

RAPID COMMUNICATION

The B7-2 (B70) Costimulatory Molecule Expressed by Monocytes and Activated B Lymphocytes Is the CD86 Differentiation Antigen

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T-cell activation is initiated after T-cell receptor binding to antigen, but also requires interactions between costimulatory molecules expressed on antigen-presenting cells. An important costimulatory molecule expressed by monocytes and activated B lymphocytes has been recently identified and termed B7-2 or B70. Independently, a new Cluster of Differentiation was defined in the Fifth International Leukocyte Differentiation Antigen Workshop as CD86, a molecule predominantly expressed by monocytes and activated B

lymphocytes. In this study, the two monoclonal antibodies that defined CD86, FUN-1 and BU-63, were shown to bind to cDNA transfected cells expressing B7-2/B70. The FUN-1 monoclonal antibody also completely blocked the costimulatory activity of B7-2/B70 in functional assays. Therefore, the serologically defined CD86 differentiation antigen is the B7-2/B70 molecule.

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INTERACTIONS between costimulatory molecules on the surface of T lymphocytes and antigen-presenting cells are critical for lymphocyte proliferation in response to antigen.¹ These costimulatory molecules include CD28 expressed by T cells, which interacts with the B7-1 (BB1) molecule expressed by antigen presenting cells.^{2,3} The B7-1 molecule is now known as the CD80 differentiation antigen.⁴ Engagement of T cell CD28 by monoclonal antibody (MoAb) or by interactions with CD80 results in enhanced production of cytokines by activated T cells and costimulation of antitumor immunity.⁵⁻¹⁰ Blockage of this important costimulatory pathway during T-cell activation results in the development of antigen-specific tolerance.^{11,12} Interactions between CD28 on T cells and CD80 on activated B cells also regulate B-cell differentiation.¹³ CD80 is expressed by activated B lymphocytes,^{7,14-17} interferon-treated monocytes,¹⁸ activated T cells,¹⁹ and HTLV-I transformed T cells.²⁰ T cells also express a cell-surface molecule called CTLA-4 that is structurally related to CD28, which also binds CD80.²¹

Activated B lymphocytes express ligands for CD28/CTLA-4 in addition to CD80, which have been termed B7-2 and B7-3.²²⁻²⁴ Recently, a cDNA encoding B7-2 was cloned²⁵ as well as a cDNA encoding the identical costimulatory molecule termed B70.²⁶ B7-2/B70 belongs to the Ig superfamily and consists of an extracellular region containing a V- and a C-type Ig-like domain, a hydrophobic

transmembrane domain, and a cytoplasmic tail. An MoAb that binds B7-2/B70 (IT2) reacts with resting monocytes and dendritic cells, and activated B and T lymphocytes.²⁶ This MoAb also inhibits the binding of CTLA-4-Ig fusion proteins (CTLA-4-Ig) to human B cell lines, and together with anti-CD80 MoAb completely blocks CTLA-4-Ig binding. The IT2 MoAb immunoprecipitates a single-chain cell surface glycoprotein of ~70,000 M_r.

CD86 is a new Cluster of Differentiation that was established during the Fifth International Leukocyte Differentiation Antigen Workshop.²⁷ Two MoAbs, FUN-1 and BU63, defined CD86 as a single-chain 80,000 M_r cell-surface protein expressed by blood monocytes and activated B cells.²⁷ Because the expression pattern and biochemical characteristics of CD86 were similar to those described for the B7-2/B70 molecule, the possibility that CD86 and B7-2/B70 were the same molecule was examined in this study.

MATERIALS AND METHODS

MoAb. The FUN-1 MoAb (CD86, IgG₁) was as described.²⁸ The anti-CD80 MoAbs used were anti-B7-g (IgG₁) and C4 (IgG_{2a}) as described.¹⁴ The IT2 MoAb²⁶ (IT2.2; IgG_{2b}) was obtained from Pharmingen (San Diego, CA). The HB11 MoAb (CD45RA, IgG₁) was as described.²⁹ The BU63 MoAb (CD86, IgG₁) was obtained from the Fifth International Workshop on Human Leukocyte Differentiation Antigens and was provided to the Workshop by Dr D.L. Hardie (University of Birmingham, Birmingham, UK).

Cells. Blood mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation of heparinized blood obtained from healthy donors according to protocols approved by the Human Use Committees of Dana-Farber Cancer Institute and Duke University Medical Center. CD28⁺ T cells were purified by negative selection as described,⁶ and monocytes were isolated by adherence to plastic. Blood mononuclear cells and cell lines were cultured in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal calf serum, glutamine, and penicillin/streptomycin. Chinese hamster ovary (CHO) cells stably transfected with CD80 cDNA and B7-2 cDNA were produced as described¹² and cultured in the presence of G418 (400 µg/mL; GIBCO-BRL, Grand Island, NY).

Immunofluorescence analysis. Indirect immunofluorescence staining was performed after washing the cells twice. The cells were incubated for 20 minutes on ice with each test MoAb as either ascites fluid or purified antibody diluted to the optimal concentration for immunostaining. After washing, the cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated goat-antimouse Ig antibodies (TAGO, Burlingame, CA) for 20 minutes at 4°C. Single-

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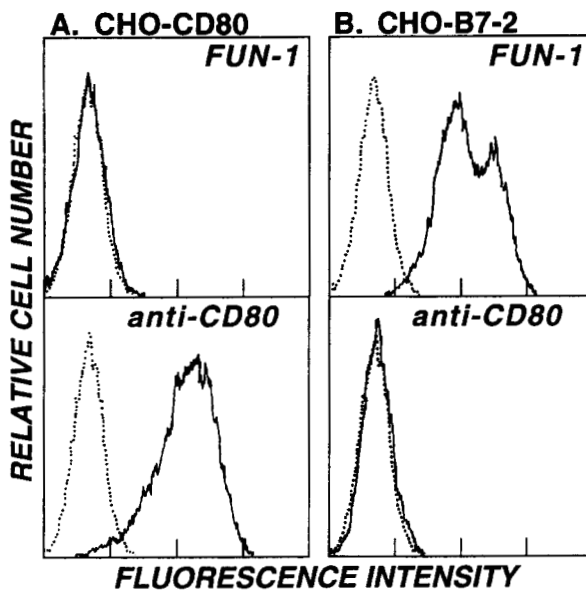


Fig 1. The FUN-1 MoAb reacts with CHO cells expressing the B7-2/B70 molecule. CHO cells stably transfected with CD80 (CHO-CD80) or B7-2/B70 (CHO-B7-2) cDNA were cultured with the FUN-1 (CD86) or anti-CD80 (B7-g) MoAbs with reactivity assessed by indirect immunofluorescence staining with flow cytometry analysis. Reactivity of the test antibody is indicated by solid lines, whereas the reactivity of an unreactive isotype-matched control MoAb is indicated as a dotted line. Results identical to those obtained with the FUN-1 MoAb were obtained with the BU-63 MoAb.

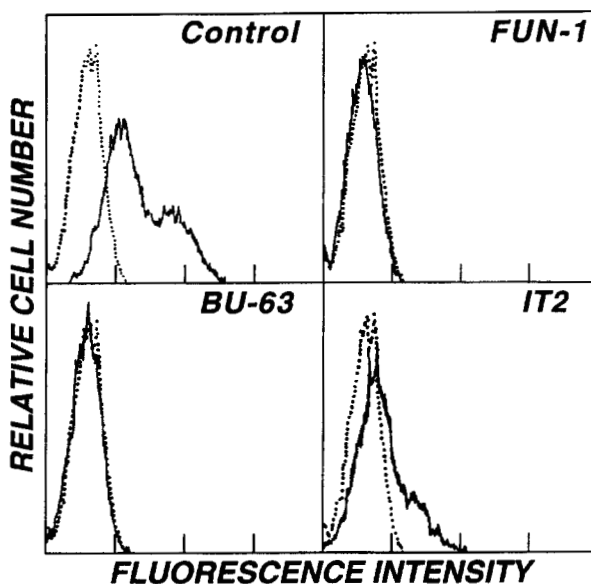


Fig 2. Analysis of the antibody-binding epitopes on the B7-2/B70 molecule. CHO-B7-2 cells were incubated with media alone (Control), FUN-1, BU63, or IT2 MoAbs, before treatment with FITC-conjugated FUN-1 MoAb (0.5 $\mu\text{g}/\text{mL}$). Solid lines indicate staining intensity of FITC-conjugated FUN-1 MoAb assessed by flow cytometry analysis, whereas dotted lines indicate background staining with an unreactive FITC-conjugated isotype-matched control MoAb. Results obtained with CHO-B7-2 cells incubated with saturating amounts of unlabeled anti-CD45RA (HB-11) before treatment with FITC-conjugated FUN-1 MoAbs were identical to the Control results shown.

color immunofluorescence analysis of 10,000 cells was performed on an Elite flow cytometer (Coulter Electronics, Hialeah, FL).

MoAb cross-blocking experiments. CHO-B7-2 cells or cells of the BJAB B cell line (1×10^6) were first incubated with saturating concentrations of test MoAb as either diluted ascites fluid or as purified MoAb for 20 minutes on ice. After this incubation, FITC-conjugated FUN-1 MoAb was added to the tube at a suboptimal concentration for immunofluorescence staining (0.5 $\mu\text{g}/\text{mL}$). After 20 minutes of further incubation, the cells were washed twice and immunofluorescence staining was assessed immediately by flow cytometry analysis.

CTLA-4-Ig binding assays. The human CTLA4-Ig fusion protein was prepared as described.¹² CTLA4-Ig blocking experiments were performed on CHO-B7-2 cells and cells of the BJAB B cell line (1×10^6). The cells were first incubated with media or saturating concentrations of FUN-1, BU63, IT2, and irrelevant control MoAb (HB11, CD45RA) for 20 minutes at 4°C before adding CTLA4-Ig for another 20 minutes. After this incubation, the cells were washed twice and incubated with FITC-conjugated goat-antihuman IgG anti-serum (TAGO). After 20 minutes of further incubation, the cells were washed twice and immunofluorescence staining assessed immediately by flow cytometry analysis.

Costimulation assays. Costimulatory assays were performed as described.⁶ Briefly, CHO cells stably transfected with CD80 and B7-2 cDNA were fixed with 0.4% paraformaldehyde, washed extensively, and incubated with CD28⁺ T cells in flat-bottom 96-well plates. The culture plates were either untreated, coated with anti-CD3 MoAb (OKT3, 1 $\mu\text{g}/\text{mL}$), or phorbol 12-myristate 13-acetate (PMA) was added to the cultures at a final concentration of 0.75 ng/mL. Anti-CD80 (C4) and IT2 MoAb were added at a concentration of 1 $\mu\text{g}/\text{mL}$ and the FUN-1 MoAb was used as a 1:500 dilution

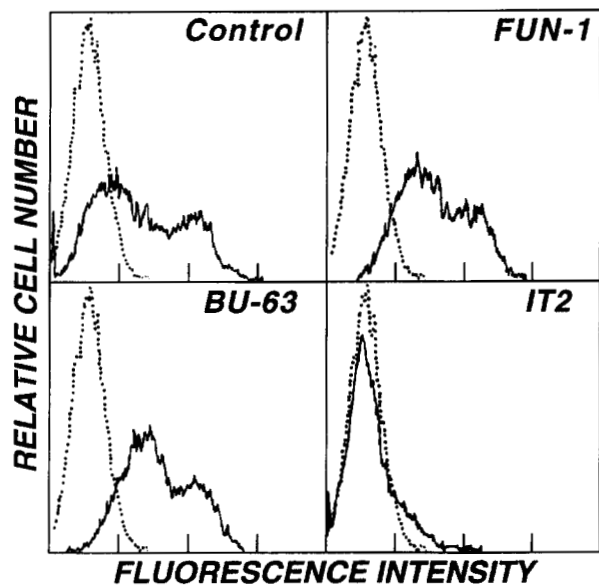


Fig 3. Ability of the anti-CD86 and IT2 MoAbs to block binding of CTLA-4-Ig to CHO-B7-2 cells. CHO-B7-2 cells were incubated with media or saturating amounts of FUN-1, BU-63, or IT2 MoAbs before treatment with CTLA-4-Ig. Solid lines indicate the fluorescence intensity of CTLA-4-Ig-treated cells stained with FITC-labeled goat-antihuman IgG antibodies as assessed by flow cytometry analysis. Dotted lines indicate background staining with the FITC-labeled goat-antihuman IgG antibodies alone. Results obtained with CHO-B7-2 cells incubated with saturating amounts of unlabeled anti-CD45RA (HB-11) MoAb before treatment with CTLA-4-Ig were identical to the Control results shown.

of ascites fluid. Control isotype-matched MoAbs were added at the same concentration or dilution. Cell proliferation was examined after 72 hours of culture and interleukin-2 (IL-2) secretion was assessed at 48 hours. Cell proliferation during the last 12 hours of culture was quantified by incubating the cells with 1 μ Ci (37 kBq) of [*methyl*-³H]thymidine (NEN-DuPont, Boston, MA). The cells were harvested onto filters and radioactivity was measured in a Beta Plate scintillation counter (Pharmacia, Uppsala, Sweden). IL-2 concentrations present in supernatant fluid were determined in duplicate using an IL-2 enzyme-linked immunosorbent assay (ELISA) (R&S Systems, Minneapolis, MN).

Statistical analysis. Significance of results was determined using the paired Student's *t*-test.

RESULTS

CD86 MoAbs bind the B7-2/B70 molecule. CHO cells stably transfected with CD80 or B7-2 cDNA were stained with CD86 MoAb or the IT2 MoAb in indirect immunofluorescence assays to determine whether CD86 and B7-2/B70 were the same molecule. The FUN-1 (CD86) MoAb specifically stained B7-2/B70 cDNA-transfected CHO cells, but failed to stain CD80 cDNA-transfected cells (Fig 1). Identical results were obtained with the IT2 and BU-63 MoAbs (data not shown). In contrast, an anti-CD80 MoAb reacted with CHO cells transfected with CD80 cDNA, but not with cells transfected with B7-2/B70 cDNA (Fig 1). These results

show that CD86 MoAb identify the protein encoded by the B7-2/B70 cDNA and that the IT2 MoAb belongs to the CD86 cluster.

Epitope analysis of the CD86 and IT2 MoAbs. Antibody cross-blocking studies have shown that the FUN-1 and BU63 MoAbs bind to a similar, if not identical, epitope on CD86.²⁷ In similar studies, binding of the FUN-1 or BU63 MoAbs completely inhibited the subsequent binding of fluoro-chrome-labeled FUN-1 MoAb (Fig 2), whereas treatment of B7-2 cDNA-transfected cells with an isotype-matched CD45RA MoAb had no effect. In contrast, the IT2 MoAb only partially blocked binding of the FUN-1 MoAb (Fig 2). This partial blocking effect was not attributable to a low concentration of IT2 MoAb because higher concentrations of the MoAb preparation blocked to a similar extent. Identical results were obtained when the above experiments were performed with the CD86⁺ BJAB B cell line (data not shown). These results indicate that the IT2 MoAb binds a different epitope than that identified by the FUN-1 and BU-63 MoAb, but both epitopes are spatially related.

The FUN-1 and BU-63 MoAbs do not block CTLA-4-Ig binding to B7-2/B70. The ability of CD86 MoAb to block interactions between soluble CTLA-4 and B7-2/B70 was examined using a fusion protein of CTLA-4 and the Fc portion of human IgG. As previously reported, the IT2 MoAb

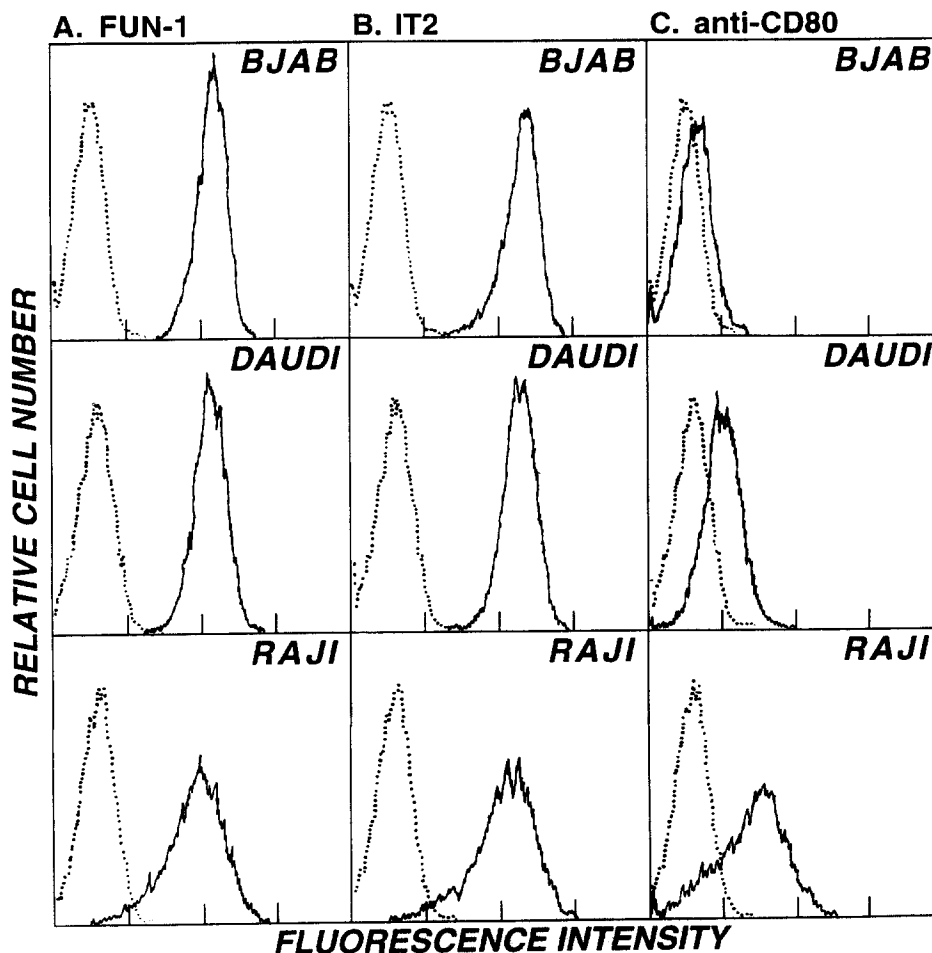


Fig 4. Reactivity of the FUN-1, IT2, and anti-CD80 MoAbs with various B-cell lines. Solid lines indicate the indirect immunofluorescence staining intensity of test MoAbs with FITC-conjugated goat-antimouse IgG antibodies as assessed by flow cytometry analysis, whereas dotted lines indicate background staining with the FITC-conjugated antibody alone.

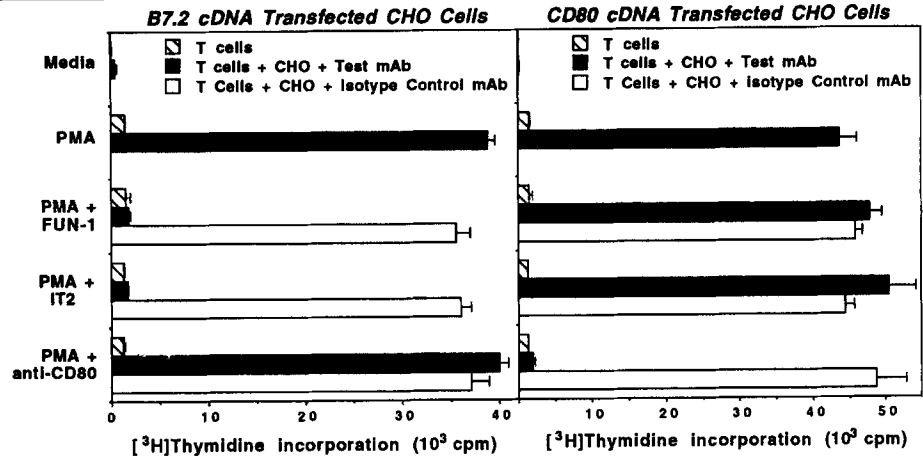
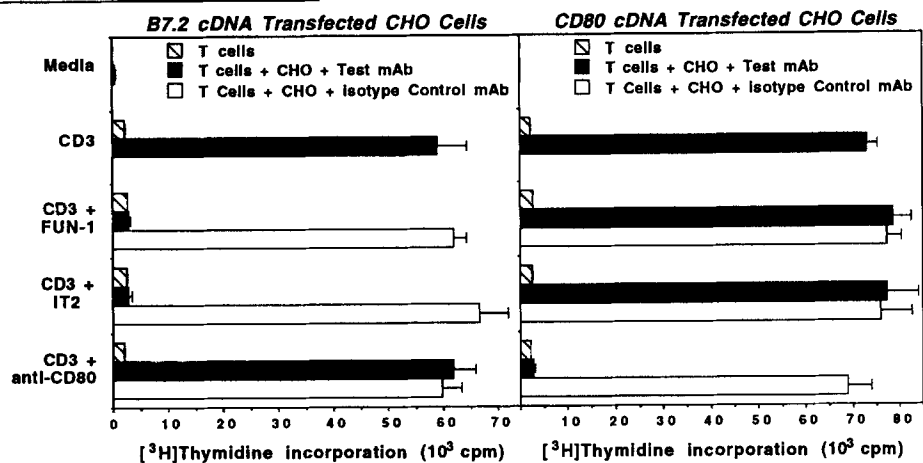
A. PMA Stimulation

Fig 5. The costimulatory function of B7-2/B70 is blocked by the FUN-1 MoAb. T cells were either cultured in medium alone or stimulated with sub-mitogenic concentrations of PMA (A) or anti-CD3 MoAb (B) and cultured alone or with fixed CHO cells expressing either B7-2/B70 or CD80. The FUN-1, IT2, anti-CD80, or isotype-matched control MoAbs were added to the cultures as indicated. Cell proliferation was assessed by [³H]-thymidine incorporation. Data are presented as the mean cpm (\pm SD) obtained from triplicate cultures. Results from one of two experiments with similar findings are shown.

B. anti-CD3 Stimulation

completely blocked the binding of CTLA-4-Ig to B7-2 cDNA-transfected cells (Fig 3). In contrast, the FUN-1 and BU-63 MoAbs not only failed to block CTLA-4-Ig binding, but actually enhanced binding (Fig 3). In similar experiments, binding of the FUN-1 and BU63 MoAbs to BJAB cells also induced increased binding of the CTLA-4-Ig protein, whereas the IT2 MoAb completely blocked binding (data not shown). The increase in CTLA-4-Ig binding observed after FUN-1 and BU-63 MoAb binding was consistently observed in six separate experiments using cDNA-transfected cells and B-cell lines.

The cellular reactivity of CD86 MoAbs and the IT2 MoAb is identical. In general, the reported cellular distribution of CD86 and the B7-2/B70 molecule are identical. Therefore, the side-by-side staining of different cell lines with these MoAbs was examined. Strong staining for the FUN-1, BU-63, and IT2 MoAbs was observed for monocytes, Burkitt's lymphoma-derived B-cell lines (Daudi, Raji), and the B-cell lines RPMI 8866 and BJAB (Fig 4, and data not shown). Intermediate levels of expression were observed on tonsillar B lymphocytes, with buoyant tonsillar B cells expressing the highest levels. Low to negative expression levels were observed on blood lymphocytes, the NALM-6 pre-B cell line, T-cell lines (Jurkat, Molt-4, CEM), and myelomono-

cytic cell lines (HL60, U937). The pattern and level of expression observed for the FUN-1, BU-63, and IT2 MoAbs was identical in all cells and cell lines examined. There was differential expression of CD80 compared with B7-2/B70 expression (Fig 4), suggesting that expression of CD80 and CD86 are independently regulated.

The FUN-1 MoAb blocks T-cell costimulation through B7-2/B70. CHO cells transfected with B7-2 cDNA augment T-cell proliferation and IL-2 secretion induced by suboptimal concentrations of PMA and anti-CD3 MoAb. Therefore, the capacity of the FUN-1 and IT2 MoAbs to block B7-2-mediated costimulation was assessed using purified CD28⁺ T cells incubated with fixed CHO-B7-2 or CHO-CD80 cells. The proliferation of T cells alone, or with low levels of PMA (Fig 5A) or anti-CD3 MoAb (Fig 5B), was minimal. However, culturing the stimulated T cells in the presence of CHO cells expressing B7-2 or CD80 induced substantial and similar levels of proliferation. Both the FUN-1 and IT2 MoAbs significantly blocked (95%, $P < .001$) B7-2/B70-induced T-cell proliferation, but had no effect on CD80-induced proliferation (Fig 5). Culturing the cells in the presence of an anti-CD80 MoAb significantly blocked (95%, $P < .001$) CD80-induced proliferation, but had no effect on B7-2/B70-induced proliferation. In similar experiments,

Table 1. FUN-1 MoAb Blocks IL-2 Production by B7-2-Stimulated T Cells

MoAb	Mitogen	IL-2 Production (U/mL)		
		T Cells	T Cells + CHO-B7-2	T Cells + CHO-CD80
Medium	PMA	<15	650	1,020
FUN-1	PMA	<15	<15	830
IgG ₁ control	PMA	<15	610	950
IT2	PMA	<15	<15	780
IgG _{2b} control	PMA	<15	600	880
Anti-CD80	PMA	<15	680	<15
IgG _{2a} control	PMA	<15	720	850
Medium	Anti-CD3	<15	700	760
FUN-1	Anti-CD3	<15	<15	810
IgG ₁ control	Anti-CD3	<15	620	860
IT2	Anti-CD3	<15	<15	850
IgG _{2b} control	Anti-CD3	<15	600	800
Anti-CD80	Anti-CD3	<15	600	20
IgG _{2a} control	Anti-CD3	<15	650	750

T cells were cultured alone or with fixed CHO cells expressing either B7-2/B70 or CD80 (as indicated) in the presence of sub-mitogenic concentrations of PMA or anti-CD3 MoAb. The FUN-1, IT2, anti-CD80 (C4), or appropriate isotype-matched control MoAbs were added to the cultures as indicated. Data represent the IL-2 levels obtained from pooled duplicate cultures.

costimulation of activated T cells with B7-2/B70 or CD80 induced substantial and similar levels of IL-2 production (Table 1). Both the FUN-1 and IT2 MoAbs significantly blocked (98%, $P < .001$) B7-2/B70-induced IL-2 production, but had no effect on CD80-induced IL-2 production (Table 1). Culturing the cells in the presence of an anti-CD80 MoAb significantly blocked (98%, $P < .001$) CD80-induced IL-2 production, but had no effect on B7-2/B70-induced IL-2 production.

DISCUSSION

The reactivity of the two CD86 MoAbs, FUN-1 and BU-63, with CHO cells expressing B7-2 showed that CD86 and B7-2/B70 are the same molecule (Fig 1). Consistent with this, the biochemical characteristics of CD86 and B7-2/B70 are similar with both molecules being described as 70,000 to 80,000 M_r single chain proteins.^{26-28,30} Further, both CD86 and B7-2/B70 are expressed on resting monocytes, activated B cells, and B-cell lines.²⁶⁻²⁸ CD86 and B7-2/B70 are not expressed by most blood lymphocytes, pre-B cell lines, or most T-cell lines. Conflicting findings on CD86 expression by activated T cells were noted in the Leukocyte Differentiation Antigen Workshop, with only some laboratories finding CD86⁺ T cells.²⁷ Our studies showed that lymphocytes stimulated for 3 days with phytohemagglutinin had modest levels of CD86 expression on ~30% of the cells.²⁷ In studies with the IT2 MoAb, antibody reactivity with activated T cells and some T-cell clones was observed, although the levels of expression were lower than those observed for activated B cells.²⁶ Similar patterns of B7-2/B70 expression have been observed in mice.³⁰ Therefore, all of the serologic and biochemical data available show that B7-2/B70 and CD86 are the same molecule.

Further proof that CD86 is B7-2/B70 was obtained with antibody cross-blocking experiments. The FUN-1 and BU-63 MoAbs recognized a similar epitope on CD86, whereas the IT2 MoAb recognized a close but nonoverlapping epitope (Fig 2). In contrast, binding of the IT2 MoAb blocked CTLA-4-Ig fusion protein binding to B7-2/B70 as reported by others,²⁶ whereas binding of the FUN-1 and BU-63 MoAbs induced a higher level of binding by the chimeric molecule (Fig 3). One possible explanation for this unexpected phenomenon is that binding of the FUN-1 or BU63 MoAb induces a conformational change in CD86 that increases the binding affinity of soluble CTLA-4. Because this was observed for CD86 cDNA-transfected CHO cells, it is unlikely that MoAb binding to CD86 leads to upregulation of a third CTLA-4 ligand. Nonetheless, the FUN-1 and IT2 MoAbs were both able to completely block the proliferation and IL-2 secretion of CD28⁺ T cells induced by CHO-B7-2 cells when cocultured in the presence of submitogenic levels of PMA or anti-CD3 MoAb (Fig 5, Table 1). These effects were specific because these two MoAbs did not affect the proliferation or IL-2 secretion of T cells cultured with CHO-CD80 cells. Although some activated T cells express CD86, these experiments showed that binding of the FUN-1 and IT2 MoAbs to activated T cells did not have a negative or enhancing effect on T-cell proliferation or IL-2 production (Fig 5, Table 1). Therefore, the functional effect of the CD86 MoAb appeared to derive from blocking CTLA-4 or CD28 interactions with CD86 rather than generating signals through CD86 expressed by T cells.

In conclusion, the results of this study show that CD86 MoAbs identify the B7-2/B70 molecule and that these MoAbs can be useful tools for studying the regulation of T-cell proliferation. The unique features of CD86 suggest that it may regulate T-cell activation in a manner different from that of CD80. For example, in contrast to CD80, CD86 is expressed by resting monocytes and appears immediately on the cell surface after B-cell activation. Thus, the differential regulation and expression of the CD80 and CD86 genes at various stages of differentiation and activation may be important for the regulation of immune responses. CD86 is also expressed by B-cell lymphomas and Reed-Sternberg cells of Hodgkin's disease, suggesting that it may play a role in the clinical features of these malignancies.²⁸

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