificial allo-APCs and LBL-DR7 cells were treated with 20 μg/ml mitomycin-C (Sigma Chemical Co., St. Louis, MO) at 37°C for 2 h and then washed extensively before use. For some experiments, LBL-DR7 cells were irradiated at 9,600 rads or fixed with paraformaldehyde according to a previously described protocol (7).

IL-2 Culture and Reversal of Anergy. T cell clones were first anergized by primary stimulation with t-DR7 and then cultured in IL-2 for 3 to 21 d. At several time intervals of IL-2 culture, T cell clones were rechallenged in a secondary stimulation as above, with either (*a*) allo-APCs (t-DR7, t-DR7/B7, t-DR7/ICAM-1); (*b*) LBL-DR7 alone or in the presence of each one of the following mAbs or fusion proteins: anti-DR, anti-LFA-1, anti-LFA-3, anti-B7-1 (133), BB-1, CTLA4-Ig, anti-CD24, anti-CD40, anti-CD72; or (*c*) COS-mock, COS-DR7, COS-B7-1/B7-2, COS-LFA-3, COS-DR7/B7-1/B7-2, COS-DR7/LFA-3, COS-DR7/LFA-3/B7-1/B7-2. To determine whether alloantigen-specific responsiveness can be fully restored after IL-2 culture and examine the role of several costimulatory pathways in the reversal of anergy, T cell clones cells were anergized by primary culture with t-DR7, maintained in IL-2 (100 IU/ml) for 7 d, and rechallenged in a secondary stimulation with LBL-DR7, COS/DR7/LFA-3, or COS/DR7/B7-1/B7-2. For these experiments, secondary stimulation was performed in 24-well plates; TC-1 cells were isolated from LBL-DR7 by Ficoll separation and from COS transfectants by Percoll gradient centrifugation and subsequently rechallenged with each one of the following stimuli: (*a*) allo-APCs (t-DR7, t-DR7/B7, t-DR7/ICAM-1), (*b*) LBL-DR7, or (*c*) IL-2 (100 IU/ml). Stimulation with artificial allo-APCs or LBL-DR7 was performed in 1:1 ratio (2.5×10^4 cells/well).

Samples were cultured in triplicate at 37°C for 72 h in 5% CO_2.

Thymidine Incorporation. Thymidine incorporation was used as an index of mitogenic activity. During the last 16 h of a 72-h culture period, cells were incubated with 1 μCi of [methyl-^{3}H]thymidine (DuPont, Boston, MA). The cells were harvested onto filters, and the radioactivity on the dried filters was measured in a β-plate liquid scintillation counter (Pharmacia LKB, Uppsala, Sweden).

IL-2 Assay. IL-2 accumulation was measured by ELISA (Biosource, Camarillo, CA) in supernatants harvested 24 h after the initiation of the cultures.

Results

Absence of Costimulation by B7 Family Members Induces Human Alloantigen-specific Clonal Anergy. Artificial allo-APCs were made by transfection of HLA-DR7 alone (t-DR7) or with B7-1 (t-DR7/B7-1) or ICAM-1 (t-DR7/ICAM-1) in NIH-3T3 cells (36). t-DR7/B7-1, t-DR7/ICAM-1, and a HLA-DR7 homozygous lymphoblastoid cell line (LBL-DR7) induced equally potent T cell clonal proliferation (data not shown). T cell clonal proliferation was alloantigen mediated since no response was observed in the absence of HLA-DR7 (transfectants expressing only B7-1 or ICAM-1 alone) or blocking DR on t-DR7/B7-1, t-DR7/ICAM-1, or LBL-DR7 stimulators with anti-DR mAb (data not shown). All studies described here were conducted simultaneously with two helper T cell clones (TC-1 and TC-2) (CD4$^+$ CD8$^-$ CD28$^+$). Since the results with both clones were consistently comparable, only results with TC-1 are presented.

To determine whether TCR signaling in the absence of costimulation induced by members of the B7 family was sufficient to induce T cell clonal anergy, TC-1 clones were cocultured for 72 h with either t-DR7, t-DR7/B7, or LBL-DR7 cells alone or in the presence of anti-B7-1 mAb or CTLA4-Ig. After primary culture, TC-1 cells were then rechallenged with either artificial allo-APCs, LBL-DR7 cells, or IL-2. Primary culture with t-DR7 resulted in an anergic response on rechallenge (absence of [^{3}H]thymidine incorporation and IL-2 accumulation) (Fig. 1 *A*). In contrast, primary culture with either t-DR7/B7-1 or LBL-DR7 resulted in a significant secondary proliferative response and IL-2 accumulation (Fig. 1, *B* and *C*). Addition of anti-B7-1 to LBL-DR7 cells in the primary culture resulted in reduced proliferation and IL-2 accumulation but did not induce unresponsiveness on rechallenge (Fig. 1 *D*). However, primary culture with LBL-DR7 in the presence of CTLA4-Ig resulted in anergy (Fig. 1 *E*). In all instances, anergized and nonanergized cells responded equally well to exogenous IL-2. These results demonstrate that blockade of the B7 family of costimulatory molecules is necessary to induce anergy. Since all other costimulatory molecules expressed on LBL-DR7 cells that are not blocked by CTLA4-Ig did not prevent the induction of anergy, these results also suggest that blockade of the B7 family is sufficient to induce anergy. Moreover, after the induction of anergy, neither B7 family members nor other non-B7 costimulatory molecules expressed on LBL-DR7 cells appear to be capable of reversing this state.

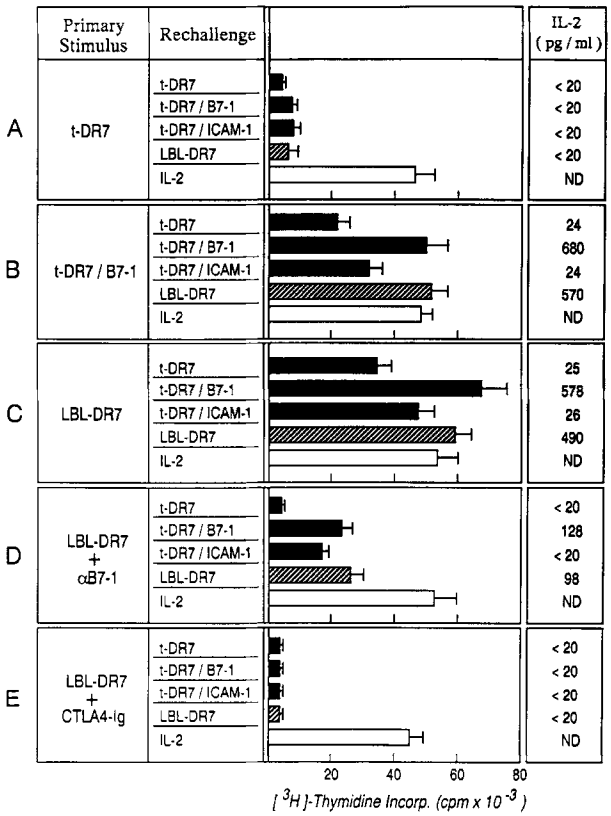

Figure 1. TCR signaling in the absence of costimulation by B7 family members induces alloantigen-specific clonal anergy. Alloantigen-specific TC-1 clone was primarily cultured with either t-DR7, t-DR7/B7-1, LBL-DR7, LBL-DR7 + anti-B7-1 (133) mAb, or LBL-DR7 + CTLA4-Ig fusion protein. After 72 h of primary culture, TC-1 cells were isolated from artificial allo-APCs by Percoll gradient and from LBL-DR7 by Ficoll separation, washed extensively, and cultured in media without IL-2 for 24 h. Each population was subsequently restimulated with a secondary stimulus as shown. IL-2 concentration was assessed in supernatants of 24 h of culture, and thymidine incorporation was measured for the last 16 h of a 72-h culture period. Results are representative of seven experiments. *Solid bars* represent response of TC-1 cells to artificial allo-APCs, *stripped bars* represent response to LBL-DR7, and *open bars* represent response to exogenous recombinant human IL-2.

Restoration of Alloantigen-specific Responsiveness After Culture in IL-2. TC-1 cells were anergized by primary culture with t-DR7 and then cultured in IL-2 for 3–21 d to determine whether IL-2 could restore responsiveness to specific alloantigen. At any time up to 21 d after IL-2 incubation, rechallenge in the secondary culture with t-DR7, t-DR7/B7-1, or t-DR7/ICAM-1 did not induce response (Fig. 2 *A*) at a wide range of stimulator/responder ratios (data not shown). In contrast, after at least 7 d of IL-2 culture, rechallenge with LBL-DR7 induced a proliferative response (Fig. 2, *A* and *B*), which was associated with significant accumulation of IL-2 (Fig. 2 *B*). Response induced by LBL-DR7 cells was alloantigen specific since it was inhibited by anti-DR mAb, and two other DR homozygous LBLs (LBL-DR1 and LBL-DR8) did not induce proliferation or IL-2 accumulation (Fig. 2 *B*). After prolonged IL-2 culture, alloantigen-specific re-

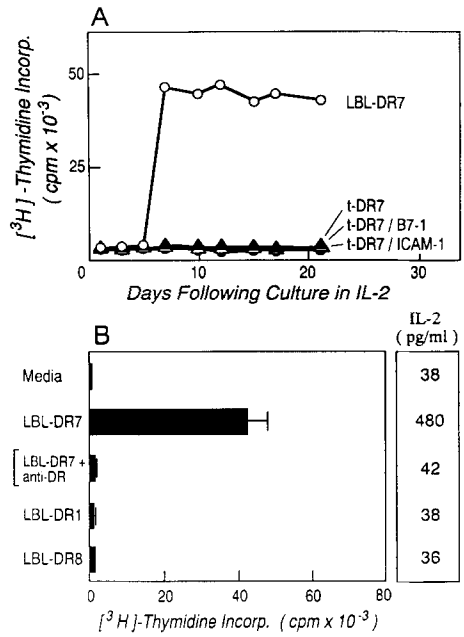

Figure 2. (*A*) After prolonged culture in IL-2, LBL-DR7 but not artificial allo-APCs can restore responsiveness of anergized TC-1 cells to specific alloantigen. TC-1 cells were first anergized by primary stimulation with t-DR7, and then cultured in IL-2 for 7 to 21 d. At the indicated time intervals of IL-2 culture, these TC-1 cells were rechallenged with equal numbers of each type of artificial allo-APCs or LBL-DR7 as stimulators. Proliferation was measured as in Fig. 1. Results are representative of five experiments. (*B*) Reversal of anergy by LBL-DR7 stimulators after IL-2 culture is alloantigen specific. After induction of anergy with t-DR7 allo-APCs followed by culture in IL-2 for 7 d, TC-1 cells were rechallenged with either LBL-DR7, LBL-DR7+anti-DR mAb, LBL-DR1, or LBL-DR8. Proliferation and IL-2 accumulation were measured as in Fig. 1. Results are representative of three experiments.

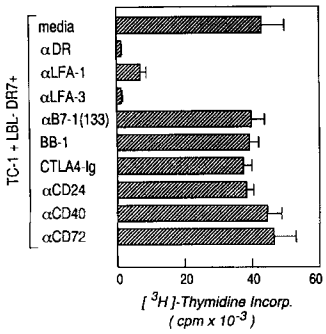

Figure 3. LFA-3, but not other known costimulatory molecules, are involved in the reversal of alloantigen-specific clonal anergy. After anergy induction with t-DR7 allo-APCs followed by culture in IL-2 for at least 7 d, TC-1 cells were rechallenged with LBL-DR7 either alone or in the presence of each of the following mAbs or fusion proteins: anti-DR, anti-LFA-1, anti-LFA-3, anti-B7-1 (133), BB-1, CLTA4-Ig, anti-CD24, anti-CD40, and anti-CD72. Proliferation was measured as in Fig. 1. Results are representative of four experiments.

sponsiveness can be restored, but neither B7-1 nor ICAM-1 appear to be sufficient to provide costimulation.

The capacity of LBL-DR7 cells to restore alloantigen-specific response was not mediated by a secreted soluble factor since rechallenge with t-DR7/B7 or t-DR7/ICAM-1 in the presence of LBL-DR7 culture supernatant did not restore responsiveness (data not shown). In contrast, paraformaldehyde-fixed LBL-DR7 induced the identical proliferative response as mitomycin C–treated or irradiated LBL-DR7 cells, suggesting that a cell surface molecule and not a soluble factor is involved in reversal of anergy (data not shown). Taken together, these results suggest that a paraformaldehyde-resistant cell surface molecule that is neither B7-1 nor ICAM-1 costimulates with alloantigen to restore responsiveness after IL-2 culture.

To determine whether a known costimulatory molecule(s) is responsible for the restoration of alloantigen-specific responsiveness by LBL-DR7 stimulators, blocking mAbs or fusion proteins directed against a variety of costimulatory molecules expressed on APCs were used. Only anti-DR, anti-LFA-1, and anti-LFA-3 mAbs inhibited the proliferative response (Fig. 3). Neither B7 family members nor other costimulatory molecules, including CD24, CD40, and CD72, appear to be important for the reversal of anergy. These results are consistent with the hypothesis that LFA-3 and alloantigen are the molecules responsible for the observed reversal of anergy. We doubt that LFA-1 and alloantigen are involved in the reversal of anergy, since t-DR7/ICAM-1 did not restore proliferation of anergized cells after culture with IL-2 (Fig. 2 *A*). However, we cannot exclude the possibility that other LFA-1 ligands (i.e., ICAM-2 or ICAM-3) also might contribute to the reversal of anergy.

It has been shown that LFA-3 can provide costimulation to antigen-specific T cell activation (5, 47, 48). To examine whether LFA-3 costimulation is also sufficient to reverse the state of established alloantigen-specific clonal anergy, COS transfectants expressing DR7 alone (COS-DR7), costimulatory molecules alone (COS-B7-1/B7-2 or COS-LFA-3), or DR7 in combination with one or more of these costimulatory molecules (COS-DR7/B7-1/B7-2, COS-DR7/LFA-3, COS-DR7/LFA-3/B7-1/B7-2) were made. TC-1 cells were anergized with t-DR7 allo-APCs, cultured in IL-2 for 7 d, and then rechallenged with the different populations of COS transfectants. As shown in Fig. 4 *A*, challenge with COS-DR7, COS-B7-1/B7-2, COS-LFA-3, and COS-DR7/B7-1/B7-2 did not induce proliferation or IL-2 accumulation. In contrast, transfectants coexpressing DR7 and LFA-3 (COS-DR7/LFA-3 and COS-DR7/LFA-3/B7-1/B7-2) induced equivalent levels of proliferation and IL-2 accumulation, indicating that after prolonged IL-2 culture, responsiveness to alloantigen can be restored in the presence of LFA-3 costimulation. Attempts to culture anergized TC-1 cells in media without IL-2, in order to determine whether exogenous IL-2 is necessary before rechallenge for the reversal of anergy, were unsuccessful since all our alloantigen-specific clones were IL-2 dependent and so underwent apoptosis after IL-2 deprivation for longer than 72 h (data not shown).

Reversal of Anergy by Specific Alloantigen and LFA-3 Costimulation. We then sought to determine whether LFA-3 costimulation had reversed anergy and fully restored alloantigen-specific responsiveness of anergized cells. TC-1 cells were anergized by primary culture with t-DR7, cultured in IL-2 for 7 d, and co-cultured with either LBL-DR7, COS-DR7/LFA-3, or COS-DR7/B7-1/B7-2 for an additional 3 d, as above. These

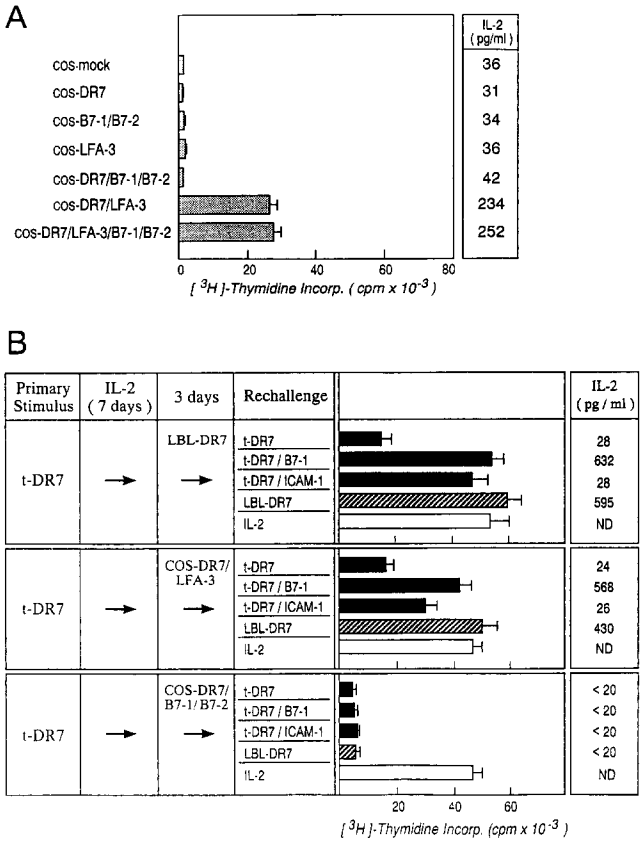

Figure 4. (*A*) Alloantigen-specific anergy can be partially reversed by artificial APCs expressing alloantigen and LFA-3. After anergy induction with t-DR7 allo-APCs followed by culture in recombinant human IL-2, TC-1 cells were rechallenged with the indicated types of COS transfectants. Proliferation and IL-2 accumulation were measured as in Fig. 1. Results are representative of three experiments with COS-DR7/LFA-3, COS-DR7/B7-1/B7-2, and COS-mock, and two experiments with COS-DR7, COS-LFA-3, COS-B7-1/B7-2, and COS-DR7/LFA-3/B7-1/B7-2 transfectants. (*B*) Alloantigen and LFA-3 restore the capacity of anergized cells to respond to subsequent rechallenge with specific alloantigen in the presence of previously insufficient costimulatory molecules. After anergy induction with t-DR7 allo-APCs followed by culture in IL-2, TC-1 cells were rechallenged with either LBL-DR7, COS-DR7/LFA-3, or COS-DR7/B7-1/B7-2 for 72 h. Subsequently, they were isolated from LBL-DR7 by Ficoll separation and from COS transfectants by Percoll gradient and rechallenged with the shown stimuli. Proliferation and IL-2 accumulation were measured as in Fig. 1. Results are representative of four experiments when rechallenge was done with LBL-DR7 stimulators and two experiments when rechallenge was done with COS transfectant stimulators.

cells were then rechallenged with either t-DR7, t-DR7/B7-1, t-DR7/ICAM-1, or LBL-DR7. After sequential culture with IL-2 and LBL-DR7 or COS-DR7/LFA-3, previously anergized TC-1 cells proliferated in response to t-DR7/B7-1, t-DR7/ICAM-1, and LBL-DR7 (Fig. 4 *B*, *top and middle*). In contrast, when COS-DR7/B7-1/B7-2 were used instead of LBL-DR7 after IL-2 culture, no response on rechallenge was seen (Fig. 4 *B*, *bottom*). In all instances, TC-1 cells proliferate equally to exogenous IL-2. Culture in IL-2 for at least 7 d followed by presentation of alloantigen and costimulation with LFA-3 is sufficient to reverse alloantigen-specific anergy and restore responsiveness to alloantigen in the presence of previously insufficient costimulatory signals.

Phenotypic and Functional Characterization of the CD2 Epitopes During the Induction, Maintenance, and Reversal of Anergy. LFA-3 mediates activation of T cells via the CD2 molecule (5, 47–50), and distinct functional CD2 epitopes regulate this alternative pathway of T cell activation (46, 51–53). Since anergized TC-1 cells did not respond to LBL-DR7 immediately after the induction of anergy with t-DR7 (Fig. 1) but did respond to LBL-DR7 or COS-DR7/LFA-3 after 7 d of IL-2 culture (Figs. 2 *A* and 4 *A*), we attempted to determine whether induction and reversal of anergy correlated with the expression of distinct epitopes of the CD2 molecule. As shown in Fig. 5, TC-1 clones expressed CD2 (T11.1 and T11.2), and CD2R (T11.3) epitopes. After induction of anergy with t-DR7, TC-1 cells still expressed T11.1 and T11.2 but CD2R (T11.3) was no longer detectable. The T11.3 epitope was reexpressed after 7 d of culture in IL-2, precisely when these cells regained responsiveness to alloantigen and LFA-3 costimulation. Moreover, although nonanergic TC-1 cells proliferated to the mitogenic combination of T11.3 with either T11.1 or T11.2 mAbs (Fig. 6 *A*), anergic cells did not (Fig. 6 *B*). After 7 d of culture with IL-2 and accompanying the reappearance of the T11.3 epitope, anergic T cells regained responsiveness to mitogenic combinations of anti-CD2 mAbs (Fig. 6 *C*). These results suggest that the inability of anergized TC-1 cells to respond to LBL-DR7 appears to be associated with the downregulation of the CD2R epitope. Moreover, the recovery of LFA-3 responsiveness after 7 d of IL-2 culture coincided with the reexpression of T11.3.

We have demonstrated previously that although ICAM-1 is as potent as B7-1 costimulation in its ability to induce

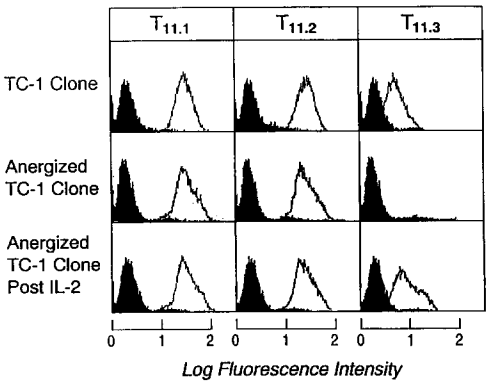

Figure 5. Expression of CD2R (T11.3) epitope of CD2 molecule is downregulated during anergy induction and restored after IL-2 culture. TC-1 cells before and after induction of anergy and after culture with IL-2 for 7 d were stained with antibodies for the T11.1, T11.2, or T11.3 epitopes of the CD2 molecule or the appropriate isotype-matched controls and were analyzed by flow cytometric analysis (Epics Elite Flow cytometer; Coulter Corp., Hialeah, FL). Shaded peak represents staining with isotype-matched mAb, and unshaded peak represents staining with mAbs for either T11.1, T11.2, or T11.3 epitopes, as indicated. Data are representative of three experiments.

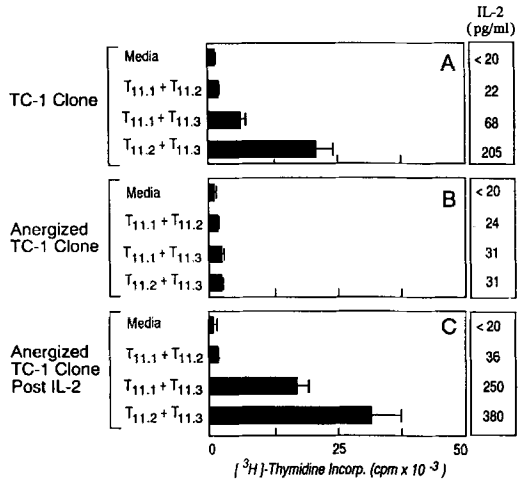

Figure 6. Unstimulated and anergic TC-1 cells after IL-2 culture proliferate to the mitogenic combination of T11 mAbs but anergic cells do not. TC-1 cells before stimulation, after anergization with t-DR7 allo-APCs, or after anergization and IL-2 culture were stimulated with mitogenic combinations of T11 mAbs as shown. Proliferation and IL-2 accumulation were measured as in Fig. 1. Results are representative of three experiments.

alloantigen-specific proliferative response by unprimed CD4$^+$ human T cells (36) and T cell clones (Boussiotis, V. A., unpublished results), it cannot prevent the induction of alloantigen-specific anergy. To determine whether the absence of CD2R was related directly to the induction of anergy or simply reflected lack of T cell proliferation, we examined whether anergization with t-DR7/ICAM-1 allo-APCs was also accompanied by downregulation of CD2R expression. As was observed after anergization with t-DR7, CD2R was lost by 24 h and was still absent at 5 d of culture (data not shown) despite the fact that these cells were proliferating. These data suggest that the observed downregulation of CD2R is associated with the induction of anergy.

Although Involved in the Reversal of Anergy, LFA-3 Costimulation During Primary Culture Does Not Prevent the Induction of Anergy. In light of the evidence that antigen-specific LFA-3–mediated costimulation appeared to be critical for the reversal of anergy, we sought to determine whether LFA-3–mediated costimulation during primary stimulation is involved in the prevention of anergy. LBL-DR7 strongly express both alloantigen and LFA-3, and TC-1 cells express all three epitopes of the CD2 molecule. However, LFA-3 did not appear to be able to prevent the induction of anergy since inhibition of B7-mediated costimulation by CTLA4-Ig during primary stimulation was sufficient to induce anergy (Fig. 1 *E*). Similar to our previous results with anti-ICAM-1 mAb, anti-LFA-1 and anti-LFA-3 mAbs significantly inhibited the capacity of LBL-DR7 cells to induce proliferation and IL-2 accumulation during primary stimulation (Fig. 7 *A*). However, secondary stimulation with t-DR7/B7-1 or LBL-DR7 induced proliferation and IL-2 accumulation, whereas t-DR7 and t-DR7/ICAM-1 induced proliferation without significant IL-2 accumulation (Fig. 7, *B* and *C*). These results show that addition of anti-LFA-1 or anti-LFA-3 during primary stimulation did not result in antigen-specific anergy on secondary stimulation. Moreover, these results suggest that ICAM-1, LFA-1, and LFA-3 largely serve adhesive functions during primary stimulation and that blockade of these pathways completely inhibits recognition of antigen. Therefore, on rechallenge, alloreactive T cells appear to respond as if they were mounting a primary response to alloantigen.

Discussion

In this report, we demonstrate that alloantigen presentation without costimulation provided by B7 family members is sufficient to induce human alloantigen-specific clonal anergy. Once induced, anergy cannot be reversed by alloantigen and a variety of costimulatory molecules expressed on LBL-DR7, including B7-1, B7-2, ICAM-1, and LFA-3. After prolonged culture in IL-2, anergized cells cannot proliferate or secrete cytokines in response to B7-1, B7-2, or ICAM-1 costimulation. However, under these conditions, anergized cells can proliferate and secrete IL-2 in response to alloantigen and LFA-3. Alloantigen and LFA-3 costimulation subsequently fully restores alloantigen-specific responsiveness. Induction of anergy is temporally related to the downregulation of the LFA-3 counterreceptor epitope CD2R, whereas the capacity of alloantigen and LFA-3 to reverse anergy coincides with the reappearance of this epitope. Therefore, the expression of CD2R appears to play a critical role in the induction, maintenance, and reversal of human alloantigen-specific anergy.

Based on these data, we can now postulate which signaling pathways appear to be defective during the induction, maintenance, and reversal of alloantigen-specific anergy. As summarized in Table 1, alloreactive T cell clones express competent TCR complex, CD28, IL-2 receptor, and CD2. These

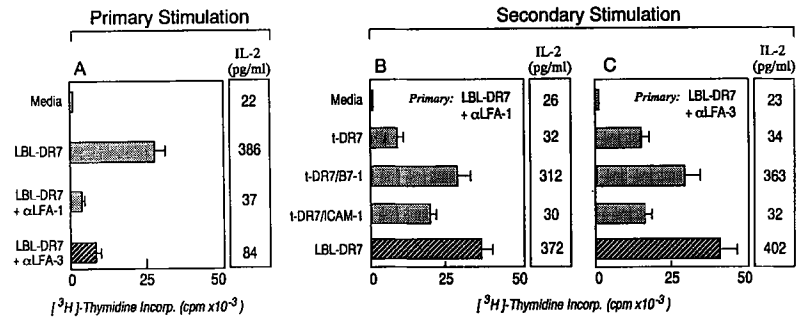

Figure 7. Anti-LFA-1 and anti-LFA-3 mAbs inhibit primary response but do not result in induction of alloantigen-specific anergy. TC-1 cells were stimulated with LBL-DR7 in the presence of anti-LFA-1 or anti-LFA-3 mAb. IL-2 accumulation and thymidine incorporation were measured as described in Fig. 1. After 72 h of primary culture, TC-1 cells were isolated from LBL-DR7 by Ficoll separation, washed extensively, and cultured in media without IL-2 for 24 h. Each population was subsequently restimulated with a secondary stimulus as shown. Results are representative of two experiments.

Table 1. *Stepwise Induction and Reversal of Anergy*

	Untreated	Anergized	Anergized after IL-2	Anergized after IL-2 after HLA-DR7/LFA-3
TCR signaling	+	−(?)*	+	+
CD28 signaling	+	−(?)*	−	+
IL-2 secretion	+	−	+	+
IL-2 receptor signaling	+	+	+	+
CD2 signaling	+	−	+	+

* Since TC-1 cells do not respond to t-DR7/B7-1 or LBL-DR7, it is not possible to determine whether the defective signaling resides in the TCR or CD28 pathways.
+, intact and −, defective signaling.

cells respond to alloantigen when costimulated via the CD28 pathway and induce IL-2 secretion. Similarly, they proliferate and secrete IL-2 when stimulated via the CD2 pathway with anti-T11.3 and either anti-T11.1 or anti-T11.2 mAbs. After alloantigen signaling via the TCR without B7 family costimulation, alloreactive T cell clones are anergized. Anergized cells do not proliferate or secrete IL-2 in response to either alloantigen and costimulation by B7 family members or additional costimulatory molecules expressed on LBL-DR7. These results do not permit us to dissect whether the signaling defect in anergized cells resides in the TCR pathway, CD28 pathway, or both. Although anergized cells cannot secrete IL-2 in response to any stimuli, the IL-2 receptor pathway is intact since these cells can proliferate in the presence of exogenous IL-2. Moreover, anergized cells cannot proliferate via the alternative CD2 pathway of T cell activation, and this lack of responsiveness appears to be associated with a downregulation of the functionally critical CD2R epitope. Taken together, these observations are consistent with the notion that anergy results in a combination of defective signaling via the TCR, CD28, and CD2 pathways.

The reversal of alloantigen-specific anergy requires both prolonged culture in IL-2 and simultaneous exposure to alloantigen and LFA-3. The ability of previously anergized cells to respond to alloantigen and LFA-3 costimulation coincides temporally with the reappearance of CD2R and may depend on the ability of the anergized cells to reexpress CD2R. In contrast, after culture in IL-2, anergized T cells do not respond to t-DR7/B7-1, demonstrating that CD28 costimulation is not involved in the reversal of anergy. These results are consistent with the hypothesis that reversal of anergy requires signaling via the IL-2 receptor pathway and reexpression of the critical CD2R epitope, followed by simultaneous activation via the TCR and CD2 pathways. After reversal of anergy with IL-2, alloantigen, and LFA-3, alloreactive T cells are again fully competent to respond to alloantigen and a variety of costimulatory signals.

Complete blockade of B7 family members with CTLA4-Ig appears to be necessary and sufficient to induce alloantigen–helper T cell–specific anergy in our in vitro model system. In contrast, partial blockade of B7-mediated costimulation with anti-B7-1 mAb resulted in reduced proliferation and IL-2 accumulation but did not result in unresponsiveness on rechallenge. These results are consistent with previous reports in both murine and human systems that demonstrated that CTLA4-Ig was more efficient than anti-B7-1 mAb in the induction of alloantigen–specific anergy (21) and suggest that B7-2 costimulation can prevent the induction of anergy. Since LBL-DR7 cells express HLA-DR7 and many potentially important candidate costimulatory molecules, our results demonstrate that all non-B7 family costimulatory molecules expressed on these cells are not effective in preventing the induction of anergy. The observed inhibition of LBL-DR7–mediated proliferation in primary culture by anti-LFA-1 and anti-LFA-3 mAbs is likely due to the disruption of T cell clone/APC conjugate formation by blockade of LFA-1/ICAM-1– and LFA-3/CD2–mediated adhesion. However, it is unclear why CD2 ligation by LFA-3 on the LBL-DR7 cells is not capable to preventing the induction of anergy. Although anti-CD2 mAbs are capable of inducing modest levels of IL-2 accumulation in TC-1 cells, culture with LBL-DR7 cells in the presence of CTLA4-Ig did not result in IL-2 accumulation (data not shown), suggesting that LFA-3 on the cell surface may not be capable of inducing sufficient costimulation and IL-2 production to prevent the induction of anergy.

The stepwise in vitro model described here should facilitate the dissection of the biochemical and molecular events that regulate the induction, maintenance, and reversal of anergy. It should be possible now to determine more precisely the defect(s) in the TCR, CD28, and CD2 pathways that are involved in the induction of anergy. Moreover, this model may provide a basis for design of novel therapeutic strategies to maintain or reverse the state of tolerance. Induction and maintenance of a state of stable alloantigen-specific anergy will likely improve the results of transplantation by decreasing the need for nonspecific immunosuppression. Our results suggest that although blockade of the B7/CD28/CTLA4 pathways may be useful to induce tolerance, blockade of additional costimulatory pathways, which might reverse tolerance, will also be required. In addition, our in vitro human model may provide insight into the mechanism(s) of autoimmunity. Autoimmunity might result from reversal of self-tolerance by microenvironmental IL-2 and self-antigen presentation with the appropriate costimulation. Novel approaches to reverse active autoimmunity might be based on these data. Finally, anergy to tumor antigens might obstruct the development of important T cell–mediated tumor immune responses, and therefore reversal of tumor antigen–specific anergy might be essential to mount an efficient

antitumor immune response. Attempts to restore tumor-specific immunity may need to be preceded by restoration of CD2 function in anergized T cells followed by rechallenge with tumor cells transfected with the essential costimulatory molecules. This study provides the rationale for preclinical murine studies to address these hypotheses directly.

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References

1. Mueller, D.L., M.K. Jenkins, L. Chiodetti, and R.H. Schwartz. 1990. An intracellular calcium increase and protein kinase C activation fail to initiate T cell proliferation in the absence of a costimulatory signal. *J. Immunol.* 144:3701.
2. van Seventer, G., Y. Shimizu, K. Horgan, and S. Shaw. 1990. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. *J. Immunol.* 144:4579.
3. Staunton, D.E., M.L. Dustin, and T.A. Springer. 1989. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature (Lond.).* 339:61.
4. Fougerolles, A.R., and T.A. Springer. 1992. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function–associated molecule 1 on resting lymphocytes. *J. Exp. Med.* 175:185.
5. Moingeon, P., H.C. Chang, B.P. Wallner, Ch. Stebbins, A.Z. Frey, and E.L. Reinhertz. 1989. CD2-mediated adhesion facilitates T lymphocyte antigen recognition function. *Nature (Lond.).* 339:312.
6. 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.
7. Gimmi, C.D., G.J. Freeman, J.G. Gribben, K. Sugita, A.S. Freedman, C. Morimoto, and L.M. Nadler. 1991. B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc. Natl. Acad. Sci. USA.* 88:6575.
8. Young, J.W., L. Koulova, S.A. Soergel, E.A. Clark, R.M. Steinman, and B. Dupont. 1992. The B7/BB1 antigen provides one of several costimulatory signals for the activation of $CD4^+$ T lymphocytes by human blood dendritic cells in vitro. *J. Clin. Invest.* 90:229.
9. Armitage, R.J., W.C. Fanslow, L. Strockbine, T.A. Sato, K.N. Clifford, B.M. Macduff, D.M. Anderson, S.D. Gimpel, T. Davis-Smith, C.R. Maliszewski et al. 1992. Molecular and biological characterization of a murine ligand for CD40. *Nature (Lond.).* 357:80.
10. Liu, Y., B. Jones, A. Aruffo, K.M. Sullivan, P.S. Linsley, and C.A. Janeway, Jr. 1992. Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. *J. Exp. Med.* 175:437.
11. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science (Wash. DC).* 248:1349.
12. Jenkins, M.K., J.D. Ashwell, and R.H. Schwartz. 1988. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. *J. Immunol.* 140:3324.
13. Jenkins, M.K., D.M. Pardoll, J. Mizuguchi, T.M. Chused, and R.H. Schwartz. 1987. Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proc. Natl. Acad. Sci. USA.* 84:5409.
14. Dallman, M.J., O. Shiho, T.H. Page, K.J. Wood, and P.J. Morris. 1991. Peripheral tolerance to alloantigen results from altered regulation of the interleukin 2 pathway. *J. Exp. Med.* 173:79.
15. Go, C., and J. Miller. 1992. Differential induction of transcription factors that regulate the interleukin 2 gene during anergy induction and restimulation. *J. Exp. Med.* 175:1327.
16. Kang, S.M., B. Beverly, A.C. Tran, K. Brorson, R.H. Schwartz, and M.J. Lenardo. 1992. Transactivation of AP-1 is a molecular target of T cell clonal anergy. *Science (Wash. DC).* 257:1134.
17. Harding, F.A., J.G. McArthur, J.A. Gross, D.H. Raulet, and J.P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature (Lond.).* 356:607.
18. 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.
19. Lenschow, D.J., Y. Zeng, J.R. Thistlethwaite, A. Montag, W. Brady, M.G. Gibson, P.S. Linsley, and J.A. Bluestone. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science (Wash. DC).* 257:789.
20. Turka, L.A., P.S. Linsley, H. Lin, W. Brady, J.M. Leiden, R.Q. Wei, M.L. Gibson, X.G. Zheng, S. Myrdal, D. Gordon, et al., 1992. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. *Proc. Natl. Acad. Sci. USA.* 89:11102.

21. Tan, P., C. Anasetti, J.A. Hansen, J. Melrose, M. Brunvard, 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.
22. Lin, H., S.F. Bolling, P.S. Linsley, R.-Q. Wei, D. Gordon, C.B. Thompson, and L.A. Turka. 1993. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J. Exp. Med.* 178:1801.
23. Freedman, A.S., G.J. Freeman, J.C. Horowitz, J. Daley, and L.M. Nadler. 1987. B7, a B cell restricted antigen which identifies pre-activated B cells. *J. Immunol.* 137:3260.
24. 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.
25. Freedman, A.S., G.J. Freeman, K. Rhynhart, and L.M. Nadler. 1991. Selective induction of B7/BB-1 on interferon-γ stimulated monocytes: a potential mechanism for amplification of T cell activation. *Cell Immunol.* 137:429.
26. Symington, F.W., W. Brady, and P.S. Linsley. 1993. Expression and function of B7 on human epidermal Langerhans cells. *J. Immunol.* 150:1286.
27. Augustin, M., A. Dietrich, R. Niedner, A. Kapp, E. Schopf, J. Ledbetter, W. Brandy, P. Linsley, and J. Simon. 1993. Phorbol-12-myristate-13-acetate-treated human keratinocytes express B7-like molecules that serve a costimulatory role in T cell activation. *J. Invest. Dermatol.* 100:275.
28. Nickoloff, B., R. Mitra, K. Lee, L. Turka, J. Green, G. Thompson, and Y. Shimizu. 1993. Discordant expression of CD28 ligands, BB1, and B7 on keratinocytes in vitro and psoriatic cells in vivo. *Am. J. Pathol.* 142:1029.
29. Boussiotis, V.A., G.J. Freeman, J.G. Gribben, J. Daley, G. Gray, and L.M. Nadler. 1993. Activated human B lymphocytes express three CTLA4 binding counter-receptors which costimulate T cell activation. *Proc. Natl. Acad. Sci. USA.* 90:11059.
30. Lenschow, D.J., G. Huei-Ting Su, L.A. Zuckerman, N. Nabavi, C.L. Jellis, G.S. Gray, J. Miller, and J.A. Bluestone. 1993. Expression and functional significance of an additional ligand for CTLA-4. *Proc. Natl. Acad. Sci. USA.* 90:11054.
31. Hansen, J.A., P.J. Martin, and R.C. Nowinski. 1980. Monoclonal antibody identifying a novel T-cell antigen and Ia antigen of human lymphocytes. *Immunogenetics.* 10:247.
32. Freeman, G.J., J.G. Gribben, V.A. Boussiotis, J.W. Ng, V. Restivo, L. Lombard, G.S. Gray, and L.M. Nadler. 1993. Cloning of B7-2: a CTLA4 counter-receptor that costimulates human T cell proliferation. *Science (Wash. DC).* 262:909.
33. Freeman, G.J., F. Borriello, R.J. Hodes, H. Reiser, K.S. Hathcock, G. Laszlo, A.J. McKnight, J. Kim, L. Du, D.B. Lombard, et al. 1993. Uncovering the functional alternative CLTA4 counter-receptor in B7-deficient mice. *Science (Wash. DC).* 262:907.
34. Harper, K., C. Balzano, E. Rouvier, M.G. Mattei, M.F. Luciani, and P. Golstein. 1991. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. *J. Immunol.* 147:1037.
35. Linsley, P.S., W. Brady, M. Urnes, L.S. Grosmaire, N.K. Damle, and J.A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* 174:561.
36. Boussiotis, V.A., G.J. Freeman, G. Gray, J. Gribben, and L.M. Nadler. 1993. B7 but not intercellular adhesion molecule 1 costimulation prevents the induction of human alloantigen-specific tolerance. *J. Exp. Med.* 178:1753.
37. Beverly, B., S. Kang, M. Lenardo, and R. Schwartz. 1992. Reversal of in vitro T cell clonal anergy by IL-2 stimulation. *Int. Immunol.* 4:661.
38. Essery, G., M. Feldmann, and J. Lamp. 1988. Interleukin-2 can prevent and reverse antigen-induced unresponsiveness in cloned human T lymphocytes. *Immunology.* 64:413.
39. Goronzy, J., C. Weyland, and C.G. Fathhman. 1987. Cloning of human alloreactive T cells. *Methods Enzymol.* 150:333.
40. Clark, E., and T. Yokochi. 1984. Human B cell and B cell blast-associated surface molecules defined with monoclonal antibodies. *In* Leukocyte Typing. A. Bernard, L. Boumsell, J. Dausset, C. Milstein, and S.F. Sclossman, editors. Springer-Verlang, New York. 339.
41. Todd, R.I., S. Meuer, P. Romain, and S. Schlossman. 1984. A monoclonal antibody that blocks class II histocompatibility-related immune interactions. *Hum. Immunol.* 10:23.
42. Rothlein, R., M. Dustin, S. Martin, and T. Springer. 1986. *J. Immunol.* 137:1270.
43. Sanchez-Madrid, F., A.M. Krensky, C. Ware, E. Robbins, J. Strominger, S. Burakoff, and T. Springer. 1982. Three distinct antigens associated with human T lymphocyte–mediated cytolysis: LFA-1, LFA-2, and LFA-3. *Proc. Natl. Acad. Sci. USA.* 79:7489.
44. Abramson, C.S., J.H. Kersey, and T.W. Lebien. 1981. A monoclonal antibody (BA-1) reactive with cells of human B lymphocyte lineage. *J. Immunol.* 126:83.
45. Dorken, B., A. Moller, A. Pezzutto, R. Schwartz-Albeiz, and G. Moldenhauer. 1989. B-cell antigens: CD72. *In* Leucocyte Typing IV. W. Knapp, B. Dorken, W.R. Gilks, E.P. Rebier, R.E. Schmidt, H. Stein, and A.E.G.Kr. von dem Borne, editors. Oxford University Press, Oxford. 99.
46. Meuer, S., R. Hussey, M. Fabbi, D. Fox, O. Acuto, K. Fitgerald, J. Hodgdon, J. Protentis, S. Schlossmann, and E. Reinherz. 1984. An alternative pathway of T-cell activation: a functional role for the 50kd T11 sheep erythrocyte receptor protein. *Cell.* 36:897.
47. Bierer, B., A. Peterson, J. Gorga, S. Herrmann, and S. Burakoff. 1988. Synergistic T cell activation via the physiologic ligands for CD2 and the T cell receptor. *J. Exp. Med.* 168:1145.
48. Koyasu, S., T. Lawton, D. Novick, M.A. Recny, R.F. Siliciano, B.P. Wallner, and E.L. Reinherz. 1990. Role of interaction of CD2 molecules with lymphocyte function–associated antigen 3 in T-cell recognition of nominal antigen. *Proc. Natl. Acad. Sci. USA.* 87:2603.
49. Selvaraj, P., M.L. Plunkett, M. Dustin, M.E. Sanders, S. Shaw, and T.A. Springer. 1987. The T lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3. *Nature (Lond.).* 326:400.
50. Bierer, B., J. Barbarosa, S. Herrmann, and S.J. Bourakoff. 1988. Interaction of CD2 with its ligand, LFA-3, in human T cell proliferation. *J. Immunol.* 140:3358.
51. Peterson, A., and B. Seed. 1987. Monoclonal antibody and ligand binding sites of the T cell erythrocyte receptor (CD2). *Nature (Lond.).* 329:842.
52. Yang, S.Y., S. Chouaib, and B. Dupont. 1986. A common pathway for T lymphocyte activation involving both the CD3-Ti complex and CD2 sheep erythrocyte receptor determinants. *J. Immunol.* 137:1097.
53. Meuer, S.C. 1989. T11 cluster report: CD2. *In* Leucocyte Typing IV. W. Knapp, B. Dorken, W.R. Gilks, E.P. Rebier, R.E. Schmidt, H. Stein, and A.E.G.Kr. von dem Borne, editors. Oxford University Press, Oxford. 270.