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A Butyrophilin Family Member Critically Inhibits T Cell Activation

Tomohide Yamazaki,* Iñigo Goya,^{†,1} Daniel Graf,[‡] Suzanne Craig,[§] Natalia Martin-Orozco,* and Chen Dong*

The costimulatory molecules in the B7-CD28 families are important in the regulation of T cell activation and tolerance. The butyrophilin family of proteins shares sequence and structure homology with B7 family molecules; however, the function of the butyrophilin family in the immune system has not been defined. In this study, we performed an analysis on multiple butyrophilin molecules and found that butyrophilin-like (BTNL)1 molecule functions to dampen T cell activation. BTNL1 mRNA was broadly expressed, but its protein was only found in APCs and not T cells. The putative receptor for BTNL1 was found on activated T cells and APCs. Also, recombinant BTNL1 molecule inhibited T cell proliferation by arresting cell cycle progression. The administration of neutralizing Abs against BTNL1 provoked enhanced T cell activation and exacerbated disease in autoimmune and asthma mouse models. Therefore, BTNL1 is a critical inhibitory molecule for T cell activation and immune diseases. *The Journal of Immunology*, 2010, 185: 5907–5914.

T cell activation is regulated simultaneously by positive and negative signals, in part but importantly via the interaction of costimulatory molecules between receptors on T cells and their ligands on APCs (1–3). In addition to CD28 and ICOS, which provide positive costimulation to enhance T cell activation, numerous inhibitory ligands or receptors have been identified that regulate immune tolerance and/or the magnitude of T cell immune responses.

In particular, the genes of the butyrophilin (*BTN*) family suggested an immunoregulatory function because of their sequence and structure similarity to the B7 family molecules. Butyrophilin (*BTN1A1*) is a type I membrane glycoprotein originally identified as a major component of milk fat globule membrane (4), and it is the founding member of the BTN Ig superfamily of molecules. Similar to the B7 molecules, BTN molecules typically contain an IgV-like and an IgC-like domain in the extracellular region (4, 5), but some unique BTN molecules have an intracellular B30.2/SPRY domain, which is involved in the formation of milk fat globules (5).

In mouse there are two *Btn* genes, *Btn1a1* and *Btn2a2*, however in human there are three BTN groups of genes, *BTN1A1*, *BTN2*, and *BTN3*. The *BTN2* and *BTN3* groups comprise three genes each: *BTN2A1*, *BTN2A2*, *BTN2A3* and *BTN3A1*, *BTN3A2*, *BTN3A3*, respectively (6).

Another set of Ig family members that has similarity to BTNs has been called BTN-like (BTNL) molecules.

Some of the functions of mouse BTNs have recently been described. *BTN1A1* is predominately expressed in mammary gland and at low levels can be detected in several tissues, such as spleen, thymus, small intestine, and so forth (6). *BTN2A2* is broadly expressed in lymphoid and nonlymphoid organs, and both *BTN1A1* and *BTN2A2* are expressed by thymic stromal cells (6). Between the lymphoid cells, *BTN2A2* is expressed in B cells, NK cells, macrophages, and dendritic cells (DCs). Both *BTN1A1* and *BTN2A2* inhibit proliferation and cytokine production of stimulated CD4⁺ and CD8⁺ T cells (6), but the receptors of each have not been identified.

Few of the human BTNs have been analyzed. *BTN2A1* is highly expressed by endothelial cells and can also be found in several tissues. DC-SIGN has been reported to be a binding partner for *BTN2A1* (7), which allows the interaction with monocyte-derived DCs. *BTN3* gene, which is only present in humans and not in mouse, is widely expressed in immune cells, such as T cells, B cells, NK cells, monocytes, and DCs (8). Also, endothelial cells express *BTN3*, and this expression can be enhanced by proinflammatory cytokines, such as TNF- α and IFN- γ . The receptor of *BTN3* has not been identified; however, a report from Compe et al. (8) has shown that the fusion protein consisting of human Fc-Ig and extracellular *BTN3* does not bind to the known receptors for B7 family molecules, such as CD28, CTLA-4, ICOS, programmed cell death 1 (PD-1), and B and T lymphocyte attenuator. In contrast, the *BTN3* Ig fusion protein showed binding to T cell leukemias (8), which suggests that the putative receptor for *BTN3* may be present in leukocytes undergoing cell division, but further studies are required to describe the function of *BTN3*.

Among the BTNL molecules, human and mouse *BTNL2* (9) has been reported to be a negative regulator for T cell activation (10, 11). *BTNL2* was highly expressed on small intestine and Peyer's patches, suggesting *BTNL2* function in mucosal immunity.

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Abbreviations used in this paper: BALF, bronchoalveolar lavage fluid; BMDC, bone marrow-derived DC; BTN, butyrophilin; BTNL, butyrophilin-like; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; IFA, incomplete Freund's adjuvant; KLH, keyhole limpet hemocyanin; MOG, myelin oligodendrocyte glycoprotein; PD-1, programmed cell death 1.

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Similar to BTN3, BTNL2 does not bind to known B7 family receptors. However, it has been suggested that BTNL2 receptor was expressed on sinusoidal endothelium in the liver and in the vascular endothelium in Peyer's patches (10). Notably, mutation of BTNL2 has been associated with human sarcoidosis and myositis (12–14).

Thus, BTN family members are emerging as potential regulators in the immune system. In this study, we have analyzed several mouse BTN molecules and identified BTNL1 (also called BTNL3, Gm33, Gm316, or MGC56990) as a novel, to our knowledge, T cell regulator. BTNL1 is expressed on APCs, and its putative receptor is expressed on activated mouse T cells as well as APCs. BTNL1 inhibits CD4⁺ T cell proliferation via cell cycle arrest. Treatment with a neutralizing anti-BTNL1 mAb enhances T-dependent immune responses and exacerbates autoimmune and allergic diseases in vivo. Therefore, BTNL1 is a novel negative regulator for T cell activation and immune diseases.

Materials and Methods

Cloning and sequence analysis of BTNL1

Mouse BTNL1 gene was identified by searching the homology with BTNL9 in the National Center for Biotechnology Information database. Full-length BTNL1 was cloned by using PCR primers (forward primer with BglII cutting site, 5'-CAAGATCTAAGAAGGGCTCCCCCTCC-3', and reverse primer with XhoI cutting site, 5'-AATTCCTCGAGTCAAGGTTTATG-CAGAAG-3'). Transmembrane, Ig-like domain, SPRY domain, and leader peptide were predicted with TMHMM Server version 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0>), Pfam (<http://pfam.sanger.ac.uk/>), and Signal IP V2.0b2 (<http://www.cbs.dtu.dk/services/SignalP/>).

Quantitative real-time polymerase chain reaction

By using TRIzol reagent (Invitrogen), total RNA from immune cells or tissues of C57BL/6 mice was prepared and used for cDNA synthesis (Invitrogen, Carlsbad, CA). Gene expression was examined with a Bio-Rad iCycler Optimal System using an iQ SYBR Green real-time PCR kit (Bio-Rad, Hercules, CA). The primers used were as follows: BTNL1, forward, 5'-GGAATCTGGAAAGAATCAAG-3', and reverse, 5'-ATCTAGGTC-GGCCTGGA-3'. The data were normalized to β -actin gene expression as a reference.

Construction and purification of Ig fusion protein

The sequence coding the extracellular region of BTN1A1, BTNL9, BTNL1, or B7-1 was amplified by PCR and subcloned into the vector pMT/Bip/V5-HisA (Invitrogen) backbone, which contained human IgG1 Fc tag and the dimerization sequence (15). Their expression vectors were stably transfected into *Drosophila* S2 cells, and the fusion protein production was induced by CuSO₄ treatment. The secreted fusion proteins were purified by a protein A column, and purity was assessed by SDS-PAGE followed by Coomassie blue staining.

Generation of anti-BTNL1 mAb

A female Lewis rat (6–8 wk old) was immunized s.c. with 100 μ g BTNL1-Ig in CFA and then boosted every third day a total of five times using the same protein quantity with Ag in incomplete Freud's adjuvant (IFA). The draining lymph node cells were harvested 1 d after the last boost and fused with Ag8.653 cells by polyethylene glycol 1500 as previously described (16). Screening was performed with BTNL1-Ig and human IgG by ELISA, and 293T cells were transfected with BTNL1 by flow cytometry.

Flow cytometric analysis

BTNL1-Ig, BTN1A1-Ig, BTNL9-Ig, anti-BTNL1, and rat IgG (Jackson ImmunoResearch, West Grove, PA) were biotinylated by sulfo-succinimidyl-6-(biotinamido) hexanoate (Thermo Scientific, Rockford, IL). Secondary Ab, allophycocyanin-labeled streptavidin, was purchased from Jackson ImmunoResearch. Anti-CD16/CD32 mAb (BD Pharmingen, San Diego, CA) was used to reduce nonspecific staining. For T and B cell staining, splenocytes were stimulated with ConA (10 μ g/ml; 48 h for BTNL1 expression, 72 h for BTNL1-Ig binding) or LPS (1 μ g/ml; 72 h for BTNL1 expression, 48 h for BTNL1-Ig binding). Peritoneal macrophages were harvested by extracting peritoneal lavage and stimulated with LPS (100 ng/ml) for 24 h. Bone marrow cells were cultured with GM-CSF (20 ng/ml) for 7 d. The cells were stimulated with LPS (100 ng/ml; 72 h for

BTNL1 expression, 24 h for BTNL1-Ig binding). The cells were gated with CD4-FITC, CD8-FITC, B220-PE, F4/80-FITC, or CD11c-FITC (all purchased from eBioscience, San Diego, CA). For B7-1-Ig blocking experiment, splenocytes were preincubated with 20-fold excess of B7-1-Ig before being stained with biotinylated BTNL1-Ig. The 293T cells were transfected with ICOS, PD-1, or TLT2 expression vector using calcium phosphate and stained with biotinylated BTNL1-Ig, PE-Cy5-labeled ICOS, and PE-labeled PD-1.

In vitro T cell assay

CD4⁺ T cells from C57BL/6 mice were purified with anti-CD4 microbeads (Miltenyi, Auburn, CA) using an auto-MACS separator, and the purity of the cells was usually >95%. T cells were stimulated with anti-CD3 (2c11) and/or anti-CD28 (37.51) (BD Pharmingen) in the presence of BTNL1-Ig, BTN1A1-Ig, BTNL9-Ig, B7-1-Ig, or human IgG. Proliferative response was assessed by pulsing the culture with [³H]thymidine before 6 h of the final 72 h culture, and IL-2 was measured in culture supernatants at 24 h.

Carboxyfluorescein diacetate succinimidyl ester division assay

FACS-sorted naive CD4⁺ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (Invitrogen) and stimulated with anti-CD3 in the presence of BTNL1-Ig or human IgG for 48 h. The cells were analyzed by flow cytometry.

Cell cycle analysis

FACS-sorted naive CD4⁺ T cells were restimulated with anti-CD3 and BTNL1-Ig or human IgG. After 72 h culture, cells were washed with PBS, fixed with 70% ethanol for 1 h on ice, and resuspended in PBS containing RNase (10 μ g/ml) and propidium iodide (50 μ g/ml).

Keyhole limpet hemocyanin immunization

C57BL/6 mice were immunized with 0.5 mg/ml keyhole limpet hemocyanin (KLH) emulsified with 0.5 mg/ml CFA at the base of the tail (100 μ l/mouse). Seven days after immunization, the mice were euthanized and analyzed individually.

Experimental autoimmune encephalomyelitis induction

C57BL/6 mice were immunized s.c. at the dorsal flanks with 150 μ g myelin oligodendrocyte glycoprotein (MOG) peptide (35–53) in CFA at day 0. At day 7, the mice were immunized with 150 μ g MOG in IFA. Pertussis toxin, 500 μ g/mouse, was given i.p. at days 1 and 8. Signs of experimental autoimmune encephalomyelitis (EAE) were assigned scores on a scale of 1–5 as follows: 0, none; 1, limp tail or waddling gait with tail tonic; 2, wobbly gait; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, death. Both the brain and spinal cord were harvested from perfused mice, and mononuclear cells were prepared by Percoll gradient.

Asthma induction

C57BL/6 mice were immunized twice at 2-wk intervals with 0.2 ml saline containing 100 μ g OVA in aluminum hydroxide. Mice were sensitized at day 14 and rechallenged intranasally three more times at days 25, 26, and 27 with 100 μ g OVA. One day after the last challenge, mice were sacrificed, and bronchoalveolar lavage fluid (BALF) was harvested. BALF was analyzed for cellular composition and cytokine production. The spleen and mediastinal lymph node cells were further cultured with OVA for 3 d. The culture supernatants were measured for cytokine production by ELISA.

Statistical analysis

Significant differences between two groups were analyzed by Mann-Whitney U test. The *p* values <0.05 and <0.01 were considered significant.

Results

Expression of BTNL1

We first assessed the expression of BTNL1 in mouse tissues by real-time RT-PCR and found that BTNL1 was broadly expressed in lymphoid and nonlymphoid tissues. The lung and stomach had the highest expression of BTNL1 compared with that in spleen, lymph nodes, and thymus (Fig. 1A). In addition, the expression of BTNL1 mRNA was found in CD8⁺ T cells, B cells, DCs, and macrophages but not in CD4⁺ T cells (Fig. 1B).

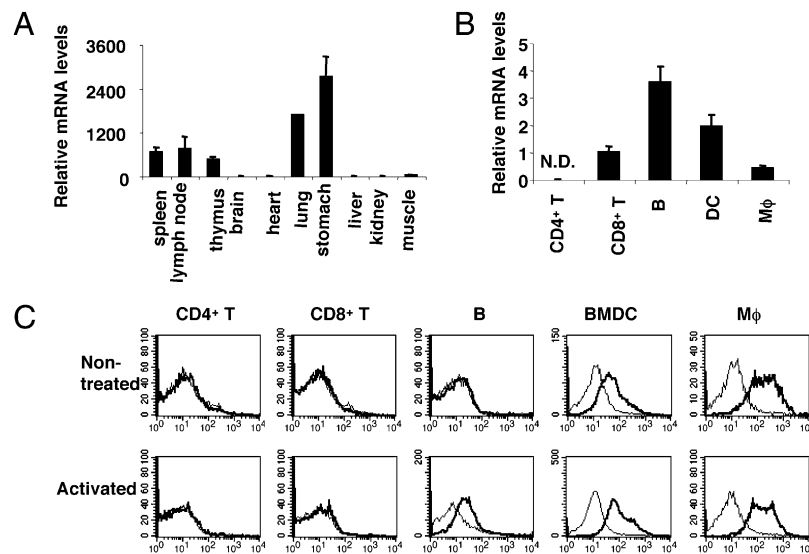


FIGURE 1. Expression of BTNL1 in tissues and immune cells. *A*, Expression of BTNL1 mRNA in tissues. Real-time PCR analysis was performed using primers specific for the transmembrane and intercellular regions of BTNL1. Tissues from C57BL/6 mouse were analyzed for BTNL1 mRNA expression, and the expression level in the liver was considered to be 1. The data are expressed as the mean $\pm$ SD of triplicate samples. These data are representative of two independent experiments. *B*, CD4⁺ T cells, CD8⁺ T cells, and B cells isolated from spleens of C57BL/6 mice were obtained by positive selection using magnetic beads (>95% purity). DCs were prepared by culturing bone marrow cells with GM-CSF. Thioglycolate-elicited macrophages were isolated from the peritoneal cavity. The expression level of CD8⁺ T cell was set as 1. The data are expressed as the mean $\pm$ SD of triplicate samples. Real-time PCR results in *A* and *B* were normalized to *Actb* gene expression, and relative gene expression levels are indicated. These data are representative of two independent experiments. *C*, BTNL1 expression on immune cells. Purified splenic CD4⁺ or CD8⁺ T cells were stimulated with ConA (10 μ g/ml). Purified splenic B cells, BMDCs, and macrophages were stimulated with LPS (for B cell, 1 μ g/ml; for DCs and macrophages, 100 ng/ml). Cells were stained with biotinylated anti-BTNL3 or rat IgG followed by allophycocyanin-labeled streptavidin. The bold histograms indicate staining with anti-BTNL3, and the thin histograms indicate background staining with rat IgG. These data are representative of three independent experiments.

We aligned mouse (Supplemental Fig. 1) and human reported sequences for BTNL and found that human and mouse BTNL proteins share only 33% homology.

To investigate the function of BTNL1 protein, we generated anti-mouse BTNL1 mAbs. First, we immunized rats with BTNL1-Ig, and after the fusion and selection, we chose clones that produced Abs that reacted with BTNL1 transfectant and BTNL1-Ig but not with parental cells, human IgG, B7, or other BTN family Ig fusion protein (Supplemental Fig. 2A). We found that clone No. 102 specifically labeled BTNL1 transfectant but not BTNL1A1, BTNL2, PD-L2, B7-H3, B7S1, or vector transfected cells (Supplemental Fig. 2A, 2B).

We then used clone No. 102 mAb (henceforth called anti-BTNL1) to analyze the expression and function of BTNL1. We found binding of anti-BTNL1 to nonactivated and LPS activated bone marrow-derived DCs (BMDCs) and macrophages. Also, BTNL1 was detected only in activated B cells. However, CD4⁺ and CD8⁺ T cells did not express BTNL1 (Fig. 1C). These results suggested that BTNL1 is expressed widely on APCs, and its mRNA expression is differently regulated in steady state.

Characterization of putative receptors of BTN family members

To evaluate the potential immunological function of BTNL1 and other BTN family members, we constructed quimeric soluble BTN proteins, which consisted of the extracellular region of BTNL1A1, BTNL9, or BTNL1 fused with the Fc portion of human IgG. Using these proteins, we assessed the expression of putative receptors of BTNL1A1, BTNL9, and BTNL1 on steady-state or activated T cells, B cells, BMDCs, and macrophages.

BTNL1A1-Ig bound strongly to macrophages and LPS-activated macrophages, and some binding was also found in DCs; however, BTNL1A1 bound weakly to CD4⁺ T cells and did not bind CD8⁺ T cells or B cells (Fig. 2). BTNL9-Ig bound broadly to all cells, but notably, the binding was increased in activated cells, except

for BMDCs where the binding did not change with activation (Fig. 2). BTNL1-Ig bound slightly to steady-state CD4⁺ T cells, B cells, and macrophages, but it bound considerably to activated CD4⁺, CD8⁺, B cells, and macrophages. Also, BTNL1-Ig bound in a similar level to both steady-state and activated DCs (Fig. 2).

These results suggested that there might be putative receptors for BTNL1A1, BTNL9, and BTNL1 expressed in immune cells, suggesting that BTN molecules might have immune functions.

BTNL1 inhibits T cell activation through cell cycle arrest

Because we found that BTNL9-Ig and BTNL1-Ig bind to activated T cells, we tested whether BTNL9 and BTNL1 could regulate T cell functions. We evaluated cell proliferation of sorted naive CD4⁺ T cells when activated with anti-CD3 in the presence of human IgG, BTNL1A1-Ig, BTNL9-Ig, BTNL1-Ig, or B7-1-Ig. As expected, the activation with B7-1-Ig greatly induced T cell proliferation compared with that of human IgG, whereas BTNL1-Ig activation showed reduced T cell proliferation, but this effect was not observed at high concentration of anti-CD3 (10 μ g/ml) (Fig. 3A). BTNL1A1-Ig or BTNL9-Ig did not have an effect on the proliferation of T cells. We also tested the effects of BTN-Ig molecules on anti-CD3 and anti-CD28 activated CD4⁺ T cells and found that only BTNL1-Ig inhibited CD4⁺ T cell proliferation at low doses of anti-CD3 (Fig. 3B). Also, BTNL1-Ig stimulation reduced the production of IL-2 from anti-CD3 and CD28-stimulated CD4⁺ T cells (data not shown). Furthermore, BTNL1-Ig inhibition of CD4⁺ T cell proliferation was dose-dependent (Fig. 3C), and BTNL1-Ig inhibited the proliferation and IL-2 production of naive and memory CD4⁺ T cells (Supplemental Fig. 3). We also found that BTNL1-Ig inhibited anti-CD3-stimulated CD8⁺ T cell proliferation and IL-2 production (Fig. 3D).

To determine the mechanism whereby BTNL1-Ig inhibits CD4⁺ T cell proliferation, we analyzed cell cycle and cell death during T cell activation. First, we labeled naive CD4⁺ T cells with car-

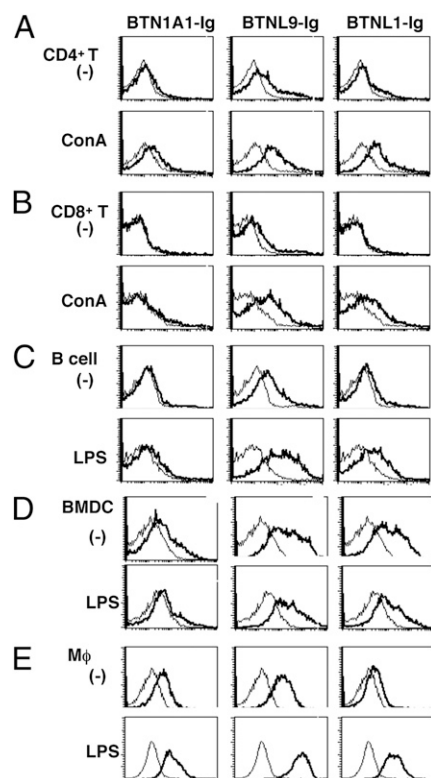


FIGURE 2. Expression of a BTNL1 putative receptor on immune cells. The binding of indicated Ig fusion proteins was assessed on CD4⁺ (A) and CD8⁺ T cells (B), B cells (C), BMDCs (D), and macrophages (E) with or without indicated stimulation. The bold histograms indicate staining with the indicated Ig proteins, and the thin histograms indicate background staining with human IgG. The indicated data are representative of more than three independent experiments.

boxyfluorescein diacetate succinimidyl ester and cultured them as before with anti-CD3 in the presence of BTNL1-Ig or human IgG. After 48 h, we found that BTNL1-Ig profoundly inhibited cell division (Fig. 3E). We further assessed cell cycling and cell death of BTNL1-Ig-stimulated CD4⁺ T cells by propidium iodide staining. We observed that BTNL1-Ig treatment led to cell cycle arrest of the activated cells, provoking an increase in cells in the G₀-G₁ phase, a reduction in cells in S/G₂/M phase, and a slight increase in apoptotic cells in sub-G₁ (Fig. 3F).

The above results suggested an inhibitory function of native BTNL1 in T cell activation. To better assess the function of BTNL1 in APCs, BMDCs were transduced with a retrovirus overexpressing BTNL1. When cocultured with CD4⁺ T cells in the presence of anti-CD3, BTNL1-overexpressing BMDCs exhibited reduced ability to induce CD4⁺ T cell proliferation and IL-2 production compared with those DCs transduced with an empty vector (Supplemental Fig. 4). Thus, BTNL1 inhibits T cell activation.

We then went on to explore the receptor for BTNL1 on T cells. First, we evaluated whether receptors of the CD28/CTLA-4 family of molecules could bind BTNL1-Ig. Pretreatment of ConA-activated CD4⁺ T cells with B7-1-Ig did not affect BTNL1-Ig binding (Supplemental Fig. 5A), suggesting that BTNL1 may not bind to CD28 or CTLA-4. Also, BTNL1-Ig binding was found on B and T lymphocyte attenuator knockout ConA-activated CD4⁺ T cells (Supplemental Fig. 5B). In 293T cells transfected with ICOS or PD-1 expression vectors, no binding of BTNL1-Ig was found (Supplemental Fig. 5C). Because TLT2 has recently been reported as the receptor for mouse B7-H3 (17), we assessed whether BTNL1-Ig could bind to TLT2 and found no binding

(Supplemental Fig. 5C). These results indicate that the receptor of BTNL1 is not a known member of the CD28/CTLA-4 family of molecules or TLT2.

Blockade of BTNL1 enhanced Ag-specific immune responses

The hybridoma clones producing anti-BTNL1 (Nos. 15, 102, and 104) were also screened to assess neutralizing effect on BTNL1-Ig. Anti-CD3-stimulated CD4⁺ T cells were cultured with human IgG or BTNL1-Ig in the presence of rat IgG or the different mAbs from clones of anti-BTNL1. Clone No. 102, but not Nos. 15 or 104, reversed the inhibitory effect of BTNL1-Ig (Supplemental Fig. 2C). Therefore, we also used mAb No. 102 as an anti-BTNL1 blocking Ab for the studies in vivo.

To assess BTNL1 function in vivo, we analyzed Ab and T cell responses in mice that were immunized with KLH in CFA and received 3 doses of anti-BTNL1 or rat IgG every 2 d. After 7 d, KLH-specific T cell proliferation as well as cytokine production (IL-17, IFN- γ , IL-4, IL-5, and IL-13) was evaluated from spleens restimulated with KLH. We found that anti-BTNL1 treatment increased T cell proliferation and cytokine production compared with that for rat IgG treatment, except for IFN- γ , which was less than that in mice treated with rat IgG (Fig. 4A–F). These results supported our notion that BTNL1 negatively regulates T cell immune responses in vivo and also suggested that BTNL1 can regulate T cell effector differentiation.

Blockade of BTNL1 enhanced EAE disease

Th17 cells have been shown to mediate EAE (18, 19). To assess the role of BTNL1 in autoimmune disease, rat IgG or anti-BTNL1 mAbs were administered to MOG peptide-immunized C57BL/6 mice. Because BTNL1 has inhibitory T cell activity, we expected that neutralizing BTNL1 would enhance disease severity. Because the immunization with MOG peptide in CFA generates a severe disease, we could not find an effect of anti-BTNL1 treatment. Therefore, the strength of MOG immunization was titrated, and we selected a mild immunization protocol that could allow us to observe an effect of the anti-BTNL1 treatment. For this we used IFA for MOG boosting (20) and found no significant EAE disease development in all the control mice (Fig. 5A). However, the anti-BTNL1-treated mice showed significant EAE disease. To understand the underlying causes of the different disease scores in the two groups of mice, infiltrating cells in the CNS and the cytokine production by CD4⁺ cells in the spleen cells were analyzed. In the CNS, the frequency of CD4⁺IL-17⁺ cells in anti-BTNL1-treated mice was significantly increased compared with that in the CNS of rat IgG-treated mice (Fig. 5B, 5C). However, the frequency of CD4⁺IFN- γ ⁺ cells in the CNS was moderately increased in anti-BTNL1-treated mice but did not reach statistical significance over those receiving rat IgG (Fig. 5B, 5D). In contrast, analysis of the cytokine production from spleen cells showed that mice treated with anti-BTNL1 had increased IL-17 production compared with that in rat IgG-treated mice (Fig. 5E). IFN- γ production was not significantly different between these two groups (Fig. 5F). These results indicate that BTNL1 negatively regulates Th17-mediated autoimmune disease.

Blockade of BTNL1 enhanced allergic responses in vivo

To assess BTNL1 function in a Th2-type immune response model, C57BL/6 mice were immunized with chicken OVA mixed in with aluminum hydroxide, and anti-BTNL1 mAb or rat IgG was administered. Seven days after immunization, analyses of OVA-specific Ig production from sera and of proliferation and cytokine production from spleen cells were performed. Blockade of BTNL1 in vivo increased OVA-specific T cell proliferation compared with

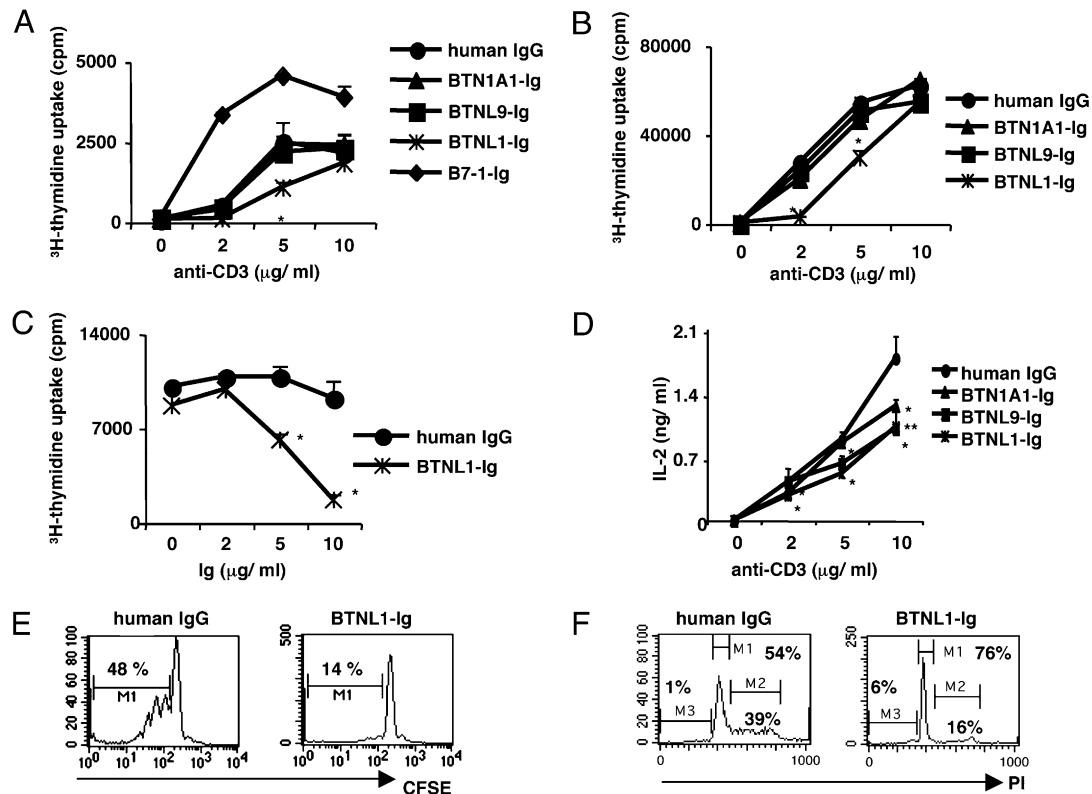


FIGURE 3. BTNL1 inhibits T cell proliferation by cell cycle arrest. *A*, CD4^+ T cells were cultured with anti-CD3 and indicated Ig proteins (10 $\mu\text{g/ml}$). *B*, CD4^+ T cells were cultured with anti-CD3, anti-CD28 (2 $\mu\text{g/ml}$), and indicated Igs (10 $\mu\text{g/ml}$). *C*, CD4^+ T cells were cultured with anti-CD3 (2 $\mu\text{g/ml}$) and different doses of indicated Ig. *A–C*, Proliferative response was assessed at 72 h by pulsing the culture with [^3H]thymidine for the last 6 h. The data are expressed as the mean $\pm$ SD of triplicate wells. *D*, Purified CD8^+ T cells were stimulated with anti-CD3 in the presence of indicated Igs (10 $\mu\text{g/ml}$). Production of IL-2 was measured after 24 h of culture. The data are expressed as the mean $\pm$ SD of triplicate wells. These data are representative of two independent experiments. *E*, Naive CD4^+ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (10 μM) and cultured with anti-CD3 (3 $\mu\text{g/ml}$) in the presence of human IgG or BTNL1-Ig (10 $\mu\text{g/ml}$) for 48 h. *F*, Naive CD4^+ T cells were cultured with anti-CD3 (3 $\mu\text{g/ml}$) in the presence of human IgG or BTNL1-Ig (10 $\mu\text{g/ml}$) for 72 h. The cells were fixed with 70% ethanol and resuspended with propidium iodide solution. Diploid (M1), supradiploid (M2), and subdiploid (M3) populations are indicated. All data are representative of more than three independent experiments. * $p < 0.01$ (compared with human IgG).

that for rat IgG treatment (Fig. 6A). Also, production of IL-2, IL-4, and IL-5 was increased by anti-BTNL1 treatment (Fig. 6B–D). Low levels of IFN- γ were detected in the culture supernatants; however, no difference between rat IgG treatment and anti-

BTNL1 treatment was found (data not shown). We also found that OVA-specific IgG1 and IgG2b levels were strongly increased by anti-BTNL1 treatment compared with those for rat IgG treatment (Fig. 6E, 6F). OVA-specific Ig levels of IgG2a and IgE

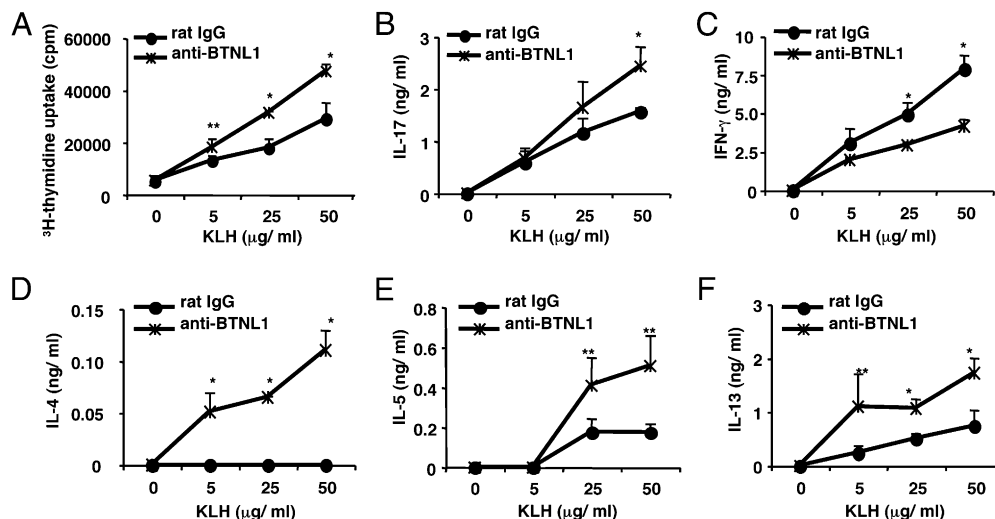
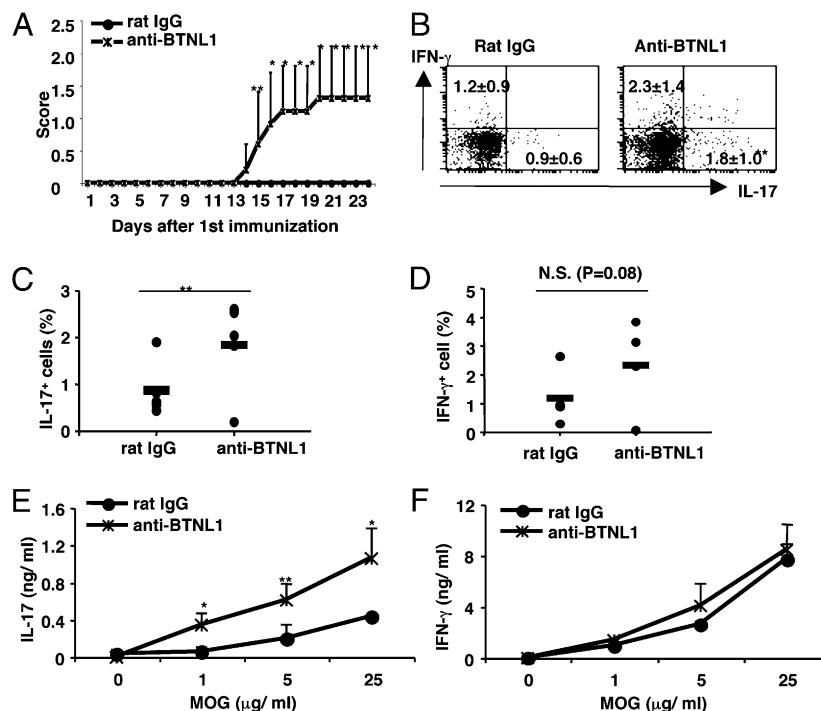


FIGURE 4. Blockade of BTNL1 induced a strong Th2 response after immunization with KLH. *A–F*, C57BL/6 mice (five mice in each group) immunized with KLH in CFA were treated with anti-BTNL1 or rat IgG. One week later, the spleen cells were harvested and restimulated with different doses of KLH. *A*, Proliferative response was assessed at 72 h by pulsing the culture with [^3H]thymidine for the last 6 h. *B–F*, Production of IL-17 (*B*), IFN- γ (*C*), IL-4 (*D*), IL-5 (*E*), and IL-13 (*F*) in the culture supernatants was measured by ELISA at 72 h of culture. These data are representative of two independent experiments. The data are expressed as the mean $\pm$ SD of five mice. * $p < 0.01$; ** $p < 0.05$ (compared with rat IgG).

FIGURE 5. Blockade of BTNL1 exacerbated EAE disease. C57BL/6 mice (five mice in each group) were treated with rat IgG or anti-BTNL1 and immunized with MOG peptide twice (once with CFA and once with IFA) to induce EAE. A, Disease scores in five mice of each group were evaluated. The data are expressed as the mean $\pm$ SD of five mice. B–D, IL-17⁺ or IFN- γ ⁺ cells in mononuclear cells in the CNS were analyzed in a CD4⁺ gate. A representative data from flow cytometry is shown in B. The data of cell percentages are expressed as the mean $\pm$ SD of five mice. C, D, The frequencies of IL-17⁺ cell and IFN- γ ⁺ cells in CNS are shown. E, F, Spleen cells from EAE-developing mice (five mice in each group) were restimulated with MOG peptide. Production of IL-17 (E) and IFN- γ (F) in the culture supernatants was measured by ELISA at 72 h of culture. The data are expressed as the mean $\pm$ SD of triplicate samples. All data are representative of three independent experiments. N.S., not significant; * p < 0.01; ** p < 0.05 (compared with rat IgG).



were not detected by ELISA. These results suggested that blockade of BTNL1 enhanced Th2-type T cell responses and humoral immunity.

We thus further investigated the role of BTNL1 in an asthma model (21). For this, rat IgG or anti-BTNL1-treated C57BL/6 mice were immunized i.p. twice with OVA in aluminum hydroxide and subsequently challenged intranasally with OVA in PBS. Anti-BTNL1-treated mice showed significantly increased total BALF cells (data not shown) and in particular increased eosinophils and lymphocytes (Fig. 7A). Because the degranulation of eosinophils is regulated by Th2 cytokines (22), we evaluated cytokine production from BALF cells by ELISA and found that blockade of BTNL1 increased IL-4 and IL-5 production by BALF

cells (Fig. 7B) compared with that for rat IgG-treated mice. The lung lymph node cells or spleen cells were also harvested and restimulated with OVA to measure Th2 cytokine production. We also found that blockade of BTNL1 increased IL-4, IL-5, and IL-13 production from lung lymph node (Fig. 7C) or spleen cells (Fig. 7D). These results confirmed that BTNL1 plays a negative role in Th2 cytokine production and Th2 cell-mediated airway inflammation.

Discussion

T cell activation or tolerance is dependent on appropriate costimulatory signals provided by APCs. B7 family molecules are well known as positive and negative costimulatory molecules for

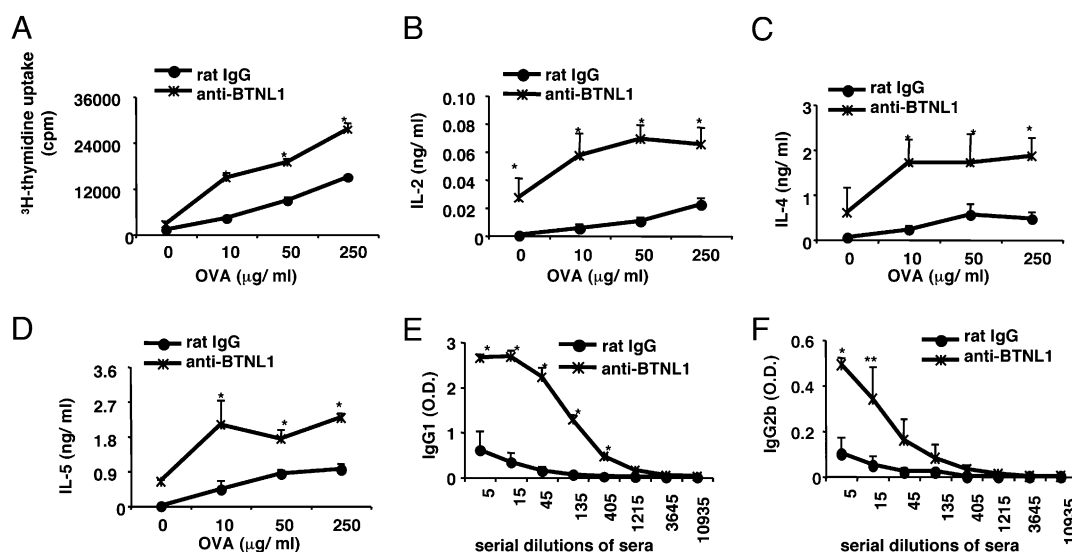


FIGURE 6. Anti-BTNL1 treatment enhanced Th2-type responses. C57BL/6 mice (five mice/group) were immunized with OVA (0.5 mg/ml) in aluminum hydroxide and were treated with rat IgG or anti-BTNL1. Seven days after immunization, Abs from serum and T cell responses from splenocytes were evaluated. A–D, Spleen cells were restimulated with OVA. A, Proliferative response was assessed at 72 h by [³H]thymidine incorporation. B–D, Production of IL-2 (B), IL-4 (C), and IL-5 (D) in the culture supernatants was measured by ELISA at 72 h of culture. E and F, Abs specific against OVA were measured in the serum, IgG1 (E), and IgG2b (F). All data are expressed as the mean $\pm$ SD of five mice. The data are representative of two independent experiments. * p < 0.01; ** p < 0.05 (compared with rat IgG).

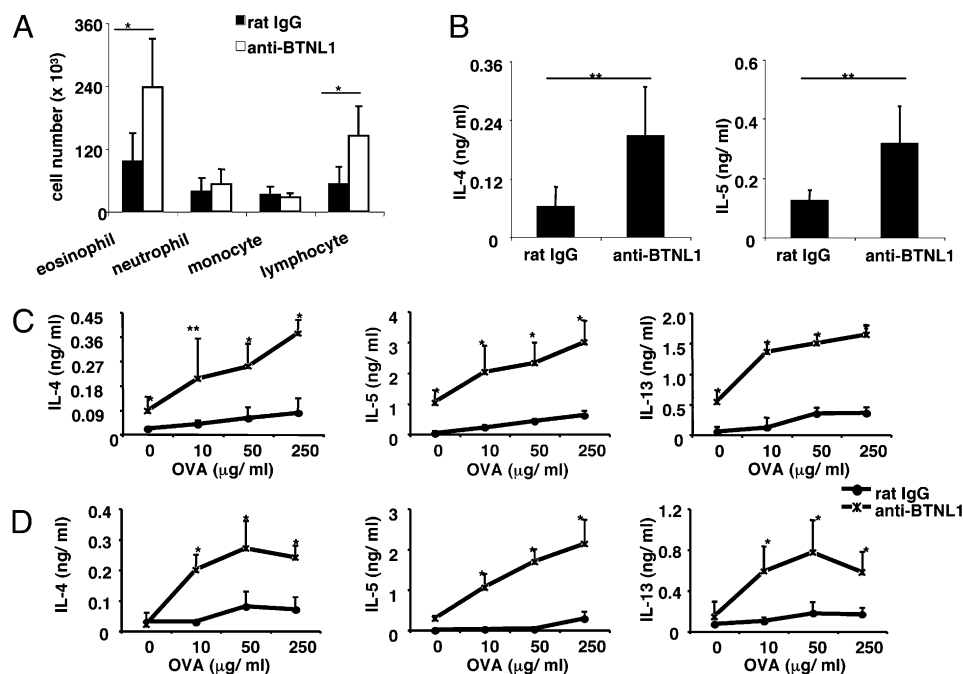


FIGURE 7. Anti-BTNL1 treatment resulted in enhanced allergic responses. C57BL/6 mice (five mice in each group) were immunized twice at 2-wk intervals with OVA in aluminum hydroxide. Ten days after the second immunization, the mice were challenged intranasally with OVA for 3 d. The mice were treated with rat IgG or anti-BTNL1. *A*, Cells from BALF were harvested and stained with May-Grünwald Giemsa, then counted under a microscope. The data are expressed as the mean $\pm$ SD of five mice. *B*, IL-4 and IL-5 were measured by ELISA from cultured supernatants of BALF cells. The data are expressed as the mean $\pm$ SD of five mice. *C* and *D*, The lung lymph nodes (*C*) and spleens (*D*) were harvested and restimulated with OVA. Production of IL-4, IL-5, and IL-13 in the culture supernatants was measured by ELISA at 72 h. The data are expressed as the mean $\pm$ SD of triplicate samples. All data are representative of two independent experiments. * p < 0.01; ** p < 0.05 (compared with rat IgG).

T cell activation and function. Although significant homology is found between B7 and BTN family molecules, the function and expression of the BTN family in the immune system have not been well characterized. To our knowledge, we show for the first time that BTNL1 is a negative regulator of T cell activation and function.

We started out to characterize multiple murine BTN and BTNL molecules. BTNL1 was found to have close homology to BTN1A1 and BTNL9. Interestingly, BTNL9 was previously called BTN3 and was thought to be the equivalent to human BTN3. However, according to the description of the BTN gene locus, there is no BTN3 gene in mouse (6). From our protein alignments of BTN molecules, we found that murine BTNL1 had ~31% homology with human BTN3, and at the time we started our studies, human BTN3 was reported to have some connection with immune function due to its expression on T cells, B cells, NK cells, monocytes, and DCs (8). Therefore, we decided to study murine BTNL1 expression pattern and immunological functions and also to search for some functions of murine BTNL9 and BTN1A1.

Similar to B7h (23), PD-L1 (24), PD-L2 (25), B7-H3 (20), B7S1 (26), and BTNL2 (11), the expression of BTNL1 mRNA was found in several peripheral organs and immune tissues. Notably, the protein expression of BTNL1 was found only in APCs and not in T cells, suggesting that activated T cells expressing BTNL1 receptor would be regulated by interaction with various APCs expressing BTNL1. When we searched for the possible receptor of BTNL1, we found that unlike mouse BTNL2 (11), BTNL1 and BTNL9 Ig fusion proteins can interact with activated T cells and APCs, thus suggesting a possible interaction of BTNL1 ligand-receptor between APC and T cells and also between APCs.

We also found that the receptor for BTNL1 was not a known inhibitory receptor of the CD28/CTLA-4 family of molecules described to date, but the activation of T cells in the presence of BTNL1 provided a negative costimulation, which resulted in re-

duced proliferation by an arrest of the cell cycle but not induction of apoptosis. This inhibitory mechanism is similar to what has been reported for PD-L1 (24), PD-L2 (25), and B7S1/B7-H4 (27) inhibitory functions. All of the BTN proteins we tested, BTN1A1, BTNL9, and BTNL1, showed inhibitory activity toward CD8⁺ T cell proliferation, and it seems that CD8⁺ T cells might be more sensitive to BTN inhibitory signal than CD4⁺ T cells.

We have examined the physiological significance of BTNL1 *in vivo* by using a neutralizing anti-BTNL1 Ab. In KLH immunization, the blockade of BTNL1 resulted in enhanced KLH-specific T cell proliferation and cytokine production of IL-2, IL-17, IL-4, IL-5, and IL-13, suggesting that BTNL1 signaling on T cells may inhibit T cell priming and may reduce IL-17 and Th2 cytokine production. Notably, blockade of BTNL1 inhibited IFN- γ production, which indicates that activated APCs that express high levels of BTNL1 participate in the induction of a Th1 response. These results suggested that BTNL1 might have a function to modulate effector differentiation of CD4⁺ T cells. This idea was supported by our results where exacerbation of EAE and airway inflammation models occurred after BTNL1 blockade. The molecular basis for BTNL1 function in effector Th differentiation has not been described. However, we previously showed that Th2 and Th17 differentiation is more dependent on positive costimulation than Th1 development (18, 28). Therefore, BTNL1 may function to fine tune the signaling strengths in T cells to favor Th1 versus Th2 and Th17 development.

In a different set of experiments, we assessed the effect of BTNL1 in a CD8⁺ T cell-driven disease model where we transferred OT-I cells into mice expressing OVA in islet β -cells (rat insulin promoter RIP-OVA) (29). We found no influence of BTNL1 blockade in diabetes development (data not shown). Therefore, BTNL1 function may differ in specific contexts and cell targets. Further characterization of BTNL1 function in other murine models

will help us better understand the specificity of negative costimulation.

Overall, we identified what we believe is a novel butyrophilin family molecule, BTNL1, that functions as a negative regulator for T cell activation and function. This study suggests a possibility of targeting BTNL1 in immune and inflammatory diseases.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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Supplementary Figure 1

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mBTN1A1  -----MAVPTNSCLLVCLLTTLTVLQLPTLDSAAPFDVTAPQEPVLALVGSDAELTCGFSPNASSEYMELLWFRQTRSTAVLLYRDGQEQEGQQMTEYRGRATLATAGLLDGRATLL
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mBTNL1   -----MMKGSPSVPPAGCLPLLLLLFTGVSGEVSWFSVKGAPEPITVLLGTEATLPCQLSPEQSAARMHIRWYRAQPTPAVLVFHNGQEQGEVQMPEYRGRTQMVRQAIDMGSAVLQ
mB7-1    -----MACNCQLMQDTPLLKFCPCPLILLFVLLIRLSQVSSDVEQLSKSVKDKVLLPCRYNSPHEDESEDRIYWQKHKDKVLSVIAG-----KLKVWPEYKNRTLYDN-----TTYSLI
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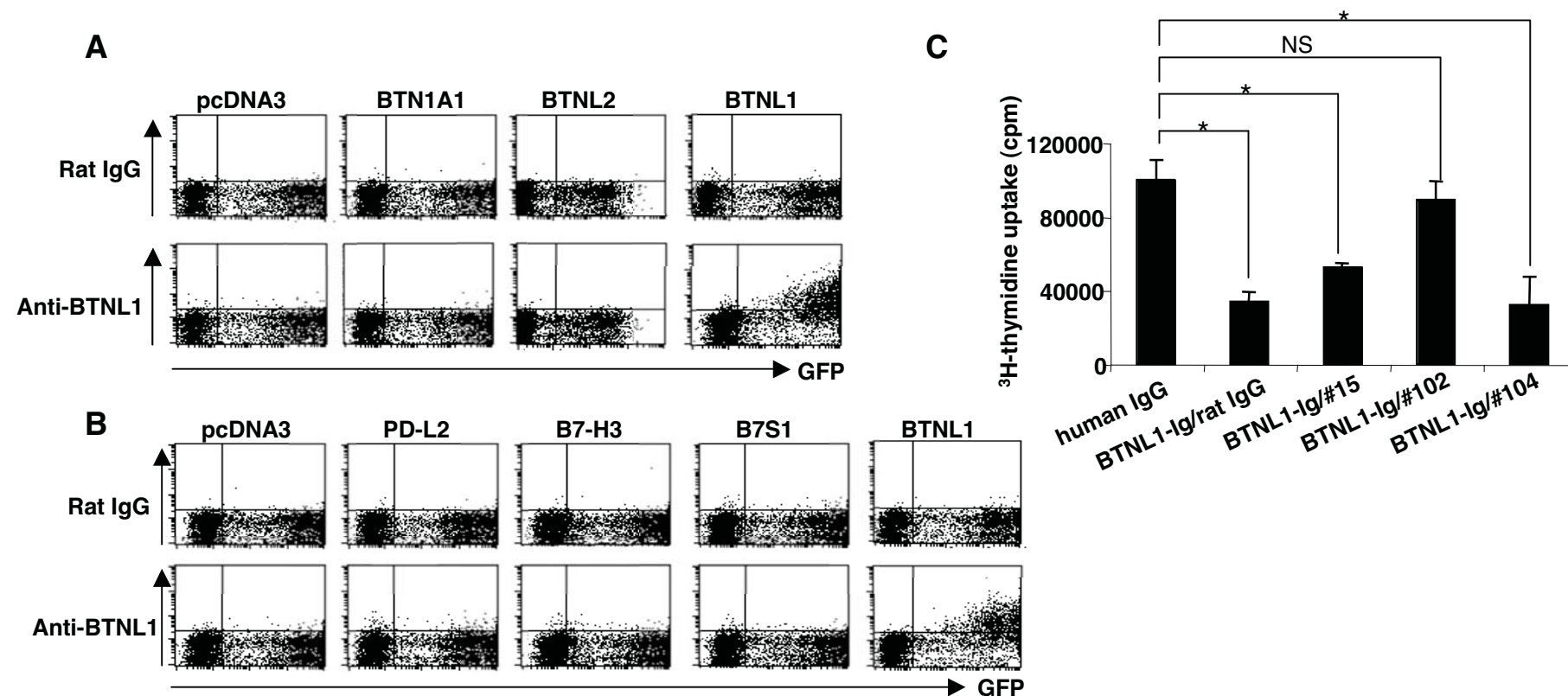
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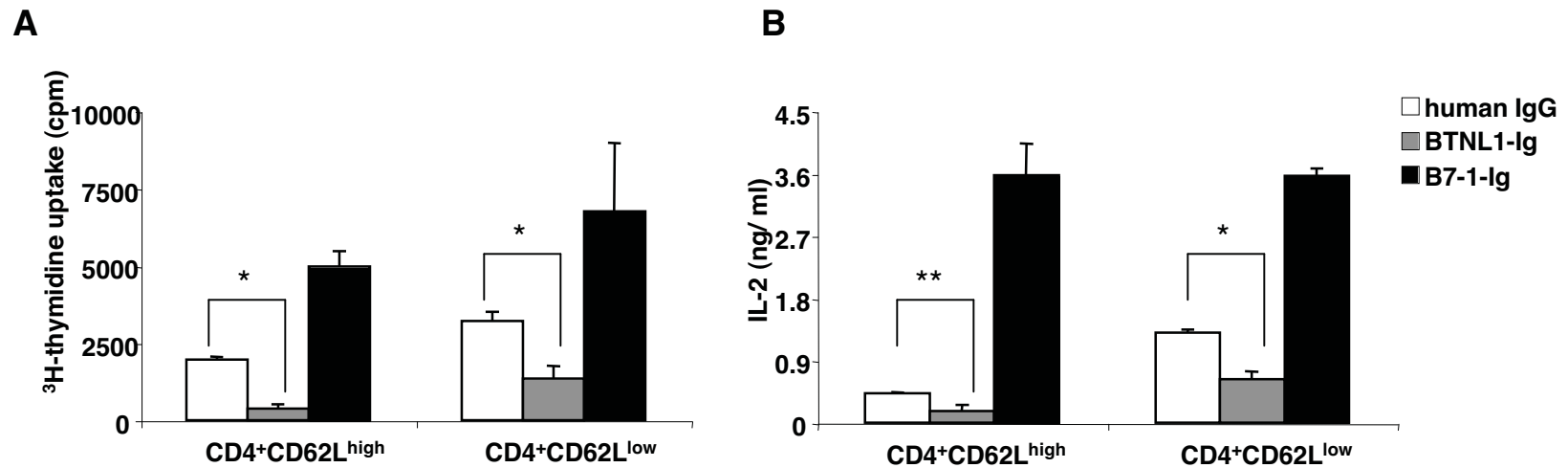
Supplementary Figure 1. Alignment of amino acid sequence of human and mouse BTNL1. Predicted amino acid sequence of mouse BTNL1 (TrEMBL; A6X8K2) was compared with mouse BTN1A1 (Swiss-Prot; Q62556), mouse BTNL9 (TrEMBL; Q8BJE2), and mouse B7-1 (Swiss-Prot; Q00609). Predicted leader peptide (thin line), Ig-like regions (bold line), transmembrane region (doublet), and B30.2 region (dashed line) was showed on the alignment. An asterisk (*) indicates identity, a colon (:) indicates conservation of strong groups, and period (.) indicates conservation of weak groups between mouse and human amino acid sequence of BTNL1.

Supplementary Figure 2



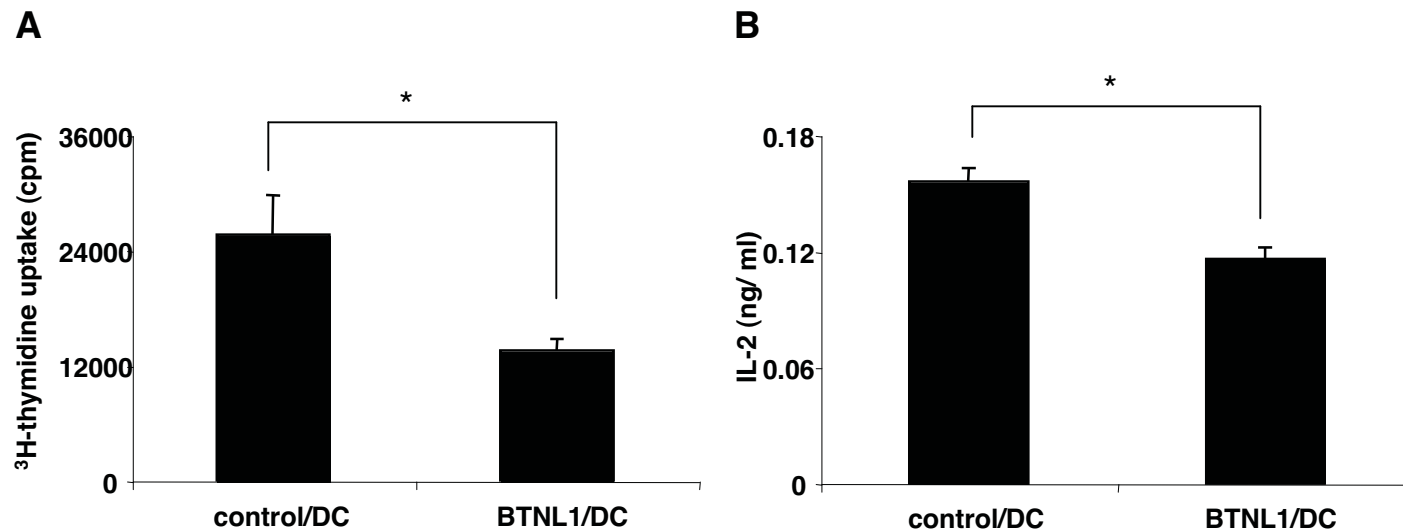
Supplementary Figure 2. Characterization of rat-derived anti-mouse BTNL1. **A-B.** Reactivity of anti-BTNL1 to mouse BTN family molecules and B7 family transfectants. 293T-derived BTN (**A**) or B7 (**B**) family molecule transfectants and empty vector transfectants (**A, B**) were stained with biotinylated rat IgG or anti-BTNL1 (clone #102) followed by APC-labeled streptavidin. **C.** Blocking activity of anti-BTNL1. Anti-CD3 (2 μ g/ml)-stimulated CD4⁺ T cells were cultured with indicated Igs in the presence of different clones of anti-BTNL1 or rat IgG. Proliferative response was assessed at 72 h by pulsing the culture with [³H]thymidine for last 6 h. The data are expressed as the mean $\pm$ SD of triplicate wells. All data are representative of three independent experiments. *, $p < 0.01$; **, $p < 0.05$; NS, not significant.

Supplementary Figure 3



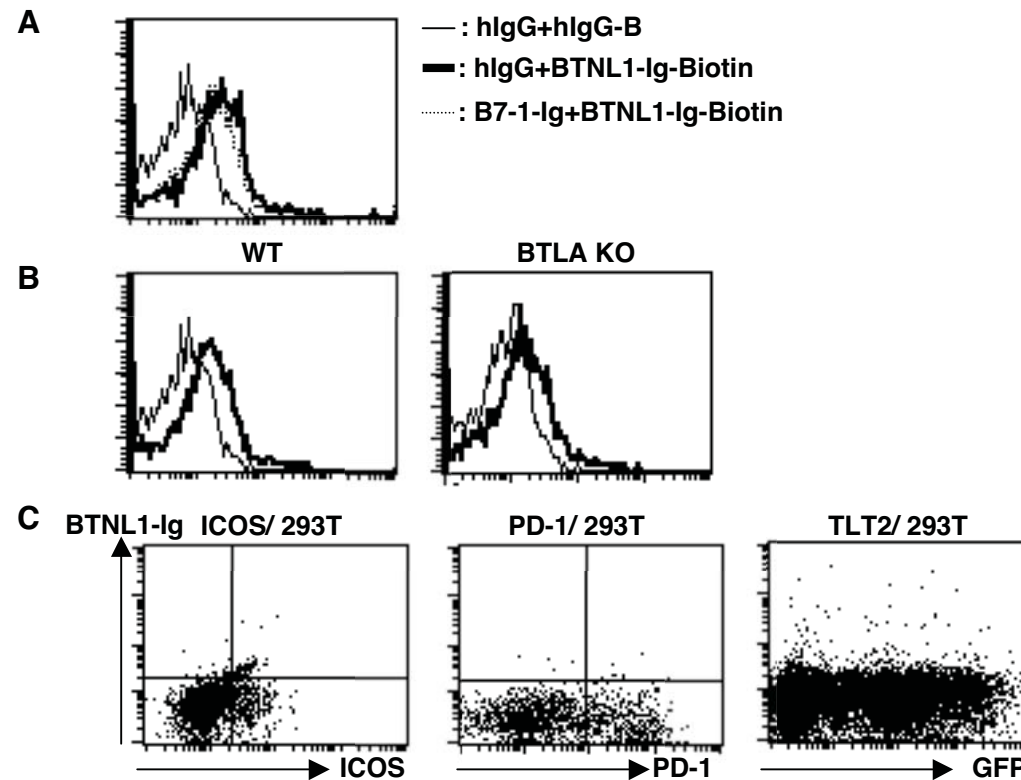
Supplementary Figure 3. BTNL1 inhibits naive and memory T cell activation. Naive (CD4⁺CD62L^{high}) and memory (CD4⁺CD62L^{low}) CD4⁺ T cells were sorted by flow cytometry. The cells were stimulated with anti-CD3 (2 µg/ml) in the presence of indicated Igs (10 µg/ml). **A.** Proliferative response was assessed at 72 h by pulsing the culture with [³H]thymidine for last 6 h. **B.** Production of IL-2 in the culture supernatants was measured at 24 h of the culture by ELISA. The data are expressed as the mean ± SD of triplicate wells. These data are representative of two independent experiments. *, $p < 0.01$; **, $p < 0.05$.

Supplementary Figure 4



Supplementary Figure 4. Overexpressed BTNL1 on BMDC inhibits CD4^+ T cell proliferation and IL-2 production. Bone-marrow cells were infected *IRES-GFP*-containing bicistronic retrovirus expressing BTNL1 or vector control retrovirus and cultured with GM-CSF. $\text{GFP}^+\text{CD11c}^+$ cells were isolated by FACS-sorting. Purified CD4^+ T cells were cocultured with BTNL1 transduced DC (BTNL1/DC) or control vector transduced DC (control/DC) in the presence of anti-CD3. **A.** Proliferative response was assessed at 72 h by pulsing the culture with [^3H]thymidine for last 6 h. **B.** Production of IL-2 in the culture supernatants was measured at 24 h of the culture by ELISA. The data are expressed as the mean $\pm$ SD of triplicate wells. These data are representative of three independent experiments. *, $p < 0.01$.

Supplementary Figure 5



Supplementary Figure 5. CD28/ CTLA-4 family molecules and TLT2 are not the receptor for BTNL1. **A.** ConA-activated CD4⁺ T cells from C57BL/6 mice were incubated with B7-1-Ig. The cells were stained with biotinylated BTNL1-Ig followed by APC-labeled streptavidin. **B.** CD4⁺ T cells from C57BL/6 or BTLA KO mice were activated with ConA and stained with biotinylated BTNL1-Ig or human IgG, followed by APC-labeled streptavidin. The bold histograms indicate staining with BTNL1-Ig and the thin histograms indicate background staining with human IgG. **C.** ICOS, PD-1, or TLT2 transfected 293T cells were stained with biotinylated BTNL1-Ig or human IgG followed by APC-labeled streptavidin. All data are a representative of two independent experiments.