B70/B7-2 Is Identical to CD86 and Is the Major Functional Ligand for CD28 Expressed on Human Dendritic Cells

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Summary

Dendritic cells comprise a system of highly efficient antigen-presenting cells involved in the initiation of T cell responses. Herein, we investigated the role of the CD28 pathway during alloreactive T cell proliferation induced by dendritic-Langerhans cells (D-Lc) generated by culturing human cord blood CD34+ progenitor cells with granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α. In addition to expressing CD80 (B7/BB1), a subset of D-Lc expressed B70/B7-2. Binding of the CTLA4-Ig fusion protein was completely inhibited by a combination of monoclonal antibodies (mAbs) against CD80 and B70/B7-2, indicating the absence of expression of a third ligand for CD28/CTLA-4. It is interesting to note that mAbs against CD86 completely prevented the binding of CTLA4-Ig in the presence of mAbs against CD80 and bound to a B70/B7-2-transfected fibroblast cell line, demonstrating that the B70/B7-2 antigen is identical to CD86. CD28 triggering was essential during D-Lc-induced alloreaction as it was inhibited by mAbs against CD28 (9 out of 11 tested). However, none of six anti-CD80 mAbs demonstrated any activity on the D-Lc-induced alloreaction, though some were previously described as inhibitory in assays using CD80-transfected cell lines. In contrast, a mAb against CD86 (IT-2) was found to suppress the D-Lc-dependent alloreaction by 70%. This inhibitory effect was enhanced to >90% when a combination of anti-CD80 and anti-CD86 mAbs was used. The present results demonstrate that D-Lc express, in addition to CD80, the other ligand for CTLA-4, CD86 (B70/B7-2), which plays a primordial role during D-Lc-induced alloreaction.

Although presentation of antigen to TCR is necessary for the initiation of T cell activation, costimulatory signals delivered by the APC permit a full immune response. The CD28 pathway is regarded as an essential costimulus of T cell proliferation (1). The coligation of the T cell CD28 receptors by the APC counter-receptors CD80 (B7/BB1) or the recently identified B70/B7-2 (2-5) results in IL-2 production and T cell proliferation (1, 2, 5).

B70/B7-2 is expressed on resting monocytes and activated B and T cells and is upregulated on monocytes upon activation (2-6). B7/BB1 and B70 expression on B cells is upregulated by cross-linking MHC class II molecules (7), surface membrane Ig (6), or CD40 antigen (2, 8) and cytokines such as IL-2 and IL-4 (9). In vivo, blocking of the CD28/CTLA4 pathway results in T cell anergy, prolongation of graft survival, and blocking of B cell responses (10-12).

Dendritic cells (DC) comprise a system of highly efficient APC that function to initiate immune responses (13). In vivo, DC are involved in alloreactive T cell activation as occurs during transplant rejection, and in vitro, primary alloreactive T cell activation appears to be essentially mediated by DC (14). Dendritic/Langerhans cells (D-Lc), generated by culturing human hematopoietic progenitor cells with GM-CSF and TNF-α, can induce strong proliferation of allogeneic T cells (15). The present study was designed to investigate the expression and the function of CD28 counter-receptors on those in vitro-generated D-Lc. D-Lc were found to express both B7/BB1 and B70, the latter being identical to the recently clustered B cell antigen CD86. Importantly, this study also concluded that B70/CD86 is the crucial CD28 ligand involved in D-Lc-induced T cell alloactivation.

Materials and Methods

Hematopoietic Factors, Reagents, and Antibodies

rhGM-CSF (sp act, 2 × 10^6 U/mg; Schering-Plough Research Institute, Kenilworth, NJ) was used at 100 ng/ml. rhuTNF-α (sp...
Collection and Purification of Cord Blood CD34+ Cells and Adult Blood T Cells

CD34* Cells. Umbilical cord blood samples were obtained according to institutional guidelines. Cells bearing CD34 antigen were isolated from nonadherent mononuclear fractions through positive selection by indirect immune "panning" using anti-CD34 mAb (Imu-133.3; Immunotech). A second purification step was performed using a cocktail of mAbs, as described (20). Thus, in all experiments, isolated cells were 95-99% CD34* as judged by staining with anti-CD34 mAb.

CD4+ T Cells. Mononuclear cells, isolated from adult blood, were depleted of adherent cells. CD4+ T lymphocytes were purified by immunomagnetic depletion using a cocktail of mAbs IOM2 (CD14), ION16 (CD16), ION2 (HLA-DR) (Immunotech), NKH1 (CD56), OKT8 (CD8) (Ortho), 4G7 (CD19), and mAb 89 (CD40). After two rounds of bead depletion, purity was routinely >95%.

Cell Cultures

Generation of D-Lc. CD34+ cell cultures were established in the presence of GM-CSF and TNF-a in medium consisting of RPMI-1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% (vol/vol) FBS, 10 mM Hepes, 2 mM l-glutamine, 5 x 10^-5 M 2-β ME, penicillin (100 U/ml), and streptomycin (100 μg/ml) (referred to as complete medium). CD34+ cells were seeded in 24-well culture plates at 2 x 10^4 cells/ml and cultures were split every 4-5 d. After 12 d, cultures contained between 40 and 80% of CD34+ D-Lc (15) and cells were collected at that time point.

T Cell Proliferation Assay. After 12 d of culture, CD4+ hematopoietic progenitor cells (HPC)-derived cells were collected and, after irradiation (40 Gy), were used as stimulator cells for resting allogeneic adult peripheral blood CD4+ T cells (2 x 10^4 per well). 10-3 x 10^9 stimulator cells were added to T cells in 96-well round-bottomed tissue culture plates. Control T cell proliferation was performed using PHA (0.1 μg/ml) and IL-2 (10 U/ml). Except for kinetic experiments, cultures lasted 5 d. After incubation, cells were pulsed with 1 μCi of [3H]Tdr for the last 8 h, harvested, and counted. Tests were carried out in triplicate, and results were expressed as cpm ± SD.

Cytfluorometric Cell-surface Phenotyping

Double-color fluorescence was carried out by sequential incubation of cells with unconjugated mAbs, PE-conjugated goat F(ab')2 anti-mouse Ig, normal mouse sera, and FITC-coupled OKT6 (CD1a) (all from Ortho). For CTLA4-Ig fusion protein staining, as previously described (2), cells were incubated with anti-CD80 mAbs (L307, L308, or mAb 104), anti-B70 mAb (IT-2), anti-CD86 (Fun-1 or BU63), unrelated mAbs, or combinations of these. CTLA4-Ig was revealed by PE-conjugated goat F(ab')2, anti-human IgG (Fc specific), absorbed on mouse Ig (Jackson Immunoresearch Labs., Inc., West Grove, PA). Cells were then double stained with FITC-coupled OKT6 (CD1a). Negative controls were performed with unrelated murine mAbs and mIL4R-Ig fusion protein. Fluorescence was measured with a FACSScan® (Becton Dickinson & Co., Mountain View, CA).

B70 Transfectants

Human B70 cDNA (2) in the pBJ expression vector (21) was used to stably transfect murine L cells expressing human CD32,
as described (22), B70-transfected L cells were selected for growth in 1 mg/ml G418 (Schering-Plough Research Institute), and transfectants with the highest cell surface density of B70 expression were isolated by flow cytometry.

Results

In Vitro-generated CD1a+ D-Lc Bind CTLA4-Ig through both B7/BB1 and B70/B7-2. We wondered whether D-Lc generated in vitro by culturing CD34+ cells for 12 d with intensity levels (low and high) (Fig. 1 B). In contrast, only 1 mg/ml G418 (Schering-Plough Research Institute), and trans-effects with the highest cell surface density of B70 expression were isolated by flow cytometry.

Double staining with anti-CDS0 and anti-B70 mAbs showed that B70/B7-2+ cells comprised all CD80+ and that all B70/B7-2+ cells were CD80low (Fig. 1 D). In 10 independent experiments, 90 ± 8% (80–100%) of CD1a+ D-Lc were found to express CD80 whereas only 55 ± 20 (20–70%) expressed B70/B7-2.

As activated human B cells have been reported to express a third ligand for CD28/CTLA4 (6), we searched for this structure on D-Lc by evaluating the effects of anti-CD80 and anti-B70 mAbs on the binding of CTLA4-Ig. 12-d D-Lc were first incubated with one anti-CD80 mAb (L307, L308, mAb 104) or isotype control without or with the B70-specific mAb IT-2, and then labeled with CTLA4-Ig (or mIL4R-Ig), revealed by PE-conjugated anti-human IgG. Cells were finally first incubated with one anti-CD80 mAb (L307, L308, mAb 104) or isotype control without or with the B70-specific mAb IT-2, and then labeled with CTLA4-Ig (or mIL4R-Ig), revealed by PE-conjugated anti-human IgG. Cells were finally tested. Kinetics studies of B70/B7-2 expression indicated that the two molecules appeared concomitantly during generation and maturation of D-Lc (data not shown). Furthermore, as previously shown for mAb IT-2, mAbs against CD86 (FUN-1 is shown) used alone induced a slight decrease in intensity of CTLA4-Ig binding (50% decrease in MFI) (Fig. 2 C), and combination of anti-CD86 and anti-CD80 mAbs completely prevented the binding of CTLA4-Ig (Fig. 2 C). Finally, the anti-CD86 mAbs (FUN-1 and BU63) bound to a B70-transfected L cell line but not to the control L cell line (Fig. 3). Taken together, these results demonstrate that B70/B7-2 is identical to CD86.

CTLA4-Ig Suppresses D-Lc–induced Alloreactivity Mainly by Blocking CD28/CD86 Interaction. Since, interactions between CD28/CTLA4 and their counter-receptors have been described as necessary for APC-dependent T cell activation, we investigated whether blocking of these interactions would also inhibit a D-Lc–induced T cell alloreactivity. First, the CTLA4-Ig fusion molecule was used to interfere with CD28/CD28 ligand interactions that may occur during the D-Lc-induced alloreactive CD4+ T cell proliferation. As shown in Fig. 4 A (Exp. 1), CTLA4-Ig inhibited D-Lc-induced alloreactivity in a dose-dependent manner with half-maximal inhibition at 2.5 μg/ml and maximal inhibition at 10 μg/ml. At high numbers of D-Lc (3 × 105 D-Lc for 2 × 105 CD4+ T cells), CTLA4-Ig caused a 60% inhibition (45–70%, n = 6) of T cell proliferation (Fig. 4 B). At lower D-Lc concentrations (3 × 104 D-Lc), CTLA4-Ig inhibited T cell proliferation by 85% (75–95%, n = 6). A mIL4R-Ig fusion protein, included as a control, did not inhibit the D-Lc-induced alloreactive CD4+ T cell proliferation (data not shown). Results are representative of six experiments.

Figure 2. CD80 and B70/B7-2 expression account for all CTLA4-Ig binding and B70/B7-2 is linked to CD86. Inhibition of CTLA4-Ig binding on D-Lc by anti-CD80 (L307), anti-B70 (IT-2), or anti-CD86 (FUN-1) mAbs was tested. D-Lc were preincubated in the presence of: (A) Isotype control ; (B) anti-CD80 mAb ; and (C) anti-CD86 mAb before CTLA4-Ig + PE goat anti-human Ig staining. Gating was then set on CD1a+ (OKT6-FITC stained) cells. Anti-CTLA4-Ig staining on anti-CD80 + anti-B70 mAbs preincubated cells was identical to mIL4R-Ig staining (data not shown). Results are representative of six experiments.
a control, never significantly affected any of the assays (Fig. 4 A, Exp. 1, and Fig. 4 B).

We then wondered whether a CD28-specific mAb (IOT28) would also suppress a D-Lc-mediated alloreaction (Fig. 4). Depending on the D-Lc concentration, IOT28 caused a 50–85% inhibition (35–90%, n = 6) of T cell DNA synthesis (Fig. 4 B). This inhibitory effect was dose dependent with half-maximal effect at 0.5 µg/ml and maximal effect at 2.5 µg/ml (Fig. 4 A).

It is interesting to note that the anti-CD80 mAb L307, which suppresses the development of CD8 cell cytotoxicity against CD80-transfected target cells (17), failed to inhibit D-Lc–induced alloreaction even at concentrations as high as 10 µg/ml (Fig. 4, A and B). To further strengthen these findings, all anti-CD28 and anti-CD80 antibodies available from the V Workshop on Human Leukocyte Differentiation Antigens were tested for their ability to affect D-Lc–induced alloreaction and to alter PHA + IL-2–dependent T cell proliferation. As shown in Fig. 5, although able to bind to D-Lc (data not shown), none of the mAbs against CD80 demonstrated any activity on D-Lc–induced alloreaction (Fig. 5, left). Furthermore, these CD80-specific antibodies did not affect PHA + IL-2–induced T cell proliferation (Fig. 5, right). In contrast, the CTLA4-Ig and 9 out of 11 mAbs against CD28 were found to inhibit by 55–85% the D-Lc–induced alloreaction. The CTLA4-Ig molecule has no effect on PHA + IL-2–dependent T cell proliferation, whereas, in accordance with their known costimulatory effects, the anti-CD28 mAbs that demonstrated blocking effect on D-Lc–induced alloreaction, costimulated T cell proliferation (1.8–2.2 fold enhancement) in the presence of PHA + IL-2. All anti-CD28 mAbs bound to T cell clones (data not shown), suggesting that the two mAbs lacking activity (M-T281 and CD28.4) recognized epitope(s) not involved in ligand binding.

Figure 3. B70/B7-2 is identical to CD86. Staining with anti-CD80 (L307 and mAb104) (A), anti-B70 (IT2) (B), anti-CD86 (FUN-1) (C), and anti-CD86 (BU63) (D) was performed on a murine L cell line (unfilled histograms) and a murine L cell line stably transfected with B70 (filled histograms).

Figure 4. D-Lc–induced alloreaction is blocked by CTLA4-Ig, anti-CD28, and anti-CD86 mAbs. D-Lc were used after irradiation (40 Gy) as stimulator cells for T cell proliferation. 2 × 10⁴ CD4⁺ T cells were seeded in the presence of either 12-3 × 10³ stimulator cells per well in 96-well round-bottomed tissue culture plates. CTLA4-Ig fusion protein and mAbs against CD80 (L307), CD86 (IT2), and CD28 (IOT28) were added at 10 µg/ml for most experiments. mIL4R-Ig fusion protein and unrelated IgG1 were used as controls at the same concentrations. (A) The dose-dependent effects of mAbs and fusion proteins on D-Lc–induced alloreaction. The proliferation was revealed by [³H]Tdr incorporation after 4 d of culture. (B) The effects of mAbs and fusion proteins on a dose-dependent D-Lc–induced alloreaction. The proliferation was revealed by [³H]Tdr incorporation after 4 d of culture. (C) The effects of mAbs and chimeric molecules (10 µg/ml) on the kinetics of D-Lc (10⁴/well)–induced T cell alloreaction. The proliferation was revealed by [³H]Tdr incorporation at the indicated time points. Results are representative of six experiments. SD were below 10% of variation.
Control isotype-matched mAbs did not significantly affect assays (Fig. 5). In contrast, the anti-CD86 mAb IT-2 suppressed D-Lc-dependent alloreaction (60–80%, n = 3) by 70%. The inhibitory effect was dose-dependent with half-maximal effect at 0.5 μg/ml and maximal effect at 2.5 μg/ml (Fig. 4 A, Exp. 2). In addition, a combination of anti-CD80 and anti-CD86 mAbs induced a nearly complete inhibition of T cell proliferation (88–100%, n = 6), even at high D-Lc numbers (data not shown).

The suppressive effect of CTLA4-Ig (data not shown), anti-CD28, and anti-CD86 mAbs could be detected as early as after 2 d of coculture and was maintained throughout the culture period (Fig. 4 C). The blocking effect of CTLA4-Ig and anti-CD86 mAb was specific for the D-Lc-induced alloreaction as they had no influence on PHA + IL-2-mediated T cell proliferation at any concentration or at any time tested (data not shown). Taken together, those results demonstrate that CD28 triggering is crucial during D-Lc-induced alloreaction, and occurs primarily through CD86 and secondarily through CD80 interactions.

Discussion

The present study was designed to determine the expression of the CD80 (B7/BB1) and B70/B7-2 accessory molecules on CD34+ progenitor-derived D-Lc and their contribution during D-Lc-induced T cell alloreactive proliferation. Using specific mAbs, CD80 was found to be expressed on virtually all CD1a+ D-Lc whereas B70/B7-2 expression was restricted to a smaller subset of CD1a+ D-Lc (50–70%). This study also demonstrates formally the identity of B70/B7-2 with CD86, a newly clustered antigen initially identified as a B cell activation antigen with the FUN-1 (23) and BU63 mAbs. Functional studies demonstrated that CD28 triggering was a crucial event during D-Lc-mediated T cell alloreaction, a finding consistent with previous reports (24–26). The present results further showed B70/CD86 as the major counter-receptor involved in CD28 triggering as a mAb against B70/B7-2 (IT-2) blocks by 50–80% the D-Lc-induced T cell alloreaction whereas none of the nine tested anti-CD80 mAbs directly affect such a biological function. Yet, CD80 does play a secondary role as a combination of anti-CD86 and anti-CD86 mAbs results in a complete abrogation of T cell activation. Such a finding suggests that no other accessory molecule, e.g., a third CD28 ligand (4, 6), can replace these known CD86 ligands. Indeed, phenotypic analysis indicated that all CTLA4-Ig ligands on CD1a+ D-Lc were accounted for by CD80 and CD86 as a combination of mAbs directed against these two antigens completely inhibited the binding of the CTLA4-Ig fusion protein. Furthermore, although CD80 and CD86 antigens are upregulated on D-Lc during CD40 triggering, a third CTLA4/CD28 counter-structure could not be detected after such activation (27).

The rationale for expression of two (or more) molecules mediating apparently similar effects on T cell activation is currently unknown. However, differences in the kinetics of upregulation during B cell activation (4, 6), as well as its restricted expression on germinal center B cells (23) may argue for specific roles of CD86 versus CD80. During maturation of CD34+ derived D-Lc, CD80 appears earlier than CD86 (data not shown) and expression of CD86 delineates two
subsets among CD1a+ D-Lc (CD80+, CD86+ and CD80+, CD86-) which might represent different maturation or activation stages of D-Lc. In addition, CD86 is more strongly upregulated than CD80 during CD40 activation of CD1a+ D-Lc (27). Such differential kinetics of expression during maturation and activation might suggest specific roles for CD80 versus CD86 during a T cell immune response.

The identification of CD86 is the major ligand for CD28 expressed on dendritic cells, will enable further manipulation of the CD28 pathway, potentially leading to a therapeutic strategy for the control of autoimmunity or transplantation tolerance. The importance of the CD28 counter-receptors in tumor immunity (28, 29) and the fact that D-Lc express spontaneously high levels of CD80 and CD86 antigens, represent new arguments to consider these in vitro generated D-Lc as an interesting material for development of immunotherapy strategies using tumor-associated antigens.

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