Published in final edited form as:
J Immunol. 2006 June 15; 176(12): 7354–7360.

BTNL2, a Butyrophilin-Like Molecule That Functions to Inhibit T Cell Activation[1]

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Abstract

B7 family members regulate T cell activation and tolerance. Although butyrophilin proteins share sequence homology with the B7 molecules, it is unclear whether they have any function in immune responses. In the present study, we characterize an MHC class II gene-linked butyrophilin family member, butyrophilin-like 2 (BTNL2), the mutation of which has been recently associated with the inflammatory autoimmune diseases sarcoidosis and myositis. Mouse BTNL2 is a type I transmembrane protein with two pairs of Ig-like domains separated by a heptad peptide sequence. BTNL2 mRNA is highly expressed in lymphoid tissues as well as in intestine. To characterize the function of BTNL2, we produced a BTNL2-Ig fusion protein. It recognized a putative receptor whose expression on B and T cells was significantly enhanced after activation. BTNL2-Ig inhibited T cell proliferation and TCR activation of NFAT, NF-κB, and AP-1 signaling pathways. BTNL2 is thus the first member of the butyrophilin family that regulates T cell activation, which has implications in immune diseases and immunotherapy.

The B7 superfamily members are crucial regulators of T cell activation and tolerance (1–3). B7.1 and B7.2, which engage CD28 on naive T cells, are highly up-regulated on APC by infectious agents (4). Mice deficient in CD28 or both B7.1 and B7.2 were found to exhibit severe impairments in CD4 T cell activation and function (5,6). A second receptor for B7, CTLA4, is induced on activated T cells (7). Mice deficient in CTLA4 died at neonatal stage due to massive T cell activation and infiltration into tissues, indicating a crucial role of CTLA4 in immune tolerance. B7h/B7RP-1 binds to ICOS, the third member of the CD28 family on activated T cells (8,9). Analysis of mice deficient in ICOS or its ligand, B7h revealed that this pathway regulates T cell activation, differentiation, and effector function (10,11). Programmed death 1 (PD-1)[4] is an inhibitory receptor expressed on activated T and B cells, which binds to the B7 family members PD-L1 (B7-H1) and PD-L2 (B7-DC) (12). The spontaneous auto-immunity seen in PD-1-deficient mice indicates its critical function in immune tolerance (13). B7-H3 is widely expressed in both lymphoid and nonlymphoid tissues, with a putative receptor expressed on activated but not naive T cells (14,15). Human B7-H3 was first found to be a positive costimulator (15); recent studies by others suggested that mouse B7-H3 may

[1]This work was supported by grants from National Institute of Health (to C.D.). C.D. is a Cancer Research Institute Investigator, an Arthritis Investigator of the Arthritis Foundation, and an M. D. Anderson Cancer Center Trust Fellow. T.N. was supported by a National Institutes of Health grant, and Y.Z. is an Odyssey Scholar of the M. D. Anderson Cancer Center.

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Disclosures
The authors have no financial conflict of interest.

[4]Abbreviations used in this paper: PD-1, programmed death 1; BTNL2, butyrophilin-like 2.

serve as a negative regulator in T cell activation (16,17). B7S1/B7x/B7-H4, a recently identified B7 family member, works through a putative receptor on activated T cells to inhibit their proliferation and IL-2 production (18–20).

Notably, the new B7 family members share considerable sequence homology with butyrophilin family proteins. The prototypical butyrophilin is a milk protein important in regulating secretion of milk droplets (21). LoucsLink annotates 15 butyrophilin-like genes in mice and 16 in humans. Butyrophilin proteins typically have a signal peptide, an IgV-like and IgC-like domain, and a transmembrane and cytoplasmic domain. In addition, they often possess a heptad repeat which is a 7-aa sequence encoded by a single exon (22–24). Many butyrophilin molecules also contain in the cytoplasmic region a B30.2 domain, comprising ~170 aa found also in tripartite motif (TRIM) proteins and stonutoxin (25,26). The precise function of B30.2 domain in butyrophilin is unclear; it has been recently found that TRIM5α protein interacts with human HIV via its B30.2 domain (27). Because the IgV-like domains of B7.1 and B7.2 are most similar to the IgV-like domain of butyrophilin than to any other sequence, Linsley et al. (28) proposed that B7 and butyrophilin molecules might have evolved from a common ancestral gene to compose a subfamily within the Ig superfamily. It is unclear whether B7 and butyrophilin molecules share common functions in regulating immune responses.

Several recent studies have associated mutations of butyrophilin-like 2 (BTNL2; also called BTL-II), an MHC class II gene-linked butyrophilin-like molecule, with the human autoimmune diseases sarcoidosis and myositis (29–31). Valentonyte et al. (30) and others reported that a truncating splice site mutation in BTNL2 disrupted its membrane localization and associated this mutation with sarcoidosis, a disease characterized by increased inflammatory activity of macrophages and $CD4^+$ Th cells (32,33). However, the immunological basis of these observations was not understood.

In this paper, we characterize mouse BTNL2 structure and expression, and show that a putative receptor for BTNL2 is expressed on activated T and B cells. BTNL2 inhibited T cell proliferation. BTNL2 is thus the first butyrophilin family member that possesses an immunoregulatory function, which will likely add another layer of complexity to the regulation of T cell activation and tolerance.

Materials and Methods

Cloning and sequence analysis of BTNL2

A mouse spleen cDNA library obtained from Dr. M. Bevan's laboratory (University of Washington, Seattle, WA) was used to clone full-length BTNL2 using PCR primers (forward, ATGGTGGATTGCCCACGG TATAG, and reverse, TCATCTGAGCCTCTCATCAGAAG). The full-length cDNA of BTNL2 was cloned by high-fidelity DNA polymerase. And the PCR products were directly sequenced multiple times. Then, the sequence results were compared with existing sequence in the National Center for Biotechnology Information (NCBI) database, and the polymorphic differences were indicated in Fig. 1*A*. Comparison of BTNL2 amino acid sequence with other B7 and butyrophilin family members was performed using CLUSTAL W. Full-length protein sequences of murine B7 family members and butyrophilins were used for phylogenetic analysis with the exception of human BT3.1, which does not have a murine homolog. The location of the intron/exon boundary of the *BTNL2* gene was identified using the NCBI databases. Transmembrane prediction was performed with TMHMM Server v. 2.0 (www.cbs.dtu.dk/services/TMHMM-2.0) and the leader sequence was predicted with SignalP V2.0.b2 (www.cbs.dtu.dk/services/SignalP-2.0/#submission).

Real-time PCR analysis

Primers spanning the third/fourth Ig domains (forward, TTCACAATGC CAGAACTTCG (983–1002 nt); reverse, TTCCATCTCTGTCCCTCCAC (1179–1198 nt)) and the transmembrane/intracellular coding sequences (forward, CTCTGGGCCAGGAGAAAAC (1319–1337 nt); reverse, TGAGCCTCTCATCAGAAGGAA (1520–1540 nt)) were used to analyze the expression of BTNL2 mRNA. 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 iCyclere iQ real-time detection software (Bio-Rad).

Production of BTNL2-Ig protein

The sequence coding the extracellular portion of BTNL2 protein (aa 26–457) was PCR amplified and subcloned into DES-hIg vector consisting of an insect expression plasmid pMT/Bip/V5-HisA (Invitrogen Life Technologies) backbone and a human IgG1 Fc tag containing the dimerization sequence (14). The PCR primers, with added restriction sites, were as follows: forward, AGATCTCACCCAGATGACTTCAGAGTGGTC (73–96 nt), and reverse, ACTAGTTATCTTGGAGTCTGAGAGAGGGAAAC (1343–1368 nt). The BTNL2-Ig expression vector was stably transfected into *Drosophila* S2 cells and induced to secrete BTNL2-Ig fusion protein upon $CuSO_4$ treatment. BTNL2-Ig fusion protein was further purified by a protein A column. SDS-PAGE and Coomassie blue staining were used to analyze the protein preparation, which showed only one major band at the predicted m.w.

Flow cytometry analysis

BTNL2-Ig and a control human IgG were biotinylated with Sulfo-NHS-LC-Biotin (Pierce) and used in conjunction with anti-CD4-FITC (L3T4), CD8-Percy5.5 (Ly-2), CD11b-PE (3A33), CD11c-PE (HL3), and B220-FITC (RA3-6B2) Abs (BD Pharmingen) for analysis of various populations of immune cells by flow cytometry. Total splenocytes were activated with 5 μg/ml LPS for 2 days before B cell analysis. Total splenocytes were activated with 2.5 μg/ml Con A for 2 days before T cell analysis. For B7.1-Ig blocking experiment, splenocytes were preincubated with 20-fold excess of B7.1-Ig before being stained with biotinylated BTNL2-Ig. 293T cells were transfected with a PD-1 expression vector using calcium phosphate, and subsequently stained with biotinylated PD-L1-Ig or BTNL2-Ig. For activation-induced cell death assay, $CD4^+$ T cells were activated with indicated doses of plate-bound anti-CD3 and 5 μg/ml BTNL2-Ig or human Ig for 24 h before staining with propidium iodide followed by flow cytometry analysis. The percentage of cells stained positive for propidium iodide was determined as an estimate of apoptosis, and plotted against anti-CD3 concentration.

In vitro T cell assays

$CD4^+$ T cells from naive C57BL/6 mice were purified by autoMACS with anti-CD4 microbeads to 90–95% purity. T cells were treated with plate-bound anti-CD3 (2C11) and/or anti-CD28 (37.51) Abs (BD Pharmingen) in the absence or presence of a human IgG or BTNL2-Ig protein. Complete RPMI 1640 medium containing 10% FBS was used for T cell culture. IL-2 production was measured 24 h after T cell activation, and cell proliferation was measured at 72 h with the addition of $[^3H]$thymidine in the last 6–8 h.

Luciferase assay

DO11.10 T cell hybridoma cells were maintained in complete RPMI 1640 medium supplemented with 10% FBS (HyClone). A total of 5×10^6 cells was transfected with 1 μg of luciferase promoter reporter plasmids of NFAT, NF-κB, or AP-1 (gifts from Dr. R. Flavell at

Yale University, New Haven, CT) and 0.25 μg of PRL-null (Promega) by electroporation at 960 microfarads and 250 V as the manufacturer recommended (Bio-Rad). After overnight culture, the cells were stimulated with 1 μg/ml plate-bound anti-CD3 Ab with or without human Ig fusion protein for 4 h. Cells were lysed and luciferase activity was measured by using the Dual-Luciferase system as the manufacturer recommended (Promega).

Results

Characteristics of mouse BTNL2 molecule

Although butyrophilin molecules share significant sequence homology with B7 family members, their functions in the immune system have not been studied. We chose to initiate our investigation on BTNL2, because it is located in the MHC class II locus between I-Ea and Notch on mouse chromosome 17. Full-length murine BTNL2 cDNA was amplified from a spleen cDNA library and sequenced. Predicted mouse BTNL2 protein has a distinct protein structure when compared with B7 family members. It has four extracellular Ig domains, consisting of two IgV-IgC pairs (IgVa-IgCa and IgVb-IgCb), unlike most B7s, which have two. Human and monkey B7-H3 uniquely have two-Ig and four-Ig isoforms, and the latter is predominantly expressed in most tissues (14). The Ig domains of BTNL2 have conserved cysteines and the DxGxYxC motif in the two IgV-like domains (Fig. 1*A*). Phylogenetic analysis of BTNL2 shows it to be more similar to the butyrophilin family members than to B7 family molecules (Fig. 1*B*). This is further affirmed by the presence of a heptad sequence, characteristic for many butyrophilins, which is located between the two pairs of IgV-IgC domains (Fig. 1*C*). Unlike many other butyrophilins such as BT3.1, BTNL2 does not have a B30.2 domain in the intracellular region (34).

We identified the transmembrane and intracellular domains of mouse BTNL2 (Fig. 1*A*), which were not reported by Stammers et al. (35). Valentonyte et al. (30) had previously shown the transmembrane and intracellular regions in humans. Overall, human and mouse BTNL2 proteins are 63% identical (Fig. 1*A*). We confirmed that human BTNL2 mRNA obtained from peripheral blood cells lacked the IgCa domain as reported by Valentonyte et al. (30). However, human and mouse BTNL2 may function similarly, because the IgV domain of B7 family member mediates receptor binding (36). The NCBI database established all of the intron/exon boundaries for mouse *BTNL2* gene. As with all B7 members, each domain is encoded by a separate exon (Fig. 1*C*). In mice, five other butyrophilin-like proteins are found within this same region; however, these five genes are not conserved in humans (Fig. 1*C*) (35).

Expression of BTNL2 mRNA

To examine BTNL2 expression, we used two pairs of PCR primers that span either the third/fourth Ig domains or the transmembrane/intracellular coding regions of *BTNL2* gene. RT-PCR analysis revealed that BTNL2 is widely expressed in tissues we examined. To compare their relative expression levels of BTNL2 in different tissues, real-time PCR analysis was used. BTNL2 mRNA was found expressed in nonlymphoid tissues, most abundantly in intestine and at reduced levels in lung and stomach (Fig. 2*A*). In addition, BTNL2 mRNA was also expressed in lymphoid organs thymus, spleen, and lymph nodes. We then performed PCR analysis on purified immune cells. BTNL2 was expressed in T cells, B cells, and macrophages (Fig. 2*B*).

Expression of a putative receptor for BTNL2

To study the function of BTNL2, we produced a soluble BTNL2-Ig fusion protein that contains the extracellular portion of BTNL2 and the Fc domain of human IgG1. BTNL2-Ig was purified by protein A, biotinylated, and used to determine where a putative receptor for BTNL2 was expressed. A BTNL2 receptor, bound by BTNL2-Ig, was found constitutively expressed on splenic $B220^+$ B cells (Fig. 3*A*). B cells appeared to express a higher level of the receptor after

LPS stimulation for 2 days. Bone marrow dendritic cells, bone marrow macrophages, peritoneal macrophages, granulocytes, and NK cells were not significantly bound by BTNL2-Ig (data not shown). In contrast, Con A-activated T cells but not unstimulated T cells were also significantly stained by BTNL2-Ig (Fig. 3*B*). Staining of BTNL2-Ig was also observed in activated but not resting DO11.10H hybridoma cells (data not shown).

B7 family members signal through members of the CD28 superfamily. Whereas CD28 is expressed by naive T cells, CTLA4, ICOS, and PD-1 are expressed by activated T cells. BTNL2 receptor expression on both T and B cells is similar to PD-1, which is expressed on activated T and B cells (37,38). BTNL2-Ig bound to T cells from CD28- or ICOS-deficient mice after Con A activation (Fig. 3*B*). B7.1-Ig did not block binding of BTNL2-Ig to activated T cells (Fig. 3*C*), suggesting that BTNL2 does not bind to CD28 or CTLA4. In addition, BTNL2-Ig did not stain PD-1 transfected 293 cells, whereas PDL1-Ig could (Fig. 3*D*). All of these indicate that a putative receptor for BTNL2 is distinct from CD28, CTLA4, ICOS, and PD-1.

Immobilized BTNL2-Ig inhibits T cell activation

Although BTNL2 receptor is expressed on B cells, it does not appear to affect B cell proliferation in contrast to PD-1. BTNL2-Ig, when given either in solution or plate bound, did not affect B cell proliferation when these cells were activated by either LPS or the combination of anti-IgM and anti-CD40 (data not shown). BTNL2-Ig did not induce B cell proliferation by itself (data not shown), which also indicates that there is no significant endotoxin contamination in our protein preparation.

In T cells, the receptor of BTNL2 is up-regulated upon activation. When $CD4^+$ T cells were activated with anti-CD3 and anti-CD28, a dose-dependent inhibition of proliferation was observed in the presence of plate-bound BTNL2-Ig compared with human IgG control (Fig. 4*A*). Activation-induced cell death was not increased by BTNL2-Ig treatment (Fig. 4*B*). When BTNL2-Ig was given in soluble form instead of plate bound, it could not inhibit anti-CD3- and anti-CD28-induced proliferation of $CD4^+$ T cells (Fig. 4*C*). This implies that BTNL2, like PD-L1 (39,40), inhibits proximal TCR signaling events and that BTNL2 receptor must be in proximity of the TCR to achieve this inhibition.

Two new B7 family members, B7-H3 and B7S1, were recently reported to inhibit mouse T cell proliferation (17,18). We compared the actions of BTNL2-Ig, B7-H3-Ig, and B7S1-Ig, all prepared in the same fashion in our lab. In the absence of CD28 costimulation, BTNL2-Ig, B7S1-Ig, or B7-H3-Ig all effectively inhibited anti-CD3-stimulated T cell proliferation (Fig. 4*D*). When CD28 costimulation was provided by anti-CD28, BTNL2-Ig inhibited T cell proliferation more strongly than B7-H3-Ig but less potently than B7S1-Ig (Fig. 4*D*).

We previously showed that inhibition of T cell proliferation by B7S1 or B7-H3 was functionally associated with reduced IL-2 production (17,18). We thus tested whether BTNL2 inhibits IL-2 expression by activated T cells. Plate-bound BTNL2-Ig moderately reduced IL-2 production when $CD4^+$ T cells were activated with different doses of anti-CD3 alone (Fig. 5*A*). A modest but dose-dependent reduction of IL-2 production by BTNL2-Ig was also observed when $CD4^+$ T cells were activated with anti-CD3 together with anti-CD28 (Fig. 5*B*). Notably, the ability of BTNL2-Ig to suppress T cell proliferation was more potent than its effect on IL-2 production.

We thus tested whether inhibition of T cell proliferation by BTNL2-Ig could be restored by the addition of exogenous IL-2. In the absence of CD28 costimulation, inhibition of proliferation by BTNL2-Ig was the least responsive to IL-2, whereas proliferation of T cells treated with B7S1-Ig and B7-H3-Ig was sharply increased by IL-2 (Fig. 5*C*). Inhibition of proliferation by BTNL2 is thus in part but not solely via reduction of IL-2 production and the

mechanism of inhibition by BTNL2 may differ from that by B7-H3 and B7S1. In contrast, CD28 costimulation greatly increased proliferation in B7-H3-treated T cells, less so in BTNL2-Ig-treated cells, and had no effect on cells stimulated with B7S1-Ig (Fig. 5*C*). IL-2 and CD28 costimulation synergistically enhanced T cell proliferation in the presence of B7-H3-Ig or BTNL2-Ig but not with B7S1-Ig (Fig. 5*C*). This result suggested differential mechanisms used by these three molecules in T cell regulation.

DO11.10 hybridoma (DO11.10H) has been used to dissect TCR and coreceptor signaling mechanisms. DO11.10H expressed a receptor for BTNL2 upon activation, and BTNL2-Ig partially inhibited IL-2 production and activation-induced cell death in DO11.10H cells (data not shown). To understand the action of BTNL2, we transfected DO11.10H with luciferase reporters for AP-1, NFAT, and NF-κB pathways. BTNL2-Ig treatment resulted in at least 50% reduction of anti-CD3-mediated activation of AP-1, NFAT, and NF-κB (Fig. 6). Inhibition of all three general pathways suggests that BTNL2, like other negative B7-like costimulators, inhibits proximal TCR signaling events.

Discussion

Despite the significant homology between butyrophilin proteins and B7 family members, the function of butyrophilin family members in immune regulation is largely unknown. Recently, mutation of BTNL2 was found associated with the autoimmune diseases sarcoidosis and myositis; however, the immunological basis for this observation is unclear. In the present study, we show that BTNL2 binds to a putative receptor on activated T cells and functions to inhibit the proliferation of T cells. This is the first case where a butyrophilin molecule regulates immune responses. Because the function of butyrophilin family members is largely unknown, it is attractive in the coming years to illustrate their possible function in the immune system.

Recent years have witnessed expansion of negative costimulatory molecules in the B7 family. It is a challenge in immunology to understand whether these molecules have redundant or specific function. BTNL2, a member of the butyrophilin family, joins these B7 molecules as a potential negative regulator of T cells. We found some interesting features of BTNL2 that may suggest its potential function in immune regulation. First, by real-time PCR analysis, BTNL2 expression was not only found in lymphoid organs but also in nonlymphoid organs, most abundantly in intestine. Our RT-PCR expression analysis of murine BTNL2 differs substantially from previously published analysis by Stammers et al. (35), who showed mRNA expression of BTNL2 mainly in the skeletal muscle, placenta, and intestine. Stammers et al. used primers that were then predicted to be specific for the 3′ untranslated region but was actually localized in an intronic region based on current knowledge on the gene structure. Human BTNL2 was reported to exhibit broad expression in tissues (30); however, a quantity comparison of the expression levels was not available. Abundant expression of BTNL2 mRNA in intestine may suggest important function of BTNL2 in regulation of mucosal immunity or tolerance.

In contrast, expression of BTNL2 in lymphoid organs suggests its involvement in initiation of immune responses. Indeed, the BTNL2-Ig we constructed potently inhibited T cell proliferation, but only modestly, the IL-2 production. Addition of exogenous IL-2 only moderately improved the proliferation of T cells treated with BTNL2-Ig. This appears unique compared with T cells treated with B7-H3 or B7S1 fusion proteins. Moreover, inhibition of T cell proliferation by B7-H3-Ig or BTNL2-Ig, but not by B7S1-Ig, could be reversed by anti-CD28 costimulation. Although qualitative difference here may be due to the nature of the recombinant fusion proteins, the differential response by T cells treated with different molecules to anti-CD28 and IL-2 suggests distinct intracellular signaling mechanisms whereby the receptors for these molecules function.

The inhibitory function of BTNL2 may explain the association of BTNL2 mutation with inflammatory autoimmune diseases such as sarcoidosis and myositis (30,31). Much more work needs to be performed by using specific Abs to BTNL2 and by gene-targeting animals to illustrate the in vivo function of BTNL2 in immune tolerance. This line of findings will likely have implications in other immune diseases and immunotherapy, and also indicates possible roles of other butyrophilin molecules in immune regulation.

Acknowledgements

We thank the entire Dong laboratory, especially Dr. Roza Nurieva, Yang Yang, and Dr. Zhaoxia Li, for their help. We are grateful to Dr. Andy Farr for his support.

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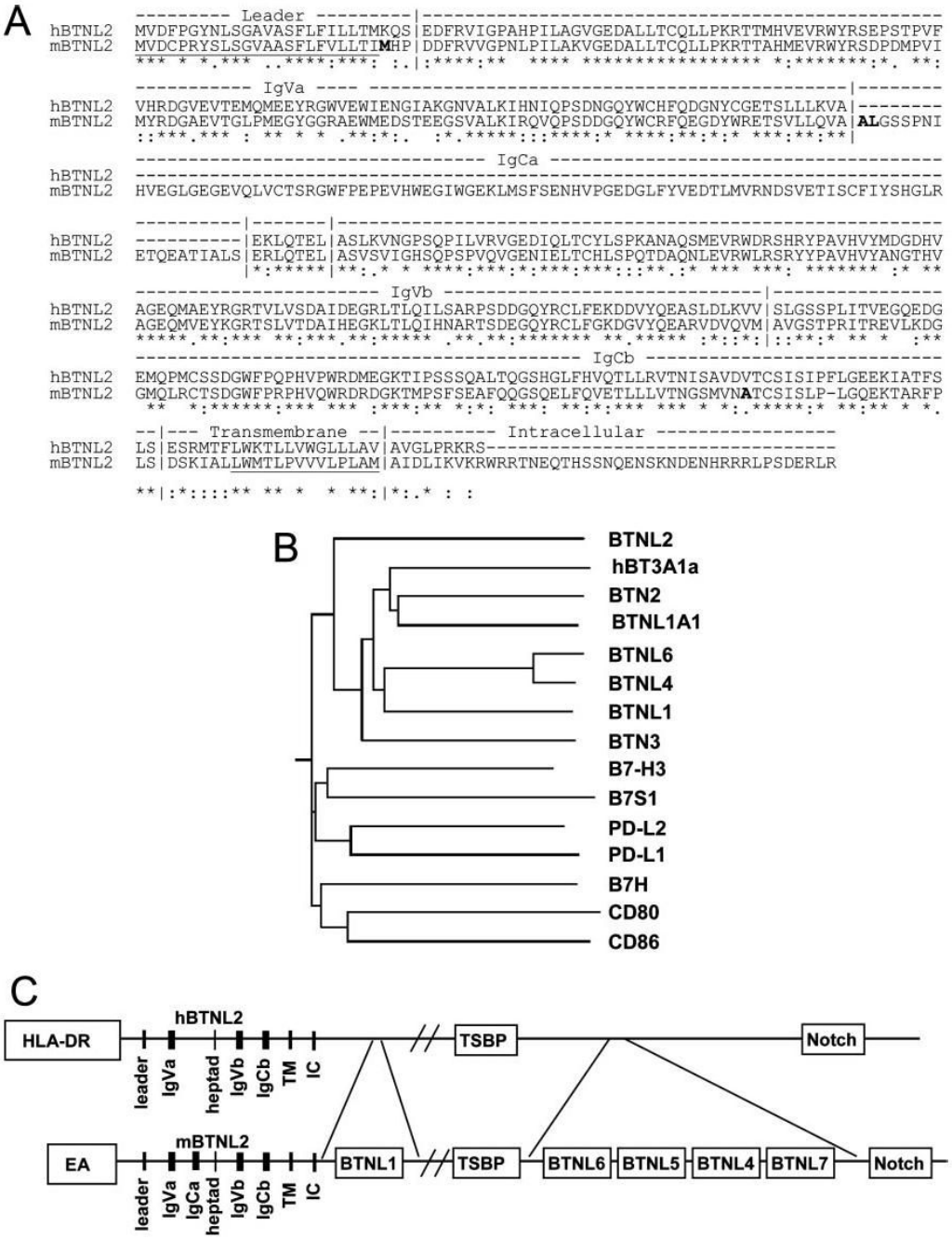

FIGURE 1.

Cloning and sequence analysis of BTNL2. *A*, Full-length BTNL2 cDNA was cloned from a spleen cDNA library. Sequence analysis revealed four polymorphic differences from the existing sequence in NCBI database, which are shown in bold. The predicted mouse BTNL2 amino acid sequence was aligned with human BTNL2 sequence. The bar symbol (|) marks the intron/exon border, and leader and transmembrane domains are underlined. An asterisk (*) denotes identity, a colon (:) denotes conservation of strong groups, and a period (.) denotes conservation of weak groups between mouse and human sequences. *B*, Phylogenetic analysis of the full-length BTNL2 protein among B7/butyrophilin superfamily members. *C*, Genomic

organization of the human and mouse *BTNL2* gene were adapted from Stammers et al. (35) and Valentonyte et al. (30) with our sequence confirmation.

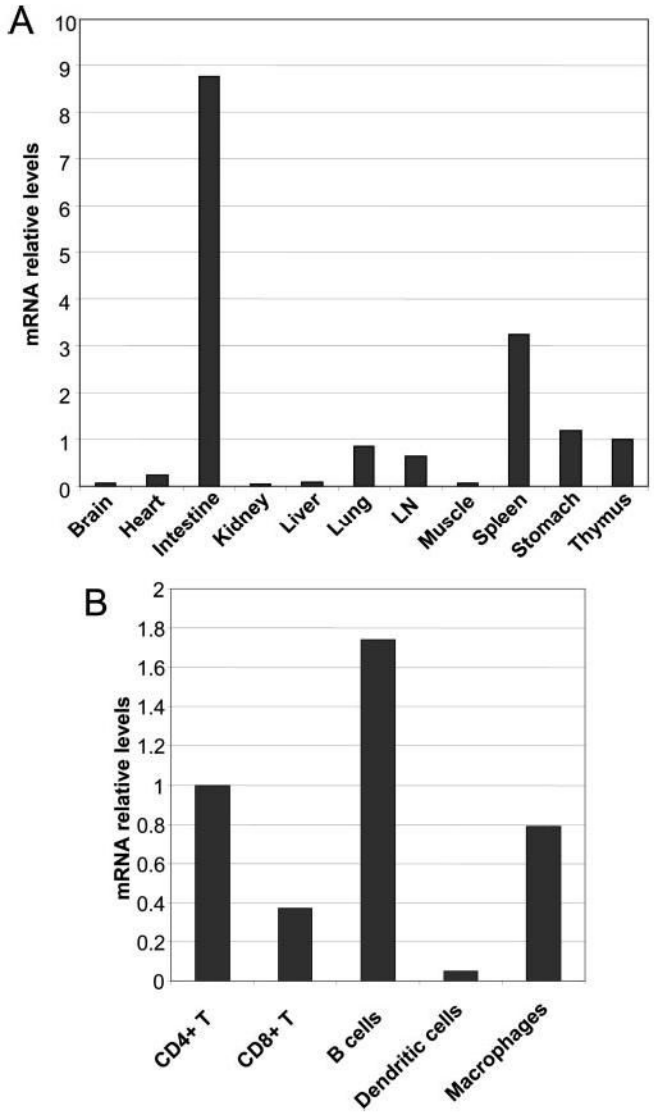

FIGURE 2.

Expression of BTNL2 mRNA. Real-time PCR analysis was conducted using primers specific for the transmembrane and intracellular regions of BTNL2. *A*, Tissues harvested from a B6 mouse were analyzed for BTNL2 expression, and the expression level in thymus was set at 1. *B*, $CD4^+$, $CD8^+$ T cells and B cells purified from spleen and lymph nodes by AutoMACS sorting (90–95% purity), bone marrow-derived dendritic cells, and peritoneal macrophages were used in the analysis. The expression level by $CD4^+$T cells was set at 1. The data are representative of three experiments.

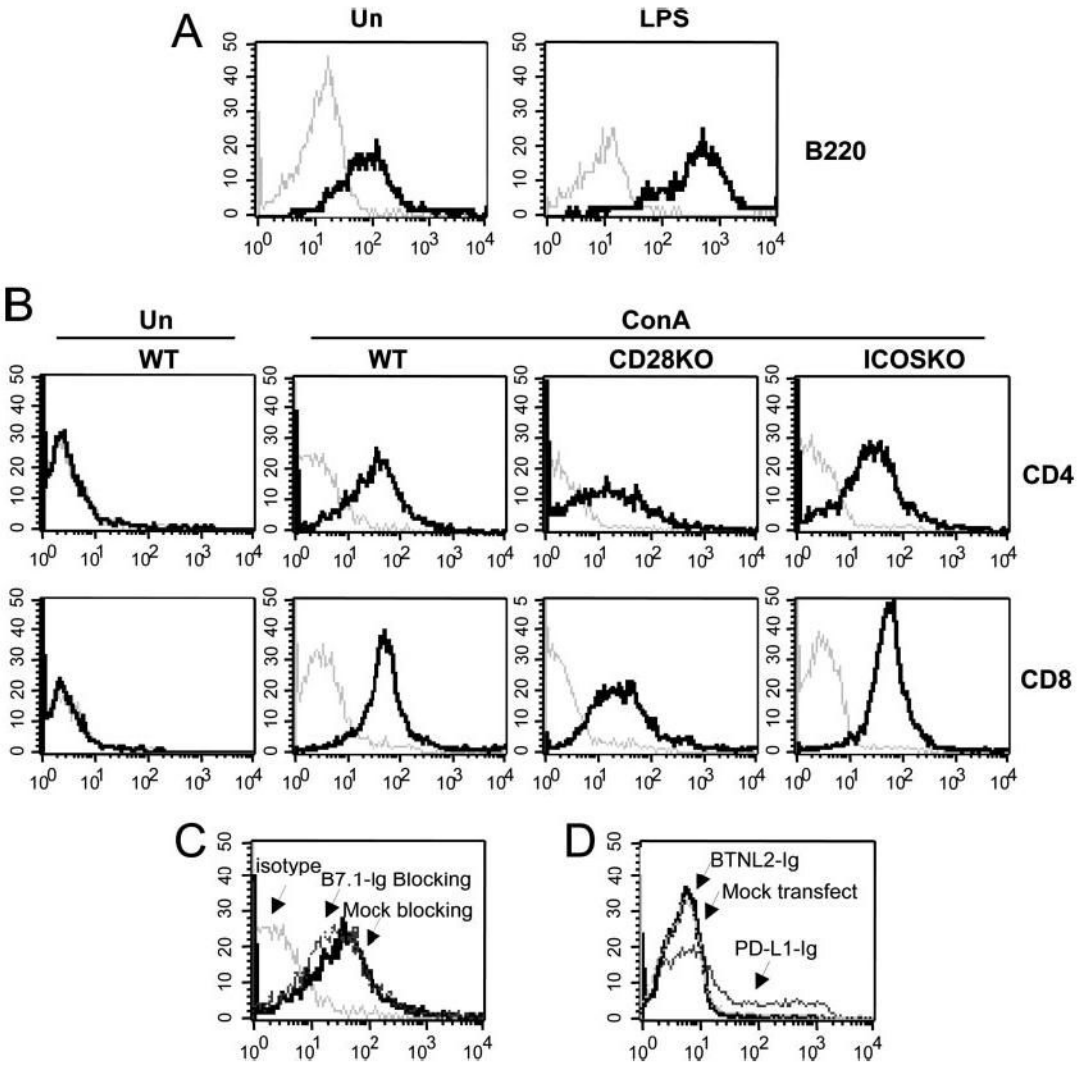

FIGURE 3.

Expression of a BTNL2 receptor in lymphocytes. BTNL2-Ig fusion protein was biotinylated to stain B cells before and after LPS treatment, and $CD4^+$ and $CD8^+$ T cells before and after activation with Con A, which was revealed by streptavidin-PE. Histogram analysis was performed on gated B cells (*A*), wild-type, CD28- or ICOS-deficient $CD4^+$ or $CD8^+$ T cells (*B*). The lighter line represents human IgG1 isotype control staining; the darker line, BTNL2-Ig staining. Con A-activated splenocytes were preincubated with 20-fold excess of B7.1-Ig before stained with biotinylated BTNL2-Ig (*C*). 293 cells were transfected with a PD-1 expression vector, and subsequently stained with biotinylated PD-L1-Ig or BTNL2-Ig (*D*). The data are a representative of at least three experiments.

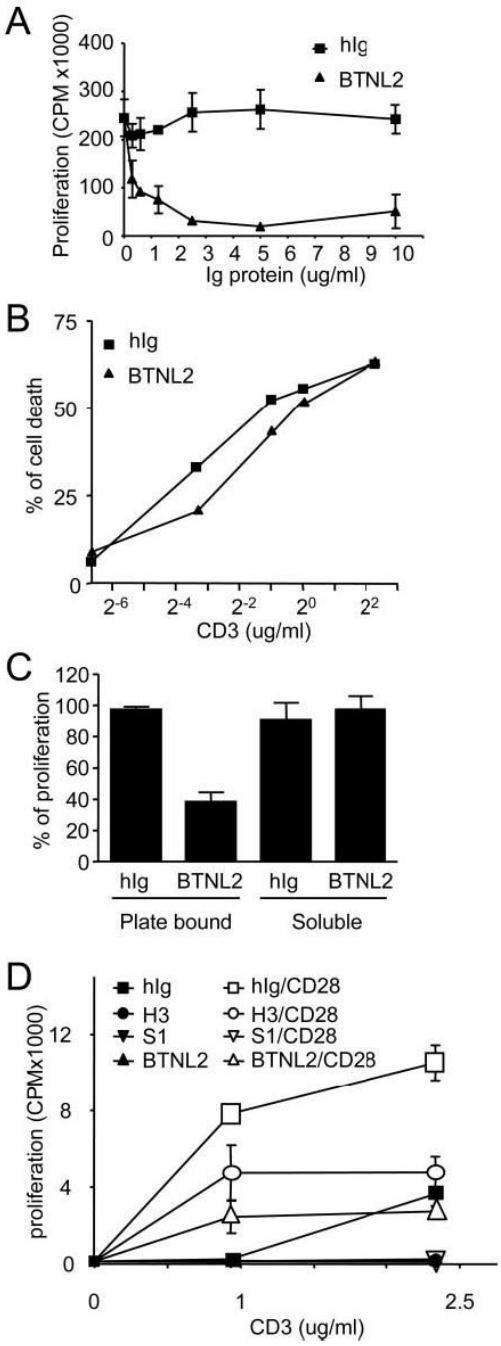

FIGURE 4.

BTNL2 inhibits T cell proliferation. Purified $CD4^+$ T cells were treated for 3 days, and [^{3}H] thymidine uptake was examined. *A*, T cells activated with 1 μg/ml plate-bound anti-CD3 and anti-CD28 in the presence of indicated doses of BTNL2-Ig or control human IgG (hIg). *B*, T cells activated with indicated doses of plate-bound anti-CD3 and 5 μg/ml BTNL2-Ig or hIg for 24 h before measuring activation induced cell death. *C*, T cells were activated with anti-CD3 and anti-CD28 as above in the presence of 10 μg/ml plate-bound or soluble BTNL2-Ig or control hIg. Proliferation of T cells treated with hIg was set at 100%. *D*, T cell proliferation after activation with indicated doses of anti-CD3 with or without 1 μg/ml CD28 and in the presence of 10 μg/ml hIg, BTNL2-Ig, B7S1-Ig, or B7H3-Ig. The data are representative of

more than three experiments, with error bars indicating the SD of triplicate samples in each experiments.

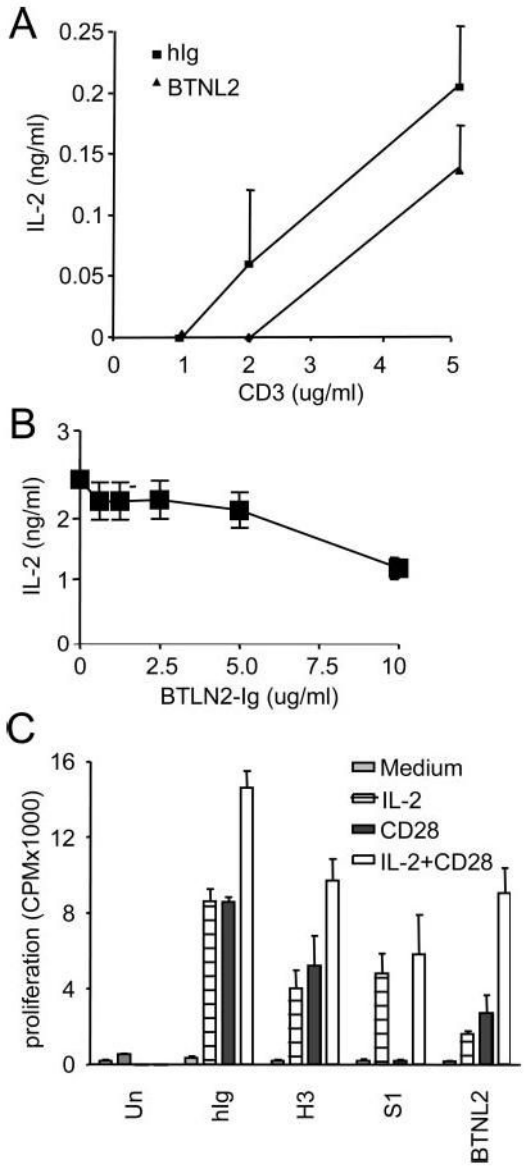

FIGURE 5.

IL-2 regulation by BTNL2. *A*, $CD4^+$ T cells were activated with different doses of plate-bound anti-CD3 in the presence of 10 μg/ml BTNL2-Ig or hIg. IL-2 production was measured at 24 h after treatment by ELISA. *B*, $CD4^+$ T cells were activated with 2 μg/ml anti-CD3 and 1 μg/ml anti-CD28 in the presence of different doses of BTNL2-Ig, and IL-2 production was examined by ELISA. *C*, $CD4^+$ T cells were treated with 1 μg/ml anti-CD3 in the presence of 10 μg/ml hIg, BTNL2-Ig, B7S1-Ig, or B7H3-Ig with or without IL-2 (100 U/ml) and/or 1 μg/ml anti-CD28 for 3 days, and [^{3}H]thymidine uptake was measured. The data are a representative of more than three experiments, with error bars indicating the SD of triplicate samples in each experiments.

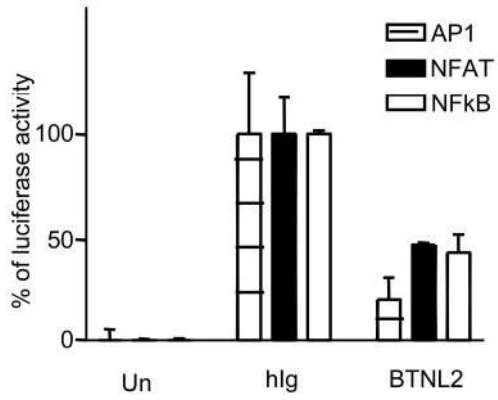

FIGURE 6.

BTNL2 inhibits TCR signaling pathways. DO11.10 hybridoma was transfected with luciferase reporter constructs for each individual signaling pathway, and stimulated with 1 μg/ml plate-bound anti-CD3 in the presence of 10 μg/ml BTNL2-Ig or hIg for 4 h before luciferase activity measurement. The data are representative of at least three experiments, with error bars indicating the SD of triplicate samples in each experiment.