B7-H4, a Molecule of the B7 Family, Negatively Regulates T Cell Immunity

Gabriel L. Sica,[1,5] In-Hak Choi,[1,2,5] Gefeng Zhu,[1]
Koji Tamada,[1] Sheng-Dian Wang,[1] Hideto Tamura,[1]
Andrei I. Chapoval,[1] Dallas B. Flies,[1]
Jürgen Bajorath,[3,4] and Lieping Chen[1,*]
[1]Department of Immunology
Mayo Medical and Graduate Schools
Mayo Clinic
Rochester, Minnesota 55905
[2]Department of Microbiology
Inje University College of Medicine
Pusan 614-735
Korea
[3]Albany Molecular Research, Inc.
Bothell Research Center
Bothell, Washington 98011
[4]Department of Biological Structure
University of Washington
Seattle, Washington 98195

Summary

We identify a B7 family molecule, B7-H4, by protein sequence analysis and comparative molecular modeling. While B7-H4 mRNA is widely distributed in mouse and human peripheral tissues, cell surface expression of B7-H4 protein is limited and shows an inducible pattern on hematopoietic cells. Putative receptor of B7-H4 can be upregulated on activated T cells. By arresting cell cycle, B7-H4 ligation of T cells has a profound inhibitory effect on the growth, cytokine secretion, and development of cytotoxicity. Administration of B7-H4Ig into mice impairs antigen-specific T cell responses whereas blockade of endogenous B7-H4 by specific monoclonal antibody promotes T cell responses. B7-H4 thus may participate in negative regulation of cell-mediated immunity in peripheral tissues.

Introduction

B7-like molecules belong to the immunoglobulin (Ig) superfamily. Based on amino acid sequence and protein structure analysis, B7-like molecules can be distinguished from other members within an extended B7 family (Linsley et al., 1994). The extracellular portion of B7-like molecules contain single IgV and IgC domains and share ∼20%–40% amino acid identity. Upon binding to their counterreceptors on T cells, B7-like molecules play critical roles in the control and fine tuning of antigen-specific immune responses. Interactions between B7-1 (CD80)/B7-2 (CD86) ligands to CD28/CTLA-4 (CD152) receptors represent a classical pathway. B7s costimulate growth, prevent anergy, and promote survival of T cells via CD28 receptor. Binding of B7s to CTLA-4, however, inhibits T cell responses by delivering

*Correspondence: chen.lieping@mayo.edu
[5]These authors contributed equally to this work.

a putative negative signal (Chambers et al., 2001). Manipulation of this pathway has profound effects on both humoral and cell-mediated immune responses. For example, B7-1/B7-2 gene transfer or administration of CTLA-4 neutralizing antibody promotes tumor immunity (Hurwitz et al., 2000) while blockade of the pathway by B7-1/B7-2 neutralizing mAb or CTLA-4Ig inhibit autoimmune diseases and transplantation rejection (Salomon and Bluestone, 2001).

A new B7-H1/B7-DC/PD-1 pathway was established recently. B7-H1 was identified by EST database searching based on their homology to B7s. Ligation of T cells by B7-H1 moderately costimulates growth and preferential secretion of IL-10 and interferon-γ in vitro, and promotes antibody and T helper cell responses in vivo (Dong et al., 1999; Tamura et al., 2001). B7-DC, a molecule with high homology to B7-H1, is originally identified by a subtractive hybridization method searching for dendritic cell specific transcripts (Tseng et al., 2001). Both B7-H1 and B7-DC are capable of binding to programmed death 1 (PD-1) receptor (Freeman et al., 2000, Latchman et al., 2001). PD-1-deficient mice spontaneously develop systemic and organ-specific autoimmune diseases (Nishimura et al., 1999), strongly supporting a role of PD-1 in negative regulation of T cell responses. While both B7-H1 and B7-DC were found to be costimulatory for naive T cells (Dong et al., 1999; Tseng et al., 2001), other laboratories showed that B7-H1 and B7-DC inhibited proliferation of activated T cells (Freeman et al., 2000; Latchman et al., 2001). Because it was proposed that both B7-H1 and B7-DC functioned through PD-1, these molecules were renamed as PD-L1 and PD-L2, respectively, to reflect this association (Freeman et al., 2000; Latchman et al., 2001), This hypothesis, however, fails to integrate all experimental findings. T cell costimulation by B7-H1 is likely mediated through a different receptor (Dong et al., 2002). This is best indicated by the fact that mutants of B7-H1 and B7-DC, which lose their ability to bind PD-1, remain costimulatory for T cells (Wang et al., 2003), and both B7-H1 and B7-DC can costimulate PD-1$^{-/-}$ T cells (Wang et al., 2003; Shin et al., 2003). Importantly, B7-H1 expresses constitutively on the majority of human cancers (Dong et al., 2002) and some mouse tumor lines (Iwai et al., 2002), promotes apoptosis of activated human T cells (Dong et al., 2002), and renders resistance of mouse tumors to T cell attack (Dong et al., 2002; Iwai et al., 2002).

B7h/B7RP-1 (B7-H2, GL-50, ICOS ligand, LICOS) was independently identified by at least six laboratories (Carreno and Collins, 2002). Considerable interest was raised when mouse (Yoshinaga et al., 1999; Mages et al., 2000) and human (Wang et al., 2000; Ling et al., 2000) receptor for B7h/B7RP-1 was identified as the inducible costimulator (ICOS) (Hutloff et al., 1999). Molecular modeling demonstrates that ICOS has a structure similar to CD28 (Wang et al., 2002) and is expressed primarily on the surface of activated T cells and resting memory T cells (Hutloff et al., 1999). Interaction of ICOS and B7h/B7RP-1 plays critical roles in the regulation of T helper-dependent antibody production, Th1 and Th2 cytokine

A

mouse MASLGQIIFWSIINIIIILAGAIALIIGFGISGKHFITVTTFTSAGNIGE
human MASLGQILFWSIISIIIILAGAIALIIGFGISGKHSITVTTVASAGNIGE

mouse DGTLSCTFEPDIKLNGIVIQWLKEGIKGLVHEFKEGKDDLSQQHEMFRGR
human DGILSCTFEPDIKLSDIVIQWLKEGVLGLVHEFKEGKDELSEQDEMFRGR

mouse TAVFADQVVVGNASLRLKNVQLTDAGTYTCYIRTSKGKGNANLEYKTGAF
human TAVFADQVIVGNASLRLKNVQLTDAGTYKCYIITSKGKGNANLEYKTGAF

mouse SMPEINVDYNASSESLRCEAPRWFPQPTVAWASQVDQGANFSEVSNTSFE
human SMPEVNVDYNASSETLRCEAPRWFPQPTVVWASQVDQGANFSEVSNTSFE

mouse LNSENVTMKVVSVLYNVTINNTYSCMIENDIAKATGDIKVTDSEVKRRSQ
human LNSENVTMKVVSVLYNVTINNTYSCMIENDIAKATGDIKVTESEIKRRSH

mouse LQLLNSGPSPCVFSSAFVAGWALLSLSCCLMLR
human LQLLNSKASLCVSS-FFAISWALLPLSPYLMLK

B

C

D

BED

A'GF
CC'C"

hCD86   hCD86   hB7-H4

Figure 1. Sequence Analysis and Molecular Models of B7-H4

(A) Amino acid alignment of mouse and human B7-H4. Gray shading indicates amino acid identity. −, signal peptide; =, transmembrane domain. Alignment was performed with ClustalW algorithm in MacVector 6.5.

(B) Amino acid comparison of human B7-H4 with the B7 family molecules. Only extracellular domains without signal peptides were compared. IgV domain, … ; IgC doman, =. The asterisks indicate conserved cysteines important for intrachain disulfide bonds. Dark gray indicates amino acid identity. Light gray indicates amino acid similarity. Comparisons have been done with ClustalW algorithm in MacVector 6.5.

(C) Structure-oriented sequence alignment. Shown is an alignment of the sequences of the IgV domains of human and mouse B7-H4 relative to human CD80 and CD86. This alignment is based on superposition of CD80 and CD86 and the conservation of structural key residues of the IgV-fold. β strands seen in CD80 and CD86 are labeled according to Ig superfamily conventions (A′-G). Residue positions that are

secretion, and cell-mediated immune responses in mouse models (Carreno and Collins, 2002). Recent studies in the patients with common variable immunodeficiency, however, indicate that homozygous loss of ICOS does not lead to defective T cell responses, although B cell responses were compromised (Grimbacher et al., 2003). More recently, a new B7-like molecule, B7-H3, has been identified (Chapoval et al., 2001). B7-H3 costimulates T cell proliferation, selectively induces IFN-γ, and promotes growth and differentiation of CD8$^+$ CTL against allogeneic antigens, indicating that B7-H3 may preferentially costimulate the cellular immune response. In this report, we describe B7-H4, a B7-like molecule with profound inhibitory function for T cell responses.

Results

Cloning and Sequence Analysis of B7-H4

When a human expressed sequence tagged (EST) database was queried with sequences of the IgV and IgC domains of the B7 family molecules, a partial EST sequence was identified. Subsequent searching of the database with the partial fragment led to additional EST sequences that overlapped the initial fragment. Using this strategy, a nucleotide sequence encoding the full-length *B7-H4* was identified and cloned using placenta cDNA as a template. The *hB7-H4* sequence encodes a putative protein of 282 amino acids with several potential N-glycosylation sites in the extracellular domain. The amino acid sequence of hB7-H4 contains a large hydrophobic transmembrane domain and a very short intracellular domain comprised of only 2 amino acids (Figure 1A). Predicted hB7-H4 is a type I transmembrane protein.

A BLAST search of the NCBI databases with *hB7-H4* sequence revealed a partial EST sequence corresponding to the 5′ sequence of the mouse *B7-H4* (*mB7-H4*) ortholog (accession number AI155439). Using a primer designed from this partial EST sequence and several primers derived from the 3′ end of the *hB7-H4* sequence, a PCR fragment containing the entire mouse B7-H4 sequence was obtained from mouse spleen cDNA. The mouse and human amino acid sequences have approximately 87% amino acid identity (Figure 1A). The level of identity between the mouse and human orthologs suggests that this molecule is highly conserved evolutionarily.

Structure Analysis of B7-H4

B7-H4 has only about 25% amino acid homology in the extracellular portion with other B7 family members (Figure 1B). Intrachain disulfide bond formed by cysteines are all conserved between B7-H4 and the other B7 family members. Since the IgV domain of the B7 family is primarily involved in the binding with the counterreceptor (Stamper et al., 2001; Schwartz et al., 2001), we analyzed the IgV domain of human and mouse B7-H4 by computer modeling. Figure 1C shows the structure-based V domain sequence alignment on which CD86 was used as the template. The V domain regions of human and mouse B7-H4 are highly homologous (91% sequence identity), whereas this region in B7-H4 shares only ∼23% sequence identity with both CD80 and CD86. In CD80 and CD86, the V domains contain the CTLA-4/CD28 binding site, and the binding site region is largely conserved in these molecules and maps to the A′GFCC′C″ face (Figure 1D). In addition, corresponding regions adjacent to but distinct from the receptor binding site mediate homodimerization of CD80 and CD86 (Figures 1C and 1D). We have generated molecular models of human (Figure 1D) and mouse B7-H4 (data not shown) in three dimensions relative to CD80/CD86. Many core or Ig superfamily consensus residue positions are conserved, or conservatively replaced, in CD80/CD86 and B7-H4, leaving little doubt that the structures of these molecules are similar overall. Essentially no residues conserved in CD80/CD86 and B7-H4 map to the A′GFCC′C″ face of B7-H4, thus predicting that B7-H4 does not bind CTLA-4 or CD28. Furthermore, only a few residues that differ in human and mouse B7-H4 map to the surface of their V domain (colored gold in Figure 1D), including the region that spatially corresponds to the receptor binding site in CD80/CD86 on the A′GFCC′C″ face. This suggests that human and mouse B7-H4 are likely to display cross-species receptor binding.

Expression Pattern of B7-H4

Northern blot analysis of B7-H4 indicates that hB7-H4 mRNA is expressed in spleen, lung, and thymus. The most prominent transcript for B7-H4 is approximately 1.8 kb (Figure 2A). By sensitive RT-PCR analysis, B7-H4 mRNA is also found in placenta, liver, skeletal muscle, kidney, pancreas, prostate, testis, ovary, and small intestine (Figure 2B). Immunohistochemistry analysis using a human B7-H4 specific mAb (hH4), however, does

---

conserved, or mostly conserved, in the compared sequence are shaded. Among these, the most important IgV-fold consensus residue positions (important for maintaining structural integrity) are labeled with asterisks. Potential N-linked glycosylation sites are boxed. Residue positions labeled with # participate in the formation of the homodimer interface seen in the X-ray structure of CD86 in complex with CTLA-4. CD86 residues that participate in CTLA-4 binding are labeled with exclamation marks and used for binding site mapping in (D). Residue numbers are given for human B7-H4.

(D) Mapping of conserved residues. The V domain of the X-ray structure of hCD86 (light blue) and the molecular model of hB7-H4 (white) are displayed with molecular surfaces and shown in two orientations. The top view focuses on the BED β-sheet surface of the domains and the bottom on the opposing A′GFCC′C″ surface (related by 180° rotation around the vertical axis). CD86 residues important for CTLA-4 binding are colored red (bottom left), and those residues that delineate the homodimerization region in CD80 and CD86, as seen in their crystal structures with CTLA-4, are shown in magenta (top left). In contrast to the receptor binding site, the dimer interface maps to the BED face of the domain. For residues involved in CTLA-4/CD28 binding and dimerization, see also (C). In the middle images, residues that are identical in hCD80 and hCD86 are colored dark blue. On the right, residues that are conserved in CD80, CD86, and B7-H4 are also colored dark blue. In addition, residues that are different in human and mouse B7-H4 are shown in gold. As can be seen, only a few nonconserved residue positions map to the surface of B7-H4.
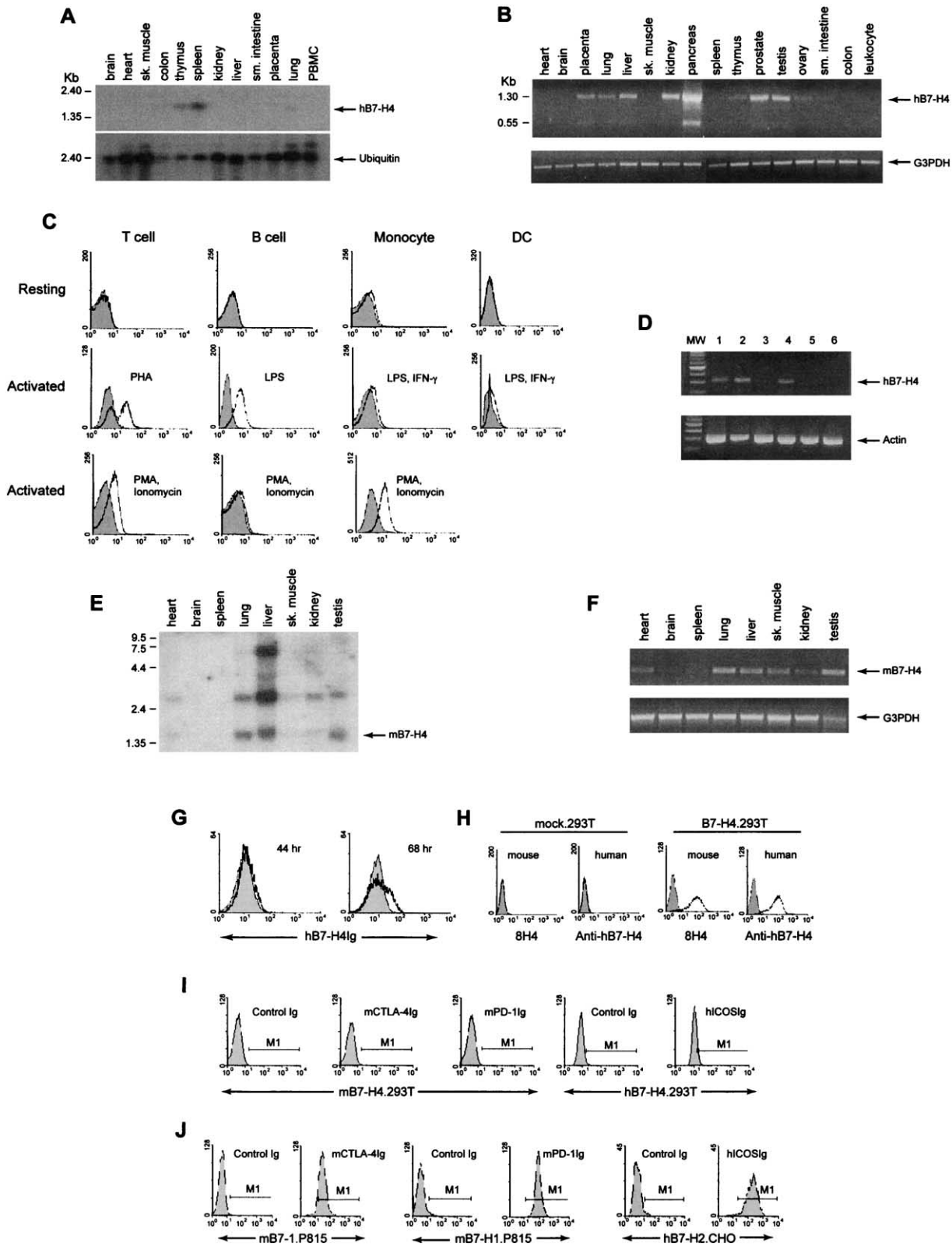
Figure 2. Expression and Putative Receptor of B7-H4

(A) Northern blot of hB7-H4 mRNA using human multiple tissue panels.
(B) PCR analysis of hB7-H4 expression using human multiple cDNA panels.
(C) FACS analysis of B7-H4 expression on the human B cells, T cells, monocytes, and DC stimulated with LPS, PHA, IFN-γ, PMA, or ionomycin for 3 days. Cells were stained with hH4 mAb.

not reveal positive staining for B7-H4 in all organs from normal individuals, including lung, colon, liver, skeletal muscle, kidney, pancreas, small bowel, breast, and uterus (data not shown). Likewise, freshly isolated human T cells, B cells, monocytes, and DC do not express B7-H4 on cell surface in FACS analysis. In contrast, B7-H4 expression can be induced on T cells, B cells, monocytes, and DC after in vitro stimulation (Figure 2C). Monocyte-derived DCs do not contain a transcript for B7-H4 while these DC express B7-H4 after stimulation with PMA and ionomycin. Human aortic endothelial cells do not express B7-H4, even after TNFα (Figure 2D) and IFN-γ (data not shown) stimulation by both FACS and RT-PCR analysis. Probing of a mouse tissue Northern blot indicated that mB7-H4 has up to four different mRNA transcripts, depending on the tissue type. The liver has both the highest level of B7-H4 and all four transcripts, varying in size from 7.5, 4, 2.6, and 1.8 kb (Figure 2E). Mouse B7-H4 transcript was detected in the heart, lung, liver, skeletal muscle, kidneys, and testis but not in brain and spleen. RT-PCR analysis, however, demonstrates the mRNA in all these organs (Figure 2F). Detection of high level B7-H4 mRNA in liver is likely due to constitutive level of the RNA in hepatocyte because all lymphocytes isolated from liver were negative by RT-PCR (data not shown).

Inducible Expression on T Cells of B7-H4 Putative Receptor

To test whether T cells express B7-H4 receptor, we first constructed vectors to express fusion proteins consisting of the extracellular domain of human or mouse B7-H4 fused to the Fc portion of mouse IgG2a or human IgG1, respectively (Chapoval et al., 2002). The purified recombinant fusion proteins were used to detect the presence of a putative B7-H4 receptor on T cells via FACS analysis. Although hB7-H4Ig did not bind freshly isolated human T cells (data not shown), 20%–30% of T cells upon stimulation with anti-CD3 mAb were stained positive (Figure 2G). This result suggests that activated human T cells express a putative receptor for B7-H4.

To determine whether B7-H4 binds to other B7 receptors, we first established 293T transfectants expressing hB7-H4 (hB7-H4.293T) or mB7-H4 (mB7-H4.293T) and examined the binding of CTLA-4Ig, ICOSIg, and PD-1Ig to these cells by FACS. Expression of B7-H4 on B7-H4-transfected, but not mock-transfected, 293 cells was confirmed by mAb specific for either mouse or human B7-H4 (Figure 2H). In FACS analysis, CTLA4Ig, ICOSIg, and PD-1Ig did not bind B7-H4 transfectants (Figure 2I), although these Ig fusion proteins are able to bind cells

transfected with B7-1, B7-H2, and B7-H1, respectively (Figure 2J). Our data indicates that B7-H4 binds to a receptor on activated T cells, which is distinct from CTLA-4, ICOS, or PD-1.

Inhibition of T Cell Responses by B7-H4 In Vitro

Using immobilized anti-CD3 mAb as antigen mimic, we tested the effect of B7-H4 ligation on T cell responses. Stimulation by an anti-CD3 mAb on freshly purified, naive CD4$^+$ T cells from DO11.10 TCR transgenic mice led to the proliferation of T cells in a dose-dependent fashion. Inclusion of immobilized mB7-H4Ig but not control Ig in the beginning of the cultures significantly inhibited T cell proliferation. Inclusion of immobilized B7-H4Ig did not affect the binding of anti-CD3 mAb on plastic plate (data not shown), thus excluding a nonspecific effect of B7-H4Ig. In the identical setting, immobilized B7-1Ig costimulated the growth of T cells (Figure 3A). Soluble mB7-H4Ig up to 20 μg/ml did not have an inhibitory effect in this assay (data not shown), which excludes the possibility of nonspecific cytotoxicity of B7-H4Ig and also suggests that crosslinking of B7-H4 is required for its inhibitory effect. Similarly, dose-dependent inhibition by B7-H4Ig was also observed using purified CD4$^+$ T cells from B6 mice (Figure 3B). To examine the effect of B7-H4Ig ligation on the production of cytokines, culture supernatants from DO11.10 T cell proliferation assays as shown previously were harvested at 24, 48, and 72 hr, and the secretion of IL-2 (Figure 3C) and IL-10 (Figure 3D) was examined. There is either a low level or no production of IL-2 and IL-10 at 24 hr whereas these cytokines can be detected in high levels at 48 or 72 hr by anti-CD3 mAb stimulation in the presence of control Ig. Inclusion of B7-H4Ig significantly inhibited the production of IL-2 and IL-10 at these time points.

Although it does not inhibit proliferation of T cell response, soluble B7-H4Ig could partially suppress the generation of allogeneic cytolytic T cells (CTL) in vitro. When purified B6 T cells were stimulated in vitro with irradiated spleen cells of BDF1 origin, high levels of cytolytic activity were generated against P815 cells (H-2^d). The induction of allogeneic CTL activity in this system was partially inhibited by inclusion of B7-H4Ig in the beginning of the cultures (Figure 3E). Our results indicate that soluble B7-H4Ig could inhibit, at least in part, induction of allogeneic CTL in vitro.

B7-CD28 costimulation represents an early activation signal for optimal T cell proliferation. To determine whether B7-H4 inhibits CD28 costimulation, we included B7-H4Ig in the beginning of DO11.10 T cell cultures that

---

(D) RT-PCR analysis of human B7-H4 mRNA from (1) PBMC, (2) plastic nonadherent PBMC, (3) dendritic cells, (4) dendritic cells stimulated with PMA and ionomycin, (5) human aortic endothelium (HAEL), and (6) HAEL stimulated with TNF-α.
(E) Northern blot of mB7-H4 mRNA using mouse multiple tissue panels. Each line contains 1 μg of poly (A)$^+$ RNA. Molecular size markers are indicated in kb. Human and mouse full-length B7-H4 cDNA were used as probes for respective tissue panels.
(F) PCR analysis of mouse cDNA from multiple tissues with mB7-H4-specific primers.
(G) Human T cells stimulated with anti-CD3 mAb at 5 μg/ml for 44 and 68 hr, stained with control human IgG (shaded) or B7-H4Ig (open) and subjected to FACS analysis.
(H) Expression of B7-H4 by 293 transfectants. 293T cells expressing human B7-H4 (hB7-H4.293T) or mouse B7-H4 (mB7-H4.293T) were stained by hH4 mAb for human B7-H4 or 8H4 for mouse B7-H4.
(I) FACS analysis of mB7-H4.293T line with mCTLA4 Ig and mPD-1Ig, and hB7-H4.293T line with hICOSIg.
(J) Positive control staining of mCTLA4Ig for mB7-1.P815 line, mPD-1Ig for mB7-H1.P815 line, and hICOSIg for hB7-H2.CHO line.
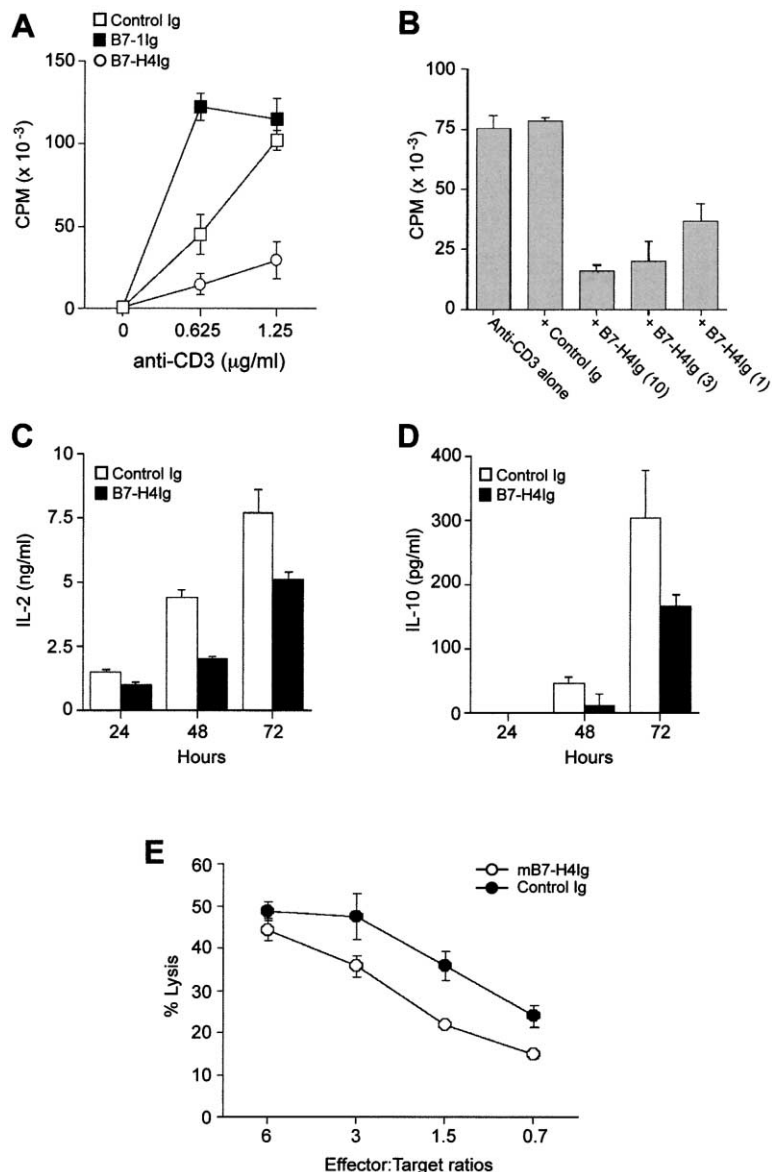
Figure 3. B7-H4 Inhibits Anti-CD3-Induced T Cell Proliferation and Cytokine Production

(A and B) Anti-CD3 mAb was plate coated at 5 μg/ml except when indicated at 4°C overnight. The plates were washed three times with PBS and coated with 10 μg/ml (A) or with the indicated concentration of the fusion protein (B) for 2 hr at 37°C. Wells were washed three times with PBS, and cells were plated at a density of 1.5 × 10⁶ cells/ml (200 μl). Cells were pulsed with 1 μCi of [³H]thymidine and harvested at 72 hr after stimulation. T cells were purified from DO11.10 (A) or B6 mice (B) by a CD4 magnetic selection column. These data are representative of three independent experiments.

(C–E) Supernatants from DO11.10 cells stimulated with 3 μg/ml anti-CD3 mAb and 10 μg/ml of B7-H4Ig (solid bars) or control Ig (open bars) were harvested at the indicated time points. Sandwich ELISA for IL-2 (C) and IL-10 (D) were performed at the same time for all time points. To determine the role of soluble B7-H4Ig in the generation of alloreactive CTL, purified T cells from B6 mice were stimulated with γ-irradiated BDF1 spleen cells at a ratio of 1:3 in the presence of 10 μg mB7-H4Ig or control Ig for 4 days. T cells were purified by LymphoLyte M and used at indicated effector:target (E:T) ratios against P815 cells in a 4 hr ⁵¹Cr-release assay (E). All assays were done with triplicated wells. These data are representative of at least two independent experiments.

were exposed to anti-CD3 mAb and B7-1Ig. While B7-1Ig costimulates a much higher level of proliferation and IL-2 secretion in the presence of anti-CD3, compared with anti-CD3 mAb alone (Figures 3A and 3C), inclusion of B7-H4Ig significantly inhibited both T cell proliferation and IL-2 production (Figure 4A). Using nylon-wool purified polyclonal T cells from B6 mice, we further demonstrated the inhibition by B7-H4Ig of IL-2, IL-4, IL-10, and IFN-γ secretion from B7-1 costimulated T cells (Figure 4B). Our results thus indicate that B7-H4-induced inhibition of T cell proliferation could not be reversed by CD28 costimulation.

We next examined whether B7-H4 on cell surface also inhibits T cell responses. To test this, we transfected EL4 cells with the mB7-H4 cDNA plasmid to establish a line that stably expresses B7-H4 (EmH4). As shown in Figure 5A, EmH4 expresses a high level of mouse B7-H4 on the surface by FACS using 8H4 mAb whereas mock.EL4 does not. Both mock.EL4 and EmH4 express similar levels of H-2Kᵇ and H-2Dᵇ. Stimulation of OT-1

TCR transgenic T cells with mock.EL4 cells which were incubated with a H-2Kᵇ-restricted OVA peptide (mock.EL4/+) led to strong proliferation of OT-1 cells while mock.EL4 cells without the peptide sensitization (mock.EL4/−) did not, indicating that proliferation of OT-1 cells is specific for OVA peptide. Importantly, the stimulation by the peptide-pulsed EmH4 cells (EmH4/+) induced significantly decreased proliferation in comparison with mock.EL4/+ cells (Figure 5B) and inhibited cytolytic activity against OVA-positive EG7 cells (Figure 5C). The inhibitory effect of B7-H4 on EL4 cells could be largely neutralized by the inclusion of 8H4 and 8H4.1 mAbs (Figure 5D). Our results indicate that, in addition to crosslinking of B7-H4, cell-associated B7-H4 could also inhibit T cell response.

Cell Cycle Arrest by B7-H4
Extensive inhibitory effect by B7-H4 in T cell proliferation and cytokine production suggests that B7-H4 may inhibit cell division and even arrest cell cycle. To test these
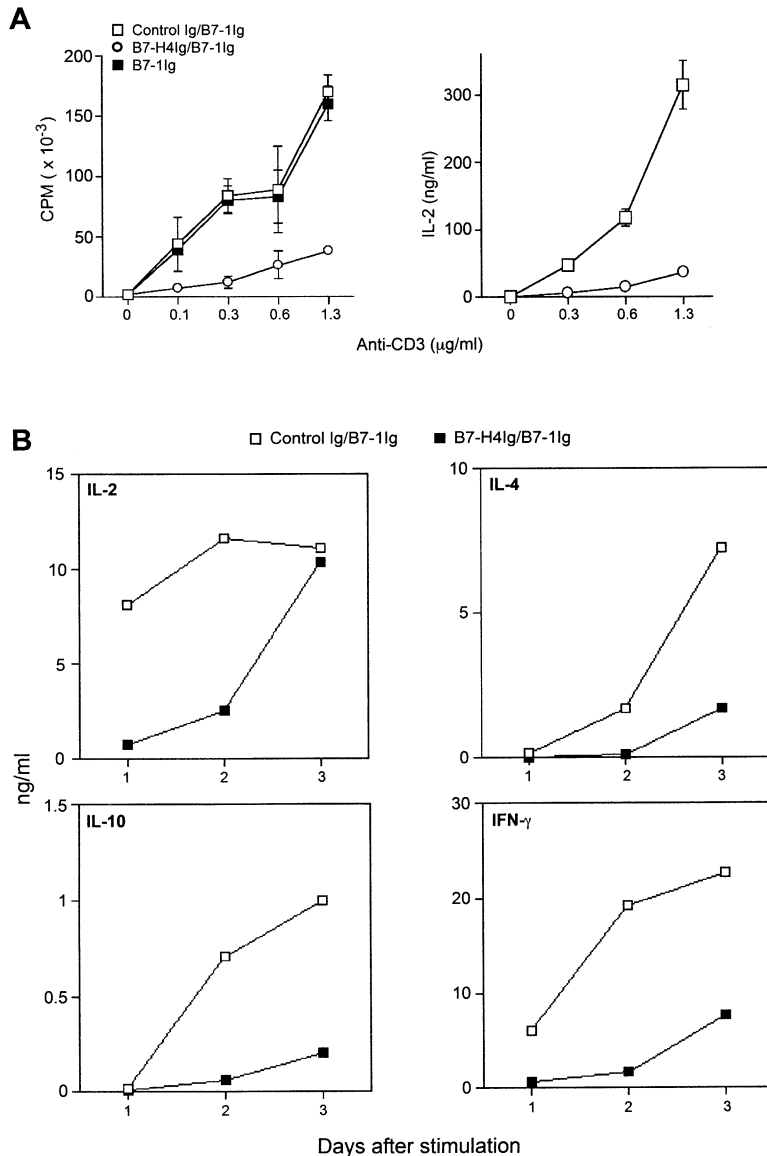
Figure 4. B7-H4 Inhibits CD28 Costimulation

CD4^{+} DO11.10 (A) or purified T cells from B6 mice (B) were stimulated for 48 hr (A) or the indicated times (B) prior to harvest. 96-well plates were coated with the indicated concentrations of anti-CD3 mAb in PBS overnight at 4°C, washed three times in PBS, and coated with the indicated recombinant protein cocktails, with each protein at 10 μg/ml, for 2 hr at 37°C. Wells were washed three times with PBS and plated with 200 μl of purified DO11.10 cells (A) or purified T cells from B6 mice (B) at a concentration of 1.5 × 10^{6} cells/ml. Cells were pulsed overnight with 1 μCi of [³H] thymidine 18 hr prior to harvesting. Supernatants were harvested at the indicated time points for cytokine ELISA. T cells were purified from DO11.10 mice by a CD4 magnetic selection column or from B6 mice by nylon wool column. These data are representative of at least two independent experiments.

possibilities, purified T cells were labeled with CFSE, incubated with immobilized anti-CD3 mAb and B7-H4Ig, and subsequently analyzed by flow cytometry. Stimulation of T cells by anti-CD3 mAb induced modest cell division (7.6%) at 48 hr, and nearly 70% of cells had more than four cell divisions at 96 hr. Significant inhibitions of cell division were observed from 48 to 96 hr after exposure to B7-H4Ig, as assessed by the lower percentage of cells in the dividing peaks and decreased number of peaks (Figure 6A). To precisely determine the effect of B7-H4 on cell cycle, we labeled the purified T cells with BrdU and 7-AAD for the analysis of cell cycle progression and apoptosis. As shown in Figure 6B, inclusion of B7-H4Ig significantly decreased the cell numbers in S phase (from 54.9% to 25.0%) while the cell numbers in G0/G1 phase increased (from 41.8% to 72.7%), in comparison to that treated with control Ig. The apoptotic cells, as determined by subdiploid population that showed negative staining of 7-AAD, did not increase significantly, indicating that programmed cell death is not a major mechanism responsible for inhibited T cell proliferation. Our results thus indicate that B7-H4 arrests cell cycle progression of T cells in G0/G1 phase.

Inhibition of T Cell Responses by B7-H4 In Vivo

To evaluate the effect of B7-H4 on the inhibition of T cell-mediated immune responses in vivo, we transferred OT-1 cells into B6 mice to facilitate the tracing of T cell responses. As a result of the transfer, 5%–7% of CD8^{+} T cells in recipient spleens have OVA tetramer reactivity. OT-1 T cells proliferate rigorously at day 6 upon immunization by OVA peptide (from 4.9% to 21.7%). Administration of B7-H4Ig induced a moderate but significant decrease of OT-1 cells from 21.7% to 14.2% in spleens (Figure 7A). Our results thus indicate that administration of B7-H4Ig could inhibit T cell proliferation in vivo.

The effect of B7-H4Ig on the inhibition of alloreactive CTL activity was also evaluated in vivo. As shown previously, transfer of B6 spleen cells into sublethally irradiated BDF1 (B6 x DBA/2) mice induced a rapid expansion and increased cytolytic activity of anti-H-2[d] CD8^{+} CTL, which ultimately leads to death of the mice due to severe
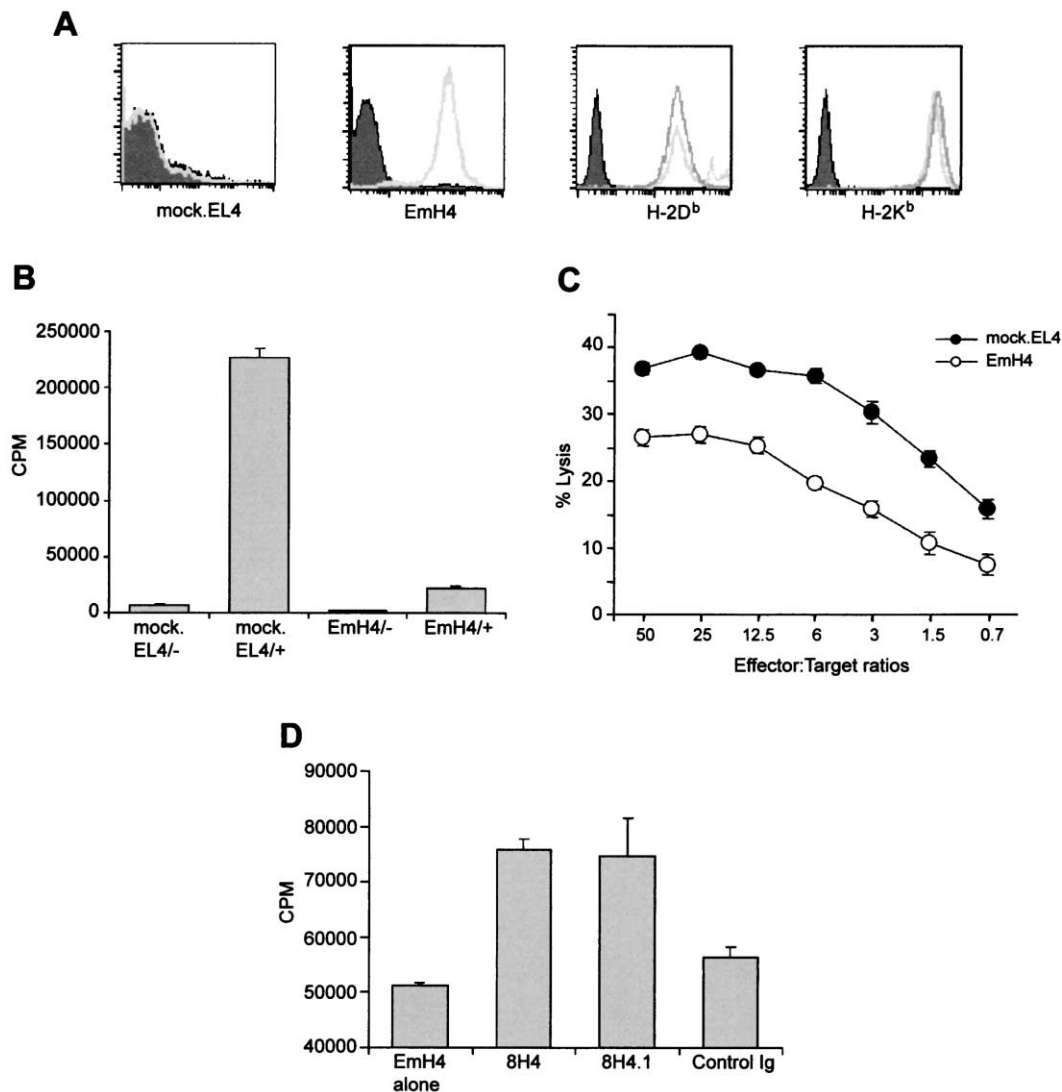
Figure 5. Cell Surface B7-H4 in the Inhibition of T Cell Responses

(A) Mock-transfected EL4 (mock.EL4) or B7-H4 transfected EL4 (EmH4) cells were stained with control hamster IgG (shaded line) or 8H4 (gray line) and analyzed by flow cytometry for the expression of mouse B7-H4, as shown in the two left panels. The staining of H-2D^b and H-2K^b molecules on mock.EL4 (gray line) and EmH4 cells (dark line) was also similarly determined by specific mAb to each MHC molecule using flow cytometry. Shaded peak represents staining with isotype-matched control mAb for both mock.EL4 and EmH4 as shown in the two right panels.

(B) Mock.EL4 or EmH4 cells were γ-irradiated and further incubated with 10 μg/ml of control peptide (mock.EL4/− and EmH4/−) or SIIN peptide (mock.EL4/+ and EmH4/+) overnight at 37°C. The SIIN peptide-pulsed cells at 6 × 10^4/well were cocultured with 3 × 10^5 OT-1 T cells/well at triplets in 96-well plates. Seventy-two hours later, the wells were pulsed with 1 μCi of [^{3}H]thymidine (TdR) for 18 hr.

(C) Purified OT-1 cells at 1.5 × 10^6/well were stimulated with either mock.EL4 or EmH4, which were pulsed with the SIIN peptide as described in (B), for 4 days in 24-well plates. At the end of the culture, OT-1 cells were purified, and their cytolytic activity against ^{51}Cr-labeled EG7 cells was examined in a standard 4 hr ^{51}Cr release assay at the indicated effector:target ratios.

(D) Purified OT-1 cells at 1.5 × 10^6/well were stimulated with OVA peptide-pulsed EmH4 together with 30 μg of anti-B7-H4 mAb from two different clones (8H4 or 8H4.1) or hamster IgG for 3 days, and the proliferation was determined as described in (B).

graft versus host diseases (GVHD) accompanied with failure of multiple organs (Tamada et al., 2000, 2002). The recipients were treated with B7-H4Ig, and alloreactive CTL activity of spleen cells was directly examined at day 10 without further in vitro stimulation. Treatment by B7-H4Ig significantly reduced CTL activity against P815 (H-2^d) in comparison with control Ig. CTL activity was specific for H-2^d because the same CTL had only minimal cytotoxicity against syngeneic EL4 cells (Figure 7B). In addition, infusion of B7-H4Ig also extended the survival of the mice undergoing GVHD (data not shown). Our results further indicate that B7-H4Ig could inhibit the proliferation and maturation of CD8$^+$ CTL.

The role of endogenous B7-H4 in the generation of allogeneic CTL was also accessed in vivo. After transfer of B6 spleen cells into F1 mice, the recipients were treated every other day with 8H4 mAb to block B7-H4. In all three experiments, the treatment by 8H4 increased
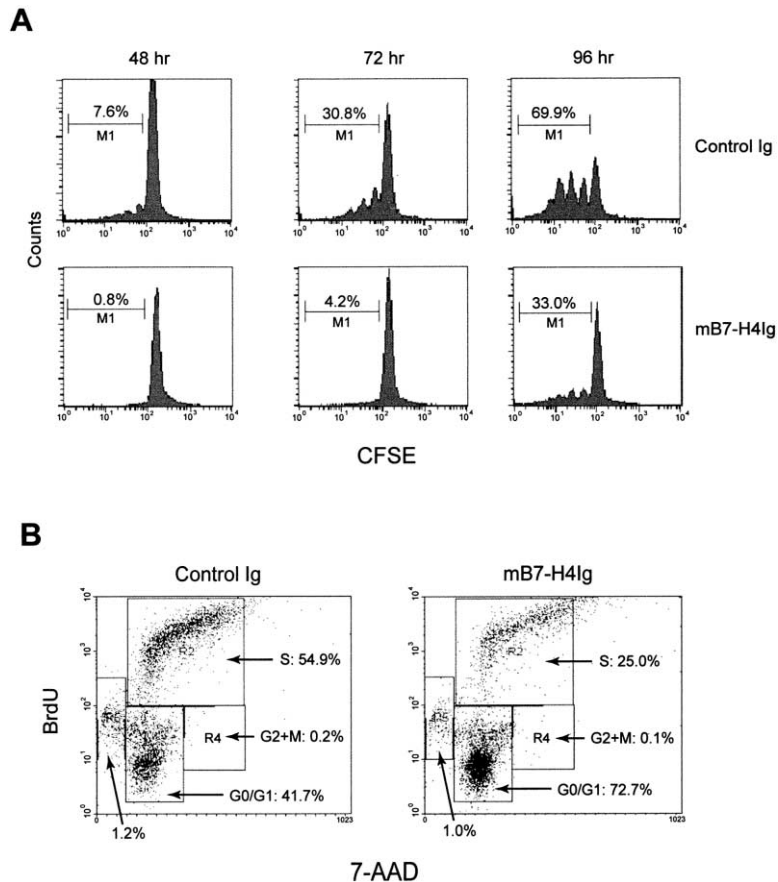
A

B

Figure 6. B7-H4 Induces Cell Cycle Arrest

(A) Nylon wool-purified naive CD4^{+} T cells (6×10^6/ml) from BALB/c mice were labeled with CFSE (1 μM) and cultured in the anti-CD3 (3 μg/ml) coated 96-well plates in the presence of coated control IgG or mB7H4 Ig (10 μg/ml). Cells were harvested at the indicated times and analyzed using flow cytometry.

(B) CD4^{+} T cells were purified from pools of spleens and lymph nodes of BALB/c using magnetic selection column and subsequently added at 3×10^5/well, which were coated with anti-CD3 mAb (3 μg/ml) and mB7H4 Ig or control IgG (10 μg/ml). After 48 hr of incubation, cells were pulsed with 10 μM BrdU for 1 hr, subsequently harvested, and stained with FITC-conjugated anti-BrdU and 7-AAD. Cells were subjected to FACS analysis. The results are representative of three independent experiments.

alloreactive CTL activity significantly in comparison with that by control hamster Ig. CTL activity was specific for P815 (H-2^d) but not for syngeneic EL4 cells (Figure 7C). Our results indicate that blockade of endogenous B7-H4 on host cells promotes the generation of allogeneic CTL. Taken together, our data support the role of endogenous B7-H4 in the inhibition of cell-mediated immunity.

Discussion

Similar to the other B7 family molecules, B7-H4 shares 20%–25% amino acid identity to other members and contains both the IgV and IgC domain in its extracellular region. Sequence analysis and computer modeling reveal structural similarity of B7-H4 to the other B7 family members. These features clearly place B7-H4 as a member of the B7 family. Many B7-like proteins have been predicted by simple homology search and alignment analysis (Fahrer et al., 2001). These molecules often, however, lack critical signature amino acids and structural features of the B7 family. In our study, a combination of primary sequence homology and structural analysis is critical for preliminary identification of B7-H4. FACS analysis using hH4 mAb indicate that B7-H4 does not constitutively express on peripheral tissues but could be induced to express on human T, B, macrophages, and DC. In a recent homology search in the NCBI and Celera databases, we found that the highest homologous sequences are B7-H1 and B7-H3. In addi-

tion, we also found that a genomic DNA sequence (FLJ22418) containing an entire open reading frame for human B7-H4 was deposited in GenBank recently.

Several lines of evidence support B7-H4's role in negative regulation of T cell responses. Immobilized B7-H4 inhibited T cell growth and cytokine secretion. Moreover, administration of B7-H4Ig led to inhibition of T cell proliferation and cytolytic activity against allogeneic antigens in vivo. Although these experiments could be interpreted as an agonistic effect of B7-H4 on its receptor, they do not exclude the possibility that B7-H4Ig also blocked the interaction between B7-H4 and its putative receptor on T cells. However, expression of membrane-bound, but not soluble form, B7-H4 on EL4 cells also inhibits proliferation of CD8^{+} T cells and development of CTL activity. Consistent with this experiment, blockade of B7-H4 by mAbs that block the inhibitory effect of B7-H4 in vitro (Figure 5D), promoted the induction of allogeneic CTL in vivo. Taken together, our results support a role of endogenous B7-H4 on host cells in inhibiting T cell responses.

Our results indicate that B7-H4 inhibits T cell responses by cell cycle arrest whereas its effect on T cell apoptosis is minimal. This finding supports the notion that the effect of B7-H4 is on a relatively early stage of T cell activation and explains the nonselective effect of B7-H4 on the inhibition of cytokine production. The inhibitory effects of B7-H4 on T cell proliferation and IL-2 secretion could only be partially reversed by increased
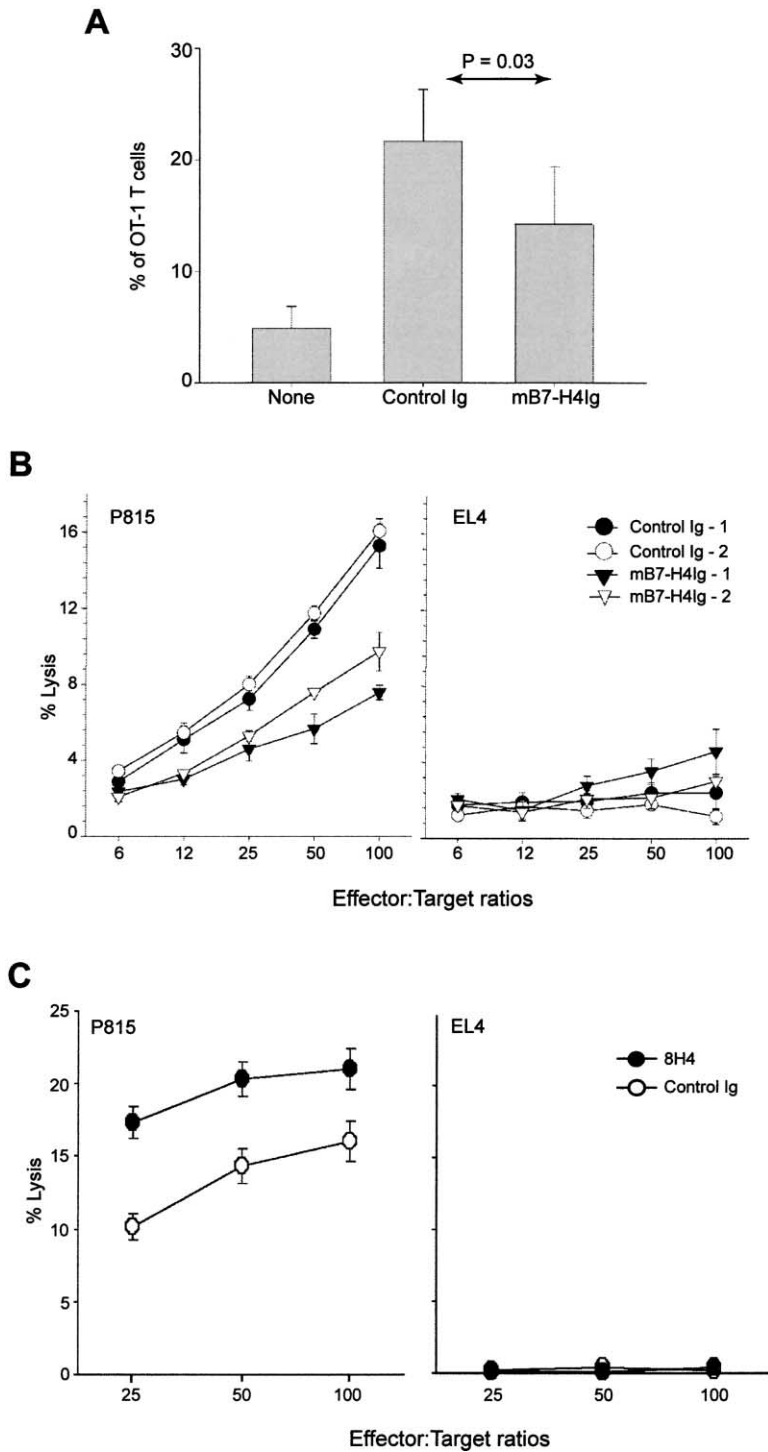
A

B

C

Figure 7. B7-H4 Inhibits T Cell Responses In Vivo

(A) B6 mice were i.v. given 1×10^7 spleen cells from OT-1 mouse and subsequently challenged with 500 μg of OVA peptide i.v. for activation of OT-1 T cells. mB7H4Ig or control IgG were given i.p. on day 0 (250 μg) and day 3 (100 μg). On day 6, spleen cells were prepared and doubly stained with OVA/tetramer and anti-CD8 mAb for FACS analysis. The mice, which were infused with OT-1 T cells but were not challenged with OVA peptide (None), were used as additional control. The results are representative of four independent experiments.

(B) BDF1 mice, in groups of two or three, were given i.v. 5×10^7 splenocytes from B6 mice as described previously (Tamada et al., 2000, 2002). On day 0 (150 μg), day 3 (100 μg), and day 6 (100 μg), mB7H4Ig or control IgG were injected i.p. On day 10, recipient splenocytes were prepared and, without further in vitro stimulation, cocultured with ^{51}Cr-labeled P815 (H-2^d) and EL-4 (H-2^b) at the indicated E:T ratios for 4 hr. Released ^{51}Cr was measured in a Microbeta TriLux liquid scintillation counter (Wallac, Finland). The data from two individual mice are presented, and the results are representative of three independent experiments.

(C) BDF1 mice were given 5×10^7 B6 spleen cells and 200 μg of control hamster IgG or 8H4 mAb via tail vein on day 0. Each antibody at 100 μg was injected subsequently on days 2, 4, and 6. On day 10, CTL activity of recipient splenocytes was measured against P815 (H-2^d) or EL4 (H-2^b) cells in the indicated effector:target cell ratios in 4 hr ^{51}Cr-release assay as described in (B). The results are representative of three independent experiments.

costimulation through CD28 signal, indicating that B7-H4 is a potent attenuator of T cell responses. It has been shown that CTLA-4 signaling arrests cell cycle progression (Krummel and Allison, 1996) and inhibits T cell receptor signaling by recruiting SHP-2 (Lee et al., 1998; Marengere et al., 1996). Similar to the inhibitory activity of the putative receptor for B7-H4, CTLA-4 inhibits TCR-induced T cell proliferation and IL-2 production (Salomon and Bluestone, 2001). Therefore, it will be in-

teresting to determine whether B7-H4 inhibition is also mediated in a fashion similar to the CTLA-4 pathway and the relationship between these two pathways.

In summary, B7-H4 has a profound effect on the inhibition of T cell responses at a relatively early stage. Therefore, engagement of early primed T cells by B7-H4 constitutes a checkpoint in negative control of T cell activation. Together with a broad and inducible expression pattern, B7-H4 may be involved in attenuation of

inflammatory responses on peripheral tissues. Further characterization of the B7-H4 pathway thus provides a new opportunity for manipulation of cell-mediated immune responses in cancers, autoimmune diseases, viral infection, and transplantation rejection.

Experimental Procedures

Bioinformatics and Molecular Cloning
Human *B7-H4* was originally identified from the NCBI database based on homology to other B7 family molecules. Full-length human *B7-H4* cDNA was amplified by PCR with PFU polymerase (Stratagene, CA) from human placental cDNA (Clontech, CA), cloned into pcDNA 3.1$^-$ vector (Invitrogen, CA), and confirmed by DNA sequencing. To isolate mouse B7-H4 homolog, several sets of primers based on mouse and human EST sequences were used to amplify mouse *B7-H4* cDNA from spleen cDNA of a C57BL/6 (B6) mouse. Full-length mouse B7-H4 cDNA was similarly cloned into pcDNA 3.1$^-$ vector and confirmed by DNA sequencing. Fusion protein constructs of human B7-H4, mouse B7-H4, and mouse B7-1 were prepared by cloning the extracellular domain of B7-H4 or B7-1 in-frame with the hinge-CH2-CH3 domain of either human IgG1 or mouse IgG2a (Chapoval et al., 2002). To enhance the secretion of fusion protein, the native signal peptide of B7-H4 was replaced with the preprotrypsin signal peptide derived from pCMV FLAG vector (Sigma, MO).

Molecular Modeling
Molecular models of the N-terminal V domains of human and mouse B7-H4 were built by homology modeling based on the X-ray structures of human CD80 and CD86 (Stamper et al., 2001; Schwartz et al., 2001) using MOE (Molecular Operating Environment, Chemical Computing Group, Quebec, Canada). Insertions and deletions in mouse and human B7-H4 relative to the structural template(s) were modeled employing a protein database segment matching procedure (Levitt, 1992; Fechteler et al., 1995) implemented in MOE. Side chain replacements were carried out using a rotamer library (Ponder and Richards, 1987) extracted from high-resolution protein databank structures (Berman et al., 2000). Intramolecular contacts and stereochemistry of the models were optimized by limited energy minimization using protein force field parameters (Engh and Huber, 1991). Residue mapping studies and computer graphical analyses were carried out with InsightII (MSI, CA).

Transfection
To produce human or mouse B7-H4Ig, 293T cells were transfected with 10 μg of mouse or human B7-H4Ig constructs by the calcium phosphate method and B7-H4Igs were purified from culture supernatant by protein G column as described previously (Dong et al., 1999). Stable 293T cell lines expressing human and mouse B7-H4 or mock-transfected lines were prepared by cotransfection of pcDNA vector containing human or mouse B7-H4 cDNA with pLXSHD, a plasmid encoding histidinol-resistant gene (a gift from Dr. Dusty Miller). Stable clones were selected with 20 mM histidinol (Sigma, MO). Clones expressing B7-H4 were screened with human or mouse B7-H4 mAb, respectively.

Preparations of Monoclonal Antibodies
The method for the generation of mAb to mouse and human B7-H4 by immunization of an Armenian hamster and a BALB/c mouse, respectively, were described previously (Wilcox et al., 2002). Two independent clones, 8H4 and 8H4.1, which secret hamster mAb (IgG) against mouse B7-H4, and clone hH4, which secretes mouse mAb (IgG1) against human B7-H4, were selected, and mAbs were purified by IgG affinity column. Specificity of the mAb was determined by negative staining of various transfectants expressing B7 family molecules including B7-1, B7-2, B7-H1, B7-DC, B7-H2, and B7-H3. Control hamster IgG and mouse IgG1 were purchased from Rockland (Gilbertville, PA). A hamster IgG mAb against DNP (clone UC8-1B9, ATCC) was also used as the control.

RNA Analysis
Human and mouse multiple tissue mRNA for Northern blots and cDNA panels (Clontech, CA) for PCR analysis of B7-H4 expression were performed according to the manufacturer's instructions (Dong et al., 1999). Human B7-H4 was amplified with forward primer (5′-GAG ATCAAAAGGCGGAGTCACCTACAGCTGC-3′) and reverse primer (5′-GCTCCCCTCTTTCCAGGCCCTTTTCTACTC-3′) binding to the transmembrane domain and 3′ UTR of hB7-H4, respectively. For amplification of mouse B7-H4, two primers (forward primer, 5′-GGC ATTTCAGGCAAGCACTTC-3′; reverse primer, 5′-GCCTCGCAGCG TAAACTCTCT-3′) covering the extracellular domain were used. For RT-PCR analysis, adherent cells were harvested by trypsin EDTA (Mediatech, VA), and RNA was isolated using the Qiagen RNeasy kit (Qiagen, CA) from cytoplasmic cell fractions. All RNA samples were digested with DNase I (Qiagen). Total RNA was reverse transcribed with Superscript II RT using random hexamers, and PCR was performed using the same primers indicated to detect B7-H4.

Cell Lines
P815 mouse mastocytoma, B7-1.P815, B7-H1.P815 (Dong et al., 2002), human 293T cells, and human B7-H2.CHO line (Wang et al., 2000) were described previously. Human peripheral blood mononuclear cells (PBMC) were prepared by density gradient centrifugation over Ficoll-Paque plus (Pharmacia, NJ). EL4 lines transfected with the plasmids containing neomycin-resistant gene (mock.EL4) or mouse B7-H4 cDNA (EmH4) were established by neomycin selection and cell cloning. EG7 is an EL4 transfectant expressing chicken ovalbumin (OVA) protein. Human DC were prepared from plastic adherent PBMC in the presence of IL-4 and GM-CSF (Chapoval et al., 2000). Human aortic endothelium cells (gift of Dr. Jeffrey Platt) were stimulated overnight with 10 ng/ml TNF-α or 1500 IU/ml IFN-γ (R&D, MN). Human and mouse T cells were purified by nylon wool column and magnetic activated cell sorting separation column. The purity of the CD4$^+$ cells from DO11.10 TCR transgenic T cells was >99%. For flow cytometry analysis, cells were stained with 10 μg of fusion protein or the indicated mAb for 30 min at 4°C. Fc receptors were blocked with anti-FcR (clone 2.4G2, ATCC) for 15 min at 4°C prior to staining. Cells were then washed extensively and further incubated with secondary mAb.

In Vitro Analysis of T Cell Responses
For T cell proliferation assays, 96-well flat bottom plates were coated with varying concentrations of anti-CD3 mAb (clone 500A2, Pharmingen, CA) in PBS at 4°C overnight. Wells were washed extensively and coated with 10 μg/ml of the indicated fusion protein for 2 hr at 37°C. When two different fusion proteins were plated, they were mixed together, each at a concentration of 10 μg/ml in PBS, prior to plating. Wells were washed extensively and plated with purified CD4$^+$ DO11.10 cells at 1.5 × 10^6 cells/ml. Alternatively, mock.EL4 or EmH4 cells were γ-irradiated (26,000 rad) and further incubated overnight with 10 μg/ml of SIINFEKL peptide. The peptide-pulsed cells at 6 × 10^4/well were cocultured with 3 × 10^5 OT-1 T cells/well, incubated for 72 hr, and pulsed with 1 μCi of [^{3}H]thymidine (TdR). For measurement of cytolytic activity of OT-1 cells, γ-irradiated and peptide-pulsed EL4 transfectants at 3 × 10^5/well were cultured with purified OT-1 cells at 1.5 × 10^6/well in 24-well plates. On day 4, OT-1 T cells were purified by Lympholyte-M (Cedarlane, Canada) and incubated at the indicated effector:target ratios with ^{51}Cr-labeled EG7 or P815 cells for 4 hr (Wilcox et al., 2002).

For cell division measurement, naive T cells were purified from spleens and lymph nodes of B6 mice using nylon wool column, labeled with CFSE (Molecular Probes, Eugene, OR) according to the manufacturer's instructions, and analyzed by flow cytometry. For cell cycle analysis, CD4$^+$ T cells were activated by anti-CD3 mAb plus 10 μg/ml control IgG or mB7H4Ig for 48 hr. Cells were incubated with bromodeoxyuridine (BrdU) at 10 μM for 1 hr at 37°C. Staining of incorporated BrdU was performed using reagents from the BrdU Flow Kit (BD Bioscience, San Diego, CA), according to the manufacturer's instructions. Cells were stained with FITC-conjugated anti-BrdU antibody for 20 min at room temperature. Twenty μg/ml 7-amino-actinomycin D (7-AAD) was added to cell suspension before flow cytometry analysis.

All primary mouse T cells and transfectants were grown in RPMI

1640-based T cell media supplemented with 2-mercaptoethanol (Gibco BRL, MD). Sandwich ELISA for indicated cytokines was performed according to the manufacturer's instructions (Pharmingen, CA).

Animal Studies
Spleen cells from OT-1 mouse (H-2^b) were prepared and suspended in Hanks' balanced salt solution (HBSS) and transferred into sex-matched naive B6 mice (H-2^b) by tail vein injection (1 × 10^7 cells/mouse). Two days later (day 0), recipients were challenged by i.v. injection of 500 μg of SIIN peptide. Control IgG or mB7H4Ig was given i.p. on day 0 (250 μg) and day 3 (100 μg). On day 6, recipient spleen cells were prepared and doubly stained with MHC class I/peptide tetramers of H-2K^b/SIINFEKL (NIH tetramer core facility, Emory University, GA) and anti-CD8 mAb (Pharmingen, San Diego, CA) to identify the OT-1 TCR transgenic T cells by flow cytometry (Beckman Coulter, San Diego, CA).

Primary allogeneic CTL in a graft versus host disease setting were generated by injection i.v. of 5 × 10^7 purified splenocytes from B6 mice into 5-week-old BDF1 mice (Tamada et al., 2000, 2002). Recipients were also given i.p. control IgG or mB7H4Ig on day 0 (150 μg), day 3 (100 μg), and day 6 (100 μg). In some experiments, recipients were injected with 100 μg 8H4 mAb or control hamster IgG at days 0, 2, 4 and 6. On day 10, CTL activity of recipient splenocytes was determined using standard 4 hr ^{51}Cr release assay by coculturing with ^{51}Cr-labeled P815 (H-2^d) or EL4 (H-2^b) at the indicated effector:target ratios without further stimulation in vitro.

References

Berman, H.M., Bhat, T.N., Bourne, P.E., Feng, Z., Gilliland, G., Weissig, H., and Westbrook, J. (2000). The protein data bank and the challenge of structural genomics. Nat. Struct. Biol. *7*, s957–s959.

Carreno, B.M.,and Collins, M. (2002). The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu. Rev. Immunol. *20*, 29–53.

Chambers, C.A., Kuhns, M.S., Egen, J.G., and Allison, J.P. (2001). CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. Annu. Rev. Immunol. *19*, 565–594.

Chapoval, A.I., Tamada, K., and Chen, L. (2000). In vitro growth inhibition of a broad spectrum of tumor cell lines by activated human dendritic cells. Blood *95*, 2346–2351.

Chapoval, A.I., Ni, J., Lau, J.S., Wilcox, R.A., Flies, D.B., Liu, D., Dong, H., Sica, G.L., Zhu, G., Tamada, K., and Chen, L. (2001). B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. Nat. Immunol. *2*, 269–274.

Chapoval, A.I., Zhu, G., and Chen, L. (2002). Immunoglobulin fusion proteins as a tool for evaluation of T-cell costimulatory molecules. Mol. Biotechnol. *21*, 259–264.

Dong, H., Zhu, G., Tamada, K., and Chen, L. (1999). B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat. Med. *5*, 1365–1369.

Dong, H., Strome, S.E., Salomao, D.R., Tamura, H., Hirano, F., Flies, D.B., Roche, P.C., Lu, J., Zhu, G., Tamada, K., et al. (2002). Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat. Med. *8*, 793–800.

Engh, R.A., and Huber, R. (1991). Accurate bond and angle parameters for X-ray protein structure refinement. Acta Crystallogr. A *47*, 392–400.

Fahrer, A.M., Bazan, J.F., Papathanasiou, P., Nelms, K., and Goodnow, C.C. (2001). A genomic view of immunology. Nature *409*, 836–838.

Fechteler, T., Dengler, U., and Schomburg, D. (1995). Prediction of protein three-dimensional structures in insertion and deletion regions: a procedure for searching databases of representative protein fragments using geometric scoring criteria. J. Mol. Biol. *253*, 114–131.

Freeman, G.J., Long, A.J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L.J., Malenkovich, N., Okazaki, T., Byrne, M.C., et al. (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J. Exp. Med. *192*, 1027–1034.

Grimbacher, B., Hutloff, A., Schlesier, M., Glocker, E., Warnatz, K., Drager, R., Eibel, H., Fischer, B., Schaffer, A.A., Mages, H.W., et al. (2003). Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nat. Immunol. *4*, 261–268.

Hurwitz, A.A., Kwon, E.D., and van Elsas, A. (2000). Costimulatory wars: the tumor menace. Curr. Opin. Immunol. *12*, 589–596.

Hutloff, A., Dittrich, A.M., Beier, K.C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., and Kroczek, R.A. (1999). ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature *397*, 263–266.

Iwai, Y., Ishida, M., Tanaka, Y., Okazaki, T., Honjo, T., and Minato, N. (2002). Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc. Natl. Acad. Sci. USA *99*, 12293–12297.

Krummel, M.F., and Allison, J.P. (1996). CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. J. Exp. Med. *183*, 2533–2540.

Latchman, Y., Wood, C.R., Chernova, T., Chaudhary, D., Borde, M., Chernova, I., Iwai, Y., Long, A.J., Brown, J.A., Nunes, R., et al. (2001). PD-L2 is a second ligand for PD-I and inhibits T cell activation. Nat. Immunol. *2*, 261–268.

Lee, K.M., Chuang, E., Griffin, M., Khattri, R., Hong, D.K., Zhang, W., Straus, D., Samelson, L.E., Thompson, C.B., and Bluestone, J.A. (1998). Molecular basis of T cell inactivation by CTLA-4. Science *282*, 2263–2266.

Levitt, M. (1992). Accurate modeling of protein conformation by automatic segment matching. J. Mol. Biol. *226*, 507–533.

Ling, V., Wu, P.W., Finnerty, H.F., Bean, K.M., Spaulding, V., Fouser, L.A., Leonard, J.P., Hunter, S.E., Zollner, R., Thomas, J.L., et al. (2000). Identification of GL50, a novel B7-like protein that functionally binds to ICOS receptor. J. Immunol. *164*, 1653–1657.

Linsley, P.S., Peach, R., Gladstone, P., and Bajorath, J. (1994). Extending the B7 (CD80) gene family. Protein Sci. *3*, 1341–1343.

Mages, H.W., Andreas, H., Heuck, C., Büchner, K., Himmelbauer, H., Oliveri, F., and Kroczek, R.A. (2000). Molecular cloning and characterization of murine ICOS and identification of B7h as ICOS ligand. Eur. J. Immunol. *30*, 1040–1047.

Marengere, L.E.M., Waterhouse, P., Duncan, G.S., Mittrucker, H.W., Feng, G.S., and Mak, T.W. (1996). Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. Science *272*, 1170–1173.

Nishimura, H., Nose, M., Hiai, H., Minato, N., and Honjo, T. (1999). Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity *11*, 141–151.

Ponder, J.W., and Richards, F.M. (1987). Tertiary templates for proteins. Use of packing criteria in the enumeration of allowed sequences for different structural classes. J. Mol. Biol. *193*, 775–791.

Salomon, B., and Bluestone, J.A. (2001). Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu. Rev. Immunol. *19*, 225–252.

Schwartz, J.C., Zhang, X., Fedorov, A.A., Nathenson, S.G., and Almo, S.C. (2001). Structural basis for co-stimulation by the human CTLA-4/B7-2 complex. Nature *410*, 604–608.

Shin, T., Kennedy, G., Gorski, K., Tsuchiya, H., Koseki, H., Azuma, M., Yagita, H., Chen, L., Powell, J., Pardoll, D.M., and Housseau, F. (2003). Cooperative B7-1/2 (CD80/CD86) and B7-DC (PD-L2) co-stimulation of CD4 T cells independent of the PD-1 receptor. J. Exp. Med., in press.

Stamper, C.C., Zhang, Y., Tobin, J.F., Erbe, D.V., Ikemizu, S., Davis, S.J., Stahl, M.L., Seehra, J., Somers, W.S., and Mosyak, L. (2001). Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses. Nature *410*, 608–611.

Tamada, K., Shimozaki, K., Chapoval, A.I., Zhu, G., Sica, G., Flies, D., Boone, T., Hsu, H., Fu, Y.X., Nagata, S., et al. (2000). Modulation of T-cell-mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway. Nat. Med. *6*, 283–289.

Tamada, K., Tamura, H., Flies, D., Fu, Y.X., Celis, E., Pease, L.R., Blazar, B.R., and Chen, L. (2002). Blockade of LIGHT-LTβ and CD40 signaling induces allospecific T cell anergy, preventing graft-versus-host disease. J. Clin. Invest. *109*, 549–557.

Tamura, H., Dong, H., Zhu, G., Sica, G.L., Flies, D.B., Tamada, K., and Chen, L. (2001). B7-H1 costimulation preferentially enhances CD28-independent T-helper cell function. Blood *97*, 1809–1816.

Tseng, S.Y., Otsuji, M., Gorski, K., Huang, X., Slansky, J.E., Pai, S.I., Shalabi, A., Shin, T., Pardoll, D.M., and Tsuchiya, H. (2001). B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. J. Exp. Med. *193*, 839–846.

Wang, S., Zhu, G., Chapoval, A.I., Dong, H., Tamada, K., Ni, J., and Chen, L. (2000). Costimulation of T cells by B7–H2, a B7-like molecule that binds ICOS. Blood *96*, 2808–2813.

Wang, S., Zhu, G., Tamada, K., Chen, L., and Bajorath, J. (2002). Ligand binding sites of inducible costimulator and high avidity mutants with improved function. J. Exp. Med. *195*, 1033–1041.

Wang, S., Bajorath, J., Flies, D.B., Dong, H., Honjo, T., and Chen, L. (2003). Molecular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction. J. Exp. Med., *197*, 1083–1091.

Wilcox, R.A., Flies, D.B., Zhu, G., Johnson, A.J., Tamada, K., Chapoval, A.I., Strome, S.E., Pease, L.R., and Chen, L. (2002). Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. J. Clin. Invest. *109*, 651–659.

Yoshinaga, S.K., Whoriskey, J.S., Khare, S.D., Sarmiento, U., Guo, J., Horan, T., Shih, G., Zhang, M., Coccia, M.A., Kohno, T., et al. (1999). T-cell co-stimulation through B7RP-1 and ICOS. Nature *402*, 827–832.

Accession Numbers

The accession numbers for the human and mouse B7-H4 sequences in GenBank are AY280972 and AY280973, respectively.