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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).

Among the BTN families, the BTN1 family (BTN1A1, BTN1A2, BTN1A3, BTN2A1, BTN2A2, BTN2A3, BTN3A1, BTN3A2, BTN3A3) and BTN2 family (BTN2A1, BTN2A2, BTN2A3, BTN3) have been well characterized at the molecular level. BTN1A1, BTN1A2, and BTN1A3 are 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 Compte 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.

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.

The online version of this article contains supplemental material.

Abbreviations used in this paper: BALF, bronchoalveolar lavage fluid; BMDC, bone marrow-derived DC; BTN, butyrophilin; BTN1L, 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.
Similar to BTN3, BTN2 does not bind to known B7 family receptors. However, it has been suggested that BTN2 receptor was expressed on sinusoidal endothelium in the liver and in the vascular endothelium in Peyer’s patches (10). Notably, mutation of BTN2 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 BTN1L1 (also called BTN3L, Gm33, Gm316, or MGC69990) as a novel, to our knowledge, T cell regulator. BTN1L1 is expressed on APCs, and its putative receptor is expressed on activated mouse T cells as well as APCs. BTN1L1 inhibits CD4+ T cell proliferation via cell cycle arrest. Treatment with a neutralizing anti-BTN1L1 mAb enhances T-dependent immune responses and exacerbates autoimmune and allergic diseases in vivo. Therefore, BTN1L1 is a novel negative regulator for T cell activation and immune diseases.

Materials and Methods

Cloning and sequence analysis of BTN1L1

Mouse BTN1L1 gene was identified by searching the homology with BTN9 in the National Center for Biotechnology Information database. Full-length BTN1L1 was cloned by using PCR primers (forward primer with BagII cutting site, 5'-CAAGATCTCAAGAAGGCTGCCACCTCC-3', and reverse primer with XhoI cutting site, 5'-AAATCTTCTAGAAGGTTATGAGCAAGG-3'), and primer with PstI cutting site, 5'-GGGAATCTGGAAAGAACATCAAG-3', and reverse, 5'-ATCTAGGTCCGGCCTGGA-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, BTN9, BTN1, BTN2, and/or anti-BTN2 (37,51) (BD Pharmingen) in the presence of BTN1L1–Ig, BTN1A1–Ig, BTN9–Ig, B7–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 naïve CD4+ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (Invitrogen) and then stimulated with anti-CD3 and human IgG. After 72 h culture, cells were washed and fixed with 70% ethanol for 1 h on ice, and resuspended in PBS containing RNase (10 μg/ml) and propidium iodide (50 μg/ml). The cells were gated for BTN1L1 expression, 72 h for BTN1L1–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–Ig blocking experiment, splenocytes were preincubated with 20-fold excess of B7–Ig before being stained with biotinylated BTN1L1–Ig. The 293T cells were transfected with ICOS, PD-1, or TLT2 expression vector using calcium phosphate and stained with biotinylated BTN1L1–Ig, PE-Cy5–labeled ICOS, and PE-labeled PD-1.

Cell cycle analysis

FACS-sorted naïve CD4+ T cells were restimulated with anti-CD3 and BTN1L1–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 μg/ml) and propidium iodide (50 μg/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 μl/mouse). Seven days after immunization, the mice were euthanized and analyzed individually.

Experimental autoimmune encephalomyelitis induction

C57BL/6 mice were immunized with 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 μg OVA in aluminum hydroxide. Mice were sensitized at day 14 and rechallenged intranasally three more times at days 25, 26, and 27 with 100 μg 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 BTN1L1

We first assessed the expression of BTN1L1 in mouse tissues by real-time RT-PCR and found that BTN1L1 was broadly expressed in lymphoid and nonlymphoid tissues. The lung and stomach had the highest expression of BTN1L1 compared with that in spleen, lymph nodes, and thymus (Fig. 1A). In addition, the expression of BTN1L1 mRNA was found in CD8+ T cells, B cells, DCs, and macrophages but not in CD4+ T cells (Fig. 1B).
We aligned mouse (Supplemental Fig. 1) and human reported sequences for BTN1 and found that human and mouse BTN1 proteins share only 33% homology.

To investigate the function of BTN1 protein, we generated anti-mouse BTN1 mAbs. First, we immunized rats with BTN1-Ig, and after the fusion and selection, we chose clones that produced Abs that reacted with BTN1 transfected and BTN1-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 BTN1 transfectant but not BTN1A1, BTN2L, PD-L2, B7-H3, B7S1, or vector transfected cells (Supplemental Fig. 2A, 2B).

We then used clone No. 102 mAb (henceforth called anti-BTN1) to analyze the expression and function of BTN1. We found binding of anti-BTN1 to nonactivated and LPS-activated bone marrow-derived DCs (BMDCs) and macrophages. Also, BTN1 was detected only in activated B cells. However, CD4+ and CD8+ T cells did not express BTN1 (Fig. 1C). These results suggested that BTN1 is expressed widely on APCs, and its mRNA expression is differentially regulated in steady state.

Characterization of putative receptors of BTN family members

To evaluate the potential immunological function of BTN1 and other BTN family members, we constructed quimeric soluble BTN proteins, which consisted of the extracellular region of BTN1A1, BTN1L9, or BTN1 fused with the Fc portion of human IgG. Using these proteins, we assessed the expression of putative receptors of BTN1A1, BTN1L9, and BTN1 on steady-state or activated T cells, B cells, BMDCs, and macrophages.

BTN1A1-Ig bound strongly to macrophages and LPS-activated macrophages, and some binding was also found in DCs; however, BTN1A1 bound weakly to CD4+ T cells and did not bind CD8+ T cells or B cells (Fig. 2). BTN1L9-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). BTN1-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, BTN1L1-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 BTN1A1, BTN1L9, and BTN1L1 expressed in immune cells, suggesting that BTN molecules might have immune functions.

**BTN1L1 inhibits T cell activation through cell cycle arrest**

Because we found that BTN1L9-Ig and BTN1L1-Ig bind to activated T cells, we tested whether BTN1L9 and BTN1L1 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, BTN1A1–Ig, BTN1L9–Ig, BTN1L1–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 BTN1L–Ig activation showed reduced T cell proliferation, but this effect was not observed at high concentration of anti-CD3 (10 μg/ml) (Fig. 3A). BTN1A1–Ig or BTN1L–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 BTN1L1–Ig inhibited CD4+ T cell proliferation at low doses of anti-CD3 (Fig. 3B). Also, BTN1L1–Ig stimulation reduced the production of IL-2 from anti-CD3 and CD28-stimulated CD4+ T cells (data not shown). Furthermore, BTN1L1–Ig inhibition of CD4+ T cell proliferation was dose-dependent (Fig. 3C), and BTN1L1–Ig inhibited the proliferation and IL-2 production of naive and memory CD4+ T cells (Supplemental Fig. 3). We also found that BTN1L1–Ig inhibited anti-CD3–stimulated CD8+ T cell proliferation and IL-2 production (Fig. 3D).

To determine the mechanism whereby BTN1L1–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-
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 increased 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
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 3. BTN1 inhibits T cell proliferation by cell cycle arrest. A, CD4+ T cells were cultured with anti-CD3 and indicated Ig proteins (10 µg/ml). B, CD4+ T cells were cultured with anti-CD3, anti-CD28 (2 µg/ml), and indicated Igs (10 µg/ml). C, CD4+ T cells were cultured with anti-CD3 (2 µg/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 ± SD of triplicate wells. D, Purified CD8+ T cells were stimulated with anti-CD3 in the presence of indicated Igs (10 µg/ml). Production of IL-2 was measured after 24 h of culture. The data are expressed as the mean ± 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 µg/ml) in the presence of human IgG or BTN1–Ig (10 µg/ml) for 48 h. F, Naive CD4+ T cells were cultured with anti-CD3 (3 µg/ml) in the presence of human IgG or BTN1–Ig (10 µg/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).

FIGURE 4. Blockade of BTN1 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-BTN1 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 ± SD of five mice. *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
BTNL1 provided a negative costimulation, which resulted in reduced 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, BTN9, and BTN1L1, 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 BTN1L in vivo by using a neutralizing anti-BTN1L Ab. In KLH immunization, the blockade of BTN1L 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 BTN1L signaling on T cells may inhibit T cell priming and may reduce IL-17 and Th2 cytokine production. Notably, blockade of BTN1L inhibited IFN-γ production, which indicates that activated APCs that express high levels of BTN1L participate in the induction of a Th1 response. These results suggested that BTN1L 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 BTN1L blockade. The molecular basis for BTN1L 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, BTN1L 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 BTN1L 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 BTN1L blockade in diabetes development (data not shown). Therefore, BTN1L function may differ in specific contexts and cell targets. Further characterization of BTN1L 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.

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Disclosures

The authors have no financial conflicts of interest.

References


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 BTNL1A (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.** 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 μg/ml)-stimulated CD4+ T cells were cultured with indicated IgGs 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 ± SD of triplicate wells. All data are representative of three independent experiments. *, \( p < 0.01 \); **, \( p < 0.05 \): NS, not significant.
Supplementary Figure 3. BTNL1 inhibits naive and memory T cell activation. Naive (CD4+CD62L\textsuperscript{high}) and memory (CD4+CD62L\textsuperscript{low}) CD4\textsuperscript{+} 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 \[^{3}\text{H}\]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.** 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. GFP⁺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 ± SD of triplicate wells. These data are representative of three independent experiments. *, p<0.01.
Supplementary Figure 5. CD28/CTLA-4 family molecules and TLT2 are not the receptor for BTN1. A. ConA-activated CD4+ T cells from C57BL/6 mice were incubated with B7-1-Ig. The cells were stained with biotinylated BTN1-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 BTN1-Ig or human IgG, followed by APC-labeled streptavidin. The bold histograms indicate staining with BTN1-Ig and the thin histograms indicate background staining with human IgG. C. ICOS, PD-1, or TLT2 transfected 293T cells were stained with biotinylated BTN1-Ig or human IgG followed by APC-labeled streptavidin. All data are a representative of two independent experiments.