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21. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
22. By cells (3×10^7/ml) were incubated with or without anti-CD3 (2C11, 1 μg/ml) or anti-CD4 (Leu3a, 1 μg/ml), or both, for 10 min on ice. Rabbit antibody to mouse immunoglobulin G (anti-mouse IgG) (10 μg/ml) was added for cross-linking, and the cells were incubated for a further 10 min on ice and then incubated at 37°C for 2 min. The cells were pelleted by a pulse spin, washed once with phosphate-buffered saline, and lysed [lysis buffer contained 1% NP-40, 50 mM tris (pH 7.6), 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, leupeptin and aprotinin (10 μg/ml of each), and 2 mM phenylmethylsulfonyl fluoride]. After the nuclei were sedimented, the lysates were immunoprecipitated with anti-Shc (2 μg per 10^7 cell equivalents, Transduction Laboratories, KY) and 40 μl of 50% protein A agarose solution for 2 hours at 4°C. The beads were washed four times [0.1% NP-40, 20 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4, 5 mM NaF, and leupeptin and aprotinin (10 μg/ml of each)], analyzed by 6 to 12% SDS-PAGE, transferred to nitrocellulose and blotted with the anti-phosphotyrosine 4G10 (Upstate Biotechnology Inc., Lake Placid, NY), and developed by enhanced chemiluminescence (ECL) (Amersham). Similar results have been obtained with the antibody to TCR, F23.1
23. Biotinylated peptides (10 μg) from ζ chain (amino acid sequence GKGHDGLYQGLSTATKDTYDALH) (*21*) or CD3 ε chain (NPDYEPIRKGQRDLYSG) were synthesized as described (*16*) with the tyrosines either phosphorylated (denoted as ζ-P and ε-P, respectively) or nonphosphorylated (ζ and ε) and were incubated with 50 μl of streptavidin-agarose beads (Oncogene Sciences) for 1 hour on ice. The beads were incubated with 4% bovine serum albumin for 15 min, washed extensively, and incubated with lysates (1×10^7 cell equivalents) from unactivated T cells for 2 hours at 4°C. After the beads were washed, the bound peptides were resolved by SDS-PAGE and immunoblotted with anti-Shc.
24. Jurkat T cells were infected with vaccinia virus containing complementary DNA (cDNA) encoding CD16-ζ chimeric molecule (CD16 extracellular, CD7 transmembrane, and ζ cytoplasmic tail) for 6 hours at 37°C (*18*). Fluorescence-activated cell sorter (FACS) analysis showed that 50% of the cells had surface expression of CD16-ζ at 6 hours. Control Jurkat cells or CD16-ζ–expressing Jurkat cells (1.3×10^7 per sample) were stimulated as in Fig. 1A with or without anti-CD16 for 2 min at 37°C. Cells were lysed and the proteins were immunoprecipitated with anti-CD16 and protein G–agarose (Oncogene Sciences). The proteins were resolved by SDS-PAGE (10%) and immunoblotted with anti-Shc.

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Identification of an Alternative CTLA-4 Ligand Costimulatory for T Cell Activation

Karen S. Hathcock,* Gloria Laszlo, Howard B. Dickler, Jeff Bradshaw, Peter Linsley, Richard J. Hodes

Stimulation of T cell proliferation generally requires two signals: The first signal is provided by the T cell receptor binding to antigen, and the second signal or costimulus is provided by a different receptor-ligand interaction. In mouse and human, the CD28-B7 interaction has been identified as a source of costimulatory signals. We have identified a cell surface molecule (GL1) that is distinct from B7 and abundantly expressed on activated B cells. On activated B cells GL1, rather than B7, is the predominant ligand for the T cell–activation molecule CTLA-4. GL1 provides a critical signal for T cell–dependent responses in vitro and in vivo.

The CD28-B7 interaction is regarded as a critical costimulus for T cell activation (*1–3*). Consistent with this model, a soluble fusion protein of CTLA-4 (CTLA4Ig), a T cell surface molecule with a high affinity for B7 (*4*), inhibits T cell–dependent responses in vivo and in vitro (*5–9*). To identify additional cell surface molecules that provide costimulatory signals to T cells, we screened monoclonal antibodies (mAbs) from rats immunized with activated mouse B cells for the ability to inhibit T cell activation and to identify ligands for CTLA-4.

The GL1 mAb identified a determinant expressed minimally on unstimulated B cells but at high density on B cells activated by lipopolysaccharide (LPS) (Fig. 1), interleukin-5 (IL-5) (*10*), or antibody to immunoglobulin D (IgD) (anti-IgD) (*10*). The B7 mAb (*11*) minimally stained activated B cells but stained B7-transfected Chinese hamster ovary (CHO) cells brightly, whereas CTLA4Ig bound strongly to both activated B cells and B7-transfected CHO cells. GL1 mAb did not react with B7-transfected cells but reacted with activated B cells from B7-deficient mice (*12*), demonstrating that the GL1 product is not encoded by the B7 gene. The GL1 mAb also brightly stained dendritic cells (*13*) but only minimally stained activated T cells (*10*).

The cell surface molecule identified by GL1 mAb was immunoprecipitated from surface-iodinated, LPS-activated B cells and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). A broad band, 65 to 100 kD, was precipitated under both reducing (Fig. 2) and nonreducing condi-

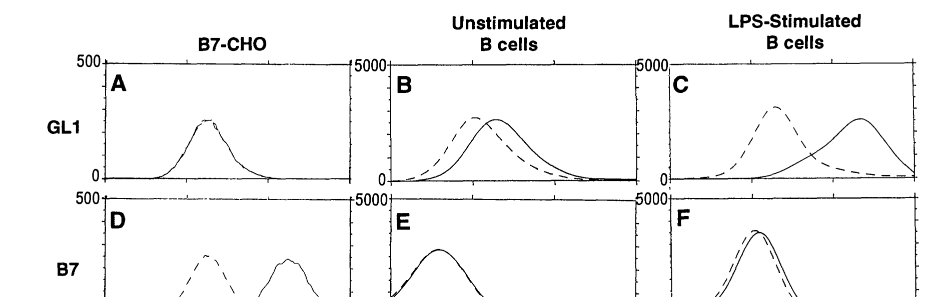

Fig. 1. Reactivity of GL1 mAb and B7 mAb with activated B cells. B7-transfected CHO cells (*14*) (**A, D,** and **G**) or T cell–depleted spleen cells that were either unstimulated (**B, E,** and **H**) or LPS-stimulated (**C, F,** and **I**) were stained with the GL1 mAb (A through C), hamster B7 mAb (D through F), (*11*), or CTLA4Ig (G through I) (*4*). The rat IgG2a hybridoma GL1 was produced by immunization with LPS-activated murine B cells, fusion, and selection as described (*15*). The DBA/2 spleen cells were T cell–depleted and cultured for 60 hours in medium alone or with LPS (15 μg/ml). Cells were stained with GL1 mAb (solid line) or control rat IgG2a (dashed line) (A through C), B7 mAb (solid line) or normal hamster Ig (dashed line) (D through F), or human CTLA4Ig (solid line) or CD7Ig (dashed line) (G through I). Cells were counterstained with antibody to B220. GL1 mAb reactivity with B cells was analyzed by electronic gating on $B220^+$ cells (*16*).

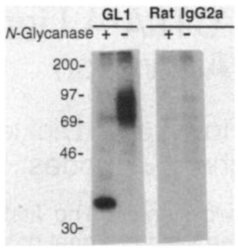

Fig. 2. Characterization of the cell surface molecule identified by GL1 mAb. Surface iodination of LPS-activated B cells, immunoprecipitation, *N*-glycanase treatment, and SDS-PAGE analysis under reducing conditions were carried out as described (*16*). Molecular sizes are indicated at left in kilodaltons.

Fig. 3. Effect of GL1 mAb on T cell activation. (**A**) APC-dependent T cell activation. The B10.A T cells (2×10^5) were cultured in the presence or absence of inhibiting Abs, in the presence of soluble anti-CD3 (4 μg/ml), and in the presence of 3×10^5 mitomycin-treated T cell–depleted spleen cells from mice that had been injected with goat antibody to mouse IgD. After 48 hours, wells were pulsed with [^{3}H]thymidine and harvested 12 hours later. (**B**) APC-independent T cell activation. T cells were cultured in the presence or absence of inhibiting antibodies in wells that had been precoated with anti-CD3 (8 μg/ml) in the absence of added APCs. Incorporation of [^{3}H]thymidine was assayed.

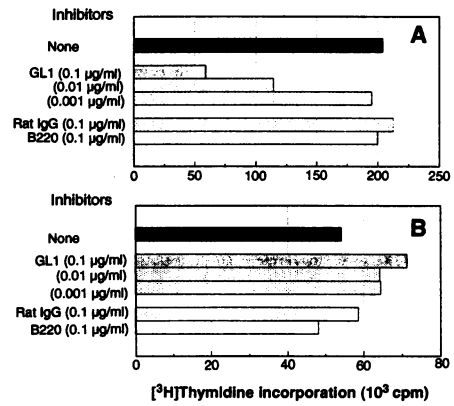

Table 1. Inhibition of in vivo T cell–dependent antibody response by GL1. Mice were treated with GL1 (50 μg) or control rat Ig intraperitoneally on the day before, the day of, and the day after immunization with FITC-MSA. Mice were bled 10 days later and serum titers to FITC were determined by enzyme-linked immunosorbent assay.

Treatment	Serum titer to FITC	
	IgG	IgM
Control	3400 ± 379	1833 ± 203
GL1	697 ± 184	1910 ± 218

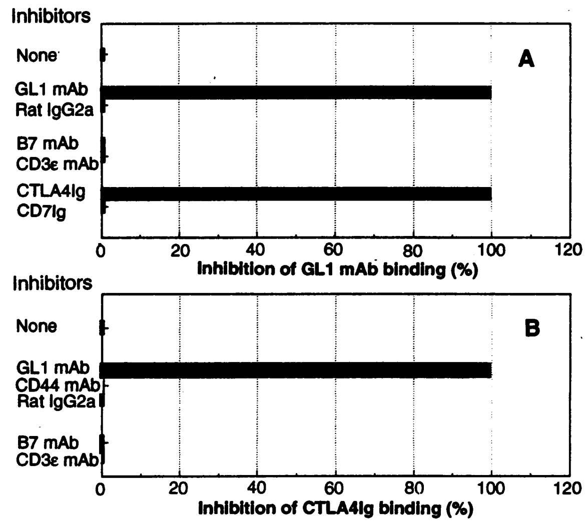

Fig. 4. GL1 is the predominant ligand for CTLA4Ig on activated B cells. (**A**) Inhibition of GL1 mAb binding on LPS-stimulated B cell blasts. LPS-activated B10.A B cells were incubated with 0.002 μg of FITC-conjugated GL1 mAb in the presence of 1 μg of GL1, normal rat IgG2a, hamster antibody to mouse B7 mAb, hamster CD3ε mAb, 0.5 μg CTLA4Ig, or control fusion protein CD7Ig (*4*). (**B**) Inhibition of CTLA4Ig binding on LPS-stimulated B cell blasts. LPS-activated B10.A B cells were incubated with CTLA4Ig (0.008 μg) in the presence of GL1 (1 μg), rat CD44 mAb, normal rat IgG2a, hamster antibody to mouse B7 mAb, or hamster CD3ε mAb. Cells were washed and incubated with FITC-conjugated mouse antibody to human Fcγ. Fluorescence and inhibition of fluorescence were calculated as described (*16*).

tions (*10*). The *N*-glycanase–treated product migrated more homogeneously with an apparent molecular mass of 35 kD. Cell surface GL1 thus appears to be a glycoprotein that is heterogeneous as a result of N-linked glycosylation.

The effect of GL1 mAb on T cell activation was examined under antigen-presenting cell (APC)–dependent or –independent conditions. The proliferative response of spleen T cells to soluble CD3 mAb (APC-dependent) was inhibited 60 to 80% by GL1 mAb but not by isotype-matched control antibody (Ab) (Fig. 3). Similar results were observed for T cell responses to stimulation from minor lymphocyte stimulating (Mls^a) superantigen and for responses of a T cell clone (*10*). In contrast, when T cells were stimulated with immobilized antibody to CD3 (anti-CD3) and no APCs, GL1 mAb did not inhibit proliferation (Fig. 3). Thus, GL1 mAb inhibited T cell proliferation only under conditions requiring signals provided by APCs. Production of IL-2 showed a pattern of inhibition similar to that observed for proliferation, whereas GL1 mAb had no effect on induction of the T cell–activation antigens CD69 and IL2Rα (*10*).

To test the possible function of GL1 in vivo, we treated mice with GL1 mAb or with control rat IgG and immunized them with fluorescein isothiocyanate conjugated to mouse serum albumin (MSA) (FITC-MSA). GL1 mAb treatment inhibited IgG responses to FITC (Table 1), indicating that costimulatory signals from GL1 can play a predominant role in vivo and that antibodies to the GL1 product or its human homolog have potential applications in manipulating immune responses in both normal and pathologic settings. The residual costimulatory activity observed in B7-deficient mice (*12*) is similarly consistent with an important functional role for additional costimulatory molecules such as GL1.

Reports that CTLA4Ig inhibits immune responses in vitro and in vivo (*5*–*8*) were interpreted as evidence that B7 is critical to these responses. However, it was not directly demonstrated that B7 is the relevant CTLA-4 ligand mediating costimulation under these conditions. Our results suggest the possibility that GL1 is an alternative ligand for CTLA-4. We found that the binding of FITC-conjugated GL1 mAb to activated B cells was specifically inhibited by CTLA4Ig or unconjugated GL1 mAb but not by control fusion protein or Ab (Fig. 4A). This indicates that GL1 identifies a ligand for CTLA4Ig. The relative contributions of B7 and the GL1 target molecule as ligands for CTLA-4 on activated B cells were evaluated next. The GL1 mAb inhibited greater than 95% of the binding of CTLA4Ig to LPS-activated B cells; in contrast, B7 mAb had minimal inhibitory effects when compared with control mAbs (Fig. 4B). As expected, B7 mAb effectively inhibited the binding of CTLA4Ig to B7-transfected CHO cells, whereas GL1 mAb had no effect (*10*). GL1, not B7, therefore appears to identify the major ligand for CTLA-4 on activated B cells.

Thus, the pathways for costimulatory signaling during immune responses may be complex, with GL1 or B7, or both, expressed on APCs potentially interacting

K. S. Hathcock, G. Laszlo, H. B. Dickler, R. J. Hodes, Experimental Immunology Branch, National Cancer Institute, and National Institute on Aging, National Institutes of Health, Bethesda, MD 20892.
J. Bradshaw and P. Linsley, Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA 98121.

*To whom correspondence should be addressed.

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

Gordon J. Freeman, Frank Borriello, Richard J. Hodes, Hans Reiser, Karen S. Hathcock, Gloria Laszlo, Andrew J. McKnight, Jinny Kim, Lina Du, David B. Lombard, Gary S. Gray, Lee M. Nadler, Arlene H. Sharpe*

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

G. J. Freeman, D. B. Lombard, L. M. Nadler, Division of Hematologic Malignancies, Dana-Farber Cancer Institute, and Department of Medicine, Harvard Medical School, Boston, MA 02115.
F. Borriello, A. J. McKnight, J. Kim, L. Du, A. H. Sharpe, Immunology Research Division, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115.
R. J. Hodes, K. S. Hathcock, G. Laszlo, Experimental Immunology Branch, National Cancer Institute and National Institute on Aging, National Institutes of Health, Bethesda, MD 20892.
H. Reiser, Division of Lymphocyte Biology, Dana-Farber Cancer Institute, and Department of Pathology, Harvard Medical School, Boston, MA 02115.
G. S. Gray, Repligen Corporation, Cambridge, MA 02139.

*To whom correspondence should be addressed.

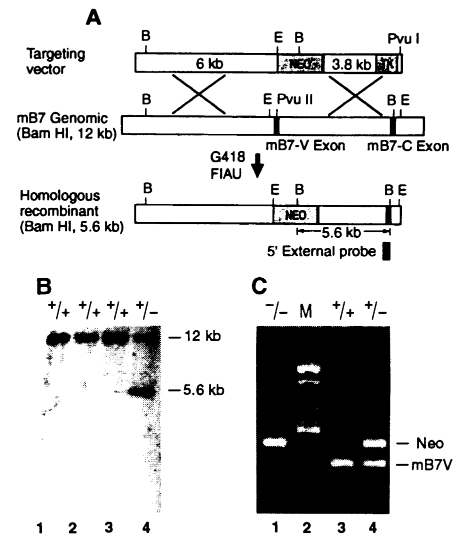

Fig. 1. (**A**) Schematic representation of the gene-targeting construct used to disrupt the B7 gene. Black boxes represent exons, and restriction sites are indicated. A targeting vector that allowed positive and negative selection (*7, 15*) was generated by insertion of the *neo* gene driven by the 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).