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Characterization of B7S3 as a Novel Negative Regulator of T Cells

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T cell activation by APCs is regulated by B7-like costimulatory molecules. In this study, we describe a new B7 superfamily member, B7S3, with two differentially spliced isoforms expressed in lymphoid and nonlymphoid tissues. A soluble B7S3-Ig protein bound to professional APC constitutively as well as to activated but not naive T cells. B7S3-Ig treatment greatly inhibited T cell proliferation and IL-2 production. B7S3-Ig also reduced cytokine production by effector T cells. Interestingly, although human genome appears to contain a single-copy B7S3 homolog, the mouse B7S3 gene has 10 relatives within a 2-Mb region constituting a B7S3 gene family. This study identifies B7S3 as a novel negative regulator of T cells, and suggests evolutionarily divergent T cell regulation mechanisms in mammals. The Journal of Immunology, 2007, 178: 3661–3667.

Costimulation is crucial in determining Ag-specific T cell activation and tolerance. The best studied costimulatory ligands are B7-1 and B7-2, which are highly expressed by activated APC as a result of innate immune signaling pathways (1). These proteins, through their positive receptor CD28, on naive and activated T cells (2) and an inhibitory receptor CTLA-4, induced after T cell activation (3), play a major role in maintaining the appropriate threshold of T cell activation. Recent identification and characterization of new B7 family members and their counter-receptors has revealed complex costimulatory regulation in T cell activation and effector function. ICOS, expressed on activated T cells, has a ligand B7h/B7RP-1, constitutively expressed in certain APC and inductively in nonlymphoid tissues and cells (4–6). The ICOS-B7h pathway plays critical roles in T cell activation, differentiation, and effector function (7). PD-1 is an inhibitory receptor that binds to the B7 family members PD-L1 (B7-H1) and PD-L2 (B7-DC); the spontaneous autoimmunity seen in PD-1-deficient mice indicates its critical function in immune tolerance (8).

B7S3 is constitutively expressed on APCs, and induced on T cells upon activation. B7S3-Ig inhibited CD4 and CD8 T cell proliferation and IL-2 production (13–15). T cell tolerance and function is determined by a combinatorial signal of these positive and negative costimulatory molecules (16). Interestingly, the new B7 family members show significant homology with butyrophilin proteins. Recently, our laboratory reported that BTNL2 as the first member of the butyrophilin family that inhibited T cell activation (17).

In this study, we describe a novel B7-like molecule, named B7 superfamily member 3 (B7S3). It has two isoforms expressed in both lymphoid and nonlymphoid tissues. A putative receptor for B7S3 is constitutively expressed on APCs, and induced on T cells upon activation. B7S3-Ig inhibited CD4 and CD8 T cell proliferation and IL-2 production, as well as effector cytokine production. Interestingly, the B7S3 genes in mouse and human have distinct structural features.

Materials and Methods

B7S3 cloning and sequence analysis

The mouse B7S3 EST clone (Incyte Genomics) was completely sequenced. The Ig-like domains of the deduced B7S3 protein were predicted by the National Center for Biotechnology Information (NCBI) CD search program. The size and location of each exon of the B7S3 gene and its homologs were determined via NCBI genomic database searches.

Real-time PCR analysis

Primers spanning the IgC and 3′ untranslated region of B7S3 short isoform (B7S3S) are forward TCATTCAAGAGGTTGATC, reverse GGCAGG TGATTCTGGTTAT, and were used to analyze the expression of B7S3S. Primers spanning the TM2 and TM3 of B7S3 long isoform (B7S3L) are forward TGGAAACGTGTTTG1AATGA, reverse TGTGTTT1AGATG CAAAATGAGCA, and were used to analyze the expression of B7S3L. cDNA samples were made from tissues of B6 mice and were subjected to real-time PCR analysis using SYBR Green Supermix according to the manufacturer’s instructions (Bio-Rad) with the following modification: denaturation for 30 s at 95°C, annealing for 20 s at 60°C and extension for 30 s at 72°C with fluorescence detection at 60°C after each cycle. The results were obtained and analyzed with iCycler iQ real-time detection software (Bio-Rad).

Confocal microscopy

293 cells were used to examine the cellular localization of B7S3. Full length of B7S3S or B7S3L, including the signal sequence, were amplified from mouse B7S3 EST clone (Incyte Genomics) or RIKEN clone (A430070M19) by PCR and subcloned into pEGFPN1 vector (Invitrogen Life Technologies). After transfection of these vectors into 293 cells, 1 million cells were stained with cholera toxin B to show the cell membrane (8 μg/ml; Sigma-Aldrich) at 4°C for 20 min. Fluorescence was detected...
Expression of B7S3-Ig protein

A soluble B7S3-Ig protein was generated by PCR amplification of a sequence coding aa 23–205, forward AAGATCTGAAAAATTCACAGTGACTGGC and reverse AACTAGTGGCTTCAATAAGAAGCGTCAT), which was then cloned into the DES-Ig vector (9). B7S3-Ig expression vector was stably transfected into Drosophila S2 cells, and the secreted B7S3-Ig fusion protein was purified with a protein A column.

Flow cytometry analysis

B7S3-Ig was biotinylated with sulfo-NHS-LC-Biotin (Pierce) and used for flow cytometry analysis. Biotinylated human IgG1 and boiled B7S3-Ig were used as staining controls. Before staining, cells were preblocked with human IgG1 (Sigma-Aldrich).

In vitro T cell assays

T cells isolated from C57BL/6, OT-I, or OT-II mice were cultured in 96-well plates (0.2 million cells/well) precoated with anti-CD3 and human IgG1 and boiled B7S3-Ig were used as staining controls. Before staining, cells were preblocked with human IgG1 (Sigma-Aldrich).

Results

Identification of B7S3 as a novel B7 family member

In a homology search of human genome database, we discovered a novel B7-like gene named B7S3 (B7 superfamily member 3), located on human chromosome 1. Using the sequence of this human gene to search the mouse EST database, we found a clone using an Olympus Fluoview FV300 confocal laser scanning biological microscope.

Luciferase assay

DO11.10 T cells transfected with 0.25 μg of PRL-null (Promega) and 1 μg of luciferase promoter reporter plasmids for NFAT or NF-κB (gifts from Dr. R. Flavell, Yale University, New Haven, CT) were stimulated with 1 μg/ml anti-CD3 Ab with 5 μg/ml human IgG or B7S3-Ig for 4 h, and luciferase activities were measured using the Dual-Luciferase system (Promega).

In vitro T cell assays

T cells isolated from C57BL/6, OT-I, or OT-II mice were cultured in 96-well plates (0.2 million cells/well) precoated with anti-CD3 and human IgG1 or B7S3-Ig. Coated plates were washed with PBS three times before seeding of the cells. IL-2 production was measured 24 h after T cell activation, and cell proliferation was measured after 72 h by incubation with [3H]thymidine in the last 8 h. A 30 U/ml exogenous IL-2 was added to the culture in some experiments. To analyze the effect of B7S3 on effector T cells, total splenocytes from OT-I and OT-II mice were stimulated with 0.01 μg/ml OT-I and 1 μg/ml OT-II peptides, respectively, in the presence of 30 U/ml IL-2. Seven days after stimulation following an extensive wash, cells were plated in 24-well plates (2.5 million/well) bound with anti-CD3 Ab (5 μg/ml) in the presence of 5 μg/ml human IgG1 or B7S3-Ig. Il-2, IFN-γ, TNF-α, and IL-4 production were measured by ELISA 24 h after secondary stimulation.
(GenBank accession no. BC042786, clone IMAGE:3603407) that may encode the mouse homolog. We completely sequenced this clone, and the deduced amino acid sequence from an open reading frame predicted a protein with the structural features similar to all of the B7 family members. It contains a leader peptide at its N terminus, an IgV-like domain, and a half IgC-like domain (Fig. 1A), all of which are encoded by a separate exon (Fig. 1B). Recently, a new EST clone derived from neonatal thymus was disclosed by RIKEN (GenBank accession no. AK040156), which shared these three domains of B7S3 followed by additional sequences encoded by three exons (Fig. 1, A and B). Therefore, we named the short form as B7S3S and the long form as B7S3L. The consensus amino acids in the IgV-like domains of the B7 family members are conserved in B7S3 (Fig. 1A). However, unlike the other known B7 molecules, the IgC-like domain of B7S3 does not appear to be complete. Recombinant full-length B7S3S protein was not secreted from Drosophila S2 or human 293 cells (data not shown). Because B7S3S does not have the characteristics of a GPI-linked protein (18), a hydrophobic region (approximately from aa 198 to 218) at the C terminus may serve as a transmembrane domain and deletion of this region allows secretion of B7S3 into supernatant (see below). Interestingly, the B7S3L contains two additional predicted transmembrane domains at its C terminus. When fusion proteins containing either B7S3S or B7S3L with a C-terminal GFP tag are expressed in 293 cells, both of them are expressed on the cell membrane (Fig. 1C).

In sequence databases, B7S3 does not possess significant homology to other Ig superfamily members, such as those in the CD28 family, CD2 family and Fc receptor family (data not shown). B7S3 shares a relatively low degree of identity (<20%) with other B7 molecules, typically the other proteins exhibit 20–30% identity with each other (data not shown). In contrast, it has ~34% identity with butyrophilin-like molecules. Linsley et al. (19) proposed an extended B7 family, which also includes butyrophilin, myelin oligodendrocyte glycoprotein, and the chicken B-G Ag based on their common sequence identity as compared with other Ig superfamily members. However, although B7S3, like other new B7 family members, shares slightly higher homology with butyrophilin proteins, the absence of the heptad structure and B30.2 domains makes it more similar in protein structure to B7 family members. Therefore, we regard B7S3 as a novel B7 family member.

**Broad expression of B7S3 isofoms in lymphoid and nonlymphoid tissues**

To examine B7S3 expression, we used several pairs of PCR primers that spanned different regions of B7S3 or B7S3L gene. RT-PCR analysis revealed that B7S3S is widely expressed in tissues we examined, but B7S3L is only expressed in several tissues with very low levels (data not shown). To determine the expression levels of B7S3 isoforms in different tissues, real-time PCR analysis was performed. B7S3S mRNA was found expressed in nonlymphoid tissues, most abundantly in lung and at reduced levels in kidney, heart, and stomach (Fig. 2A). In addition, B7S3S mRNA was also expressed in lymphoid organs spleen, lymph nodes, and thymus. In contrast, the expression of B7S3L mRNA is very restricted, with only low levels detected in the thymus, stomach, intestine, and heart (Fig. 2B). The expression levels of B7S3 in lymphoid cells, including CD4+ T cells, CD8+ T cells, B cells, bone marrow-derived dendritic cells, and peritoneal macrophages, were also examined by real-time PCR. The levels of B7S3S are very low in these cells, and B7S3L almost undetectable (data not shown). We also measured the levels of B7S3S in bone marrow-derived dendritic cells and peritoneal macrophages after different stimuli, including LPS, CpG, TLR2, polyinosinic-polycytidylic acid (poly(I:C)),4 TNF-α, and IL-17. Interestingly, B7S3S is up-regulated after stimulation with poly(I:C) or TNF-α plus IL-17 in bone marrow-derived dendritic cells (Fig. 2C), and after LPS or IL-17 stimulation in peritoneal macrophages (Fig. 2D). These data indicate potential regulation of B7S3 expression by innate and inflammatory stimuli, though the significance of this observation needs to be further investigated.

**Expression of B7S3 receptors on APC and activated T cells**

To examine the expression pattern of the B7S3 receptors and its possible immune function, we constructed a B7S3-Ig fusion protein that contains the Ig-like domains of B7S3 and a human IgG tag (9). Interestingly, when we used the biontinylated B7S3-Ig fusion protein to stain the total splenocytes, we found it bound to B220+ B cells, CD11c+high dendritic cells and Mac-1+ macrophages in spleen (Fig. 3A) as well as peritoneal macrophages (data not shown). Other Ig fusion proteins, including B7S1, B7-H3, and B7h we prepared in the same fashion did not bind to these cells (9, 13, 20). Neither did boiled B7S3-Ig (Fig. 3A). The binding of B7S3-Ig to B cells was not affected by LPS stimulation (Fig. 3A). These results indicate that the B7S3 receptor is constitutively expressed on APCs, distinct from the receptors of other B7 molecules.

CD4 and CD8 T cells, conversely, were not bound significantly by B7S3-Ig (Fig. 3B). After activation by Con A for 48 h, B7S3-Ig

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4 Abbreviation used in this paper: poly(I:C), polyinosinic-polycytidylic acid.
binding was significantly up-regulated in both CD4 and CD8 T cells (Fig. 3B). B7S3 receptor expression on T cells can also be induced by anti-CD3 and anti-CD28 stimulation (data not shown). B7S3-Ig-binding cells also express CD69 and CD25 markers (Fig. 3C), indicating their activation states. Boiled B7S3-Ig did not bind to activated T cells (data not shown).

Therefore, the B7S3 receptor on T cells shares a similar expression pattern with the receptors for other new B7 family members, B7h (ICOS), PDL1/PDL2 (PD-1), B7-H3 (unknown), and B7S1 (unknown). We activated T cells from CD28 and ICOS knockout mice and found that the B7S3-Ig binding was not altered (Fig. 3B). B7S3-Ig did not bind to 293 cells transfected with a PD-1 expression vector (data not shown). In addition, B7S3-Ig bound similarly to BTLA knockout mice (open histogram) or heterozygous littermates (gray histogram) were stained with B220 Ab together with biotinylated B7S3-Ig or human IgG1. Con A-activated T cells were stained with CD4 or CD8 Ab together with biotinylated B7S3-Ig or human IgG1 (filled histogram). Streptavidin-PE was used as secondary detection agent in A and D and streptavidin-allophycocyanin was used in B and C.

B7S3-Ig inhibits T cell activation and effector function

To study B7S3 function in the immune system, we first tested the effect of B7S3-Ig on B cells and macrophages by itself or together with other stimuli such as LPS or anti-CD40 for B cells and LPS...
FIGURE 5. B7S3-Ig inhibits IL-2 production as well as NFAT and NF-κB activities. DO11.10 cells transfected with NFAT-luciferase or NF-κB-luciferase reporter constructs were untreated or treated with plate-bound 1 μg/ml anti-CD3 Ab alone and with 5 μg/ml human IgG1 (HuIg) or B7S3-Ig for 4 h, and luciferase activity was measured. Results were shown as Relative Luciferase Activity (RLA). Supernatant IL-2 was measured by ELISA.

Distinct genomic structure of B7S3 loci in human and mouse

In addition to the two forms of B7S3, we found 10 close homologs of B7S3 within a 2-Mb region on mouse chromosome 4 (Fig. 7A). They all have a pair of exons encoding the Ig-like domains sharing various degrees of homology with those of B7S3, from 86% to 44% (Fig. 7), which are well above the homology seen between B7S3 and other B7 or butyrophilin family members. Therefore, a B7S3 gene family exists in mice. A search in the mouse EST database revealed that four genes had corresponding EST clones. To study the human B7S3 gene, we used the deduced amino acid sequence of mouse B7S3 to search back in the human genome database. A single pair of two exons bearing highest homology to mouse B7S3 was found located on human chromosome 1 (Fig. 7A). A search of the available rat genome database indicated a similar genomic B7S3 configuration as in mouse (data not shown). This information nonetheless suggests selective evolution of immune regulatory pathways within mammalian species.

Discussion

In this study, we identify a new B7 family member, B7S3, as a potential negative regulator in immune system. B7S3-Ig we constructed identified a putative receptor expressed constitutively on APC and inducibly on activated T cells. B7S3-Ig potently inhibited T cell activation as well as cytokine production by effector T cells. Consistently, B7S3-Ig greatly attenuated IL-2 production and NFAT and NF-κB activities in DO11.10 cells induced by anti-CD3 treatment. These results indicate that B7S3 inhibits the transcriptional machinery responsible for cytokine gene expression in activated T cells. B7S3 thus may play similar functional roles as the existing negative costimulatory molecules in the B7 family.

Structurally, B7S3 shares significant sequence homology with existing B7 and butyrophilin family members. They have been proposed to be included in an extended B7 family based on their sequence similarities compared with other Ig superfamily members (19). In this extended B7 family, there has been an expansion of negative costimulatory molecules. Notably, BTNL2 was reported as the first member of the butyrophilin family that inhibited T cell activation (17). The importance of BTNL2 function was revealed by its association with the inflammatory autoimmune diseases sarcoidosis and myositis in human (22, 23). The B7 and butyrophilin family members may thus have similar functional roles in immune regulation.

Two isoforms of B7S3 exist with the long form bearing additional transmembrane regions. The short form was initially regarded as a secreted protein because it did not have a long enough stretch of hydrophobic amino acids for spanning across the plasma membrane. However, recombinant full-length B7S3 protein was
not secreted from Drosophila S2 or human 293 cells. When enhanced GFP was fused to the N terminus of the B7S3 native sequence, both B7S3S and B7S3L were expressed on the membrane of 293 cells. These studies reveal that both short and long forms are expressed on the cell surface. It is possible at this stage that the surface expression of the short form may be facilitated by other proteins or mechanisms.

In addition to the two forms, we found 10 close homologs of B7S3 within a 2-Mb region on mouse chromosome 4. However, there is only one counterpart on human chromosome 1. The distinct genomic structure of B7 molecule in rodents and humans is not unique to B7S3. We previously reported that human B7-H3 has two isoforms, one of which contains four Ig-like domains (9). This four-Ig B7-H3 isoform represents the major form in most human tissues, although absent in mice. However, the differential expansion of the B7S3 gene in mammalian species is to our knowledge very rare.

The finding of two forms of B7S3, together with its 10 close family members, broadens our understanding of the B7 family negative costimulatory molecules, and likely has implications in immune regulation. The broad expression of B7S3 in lymphoid and nonlymphoid organs suggests its involvement in regulation of immune responses. Much more work needs to be performed in the future to illustrate the physiological function of B7S3 in immune responses and diseases.

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Disclosures

The authors have no financial conflict of interest.

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