with CD28 and CTLA-4 on T cells. A detailed analysis of these interactions will be required to provide an overall understanding of regulated T cell activation.

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Uncovering of Functional Alternative CTLA-4 Counter-Receptor in B7-Deficient Mice

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B7 delivers a costimulatory signal through CD28, resulting in interleukin-2 secretion and T cell proliferation. Blockade of this pathway results in T cell anergy. The in vivo role of B7 was evaluated with B7-deficient mice. These mice had a 70 percent decrease in costimulation of the response to alloantigen. Despite lacking B7 expression, activated B cells from these mice bound CTLA-4 and GL1 monoclonal antibody, demonstrating that alternative CTLA-4 ligand or ligands exist. These receptors are functionally important because the residual allogenic mixed lymphocyte responses were blocked by CTLA4Ig. Characterization of these CTLA-4 ligands should lead to strategies for manipulating the immune response.

In vitro and in vivo studies of T cell activation demonstrate that costimulatory signals delivered by antigen-presenting cells (APCs) are critical because their absence results in an abortive immune response (*1*). Although B7-transfected cells provide a potent costimulatory signal to T cells in vitro (*2–4*), the importance of B7 for regulating in vivo T cell responses has been inferred from studies with CTLA4Ig fusion protein (*5*). Blocking costimulation with CTLA4Ig prolongs graft survival. To evaluate the significance of B7, we generated a B7-deficient (−/−) mouse strain.

Murine B7 (mB7) is a 50- to 60-kD glycoprotein consisting of immunoglobulin V (Ig V)– and Ig C–like extracellular domains, a transmembrane region, and a short cytoplasmic tail (*3*). Analysis of mB7 genomic DNA has identified five exons organized 5′ to 3′: 5′ untranslated plus signal sequence, Ig V, Ig C, transmembrane, then cytoplasmic plus 3′ untranslated region together (*6*). To generate mice lacking B7, we designed a targeting vector (Fig. 1A) that replaces the B7 Ig V–like exon with the neomycin resistance gene (*neo*) (*7*). The Ig V–like exon was deleted because all antibodies blocking costimulation bind to the Ig V–like domain (*8*). Linearized B7 targeting vectors were transfected into the J1 embryonic stem (ES) cell line (*9*). We analyzed G418- and FIAU-resistant ES clones by DNA blot hybridization to identify clones in which the targeted homologous recombination event occurred. Hybridization of Bam HI–digested DNA with an external probe showed a 12-kb fragment from the wild-type locus and a 5.6-kb fragment from the targeted locus (Fig. 1). Homologous recombination occurred at a high frequency. Hybridization with a *neo* probe indicated that 95% of the clones were the result of a single integration event. ES clones carrying the B7 mutation were injected into BALB/c or C57BL/6J blastocysts (*10*) and were found to give germline transmission.

Interbreeding of B7 heterozygotes revealed that mice homozygous for the B7 mutation were viable. We examined organs

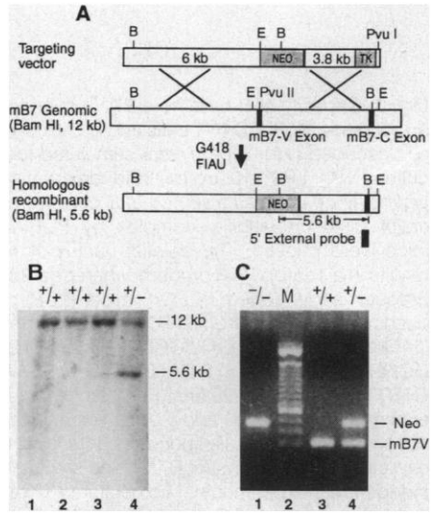

Fig. 1. (**A**) Schematic representation of the gene-targeting construct used to disrupt the B7 gene. Black boxes represent exons, and restriction sites are indicated. A targeting vector that allowed positive and negative selection (*7, 15*) was generated by insertion of the *neo* gene driven by the mouse phosphoglycerol kinase (PGK) promoter (*16*) into the Pvu II site in the Ig V–like exon. A 1.6-kb region (from Eco RI to Pvu II) 5′ of the *neo* insertion was subsequently excised, deleting the 5′ portion of the Ig V–like exon. The MC1 promoter–driven herpes thymidine kinase (TK) gene was incorporated at the 3′ end of the targeting vector to select against random insertion events (*7*). The targeting vector was linearized at a Pvu I site and transfected into the ES cell line J1 (*9*). G418^r and FIAU^r ES cell colonies were selected as described (*9*). FIAU, 1-[2-deoxy, 2-fluoro-β-D-arabinofuranosyl]-5 iodouracil (Eli Lilly). (**B**) Homologous recombination at the B7 locus. Genomic DNA was prepared from G418^r and FIAU^r ES clones and analyzed by Southern (DNA) blot with Bam HI and a 167–base pair (bp) 3′ end external probe. Sizes are indicated at the right. (**C**) Viable B7$^{-/-}$ homozygotes. PCR analysis of tail DNA utilized primers for the *neo* gene (ATTGAACAAGATGGATTGCAC and CGTCCAGATCATCCTGATC) and primers specific for the B7 Ig V–like exon (GTTGATGAACAACTGTCC and TTTGATGGACAACTTTACTA).

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and lymphocytes obtained from wild-type (+/+) and mutant (−/−) mice for B7 expression at the RNA and protein levels. RNA expression was analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR) with RNA prepared from thymus and lipopolysaccharide (LPS)-stimulated B cells of $B7^{+/+}$ and $B7^{-/-}$ animals. Analysis by RT-PCR and then nested PCR demonstrated the absence of B7 transcripts in $B7^{-/-}$ mouse-derived tissues and cells (Fig. 2A). When LPS and dextran sulfate–stimulated B cells from $B7^{+/+}$ and $B7^{-/-}$ mice were analyzed for B7 expression with B7 monoclonal antibody (mAb), expression was only observed in $B7^{+/+}$ cells (Fig. 2B), confirming a lack of B7 expression in the $B7^{-/-}$ mice. However, there was equivalent staining with CTLA4Ig on $B7^{+/+}$- and $B7^{-/-}$-activated B cells, demonstrating that additional ligand or ligands exist for CTLA-4 (Fig. 2C). The GL1 mAb stained $B7^{-/-}$-activated B cells (*11*) (Fig. 2D).

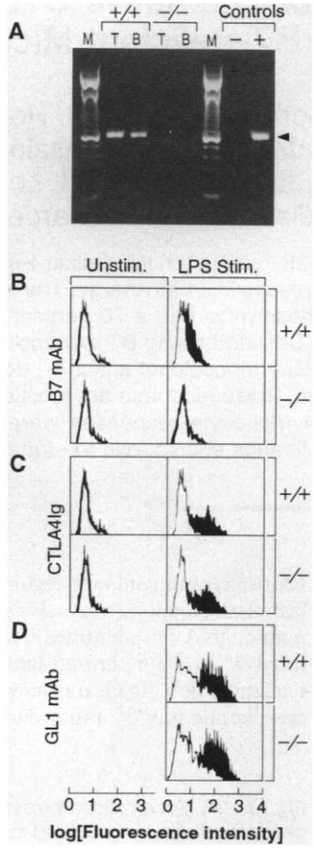

Fig. 2. (**A**) Analysis of B7 mRNA expression in $B7^{-/-}$ mice. Isolated splenic B cells were stimulated in vitro with LPS (10 μg/ml) for 3 days (*4*). RNA was prepared from spleen, thymus, and stimulated B cells with RNA-Stat-60 (Tel-Test "B," Friendswood, Texas). Random hexamer–primed reverse transcription was done. Nested PCR amplifications were done on the region spanning the signal sequence exon to the cytoplasmic domain exon. The primer sequences were CTGAGCTATGGCTTGCAATT and AATACCATGTATCCCACATGG for the primary PCR and ACAAGTGTCTTCAGATGTTGAT and CCAGGTGAAGTCCTCTGACA for the secondary PCR. Additional PCR studies confirmed the lack of intact B7 mRNA transcripts. T, thymus; B, LPS-stimulated B cells; controls, mB7 cDNA plasmid (+) and mock sample lacking RNA (−). (**B** through **D**) Fluorescence-activated cell sorting (FACS) analyses of B cells from $B7^{-/-}$ mice. Splenic B cells were stimulated in vitro with LPS (10 μg/ml) and dextran sulfate (20 μg/ml) and examined for B7 expression at 3 days poststimulation (*4*). Staining of LPS-stimulated B cells from a B7 (+/+) and (−/−) mouse are shown in the upper and lower panels, respectively. (B) Unshaded peak represents isotype-matched control mAb, hamster antibody to interferon-γ (H22) (*17*), and shaded peak represents staining of B7 with mAb 16-10A1 (*4*). (C) Unshaded peak shows staining with a control chimeric human Ig fusion protein (*2*), and shaded peak shows staining with human CTLA4Ig fusion protein (*2*). (D) Unshaded peak represents control rat IgG2a, and shaded peak represents staining with GL1 mAb (*11*).

Initial immunologic analyses of B7-deficient mice reveal normal numbers of thymocytes with normal maturation; normal numbers of splenic and lymph node $CD3^+$, $B220^+$, $CD4^+$, and $CD8^+$ cells; similar concentrations of serum immunoglobulins; and comparable responses of spleen cells to the B cell mitogen, LPS, and the T cell mitogen, concanavalin A (6).

Stimulatory activity of LPS- and dextran sulfate–stimulated B cells in an allogenic mixed lymphocyte response (MLR) is partially sensitive to mB7 mAb (*12*). To assess the stimulatory capacity of $B7^{-/-}$ APCs, we used $B7^{-/-}$ B cells that had been stimulated with LPS and dextran sulfate to stimulate an allogenic MLR. The MLR was reduced 70% compared with the response to $B7^{+/+}$ mice ($P < 0.025$, Student's t test). Addition of CTLA4Ig inhibited the MLR in both $B7^{+/+}$ and $B7^{-/-}$ mice, whereas control Ig had no effect (Fig. 3). These results indicate that additional CTLA-4 counter-receptor or -receptors are functional.

Characterization of the $B7^{-/-}$ mouse suggests that the absence of the B7 gene results only in a partial defect in the immune response. Naïve $B7^{-/-}$ mice have normal numbers of B and T cells and normal concentrations of serum immunoglobulins and respond normally to mitogens. Analysis of the B7-deficient mouse revealed a 70% reduction of costimulation of the response to alloantigen. The lack of a more dramatic phenotype can be explained by the existence of additional CTLA-4 counter-receptors. Inhibition of residual proliferation in the allogenic MLR by CTLA4Ig demonstrates that one (or more) of these ligands is functional. The expression of the GL1 molecule on activated $B7^{-/-}$ B cells suggests that GL1 is likely to be functionally important in vivo. Administration of GL1 mAb in vivo has inhibitory effects (*11*) in $B7^{+/+}$ mice. In addition, we have molecularly cloned a human, non-B7, CTLA-4–binding counter-receptor, termed B7-2 (*13*). This molecule costimulates T cells to secrete interleukin-2 and proliferate. The murine homolog of B7-2 is likely to account, at least in part, for costimulation observed in the B7-deficient mouse.

The biological relevance of additional CTLA-4 counter-receptors can only be defined in vivo. Further characterization of the B7-deficient mouse will enable us to determine the contribution of B7 to immunity and whether B7 functions to amplify, rather than initiate, a T cell immune response. Definitive understanding of non-B7 CTLA-4 counter-receptors awaits their molecular cloning and deletion in vivo. These results provide potential strategies and re-

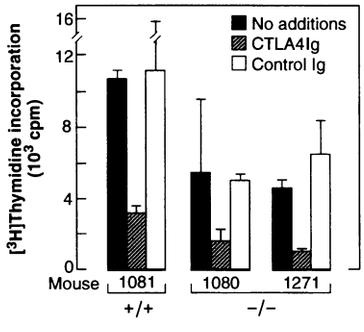

Fig. 3. Capacity of B cells from $B7^{-/-}$ mice to stimulate an allogenic MLR. $CD4^+$ T cells and B cells were purified as described (*18*). B cells were stimulated for 5 days in culture with LPS (10 μg/ml) and dextran sulfate (20 μg/ml). Mice used as a source of B cells were typed for major histocompatibility complex by PCR and $I\text{-}A^{b/b}$ mice were chosen. The deletion in Eα of $H\text{-}2^b$ mice results in a 155-bp PCR product, whereas a 780-bp PCR product is generated in $H\text{-}2^d$ mice (PCR primer sequences: Eα5′, 5′AGTCTTCCCAGCCTTCACACTCAGAGGTAC, Eα3′, 5′CATAGCCCAAATGTCTGACCTCTGGAGAG; and k5′, 5′CATGGGCATAGAAAGGGCAGTCTTTGAACT). FACS analyses demonstrated that the experimental B7 (+/+) and (−/−) mice had comparable concentrations of IA^b. Responder T cells were from BALB/c ($H\text{-}2^d$) mice. Data from a representative experiment are shown. Six $B7^{+/+}$ and six $B7^{-/-}$ mice were used as a source of stimulators in three independent experiments. Microcultures were set up in duplicate in 96-well plates with 10^5 $CD4^+$ T cells and mitomycin C–treated LPS–dextran sulfate–activated B cells. An experiment with 5×10^5 B cells per well is shown. Comparable differences in MLR were seen with 0.6×10^5 to 5×10^5 B cells per well. CTLA4Ig (5 μg/ml) or a control chimeric human fusion protein (5 μg/ml) was preincubated with B cells before the addition of $CD4^+$ T cells. Proliferation was measured by [^{3}H]thymidine incorporation for the last 6 hours of a 5-day assay.

agents for both the augmentation of immunity to microbes and tumor antigens and the inhibition of immune responses during transplantation and autoimmunity.

Note added in proof: The GL1 mAb reacts with the murine homolog of B7-2 (*14*).

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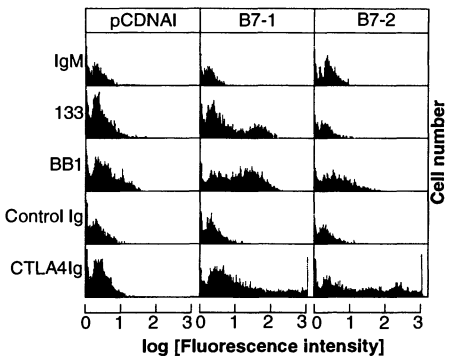

Fig. 1. B7-2 binds CTLA4Ig but not B7-1 mAb or B7-3 mAb. COS cells were transfected with cDNAs encoding B7-1 or B7-2 (clone 29) or with pCDNAI vector alone. Transfectants were stained with mAb 133 (*9*), mAb BB1 (*10*), control IgM mAb, recombinant CTLA4Ig (*12*), or control Ig fusion protein (*12*).

Cloning of B7-2: A CTLA-4 Counter-Receptor That Costimulates Human T Cell Proliferation

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Although presentation of antigen to the T cell receptor is necessary for the initiation of an immune response, additional molecules expressed on antigen-presenting cells deliver essential costimulatory signals. T cell activation, in the absence of costimulation, results in T cell anergy. The B7-1 protein is a costimulator molecule that regulates interleukin-2 (IL-2) secretion by signaling through the pathway that uses CD28 and CTLA-4 (hereafter referred to as the CD28 pathway). We have cloned a counter-receptor of CD28 and CTLA-4, termed B7-2. Although only 26 percent identical to B7-1, B7-2 also costimulates IL-2 production and T cell proliferation. Unlike B7-1, B7-2 messenger RNA is constitutively expressed in unstimulated B cells. It is likely that B7-2 provides a critical early costimulatory signal determining if the T cell will contribute to an immune response or become anergic.

After T cells receive a signal through the T cell receptor (TCR), the ligation of the CD28 and CTLA-4 receptors by the inducible counter-receptor B7-1 (originally termed B7) on antigen-presenting cells (APCs) results in IL-2 production and T cell proliferation (*1*, *2*). Because B7-1 has 20 times the affinity for CTLA-4 as it has for CD28, CTLA4Ig fusion protein is the most effective reagent for inhibiting the B7-1 CD28 costimulatory pathway (*3*–*5*). There is evidence for additional CTLA-4 counter-receptors (*6*, *7*). Activated human B lymphocytes express at least three distinct CTLA-4 counter-receptors, termed B7-1, B7-2, and B7-3 (*7*). Within 24 hours after activation, human B cells express a CTLA-4 counter-receptor (B7-2) that induces T cell proliferation (*7*). At 48 hours, two additional CTLA-4 counter-receptors are expressed including the original B7-1 identified by two monoclonal antibodies (mAbs) 133 (*8*, *9*) and BB1 (*10*) and a third CTLA-4 counter-receptor, B7-3, identified only by monoclonal antibody (mAb) BB1. These observations suggest that multiple CTLA-4 counter-receptors exist and costimulate T cells at different times after B cell activation.

A complementary DNA (cDNA) encoding a second CTLA-4 counter-receptor, termed B7-2, was isolated by cDNA expression cloning with CTLA4Ig selection from an activated human B cell cDNA library. COS cells transfected with B7-2 cDNA bound CTLA4Ig but did not bind to control immunoglobulin (Ig) fusion protein, mAb 133, or mAb BB1 (Fig. 1). In contrast, COS cells transfected with B7-1 bound to CTLA4Ig, mAb 133, and mAb BB1. Vector-transfected COS cells bound none of the above. This demonstrates that B7-2 encodes a CTLA-4 counter-receptor distinct from B7-1 and B7-3.

The B7-2 cDNA comprised 1120 nucleotides (GenBank accession number L25259) with a single, large open reading frame of 987 nucleotides. The NH_2-terminus of the B7-2 protein is a secretory signal peptide with a predicted cleavage between the alanines at positions 23 and 24. Processing at this site would result in a 34-kD membrane protein of 306 amino acids before modification. This protein would consist of a 220–amino acid extracellular region containing Ig superfamily V– and C–like domains, a hydrophobic transmembrane domain of 23 amino acids, and a cytoplasmic tail of 60 amino acids. The B7-2 extracellular domain contains eight potential N-linked glycosylation sites and, like B7-1, is probably heavily glycosylated. Comparison of both nucleotide and amino acid sequences of B7-2 with the GenBank and European Molecular Biology Laboratory databases has shown that only the human and murine B7-1 proteins are related. Alignment of the three B7 protein sequences shows that B7-2 has 26% identity with human B7-1 (Fig. 2). Because human B7-1, human B7-2, and murine B7-1 all bind to human CTLA-4 and CD28, conserved amino acids probably represent those necessary to form a CTLA-4 or CD28 binding site.

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