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B7.1 Is a Quantitatively Stronger Costimulus Than B7.2 in the Activation of Naive CD8$^+$ TCR-Transgenic T Cells[1]

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Using a TCR transgenic mouse bred onto a recombinase-activating gene-2-deficient background, we have examined the influence of B7.1 and B7.2 on activation of naive, CD8$^+$ T cells in vitro. We found that B7.1 was a more potent costimulus than B7.2 for induction of proliferation and IL-2 production by naive CD8$^+$ T cells. This difference appeared to be quantitative in nature, as determined using transfectants expressing various defined levels of B7.1 or B7.2, or using purified B7.1 or B7.2 fusion proteins. In contrast to the quantitative differences seen in stimulation of naive T cells, B7.1 and B7.2 were comparable in their ability to costimulate responses in T cells previously primed in vitro. In addition, primed, but not naive, T cells were capable of proliferating and producing IL-2 in response to a TCR stimulus alone, apparently in the absence of B7 costimulation. Lastly, we found that B7.1 and B7.2 were equivalently capable of driving differentiation of naive CD8$^+$ T cells into an IL-4-producing phenotype when exogenous IL-4 was added to the primary culture or to an IFN-γ-producing phenotype in the presence of IL-12. These results indicate that signals generated by B7.1 and B7.2 are qualitatively similar, but that B7.1 is quantitatively stronger than B7.2. Further, our results indicate that the activation state of the responding T cell may influence the efficiency with which the T cell can respond to a costimulatory signal provided by either B7.1 or B7.2. ***The Journal of Immunology,* 1998, 161: 5268–5275.**

Coligation of CD28 along with the TCR augments T cell cytokine production and proliferation, promotes peripheral T cell differentiation and cell survival, and can prevent the induction of anergy (1–4). There are two identified ligands for CD28, B7.1 (CD80) and B7.2 (CD86), that are expressed predominantly on dendritic cells, macrophages, and activated B cells and also on activated T cells (5, 6).

Whether B7.1 and B7.2 can costimulate T cells equivalently has remained controversial. Differences in the ability of these molecules to augment T cell responses have been observed in several model systems. Some published studies, involving either human or murine models, have suggested that B7.1 can provide a stronger costimulus than B7.2 for T cell activation, as measured by proliferation, lymphokine production, induction of cytolysis, and rejection of certain tumors (7–10). In addition, costimulation with B7.2 has been reported to preferentially induce differentiation of T cells into an IL-4-producing or T2-like response, suggesting a qualitative difference between the T cell signals generated by costimulation with either B7.1 or B7.2 (11, 12). In contrast, other studies have reported that these molecules appear to be functionally equivalent (13, 14). These disparate findings have been difficult to reconcile using normal lymphocyte populations.

Given the importance of B7.1 and B7.2 in regulating immune responses, a clearer understanding of the capabilities of each in directing T cell responses is critical. It seems likely that differing results have been obtained by various investigators because different model systems have been used. We postulate that at least one factor contributing to the discrepant results of previous studies is the use of mixed populations of responding T cells because naive and primed T cells may differ in their requirements for B7 costimulation (15, 16). To explore these hypotheses, we have developed a model system for examining the requirements for B7 costimulation of naive and previously stimulated CD8$^+$ T cells in vitro. A monoclonal T cell population expressing a naive surface phenotype was obtained by breeding a TCR transgenic mouse with recombinase-activating gene-2-deficient (RAG-2$^{-/-}$)[3] mice. Strict control over the B7 costimulus was achieved by generating transfectants expressing defined levels of B7.1 or B7.2 or by using B7.1-Ig or B7.2-Ig fusion proteins at defined concentrations. This system was used with the intention of 1) allowing strict control over the type and amount of B7 molecule presented to the T cells; 2) ensuring that when initially stimulated, the responding T cell population had not previously encountered a TCR stimulus in the periphery, and thus was truly naive; and 3) minimizing and controlling the potential influence of adhesion or other accessory molecules on the abilities of the B7 costimulatory molecules to drive T cell responses. We attempted to answer three questions. 1) Are there differences between B7.1 and B7.2 in the costimulation of primary CD8$^+$ T cell responses? 2) If so, are the differences quantitative or qualitative in nature? 3) Do naive T cells and T cells previously stimulated with Ag differ in their requirements for B7 costimulation?

Using this model system, we found quantitative differences in the abilities of B7.1 and B7.2 to costimulate CD8$^+$ T cell responses. In naive cells, B7.1 provided a stronger costimulus than

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[3] Abbreviations used in this paper: RAG-2$^{-/-}$, recombinase-activating gene-2-deficient; DMEM-10, DMEM supplemented with 10% FCS.

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B7.2 for proliferation and IL-2 production. The addition of exogenous IL-2 to B7.2-stimulated T cells increased their proliferative response, suggesting that the difference in the abilities of B7.1 and B7.2 to induce proliferation reflects a difference in their respective abilities to induce IL-2 production by primary T cells. In contrast to those of naive cells, we found that the responses of primed T cells to costimulation by B7.1 and B7.2 were quantitatively similar. In addition, primed T cells proliferated and produced IL-2 in response to a TCR stimulus alone in the absence of B7 costimulation. Finally, both B7.1 and B7.2 were capable of supporting the differentiation of naive $CD8^+$ T cells into an IL-4-producing, Tc2 phenotype, arguing against a qualitative difference between the fundamental effects of these two costimulatory molecules.

Materials and Methods

Mice, Abs, reagents, and cell lines

All mice were housed in the University of Chicago Barrier Animal Facility under pathogen-free conditions. The 2C transgenic mice were obtained from Dr. Dennis Loh (17). These mice are haplotype H-2^b, and 2C T cells are reactive against the H-$2L^d$ alloantigen complexed with an octapeptide, p2Ca, from α-ketoglutarate dehydrogenase. The RAG-$2^{-/-}$ mice were a gift from Dr. Celeste Simon (University of Chicago, Chicago, IL) (18). The 2C mice were bred onto a RAG-$2^{-/-}$ background, and the progeny were screened for expression of the 2C transgene and lack of B cells by immunofluorescence using FITC-coupled 1B2 (anti-2C clonotype, D. Loh) and phycoerythrin-coupled DS-1 (anti-IgM, PharMingen, San Diego, CA) Abs. Splenic T cells from these 2C $\times$ $RAG^{-/-}$ mice (2C/$RAG^{-/-}$) were purified as described below and used in experiments. FITC- or phycoerythrin-coupled Abs against murine ICAM-1, LFA-1, H-$2L^d$, CD4, CD8, CD80, CD86, CD44, CD62L, and IgM used for FACS analysis of P815 transfectants and T cells were purchased from PharMingen. The hybridoma-producing anti-CD3ϵ mAb, 145-2C11, was a gift from Dr. Jeffrey Bluestone (University of Chicago). The Ab was purified from culture supernatant using a protein A-agarose column (Pharmacia Biotech, Piscataway, NJ). Cell lines producing anti-CD11c mAb, M1/70, and anti-I-A^b mAb, 25-9-3s, were purchased from American Type Culture Collection (Manassas, VA). These Abs were used in T cell purification as culture supernatant. The H-$2L^d$-expressing mouse mastocytoma cell line, P815, was transfected with B7.1 or B7.2 and was used as a stimulator cell line (19). Recombinant human IL-2 was a gift from Cetus Oncology Corp. (Emeryville, CA). Recombinant murine IL-4 was a gift from Immunex Corp. (Seattle, WA). rIL-12 was a gift from Genetics Institute (Cambridge, MA).

P815 transfectants

cDNA constructs containing the sequences for murine B7.1 and B7.2, which were originally isolated by Dr. Gordon Freeman, were obtained from Dr. J. Bluestone (20, 21). Murine B7.1 was cloned into the eukaryotic vector pNA′, a modified form of pHβAPr-neo that contains the human β-actin promoter and confers resistance to neomycin (22, 23). The cDNA sequence was originally removed as an *Eco*RI fragment from pBSKS2-/B71 and inserted into the *Eco*RI site of pcDNA3 (Invitrogen, San Diego, CA). A subclone containing the B7.1 sequence in the correct orientation was selected; B7.1 was removed with a *Kpn*I, *Xba*I digest and ligated into pNA′ that had been digested with *Kpn*I and *Xba*I. The resulting subclones were screened for correct orientation in relation to the β-actin promoter, and one subclone was selected for transfection into P815. The cDNA sequence for murine B7.2 was removed as an *Xba*I fragment from pcDNA3/B7-2 and inserted into the *Xba*I site of pHA′, a modified form of pNA′ that confers resistance to hygromycin (22). A subclone containing B7.2 in correct orientation in relation to the β-actin promoter was selected for transfection into P815.

pNA′/B7.1 was linearized with *Sca*I, and pHA′/B7.2 was linearized with *Xmn*I. Both constructs were transfected into P815 cells by electroporation. pNA′/B7.1 transfectants were selected on 1 mg/ml G418 (Gemini Bioproducts, Calabasas, CA). pHA′/B7.2 transfectants were selected on 0.5 mg/ml hygromycin B (Boehringer Mannheim, Indianapolis, IN). Resulting antibiotic-resistant cells were screened for B7.1 and B7.2, and bulk populations of transfected cells were sorted for different levels of expression by FACS using FITC-coupled anti-B7.1 and anti-B7.2 (1G10 and GL-1, respectively; PharMingen). Surface expression of B7.1 and B7.2 on subclones expressing varying levels of the molecule was determined by immunofluorescence using saturating amounts of FITC-coupled murine CTLA-4 Ig (provided by Dr. Jeffrey Bluestone).

Preparation of B7-Ig fusion proteins, B7.1-Ig and B7.2-Ig

Murine B7.1-Ig and murine B7.2-Ig expression plasmids were constructed by joining the DNA encoding the signal and extracellular domains of murine B7.1 or B7.2 to the DNA encoding the hinge CH2-CH3 domains derived from a murine IgG2a Ab. The Ab hinge cysteines remained intact such that the expressed B7.1-Ig or B7.2-Ig was dimeric.

Recombinant Chinese hamster ovary (CHO) cell lines, 74-18/0.02 and 73-15/0.02, expressing B7.1-Ig and B7.2-Ig, respectively, were grown in DMEM/Ham's F-12 medium containing 10% FBS, 0.02 mM methotrexate, and 1.0 mg/ml G418. The cell lines were grown to confluence in roller bottles, and growth medium was replaced with serum-free secretion medium. After 24 h, the secretion medium was removed and clarified through 0.22-μm filters. This medium containing B7.1-Ig or B7.2-Ig was passed over a recombinant protein A-Sepharose Fast Flow column (Pharmacia). The column was eluted with 20 mM citrate, pH 3.0. Concentrations of B7.1-Ig and B7.2-Ig were determined by spectrophotometry (280 nm), and relative concentrations, m.w., and purity of the fusion protein preparations were confirmed with SDS-PAGE.

T cell purification and stimulation

Splenic 2C/$RAG^{-/-}$ T cells used in these studies were purified using a two-step, negative selection protocol. First, spleens from 2C/$RAG^{-/-}$ mice were macerated and washed once in DMEM supplemented with 10% FCS (DMEM-10; Life Technologies, Gaithersburg, MD). Large pieces of debris were removed by filtering the suspension through sterile Nitex mesh (Tetko, Briarcliff Manor, NY). Adherent cells were then removed by adherence to tissue culture dishes (Costar, Cambridge, MA) at 37°C for 2 h. After the plate adherence step, the cells were harvested and washed once with DMEM-10. The harvested cells were incubated with a mixture of anti-CD4 (RL1724.4), anti-MAC-1, and anti-I-A^b at 4°C for 20 min. The cells were washed once in DMEM-10 and resuspended in DMEM without FCS (10 ml/spleen). Rabbit complement (Pel-Freeze, Brown Deer, WI) was added to the suspension at a final dilution of 1/10. This mixture was incubated at 37°C for 45 min. Dead cells were then removed by centrifugation over Ficoll-Hypaque, and the purity of the live T cells was determined by immunofluorescence using anti-TCR (1B2) and anti-CD8 (24). Purified T cells were counted and used in experiments.

In some experiments, previously stimulated T cells were studied. These effector T cell populations were generated by in vitro stimulation of purified primary 2C/$RAG^{-/-}$ T cells with P815/B7.1-H. Cultures were initiated by stimulating 5×10^4 purified splenic $CD8^+$ T cells (2C/$RAG^{-/-}$) with 3.5×10^5 stimulator cells (P815/B7.1-H) in 24-well tissue culture dishes (Costar) in the presence or the absence of exogenous cytokines. After 5–7 days in culture, the T cells were removed from culture wells and purified by centrifugation over Ficoll-Hypaque for use in experiments.

Proliferation assays and detection of lymphokine production

Purified T cells were stimulated with P815, P815/B7 transfectants, or plate-bound anti-CD3 mAb in the presence or the absence of immobilized B7.1-Ig or B7.2-Ig. For stimulation with P815 and P815/B7 transfectants, 5×10^3 purified T cells were stimulated in 96-well round-bottom plates (ICN Biomedicals, Aurora, OH) with varying numbers of stimulator cells that had been treated previously with mitomycin C (50 μg/ml/10^7 cells for 90 min at 37°C; Sigma, St. Louis, MO) to inactivate the cells. At various times after the initiation of cultures, 0.5 μCi of [^{3}H]thymidine was added to the cells in microwells for 8 h, at which time the plates were frozen and thawed, and the wells were harvested for determination of [^{3}H]thymidine incorporation using a Packard cell harvester and plate reader (Packard, Meriden, CT). For lymphokine production, 5×10^4 purified T cells were stimulated with 3.5×10^5 P815 cells or P815/B7 transfectants in 24-well plates (ICN Biomedicals). Supernatant was removed at 24 and 48 h to determine levels of IL-2 and IFN-γ, respectively.

For stimulation of T cells with anti-CD3 mAb and B7-Ig fusion protein, 96-well flat-bottom tissue culture plates (Costar) were precoated for 2 h at 37°C with varying concentrations of anti-CD3 and/or B7-Ig or control Ig (murine IgG2a; Zymed, South San Francisco, CA) in Dulbecco's PBS (Life Technologies) in a final volume of 50 μl/well. After washing the wells twice with Dulbecco's PBS, 2.5×10^4 purified T cells were added to each well, and the plates were centrifuged for 2 min at 500 $\times$ *g* and placed in the incubator at 37°C. Supernatants were removed at 24 and 48 h for determination of IL-2 and IFN-γ, respectively. The amounts of these lymphokines in the supernatants were determined by ELISA (Endogen, Cambridge, MA).

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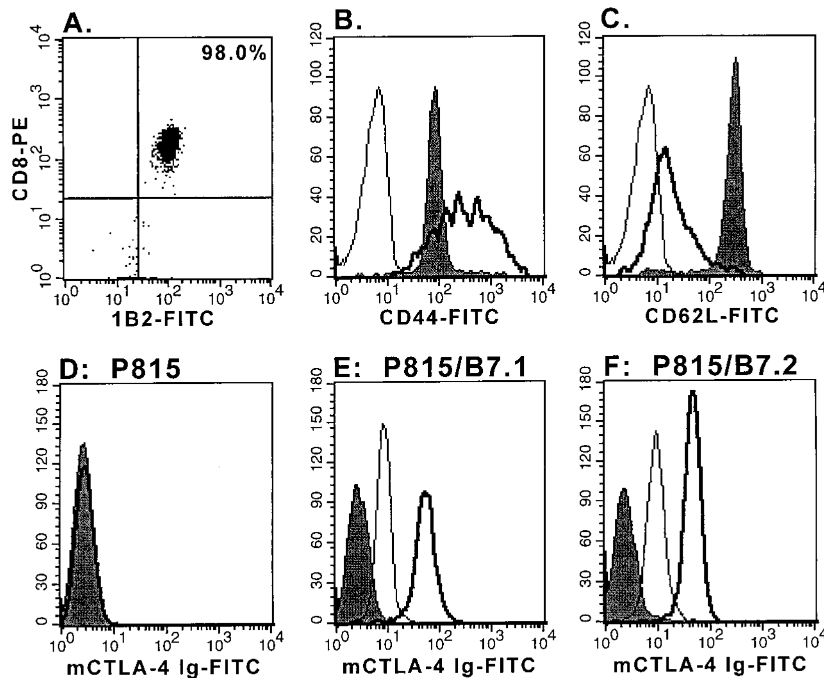

FIGURE 1. Flow cytometric analysis of 2C/RAG$^{-/-}$ T cells (*A–C*), P815 (*D*), and P815/B7 transfectants (*E* and *F*). Purified splenic 2C/RAG$^{-/-}$ T cells (*A*) were analyzed by two-color flow cytometry using anti-CD8 (*y*-axis) and 1B2 (anti-TCR clonotypic; *x*-axis). The percentage of cells expressing both is given in the *upper right* quadrant. Shown in *B* and *C* is expression of CD44 (*B*) and CD62L (*C*) on purified T cells either unstimulated (shaded figures) or stimulated for 24 h with P815 (heavy lines). Unstained unstimulated T cells are depicted by light lines (*B* and *C*). Mean fluorescence values for unstained resting or activated T cells were identical. A representative staining for splenic T cells purified from one animal is depicted. Parental P815 cells (*D*) were stained with FITC-coupled mCTLA-4 Ig. The shaded curve represents unstained cells, whereas the solid line figure is cells stained with mCTLA-4 Ig. Shown in *E* are P815/B7.1-L (light line) and P815/B7.1-H (heavy line) cell lines stained with mCTLA-4 Ig. The shaded figure is unstained P815/B7.1-L. Shown in *F* are P815/B7.2-L (light line) and P815/B7.2-H (heavy line) stained with mCTLA-4 Ig. The shaded figure is unstained P815/B7.2-L. Mean fluorescence values for unstained P815 and all P815/B7 transfectants were identical. Flow cytometry and T cell purification and stimulation were performed as described in *Materials and Methods*.

Results

Expression of cell surface markers on TCR transgenic T cells and transfected stimulator cells

To generate a model system in which all T cells had a naive phenotype and expressed the same TCR, 2C transgenic mice were bred to RAG-2 $^{-/-}$ mice. T cells from the 2C/RAG$^{-/-}$ mice express a transgenic TCR specific for the α-ketoglutarate dehydrogenase octapeptide, p2Ca; this TCR is detected by the clonotypic mAb, 1B2. The CD3$^+$ cells from these mice were 100% 1B2$^+$, confirming that the T cells both expressed the transgene and did not express detectable endogenously rearranged TCR genes (data not shown). Also, the 2C/RAG$^{-/-}$ splenocytes lacked IgM$^+$ cells, indicating that the animals were RAG-2 deficient (data not shown). After purification, the T cells used in experiments were >98% CD8$^+$1B2$^+$ by immunofluorescence analysis (Fig. 1*A*). Because the T cells of the mice were monoclonal and specific for an alloantigen, it was very unlikely that they had encountered a TCR stimulus in the periphery. Indeed, the splenic T cells from these mice were all CD44^{low} and CD62L^{high}, consistent with a naive phenotype (Fig. 1, *B* and *C*). Stimulation of these cells with the alloantigen-bearing cell line, P815, for 24 h in vitro induced an increase in CD44 and a decrease in CD62L expression (Fig. 1, *B* and *C*).

To control for the type and level of expression of B7 molecules presented to the T cells, P815 was transfected with either murine B7.1 or murine B7.2. Subclones of the transfectants were screened for B7 expression using a saturating concentration of FITC-coupled murine CTLA-4 Ig. The parental P815 line was negative for staining with CTLA-4 Ig, indicating that this cell line did not express endogenous B7.1 or B7.2 at detectable levels (Fig. 1*D*). Subclones of B7.1- and B7.2-transfected lines binding comparable amounts of CTLA-4 Ig were selected to use as stimulator lines. Two B7.1 transfectants and two B7.2 transfectants were selected that expressed either high or low levels of the molecule on the cell surface (P815/B7.1-H and P815/B7.1-L, and P815/B7.2-H and P815/B7.2-L, respectively). P815/B7.1-L and P815/B7.2-L expressed comparable levels of CTLA-4 Ig binding at the cell surface (Fig. 1, *E* and *F*). Likewise, P815/B7.1-H and P815/B7.2-H expressed comparable, yet higher, levels of CTLA-4 Ig binding at the cell surface (Fig. 1, *E* and *F*). The parental P815 and all P815 transfectants expressed comparable levels of the alloantigen, H-2L^d, as well as of ICAM-1 and LFA-1, as determined by immunofluorescence analysis (data not shown). Because these cell lines differed only in the type and amount of B7 molecule expressed at the cell surface, a quantitative assessment of the relative capacities of B7.1 and B7.2 to stimulate primary CD8$^+$ T cells could be made.

Primary stimulation of naive 2C/RAG$^{-/-}$ T cells

To determine whether B7.1 or B7.2 could equivalently costimulate primary T cells, purified naive 2C/RAG$^{-/-}$ T cells were cultured with either P815 or the transfectants expressing high or low levels of B7.1 and B7.2. Naive 2C/RAG$^{-/-}$ T cells stimulated with P815 did not proliferate appreciably (Fig. 2*A*). In contrast, the T cells did proliferate when B7 was expressed on the stimulator cells. However, quantitative differences were observed between B7.1 and B7.2 (Fig. 2, *A* and *B*). Although P815/B7.1-L and P815/B7.1-H cells stimulated comparable vigorous levels of proliferation, the

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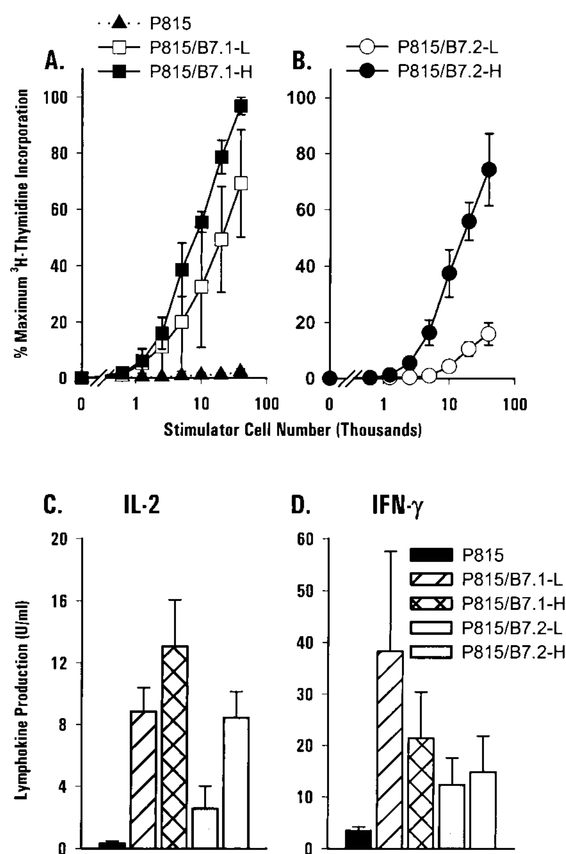

FIGURE 2. Primary stimulation of naive splenic 2C/RAG$^{-/-}$ T cells with P815 and P815/B7 transfectants. Purified 2C/RAG$^{-/-}$ T cells were stimulated, and [^{3}H]thymidine incorporation and lymphokine production were measured as described in *Materials and Methods. A* and *B*, [^{3}H]thymidine incorporation of 5×10^3 2C/RAG$^{-/-}$ T cells induced by varying numbers of P815, P815/B7.1-L, and P815/B7.1-H (*A*) and that induced by P815/B7.2-L and P815-B7.2-H (*B*) are shown. [^{3}H]Thymidine incorporation in each experiment was normalized to a percentage of the maximum response. Maximum [^{3}H]thymidine incorporation (cpm) measured at 72 h in the three experiments were 163,598, 109,783, and 94,220. *C* and *D*, In parallel cultures, 5×10^4 2C/RAG$^{-/-}$ T cells were stimulated for IL-2 (*C*) or IFN-γ (*D*) production with 3.5×10^5 P815 or P815/B7 transfectants. IL-2 and IFN-γ were measured at 24 and 48 h, respectively. Figures represent the mean ± SE of results from three independent experiments.

B7.2 transfectants were consistently inferior. In fact, stimulation with P815/B7.2-L cells resulted in levels of proliferation that were little above background in some experiments. Induction of IL-2 production paralleled proliferation, with the P815/B7.2-L transfectant stimulating marginally greater IL-2 secretion than the wild-type P815 cells (Fig. 2*C*). Induction of IFN-γ production by the T cells was slightly different. Both the B7.1 transfectants induced greater IFN-γ production than transfectants expressing comparable levels of B7.2; however, the differences were only around twofold (Fig. 2*D*). Collectively, these data suggest that B7.1 provides a quantitatively stronger costimulus than does B7.2 for IL-2 production and proliferation. Although B7.1 appeared to be more potent than B7.2 for inducing IFN-γ production, the differences were only about twofold.

A kinetic analysis was performed to assess the proliferation induced by B7.1 vs B7.2 costimulation over time. Proliferation induced by P815/B7.2-L was significantly less than that induced by P815/B7.1-L throughout the time course, demonstrating that the difference in proliferation initially observed could not be accounted for by differences in the kinetics of response (Fig. 3). The

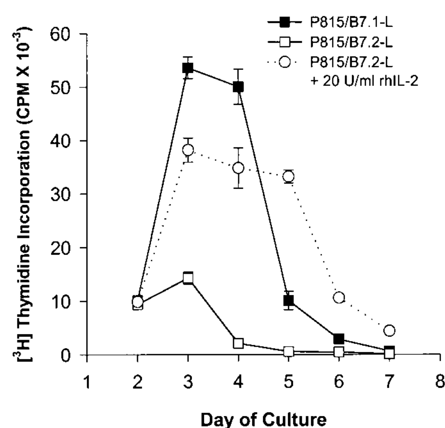

FIGURE 3. The effect of the addition of exogenous IL-2 on proliferation of 2C/RAG$^{-/-}$ T cells induced by P815/B7.2-L. Purified naive 2C/RAG$^{-/-}$ T cells (5×10^3) were stimulated with 1×10^4 P815/B7.1-L (■) or P815/B7.2-L with (□) or without (○) the addition of 20 U/ml recombinant human IL-2. [^{3}H]Thymidine incorporation was measured at various times after initiation of cultures as described in *Materials and Methods*. Depicted are the mean ± SE of triplicate cultures from one representative of three independent experiments.

inferior capacity of B7.2 transfectants to induce proliferation appeared to correlate with their decreased ability to induce IL-2 production. To test this hypothesis, we added exogenous IL-2 to cultures of T cells stimulated with P815/B7.2-L. Indeed, when exogenous IL-2 was added to P815/B7.2-L-stimulated T cells, the proliferative response of the T cells was increased to a level approaching that induced by P815/B7.1-L (Fig. 3).

Stimulation of naive CD8$^+$ T cells with anti-CD3 mAb in combination with B7.1-Ig or B7.2-Ig

Because the responses of the T cells to Ag and B7 costimulation might be influenced by other molecules on the surface of the stimulator cell, 2C/RAG$^{-/-}$ T cells were stimulated with the anti-CD3 mAb, 145-2C11, with or without B7.1-Ig or B7.2-Ig fusion proteins in an APC-free system. This system enabled us to compare B7.1 and B7.2 without potential effects of other molecules expressed on the surface of the stimulator cell line. Moreover, it allowed us to manipulate independently the intensity of the TCR/CD3 stimulus and the B7 costimulus.

Preliminary studies revealed that immobilization of the B7 fusion proteins was necessary to costimulate the T cells, because the addition of the B7 fusion proteins in soluble form caused no augmentation of IL-2 production or proliferation (data not shown). Stimulation of naive 2C/RAG$^{-/-}$ T cells with anti-CD3 mAb alone induced a proliferative response (Fig. 4*A*) and very little detectable IL-2 (Fig. 4*B*), but appreciable amounts of IFN-γ (Fig. 4*C*). The addition of either B7.1-Ig or B7.2-Ig increased all three responses in a dose-dependent fashion. However, B7.1-Ig was more potent than B7.2-Ig for induction of IL-2 production and proliferation when added at a concentration of 20 μg/ml (Fig. 4, *A* and *B*). Although IFN-γ production increased with the addition of either B7.1-Ig or B7.2-Ig, there was little difference between the two (Fig. 4*C*).

When T cells were stimulated with a fixed concentration of anti-CD3 mAb (2.0 μg/ml) plus varying concentrations of B7.1-Ig or B7.2-Ig, similar results were obtained. Low levels of proliferation were observed with anti-CD3 mAb alone, which were increased with the addition of either B7.1-Ig or B7.2-Ig (Fig. 4*D*). Anti-CD3 alone induced no detectable IL-2 (Fig. 4*E*) and low levels of IFN-γ

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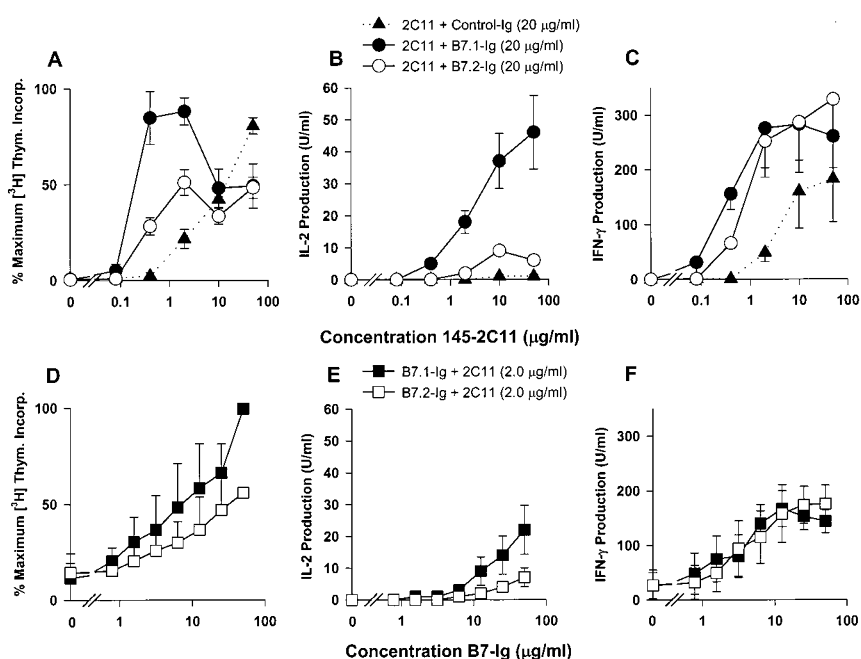

FIGURE 4. Primary stimulation of 2C/RAG$^{-/-}$ T cells with immobilized anti-CD3 mAb with or without immobilized B7-Ig fusion proteins. Naive splenic 2C/RAG$^{-/-}$ T cells were purified and stimulated in culture with anti-CD3 mAb, 145-2C11, with or without B7-Ig fusion proteins, immobilized to tissue culture plates as described in *Materials and Methods*. In *A*, *B*, and *C*, 2.5 × 10^{4} T cells were stimulated with B7.1-Ig or B7.2-Ig (each at 20.0 μg/ml) with or without varying concentrations of anti-CD3 mAb. In *D*, *E*, and *F*, 2.5 × 10^{4} T cells were stimulated with anti-CD3 mAb (2.0 μg/ml) with or without varying concentrations of B7.1-Ig or B7.2-Ig. [^{3}H]Thymidine incorporation was measured at 72 h (*A* and *D*). Supernatants were harvested at 24 h (IL-2) and 48 h (IFN-γ), and amounts of IL-2 (*B* and *E*) and IFN-γ (*C* and *F*) were determined by ELISA. [^{3}H]Thymidine incorporation in each experiment was normalized to a percentage of the maximum response. Maximum [^{3}H]thymidine incorporation (cpm) in the three experiments was 112,470, 151,390, and 162,926 (*A*) and 288,003, 200,200, and 155,703 (*D*). Depicted are the mean ± SE of results from three independent experiments (*A*–*F*).

(Fig. 4*F*), both of which were increased with the addition of either B7.1-Ig or B7.2-Ig. As observed in the previous experiments, B7.1-Ig induced significantly greater proliferation and IL-2 production than B7.2-Ig, whereas differences in IFN-γ induction were negligible (Fig. 4, *D*–*F*).

Stimulation with anti-CD3 mAb and B7-Ig allowed determination of whether a combination of B7.1 and B7.2 would be additive or synergistic. From this information, inferences about the nature of the signals generated by these two stimuli could be made. We reasoned that if the two molecules were synergistic in their ability to costimulate T cells, then the signals generated by the two molecules would surely be different. However, if the two were additive in their effects, this would suggest that B7.1 and B7.2 generate similar signals. 2C/RAG$^{-/-}$ T cells were stimulated with suboptimal concentrations of anti-CD3 mAb plus either B7.1-Ig or B7.2-Ig at concentrations that gave similar functional responses. The two molecules were additive in their ability to costimulate proliferation and IL-2 production at the anti-CD3 mAb concentrations tested (Table I). This suggests that the costimulatory signals for proliferation and IL-2 production generated by B7.1 and B7.2 are similar. A corollary to this is that the differences observed between B7.1 and B7.2 are quantitative in nature, owing to an inferior ability of B7.2 to costimulate proliferation and IL-2 production by primary CD8^{+} T cells.

Restimulation of previously activated T cells using B7.1 or B7.2

Naive and primed T cells may have different requirements for B7 costimulation. To compare the responses of naive T cells with those of previously stimulated cells, 2C/RAG$^{-/-}$ T cells were first stimulated with P815/B7.1-H for 6 days. T cells primed in this fashion (henceforth called secondary cells) were then restimulated with P815 or P815 transfected with B7.1 or B7.2. In contrast to naive T cells (Fig. 5*A*), secondary cells (Fig. 5*B*) had increased proliferative responses to both B7.1 and B7.2 transfectants. In addition, whereas B7.1 induced a significantly greater proliferative response than B7.2 by naive T cells, the secondary cells proliferated equivalently to P815/B7.1-L and P815/B7.2-L (Fig. 5*B*). Strikingly, while wild-type P815 induced no proliferation of naive cells, the secondary cells proliferated significantly in response to P815, apparently in the absence of B7 costimulation.

The differences between naive and secondary cell proliferative responses correlated with differences in IL-2 production by these two cell populations. Secondary cells produced more IL-2 than did naive cells whether stimulated with P815/B7 transfectants (Fig. 5*C*) or anti-CD3 mAb and B7-Ig fusion proteins (Fig. 6, *A* and *B*). In addition, the difference in IL-2 production induced by B7.1 vs B7.2 costimulation in naive cells was much less evident in secondary cells (Figs. 5*C* and 6, *A* and *B*). Also, in sharp contrast to naive cells, the secondary cells produced significant quantities of IL-2 in response to P815 or to anti-CD3 mAb alone, even without B7 costimulation (Figs. 5*C* and 6, *A* and *B*). Similar results upon secondary stimulation were observed when the primary stimulation was performed with P815/B7.2-H (data not shown). Neither proliferation nor IL-2 production by secondary cells induced by parental P815 could be blocked with CTLA-4 Ig, suggesting that activation of secondary T cells by P815 is truly B7 independent (data not shown). These results suggest that primed (secondary) T cells have a relaxed requirement for B7 costimulation, such that TCR ligation alone is sufficient to induce a substantial response, which is augmented comparably by either B7.1 or the weaker ligand, B7.2.

Some previous studies have suggested that costimulation of T cells with B7.2 could result in the preferential acquisition of IL-4-producing capacity compared with the effect of B7.1 (11, 12). To address this question, beginning with a homogeneous naive T cell population, 2C/RAG$^{-/-}$ T cells were stimulated with either P815/B7.1-H or P815/B7.2-H cells, each in the absence of exogenous cytokines or in the presence of recombinant murine IL-4 or recombinant murine IL-12. On day 6, T cells were harvested from each of the cultures for restimulation and determination of IL-4 or IFN-γ production (Fig. 7, *A* and *B*). T cells stimulated in the primary culture with B7.1, with or without the addition of exogenous IL-4 or IL-12, were restimulated in secondary cultures with B7.1 transfectants without the addition of any exogenous cytokines.

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Table I. *Costimulation of IL-2 production and proliferation by B7.1 and B7.2 is additive*

Concentration Anti-CD3 mAb (μg/ml)	Mean (± SEM)[a]			Measured Response
	B7.1 Ig (5 μg/ml)	B7.2 Ig (25 μg/ml)	B7.1 Ig + B7.2 Ig	
0.4	55.5 (± 4.9)	66.7 (± 6.7)	125.4 (± 10.5)	[^{3}H]Thymidine[b] incorporation
0.4	2.0 (± 0.4)	0.84 (± 0.3)	3.0 (± 0.3)	IL-2[c] production
10	9.4 (± 0.4)	5.2 (± 1.2)	13.3 (± 3.3)	

[a] Averages ± SE of three independent experiments.
[b] [^{3}H]Thymidine incorporation expressed as cpm × 10^{-3}.
[c] IL-2 values expressed as U/25,000 cells/ml.

Likewise, T cells initially stimulated with B7.2 transfectants were restimulated with B7.2 transfectants without the addition of exogenous cytokines. We found that CD8$^+$ T cells primed with either B7.1 or B7.2 in the absence of exogenous cytokines or in the presence of exogenous IL-12 produced only background levels of IL-4 upon restimulation (Fig. 7*A*). In contrast, T cells stimulated with either B7.1 or B7.2 transfectants in the presence of exogenous IL-4 produced increased amounts of IL-4 at similar levels upon restimulation (Fig. 7*A*). These data indicate that B7.1 and B7.2 were equivalent in their ability to prime naive T cells for IL-4 production when the T cells were stimulated in the presence of exogenous IL-4.

Production of IFN-γ upon restimulation of T cells stimulated in the primary culture with IL-12 was greater than that of T cells stimulated in the primary culture in the presence of IL-4 or in the absence of exogenous cytokines (Fig. 7*B*). This pattern of IFN-γ production was observed regardless of whether primary stimulation was conducted with B7.1 or B7.2 transfectants. From these observations, we conclude that both B7.1 and B7.2 can support the differentiation of naive CD8$^+$ T cells toward an IL-4-producing or IFN-γ-producing phenotype. Collectively, our results indicate that B7.1 and B7.2 are quantitatively, rather than qualitatively, different in their ability to costimulate T cells, and that this quantitative difference is particularly evident when a naive T cell population is studied.

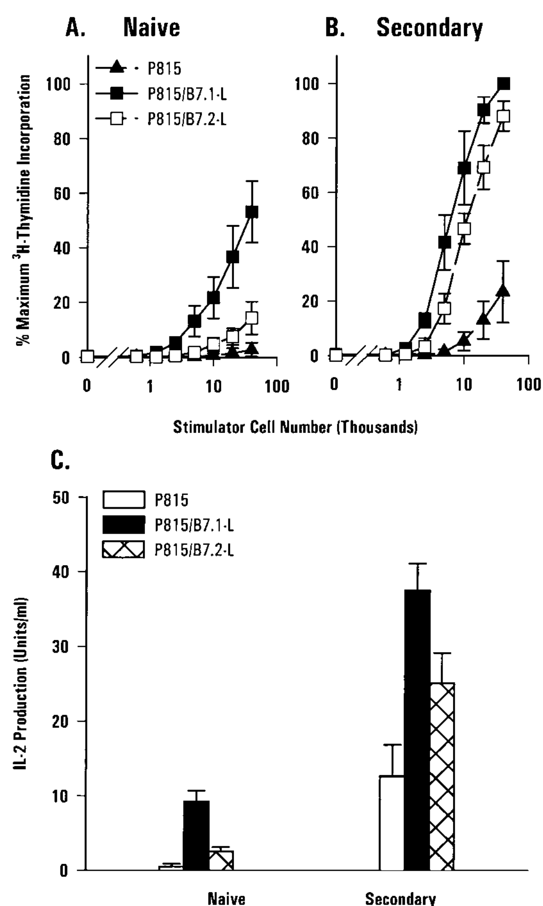

FIGURE 5. Stimulation of naive and previously activated 2C/RAG$^{-/-}$ T cells with P815 and P815/B7 transfectants. Secondary cells were produced by primary stimulation of naive T cells for 6 days with P815/B7.1-H as described in *Materials and Methods*. Isolation of naive 2C/RAG$^{-/-}$ T cells and stimulation of naive and secondary cells with P815- and P815-transfected stimulators are described in *Materials and Methods*. In *A* and *B*, [^{3}H]thymidine incorporation by 5 × 10^3 naive (*A*) and secondary (*B*) 2C/RAG$^{-/-}$ T cells stimulated with varying numbers of P815, P815/B7.1-L, and P815/B7.2-L is shown. [^{3}H]Thymidine incorporation in each experiment was normalized to a percentage of the maximum response. Maximum [^{3}H]thymidine incorporation (cpm) measured at 72 h in the three experiments was 50,170, 183,218, and 161,606 (*A* and *B*). *C*, In parallel cultures, 5 × 10^4 naive or secondary 2C/RAG$^{-/-}$ T cells were stimulated for IL-2 production with 3.5 × 10^5 P815, P815/B7.1-L, or P815/B7.2-L. Supernatants were harvested at 24 h, and IL-2 production was determined by ELISA. Figures represent the mean ± SE of results from three independent experiments (*A*–*C*).

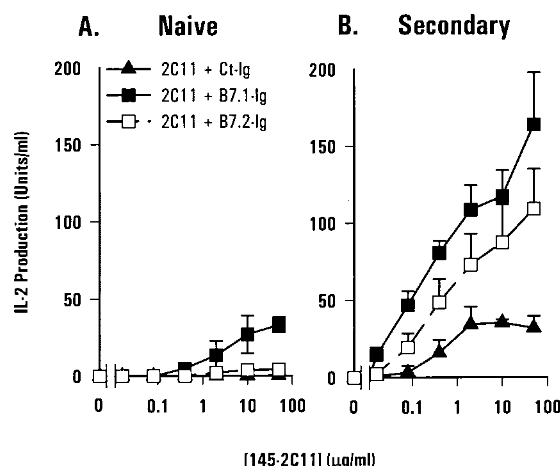

FIGURE 6. Stimulation of naive and previously activated 2C/RAG$^{-/-}$ T cells with immobilized anti-CD3 mAb with or without immobilized B7-Ig fusion proteins. Secondary cells were produced by primary stimulation of naive T cells for 6 days with P815/B7.1-H as described in *Materials and Methods.* Isolation of naive 2C/RAG$^{-/-}$ T cells and stimulation of naive and secondary 2C/RAG$^{-/-}$ T cells are described in *Materials and Methods*. In *A* and *B*, 2.5 × 10^4 naive (*A*) or secondary (*B*) 2C/RAG$^{-/-}$ T cells were stimulated with immobilized B7.1-Ig or B7.2-Ig (each at 20.0 μg/ml) in the presence or the absence of varying concentrations of immobilized anti-CD3 mAb. Supernatants were harvested at 24 h, and IL-2 was measured by ELISA. Figures represent the mean ± SE of results from three independent experiments.

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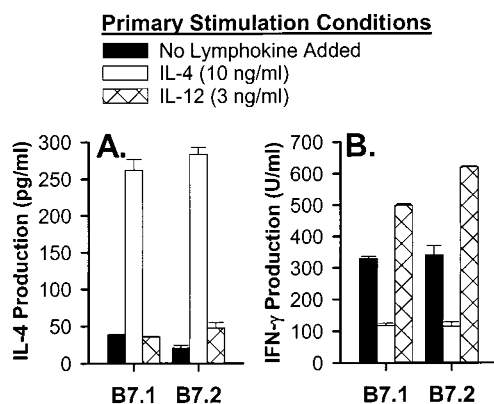

FIGURE 7. Differentiation, in vitro, of 2C/RAG$^{-/-}$ T cells into IL-4- or IFN-γ-producing CD8$^+$ T cells. Purified naive 2C/RAG$^{-/-}$ T cells (5×10^4) were stimulated in vitro with 3.5×10^5 P815/B7.1-H or P815/B7.2-H for 6 days with no added cytokines, with 10 ng/ml recombinant murine IL-4, or with 3 ng/ml recombinant murine IL-12. On day 6, the cells were washed to remove remaining cytokines and were restimulated without added cytokines with the same stimulator cell line as that used in the primary stimulation. At 24 and 48 h, supernatant was removed, and amounts of IL-4 (*A*) and IFN-γ (*B*) were determined, respectively, by ELISA. Figures show the amounts of IL-4 and IFN-γ ± SE of triplicate determinations. Shown is one representative of three independent experiments.

Discussion

In the present study, we analyzed the influence of B7.1 or B7.2 costimulation on the primary activation of naive CD8$^+$ T cells. Costimulation of naive CD8$^+$ T cells with B7.1 resulted in significantly greater IL-2 production and proliferation than costimulation with B7.2. This was true whether the T cells were stimulated with an Ag-bearing B7.1- or B7.2-expressing stimulator cell line or with immobilized anti-CD3 mAb and B7.1-Ig or B7.2-Ig fusion proteins. Several possible explanations could account for this observation. While B7.1 and B7.2 both interact with either CD28 or CTLA-4 on the surface of the T cell, an interaction with CTLA-4 is thought to deliver an inhibitory signal to the T cell, resulting in reduced T cell responses (25–27). If B7.2 preferentially triggered an inhibitory signal through CTLA-4, this might account for the reported differences. This explanation seems unlikely for two reasons. First, in the human system B7.1 has a higher affinity for CTLA-4 than does B7.2 (28). As this is likely to be the case in the murine system as well, it is expected that B7.1 would trigger a stronger inhibitory signal through interaction with CTLA-4. Second, in preliminary studies of T cells from TCR transgenic × CTLA-4$^{-/-}$ mice, eliminating the potential for interaction with CTLA-4, we found that B7.1-bearing stimulator cells still induced greater T cell proliferation than B7.2-bearing stimulators (data not shown). In addition, we found no significant differences in the ability of B7.1- or B7.2-bearing stimulator cells to induce CTLA-4 expression on T cells (M. Alegre and P. Fields, unpublished observations). Thus, it seems likely that the quantitative differences between B7.1 and B7.2 to promote T cell activation are attributable to differences in binding and stimulation via CD28.

In the human system, B7.1 binds to CD28 and CTLA-4 more avidly and with a slower off-rate compared with B7.2 (28). B7.2, which is a lower avidity ligand, would be expected to have a shorter occupancy time, resulting in the reduced probability of making a productive interaction with the receptor, thus resulting in a reduced capacity to stimulate the cell through that receptor. Several lines of evidence suggest that this is the case with B7/CD28 interactions. One recent study showed that B7.1 was significantly more effective than B7.2 at inducing the down-regulation of CD28 expression upon engagement of CD28 with comparable amounts of either ligand (29). While the functional importance of this phenomenon is unknown, the observation demonstrates a difference in a very early event that might depend on the strength of the interaction between the ligand and its receptor. In other studies using B7 fusion proteins that were mutated to affect their binding affinity to CD28, a direct correlation between strength of adhesion/binding and the ability to promote IL-2 production by T cells was demonstrated (30).

The strength or quality of the TCR signal might also influence the response of T cells to B7.1 and B7.2. Quantitative differences in B7.1 and B7.2 in inducing both proliferation and lymphokine production by T cells have been reported when the T cells were stimulated with a low-affinity weak agonist peptide, but not with a higher-affinity agonist peptide (31). These differences were attributed to the response of the T cells to the different affinity TCR ligands, a higher-strength TCR stimulus masking differences in the abilities of the two costimulatory molecules to induce T cell responses. In our present study, when the strength of TCR ligation was increased in the anti-CD3 mAb plus B7-Ig stimulation of T cells, we did not observe any diminishment of differences between B7.1 and B7.2. This may reflect a difference between stimulation with Ag/APC vs immobilized purified ligands.

Another determinant of the apparent strength with which B7 molecules influence T cell activation is the state of the responding T cell itself. As reported above, naive T cells respond differently to B7.1 and B7.2 costimulation, whereas activated T cells respond to the two molecules equivalently. It is notable that the primed T cells produced IL-2 and proliferated in response to a TCR stimulus alone, in the absence of B7. The transition of naive T cells to a primed phenotype apparently enables them to respond to subsequent TCR stimulation in a B7-independent manner. This may reflect an increased sensitivity of primed T cells to TCR ligation. In a recent study it was shown that CD44^{high}, Ly-6C$^+$ (memory) CD8$^+$ T cells were more sensitive than naive T cells (CD44^{low}, Ly-6C$^-$) to stimulation via the TCR, corroborating our findings (32). While our studies suggest that TCR ligation alone is sufficient for activation of primed T cells, it is formally possible that other CD28-independent molecules up-regulated on the T cell during priming could play a role in T cell activation via T-T contact.

We found that the effects of B7.1 and B7.2 on proliferation and IL-2 production were additive, suggesting that the signals generated by stimulation with the two molecules necessary for these responses are similar, consistent with quantitative, rather than qualitative, differences in B7.1- vs B7.2-induced cellular events. A clearer understanding of the early biochemical events induced by B7 costimulation will be necessary to definitively resolve this issue. In contrast with the findings of proliferation and IL-2 production, differences between B7.1 and B7.2 were less evident when IFN-γ production by the T cells was examined. B7.1- and B7.2-generated signals necessary for IL-2 production may be different from those that influence IFN-γ production, but the signaling threshold required for IFN-γ production by the T cell also may be lower than that required for IL-2 production. We have observed a similar phenomenon previously in anergic T cells (33). These and other data suggest that a hierarchy of signaling thresholds needed to be crossed to allow the transcription of different lymphokine genes (34). Because the quantity of signal required for IFN-γ may be less than that required for IL-2 production, costimulation provided by low levels of either B7.1 or B7.2 may be sufficient to enable maximum or near-maximum IFN-γ gene transcription.

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Previous studies reported differences in the abilities of B7.1 and B7.2 to drive differentiation of T cells into lymphokine-producing subsets. Naive T cells serially stimulated with B7.2-transfected stimulator cell lines were found to produce much more IL-4 after several rounds of stimulation (11). In our present study we found no difference after one restimulation. In fact, in our system neither B7.1 nor B7.2 costimulation induced differentiation of naive $CD8^+$ T cells into an IL-4-producing phenotype unless exogenous IL-4 was included in the primary stimulation. An alternative interpretation of the previous reports may relate to the differentiation state of the starting T cell population. Because naive T cells respond less well to B7.2 than to B7.1 costimulation, whereas activated T cells respond to the two equivalently, B7.2 costimulation might preferentially result in the expansion of previously activated T cells. If such previously activated T cells produce some IL-4, then preferential expansion of this IL-4-producing population might result in an apparent skewing of the T cell response toward a Th1/Th2 phenotype by selection. Because in our system very little IL-4 was produced in the absence of exogenous IL-4, no such apparent skewing was observed.

Our findings indicate that the differences in the abilities of B7.1 and B7.2 to costimulate naive $CD8^+$ T cells probably result from quantitative, rather than qualitative, differences in stimulation with these molecules. However, the activation state of the responding T cell has a profound effect on the ability of T cells to respond, such that primed T cells respond to B7.1 and B7.2 similarly. It appears that an activation-induced "resetting" of TCR stimulation and costimulation thresholds occurs in the T cells. An understanding of these events at a molecular level could have implications for understanding the generation of memory T cell responses.

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