Comparative Analysis of B7-1 and B7-2 Costimulatory Ligands: Expression and Function

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Summary

Antigen-specific T cell activation requires the engagement of the T cell receptor (TCR) with antigen as well as the engagement of appropriate costimulatory molecules. The most extensively characterized pathway of costimulation has been that involving the interaction of CD28 and CTLA4 on the T cell with B7 (now termed B7-1) on antigen presenting cells. Recently, B7-2 a second costimulatory ligand for CTLA4, was described, demonstrating the potential complexity of costimulatory interactions. This report examines and compares the expression and function of B7-1 and B7-2. Overall these results indicate that (*a*) B7-1 and B7-2 can be expressed by multiple cell types, including B cells, T cells, macrophages, and dendritic cells, all of which are therefore candidate populations for delivering costimulatory signals mediated by these molecules; (*b*) stimulating B cells with either LPS or anti-IgD-dextran induced expression of both B7-1 and B7-2, and peak expression of both costimulatory molecules occurred after 18–42 h of culture. Expression of B7-2 on these B cell populations was significantly higher than expression of B7-1 at all times assayed after stimulation; (*c*) blocking of B7-2 costimulatory activity inhibited TCR-dependent T cell proliferation and cytokine production, without affecting early consequences of TCR signaling such as induction of CD69 or interleukin 2 receptor α (IL-2Rα); and (*d*) expression of B7-1 and of B7-2 can be regulated by a variety of stimuli. Moreover, expression of B7-1 and B7-2 can be independently regulated by the same stimulus, providing an additional complexity in the mechanisms available for regulating costimulation and hence immune response.

Activation of T cells to cytokine production and proliferation requires at least two distinct signals (1, 2). The antigen-specific "first signal" is provided when the TCR interacts with antigen presented in the context of MHC expressed on APCs. The "second signal" or costimulatory signal is provided by a set of receptor–ligand interactions distinct from the TCR interactions. In both mice and humans, CD28 has been identified as one of the molecules on T cells through which costimulatory signals can be delivered (2–7). CTLA4 is a molecule expressed on T cells that may also be involved in APC-dependent T cell activation as suggested by the fact that a soluble CTLA4 fusion protein (CTLA4Ig)[1] inhibits costimulatory signaling (8–10). B7 (now termed B7-1), a ligand for both CD28 and CTLA4, is expressed by APCs and has been shown to be capable of delivering a costimulatory signal (4, 8, 11–14). Recently, we reported the existence of B7-2, a second CTLA4 ligand expressed on murine B cells and defined by the mAb GL1, which provides costimulatory function both in vitro and in vivo for APC-dependent T cell activation (15–17).

B7-1 and B7-2, members of the immunoglobulin supergene family, are encoded by separate genes, and both molecules can provide costimulatory function (6, 12, 15–20). APCs derived from mice in which the gene encoding B7-1 has been inactivated by homologous recombination express B7-2 and are competent to present alloantigens (16). As was previously demonstrated for B7-1 transfectants, B7-2 transfectants are capable of providing costimulatory signals for antigen activated T cells (17). Taken together, these results demonstrate the existence of two distinct costimulatory molecules, each of which can provide costimulatory signals. It is unknown

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; CTLA4Ig, soluble fusion protein of CTLA; FCM, flow cytometry; HPRT, hypoxanthine-guanine phosphoribosyl transferase; PEC, peritoneal exudate macrophages; R, receptor; Tg, transgenic; TRA, Texas Red streptavidin.

at the present time whether the costimulatory signals provided by B7-1 and B7-2 are functionally distinct or overlapping.

In this report, expression of B7-2 and B7-1 was analyzed on freshly explanted as well as activated cultures of murine B cells, T cells, and thioglycollate-induced peritoneal exudate macrophages (PEC). Further, we examined the function of B7-1 and B7-2 molecules on APC-dependent T cell activation. The functional implications of the existence of multiple costimulatory molecules in T cell activation are discussed.

Materials and Methods

Animals. Female DBA/2, BALB/c, B10.A, CBA/J, and CBA/CaH mice were obtained from Frederick Cancer Research Center (Frederick, MD). T cell receptor $V_\beta 8.1$ transgenic ($V_\beta 8.1$ Tg) mice were generously provided by Dr. Mark Greene (University of Pennsylvania, Philadelphia, PA) (21). BALB.Mls^a mice were generated by Berumen et al. (22). All mice were used at 3–12 mo of age.

Preparation and Culture of Cell Suspensions. Single cell suspensions were prepared from spleens, and erythrocytes were lysed as previously described (23). T cell–depleted B cell–enriched APCs were prepared by treating spleen cells with anti-Thy-1.2 + C and cultured in vitro at 2–4 × 10^6 cells/ml. B cells were stimulated in vitro with LPS (Sigma Chemical Co., St. Louis, MO) (15 μg/ml), IL-5 (10^4 U/ml), or anti-IgD-dextran (mouse mAb Hδ^a/1) (0.01 μg/ml) (24) generously provided by Dr. James Mond (Uniformed Services University of the Health Sciences, Bethesda, MD). Viable cultured cells were enriched by isolation on a Lymphocyte Separation Medium (Organon Teknika Corp., Durham, NC) gradient.

PECs were collected by peritoneal lavage 3–5 d after injection of 3% thioglycollate medium intraperitoneal (National Institutes of Health [NIH] Media Unit) (25). This cell preparation was >90% CD11b/MAC-1$^+$ and contained >80% latex bead ingesting macrophages (26). PECs were cultured for 18 h (37°C, humidified CO_2 incubator) on dishes from Falcon Labware (1029; Lincoln Park, NJ) in the presence or absence of either 15 μg/ml LPS or 100 U/ml recombinant IFN-γ (Genetec, South San Francisco, CA) and adherent cells were collected using trypsin-versine solution (Biofluids, Inc., Rockville, MD).

Chinese hamster ovary (CHO) cells transfected with the gene encoding murine B7-1 have previously been described (6).

T cells were enriched by passage over tissue culture plates that had been coated with rabbit anti–mouse-IgG (Organon Teknika Corp.). T cells were stimulated either with 4 μg/ml soluble anti-CD3ε (145-2C11 mAb, hamster Ig, American Type Culture Collection [ATCC] CRL-1975) (27) presented by syngeneic APC or with Mls^a expressing APC. $V_\beta 8.1$ Tg (Mls^a specific) T cells were stimulated with titrated numbers of APC isolated from either CBA/J (H-2^k, Mls^a-positive) or CBA/CaH (H-2^k, Mls^a-negative) mice that had been injected with 200 μl i.p. goat anti-IgD serum 24 h before harvest (28). Alternatively, BALB/c (H-2^d, Mls^a-negative) T cells were stimulated with titrated numbers of APCs isolated from either BALB/c.Mls^a (H-2^d, Mls^a-positive) or BALB/c mice that had been injected with goat anti-IgD serum. Goat anti-IgD serum was the kind gift of Dr. Fred Finkelman (Uniformed Services University of the Health Sciences). APCs were prepared from T-depleted spleen cells and were inactivated by mitomycin C treatment (Sigma Chemical Co.). For proliferation assays, T cells were cocultured in 96-well plates in the presence or absence of inhibitory mAb and titrated numbers of mitomycin C–treated APCs. For PCR analysis and cytokine assays, equal numbers of T cells and APCs were cocultured with the indicated mAb. Cultures intended for cytokine analysis were generated in the presence of 0.1% anti-IL-2 receptor (R) α ascites (7D4 mAb, rat IgM, ATCC CRL-1698) (29) and collected at the indicated times. Cells were harvested at 24 h for examination of activation markers (CD69 and IL-2Rα) and were harvested at 60 or 84 h for proliferation assays. For proliferation assays, cells were labeled with 1 μCi of ^3H during the final 15 h of the culture period.

Reagents. All antibodies were pretitrated and used at saturating amounts for flow cytometric studies. GL1 (anti-B7-2, rat IgG2a) (15) and FD441.8 (anti-LFA-1, rat IgG2b, ATCC TIB 213) (30) mAb were purified from culture supernatants using goat anti–rat IgG agarose columns (Sigma Chemical Co.). RA3-6B2 (31) is a CD45-specific rat IgG2a mAb that reacts with essentially all B lymphocytes, but with very few T lymphocytes (anti-B220). Purified-, FITC-, and biotin-RA3-6B2 were purchased from PharMingen (San Diego, CA) as were purified mAb 1G10 (anti-B7-1, rat IgG2a) (32), biotin-3C7 (anti-IL-2Rα, rat IgG2a) (29), biotin-H1 (anti-CD69, hamster IgG) (33), and FITC-M1/70 (anti-CD11b/MAB-1, rat IgG2b) (34). Biotin-16-10A1 (anti-B7-1, hamster IgG) (13) was the kind gift of Dr. Jeffrey Bluestone (University of Chicago, Chicago, IL). The CTLA4Igs and CD7 (CD7Ig) were produced as previously described (8). FITC goat anti–rat Ig was selected for its minimal staining of activated murine B cells and was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL). FITC mouse anti–human IgG Fcγ was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and used for detecting the binding of CTLA4Ig. 2.4G2 (anti-FcRγII) (35) was generously provided by J. Titus (NIH). Equivalent results were obtained when nonspecific staining by FITC goat anti–rat Ig was assessed either in the presence of affinity-purified normal rat IgG2a (Zymed Laboratories, Inc., South San Francisco, CA) or mAb III/10 (anti–goat Ig, rat IgG2a) (G. Laszlo, unpublished results). FITC anti-Leu 4 and biotin-anti-Leu 4 were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA) and Texas-red–conjugated streptavidin (TRA) was purchased from Life Technologies, Inc., BRL (Gaithersburg, MD).

Flow Cytometry (FCM). FCM analysis was performed as previously described (23) using a modified Dual-Laser FACStar Plus® (Becton Dickinson Immunocytometry Systems). Cells were stained at 4°C and washed in Hank's balanced salt solution containing 1% bovine serum albumin and 0.1% sodium azide. To prevent nonspecific binding of mAb via FcR interactions, 2.4G2 (35) was either added initially, when directly conjugated antibodies were used, or it was added after the FITC-conjugated goat anti–rat Ig or FITC mouse anti–human IgG Fcγ reagents. To prevent nonspecific binding of mAb to PEC, 10 μg of both normal rat IgG and 2.4G2 were added before the addition of the directly conjugated mAb. For two-color FCM analysis, cells were first stained with a saturating concentration of culture supernatant or purified mAb, washed twice, and then incubated with FITC-conjugated antibodies. Cells were then washed, and incubated with biotinylated mAbs. The antibody-labeled cells were washed before incubation with TRA.

In some experiments, one-color profiles of FITC staining were generated by electronically gating on B220$^+$ B cells, CD11b/MAC1$^+$ PEC, or CD4$^+$ or CD8$^+$ T cells. To allow direct comparison of staining intensities, values of median channel logarithmic fluorescence for the gated cell populations were converted to linear units using an empirically derived standard calibration curve for the logarithmic amplifier used. In the results presented in this report, values of background fluorescence have been subtracted after conversion to linear units.

FCM analysis of CTLA4Ig ligands was performed as described previously (15). Briefly, 10^6 cells were preincubated for 30 min at 4°C with titrated amounts of the inhibiting mAb. 0.008 μg of CTLAIg was added and the cells were incubated for an additional 30 min at 4°C in the presence of both the inhibiting mAb and CTLA4Ig. The cells were washed to remove unbound reagents and incubated with FITC mouse anti-human IgG Fcγ to assess CTLA4Ig binding. After converting median channel logarithmic fluorescence to mVolts and subtracting values of background fluorescence, percent inhibition in CTLA4Ig binding was calculated using the following formula: [(uninhibited CTLA4Ig staining − inhibited CTLA4Ig staining)/uninhibited CTLA4Ig staining] × 100%.

Cytokine Assays. Supernatants generated in the presence of 0.1% 7D4 ascites (anti-IL-2Rα mAb), added to prevent IL-2 consumption (36), were analyzed for murine IL-2 and IFN-γ using ELISA kits purchased from Endogen Inc. (Boston, MA) and Genzyme Corp. (Cambridge, MA), respectively.

Polymerase Chain Reaction (PCR). RNA preparation and subsequent cDNA synthesis and amplification by PCR was performed as previously described (37, 38). Oligonucleotide primers specific for IL-2 were prepared by David Winkler (NIH) using an automated DNA synthesizer (DuPont, Newtown, CT) and purified by Sephadex G-50 column chromatography. Primers for IL-2 were: (sense) 5′ TCCACTTCAAGCTCTACAG 3′ and (antisense) 5′ GAGTCAAATCCAGAACATGCC 3′. Primers for hypoxanthine-guanine phosphoribosyl transferase (HPRT) were: (sense) 5′ GATTCAACTTGCGCTCATCTTAGGC 3′ and (antisense) 5′ GTTGGATACAGGCCAGACTTTGTTG 3′ (39).

cDNA samples were subjected to amplification using a programmed thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The optimum number of cycles was determined experimentally and was defined as that number of cycles that could achieve a detectable concentration that was well below saturating conditions. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 2 min. To verify that equal amounts of RNA were added in each PCR reaction within an experiment, mRNA for the "housekeeping gene," HPRT, was also amplified by reverse transcriptase PCR. 20-μl samples were resolved on 1.8% agarose gels in 0.5% Tris-buffered EGTA (TBE), run at 200 V, stained with ethidium bromide, and transferred to HybondTM-N^+ (Amersham Corp., Arlington Heights, IL) for analysis by Southern blotting. The membranes were prehybridized for 1 h in QuikHyb solution (Stratagene, La Jolla, CA) and were then hybridized to ^{32}P-labeled oligonucleotide probes specific for either IL-2 or HPRT. Oligonucleotide probe for IL-2 was: 5′ CTCCCCAGGATGCTCACCTTC 3′ (256–277). Oligonucleotide probe for HPRT was: 5′ GTTGTTGGATATGCCCTTGAC 3′ (39). Membrane-bound cDNA was hybridized overnight at 49°C. After hybridization, blots were washed twice for 15 min in 6× NaCl/NaH_2PO_4/EDTA (SSPE), 0.1% SDS at room temperature and then 5 min at 45°C in 2× SSPE, 0.1% SDS. Autoradiography was performed using Phosphor Screens (Molecular Dynamics Inc., Sunnyvale, CA) and exposed screens were scanned on a Phosphoimager (Molecular Dynamics Inc.). Relative scanning values for IL-2 are presented after normalizing to HPRT values.

Results

The GL1 mAb identifies B7-2, a CTLA4 ligand distinct from B7-1, that can serve as a costimulatory molecule for T cell activation (15–17). Previous studies have shown B7-1 expression by activated B cells (11, 20, 40–42), macrophages (13, 25), dendritic cells (43, 44), and selected populations of activated T cells (45, 46). It was therefore of interest to analyze the cell types expressing B7-2 and the activation conditions required for the expression of these two costimulatory molecules.

Expression of B7-1 and B7-2 by B Cells. B7-2 expression was first examined on unstimulated B cells or on B cells stimulated with a variety of B cell–specific stimuli. Previous reports have failed to detect B7-1 expression on unstimulated B cells and have reported that LPS-stimulation or cross-linking of MHC class II or surface Ig induced low levels of expression of this costimulatory molecule (32, 40, 41). In contrast, a low level of B7-2 expression was detected on freshly explanted B cells and expression was substantially increased by LPS-, anti-IgD-dextran, or IL-5 stimulation (Fig. 1). The $B220^{lo}$ subpopulation of IL-5–stimulated B cells analyzed in the present study (Fig. 1 *D*) has previously been shown to be

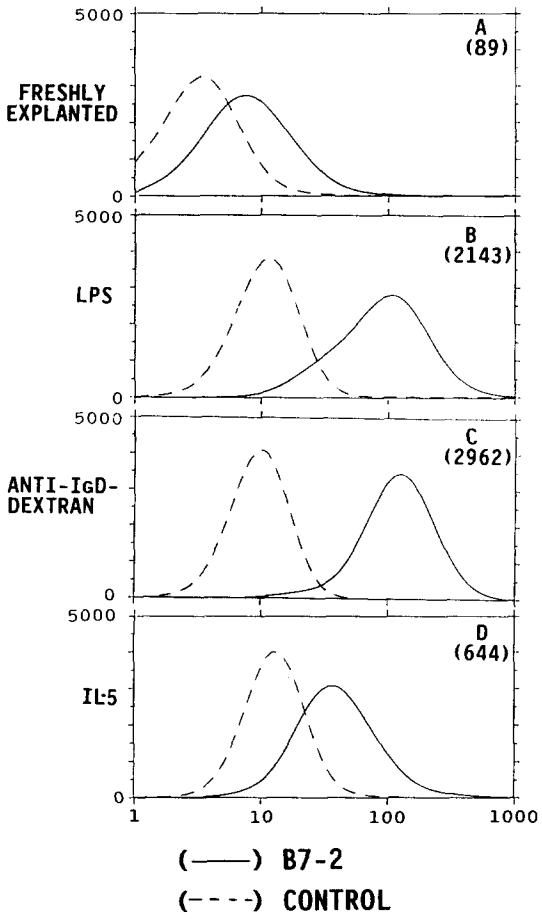

Figure 1. Expression of B7-2 on unstimulated (*A*), LPS- (*B*), anti-IgD-dextran (*C*), or $B220^{lo}$ IL-5–stimulated (*D*) DBA/2 B cells. All stimulated B cells were cultured for 2.5 d. These one-color FCM profiles were obtained by electronically gating on all $B220^+$ B cells (*A–C*) or on $B220^{lo}$ IL-5–stimulated B cells (*D*) and show B7-2 (mAb GL1) expression (———) or control (mAb Leu4) (- - - -) staining on these populations. The x-axis represents log green fluorescence and the y-axis indicates the number of cells detected at a given fluorescence intensity. The numbers in parentheses indicate the mean fluorescence intensity (mV) after the values for control staining were subtracted.

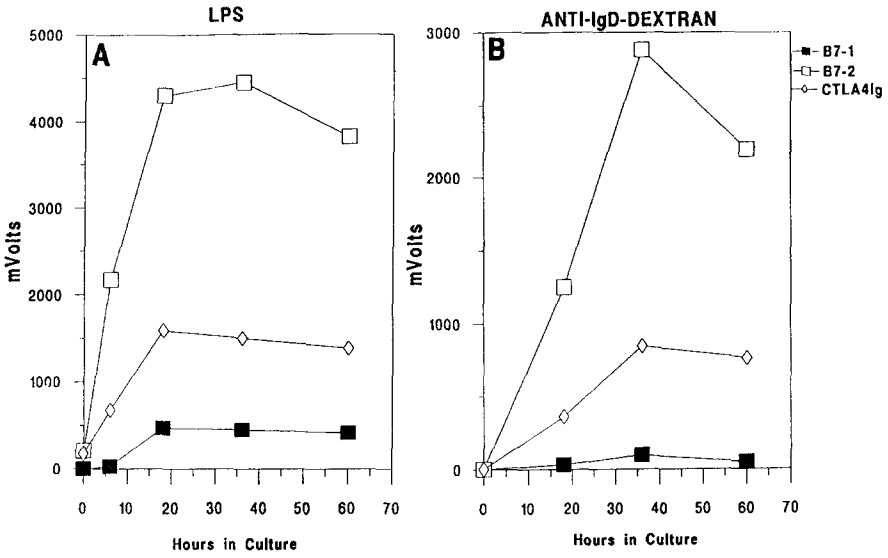

Figure 2. Kinetics of CTLA4Ig binding and expression of the costimulatory molecules, B7-1 and B7-2. Cells were first incubated with unconjugated rat mAbs or fusion proteins and subsequently stained with either goat anti-rat Ig-FITC (for B7-1 and B7-2) or mouse anti-human FcIgG-FITC (for CTLA4Ig); B cells were identified by counter-staining with anti-B220. Values of median channel logarithmic fluorescence (*green*) were generated by electronically gating on $B220^+$ cells and converted to linear units (mV) using an empirically derived formula. Values of background fluorescence obtained by staining with control rat mAb or control fusion protein have been subtracted after conversion to mV and are plotted on the y-axis. *A* depicts the relative staining intensities of B10.A B cells stimulated with LPS for 0, 6, 18, 36, or 60 h and analyzed for the expression of B7-1 (mAb 1G10) (■-■), B7-2 (mAb GL1) (□-□), or CTLA4Ig binding (◇-◇). *B* depicts the relative staining intensities of DBA/2 B cells stimulated with anti-IgD-dextran for 0, 18, 36, or 60 h and analyzed for the expression of B7-1 (mAb 1G10) (■-■), B7-2 (mAb GL1) (□-□), or CTLA4Ig binding (◇-◇).

the proliferating and Ig-secreting subpopulation of B cells present in IL-5–stimulated cultures (47). B cells stimulated with LPS or anti-IgD-dextran also expressed B7-1, although the intensity of B7-1 staining was much lower than B7-2 staining (Fig. 2).

The relative contributions of B7-1 and B7-2 to costimulation of T cells is an issue of substantial importance. It has been proposed that B7-1 and B7-2 are expressed/induced with differing kinetics and thus, may play different roles in initiating and maintaining immune response (17). To test this possibility, we analyzed the kinetics of induction of B7-1 and B7-2 expression and CTLA4Ig binding on B cells activated in vitro by either LPS or anti-IgD-dextran (Fig. 2). Freshly explanted B cells expressed low levels of B7-2 (Fig. 1) and undetectable levels of B7-1 (40–42, and data not shown). 4–6 h after stimulation with either LPS or anti-IgD-dextran, increased expression of B7-2 was routinely detected; expression of B7-1 and CTLA4Ig binding were variably detected at this early time point. It should be noted, however, that B7-2 staining was ~10-fold brighter than B7-1 staining; therefore, the ability to detect B7-2 before B7-1 may simply reflect the limits of detection by FCM analysis. Peak expression of both B7-1 and B7-2 occurred after 18–42 h of culture as did CTLA4Ig binding. By 60 h of stimulation B7-1, B7-2, and CTLA4Ig binding decreased in both stimulated B cell populations. B7-1 and B7-2 expression was not analyzed beyond 60 h of culture since cell viability and recovery were substantially reduced. Thus, for these B cell stimuli, B7-2 was detected earlier and at higher levels than B7-1, but there was no consistent difference in the kinetics of maximum expression of B7-1 and B7-2.

Although B cells stimulated with LPS or anti-IgD-dextran expressed both B7-1 and B7-2, the intensity of B7-1 staining was much lower than B7-2 staining, even when analyzed by indirect immunofluorescence with isotype-matched anti-B7-1 and anti-B7-2 mAb. Moreover, CTLA4Ig binding to these B cells was essentially completely inhibited by GL1 mAb (specific for B7-2), with little or no inhibition observed with either of two B7-1–specific mAb available (15). The failure to observe significant inhibition by B7-1 mAb under these

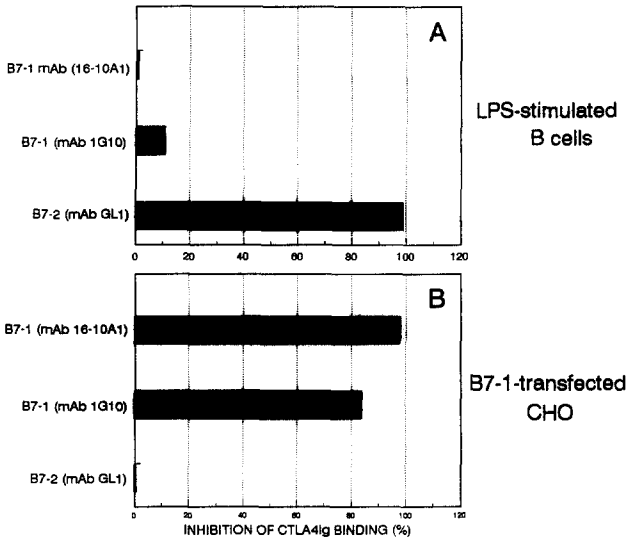

Figure 3. Inhibition of CTLA4Ig binding to 60-h LPS-activated B cells (*A*) or B7-1–transfected CHO cells (*B*). Cells were preincubated with 3 μg of either of two B7-1–specific mAb (16-10A1 or 1G10) or anti-B7-2 mAb (GL1), followed by incubation with CTLA4Ig or control CD7Ig. Cells were washed and incubated with FITC mouse anti-human IgG Fcγ to detect bound CTLA4Ig. Percent inhibition of CTLA4Ig binding was calculated as described in Materials and Methods.

conditions was not due to the inability of these mAb to compete with CTLA4Ig for binding since either of two B7-1–specific mAb (16-10A1 or 1G10) efficiently blocked binding of CTLA4Ig to B7-1–transfected (B7-2-negative) CHO cells (Fig. 3). Taken together these results indicate that activated B cells express quantitatively higher amounts of B7-2 than B7-1.

An early but transient increase in B7-2 expression (beginning 4–6 h after culture) also occurred on B cells that were cultured in vitro in the absence of defined B cell stimuli. After overnight culture B7-2 staining returned nearly to the level originally detected on freshly explanted B cells. Both this transient increase in B7-2 expression as well as the increase induced by LPS-stimulation were completely inhibited by cycloheximide (data not shown).

Expression of B7-1 and B7-2 by Activated Macrophages. We next examined macrophages for the expression of B7-1 and B7-2 costimulatory molecules. Freshly explanted MAC-1$^+$ thioglycollate-induced macrophages (PEC) (>80% latex ingesting) expressed both B7-1 and B7-2 (Table 1). Expression of both B7-1 and B7-2 was modestly increased on PEC cultured in vitro for 18 h in medium alone. Further, LPS stimulation of PEC induced substantial increases in the expression of both molecules. In contrast to the increased expression of both B7-1 and B7-2 on LPS-stimulated PEC, IFN-γ stimulation resulted in differential effects on B7-1 and B7-2 expression. Relative to expression levels observed on freshly explanted or control cultured PEC, B7-2 expression was increased by in vitro stimulation with IFN-γ, whereas B7-1 expression was decreased by the same IFN-γ stimulation. Due to technical constraints, B7-1 and B7-2 expression on PEC was analyzed using directly conjugated antibodies; therefore, differences in fluorescence intensities observed using these two mAbs may reflect differences in efficiency of biotinylation rather than differences in the expression of B7-1 and B7-2. Thus, expression of the costimulatory molecules of B7-1 and B7-2, was influenced by activation stimuli and, strikingly, these two costimulatory molecules are subject to independent regulation.

Table 1. *Expression of B7-1 and B7-2 Molecules on Thioglycollate-induced Peritoneal Macrophages Stimulated with either LPS or IFN-γ*

PEC cell culture*	B7-1‡ mV‖	B7-1‡ Percent change¶	B7-2§ mV	B7-2§ Percent change
Uncultured	545	–	173	–
Cultured/ medium	629	+15	369	+113
Cultured/ LPS	725	+33	725	+319
Cultured/ IFN-γ	425	−22	611	+253

* PEC were incubated for 18 h with media, LPS (15 μg/ml) or IFN-γ (100 U/ml).

‡ B7-1 expression was detected using biotin-conjugated 16-10A1 mAb. Differences in fluorescence intensity between B7-1– and B7-2–specific staining may reflect differences in the amount of biotin conjugated rather than actual differences in the number of B7-1 and B7-2 molecules expressed.

§ B7-2 expression was detected using biotin-conjugated GL1 mAb.

‖ One-color profiles of TRA staining with either biotin anti-B7-1 or biotin anti-B7-2 were generated by electronically gating on MAC1$^+$ PEC. The numbers in this experiment indicate the mean fluorescence intensity (mV) for MAC1$^+$ cells after the values for control staining were subtracted.

¶ Percent change in fluorescence was calculated using the following formula: [uncultured (mV) − cultured (mV)]/uncultured (mV) × 100%.

Expression of B7-2 by T Cells. B7-2 expression was next investigated on T cells. As can be seen in Fig. 4, freshly explanted spleen T cells expressed a low, but detectable level of B7-2. After stimulation with soluble anti-CD3ϵ for 18 h, both CD4$^+$ and CD8$^+$ T cells showed increased expression of B7-2. Similar results were obtained when T cells were stimulated with Concanavalin A (data not shown). Kinetics of peak expression of B7-2 by activated T cells were similar to those observed for activated B cells (data not shown). In contrast to the low level expression of B7-2 on freshly explanted peripheral T cells, B7-2 was undetectable on freshly explanted thymic T cells (data not shown).

Participation of B7-1 and B7-2 Molecules in T Cell Proliferation to Mls^a Stimulation. Since both B7-1 and B7-2 molecules have been shown to provide costimulatory signals for T cell activation, experiments were performed to analyze their individual and joint contributions to costimulatory function. In these experiments, BALB/c (Mls^a-reactive) T cells were stimulated with BALB.Mls^a-positive APCs in the presence or absence of inhibitory mAb specific for B7-1 or B7-2 or with CTLA4Ig (Fig. 5). The proliferative response of BALB/c

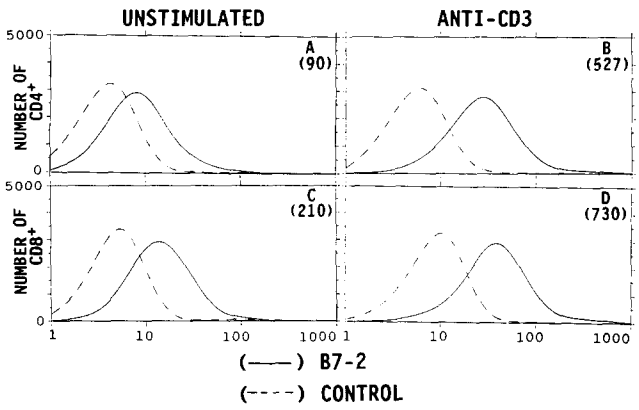

Figure 4. Expression of B7-2 on freshly explanted (*A* and *C*) or anti-CD3ϵ–stimulated (*B* and *D*) BALB/c T cells. T cells were cultured for 1.5 d in the presence of soluble anti-CD3ϵ and APCs. These one-color FCM profiles were obtained by electronically gating on CD4$^+$ T cells (*A* and *B*) or on CD8$^+$ T cells (*C* and *D*) and show B7-2 (mAb GL1) expression (——) or control (mAb Leu4) (- - - -) staining on these populations. The numbers in parentheses indicate the mean fluorescence intensity (mV) after the values for control staining were subtracted.

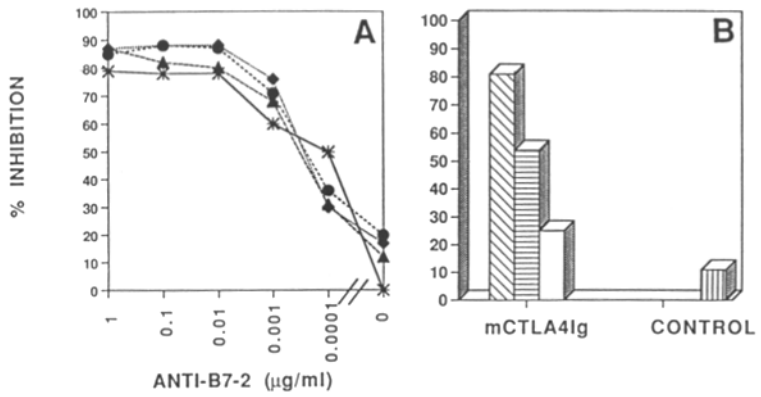

Figure 5. Inhibition of the proliferative response of BALB/c T cells to Mls^a by B7-1– and B7-2–specific mAb. *A* depicts the percent inhibition of T cell proliferation observed when 2×10^5 BALB/c T cells were stimulated with 10^5 mitomycin C–treated APC and the indicated concentrations of test mAb: anti-B7-2 (GL1 mAb) (*–*), anti-B7-2 mAb + 10 μg/ml anti-B7-1 (1G10 mAb) (◆–◆), anti-B7-2 mAb + 5 μg/ml anti-B7-1 mAb (●–●), anti-B7-2 mAb + 1 μg/ml anti-B7-1 mAb (▲–▲). *B* depicts the percent inhibition of T cell proliferation by titrated amounts of CTLA4Ig: 5 μg/ml (▨), 0.5 μg/ml (▤), or 0.05 μg/ml (□). Control isotype matched mAb (mAb III/10) was used at 10 μg/ml (▥); 5 μg/ml control CD7Ig generated −10% inhibition (data not shown). Cultures were pulsed at 72 h and harvested at 84 h. T cells cultured without APC generated 147 cpm. T cells cultured with BALB/c.Mls^a APC and no inhibitory mAb generated 132,217 cpm and T cells cultured with syngeneic BALB/c APC generated 175 cpm. Percent inhibition was calculated using the following formula: [uninhibited (cpm) − inhibited (cpm)]/uninhibited (cpm) × 100%.

T cells to Mls^a superantigen was only minimally affected by anti-B7-1 mAb alone, whereas blocking of B7-2 resulted in profound inhibition, comparable with that observed with CTLA4Ig. Further, adding B7-1 and B7-2 mAb produced little to no increase in inhibition over what was seen when B7-2 was added alone. This result suggests that B7-2 is the major costimulatory molecule involved in the activation of T cells by Mls^a presented by this anti-IgD–activated APC population.

Inhibition of B7-1 and B7-2 Costimulatory Pathways Blocks T Cell Proliferation and Cytokine Production but Not the Induction of CD69 and IL-2Rα. To further define the contribution(s) of B7-1 and B7-2 costimulatory molecules to antigen-specific T cell activation, Vβ8.1 Tg (Mls^a-reactive) T cells were stimulated with Mls^a-expressing CBA/J APCs in the presence or absence of inhibitory mAb specific for B7-2 or LFA-1. Parallel cultures were analyzed for proliferation, cytokine production, or CD69, and IL-2Rα expression. T cell proliferation was substantially inhibited by the addition of anti-B7-2 (GL1 mAb) (63% inhibition) and almost completely inhibited by the addition of anti-LFA-1 (FD441.8 mAb) (99% inhibition) (Table 2). Supernatants were generated in parallel cultures and analyzed by ELISA for secreted cytokines and by PCR for mRNA expression of the T cell cytokines IL-2 and IFN-γ. Anti-B7-2 mAb substantially inhibited IL-2 and IFN-γ generation whereas anti-LFA-1 mAb almost completely inhibited the production of these cytokines (Fig. 6). Induction of IL-2 and IFN-γ responses was investigated further by PCR analysis. The induction of IL-2 mRNA (Fig. 7) and IFN-γ mRNA (data not shown) was inhibited in a pattern similar to that observed for assays of secreted cytokine. This result suggests that the inhibition of proliferation that occurs in the presence of mAb to B7-2 or LFA-1 may be due, at least in part, to reduced IL-2 production. In contrast to the inhibitory effects of GL1 mAb on T cell proliferation and cytokine production, blocking the costimulatory signal

Table 2. *Comparative Effects of mAb Specific for B7-2 and LFA-1 on Proliferation and Expression of CD69 and IL-2Rα Antigens by Vβ8.1 Tg T Cells in Response to Mls^a*

Cell culture*				Response		
Vβ8.1 T	APC	Mls^a	Inhibitor‡	Proliferation§	Percent CD69‖	Percent IL-2Rα
+	CBA/J	+	control IgG2a	193,527	29	32
+	CBA/J	+	B7-2	71,899	30	29
+	CBA/J	+	LFA-1	2,158	5	5
+	CBA/CaH	−	−	831	3	0

* T cells were prepared by passage over plates coated with rabbit-anti-mouse Ig. APCs were prepared by T depletion of spleen cells harvested from mice that had been injected with goat anti-IgD serum and were inactivated by treatment with mitomycin C.
‡ All mAbs were used at a final concentration of 1 μg/ml.
§ Cultures were pulsed during the last 15 h of a 60-h assay with 1 μCi ^{3}H. T cells cultured without any APCs generated 1,084 cpm.
‖ Cells were cultured for 18 h and analyzed by two-color FCM analysis for CD4 expression and either CD69 or IL-2Rα expression. The results shown here are presented as the percent of CD4$^+$ T cells that are CD69$^+$ or IL-2Rα$^+$.

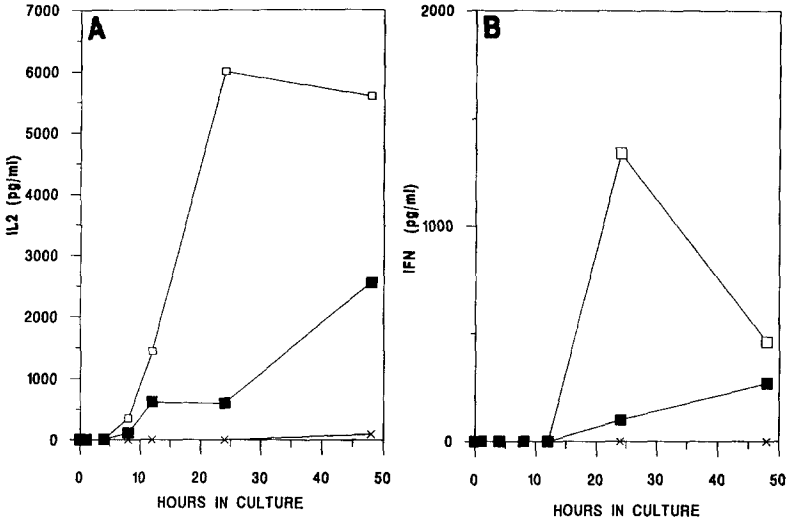

Figure 6. Effect of B7-2–specific mAb and LFA-1–specific mAb on IL-2 and IFN-γ induction by Vβ8.1 Tg T cells in response to Mls^a. Supernatants were generated in the presence of 0.1% 7D4 ascites and harvested at the indicated times. *A* shows IL-2 production (pg/ml) for Vβ8.1 Tg T cells stimulated with Mls^a-expressing CBA/J APC and cultured in the presence of control rat IgG2a mAb (III/10) (□–□), anti-B7-2 mAb (GL1) (■–■), or anti-LFA-1 mAb (F44.1) (X–X). *B* shows IFN-γ production (pg/ml) for Vβ8.1 Tg T cells stimulated with Mls^a-expressing CBA/J APC and cultured in the presence of control rat IgG2a mAb (□–□), anti-B7-2 mAb (■–■), or anti-LFA-1 mAb (X–X). Vβ8.1 Tg T cells cultured without APC or stimulated with Mls^a-negative CBA/Ca APC produced undetectable amounts of both IL-2 and IFN-γ. All mAb were used at a final concentration of 1 μg/ml that was previously shown to provide maximum inhibitory effect.

provided by B7-2 did not significantly inhibit the percent of CD4$^+$ T cells induced to express CD69 or IL-2Rα (Table 2) nor the fluorescence intensity of the induced activation antigens (data not shown). Anti-LFA-1 mAb completely inhibited the induction of these activation antigens. Blocking with either B7-1 mAb alone or with a mixture of both B7-1- and B7-2–specific mAb also failed to inhibit the induction of these activation antigens (data not shown). Similar results were obtained when T cells were stimulated by soluble anti-CD3ε under APC-dependent conditions (data not shown).

Discussion

The present study investigated the regulation of expression of B7-1 and B7-2 costimulatory molecules and analyzed their contribution to costimulatory function under selected conditions. Low levels of B7-2 were expressed by freshly explanted B and T cells and expression was substantially increased by a variety of T and B cell–specific stimuli. Analysis of B7-1 and B7-2 induction on B cells stimulated with either LPS or anti-IgD-dextran revealed a similar kinetics of peak expression for both molecules that occurred after 18–42 h of culture. Thioglycollate-induced PEC expressed both B7-1 and B7-2 molecules, and LPS stimulation resulted in increased expression of both of these costimulatory molecules. In contrast, B7-1 and B7-2 were differentially regulated in vitro in response to IFN-γ; B7-2 expression was increased while B7-1 expression was decreased on these PEC. In addition, B7-2 as well as B7-1 are expressed on dendritic cell populations (reference 47a and R. Steinman, personal communication). Thus, B7-1 and B7-2 are expressed on a broad spectrum of lymphocytes, including B cells, T cells, macrophages, and dendritic cells.

The contribution of costimulatory signals to in vitro T cell activation was analyzed by assessing the effects of B7-1, B7-2, and LFA-1 on T cell activation to Mls^a. These results

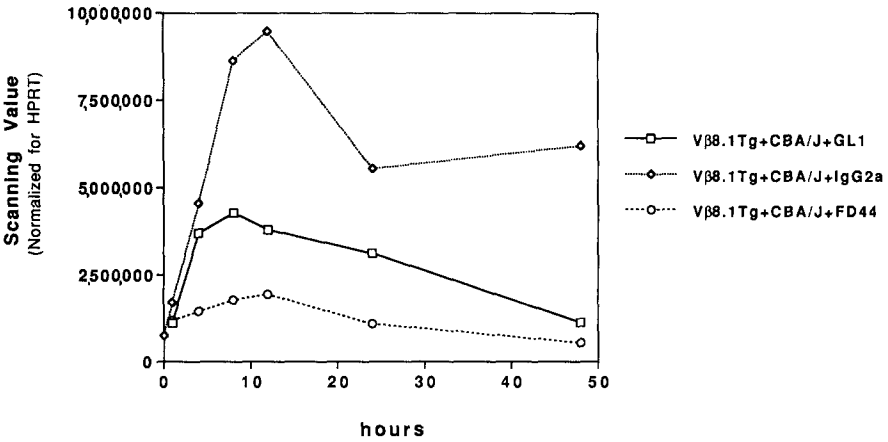

Figure 7. PCR analysis of the effect of B7-2–specific mAb and LFA-1–specific mAb on IL-2 mRNA generation by Vβ8.1 Tg T cells in response to Mls^a-expressing APCs. Cultures were harvested at the indicated times, mRNA was isolated, and the resulting cDNA was amplified in the presence of IL-2–specific oligonucleotide primers (24 cycles) or HPRT-specific oligonucleotide primers. PCR-amplified products were resolved on agarose gels, transferred to nylon membrane, and subjected to Southern analysis with either ^{32}P-labeled IL-2–specific or HPRT-specific oligonucleotide probes. Autoradiography was performed using Phosphor screens and analyzed using a Phosphoimager (Molecular Dynamics, Inc.). The scanning values for IL-2 were normalized to HPRT values and are plotted on the y-axis. These results depict relative IL-2 mRNA expression on Vβ8.1 Tg T cells stimulated with Mls^a-expressing CBA/J APC and cultured in the presence of control rat IgG2a mAb (III/10) (◇–◇), anti-B7-2 mAb (GL1) (□–□), or anti-LFA-1 mAb (F44.1) (O–O). Vβ8.1 Tg T cells cultured without APC or stimulated with Mls^a-negative CBA/Ca APC expressed undetectable IL-2. All mAb were used at a final concentration of 1 μg/ml that was previously shown to provide maximum inhibitory effect.

indicated that T cell proliferation to Mls^a expressed by anti-IgD–activated B cell–enriched splenocytes was highly dependent on costimulatory function of B7-2 with little evidence for a role of B7-1. Further, inhibition of either B7-2 or LFA-1 interactions inhibited not only antigen-induced T cell proliferation but also IL-2 and IFN-γ mRNA and protein production. Addition of B7-2 mAb failed to inhibit induction of early T cell activation antigens CD69 and IL-2Rα whereas LFA-1–specific mAb completely inhibited the induction of these antigens.

The potential for signaling through the B7:CD28:CTLA4 costimulatory pathway is more complex than had previously been appreciated. Thus, T cells can express at least two receptors for costimulation, CD28 and CTLA4 (2, 7, 48, 49), and APC can express at least two and perhaps more (15, 19, 50–53) molecules capable of interacting with one or both of these receptors on the T cell. It is not yet clear whether different costimulatory molecules such as B7-1 and B7-2 mediate distinct function in the course of immune responses. It has been proposed that B7-1 and B7-2 are expressed with different kinetics and thus may play different roles in initiating or maintaining immune responses (17). For this reason, we examined the kinetics of induction and the cellular distribution of B7-1 and B7-2 after stimulation. These experiments demonstrated that B7-2 was detected earlier than B7-1 on LPS- or anti-IgD-dextran–activated B cells and that B7-2 may be expressed at higher levels than B7-1; however, the kinetics of peak expression of these two costimulatory molecules was, in fact, not different. Further, since these experiments were performed using isotype matched mAb (rat IgG2a) and goat anti–rat Ig FITC, the 10-fold difference in fluorescence intensity of B7-1 and B7-2 staining may reflect the relative difference in the numbers of B7-1 and B7-2 molecules expressed by these B cell populations. Consistent with this interpretation, B7-2–specific mAb completely inhibited CTLA4Ig binding by all B cell populations tested, with little or no inhibition observed with either of two B7-1–specific mAb available The low level of staining observed on B cells using B7-1–specific mAb and the failure of B7-1–specific mAb to inhibit CTLA4Ig binding to B cells were not due to lower affinities of these mAb since either of two B7-1–specific mAb (16-10A1 or 1G10) effectively inhibited the binding CTLA4Ig to B7-1–transfected CHO cells. Thus, quantitative differences in the amount of B7-1 and B7-2 expressed on activated B cells may profoundly influence their contribution to costimulatory function(s). The possibility remains, however, that distinct stimuli, including physiologically relevant signals, may result in kinetics and relative expression of B7-1 and B7-2 that differ from the patterns described here.

It is of considerable interest to identify the biologically relevant signals, mediated either by soluble cytokines or by cell contact, that can regulate B7-1 and B7-2 expression. Nabavi et al. (32) recently reported that induction of B7 (B7-1) could be mediated by signaling through MHC class II molecules on the APC. In the present report we demonstrated that cytokines such as IL-5 and IFN-γ, were able to regulate the expression of costimulatory molecules. The observation that the expression of B7-1 and B7-2 on PEC is differentially regulated by IFN-γ stimulation further suggests that the costimulatory function mediated by these two molecules can be regulated differentially as well. Recently Larsen et al. (47a) examined B7-1 and B7-2 expression and costimulatory function of splenic dendritic cells cultured with either GM-CSF or IFN-γ and showed that GM-CSF–stimulation increased expression of both B7-1 and B7-2 molecules whereas IFN-γ–stimulation resulted in increased expression of B7-2 and decreased expression of B7-1. Further analysis of the relative contributions of cell contact and cytokine-mediated signals to the regulation of B7-1 and B7-2 expression and costimulatory function will be critical to an overall understanding of the roles played by these molecules.

At the current time, it is not known whether B7-1 and B7-2 mediate distinct or overlapping costimulatory functions. Several lines of experimental evidence suggest that the costimulatory signals provided by B7-1 and B7-2 may be, at least in part, redundant. It has been demonstrated that transfected cells expressing high amounts of either B7-1 or B7-2 can provide costimulatory function (6, 12, 17). Similarly, APCs derived from mice in which the B7-1 gene was inactivated by homologous recombination express B7-2 and not B7-1, and are competent APCs (16). Thus, either B7-1 or B7-2 is sufficient, in the absence of the other, to provide some costimulatory function. In systems that have examined the costimulatory function of normal human and murine APCs, presumably capable of expressing both B7-1 and B7-2, the contribution of each is not so clear. For example, Razi-Wolf et al. (13) have shown that T cell proliferation to Concanavalin A or alloantigen stimulation was dependent on expression of B7-1 as evidenced by essentially complete inhibition with anti-B7-1 mAb. In contrast, our experiments demonstrated that T cell proliferation to Mls^a expressing APC or to soluble anti-CD3 (15) presented by activated B cell populations was highly dependent upon B7-2 with little if any dependence on B7-1. The explanation for these results may involve the stimuli studied as well as the APC populations used.

The contributions of B7-1, B7-2, and LFA-1 to T cell activation were further characterized by analyzing in parallel several parameters of T cell activation. Blocking of the B7-2 costimulatory pathway on APC capable of expressing both B7-1 and B7-2 resulted in a profound reduction in T cell proliferation and in IL-2 and IFN-γ production but had no effect on the induction of the T cell activation antigens CD69 and IL-2Rα. In contrast, anti-LFA-1 mAb inhibited T cell proliferation, cytokine production, and induction of activation antigens. These results are consistent with a model in which blocking LFA-1/ligand interactions prevents TCR-mediated signaling events that are necessary for all of the measured components of T cell activation, whereas inhibition of B7-2 allowed TCR-mediated signaling events to occur (as evidenced by the induction of CD69 and IL-2Rα expression) but inhibited the distinct costimulatory signal(s) that are necessary for optimal cytokine production and cytokine-dependent proliferation. This interpretation is consistent with the previous demonstration that, in the presence of TCR signals, CD28 signaling plays a critical role in T cell cytokine production and mRNA stabilization (54–56).

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