A Pathway of Costimulation That Prevents Anergy in CD28− T Cells: B7-independent Costimulation of CD1-restricted T Cells
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Summary
A class of molecules that is expressed on antigen presenting cells, exemplified by CD80 (B7), has been found to provide a necessary costimulatory signal for T cell activation and proliferation. CD28 and CTLA4 are the B7 counterreceptors and are expressed on the majority of human CD4+ T cells and many CD8+ T cells. The signal these molecules mediate is distinguished from other costimulatory signals by the finding that T cell recognition of antigen results in a prolonged state of T cell unresponsiveness or anergy, unless these costimulatory molecules are engaged. However, nearly half of the CD8+ and CD4−CD8− T cells lack CD28, and the costimulatory signals required for the activation of such cells are unknown. To understand the pathways of activation used by CD28− T cells, we have examined the costimulatory requirements of antigen-specific CD4+CD8− TCR+α/β circulating T cells that lack the expression of CD28. We have characterized two T cell lines, DN1 and DN6, that recognize a mycobacterial antigen, and are restricted not by major histocompatibility complex class I or II, but by CD1b or CD1c, two members of a family of major histocompatibility complex-related molecules that have been recently implicated in a distinct pathway for antigen presentation. Comparison of antigen-specific cytolytic responses of the DN1 and DN6 T cell lines against antigen-pulsed CD1+ monocytes or CD1+ B lymphoblastoid cell lines (B-LCL) demonstrated that these T cells recognized antigen presented by both types of cells. However, T cell proliferation occurred only when antigen was presented by CD1+ monocytes, indicating that the CD1+ monocytes expressed a costimulatory molecule that the B-LCL transfectants lacked. This hypothesis was confirmed by demonstrating that the T cells became anergic when incubated with the CD1+−transfected B-LCL in the presence of antigen, but not in the absence of antigen. The required costimulatory signal occurred by a CD28-independent mechanism since both the CD1+ monocytes and CD1+ B-LCL transfectants expressed B7-1 and B7-2, and DN1 and DN6 lacked surface expression of CD28. We propose that these data define a previously unrecognized pathway of costimulation for T cells distinct from that involving CD28 and its counterreceptors. We suggest that this B7-independent pathway plays a crucial role in the activation and maintenance of tolerance of at least a subset of CD28− T cells.

Most human T cells express TCRs that recognize peptide antigens bound to class I or class II MHC molecules. However, formation of the TCR−peptide−MHC molecule ternary complex does not generate a signal sufficient to cause T cell activation and proliferation; additional costimulatory signals are also required (1). One such critical costimulatory signal is transmitted through the T cell surface molecule CD28 when it binds its ligands CD80 (B7-1) or CD86 (B70/B7-2) (2-7), which are expressed on APC such as activated B cells, monocytes, and dendritic cells (8, 9). If cross-linking of the TCR by peptide−MHC complexes or by anti-CD3 antibody occurs in the absence of this costimulatory signal, then a state of prolonged unresponsiveness of the T cell to antigen is induced (10, 11).

The induction of this unresponsive state, referred to as T cell anergy, may be an important mechanism of peripheral tolerance that prevents the activation of circulating autoreactive T cells that have escaped thymic deletion (12-14). Although the majority of circulating T cells expresses the TCR−α/β and either CD4 or CD8, a subset of mature peripheral blood T cells expresses neither the CD4 nor the CD8 markers and is termed double negative (DN). In general, the circulating pool of CD4−8− T cells contains most of the TCR−γ/δ+ cells, and also a consistently detect-
able subpopulation of TCR-α/β⁺ cells (15). CD4⁺ 8⁻ TCR-α/β⁺ T cells are found in bone marrow, lymph nodes, thymus, and epidermis (16), and expansions up to 20% of CD3⁺ cells are found in the peripheral blood of some normal individuals (15) and in patients with autoimmune diseases (17–21). The absence of the CD4 and CD8 coreceptors from DN T cells suggests that they may not interact with MHC class I and class II molecules during antigen presentation, and several previous studies have implicated the non-MHC-encoded CD1 family of cell surface proteins as important antigen presenting molecules for these T cells (22–28).

The human CD1 locus consists of five genes (CD1A, -B, -C, -D, and -E) that encode type I transmembrane glycoproteins that have limited detectable homology to MHC proteins, and they are expressed on many professional APC in association with β₂-microglobulin (26, 28–36). High levels of CD1 can be induced in vitro by culturing monocytes in the presence of GM-CSF and IL-4 (23, 37). Such monocytes (hereafter referred to as CD1⁺ monocytes) are able to present microbial antigens to CD1-restricted T cells and induce their proliferation (23, 24). Previously, we have shown that some CD4⁻8⁻ TCR-α/β⁺ T cells recognize antigens restricted by the non-MHC-encoded CD1b molecule (23, 24). Recently, the antigen recognized by the prototype CD1b-restricted T cell line DN1 was identified as mycolic acid, a complex and structurally variable fatty acid that is part of the mycobacterial cell wall (25).

Several studies have documented the absence of CD28 expression on a substantial fraction of circulating CD4⁺ 8⁻ and CD4⁻8⁻ T cells (38, 39), raising questions about the costimulatory requirements of these cells. This study sought to determine whether CD28⁻ T cells lack a requirement for costimulation in contrast to their CD28⁺ counterparts, or alternatively use a distinct costimulatory pathway. Here, using the CD1-restricted DN1 and DN6 T cell lines, we show that CD28⁻ 8⁻ TCR-α/β⁺ T cells required costimulation that was independent of the B7–CD28 axis. These data revealed the existence of a distinct pathway for costimulation of CD28⁻ T cells.

Materials and Methods

Antibodies. The following antibodies were used: P3 (IgG₁, nonbinding control [40]); W6/32 (anti-HLA-A, -B, -C [41]; murine IgG₂a, American Type Culture Collection, Rockville, MD); L243 (anti-HLA-DR, murine IgG₂a, American Type Culture Collection); IT2 (anti-B7-1/2-4 [4], IgG₂a, gift of Ko Okumura, Juntendo, University School of Medicine, Tokyo, Japan); BB-1 (anti-B7 [42], murine IgM; gift of Ed Clark, University of Washington, Seattle, WA); 9.3 and 11D4 (anti-CD28, murine IgG₂, and anti-CTLA4 [43], murine IgG₂, respectively; gift of Peter Linsley, Bristol-Myers Squibb, Seattle, WA).

Antigens. 200 μg of detoxicated Mycobacteria tuberculosis bacilli (strain H37Ra; Difco Laboratories Inc., Detroit, MI) was sonicated in 5 ml PBS, followed by centrifugation at 100,000 g to remove insoluble material. Protein content was assayed using the Bradford method (Bio-Rad Laboratories, Melville, NY). A protein concentration of 10 μg/ml corresponds to a 1:30–1:60 dilution of the sonicate. Recombinant C fragment of tetanus toxin (Boehringer Mannheim Corp., Indianapolis, IN) was reconstituted in PBS.

Cell Culture. DN1 (23) and DN6 (Beckman, E. M., A. Melen, S. M. Behar, P. A. Seling, D. Chatterjee, R. Matsumoto, J. P. Rosat, L. L. Modlin, and S. A. Porcelli, manuscript in preparation) are CD4⁻ 8⁻ TCR-α/β⁺ T cell lines that recognize M. tuberculosis antigens presented by CD1b and CD1c, respectively. The DN1 and DN6 T cell lines were derived independently from different human donor’s PBMC depleted of CD4⁺ and CD8⁺ T cells. The DN T cell lines were repeatedly stimulated with M. tuberculosis sonicate, using allogeneic CD1⁺ monocytes as APC that express high levels of CD1a, CD1b, and CD1c. SP-F3 and SP-F14 (gifts of Dr. Herger Spits, Netherlands Cancer Institute, Amsterdam, The Netherlands) are human CD4⁺ T cell clones that recognize the C fragment of tetanus toxoid restricted by HLA-DR (44). All T cells were grown in RPMI 1640 supplemented with 10 mM Hepes, 2 mM l-glutamine, 10 mM nonessential amino acids, 10 mM essential amino acids, 0.055 mM 2-ME (all from Life Technologies Inc., Gaithersburg, MD), 10% heat-inactivated FCS (Hyclone Laboratories Inc., Logan, UT), and 1.5 mM rL-2 (gift of Ajinomoto Company, Kawasaki, Japan).

The B lymphoblastoid cell line (B-LCL) ClR (45) was transfected with the expression vector pSRα-NEO (46) containing either the CD1a, CD1b, or CD1c cDNA (47), or with the vector alone. Stably transfected cells were first selected for G418 resistance and then for high surface expression of the relevant proteins using immunomagnetic bead selection. Clones were subsequently established by limiting dilution. ClR was grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 10 mM Hepes, 2 mM l-glutamine, 10 mM nonessential amino acids, 10 mM essential amino acids, and 0.055 mM 2-ME.

Human blood monocytes were isolated from leukocyte concentrates obtained from volunteer plateletpheresis donors and included CMV-seropositive and -seronegative individuals. Human PBMC were prepared by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation, and monocytes were enriched by isolating plastic adherent cells (23). CD1 expression was induced on the monocytes by culturing the cells with 200 U/ml of GM-CSF (gift of Genetics Institute, Cambridge, MA) and 100 U/ml of IL-4 (gift of Schering Corp., Bloomfield, NJ) for 60 h as described (23).

FACS®. 2 × 10⁵ T cells or monocytes were stained with 50 μl of ascites (diluted 1:400) or purified antibody (5 μg/ml) in FACS® buffer (5% bovine calf serum/0.01% azide) for 1 h at 4°C. The cells were washed, and 20 μl of FITC-labeled F(ab′)2 goat anti-mouse Ig (Tago, Inc., Burlingame, CA) (diluted 1:30) was added for 1 h at 4°C. After washing extensively with FACS® buffer, the cells were counterstained with propidium iodide and analyzed using a FACS® Sort® (Becton Dickinson & Co., Raritan, NJ).

Cytotoxicity. ClR lymphoblastoid cell lines stably transfected with the CD1a, CD1b, or CD1c genes, or mock transfected (vector alone) were used as targets in a standard ⁵¹Cr release assay. Targets were pulsed for 16 h with the M. tuberculosis antigen preparation (10 μg protein/ml) and then labeled with 200 μCi of ⁵¹Cr for 2 h. 2,000 target cells were incubated with effector T cells for 4 h in triplicate, and the E/T ratio varied as indicated in Fig. 1. A and B. Cr release was assessed by counting 25 μl of the supernatant in a liquid scintillation counter. Specific lysis was calculated as (sample cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) × 100.

Proliferation. T cells were cultured in triplicate at a concentration of 5 × 10⁴ cells per well in the presence of 5 × 10⁴ alloge-
neic irradiated (5,000 rad) CD1+ monocytes or 5 × 10^6 mitomycin C (Sigma Chemical Co., St. Louis, MO)-treated CD1+ B-LCL in a flat-bottomed 96-well plate in a total volume of 200 µL per well. Antigen, antibodies, or cytokines were added to the final concentrations indicated in the figures. The cultures were incubated for a total of 72 (for DN6) or 120 (for DN1) h at 37°C in 5% CO_2 and pulsed with 1 µCi of [3H]thymidine (6.7 Ci mmol⁻¹; New England Nuclear, Boston, MA) per well for the last 6 h of culture, and [3H] incorporation was measured by liquid scintillation counting to assess T cell proliferation. Cultures were harvested onto fiberglass filters (Wallac, Gaithersburg, MD) using an automated harvester (Tomtec, Orange, CT) and counted in a liquid scintillation counter (model 1205 Betaplate; Wallac, Gaithersburg, MD) per well. Antigen, antibodies, or cytokines were added to the culture supernatants 24 h after antigen stimulation and assayed at a 1:4 dilution, using 5,000 CTLL-20 cells per sample. The proliferation of the CTLL-20 T cells was assessed by measuring [3H]thymidine incorporation during the last 6 h of a 24-h culture.

Transwell Assays. The dependency of the costimulatory signal on cell–cell contact was determined using a 24-well format transwell culture system that used a polycarbonate membrane with a 0.4-µm pore size (Costar Corp., Cambridge, MA). 3 × 10^6 T cells were cultured together with 3 × 10^6 C1R.CD1b or C1R.CD1c cells and M. tuberculosis antigen in each lower (cluster) well. The upper well (transwell) contained either medium or 5 × 10^4 CD1+ monocytes and antigen. Proliferation was assessed as described above.

Production of Hybridomas. 6-wk-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were immunized intraperitoneally with 5 × 10^6 CD1+ monocytes in PBS and boosted 4 wk later in the same manner. Mice used for the production of hybridomas were given a third intraperitoneal immunization with 10^7 CD1+ monocytes in PBS and boosted 4 wk later. Daudi cells and M. tuberculosis antigen were added to spleen cultures and fused in the presence of 5,000 mitomycin C-treated CD1+ monocytes or 5 × 10^4 mitomycin C-treated CD1+ monocytes and antigen in a total volume of 200 µL per well. Antigen, antibodies, or cytokines were added to the final concentrations indicated in the figures. The cultures were incubated for a total of 72 (for DN6) or 120 (for DN1) h at 37°C in 5% CO_2 and pulsed with 1 µCi of [3H]thymidine (6.7 Ci mmol⁻¹; New England Nuclear, Boston, MA) per well for the last 6 h of culture, and [3H] incorporation was measured by liquid scintillation counting to assess T cell proliferation. Cultures were harvested onto fiberglass filters (Wallac, Gaithersburg, MD) using an automated harvester (Tomtec, Orange, CT) and counted in a liquid scintillation counter (model 1205 Betaplate; Wallac). In general, the standard deviation of the triplicates was 5–10% of the mean.

**Induction of Anergy.** The B-LCL C1R, stably transfected with CD1b or CD1c (C1R.CD1b or C1R.CD1c), was cultured without or with the appropriate antigen for 16 h and then extensively washed. The APC were treated with mitomycin C and then cultured in 24-well plates (Linbro, Horsham, PA) with resting DN1 or DN6 T cells at a ratio of 10 APC/1 T cell for 48 h. Viable cells were isolated using density centrifugation and were then restimulated with CD1+ monocytes and M. tuberculosis antigen in a proliferation assay as described above.

**IL-2 Assay.** The amount of IL-2 produced by DN1 and DN6 T cells was determined using a bioassay in which culture supernatants were tested for their ability to stimulate the growth of the IL-2–dependent CTLL-20 T cell line (48). DN1 or DN6 culture supernatants were sampled 24 h after antigen stimulation and assayed at a 1:4 dilution, using 5,000 CTLL-20 cells per sample. The proliferation of the CTLL-20 T cells was assessed by measuring [3H]thymidine incorporation during the last 6 h of a 24-h culture.

Results

The CD28+ DN1 and DN6 T Cell Lines Was Independent of the CD28–B7 Axis. Notably, neither the DN1 nor the DN6 T cell lines stained with mAb 9.3 in flow cytometric analysis, indicating that these T cells lack expression of the T cell–costimulatory molecule CD28, even 72 h after activation with antigen and CD1+ monocytes (Fig. 1, C and D). Treatment of the CD1b+ B-LCL with GM-CSF and IL-4 did not modify the ability of these APC to stimulate the proliferation of the DN1 T cell line. Similarly, DN1 and DN6 only produced IL-2 when stimulated by antigen-pulsed CD1+ monocytes, whereas no IL-2 was detected after stimulation by the appropriate CD1+ B-LCL transfected and antigen (Fig. 1, E and F). These findings suggested that the CD1+ monocytes, but not CD1+ B-LCL, provided a costimulatory signal that was required by DN1 and DN6 for proliferation and IL-2 production, but not for cytotoxic activity.
fragment of tetanus toxoid, constitutively expressed CD28 (Fig. 2).

Another T cell surface protein, CTLA4, has been identified as a counterreceptor for the B7 family of costimulatory ligands, and it was thus of interest to examine the expression of this protein by DN1 and DN6 T cells (4, 6, 52, 53). CTLA4 is expressed only by CD28+ T cells in peripheral blood, and in nearly all cases so far reported, this is also true for T cells grown in vitro (54, 55). No cell surface expression of CTLA4 was detected on resting cultures of either the DN1 or DN6 T cell lines (data not shown). In contrast to the resting phenotype of these cells, activation by PMA and ionomycin induced some surface expression of CTLA4 (Fig. 2). However, whereas CD28 clearly functions as a T cell–costimulatory molecule, recent data suggest that CTLA4 is a negative regulator of the T cell immune response and may induce apoptosis of T cells (56, 57). Thus, it was unlikely that CTLA4 would be able to substitute for CD28 in activation of DN1 and DN6, and the absence of CD28 suggested that another costimulatory pathway was operating for these T cells.

The pivotal role of the well-studied CD28–B7 pathway in the costimulation of CD4+ and CD8+ T cells and in the prevention of T cell anergy led us to assess if differences in stimulatory potential of the CD1+ monocytes compared with the C1R B-LCL was a consequence of a discrepancy in the expression of B7 on these two APC. The surface expression of CD80 (B7-1) and CD86 (B70/B7-2) on C1R cells and CD1+ monocytes was determined by flow cytometry. The CD1+ monocytes constitutively expressed both CD80 and CD86 (Fig. 3), similar to what has been reported for unstimulated monocytes and dendritic cells. Similarly, CD80 and CD86 were detected on C1R.CD1b and C1R.CD1c cells (Fig. 3). In fact, the cell surface expression of CD80 and CD86 on the C1R cell line exceeded the levels observed on the CD1+ monocytes. Since CD80 and CD86 were expressed on both C1R cell lines as well as the CD1+ monocytes, differences in the expression of the B7 family of costimulatory molecules failed to explain the divergent capacity of these APC to provide costimulation to DN1 and DN6. These findings cannot be ascribed to some functional deficit in the B7-1 or B7-2 molecules expressed by C1R, since these cells efficiently supported the proliferation of CD28+ T cells (see below).

It has been observed that B7 is functionally active even when expressed at low levels on APC, and we considered whether this could be true for CD28 as well. To be certain that DN1 proliferation was independent of the CD28–B7 pathway, we studied the effect of CTLA4-Ig on the proliferation of DN1. CTLA4-Ig binds avidly to CD80 and CD86 and blocks their interaction with CD28, thereby preventing CD28-mediated costimulation. CTLA4-Ig did not block the proliferation of DN1 (Fig. 4 A), even at concentrations that were sufficient to inhibit an MLR by 80–90% (Fig. 4 B). This indicated the existence of one or more non–CTLA4-binding costimulatory molecules required for the optimum proliferation of T cell line DN1.

The Costimulatory Signal Used by the CD28+ DN TCR-α/β+ T Cells Required Cell–Cell Contact. We next sought to determine whether the costimulatory signal provided by the monocytes was mediated by a soluble molecule, or alternatively, required cell–cell contact. Previous studies have shown that antigen-pulsed, glutaraldehyde-fixed CD1+ monocytes are able to stimulate the proliferation of the
CD28^+ T Cells Became Anergic upon Recognizing Antigen in the Absence of a Costimulatory Signal. Previous studies have shown that if a TCR-mediated signal is delivered to the T cell in the absence of a costimulatory signal, anergy rather than proliferation occurs. This paradigm predicts that, if an essential costimulatory molecule were absent from the B-LCL, DN1 and DN6 T cells should become anergic upon recognition of antigen presented by the C1R.CD1b and C1R.CD1c cell lines since their TCRs are being engaged in the absence of costimulation. On the other hand, anergy should not occur if the T cells and the C1R transfectants are cultured together in the absence of antigen, as the TCR would not be stimulated.

To test this hypothesis, DN1 T cells were cultured for 48 h with C1R.CD1b APC that had been pulsed with M. tuberculosis extract. DN1 treated in this way did not pro-
liferate when restimulated by CD1\(^+\) monocytes and *M. tuberculosis* antigen (Fig. 5A), a condition that supported this proliferation without such pretreatment (Fig. 1C). This observed hyporesponsiveness of the DN1 T cells was a consequence of T cell anergy and not due to a loss of viability of the T cells, because DN1 T cells recultured in the presence of IL-2 proliferated well. In contrast, if DN1 were first cocultured with C1R.CD1b APC in the absence of *M. tuberculosis* antigen, anergy did not occur, as indicated by a normal proliferative response to rechallenge with CD1\(^+\) monocytes and antigen. DN6 also became anergic after coculture with antigen-pulsed C1R.CD1c (Fig. 5B). Like DN1, the induction of anergy in DN6 was antigen specific and restricted by the same form of CD1 that restricted the T cell proliferative and CTL responses (i.e., CD1c in this case).

A high APC/T cell ratio during the preincubation with the C1R transfectants was required for the induction of anergy. Anergy resulted only when the appropriate form of CD1 was expressed by C1R, since the C1R mock transfectant failed to induce anergy in either T cell line, regardless of whether it was pulsed with antigen or not. As a control for antigen specificity, tetanus toxoid (recombinant C fragment)-pulsed C1R cells did not induce anergy. Furthermore, the induction of anergy was dependent on the *M. tuberculosis* antigen concentration. For DN1, pulsing C1R.CD1b with 1 \(\mu g/ml\) was as effective as 25 \(\mu g/ml\); however, DN6 required 10–25 \(\mu g/ml\) for a maximal effect. This is consistent with DN1 being more sensitive by at least an order of magnitude than DN6 to stimulation with *M. tuberculosis* and CD1\(^+\) monocytes (Fig. 1, C and D).

![Figure 3](image3.png)

**Figure 3.** The expression of CD80 (B7-1) and CD86 (B70/B7-2) on CD1\(^+\) monocytes and C1R.CD1b. The binding of mAbs BB-1 (CD80) and IT2 (CD86) were compared on the CD1\(^+\) monocytes and C1R.CD1b. A negative control and HLA-DR expression are shown as controls. The fluorescent channel number is plotted along the x-axis, and the y-axis represents the relative cell number.

![Figure 4](image4.png)

**Figure 4.** The antigen-induced proliferation of DN1 was not inhibited by CTLA4-Ig. (A) DN1 was stimulated using CD1\(^+\) monocytes and different concentrations of *M. tuberculosis* sonicate in the presence of CTLA4-Ig (10 \(\mu g/ml\)), human IgG1 (10 \(\mu g/ml\)), or no antibody. (B) At 10 \(\mu g/ml\), CTLA4-Ig blocked T cell proliferation induced by a mixed lymphocyte reaction by 80%. Human IgG1, CTLA4-Ig; no antibody.
CD1+ monocytes or CD1+ B-LCL were cultured with mycobacterial antigen and the DN1 or DN6 T cell line in the lower well (cluster well) of a transwell plate. The upper well (transwell) contained either medium alone or CD1+ monocytes and mycobacterial antigen and was separated from the lower well by a semipermeable cellulose acetate membrane (0.4 μm pore size). The cells in the lower well were pulsed with [3H]TdR for 6 h after 3 d (for DN6) or 5 d (for DN1), and the thymidine incorporation was measured. Mφ, monocytes; MTb, Mycobacterium tuberculosiis antigen.

Table 1. Requirement for Cell–Cell Contact in Costimulation of CD28− T Cells

| Upper well | Lower well | cpm (lower well) |
|------------|------------|------------------|
| CD1+ Mφ + MTb + DN1 | 39,603 ± 1,930 |
| CD1+ CIR.CD1b + MTb + DN1 | 205 ± 10 |
| CD1+ Mφ + MTb + DN1 | 194 ± 76 |
| CD1+ CIR.CD1c + MTb + DN6 | 87,624 ± 10,890 |
| CD1+ Mφ + MTb | 28 ± 6 |
| CD1+ CIR.CD1c + MTb + DN6 | 31 ± 9 |

Although the CD1 transfectants of the CIR cell line were unable to stimulate the proliferation of DN1 or DN6, B-LCLs are generally potent APC for in vitro stimulation of MHC-restricted T cell responses to soluble antigens since they are able to process antigen and express B7 and a full array of cell adhesion molecules. In fact, the CIR cell line was capable of stimulating the proliferation of human CD4+ T cells. Two tetanus toxoid–specific HLA-DR–restricted CD4+ T cell clones, SP-F3 and SP-F14, proliferated when the C fragment of tetanus toxoid was presented by the CIR transfectants (Fig. 6). In direct contrast to the results obtained with DN1 and DN6, when CIR.CD1b cells were pulsed with 10 μg/ml of the tetanus toxoid C fragment and then incubated with SP-F3, anergy was not induced in SP-F3 (Fig. 7). These experiments clearly demonstrated the existence of different requirements for the costimulation and anergy induction for these representative CD4+CD28− T cell clones (SP-F3, SP-F14), in contrast to the CD28+ DN T cell lines (DN1, DN6). Thus, the induction of anergy in DN1 and DN6 after the recognition of antigen presented by the B-LCL CD1 transfectants strongly indicated that the DN T cells have a unique costimulatory requirement.

Figure 5. Induction of anergy in CD28− T cells. (A) DN1 T cells became anergic when stimulated by antigen-pulsed CIR.CD1b cells. DN1 was cocultured with either mycobacterial antigen-pulsed (1 μg/ml) CIR.CD1b cells (■) or with unpulsed CIR.CD1b cells (○) for 48 h. The T cells were then isolated and restimulated with CD1+ monocytes in the absence (medium alone) or presence of mycobacterial antigen at the indicated concentrations, or with IL-2 (2 nM). The assay was harvested on day 3 after a 6-h pulse with 1 μCi/well of [3H]TdR. (B) DN6 became anergic when stimulated by antigen-pulsed CIR.CD1c cells. DN6 was cocultured with either mycobacterial antigen-pulsed (10 μg/ml) CIR.CD1c cells (■) or with unpulsed CIR.CD1c cells (○) for 48 h. The T cells were then isolated and restimulated as described above for DN1. The assay was harvested on day 3 after a 6-h pulse with 1 μCi/well of [3H]TdR.
Anti-CD1b mAbs Blocked the Induction of Anergy. The two-signal model of T cell activation predicts that if signal one (TCR mediated) is blocked, then neither T cell proliferation nor T cell anergy will occur. This hypothesis was tested using the anti-CD1b mAb BCD1b3.1, which blocked the proliferation of the DN1 T cell line that normally occurs when *M. tuberculosis* antigen is presented by CD1+ monocytes (data not shown). Coculture of DN1 with antigen-pulsed C1R.CD1b cells induced anergy as indicated by a decreased proliferative response upon restimulation (Fig. 8). However, when 10 μg/ml of anti-CD1b mAb was included during the preincubation phase of anergy induction, the development of DN1 T cell anergy was prevented (Fig. 8).

IL-2 Did Not Prevent the Induction of Anergy. Considerable interest has been generated by the question of whether IL-2 can prevent the induction of T cell anergy. At least one report (58) demonstrated that the addition of IL-2 to cultured T cells under conditions that would otherwise lead to the induction of anergy can prevent the development of human T cell hyporesponsiveness. However, this area remains controversial since, in other systems, the presence of IL-2 did not appear to prevent the induction of anergy (59). To clarify this point for the CD28− T cells we have studied, experiments were performed to assess the effect of IL-2 on the induction of anergy in DN1 T cells. We also examined the effect of IL-1, because it has been shown that a membrane form of IL-1 can be an important costimulatory molecule for the activation of T cells by monocytes (60). We found that the inclusion of IL-2 (Fig. 9) or IL-1 (data not shown) during the induction phase of anergy for both CD28− T cell lines was unable to provide costimulation and did not prevent the development of anergy.

### Figure 8

Anti-CD1b antibody prevented the induction of anergy. Anergy was induced as described in the legend of Fig. 3. 10 μg/ml of mAb BCD1b3.1 (anti-CD1b) was added during the preincubation where indicated. Restimulation conditions: MTb 5 ng/ml (solid bars), no antigen (open bars); 2 nM IL-2 (hatched bars).

### Figure 9

IL-2 did not prevent the induction of anergy. Anergy was induced as described in the legend of Fig. 3. 2 nM IL-2 was added during the preincubation where indicated. Restimulation conditions: MTb 10 ng/ml (solid bars); no antigen (open bars); 2 nM IL-2 (hatched bars).

### Discussion

Work in several systems has shown that the binding of CD80 or CD86 to CD28 is sufficient to provide a costimulatory signal that leads to full T cell activation and prevention of T cell anergy. However, many human T cells are CD28−, and the costimulatory requirements of these T cells are presently not known. In this study, we have examined two representative CD28− TCR-α/β+ T cell lines. DN1 and DN6 T cell lines killed antigen-pulsed CD1-transfected C1R B-LCL but failed to proliferate in response to these same APC. Furthermore, after recognition of antigen presented by B-LCL transfecants, these T cells were found to be anergic when assayed for subsequent proliferation. The ability of the CD28− T cells to perform effector functions such as killing did not appear to depend on costimulation. Such a phenomenon has been observed for Th1 and CD8+ cells and has been termed split or partial anergy (61, 62).

These data suggest that the CD28− T cells required a costimulatory signal not expressed on the B-LCL, although these cells do express the known costimulatory ligands CD80 and CD86 and were able to provide costimulation to human CD28+ T cell clones. The costimulatory ligand required by CD28− TCR-α/β+ T cells was present on CD1+ monocytes, since this APC induced a brisk proliferative response without inducing anergy. The costimulatory signal required by CD28− TCR-α/β+ T cells was functionally similar to B7–CD28, since antigen-specific activation of DN1 and DN6 in the absence of the costimulatory signal resulted in pronounced T cell anergy. Several lines of evidence lead to the conclusion that the costimulatory signal required by DN1 and DN6 must be distinct from that generated by the CD28–B7 axis. First, DN1 and DN6 did not express CD28. Second, the costimulatory ligand required by DN1 and DN6 was unlikely to be CD80 or CD86 since both were normally expressed on C1R cells, which induced anergy and not proliferation or IL-2 production upon antigen presentation to these T cells. Thus, the CD80 or CD86 molecules expressed by the CD1-transfected C1R B-LCLs were not sufficient to prevent the induction of anergy when DN1 or DN6 was cocultured with these APC. In contrast, it has been previously shown that CD80 is sufficient to prevent the development of T cell anergy in human CD4+ T cell clones dependent on the CD28–B7 pathway (11), and our own studies showed that the CD80+86+ C1R B-LCLs were able to fully activate CD28+ T cell clones without inducing anergy. Lastly,
CTLA4-Ig, which binds with high avidity to CD80 and CD86 and competitively inhibits costimulation by this pathway, could not inhibit DN1 T cell proliferation that occurred when antigen was presented by the CD1+ monocytes. Together, these data indicate that a distinct costimulatory pathway exists and is critical for the proliferation of CD28- T cells such as DN1 and DN6.

Experimentally, T cell anergy has been induced by chemically modifying the APC using paraformaldehyde or the cross-linking agent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (10). Alternatively, investigators have used artificial APC such as COS or Chinese hamster ovary cells transfected with CD80 or CD86 to provide a costimulatory signal (3). These systems have been useful for dissecting the function of the CD80 and CD86 costimulatory ligands, but their in vivo relevance is limited. The APC used in the experiments described here are closely analogous to a major population of APC in vivo (i.e., B lymphoblasts, activated monocytes, and tissue macrophages), and neither the surface of the lymphoblastoid cell lines nor the CD1+ monocytes were chemically modified. Thus, anergy was induced by live APC. In addition, the system described here provides an example of a costimulatory molecule that is expressed on cells of the myeloid lineage but not on cells of the B cell lineage. Johnson and Jenkins reported that freshly isolated monocytes and the U937 human monocytic leukemia cell line can provide a costimulatory signal to T cells that is independent of the CD28-B7 axis (63). Freshly isolated monocytes and myeloid tumor cell lines lack expression of CD1, which is required for antigen recognition by the DN1 and DN6 T cell lines, and, consequently, we have been unable to ascertain whether the costimulatory signal provided by these APC is similar in function to the ligand expressed by the CD1+ monocytes. Nevertheless, these experiments highlight important differences between types of APC. Specialized APC such as macrophages, dendritic cells, and Langerhans cells express a full array of antigen-presenting molecules, adhesion molecules, and costimulatory molecules. The expression of CD1 and novel costimulatory molecules by the GM-CSF/IL-4-treated monocytes is critical for the activation and expansion of certain T cell subsets such as CD4-8- TCR-α/β+ T cells, as exemplified by the CD28- DN1 and DN6 T cell lines.

The role of the B7–CD28 axis in peripheral tolerance and T cell activation and its therapeutic potential for treating cancer, transplant rejection, and autoimmune disease are rapidly advancing areas of investigation. However, most in vivo studies have been carried out in mice, and there exist important differences between human and murine T cell costimulation. Whereas most murine T cells express CD28 (64), many human T cells do not. These CD28- T cells include approximately half of the circulating CD8+ and CD4-8- T cells (38, 39, 51, 65). All of these T cell subsets have been proposed to be important in human cancer immunity (66-69), graft rejection, and autoimmunity—situations in which manipulation of the CD28–B7 axis has been associated with beneficial effects in the murine system. Clonal expansions of CD28- CD8+ T cells have been reported in the peripheral blood of normal individuals (70) and patients with autoimmune diseases (71, and Behar, S. M., unpublished observations). The numbers of CD28- CD8+ T cells and CD28- CD4+ T cells are increased in the peripheral blood and lung of HIV+ patients (72). Among human CD4+ T cells, there are CD28-high and -low expressors, and 1–5% of the peripheral blood CD4+ T cells are CD28-; these seem to form a distinct subset of T cells that have a limited TCR repertoire and poor proliferative capacity to alloantigen (73, 74). Although CD28- and CD28+ T cells have similar cloning efficiency (65), the difficulty establishing long-term CD28- T cell lines and clones may reflect a requirement for a costimulatory signal other than CD28, which may be provided only by specialized APC such as the CD1+ monocytes we have used in this study. Furthermore, evidence from studies in the mouse also suggests the existence of costimulatory molecules other than CD28. Several groups have demonstrated that the generation of primary T cell responses is not impaired in CTLA4-Ig-transgenic mice or in mice made deficient in CD28 by targeted gene disruption (75, 76), both of which have abnormal T cell dependent antibody production presumably as a result of deficient CD28–B7 signaling. Thus, a role for CD28-independent T cells in both normal immunity and in a variety of disease states is strongly indicated, and a better understanding of CD28-independent costimulatory pathways is essential. The abundance of human CD28- T cells together with the functional data presented in this report suggest the existence of alternate costimulatory pathways independent of the B7–CD28 axis, which may be of general importance to CD28- T cells.

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