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B7-1 and B7-2 Do Not Deliver Identical Costimulatory Signals, Since B7-2 but Not B7-1 Preferentially Costimulates the Initial Production of IL-4

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Summary

The functional necessity for two CD28 counterreceptors (B7-1 and B7-2) is presently unknown. B7-1 and B7-2 equivalently costimulate IL-2 and interferon-γ (IFNγ) production and IL-2 receptor α and γ chain expression. B7-2 induces significantly more IL-4 production than B7-1, with the greatest difference seen in naive T cells. Repetitive costimulation of $CD4^+CD45RA^+$ T cells with B7-2 results in moderate levels of both IL-4 and IL-2, whereas repetitive costimulation with B7-1 results in high levels of IL-2 and low levels of IL-4. Therefore, B7-1 and B7-2 costimulation mediate distinct outcomes, since B7-2 provides an initial signal to induce naive T cells to become IL-4 producers, thereby directing the immune response more towards Th0/Th2, whereas B7-1 is a more neutral differentiative signal.

Introduction

The B7 family of CD28/CTLA4 counterreceptors is composed of at least two members of the immunoglobulin supergene family, B7-1 (CD80) (Freedman et al., 1987; Freeman et al., 1989) and B7-2 (CD86) (Freeman et al., 1993b; Azuma et al., 1993), which demonstrate only modest amino acid conservation. In spite of their structural differences, both B7-1 and B7-2 have been shown to signal through CD28 and equivalently costimulate T cell proliferation and interleukin-2 (IL-2) production (Freeman et al., 1993b). However, B7-1 and B7-2 are differentially expressed on populations of antigen-presenting cells (APCs). Monocytes constitutively express B7-2 (Azuma et al., 1993; Nozawa et al., 1993), whereas B7-1 is induced after culture with interferon-γ (IFNγ; Freedman et al., 1991). On B cells, B7-2 is rapidly expressed following activation, whereas B7-1 expression appears significantly later (Boussiotis et al., 1993b; Freeman et al., 1993b; Hathcock et al., 1994; Lenschow et al., 1994). B7-2 is expressed at low levels on unstimulated dendritic cells and expression of both B7-1 and B7-2 is up-regulated by granulocyte–macrophage colony-stimulating factor (GM-CSF) (Hart et al., 1993; Caux et al., 1994; Hathcock et al., 1994; Larse al., 1994)

Increasing evidence suggests that CD28-mediated stimulatory signals are important at several stages cell differentiation. To initiate their first proliferative cy naive T cells require T cell receptor (TCR) signaling a second signal, which can be provided by CD28, resul in secretion of IL-2 (Ehlers and Smith, 1991; Sagest et al., 1993; McKnight et al., 1994). Following additi exposures to TCR and CD28-mediated signaling, IL-2 creting T cells differentiate into Th0 T cells capabl secreting multiple cytokines. The evolution of an imm response is regulated by specific cytokines present in microenvironment (Mosmann and Coffman, 1989). Th cytokines direct $CD4^+$ T cells to differentiate into sub capable of secreting distinct patterns of lymphokines der and Paul, 1994). Increasing evidence demonstr that the monokine IL-12 (Kubin et al., 1994; Murphy e 1994) and, to a lesser extent IFNγ, direct $CD4^+$ T to differentiate into Th1 cells, which secrete lymphoki (IL-2, IFNγ, tumor necrosis factor-β [TNFβ]) critical fo generation of a cellular immune response and, in m for immunoglobulin G2a (IgG2a) antibody productio contrast, IL-4 priming is necessary to direct $CD4^+$ T to differentiate into Th2 cells, which secrete IL-4, IL-5, IL-10, which, in mice, are critical for IgG1 and IgE antib production and immunity against helminthic para (Swain et al., 1990; Hsieh et al., 1992; Seder et al., 19 IL-4 and IL-10 also inhibit macrophage activation and gen presentation, thereby down-regulating the cellula mune response (Fiorentino et al., 1991; Hsieh et al., 1 Ding et al., 1993; Powrie et al., 1993). When both IL-4 IL-12 are added to in vitro cultures, IL-4 dominates IL-12, driving naive $CD4^+$ T cells toward Th2 cells (Hsie al., 1993); however, in vivo, administration of IL-12 inh Th2 development (Oswald et al., 1994). Taken toge these observations suggest that signaling via both C and specific cytokine receptors is critical to direct the larization of T cells toward $CD4^+$ Th subsets.

Several recent murine studies demonstrate tha CD28 pathway is critical for the development of producing T cells. Using an in vivo model of helmi parasitic infection, Lu et al. (1994) demonstrated CTLA4-Ig blocked T cell IL-4 production and thereby i ited both B cell activation and IgE secretion. Leishm infection of BALB/c mice results in fatal disease as ated with the generation of Th2 cells, which inhibi differentiation of protective Th1 cells. In Leishm infected mice, early short-term blockade with CTL inhibits IL-4 production and protects against letha ease, suggesting that the priming of Th2 is deper upon the CD28 pathway (Corry et al., 1994). Similarly transgenic model of autoantibody production (of prin IL-4-dependent IgG1 isotype), early short-term bloc with CTLA4-Ig inhibited autoantibody production (et al., 1994). These studies provide indirect, but in

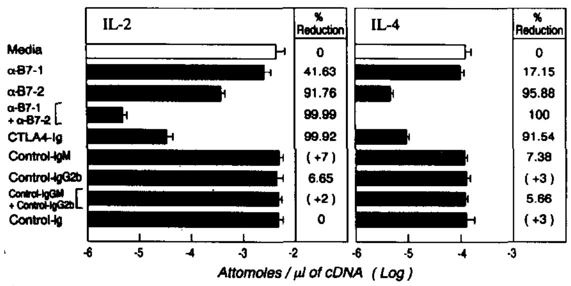


Figure 1. Inhibition of IL-2 and IL-4 mRNA Synthesis in an MLR by CTLA4-Ig or anti-B7-1 or anti-B7-2

MLR of fully mismatched allogeneic donors and recipients were undertaken in the presence or absence of 10 µg/ml CTLA4-Ig, anti-B7-1 MAb (clone 133), and/or anti-B7-2 MAb (clone IT2.2) or isotype-matched control antibodies. IL-2 and IL-4 mRNA levels were determined by quantitative RT–PCR and are expressed as attomoles of specific mRNA per µl of cDNA. The percent reduction in mRNA levels is indicated to the right. Error bars indicate SD. Results are the average of three experiments that all had similar results. Similar results were also observed using either of two different anti-B7-1 MAbs and three different anti-B7-2 MAbs.

evidence that the CD28 pathway is involved in IL-4 production.

These studies, in conjunction with differences in the structure, expression, and ligand binding of B7-1 and B7-2, suggested the possibility that B7-1 and B7-2 might deliver distinct signals. The present studies were undertaken to address these issues directly using B7-1 and B7-2 transfectants. Here, we demonstrate that both B7-1 and B7-2 are equivalent in their ability to induce IL-2 and IFNγ production and interleukin-2 receptor (IL-2R) expression. More importantly, we demonstrate that B7-2 more effectively costimulates the production of IL-4 and appears to be capable of directing the differentiation of T cells towards a more Th2-like phenotype.

Results

Blockade of Costimulation Mediated by B7-2, but Not B7-1, Greatly Reduces IL-4 mRNA Synthesis During a Primary Allogeneic Mixed Lymphocyte Reaction

Previous work has shown that anti-B7-2 monoclonal antibody (MAb) blocks proliferation of a mixed leukocyte reaction (MLR) more effectively than does anti-B7-1 MAb (Azuma et al., 1993). We sought to determine the relative contributions of B7-1 and B7-2 for IL-2 and IL-4 production in a primary MLR. Both IL-2 and IL-4 mRNAs were induced in one-way MLR of fully mismatched allogeneic donors and recipients (Figure 1, media alone). IL-2 and IL-4 mRNA levels were quantitated by competitive polymerase chain reaction (PCR) using PCR MIMICs as described in Experimental Procedures. The addition of anti-B7-1 MAb reduced the level of IL-2 mRNA by 42%, and this was statistically significant ($p < 0.05$) compared with the isotype-matched control MAb. Anti-B7-1 MAb did not significantly reduce IL-4 mRNA levels compared with an isotype-matched control (17%; $p = 0.205$). In contrast, blockade of the MLR with anti-B7-2 MAb greatly reduced the levels of both IL-2 mRNA (91.76%; $p < 0.005$) and IL-4 mRNA (95.88%; $p < 0.005$). These results confirm that B7-2 is the major costimulatory molecule in an MLR. The combination of anti-B7-1 and anti-B7-2 MAbs reduced IL-2 mRNA levels by log 3 (99.99%) and IL-4 mRNA to undetectable levels. The combination of anti-B7-1 and anti-B7-2 MAbs was consistently more effective than CTLA4-Ig at reducing both IL-2 ($p < 0.032$) and IL-4 mRNA levels ($p < 0.05$). The more effective blockade by anti-B7-1 plus anti-B7-2 MAbs may be explained by the rapid on–off rate of CTLA4-Ig binding to B7-2 (Linsley et al., 1994).

Differential Induction of Cytokines in CD4+ T Cells by B7-1 and B7-2 Costimulation

The inhibition of IL-4 mRNA synthesis by anti-B7-2 MAb might be a direct consequence of blocking a B7-2-mediated signal for IL-4 production or a secondary consequence of blocking IL-2 synthesis. To examine whether B7-1 and B7-2 mediate the same or different costimulatory signals, we prepared Chinese hamster ovary (CHO) cell transfectants expressing high levels of B7-1 or B7-2. Immunophenotyping showed very similar levels of expression with a mean fluorescence intensity for B7-1 and B7-2, respectively, of 32 and 28 with isotype-matched MAbs and 173 and 79 with CTLA4-Ig (Figure 2a). The 2-fold difference in CTLA4-Ig binding compared with isotype-matched MAbs most likely reflects the higher on–off rate of CTLA4-Ig binding for B7-2 (Linsley et al., 1994). To determine whether costimulation mediated by B7-1 and B7-2 differentially regulated the production of cytokines, we provided a first signal to human $CD4^+$ T cells with anti-CD3 MAb and a costimulatory signal with either CHO/B7-1 or CHO/B7-2. Protein accumulation of IL-2, IFNγ, TNFβ, GM-CSF, and IL-4 was examined by enzyme-linked immunosorbent assay (ELISA; Table 1). B7-1 and B7-2 equivalently costimulated production of IL-2 and IFNγ. In contrast, B7-2 costimulated 3-fold higher levels of TNFβ production but one-half the level of GM-CSF compared with B7-1. Only B7-2 induced expression of IL-4 protein, albeit at low levels. These differences were consistently observed. CTLA4-Ig inhibited cytokine production to levels equivalent to that observed for anti-CD3 alone (Table 1).

The dose–response of IL-4 production by $CD4^+$ T cells in response to anti-CD3 MAb plus increasing numbers of CHO/B7-1 or CHO/B7-2 transfectants was examined. Only CHO/B7-2 induced IL-4 accumulation with increasing production up to 20,000 CHO/B7-2 cells per 50,000 T cells (Figure 3). IL-4 production declined with very high numbers of CHO/B7-2 cells, probably because of toxicity caused by the high number of CHO cells as T cell proliferation also declined. CHO/B7-1 did not induce IL-4 production at any number of CHO/B7-1 cells tested (2,500–80,000).

IL-4 and G3PDH (positive control) mRNA expression was examined by reverse transcriptase (RT)–PCR (Figure 4). No IL-4 transcripts were detectable when $CD4^+$ T cells were cultured in media in the absence or presence of anti-CD3 MAb. Anti-CD3 MAb plus CHO/B7-2 induced expression of IL-4 mRNA (Figure 4). Quantitative PCR of IL-4 mRNA using MIMICS gave an estimate of approximately 5×10^{-4} attomol/µl of cDNA (data not shown). Blockade

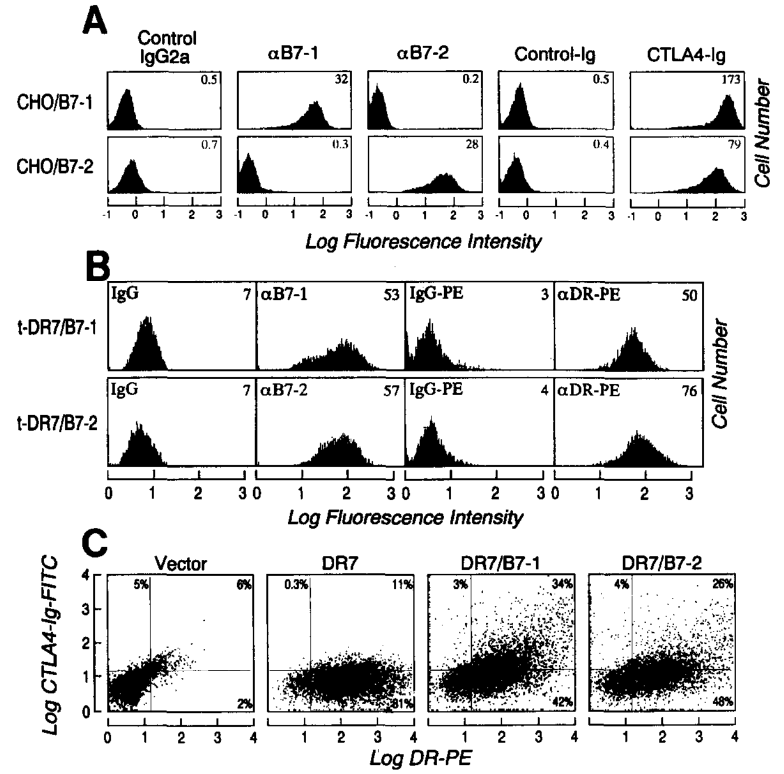


Figure 2. Phenotypes of Transfectants

(A) CHO cells transfected with the cDNA B7-1 or B7-2 or (B) NIH 3T3 cells transfe with the cDNAs for DR7 and either B7-1 or were stained with anti-DR MAb coupled to coerythrin or with isotype-matched (Ig MAbs for B7-1 (4B2.C4), B7-2 (HF2.3D with CTLA4-Ig, or isotype-matched control bodies as indicated and reactivity detern by indirect immunofluorescence and flo tometry analysis. Mean fluorescence inte is indicated in the upper right of each pane COS cells transiently transfected with pCI vector or with cDNAs encoding DR7 and e B7-1 or B7-2 were stained with anti-DR coupled to phycoerythrin and CTLA4-Ig pled to FITC and reactivity determine immunofluorescence and flow cytometry ysis. The percent cells in each quadran indicated. The mean fluorescence int (CTLA4-Ig-FITC/anti-DR-PE) of the transfectants was 6/5, 6/125, 13/51, 11/ vector, DR7, DR7/B7-1, DR7/B7-2, re tively.

of B7-2 costimulation with anti-CD28 Fab reduced IL-4 mRNA levels to undetectable levels. In contrast, anti-CD3 plus CHO/B7-1 did not result in the production of any IL-4 mRNA detectable by PCR.

B7-1- and B7-2-Mediated Costimulation Equivalently Up-Regulate IL-2Rα and IL-2Rγ Chain Expression

Since accumulation of IL-2 and expression of sufficient numbers of high affinity receptors are critical for T cell clonal expansion, we sought to determine whether costimulation mediated by B7-1 and B7-2 would induce the α and γ chains of the IL-2R. IL-2Rα$^+$ and IL-2Rγ$^+$ T cells were first removed from CD4$^+$ T cell populations by MAb and magnetic bead depletion. IL-2Rα$^-$ IL-2Rγ$^-$ CD4$^+$ T cells were subsequently cultured with either anti-CD3 alone or anti-CD3 in the presence of CHO/B7-1 or CHO/B7-2 cells. Stimulation of the IL-2Rα$^-$ IL-2Rγ$^-$ CD4$^+$ T cells in the presence of either B7-1 or B7-2 resulted in significant up-regulation of IL-2Rα and IL-2Rγ within 12 hr of culture. At 48 hr, most T cells coexpressed IL-2Rα and IL-2Rγ (Figure 5, middle and bottom). In contrast, culture of IL-2Rα$^-$ IL-2Rγ$^-$ CD4$^+$ T cells with anti-CD3 alone resulted in up-regulation of IL-2Rα and IL-2Rγ only after 48 hr of culture and on only a minority of cells (Figure 5, top). These results further explain the mechanism by which CD28 costimulation may prevent the induction of anergy by hastening and increasing the production of both IL-2 and the IL-2Rα (Cerdan et al., 1992; Reiser et al., 1992), β (Cerdan et al., 1995), and common γ chains. Induction of common γ chain by B7-1- and B7-2-mediated costimulation may also pro-

Table 1. Differential Induction of Cytokines in CD4$^+$ T Cells by and B7-2 Costimulation

CD4$^+$T	No Inhibitors	+CTLA4-Ig	+αCD28 I
IL-2 (pg/ml)			
+media	<16	–	–
+αCD3	<16	–	–
+αCD3 + CHO/B7-1	620	<16	<16
+αCD3 + CHO/B7-2	640	<16	<16
IFN-γ (pg/ml)			
+media	<20	–	–
+αCD3	<20	–	–
+αCD3 + CHO/B7-1	320	<20	32
+αCD3 + CHO/B7-2	440	<20	52
TNF β (pg/ml)			
+media	25	–	–
+αCD3	28	–	–
+αCD3 + CHO/B7-1	123	22	26
+αCD3 + CHO/B7-2	420	28	32
GM-CSF (pg/ml)			
+media	22	–	–
+αCD3	88	–	–
+αCD3 + CHO/B7-1	400	75	–
+αCD3 + CHO/B7-2	220	26	–
IL-4 (pg/ml)			
+media	<3	<3	–
+αCD3	<3	<3	–
+αCD3 + CHO/B7-1	<3	<3	–
+αCD3 + CHO/B7-2	32	<3	–

< denotes below the indicated lower limit of detection of the and – indicates not done. Similar results were obtained in fou pendent experiments.

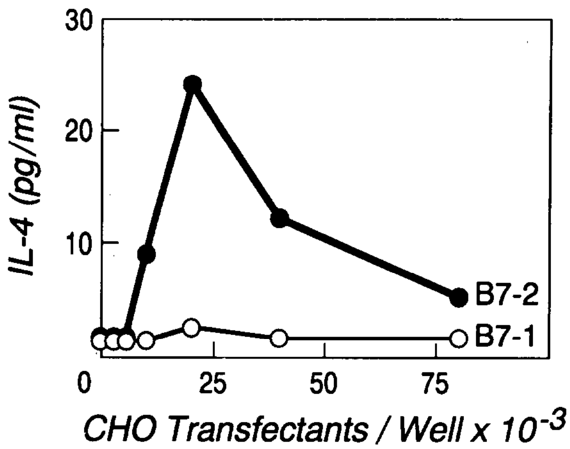


Figure 3. Dose Response of IL-4 Production in Response to CHO/B7-2

CD4+ T cells (5×10^4) were stimulated with submitogenic concentrations of anti-CD3 MAb in the presence of increasing numbers of CHO/B7-1 or CHO/B7-2. Supernatants were harvested after 24 hr and assayed for IL-4 by ELISA.

vide one explanation for CD28 costimulation regulating responsiveness to IL-4 (Damle and Doyle, 1989), as the common γ chain is shared by the IL-2, IL-4, and IL-7 receptors (Russell et al., 1993).

Differential Induction of Cytokines in a $CD4^+$ Alloreactive T Cell Clone by B7-1 and B7-2 Costimulation

Similar differences in lymphokine production were observed when the responding cell population was a Th0 T cell clone, TC-3. This alloreactive T cell clone produced both IL-2 and IL-4 in response to a B lymphoblastoid cell line that coexpresses DR7 alloantigen, B7-1, and B7-2 (Boussiotis et al., 1994a). To examine the effects of B7-1 versus B7-2 costimulation, TC-3 cells were stimulated using COS cells cotransfected with DR7 and either B7-1 or B7-2. Approximately 30% of the transiently transfected COS cells coexpressed DR7 and either B7-1 or B7-2 with B7-1 being expressed at slightly higher levels (see Figure 2C). Alloantigen plus either B7-1 or B7-2 equivalently costimulated IL-2 and IFNγ protein production by TC-3 cells (Table 2). B7-2 induced a moderate level of IL-4 protein and this level was 11-fold higher than that induced by B7-1, which was just above the lower limit of detection in this experiment and was below the level of detection in three other experiments (Table 2). B7-2 costimulation induced 4-fold higher levels of TNFβ protein than did B7-1 costimulation.

Both B7-1 and B7-2 Costimulate IL-4 Production in $CD4^+CD45RO^+$ T Cells but Only B7-2 Costimulates $CD4^+CD45RA^+$ T Cells to Produce IL-4

$CD4^+$ T cells were divided into $CD45RA^+$ (naive) and $CD45RO^+$ (memory) subsets (Morimoto and Schlossman, 1993), stimulated with anti-CD3 MAb, and examined for the capacity of B7-1 and B7-2 to costimulate cytokine production and proliferation. CHO/B7-2 costimulated slightly higher levels of proliferation and IL-2 production in $CD4^+CD45RA^+$ T cells than did CHO/B7-1. Only B7-2 costimulated $CD4^+CD45RA^+$ T cells to secrete IL-4 (Figure 6), albeit at low levels. B7-1 and B7-2 costimulated nearly equivalent levels of proliferation and IL-2 production in $CD4^+CD45RO^+$ T cells (Figure 6). Both B7-1 and B7-2 costimulated IL-4 production by $CD4^+CD45RO^+$ T cells and B7-2 consistently induced 3-fold more IL-4 in $CD4^+CD45RO^+$ T cells than did B7-1. Cytokine production by both $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ T cells was blocked by CTLA4-Ig.

Repetitive Costimulation by B7-2 Leads to Increased Production of IL-4

Since B7-1 and B7-2 equivalently costimulate IL-2 production, but only B7-2 costimulates IL-4 production by $CD4^+CD45RA^+$ T cells, we sought to determine the consequences of B7-1 or B7-2 costimulation on the evolution of IL-2 and IL-4 production following repetitive stimulation with alloantigen. $CD4^+CD45RA^+$ T cells were stimulated with NIH 3T3 cells transfected with DR7, DR7 and B7-1,

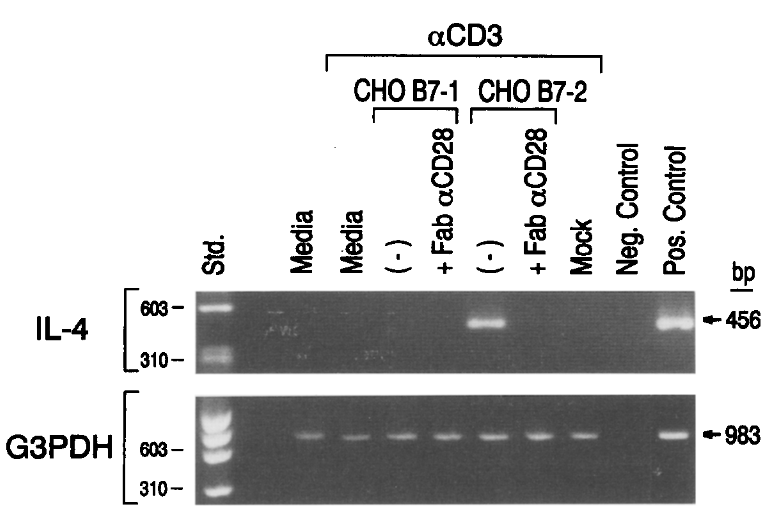


Figure 4. B7-2 but Not B7-1 Costimulation Induces Detectable IL-4 mRNA in Unprimed $CD4^+$ T Cells

$CD4^+$ T cells were stimulated with submitogenic concentrations of anti-CD3 MAb alone or in the presence of CHO/B7-1 or CHO/B7-2 costimulation with or without anti-CD28 Fab. Cells were harvested 6 hr after the initiation of culture, and RNA preparation and reverse transcription were performed as described in the Experimental Procedures. PCR amplification of 2 μg of these cDNA was performed using oligonucleotides specific for the indicated genes, and equal aliquots of the reaction products were electrophoresed on a 2.5% agarose gel containing ethidium bromide. Results are representative of four experiments.

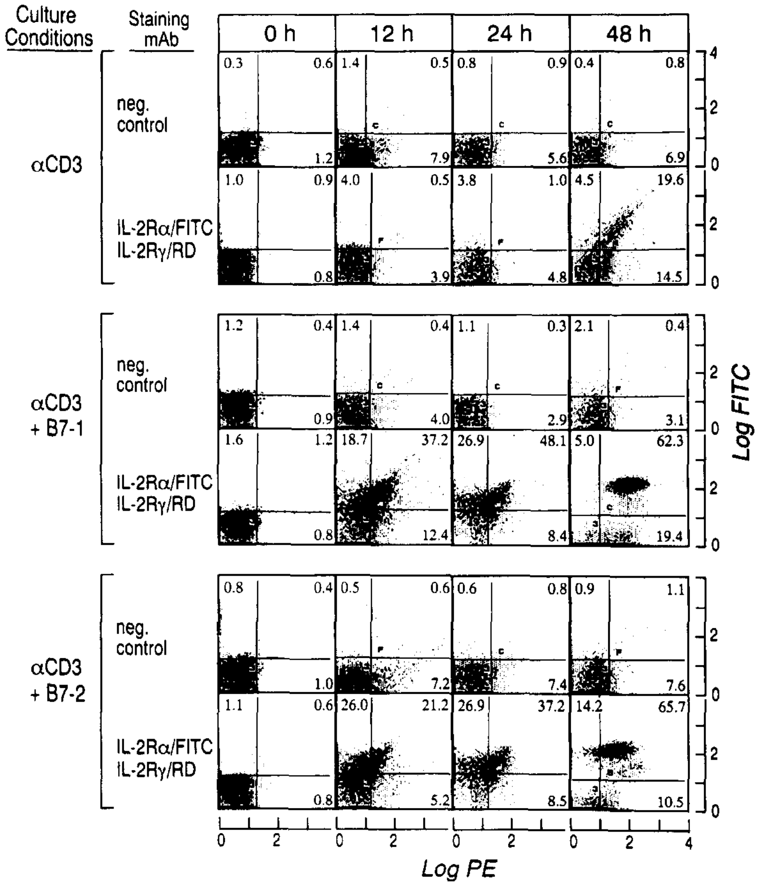


Figure 5. B7-1 and B7-2 Costimulation Rapi Up-Regulates IL-2Rα and IL-2Rγ Expressic

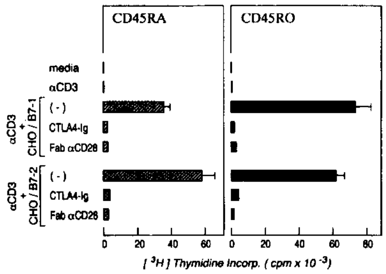
Purified CD4⁺ IL-2Rα⁻ IL-2Rγ⁻ T cells we stimulated with submitogenic concentratic of anti-CD3 MAb alone or in the presence either CHO/B7-1 or CHO/B7-2. At the indicat time intervals, cells were harvested and ‹ pression of IL-2Rα and IL-2Rγ was examin as described in Experimental Procedures. T percent cells in each quadrant are indicat‹ Results are representative of three exp‹ ments.

or DR7 and B7-2. The expression of DR7 with either B7-1 or B7-2 in NIH 3T3 cells was comparable (see Figure 2B). T cells from each primary stimulation were restimulated with the identical transfectant for an additional four cycles and IL-2 and IL-4 accumulations were quantitated. In the first round of stimulation, only DR7/B7-2 transfectants induced IL-4 production, albeit at low levels (Figure 7). With further rounds of stimulation, DR7/B7-2 transfectants stimulated progressively increasing levels of IL-4 production (peak level 140 pg/ml), whereas DR7/B7-1 transfectants did not costimulate any IL-4 production during the first or second round and low levels of IL-4 were detected with additional rounds of stimulation (peak level 34 pg/ml). In contrast, both DR7/B7-1 transfectants and DR7/B7-2 transfectants costimulated equivalent levels of IL-2 production in the first and second rounds of stimulation. Stimulation with DR7/B7-1 transfectants in subsequent rounds resulted in increasing levels of IL-2 production (peak 2000 pg/ml), whereas additional rounds of stimulation with DI B7-2 transfectants did not lead to further increases in l els of IL-2 production. T cells stimulated with DR7/B transfectants or DR7/B7-2 transfectants proliferated vig ously. In contrast, T cells stimulated with DR7 transf tants did not proliferate or produce IL-2 or IL-4 and bar enough cells remained viable to perform the assay. Wh T cells were stimulated multiple rounds with either DI B7-1 transfectants or DR7/B7-2 transfectants and th challenged with DR7 transfectants alone, the T cells not produce IL-4 (data not shown). Similar results w seen in identical experiments performed with COS ‹ transfectants (data not shown). These results are con: tent with the hypothesis that B7-2 costimulation can p vide a signal for production of low levels of IL-4, and t IL-4 is sufficient to prime for subsequent production of l upon restimulation but is not sufficient to drive T cells terminal Th2 differentiation.

Table 2. Differential Induction of Cytokines in a CD4⁺ Alloreactive T Cell Clone by B7-1 and B7-2 Costimulation

T Cell Clone (TC-3)	IL-2 (pg/ml)	IFN γ (pg/ml)	IL-4 (pg/ml)	TNF β (pg/ml)
+media	<16	<20	<3	22
+COS DR7/B7-1	120	220	6	98
+COS DR7/B7-2	130	240	65	380
+COS mock	<16	<20	<3	16

< denotes below the indicated lower limit of detection of the assay. Similar results were obtained in four independent experiments.



	CD45RA IL-2 (pg/ml)	CD45RA IL-4 (pg/ml)	CD45RO IL-2 (pg/ml)	CD45RO IL-4 (pg/ml)
media	< 16	< 3	< 16	< 3
αCD3	< 16	< 3	34	4
αCD3 + CHO/B7-1 (-)	420	< 3	760	28
αCD3 + CHO/B7-1 CTLA4-Ig	28	< 3	48	< 3
αCD3 + CHO/B7-1 Fab αCD28	nd	nd	nd	nd
αCD3 + CHO/B7-2 (-)	760	26	640	75
αCD3 + CHO/B7-2 CTLA4-Ig	32	< 3	40	8
αCD3 + CHO/B7-2 Fab αCD28	nd	nd	nd	nd

Figure 6. Both B7-1 and B7-2 Can Costimulate IL-4 Production by CD4$^+$CD45RO$^+$ T Cells, but Only B7-2 Can Costimulate IL-4 Production by CD4$^+$CD45RA$^+$ T Cells

CD4$^+$ T cells were divided into CD45RA$^+$ and CD45RO$^+$ subsets and cultured with submitogenic concentrations of anti-CD3 MAb alone or in the presence of CHO/B7-1 or CHO/B7-2 with or without anti-CD28 Fab. IL-2 and IL-4 concentrations were assessed in supernatants after 24 hr of culture and [^{3}H]thymidine incorporation was measured for the last 16 hr of a 72 hr culture period. Error bars indicate SD. Results are representative of three experiments.

Discussion

In the present report, we show that the functional outcomes of costimulation mediated by B7-1 and B7-2 are different. Although B7-1 and B7-2 costimulation were equivalent at inducing IL-2 and IFNγ production and expression of IL2-Rα and IL-2Rγ chains, B7-2 more effectively costimulated IL-4 and TNFβ production, whereas B7-1 more effectively costimulated GM-CSF production. The most striking and functionally significant difference between B7-1 and B7-2 was that B7-2 costimulation more effectively induced IL-4 production. The more effective induction of IL-4 by B7-2 costimulation has been consistently observed in different T cell populations (CD4$^+$CD45RA$^+$ T cells, CD4$^+$CD45RO$^+$ T cells, and an alloreactive T cell clone), in response to either anti-CD3 MAb or alloantigen, and with B7-2 expressed in either CHO, COS, or NIH 3T3 cells. Similarly, anti-B7-2 MAb but not anti-B7-1 MAb significantly reduced the induction of IL-4 mRNA in a primary human allogeneic MLR. The magnitude of the difference in IL-4 induction by B7-1 and B7-2 was dependent upon the differentiative state of the T cell. In unprimed CD4$^+$CD45RA$^+$ T cells (naive; Morimoto and Schlossman, 1993), B7-2 induced 10-fold or more higher levels of IL-4 than did B7-1. In previously stimulated T cells or CD4$^+$CD45RO$^+$ T cells, B7-2 induced 3- to 11-fold more IL-4 than did B7-1. These data support the conclusion that the differences between B7-2 and B7-1 costimulation will be greatest at the initiation of an immune response and less pronounced thereafter. It should be emphasized that the levels of IL-4 induced by B7-2 costimulation are low and probably not sufficient to induce all of the biologic effects mediated by high levels of IL-4.

Previous investigators have shown that IL-4 is a dominant cytokine and IL-4 priming directs differentiation toward the Th2 subset (Swain et al., 1990; DeKruyff et al., 1992; Hsieh et al., 1992; Seder et al., 1992; Seder and Paul, 1994; McKnight et al., 1994). The signals initiating the production of IL-4 as well as the cellular populations that initiate IL-4 secretion are much less well understood

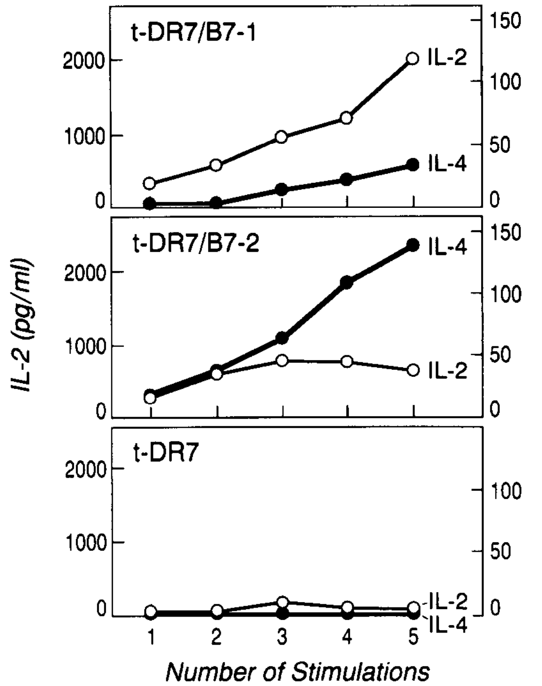


Figure 7. Evolution of IL-2 and IL-4 Production in Response to Repetitive Costimulation with B7-1 or B7-2

CD4$^+$CD45RA$^+$ T cells were stimulated with NIH 3T3 cells transfected (abbreviated t-) with DR7, DR7 and B7-1, or DR7 and B7-2. At weekly intervals, T cells were restimulated with the identical transfectants. Supernatants were harvested after each stimulation and levels of IL-2 and IL-4 were determined by ELISA.

(van der Pouw-Kraan et al., 1992, 1993; Seder and Paul, 1994). It has been proposed in murine systems that either mast cells or a subpopulation of NK cells may provide the initial source of IL-4 (Seder and Paul, 1994). Alternatively, we now show in a human in vitro system that B7-2 can costimulate naive T cells to produce low levels of IL-4, and this amount of IL-4 appears to be sufficient to prime these cells for further IL-4 production. When CD4$^+$CD45RA$^+$ T cells were repetitively stimulated with alloantigen and B7-2, IL-4 was induced in the first round of stimulation and increased steadily thereafter. IL-2 levels increased for the first two rounds but declined slightly thereafter. In contrast, repetitive stimulation with alloantigen and B7-1 did not lead to any detectable IL-4 production until the third round and low levels were produced thereafter. IL-2 production steadily increased with each round of B7-1 costimulation to very high levels. Taken together, these results demonstrate that a major difference between the outcomes of B7-1- and B7-2-mediated costimulation is the ability of B7-2 to initiate and amplify IL-4 secretion by naive T cells.

Although repetitive costimulation with B7-2 led to progressively increased, albeit low, levels of IL-4, IL-2 was still produced, demonstrating that B7-2 costimulation was not sufficient to drive the entire population to Th2 differentiation. These results are consistent with previous studies, which have shown that exogenous IL-4 drives T cell differentiation towards Th2 in an IL-4 dose-dependent fashion and that low levels of exogenous IL-4 lead to a mixed population of T cells that secrete both IL-2 and IL-4 (Swain et al., 1990). Additional signals are undoubtedly required for higher levels of IL-4 production and terminal differentiation into Th2. A role for B7-2 in the initiation of an immune response by naive T cells may be to costimulate an initial low level of IL-4 that gives T cells the option of responding to further differentiative signals. Since B7-2 is constitutively expressed on monocytes and dendritic cells but B7-1 is not (Freedman et al., 1991; Azuma et al. 1993; Hart et al., 1993; Caux et al., 1994), the initial costimulation of an immune response will usually be by B7-2, perhaps explaining why the "default" response of the immune system is towards Th0/Th2 (Hsieh et al., 1992). In contrast, B7-1 does not induce IL-4 production in the initial rounds of stimulation and is thus a more neutral differentiative signal, perhaps leaving T cells particularly sensitive to Th1 differentiative signals such as IL-12.

Additional recent work supports the idea that B7-1 and B7-2 are not equivalent in their in vivo biological function and that CD28 signaling is critical for IL-4 production. The antibody isotypes induced by adoptive transfer of antigen-pulsed APCs (De Becker et al., 1994) is consistent with our hypothesis that B7-2 costimulates IL-4 secretion and provides a moderate signal towards Th2 differentiation. In these studies, B7-2$^+$, B7-1$^-$ murine monocytes preferentially induced the secretion of IgG1 and IgE (Th2-dependent isotypes), whereas B7-2$^+$, B7-1$^+$ murine dendritic cells induced both IgG2a and IgG1 antibodies (Th1- and Th2-dependent isotypes). In mice, challenge with B7-1-transfected tumor cells leads to tumor rejection and subsequent immunity against untransfected tumor cells (Chen et al., 1992; Baskar et al., 1993; Townsend and Allison, 1993). These results have been replicated in a B7-1-transfected myeloid tumor cell line; however, the B7-2-transfected tumor has a much lower rate of tumor rejection and survivors often cannot reject untransfected tumor upon rechallenge (Matulonis et al., 1995; unpublished data). Studies of the development of T helper subsets in a TCR αβ transgenic system have shown that splenic adherent cells stimulate the development of a mixed population of Th0 but that the B7-1$^-$, B7-2$^+$ TA3 B cell line (Freeman et al., 1993a) stimulates the development of Th2 (Hsieh et al., 1992). In a murine model of experimental allergic encephalomyelitis (EAE), in vivo administration of anti-B7-1 MAb (allowing B7-2 to dominate) reduced the severity of disease and led to increased production of IL-4 (Kuchroo et al., 1995). T cell clones derived from anti-B7-1 MAb-treated mice with EAE were primarily of Th2 phenotype, instead of the primarily Th1 clones derived from untreated animals. In contrast, in vivo administration of anti-B7-2 MAb (allowing B7-1 to dominate) increased EAE severity. Previous work has shown that for the generation of IL-4-producing T cells, stimulation of uncommitted T cells by both IL-2 and IL-4 is necessary. IL-2 either provides a necessary signal for IL-4 production or simply a viability signal (Le Gros et al., 1990; Swain et al., 1990; DeKruyff et al., 1992; Seder et al., 1992; McKnight et al. 1994). The results of in vivo blocking with anti-B7-2 in the EAE model, therefore, could be due to direct blocking of a signal for IL-4 production or alternatively an indirect effect of blocking the major costimulator of IL-2 production. Our in vitro hum results are consistent with B7-2 providing a direct sig for IL-4 production. Taken together, these studies sugg that B7-1 and B7-2 are not equivalent and that CD mediated signaling by B7-2 has a role in the differentiati of CD4$^+$ T cells capable of secreting IL-4.

At first, it would seem surprising that B7-1 and B7-2 c bind to the same receptor but lead to differential induct of some lymphokines. However, several immunologi receptors have been shown to transmit signals leading different outcomes depending on the ligand bound. example, peptide–MHC binding to TCR normally sign for T cell activation but certain "altered" peptide–M combinations can deliver signals for T cell anergizat (Sloan-Lancaster et al., 1993) and these may critically fer in their on–off rates (Matsui et al., 1991). Linsley et (1994) have recently shown that B7-1 and B7-2 have si lar low affinities for CD28 and high affinities for CTL but very different kinetics of binding. B7-2 binds faster also falls off faster than does B7-1. In addition, the bind determinants on CTLA4 for binding to B7-1 and B7-2 di (Linsley et al., 1994). These differences in the binding and on–off rates may permit B7-1 and B7-2 to recruit dif ent intracellular signaling pathways. Nunes et al. (19 identified a number of intracellular signals induced cross-linking with anti-CD28 MAb but only some of th were duplicated by B7-1 binding to CD28. They hypot sized that CD28 signals not induced by B7-1 may be duced by B7-2.

It is becoming increasingly apparent that subpop tions of APC can direct the differentiation of a T cell wards Th1 or Th2 by expressing distinct costimulatory surface proteins, secreting cytokines, or both. A crit question will be to understand the natural mechanis whereby APCs differentially express costimulatory m cules in response to different pathogens and immune c lenges. Moreover, these observations may be clinic relevent in attempts to induce immunity to pathogens tumors or to control autoimmunity.

Experimental Procedures

MAbs and Immunoglobulin-Fusion Proteins

MAbs were used as purified immunoglobulin unless indicated o wise: anti-CD3: OKT3, IgG1, was from ATCC; anti-CD8: 7PT IgG2a; anti-CD11b: Mo1, IgM and anti-CD14: Mo2, IgM; anti-C 9.3, IgG2a (Dr. C. June, Naval Research Institute, Bethesda, M land); anti-CD16: 3G8, IgG1 (used as ascites); anti-IL-2Rγ: 3B5, I (Nakarai et al., 1994; provided by Drs. T. Nakarai and J. Ritz, D Farber Cancer Institute, Boston, Massachusetts); anti-CD45RA: IgG1 and anti-CD45RO: UCHL1, IgG1 (Dr. P. Beverly, University lege, London, and Dr. C. Morimoto, Dana-Farber Cancer Insti Boston, Massachusetts); anti-B7-1: 133, IgM (Freedman et al., 1 anti-B7-2/B70/CD86: IT2.2, IgG2b (Pharmingen, San Diego, Ca nia) and Fun-1 (Nozawa et al., 1993; obtained through the Fifth Int tional Conference on Human Leukocyte Differentiation Antigens) anti-CD25 (IL-2Rα), IgG1 (Coulter Corporation, Hialeah, Florida). CD28 Fab fragments were generated in our laboratory from th MAb by papain digestion and purification on a protein A column cording to the instructions of the manufacturer (Pierce, Rockfor nois). Human CTLA4-Ig and control fusion protein were prepare previously described (Gimmi et al., 1993; McKnight et al., 1994

Cell Transfections

CHO/B7-1 were prepared as described, and fixed with paraform hyde prior to use (Gimmi et al., 1991). CHO/B7-2 were made as described (Engel et al., 1994) by cotransfecting the B7-2 cDNA in the pCDM8 expression vector and the pPGK–Hygro vector expressing hygromycin resistance. Transfectants were sorted for CTLA4-Ig binding twice and cloned. Expression of B7-2 was confirmed by staining with anti-B7-2/B70/CD86 MAbs IT2.2 (Azuma et al., 1993) and Fun-1 (Nozawa et al., 1993). CHO/B7-2 cells were fixed with 0.4% paraformaldehyde prior to use.

NIH 3T3 cells stably transfected with DR7 (DR7 transfectants) or DR7 and B7-1 (DR7/B7-1transfectants) have been described (Gimmi et al., 1993). NIH 3T3 cells stably transfected with DR7 and B7-2 (DR7/B7-2 transfectants) were prepared by cotransfecting DR7 transfectants cells with a B7-2 cDNA in the SRα plasmid and the pPGK–Hygro plasmid expressing the hygromycin-resistance gene. Transfectants were selected in media containing 200 μg/ml hygromycin and 200 μg/ml G418. Transfectants were sorted with an anti-MHC class II MAb-coupled to phycoerythrin (I3, Coulter Corporation, Hialeah, Florida) and CTLA4-Ig coupled to fluorescein isothiocyanate (FITC). Positive cells were grown up, resorted and cloned. A transfected DR7/B7-2 cloned cell line expressing equivalent amounts of MHC class II and CTLA4 ligand to that of the DR7/B7-1 transfectants was selected for use.

$CD4^+$ Human T Cells

Peripheral blood mononuclear cells were isolated from healthy donors by density gradient centrifugation on Ficoll–Hypaque. Monocytes were depleted by adherence on plastic. The $CD4^+$ T cell population was further enriched by separation from residual monocytes, B cells, NK cells and $CD8^+$ T cells by MAb and anti-mouse immunoglobulin-coated magnetic beads, using anti-CD14 (Mo2), anti-CD11b (Mo1), anti-CD20 (B1), anti-CD16 (3G8), and anti-CD8 (7PT 3F9) MAbs. The efficiency of the purification was analyzed in each case by flow cytometry (Coulter, EPICS Elite), using anti-CD3, anti-CD4, anti-CD8, and anti-CD14 MAbs followed by FITC-conjugated goat anti-mouse immunoglobulin (Fisher, Pittsburgh, Pennsylvania). The final cell preparation was always >99% $CD3^+$, >99% $CD4^+$, <1% $CD8^+$, and <1% $CD14^+$. $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ T cell subsets, were prepared as described above, but with the additional use of anti-CD45RO (UCHL1) for the preparation of the $CD4^+CD45RA^+$ cells and the addition of anti-CD45RA (2H4) for the preparation of the $CD4^+CD45RO^+$ cells.

T Cell Cultures

For proliferation assays and assessment of cytokine accumulation in the culture supernatants, T cells were cultured at a concentration of 5×10^4 cells/well in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml) and gentamycin sulfate (5 μg/ml) in 96-well flat-bottomed microtiter plates at 37°C in 5% CO_2. For submitogenic stimulation of unprimed $CD4^+$ T cells, anti-CD3 MAb was precoated onto plates at a concentration of 0.5 μg/ml for 1 hr at room temperature. After incubation, plates were washed with phosphate-buffered saline three times. CHO/B7-1 or CHO/B7-2 cells were added at a concentration of 2×10^4 cells/well. Factors under study were added to the required concentration for a total final volume of 200 μl/well. Where indicated, T cells were incubated with anti-CD28 Fab (final concentration of 15 μg/ml) for 30 min at 4°C, prior to addition in experimental plates. Cells were pulsed with 1 μCi [methyl-^{3}H]thymidine (37 kBq; Du Pont, Boston, Massachusetts)/well and incorporation during the last 18 hr of culture was used as an index of mitogenic activity. The cells were harvested onto filters and the radioactivity on the dried filters was measured in a β plate liquid scintillation counter (Pharmacia, Sweden). When the alloreacive T cell clone was used as the responder population, stimulation at a 1:1 ratio was performed using COS cells transfected with cDNAs encoding the specific alloantigen (DR7) and either B7-1 or B7-2.

Repetitive Stimulations

$CD4^+CD45RA^+$ cells (5×10^4) per well were cultured in 96-well flat-bottomed microtiter plates at 37°C in 5% CO_2, with 2×10^4 each of mitomycin-treated NIH 3T3 transfectants (DR7 transfectants, DR7/B7-1 transfectants, DR7/B7-2 transfectants) in a primary allostimulation. Following 7 days of culture, alloreactive T cell populations were separated from the transfectants by percoll gradient as described (Boussiotis et al., 1993a), rested in media overnight, and subsequently 5×10^4 T cells were rechallenged with 2×10^4 of each of the transfectants. Sequential (repetitive) stimulations were performed 5 times. Supernatants were harvested 48 hr after the primary stimulation and at 24 hr after each restimulation and assayed for IL-4 and IL-2 accumulation by ELISA.

Alloantigen-Specific T Cell Clones

HLA-DR7 alloantigen-specific helper T cell clones were generated using standard methodology (Goronzy et al., 1987). T cell clones were maintained by cycles of antigen stimulation and rest. Prior to use, T cell clones were maintained for 10–15 days without antigenic stimulation.

MLR

For MLR responses, normal donor peripheral blood mononuclear cells were cultured with irradiated (2.5 Gy) normal donor peripheral blood mononuclear cells from HLA disparate individuals. Cells were cultured in RPMI 1640, 5% heat-inactivated human AB serum at 37°C in 5% CO_2 at a final concentration of 10^6 cells/ml. Cells were cultured as indicated in the absence or presence of anti-B7-1 MAb, anti-B7-2 MAb, CTLA4-Ig, or isotype control antibodies, all at a final concentration of 10 μg/ml. Cells were cultured in 25 cm^2 tissue culture flasks and harvested after 48 hr for RNA extraction. Proliferation was assessed in parallel experiments by measuring thymidine incorporation for the last 16 hr of a 5 day assay performed in 96-well plates.

Cytokine Assays

Cytokine concentrations in culture supernatants were assayed by ELISA using commercially available kits for IL-2 (BioSource, Camarillo, California), IL-4 (Endogen, Cambridge, Massachusetts), IFNγ (BioSource, Camarillo, California), TNFβ (Boehringer Mannheim, Indianapolis, Indiana), and GM-CSF (R & D Systems, Minneapolis, Minnesota). Lymphokine levels were determined by comparison with a standard curve, which was linear down to the indicated lower limit of detection.

Immunofluorescence and Flow Cytometry

T cells activated with anti-CD3 cross-linking alone, or in the presence of anti-CD3 and either CHO/B7-1 or CHO/B7-2 for 12, 24, and 48 hr were analyzed for the coexpression of IL-2Rα and IL-2Rγ. Cells were stained with FITC-conjugated anti-IL-2Rα and biotinylated anti-IL-2Rγ MAbs or the appropriate controls (isotype-matched FITC-conjugated or biotinylated MsIg). Specific immunoreactivity of the biotinylated MAbs was determined using phycoerythrin-conjugated streptavidin as secondary reagent.

RT–PCR

$CD4^+$ T cells were cultured at 1×10^6 cells/well in 24-well plates precoated with anti-CD3 MAb as described above, in the presence of CHO/B7-1- or CHO/B7-2-transfected cells and harvested for RNA preparation after 6 hr (Chomczynski and Sacchi, 1987). RNA (2 μg) was used for reverse transcription as previously described (Boussiotis et al., 1994b). PCR amplification of cDNA from 2 μg of mRNA was performed using specific oligonucleotides for IL-2 or IL-4 (Clontech, Palo Alto, California) for 34 cycles in a Perkin Elmer–Cetus thermal cycler (Cetus, Emoryville, California) in a 50 μl final volume as previously described (Siebert and Larrick, 1993). A 20 μl aliquot of each of the final reaction products was electrophoresed on a 2.5% agarose gel containing ethidium bromide.

RNA was prepared from an MLR reaction after 48 hr, the time determined to be maximal for IL-2 and IL-4 mRNA expression. Levels of IL-2 and IL-4 mRNA were determined by competitive RT–PCR using a MIMIC template according to the instructions of the manufacturer (Siebert and Larrick, 1993; Clontech, Palo Alto, California). mRNA (1 μg) was reverse transcribed and equal 1/20 aliquots added to PCR reactions containing serial 10-fold dilutions of PCR MIMICs comprised of the primer sequence for IL-2 or IL-4 separated by a nonhomologous DNA. After PCR amplification, the products derived from the MIMIC template and cDNA were resolved on an agarose gel and the relative ethidium bromide staining intensities of the target and MIMIC DNAs compared. The PCR reaction was then repeated with a constant amount of cDNA and serial 2-fold dilutions of the MIMIC covering the appropriate range and the DNA products were separated by gel electrophoresis. The amount of target cDNA was measured by determining how much MIMIC is required to produce equal molar quantities of both PCR products. The data was analysed for statistical significance using the paired Student's t test.

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